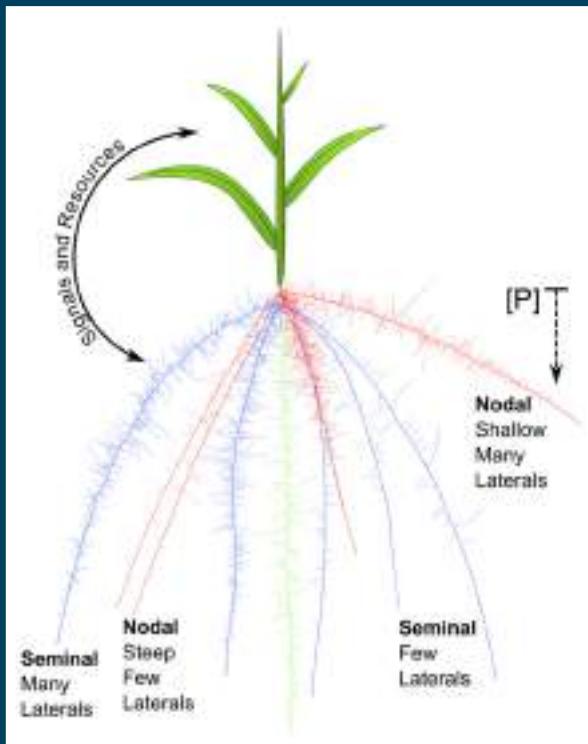


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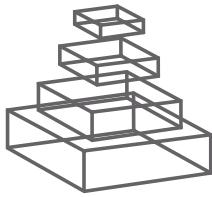
ECOPHYSIOLOGY OF ROOT SYSTEMS-ENVIRONMENT INTERACTION

Topic Editors

Boris Rewald, Omer Falik, Douglas Godbold
and Shimon Rachmilevitch



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ECOPHYSIOLOGY OF ROOT SYSTEMS-ENVIRONMENT INTERACTION

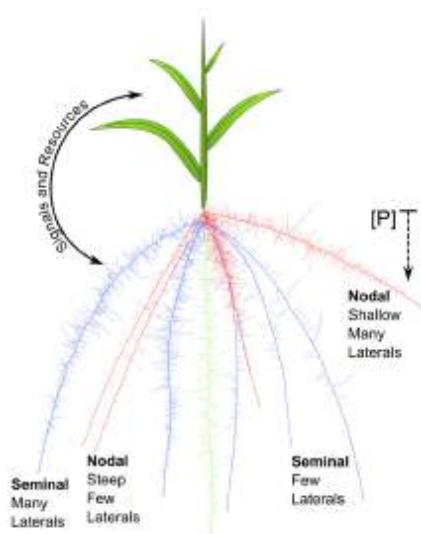
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A maize seedling is depicted.

Figure taken from: York LM, Nord EA and Lynch JP (2013) Integration of root phenes for soil resource acquisition. *Front. Plant Sci.* 4:355. doi: 10.3389/fpls.2013.00355

soil interface and in regard to the potential adaptive plasticity of root-rhizosphere interactions under abiotic stress and/or competition. It is currently unknown whether adaptations in microbe communities occur, for example due to modified exudation rates, and what are the subsequent influences on nutrient mobilization and uptake. Furthermore, uncovering the mechanisms by which roots perceive neighboring roots may not only contribute to our understanding of plant developmental strategies, but also has important implications on the study of competitive interactions in natural communities, and in optimizing plant performance and resource use in agricultural and silvicultural systems.

Plant sciences research focuses predominantly on aboveground parameters. There is a scarcity of detailed information regarding the ecophysiology of root systems and the way root system functioning is affected by both internal and external factors. Furthermore, global climate change is expected to increase the intensity of climate extremes, such as severe drought, heat waves and periods of heavy rainfall; in addition other stresses such as salinization of soils are increasing world-wide. Recently an increasing awareness has developed that understanding plant traits will play a major role in breeding of future crop plants. For example, there is increasing evidence that the traits of root systems are defined by the properties of individual roots. However, further knowledge on the functional importance of root segments and the molecular/physiological mechanisms underlying root system functioning and persistence is needed, and would specifically allow modifying (crop) root system functionality and efficiency in the future. Another major gap in knowledge is localized at the root-

In this Research Topic, we aimed to provide an on-line, open-access snapshot of the current state of the art of the field of root ecology and physiology, with special focus on the translation of root structure to function, and how root systems are influenced by interplay with internal and external factors such as abiotic stress, microbes and plant-plant interaction. We welcomed original research papers, but reviews of specific topics, articles formulating opinions or describing cutting-edge methods were also gladly accepted.

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Root and rhizosphere processes—high time to dig deeper

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The purpose of the research topic “Ecophysiology of root systems–environment interactions” was to shed light on belowground processes—in an effort to further enhance its understanding, but also to increase the awareness of the research community and funding bodies toward this utmost important part of plants and ecosystems.

Why is it important to increase our understanding and awareness? The new challenge of global climate change is driven by an increase in atmospheric CO₂ levels, a factor which in itself affects plant and root growth, but is also expected to increase the intensity of climatic and edaphic extremes. In addition, other stresses such as salinization and heavy metal contamination of soils are either increasing or continue to persist world-wide. To secure crop yield and soil quality, and to understand the functioning, and thus the resilience and resistance of pristine ecosystems under changing environmental conditions, an increased understanding of acclimation and adaptation processes is imperative. In the past, plant sciences’ research has focused predominantly on parameters above ground—resulting in disproportional less knowledge regarding root systems and the way root system functioning is affected by both internal and external factors. Similarly, soil scientists have often preferred studying bulk soil over the rhizosphere and as a consequence root-driven soil processes are still far less studied. Because it is “better to light a candle than curse the darkness” (Herron et al., 2013), this research topic puts research on root and rhizosphere into the spotlight.

Increased net photosynthesis and decreased shoot nitrogen and water use under elevated CO₂ can alter source–sink relations of plant organs. In this research topic, Easlon and Bloom (2013) emphasize the important role of root-shoot signaling for plant acclimation to increasing CO₂ levels. Addressing water availability, Carminati (2013) shows that fast and almost immediate rewetting after soil drought took place in the rhizosphere of distal maize root segments while the rhizosphere of higher root orders possessed slower rewetting. The difference in the speed of rewetting for different root orders, may possibly be an adaptation strategy to drought periods, increasing the water uptake by young root segments and hydraulically disconnecting the older ones. Two comprehensive reviews shed light on the influence and interplay of heavy metals on/with root systems, and influence of root traits on whole plant stress resistance. In one review,

Fahr et al. (2013) address the wide range of tolerance mechanism of roots against lead exposure, while in another review Brunner and Sperisen (2013) focus on the current understanding of aluminium exclusion and tolerance mechanisms in woody plants.

The response of roots to abiotic stress can be modified by some root-associated bacteria and fungi. This is expressed as modification of root morphology and whole plant ecophysiology, which often enhances plant growth under stress (Alavi et al., 2013; Vacheron et al., 2013). Increasing knowledge of bacterial nutrition in the rhizosphere will further increase the understanding of the role of certain bacteria as plant-growth promoters (Lopez-Guerrero et al., 2013). Similarly, mycorrhizal symbionts are very important for soil exploration; in this context, Lang et al. (2013) report on spatial structuring of ectomycorrhizal assemblages within beech root systems. While earthworms often improve soil structure and nutrient availability, Arnone and Zaller (2014) report decreasing grass root length densities under increasing earthworm densities, with yet unknown consequences for nutrient foraging.

Besides abiotic stress, root-microbe and root-fauna interactions, plant-plant interactions below ground are common and can influence plant performance considerably. For example, Bolte et al. (2013) show that beech fine roots are facilitated in the presence of spruce roots—possibly by lowering the competitive pressure (for resources) compared to intraspecific competition. While information on the mechanisms of belowground neighbor perception is rare, Schmid et al. (2013) outline that *Arabidopsis* roots perceive neighboring roots or their associated microorganisms by a mechanism that involves the induction of pathogenesis-related proteins. Their findings reveal that belowground neighbor detection may occur independently of resource depletion, possibly allowing roots to anticipate future competition.

In the past years an increasing awareness has developed that understanding root traits will help to understand plant functioning. Since resources are acquired by the root system, breeding for crops with root traits/phenotypes increasing water and nutrient acquisition should increase yields on infertile soils and under a range of other abiotic stresses such as drought (Comas et al., 2013; White et al., 2013). In doing so, a better understanding of how root and root system traits interact to affect soil resource

acquisition is needed (York et al., 2013). In woody plants, our understanding of species- or even variety-specific root trait plasticity under variable environments and the importance of specific traits such as deep rooting (Laclau et al., 2013; Maeght et al., 2013) is even more scarce than in crop plants. A primary reason for this difference in understanding being the challenges caused by more complex, difficult-to-access, perennial root systems. Fortunately, several studies of this research topic shed light on the variability/plasticity of (some) fine root traits of several tree species with ontogeny, and/or under different environmental or management conditions. For example, Noguchi et al. (2013) showed that N-fertilization had a more pronounced effect on *Cryptomeria japonica* root morphology than on root biomass. Studying ectomycorrhizal short roots, Ostonen et al. (2013) found that morphological root traits of late-successional spruce are as plastic as that of pioneer silver birch, and that differences between root traits of the two species was less under more temperate conditions compared to more boreal conditions. The work of Tobner et al. (2013) evidenced that the responses to ontogeny or soil conditions are species but also trait dependent. Hajek et al. (2013) found distinct intraspecific variation in most root traits among seven *Poplar* demes. As highlighted by Fort (2013), their results challenge the existence of well-defined species-specific trait values, but rather highlight the existence of pronounced within-species trait diversity linked to genetic differentiation. While manuscripts in this research topic address a plethora of root traits, increasing efforts are also required to understand root system branching *in situ* and *in silico* (Bodner et al., 2013) and to develop meaningful classification approaches for functional units within root systems. Bodner and colleagues showed that statistical classification methods can integrate knowledge on morphological traits obtained with different methods and at various scales. Currently morphology seems to be the most promising basis for classification approaches due to its wide use. However, the lack of consensus about fine root classification (and a clear nomenclature), and the importance of specific traits constrains the development of a unified framework toward a “root economics spectrum” as was achieved for both leaves (see e.g., Poorter et al., 2014 and references therein) and wood. In addition, more suitable methods are needed allowing advanced root research; here Danjon et al. (2013) describe a modeling approach to estimating root loss during tree root system sampling, and Faget et al. (2013) introduce a combined root fluorescence and planar Optode technique which allows to distinguish between different plant species grown in natural soil and to measure the impact of root (exudates) on the soil environment. Using light-emitting soil microbial “biosensors,” Herron et al. (2013) were able to determine hotspots of microbial growth along the growing root. The growth of these microbial hotspots was supported by high carbon availability.

In addition to articles on basic research which often accentuate the uncertainties in the field of root research, some articles already outline the application of knowledge of root traits in applied plant production systems. The study by Kerbiriou et al. (2013) provides first evidence that “robustness” and head growth rates of lettuce cultivars are related to the size of the root system. Terzaghi et al. (2013) investigated C and N concentrations in *Fagus sylvatica* fine

roots in relation to different stand characteristics resulting from conversion of coppiced forests to high forests. The fine-root C:N ratio was higher in coppiced than in converted stands and showed an inverse relationship with fine-root turnover rate, illustrating a significant change of fine-root status under different management practices—likely influencing e.g., the C sequestration potential of stands. Chairungsee et al. (2013) revealed a significant negative correlation between fine root dynamics and production in rubber plantations. Latex harvesting might disturb carbon dynamics in the whole tree, far beyond the trunk; the results emphasize the impact of root systems on the carbon budget and thus yield of tree crops plantations.

When taken as a whole, the 28 contributions to this research topic cover many, although by no means all, aspects of root and rhizosphere research. The number of articles collected within a relatively short period of time, and other recently published special issues addressing root-environmental interactions (e.g., Annals of Botany, 2012, 2013; New Phytologist, 2013), demonstrate that the awareness about root and rhizosphere research and its applicability is rising.

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The effects of rising atmospheric carbon dioxide on shoot–root nitrogen and water signaling

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Terrestrial higher plants are composed of roots and shoots, distinct organs that conduct complementary functions in dissimilar environments. For example, roots are responsible for acquiring water and nutrients such as inorganic nitrogen from the soil, yet shoots consume the majority of these resources. The success of such a relationship depends on excellent root–shoot communications. Increased net photosynthesis and decreased shoot nitrogen and water use at elevated CO₂ fundamentally alter these source–sink relations. Lower than predicted productivity gains at elevated CO₂ under nitrogen or water stress may indicate shoot–root signaling lacks plasticity to respond to rising atmospheric CO₂ concentrations. The following presents recent research results on shoot–root nitrogen and water signaling, emphasizing the influence that rising atmospheric carbon dioxide levels are having on these source–sink interactions.

Keywords: carbon dioxide, nitrogen, nitrate assimilation, water, drought, salinity, chilling

INTRODUCTION

Land plants occupy highly dissimilar aboveground and below-ground environments and face the basic allocation dilemma of where to invest resources (Bloom et al., 1985). Too little investment in roots leads to nutrient- or water-limited growth, whereas too much investment compromises shoot growth, reproduction, and photosynthesis. Excellent communications between roots and shoots are paramount for meeting the immediate demands of distal organs to optimize resource supply from them, while avoiding superfluous distribution of resources.

For example, the dependence of photosynthesis on nitrogenous compounds and the inevitability of water loss during CO₂ uptake (Field and Mooney, 1986) makes communicating N and water availability from roots to shoots essential to maintain shoot productivity (Boyer, 1982; Bloom, 1997). Conversely, shoot to root communication of leaf N status is necessary to optimize carbohydrate allocation in roots among growth, N uptake, and inorganic N assimilation. Coordination of N transport from root to shoot and of carbohydrate transport from shoot to root is fundamental for maintaining a C/N ratio throughout the plant that is optimal for plant growth and development (Martin et al., 2002; Zheng, 2009).

Climate change, in particular rising CO₂, is likely to alter root–shoot communications. Atmospheric CO₂ concentrations have remained relatively low, between 180 and 300 μmol mol⁻¹ over the last 400,000 years (Petit et al., 1999) and between 140 and 320 μmol mol⁻¹ over the last 23 million years (Pearson and Palmer, 2000). Flowering plants have evolved specific adaptations to this low CO₂ environment including increased stomatal density (Beerling and Chaloner, 1993), increased leaf vein density (Boyce and Zwieniecki, 2012), and C₄ photosynthesis (Ehleringer et al., 1991). This concentration has increased from 280 to 400 μmol mol⁻¹ since 1800 from the burning of fossil fuels (Whorf and Keeling, 1998) and is projected to reach between 500

and 900 μmol mol⁻¹ by the end of the century (Joos et al., 1999). This CO₂ enrichment will increase photosynthesis in C₃ plants and will decrease shoot N and water requirements for photosynthesis. This frequently results in increased biomass and productivity in the short-term that is not sustained in the long-term (Dukes et al., 2005; Körner, 2006; Kimball et al., 2007). Only after long-term growth at elevated CO₂ do limitations from N deficiencies, carbohydrate transport, and altered shoot/root allocation patterns become apparent. Unknown is whether the mechanisms of long distance communication between roots and shoots that evolved under low CO₂ will have the plasticity to optimize coordination of root and shoot growth under long-term exposure to elevated CO₂.

The goal of this review is to describe shoot–root signaling for N and water and to examine the observed and predicted responses of these signaling mechanisms to rising atmospheric CO₂ concentrations. First, we discuss shoot–root N signaling, changing C and N demand, and the breakdown of N signaling at elevated CO₂. Then, we explore the common and distinctive features of drought, salinity, chilling, and high vapor pressure deficit and the opposing effects of elevated CO₂ on chemical and hydraulic water stress signaling. Finally, we consider the effects of non-optimal shoot–root coordination on plant growth at elevated CO₂.

NITROGEN: COMMUNICATING ROOT AVAILABILITY AND SHOOT DEMAND

For most plants, growth and productivity is highly dependent upon N obtained from root absorption of soil inorganic and organic N. In most temperate soils, the primary form of N available to plants is nitrate (NO₃⁻; Epstein and Bloom, 2005). Therefore, this review focuses on this form.

Many studies have shown that elevated CO₂ stimulates photosynthesis, plant growth, and demand for mineral nutrients.

High variability in plant growth and photosynthetic responses to elevated CO₂ may result from vast experimental differences in soil NO₃⁻ concentration. In natural systems, soil NO₃⁻ is typically around 1 mM (Andrews, 1986b), but in fertilized agricultural soils, NO₃⁻ can be much higher, ranging from 10 to 70 mM (Reisenauer, 1966). The negative charge of NO₃⁻ prevents it from binding to most soil particles, and this contributes to substantial spatial and temporal heterogeneity in soil NO₃⁻ availability (Jackson and Caldwell, 1993). Plants have responded to soil NO₃⁻ variability with adaptations to increase NO₃⁻ uptake rapidly when it is available. In response to high soil NO₃⁻, individual roots increase NO₃⁻ uptake (Forde, 2002a) and alter root hydraulic properties to increase mass flow (Gorska et al., 2008). These adaptations allow a few roots in a high NO₃⁻ region of the soil to provide all the N that the shoot requires (Laine et al., 1995).

ROOT TO SHOOT N SIGNALING

Root to shoot communication of soil N availability may be as simple as NO₃⁻ delivery from roots to shoots in xylem sap (Takei et al., 2002). When soil NO₃⁻ is low, root C/N ratios are high and roots have sufficient carbohydrate to assimilate most of the NO₃⁻ that they absorb (Andrews et al., 1992) and thus deliver little NO₃⁻ to shoots. As soil NO₃⁻ increases, a greater proportion of absorbed NO₃⁻ remains unassimilated in the root and is transported to the shoot (Andrews, 1986a; Agrell et al., 1994). Xylem sap NO₃⁻ directly links soil N availability to the shoot and thereby serves as an ideal signal for such a temporally and spatially variable nutrient. High shoot NO₃⁻ stimulates shoot growth and low shoot NO₃⁻ inhibits shoot growth even when total shoot N is high (Walch-Liu et al., 2000; Rahayu et al., 2005). Species that predominantly transport N from root to shoot as amino acids instead of NO₃⁻ may not use xylem sap NO₃⁻ for root to shoot N signaling (Sprent and Thomas, 1984). Indeed, leaf growth is not always proportional to leaf NO₃⁻ concentration (Rahayu et al., 2005), indicating the importance of other signals such as phytohormones for root to shoot communication of root N supply.

One class of phytohormones involved in root to shoot signaling is cytokinins. Stimulation of leaf growth by N supply is associated with increased concentrations of active forms of cytokinins (Rahayu et al., 2005). Root cytokinin production and xylem sap delivery of cytokinins to shoots increase with NO₃⁻ fertilization (Takei et al., 2001; Forde, 2002b). Cytokinins stimulate leaf growth, increase shoot sink strength (Werner et al., 2008), and delay leaf senescence (Gan and Amasino, 1995), while they inhibit root elongation. Xylem sap transport of cytokinins increases expression of N responsive genes in leaves (Sakakibara et al., 1999; Takei et al., 2001; Kiba et al., 2011; Ruffel et al., 2011). All of these responses to cytokinins suggest that these phytohormones serve as root to shoot signals for root N availability.

ELEVATED CO₂ EFFECTS ON ROOT TO SHOOT N SIGNALS

CO₂ enrichment influences root to shoot N signaling through its effects on xylem sap flow rate, NO₃⁻ assimilation, and root allocation.

Root to shoot signals of N availability depend upon xylem sap flow for rapid signal delivery, and elevated CO₂ affects xylem flow rates. Elevated CO₂ decreases transpiration rates between

5 and 20% as stomata close in response to higher intercellular CO₂ concentration (Leakey et al., 2009). Stomatal closure slows water uptake and thereby xylem sap flow rate. Decreased transpiration may impede mass flow of NO₃⁻ in the soil solution to roots (McDonald et al., 2002), but this decrease may not slow delivery of N to shoots (Schulze and Bloom, 1984) because N concentration in the xylem sap increases as xylem sap flow decreases, maintaining N delivery rates (Shaner and Boyer, 1975; Schulze and Bloom, 1984). Increasing xylem loading of N in roots does not require substantial energy in that xylem solute N concentrations are relatively low. Xylem concentrations of cytokinins are in the nanomolar range (Foo et al., 2007), and so are even less likely to be affected by xylem sap flow rates.

Elevated CO₂ may disrupt root to shoot N signaling through shifting the location of NO₃⁻ assimilation. Greater rates of photosynthesis at elevated CO₂ increase carbohydrate flux to roots (Grimmer and Komor, 1999). In the root, higher carbohydrates increase NO₃⁻ assimilation (Matt et al., 2001), growth, and local demand for N (Kircher and Schopfer, 2012). Consequently, the root transports less NO₃⁻ to the shoot, and xylem sap NO₃⁻ becomes less effective as a signal of root N availability.

Plant allocation of carbohydrate to roots varies greatly with CO₂ enrichment (Rogers et al., 1996). For species in which carbohydrate flux to roots is insensitive to CO₂, the relationship among root NO₃⁻ assimilation, root N utilization, and xylem sap NO₃⁻ transport could indicate the potential for improving root to shoot N signaling at elevated CO₂. For species in which CO₂ enrichment increases carbohydrate flux, elevated CO₂ may disrupt cytokinin signaling. A low baseline level of root cytokinin production at low root available NO₃⁻ (Samuelson and Larsson, 1993) may result in greater root xylem cytokinin loading when root allocation is high under long-term growth at elevated CO₂ (Yong et al., 2000). High rates of cytokinin delivery to shoots could induce shoot growth in excess of what can be supported by root N supply. This could partially explain the decline in leaf N after prolonged exposure to elevated CO₂ (Oren et al., 2001). Additional study of xylem sap and leaf cytokinins at elevated CO₂ are necessary to determine if this disruption in cytokinin signaling is responsible for declining leaf N content.

SHOOT TO ROOT N SIGNALING

When soil NO₃⁻ is high, a few roots – 3.5% of the root system in spring wheat (Robinson et al., 1991) and 12% in lettuce (Burns, 1991) – can supply leaves with all of their N. When leaf N becomes limiting, plants may enhance root uptake by increasing (1) root growth, (2) root transporters to absorb soil N, and (3) root exudation to stimulate soil microbe activity that accelerates mineralization (Hawkes et al., 2005). All of these N acquisition strategies expend carbohydrate exported from shoots, and coordination of these processes is essential for optimal plant growth. Signals that stimulate root growth when leaf N is low or that repress root growth when leaf N is high balance root N acquisition and shoot demand.

A significant portion of N transported to shoots is recycled to roots via phloem transport of amino acids (Forde and Clarkson, 1999). It has been hypothesized that this transport of amino acids from shoots to roots in phloem could allow for feedback inhibition

of root growth and NO_3^- assimilation (Marschner, 1986; Imsande and Touraine, 1994; Marschner et al., 1996). Although exogenously supplied amino acids can inhibit root growth and NO_3^- uptake (Orsel et al., 2002; Forde and Walch-Liu, 2009), composition and transport of amino acids in phloem often do not correlate with shoot N status or root NO_3^- uptake (Forde, 2002a). In split root experiments, amino acids were preferentially transported to portions of root systems supplied with NO_3^- rather than those deprived of exogenous N, and the roots receiving more amino acids had higher growth rates (Tillard et al., 1998). This supports that amino acids delivered via the phloem stimulate root growth rather than inhibit it (Marschner et al., 1996).

Auxins are primarily synthesized in shoots and inhibit shoot branching (Normanly et al., 1995; Ljung et al., 2001). They are transported to roots through polar transport in the phloem (Baker, 2000) and promote proliferation of lateral roots. Phloem and root auxin concentrations decrease when plants are grown at high NO_3^- (Caba et al., 2000; Tian et al., 2008) and increase in roots when N is limiting (Walch-Liu et al., 2006). Therefore, auxins are prime candidates for signals that communicate shoot NO_3^- levels to roots (Forde, 2002b). Roots rely on photosynthesizing organs for carbohydrates, and thus, auxin-induced increases in root growth depend upon root carbohydrate supply (Reed et al., 1998; Bhalerao et al., 2002; Zhang et al., 2007).

The amount of carbohydrate transported in phloem sap from shoots to roots may also signal shoot N status, and this carbohydrate signaling mechanism appears to be independent of phloem transport of auxin (Bingham et al., 1998). At high leaf N, shoot growth acts as a sink for shoot produced carbohydrates and relatively little carbohydrate is transported to roots. If leaf N is low, shoot growth is limited and more carbohydrate is transported to roots (Brouwer, 1967; Brouwer and DeWit, 1969; Bloom et al., 1993; Kallarackal et al., 2012). High root carbohydrates increases root elongation and lateral root initiation (Bingham et al., 1998; Kircher and Schopfer, 2012), increases root area for N acquisition, and upregulates NO_3^- uptake and assimilation (Lejay et al., 1999; Ono et al., 2000; Matt et al., 2001).

ELEVATED CO₂ EFFECTS ON SHOOT TO ROOT N SIGNALING

Leaf N concentrations decline under prolonged growth at elevated CO₂ (Oren et al., 2001). Photosynthetic acclimation can account for some of this decrease (Long et al., 2004), but fertilization with NH₄NO₃ eliminates it (Crous et al., 2010; Liu et al., 2011), showing that increased N supply can compensate for the effects of elevated CO₂ through enhanced root N uptake and plant N assimilation. This suggests that elevated CO₂ interrupts shoot to root N signaling.

Amino acids in the phloem, potential signals of shoot N status, do not show a consistent response to elevated CO₂ (Docherty et al., 1997; Sicher, 2008). By contrast, leaf and root auxins increase under elevated CO₂ and stimulate root growth (Teng et al., 2006; Wang et al., 2009; Niu et al., 2011). Other processes, however, such as carbohydrate transport or shoot NO_3^- assimilation, may limit the ability of increased root auxins to stimulate root N uptake.

Carbohydrate transport through the phloem is driven by a carbohydrate concentration gradient (van Bel, 2003). Higher rates

of net photosynthesis under elevated CO₂ increase carbohydrate delivery to roots and can increase root respiration and root NO_3^- assimilation (Bassirirad et al., 1996; Fonseca et al., 1997; Kruse et al., 2002). High carbohydrate delivery to roots of C₃ plants under long-term growth at elevated CO₂ can also increase root growth (Berntson and Bazzaz, 1996; Kimball et al., 2002) and root carbohydrate exudation (Berntson et al., 1997). Carbohydrate flow from shoots to roots, however, does not increase proportionally to photosynthesis at elevated CO₂. For example, elevated CO₂ increases photosynthesis in C₃ species, but carbohydrate export from the leaves may not increase proportional to this carbon fixation (Grodzinski et al., 1998). This probably derives from leaf carbohydrate production under elevated CO₂ exceeding phloem export capacity (Korner et al., 1995; Komor, 2000).

In most tropical and subtropical plants and in temperate plants at high soil NO_3^- , most NO_3^- assimilation occurs in shoots because NO_3^- photoassimilation in shoots is more energy efficient than respiratory-driven NO_3^- and NO_2^- reduction in roots (Andrews, 1986b). Elevated CO₂ inhibits shoot NO_3^- assimilation in C₃ plants (Rachmilevitch et al., 2004; Bloom et al., 2010), necessitating a greater reliance on root NO_3^- assimilation to maintain plant capacity for NO_3^- assimilation. In tobacco, 3 weeks of CO₂ enrichment enhances root NO_3^- assimilation and may compensate for decreasing shoot NO_3^- assimilation when there is sufficient root carbohydrate (Kruse et al., 2002). A shift from shoot NO_3^- assimilation to root NO_3^- assimilation requires translocation of more carbohydrate to the roots to provide sufficient energy and carbon skeletons for these processes (Zheng, 2009). NH₄⁺ fertilization decreases the limitations of phloem carbohydrate transport on plant N status because NH₄⁺ assimilation requires less carbohydrate.

WATER STRESS SIGNALING

Photosynthesis in land plants results in the inevitable water loss during CO₂ uptake because both diffusion of CO₂ into leaves and water vapor out of leaves occur through stomata. Soil drought, salinity, and chilling can result in an inability of water transport from roots to match shoot water loss. To maintain leaf photosynthesis, shoot turgor, and shoot growth, plants under water stress rely on local root responses that increase water uptake as well as shoot responses that reduce water use.

During drought or salt stress, xylem tension acts as an integrative hydraulic signal of soil water potential that rapidly communicates soil water stress to leaves (Malone, 1993). Likewise, low root hydraulic conductance during root chilling results in rapidly increasing xylem tension and declining leaf turgor (Bloom et al., 2004). Turgor loss causes stomatal closure through either passive or active regulation (Tardieu and Davies, 1993) and inhibits leaf growth as leaf cell turgor declines below the threshold for cell wall expansion (Hsiao and Acevedo, 1974). Smaller leaf area and stomatal closure resulting from decreased leaf turgor protect leaves from desiccation. During slowly developing soil drought, soil moisture content has substantial heterogeneity, but hydraulic signals are integrative; that is, xylem tension in leaves is affected by xylem tension in all connected roots. Roots in drier regions experience greater decreases in water potential before hydraulic signals are transmitted to leaves. Non-hydraulic

signals can be generated in these roots with lower water potential, allowing shoots to preemptively reduce shoot water use before leaf water deficit develops (Dodd et al., 2008). During root chilling, chilling tolerant species close stomata before declines in leaf water potential occur, indicating that non-hydraulic chemical signals are also important in response to this type of water stress (Bloom et al., 2004).

Abscisic acid (ABA) increases with drought and salinity, induces stomatal closure, and inhibits transpirational water loss (Davies and Zhang, 1991; Bahrin et al., 2002; Jia et al., 2002). Low root water potential increases both root ABA production (Simonneau et al., 1998) and xylem sap transport of ABA from root to shoot (Zhang and Davies, 1989). ABA production also increases during chilling stress in the long-term (Melkonian et al., 2004), but the rapidity of stomatal closure during root chilling indicates that other, more rapidly produced root to shoot signals are involved in root chilling.

Abscisic acid-induced stomatal closure is not solely dependent on root ABA production. Shoot vascular tissue ABA production (Endo et al., 2008) and ABA uptake by leaf symplast also affect guard cell ABA concentration. Xylem sap pH increases with soil drought, salinity, and root chilling, slows leaf symplastic ABA uptake, and increases guard cell ABA concentration, thereby promoting stomatal closure (Vernieri et al., 2001; Wilkinson and Davies, 2002; Felle et al., 2005; Wilkinson et al., 2007).

Evidence is mounting for non-hydraulic signals other than ABA and pH in xylem sap that also affect stomatal regulation during water stress (Munns, 1992; Chen et al., 2002; Holbrook et al., 2002). For example, salts carried in the transpiration stream can also act as long distance root to shoot signals. During salinity stress Na^+ and Cl^- are transported in xylem sap and concentrated at sites of evaporation in leaves. High leaf apoplastic Na^+ and Cl^- decrease water potential, prompting osmotic adjustment and, in some halophytes, stomatal closure (Very et al., 1998).

Shoot to root signaling is also important for responses to chilling and high vapor pressure deficit stresses that do not directly affect root water potential. During both of these stresses, transpiration exceeds the capacity for root water transport. High root ABA increases root hydraulic conductance and water flow during chilling or at high vapor pressure deficit to ameliorate shoot water deficit (Markhart, 1984; Kudoyarova et al., 2011). This increase in root ABA requires water stress signaling from shoots; for example, if leaf water potential is maintained during chilling, there is no increase in root ABA (Vernieri et al., 2001). Shoot to root communication of shoot water deficits may be communicated hydraulically or through phloem transport of ABA or other signals.

ELEVATED CO₂ EFFECTS ON WATER STRESS SIGNALING

The primary effect of elevated CO₂ on water stress signaling derives from stomatal closure in response to high intercellular CO₂ and the resulting lower transpiration rates (Leakey et al., 2009). Lower transpiration rates under elevated CO₂ may decrease both accumulation of ABA at sites of evaporation near guard cells (Zhang and Outlaw, 2001) and foliar ABA concentration in general (Teng et al., 2006). Moreover, stomatal closure in response to root

ABA application and osmotic stress are greater at elevated CO₂ (Leymarie et al., 1999) and may result from higher intercellular CO₂. At ambient CO₂, when stomata begin to close during water stress, low intercellular CO₂ can partially reverse stomatal closure. At elevated CO₂, intercellular CO₂ remains high even after stomatal closure, and this can prevent reversal of stomatal closure.

Hydraulic signaling is also affected by lower transpiration rates at elevated CO₂. Slower transpiration reduces leaf xylem tension and improves leaf water potential during drought (Xiao et al., 2005). This may mitigate midday declines in leaf water potential during early stages of drought that are necessary for shoot perception of water stress. Slower transpiration at elevated CO₂ delays hydraulic signaling of declining root water potential, but does not delay non-hydraulic signaling. Non-hydraulic signals like ABA are still delivered to shoots at elevated CO₂, decreasing shoot water use and further delaying hydraulic signaling of declining root water potential. Slower transpiration also minimizes development of leaf water deficit during chilling at elevated CO₂ (Boese et al., 1997), which may inhibit root ABA production (Vernieri et al., 2001) that is important for root acclimation to chilling.

CONCLUSION

Leaf N concentration declines under prolonged growth at elevated CO₂ (Oren et al., 2001) unless plants are heavily fertilized with NH₄NO₃ (Crous et al., 2010; Liu et al., 2011). This suggests that mechanisms for long distance root–shoot communication of root N availability and shoot N status, which evolved under low CO₂, may lack plasticity to maintain root–shoot coordination under elevated CO₂. Leaf and root auxin concentrations increase in response to low leaf N under elevated CO₂ which should increase root growth, root NO₃⁻ uptake, and root NO₃⁻ assimilation (Teng et al., 2006; Wang et al., 2009; Niu et al., 2011). However, root organic N supply to shoots may be limited by phloem carbohydrate transport from shoots to roots (Grodzinski et al., 1998); although these effects may not affect growth until stored leaf N is depleted. The accumulation of non-structural carbohydrates in leaves at elevated CO₂ that is often observed (Long et al., 2004) may result from an inability to transport carbohydrate out of leaves or to obtain enough N from roots for shoot growth. Photosynthetic acclimation, whereby carbon fixation per unit leaf area declines under prolonged exposure to elevated CO₂, decreases leaf N requirements and increases leaf phloem export capacity. This may mitigate phloem carbohydrate export limitations and thus improve shoot–root N signaling.

The improvement in leaf water potential and water use efficiency resulting from higher intercellular CO₂ concentration are predicted to benefit plant growth under elevated CO₂, but productivity gains at elevated CO₂ under water limitation are often lower than predicted (Nowak et al., 2004; Newingham et al., 2013). Slower transpiration impedes development of leaf water deficits important for shoot water stress perception as soil water potential declines. Plants generate ABA and other non-hydraulic signals of low root water potential, and these can decrease stomatal conductance and shoot growth before declines in leaf water

Table 1 | Root–shoot N and water signal responses to elevated CO₂.

Signal	Role	Response to elevated CO ₂
NO ₃ ⁻	Root to shoot signal of root NO ₃ ⁻ availability	Root NO ₃ ⁻ assimilation, local root demand for N increase, and xylem transport of NO ₃ ⁻ decreases
Cytokinin	Root to shoot signal of root NO ₃ ⁻ availability	Cytokinin production and xylem transport increases even at low root available NO ₃ ⁻
Auxin	Shoot to root signal of leaf N availability	Auxin production and transport to roots increases in response to low leaf N
Carbohydrate	Shoot to root signal of leaf N availability	Increased carbohydrate delivery to roots, but delivery does not increase proportionally with leaf carbohydrate production
Xylem tension	Bidirectional signal of root or shoot water stress	Stomatal closure reduces leaf xylem tension delaying shoot perception of water stress
ABA	Bidirectional signal of root or shoot water stress	Transpirational accumulation of leaf and guard cell ABA decreases and stomatal sensitivity to ABA increases

potential occur. While stomatal closure from these non-hydraulic water stress signals has less negative impact on photosynthesis at elevated CO₂ as compared to ambient CO₂, these signals can still unnecessarily limit shoot growth (Leymarie et al., 1999). Greater stomatal sensitivity to osmotic and drought stress results in high water use efficiency and less negative leaf water potential, but more conservative shoot growth and lower potential productivity (Warren et al., 2011).

Shoot–root N and water signaling involve both resource and phytohormone transport from source organs to distant sink

organs to achieve a functional equilibrium between roots and shoots. Rising atmospheric CO₂ concentrations will increase net photosynthesis, decrease water use, and may alter source–sink interactions beyond the capability of signaling mechanisms that evolved at the lower atmospheric CO₂ concentrations, which have prevailed throughout recent history (Table 1). Critical assessment of limitations in shoot–root signaling at elevated CO₂ and careful genetic manipulations of N and water signaling could enhance crop response to rising atmospheric CO₂ and avoid declines in plant N.

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Root traits contributing to plant productivity under drought

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Geneticists and breeders are positioned to breed plants with root traits that improve productivity under drought. However, a better understanding of root functional traits and how traits are related to whole plant strategies to increase crop productivity under different drought conditions is needed. Root traits associated with maintaining plant productivity under drought include small fine root diameters, long specific root length, and considerable root length density, especially at depths in soil with available water. In environments with late season water deficits, small xylem diameters in targeted seminal roots save soil water deep in the soil profile for use during crop maturation and result in improved yields. Capacity for deep root growth and large xylem diameters in deep roots may also improve root acquisition of water when ample water at depth is available. Xylem pit anatomy that makes xylem less "leaky" and prone to cavitation warrants further exploration holding promise that such traits may improve plant productivity in water-limited environments without negatively impacting yield under adequate water conditions. Rapid resumption of root growth following soil rewetting may improve plant productivity under episodic drought. Genetic control of many of these traits through breeding appears feasible. Several recent reviews have covered methods for screening root traits but an appreciation for the complexity of root systems (e.g., functional differences between fine and coarse roots) needs to be paired with these methods to successfully identify relevant traits for crop improvement. Screening of root traits at early stages in plant development can proxy traits at mature stages but verification is needed on a case by case basis that traits are linked to increased crop productivity under drought. Examples in *lesquerella* (*Physaria*) and rice (*Oryza*) show approaches to phenotyping of root traits and current understanding of root trait genetics for breeding.

Keywords: root morphology, root architecture, hydraulic conductance, hydraulic conductivity, QTL, drought tolerance, MAS

INTRODUCTION

Water shortages are responsible for the greatest crop losses around the world and are expected to worsen, heightening international interest in crop drought tolerance. Within the U.S. alone, about 67% of crop losses over the last 50 years have been due to drought. The 2012 drought in the U.S. was the worst in 60 years and more frequent occurrences of water shortages are expected due to climate projections and increasing competition for water among urban, industrial, and agricultural demand (IPCC, 2012; Haro von Mogel, 2013). Geneticists and breeders are in position to make strides in breeding plants for better yields under drought. Drought tolerance is most desirable as the maintenance of crop productivity under drought (definition of drought tolerance in this paper; Passioura, 2007), which can be accomplished in a variety of ways, including drought avoidance or desiccation prevention, potentially in combination, through matching crop water use with water availability, and recovery of growth following rewetting (Passioura, 2012). While the shoot drives water uptake through a plant, root system size, properties, and distribution ultimately determine plant access to water, and thus, set

limits on shoot functioning, similar to an analogy of a horse driving a cart and the cart setting limits on the capacity of the horse (Nardini et al., 2002; Sperry et al., 2002). Thus, an area of recent interest is improvements of root traits that increase efficient deployment of tissues for foraging of soil water and, expressly, the maintenance of productivity under water deficit. However, key questions remain: *which root traits help most and under what conditions?*

Past efforts in improvement of germplasm for water-limited environments have been accomplished by focusing on specific traits for particular crops and drought conditions, which appear more clearly when viewed through a framework that dissects the benchmark of water-limited yield potential into independent components (Passioura and Angus, 2010). An appreciation of the growth strategies of individual crops and specifics of particular drought conditions crops face will need to continue to be at the forefront of successful breeding programs. In agricultural systems without irrigation (dryland systems), drought may be episodic in varying degrees or extend through the majority of the growing season. These different scenarios of drought will have

different impacts on crop growth and development above and below ground (Passioura, 2012). In irrigated agriculture, water may be applied in varying degrees of deficit irrigation throughout the season, as full irrigation during strategic periods of the season, or applied in different combinations of deficit and full irrigation during different periods of the growing season. Breeding efforts will also be more successful if coupled to advances being made in management (Kirkegaard and Hunt, 2010). It is widely recognized that breeding efforts need to account for the genotype by environment by management ($G \times E \times M$) interaction because improving crop productivity will require breeding for different plant traits and growth strategies in different environments and under different management (Sinclair et al., 2010; Passioura, 2012; Reynolds et al., 2012). Nevertheless, a few generalizations in root traits associated with crop productivity under drought are beginning to emerge (Wasson et al., 2012). Discussion of these root traits and others resulting from advances in the plant ecophysiological arena are the subject of this review and will be discussed at the organism, organ system, organ, and tissue and cellular level (**Figure 1**).

ROOT SYSTEMS, TRAITS, AND FUNCTIONING IN WATER UPTAKE

Before considering specific root traits, it is worth discussing root systems as a whole. There is a level of complexity in root systems of both woody and herbaceous plants that is crucial to root system functions but often goes unacknowledged: the root system is not one organ but rather composed of two, and sometimes three, main types of root organs. For woody plants, coarse woody roots, mirroring stems aboveground, serve functions of perennial structures, anchorage, carbohydrate and nutrient storage during the season, and transport of nutrients and water. The fine roots of woody plants, which are limited to the terminal two root segments (referred to as first and second branch orders counting back from root tips), serve ephemeral roles in foraging for belowground resources (Guo et al., 2008; Xia et al., 2010). The root system of herbaceous plants, crop and non-crop alike, is also comprised of coarse and fine roots, which may correspond to tap versus lateral roots in a tap root system or seminal and nodal versus lateral roots in a fibrous root system (Fitter, 2002). Like in woody plants, coarse and fine roots of herbaceous plants can be distinguished by a jump in diameter class, which tends to occur between the terminal two root orders and the rest of the root system. Coarse roots of herbaceous plants serve functions of anchorage and typically establish overall root system architecture, controlling ultimate rooting depth, and the ability of plants to grow into compacted soil layers (e.g., Henry et al., 2011). In addition to coarse seminal roots, nodal roots (or brace roots in maize, *Zea mays*) developing from lower portions of the stem provide additional opportunities for plant foraging of late-season precipitation with different responses to soil water than the primary root system (Rostamza et al., 2013). Finally, fine (or lateral) roots are the most active portion of the root system in water uptake, and comprise the majority of the length and surface area of these root systems in herbaceous and woody plants alike (Bauhus and Messier, 1999; Rewald et al., 2011).

ROOT SYSTEM SIZE AND ALLOMETRY

The size of a plant's root system is a key trait of interest related to acquisition of soil resources but only when considered in relation to the size of the remainder of the plant, either relative to leaf area, shoot, or whole plant size. Shifts in allometry (metrics of root to shoot relationships) and shoot stature can compensate for water shortage, and, along with shifts in stand densities, can maintain stomatal conductance under xeric conditions similar to levels under mesic conditions (Mencuccini, 2003; Addington et al., 2006; Maseda and Fernandez, 2006 and references within). Allometry is typically measured as root:shoot ratio of dry mass. When determined from biomass, root biomass per total plant biomass (i.e., root mass fraction, RMF) is a more robust quantification of the relative size of root systems for statistical reasons but has been less frequently used (Reich, 2002). Ultimately, ratios of root to leaf surface area ($A_R:A_L$) or root length:leaf area ratio are more functionally descriptive than mass fractions of tissues and can be used as a surrogate for water uptake capacity in proportion to capacity for light interception, as well as providing the surface area of water uptake versus transpiration loss (e.g., Sperry et al., 2002; Diaz-Espejo et al., 2012).

Functional equilibrium theory suggests that plants shift allocation among absorptive tissues to acquire resources that most limit growth (Brouwer, 1983). Optimal partitioning theory takes this idea one step further and suggests that plants allocate resources among organs to optimize whole plant growth (Thornley, 1969; Bloom et al., 1985). These theories suggest plants may be adapted to produce a particular root:shoot ratio but this ratio will shift to balance resources limiting growth with a degree of plasticity, or responsiveness, which is a trait of interest in and of itself (Shipley and Meziane, 2002; but see Reynolds and D'Antonio, 1996). Root:shoot ratio changes with plant growth and development in addition to shifting in response to limiting resources above versus below ground. Therefore, care must be taken to control for plant size and ontology, especially when assessed on young plants (Müller et al., 2000). When ratios of dry mass fractions (e.g., root:shoot ratio; RMF) are taken instead of $A_R:A_L$, these ratios may be too coarse of a measure to be meaningful in many cases (Reynolds and D'Antonio, 1996 and references within). Ratios of dry mass fractions do not account for the more plastic response of tissue morphology, architecture, and physiology (e.g., Boot and Mensink, 1990; Jackson et al., 1990; Aerts et al., 1991; Van der Vijver et al., 1993; Berntson et al., 1995; Ryser and Lambers, 1995). This is crucial because root dry mass fractions can mask shifts in root morphology or architecture by remaining constant while the total length or surface area of a root system increases or decreases dramatically with relatively small shifts in root diameter, specific root length (SRL; root length per dry mass), specific surface area (SSA; root surface area per dry mass), or proportion of coarse to fine roots.

ORGAN, TISSUE, AND CELLULAR LEVEL TRAITS

At the organ level, several root morphological traits for both fine and coarse portions of root systems have been found to be associated with increased productivity under drought. Key morphological traits seem to be traits that influence total root length

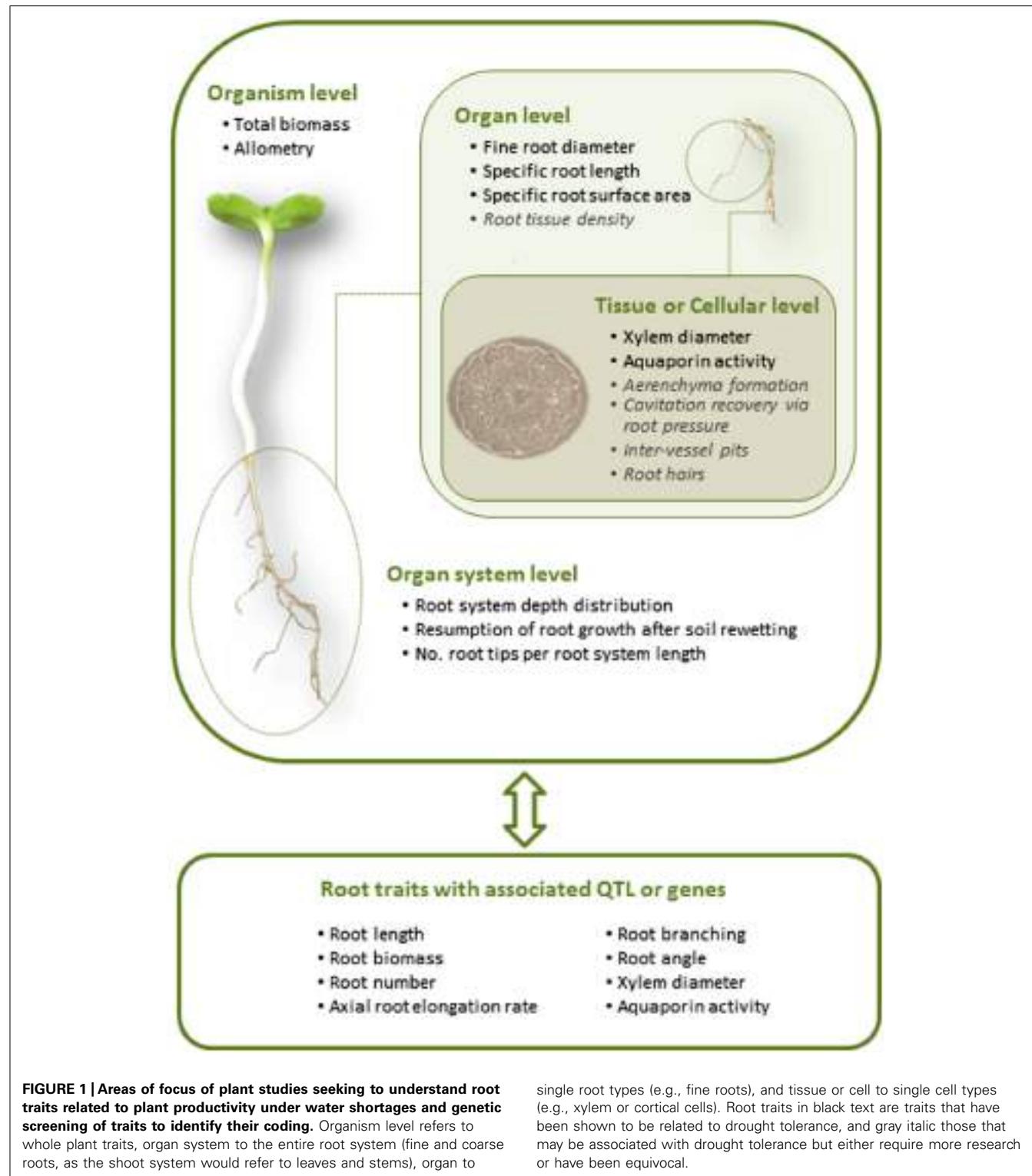


FIGURE 1 | Areas of focus of plant studies seeking to understand root traits related to plant productivity under water shortages and genetic screening of traits to identify their coding. Organism level refers to whole plant traits, organ system to the entire root system (fine and coarse roots, as the shoot system would refer to leaves and stems), organ to

single root types (e.g., fine roots), and tissue or cell to single cell types (e.g., xylem or cortical cells). Root traits in black text are traits that have been shown to be related to drought tolerance, and gray italic those that may be associated with drought tolerance but either require more research or have been equivocal.

and surface area of root systems and include root diameter, root tissue density, SRL, and SSA (Fitter, 2002; Nardini et al., 2002). Root diameter and tissue density control the length and surface area of root systems for a given biomass allocated to the root system (Fitter, 2002), which not only controls the amount of surface

directly interacting between roots and soil, but also the amount of root surface colonized by mycorrhizal fungi assisting in plant nutrient acquisition (Smith and Read, 2008). SRL and SSA summarize the overall effect of both root diameter and tissue density in terms of root length per dry biomass invested in the tissue (Fitter,

2002). For woody plants, root diameter predominately controls differences in SRL and SSA among species, with root tissue density affecting plasticity within species due to plant responses to edaphic factors such as soil water (Comas et al., 2002; Comas and Eissenstat, 2009). Small diameter roots with greater SRL enable plants to efficiently increase hydraulic conductance by increasing surface area in contact with soil water, increasing the volume of soil that can be explored for water, and, also, increasing root hydraulic conductivity by decreasing the apoplastic barrier of water entering the xylem (Eissenstat and Achor, 1999; Rieger and Litvin, 1999; Huang and Eissenstat, 2000; Solari et al., 2006; Hernández et al., 2010; Comas et al., 2012). Accordingly, decrease in root diameter has been proposed as a trait for increasing plant acquisition of water and productivity under drought (Wasson et al., 2012). In addition to root morphological traits affecting water and nutrient acquisition through control of root length and surface area, root morphology also affects resource acquisition by influencing root growth rate, with finer roots associated with faster root growth rate (Eissenstat, 1991; Robinson et al., 1991, 1999). Both woody and herbaceous plants adapted to dry conditions are found to have smaller diameter fine roots with greater SRL (Hernández et al., 2010; Henry et al., 2012).

A few additional root attributes have been associated with increased productivity under drought. Root tissue density was found to primarily control differences in SRL and SSA among several grass species (Ryser and Lambers, 1995; Wahl and Ryser, 2000). Aerenchyma formation in the root cortex can decrease root tissue density, increasing SRL and SSA (Zhu et al., 2010). Induction of root aerenchyma has been proposed to increase plant performance and improve carbon economy under drought in maize (Zhu et al., 2010). However, aerenchyma impeded radial movement of water through the root cortex and decreased water uptake in water-stressed rice (Yang et al., 2012a). Root hairs produced in many species can also substantially increase root surface area and are particularly responsive to reductions in soil water and nutrient availability (Bhat et al., 1979; Claassen and Jungk, 1982; Mackay and Barber, 1985; Bates and Lynch, 2001), although benefits under low soil water may not be found for all plants or conditions (Wen and Schnable, 1994; Suzuki et al., 2003). Root hairs in rice, for example, were found to be more important for nutrient uptake and provided no significant impact on water uptake (Suzuki et al., 2003). However, increases in root surface area *via* root hairs may compensate for reductions in root elongation occurring in extremely dry soils (Mackay and Barber, 1985). Root hairs may also promote root contact with soil particles as soil dries and may thus assist roots in acquiring soil water (Wasson et al., 2012 and references within). Additionally, increased abundance and conductance of aquaporins, which regulate the passage of water uptake, may increase root hydraulic conductivity (conductance per length of root) to meet shoot demand and compensate for reduced root surface area (e.g., Kaldenhoff et al., 1998; Parent et al., 2009; Vandeleur et al., 2009; Laur and Hacke, 2013).

New root tips, and, thus, continual root growth to produce these tips, may be more important for the uptake of mobile resources than the total amount of root length and surface area (Robinson et al., 1991). The main zones of water uptake are young

root tips (Sanderson, 1983; Haussling et al., 1988; Peterson et al., 1993; Varney et al., 1993; Kramer and Boyer, 1995). Although, even for mobile soil resources, total root length and surface area may matter when plants compete (Newman and Andrews, 1973). Roots increase apoplastic barriers and take up less water with age and exposure to dry soil (Steudle, 2000), which may appear unfavorable. However, models show greater water uptake for the same amount of root length when a small proportion of the root system is unshrubberized (e.g., when only root tips are unshrubberized) because there is greater hydraulic conductance along the root axis, in contrast to that of a “leaky pipe” (Zwieniecki et al., 2003).

In addition to root diameter, xylem diameter also affects root hydraulic conductivity and can affect plant productivity under drought (Zimmermann, 1983; Tyree et al., 1994). Research to some degree supports generalizations that plants with large diameter xylem vessels have greater hydraulic conductivity, but less conservative water use and greater risk of cavitation than those with small diameter vessels (Richards and Passioura, 1989; Sperry and Saliendra, 1994; Tyree et al., 1994; Alder et al., 1996; Gallardo et al., 1996) but exceptions can be found (Pockman and Sperry, 2000). Cavitation and embolism formation set thresholds on stomatal closure, with safety margins needed varying with frequency and amount of drought that plants are adapted to handle (Choat et al., 2012). As a breeding strategy, a general reduction in root xylem diameter can reduce total plant hydraulic conductance under well-watered conditions and limit plant maximum growth potential, therefore, when breeding these traits, programs have targeted their expression specifically in roots that function in water uptake primarily under dry conditions (Passioura, 1983). An Australian wheat (*Triticum aestivum*) breeding program successfully developed wheat varieties with more conservative hydraulic architecture in seminal roots to save soil water under drought for critical stages in crop yield development later in the field season (Passioura, 1972; Richards and Passioura, 1989). In this case, a general decrease in root hydraulic conductance was not manifested under well-watered conditions when seminal roots played a minor role and nodal roots predominately acquired water for the plant (Richards and Passioura, 1989).

Exceptional species with large diameter xylem adapted to dry environments have been found (Pockman and Sperry, 2000). These species are able to maintain high transpiration rates and conductivity but have high resistance to cavitation (Smith et al., 1996; Pockman and Sperry, 2000). Identifying mechanisms in such examples may be of special interest to breeding programs because such mechanisms would avoid reduced maximum yield potential under favorable growing condition. Mechanisms at work in such examples may be related to the anatomy of intervessel pit areas and greater rarity of “leaky” pits, which minimizes the initiation of cavitation (Wheeler et al., 2005; Christman et al., 2009).

ROOT SYSTEM GROWTH AND DISTRIBUTION UNDER DROUGHT: NUANCES RELATED TO FIELD CONDITIONS AND GENOTYPES

Of all root traits of potential importance, plant allometry and hydraulic conductance during drought have been of keen interest and the subject of several reviews (Mencuccini, 2003; Maseda and Fernandez, 2006; Wasson et al., 2012). Although shifts in

root growth and allometry may increase plant hydraulic conductance and productivity under drought (Mencuccini, 2003; Addington et al., 2006; Maseda and Fernandez, 2006), plant allometric responses partially depend on soil properties and spatio-temporal formation of drought. The “balanced growth” hypothesis (sensu Bloom et al., 1985) suggests that some plants respond to drought by stimulating or maintaining root growth while reducing shoot growth. Increased root versus shoot growth should improve plant hydraulic status under mild or moderate drought stress due to increased root to leaf surface, continued production of new root tips, and enhancement of plant capacity for acquiring water to support existing shoots. The underlying mechanisms behind the shift in allometry are difference in the sensitivity of root and shoot growth to water stress (Hsiao and Xu, 2000). Even partial drying of root systems can lead to decreased allocation to vegetative shoots (e.g., Dry et al., 2001). It has been observed, however, that under severe water deficits, limited root growth may occur because of very low soil water availability and high soil impedance (Taylor and Gardner, 1963; Cornish et al., 1984; van Zyl, 1984; Comas et al., 2005). In this case, as mentioned in the previous section, increased root hair and aquaporin production may play particularly important roles in compensating for reductions in root elongation and surface area production.

Additionally, the ability of plants to grow roots according to distribution of available soil water profoundly increases plant productivity under drought. Root traits for water acquisition from deep in the soil profile and methods of such trait assessment have been well described in recent reviews (Wasson et al., 2012). Plants are inherently somewhat plastic in their root distribution, especially deep-rooted species such as maize and sunflower (*Helianthus annuus*; Figure 2). Irrigation reached to approximately 30 cm soil depth in the crops illustrated but roots of these crops were found below 1 m. Deep roots for water acquisition deep in the soil profile may be especially important for smaller statured plants, such as wheat, rice, and common bean (*Phaseolus vulgaris*), but have generally conferred advantages for plants growing under limited soil water in agricultural and natural systems (Ho et al., 2005; Schenk and Jackson, 2005; Hund et al., 2009; Lopes and Reynolds, 2010; Henry et al., 2011; but see Sun et al., 2011). As soil dries at the surface, water may be available deeper in the profile than many agricultural species are adapted to reach, and require root system development deeper in the profile to access this water. In this case, breeding for plants with less root length density (RLD, root length per soil volume) in shallow soil layers and increased RLD in medium and deep layers has been proposed as an efficient growth strategy in environments where deep water could be available to crops later in the growing season (Wasson et al., 2012; Lynch, 2013). In addition to root distribution, root architecture that includes greater hierarchical structure may promote hydraulic lift and allow for greater utilization of water available deep in the soil profile (Doussan et al., 2006). In cases where deep water availability could promote crop productivity directly or via hydraulic redistribution, larger diameter xylem vessels may be advantageous to increase axial hydraulic conductivity of roots growing in deeper soil layers (Wasson et al., 2012). Transpiration supplied by hydraulic lift or redistribution

may be large enough to support plants through extreme drought episodes even if the total amount of water redistributed is small.

Where drought is episodic, plant response to rewetting of soil is equally important for maintenance of yield under drought as water extraction and hydraulic functioning in drying soil (Sperry et al., 2002). In many woody plants, hydraulic failure occurs in roots rather than shoots because xylem in roots is more prone to cavitation than in shoots (Pockman and Sperry, 2000 and references within). Structural impediments to water uptake in root systems that develop under stress may require regrowth of roots with plant recovery contingent on this regrowth (Lo Gullo et al., 1998). Recovery through new root growth may be species specific, as demonstrated by examples of evergreen tree species unable to repair extensive loss of root hydraulic conductance to resume water uptake (Hacke et al., 2000), whereas drought-adapted genotypes of wheat respond rapidly to rewetting by producing “rain roots,” similar to desert succulents (North and Nobel, 1991; Sadras and Rodriguez, 2007). Where drought is episodic but perhaps less severe, nocturnal refilling of embolized xylem via root pressure appears to play an critical role for resumption of hydraulic conductance in herbaceous crops, potentially providing an important additional area for breeders to improve drought tolerance (Sperry et al., 2003; Stiller et al., 2003, 2005; Sperry, 2011).

Root allocation and distribution may depend on plant growth strategies and their general response to water deficits and distribution of available soil water. Maize has high water use efficiency (WUE) but is sensitive to water shortages (Figure 3; Ghannoum, 2009). Maize, which has more conservative hydraulic conductance compared to sunflower, decreases transpiration more quickly than sunflower, which maintains carbon assimilation during drought, even during the course of wilting (Comas, personal observation). Both maize and sunflower decrease shoot size, and increase $A_R:A_L$ and relative root distribution to deeper depths in response to water deficits, although sunflower, emblematic of a drought avoider, has a generally deeper root system than maize and redistributes an even greater percentage of its roots to deeper soil depths (Figure 2). Root growth in both maize and sunflower continues longer into the season than shoot vegetative growth and the onset of reproduction, with the capacity for even greater overlap of root growth with reproduction under water deficit (Figure 4). As breeding for plant productivity under drought advances, it may be advantageous to consider whole plant strategies and root traits and patterns of spatio-temporal growth with a systems approach. Working with two crops with contrasting hydraulic responses, we might expect different traits to improve productivity under drought in these crops, which highlights the need to take specifics of the genotype, as well as environment and management, into account.

GENETICS OF ROOT TRAITS UNDER DROUGHT CHALLENGES IN UNDERSTANDING AND UTILIZING GENETIC ANALYSES OF ROOT TRAITS

Most root traits are controlled by multiple genes, each governing small effects and often with a degree of epistasis or interaction effects that can change with environmental conditions (de Dorlodot et al., 2007; Cooper et al., 2009). The quantitative

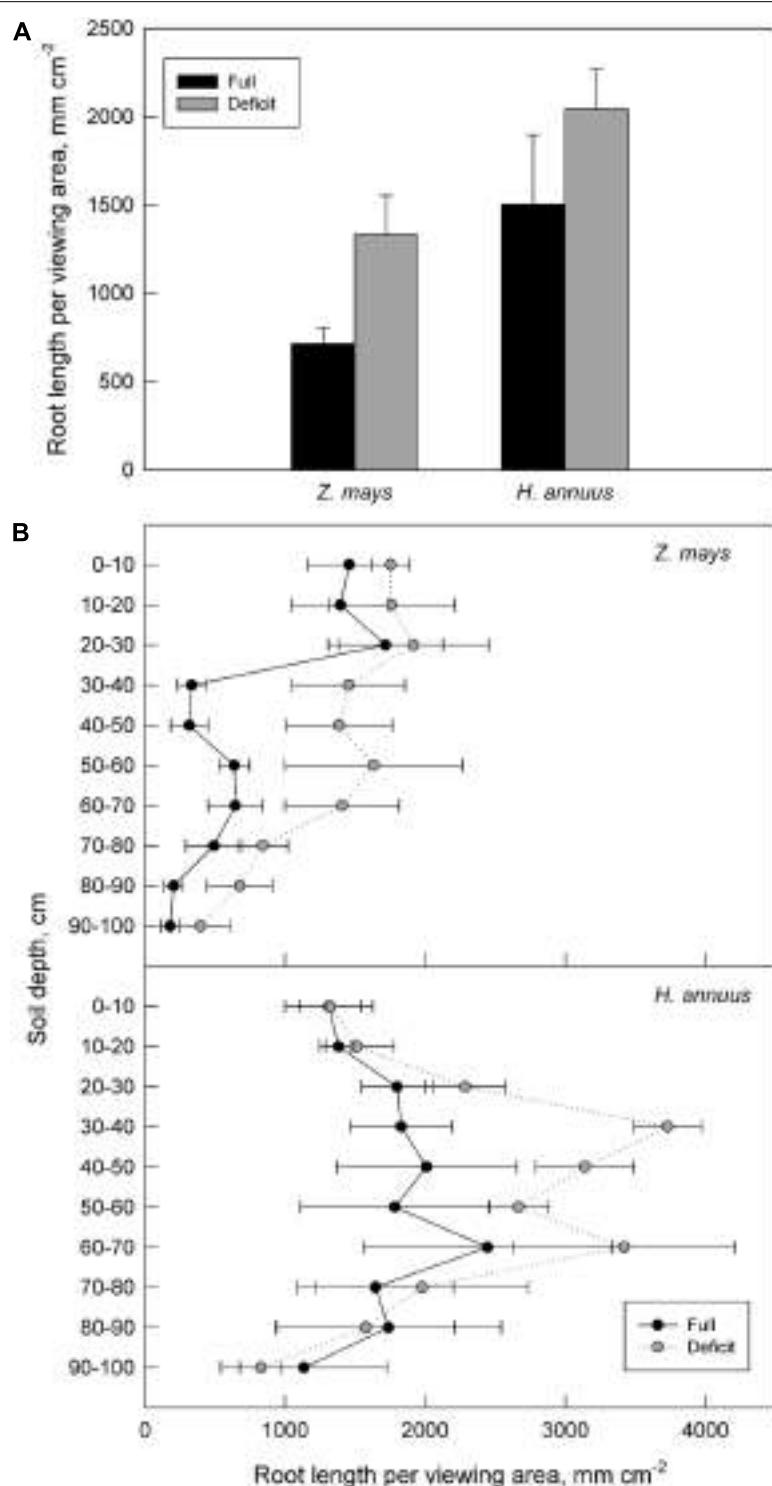


FIGURE 2 |The production of root length and its distribution for fully and deficit irrigated maize and sunflower over the 2012 growing season in minirhizotron windows at the USDA-ARS Limited Irrigation Research Farm in Greeley, CO, USA (40.45° , -104.64° , 1430 m). Root growth is expressed in terms of root length per viewing area of minirhizotron window for two crops contrasting in hydraulic strategies grown under full and deficit irrigation. Total annual root growth in viewing windows down to 100 cm **(A)** as well as in 10 cm increments of soil depth

(B) are given. Each bar and point represents root growth averaged among four minirhizotron tubes per treatment, with each tube installed in a different treatment plot. Soil at the site is a sandy loam. Annual precipitation is approximately 350 mm. Irrigation is applied with pressure-compensated surface drip. Deficit irrigation is applied to achieve a targeted 40% of full evapotranspiration (ET) irrigated treatment during deficit periods in late vegetative and maturation growth phases (V7-V21 and R3-R6 in maize; V8-R2 and R6-R9 in sunflower).

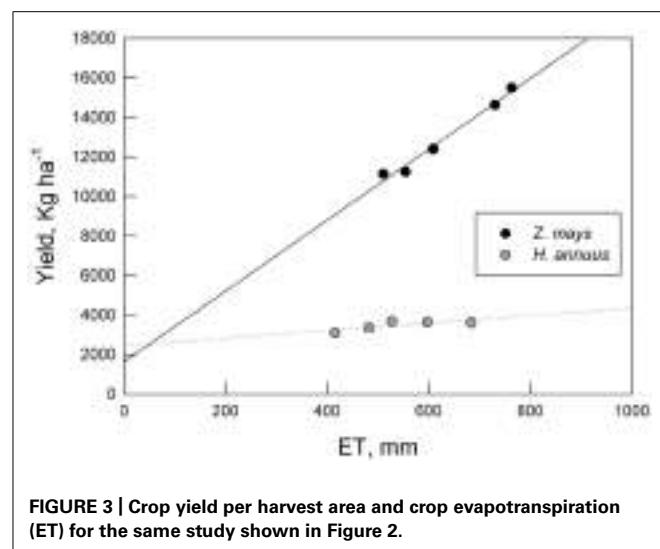


FIGURE 3 | Crop yield per harvest area and crop evapotranspiration (ET) for the same study shown in Figure 2.

trait loci (QTL) that contribute to root traits can be considered either constitutive or adaptive, the classification of which can be useful in selecting traits most beneficial in the target environment (Collins et al., 2008).

Both adaptive and constitutive root traits can be difficult to phenotype. Therefore, it is not surprising that a majority of genetic research has focused on above-ground traits while the “hidden half” of the plant is much less represented in recent research (Herder et al., 2010). A search for rice (*Oryza sativa* L.) QTL associated with drought in the database TropGene (Hamelin et al., 2013) revealed 139 QTL in only five studies for root traits under drought stress, while non-root traits consisted of 387 QTL in 15 studies. A common approach to phenotyping for genetic research is the use of controlled growing environments such as greenhouse pots or tubes, growth chambers, hydroponic systems, and agar gel. However, caution must be used when applying these procedures to root morphology studies, as frequent inconsistencies of QTL and gene locations are often caused by a lack in quality and quantity of phenotypic information (Collins et al., 2008; Xu and Crouch, 2008; Hargreaves et al., 2009; Wojciechowski et al., 2009). In a maize study for gene expression under drought, Barker et al. (2005) reported that 27% of gene expression was up- or down-regulated when stressed for 5 days in buckets as compared to only 5% differential regulation when plants were stressed over 5 weeks in the field. The same study also reported that genes regulated in buckets tended to differ from those regulated in field conditions. These differences may be related to the involvement of different mechanisms in short-, medium-, and long-term response

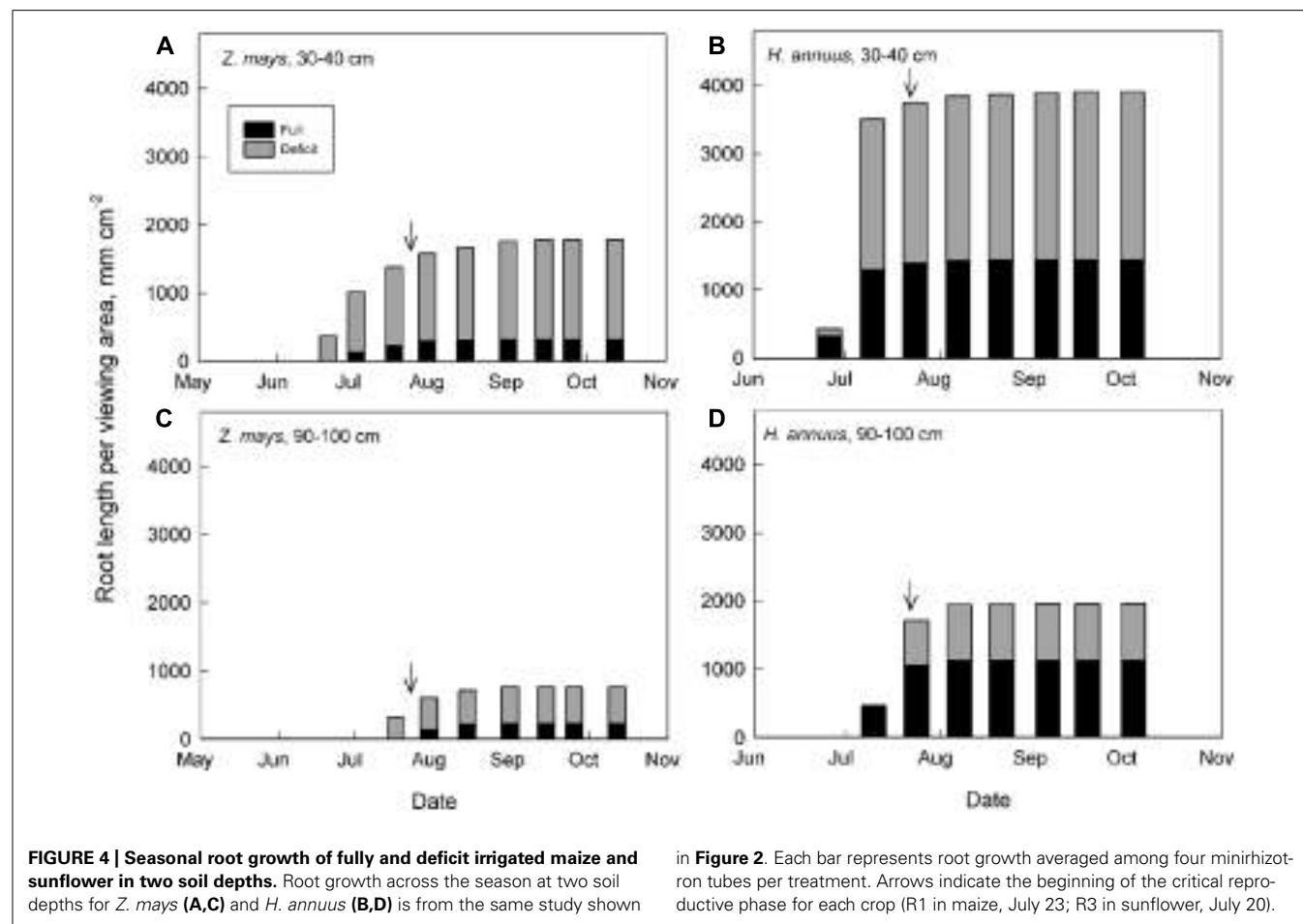


FIGURE 4 | Seasonal root growth of fully and deficit irrigated maize and sunflower in two soil depths. Root growth across the season at two soil depths for *Z. mays* (A,C) and *H. annuum* (B,D) is from the same study shown

in Figure 2. Each bar represents root growth averaged among four minirhizotron tubes per treatment. Arrows indicate the beginning of the critical reproductive phase for each crop (R1 in maize, July 23; R3 in sunflower, July 20).

to drought (Maseda and Fernandez, 2006). To the extent that differences among studies are related to environmental differences, the compilation of these studies could lead to the identification of constitutive gene and trait expressions that are crucial across multiple environments for improving drought tolerance in the field.

Traits such as rooting depth and RLD in wheat and chickpea (*Cicer arietinum*), respectively, have shown high heritability across different environments and have also been related to improvements in grain yield under certain conditions (Kashiwagi et al., 2005; Sayar et al., 2007). Phenotypic and genotypic variation for highly heritable traits such as these in controlled environments is more likely to be similar to variation under field conditions. However, cases where phenotypes at mature developmental stages were highly responsive to soil and climatic conditions, and showed different results from those in controlled conditions emphasize the need for thorough field validation (Watt et al., 2013).

GENES AND QTL ASSOCIATED WITH ROOT TRAITS UNDER DROUGHT

A number of studies have reported QTL linked to traits associated with increasing the foraging capacity of root systems. These include in rice: increased root length (Price et al., 2002; MacMillan et al., 2006; Courtois et al., 2009), root biomass (Courtois et al., 2003), and root number (Zheng et al., 2000, 2003; Courtois et al., 2009); in wheat: increased total root biomass, length and number of roots (Sharma et al., 2011), seminal root angle and number (Christopher et al., 2013; but see Giuliani et al., 2005b for contrasting strategy in maize), and deep root growth and seminal root number (Hamada et al., 2012); and in maize: increased root number, branching, dry mass, and decreased diameter and root angle (Giuliani et al., 2005b), and lateral and axial root length, and axial root elongation rate (Ruta et al., 2010). Increased root biomass, RLD and rooting depth are often considered to be primary drivers of drought avoidance (Kashiwagi et al., 2005). It is also possible that these traits are associated with stable QTL that are expressed in multiple environments. In a meta-QTL analysis, Courtois et al. (2009) identified 119 root QTL in rice from 24 studies. Many of these QTL, primarily for maximum root length, were associated with “hot spots” on chromosomes 1 and 9, which contained QTL detected in multiple populations and environments.

In addition to QTL, some specific genes or mechanisms have been associated with variation for root traits in major cereal crops. Reduced height and semi-dwarfing genes are common in many modern wheat (Evans, 1998) and barley (*Hordeum vulgare*) varieties (Chloupek et al., 2006). Semi-dwarfing genes of barley have been shown to contribute to greater root system size (measured by electrical capacitance) than non-semi-dwarf alleles at the same loci (Chloupek et al., 2006). However, Wojciechowski et al. (2009) found inconsistent effects of these genes for root length and root architecture traits in different types of growing media.

Genotypic variation or plasticity in deep rooting capacity in rice has been associated with productivity under drought stress (Kato et al., 2006; MacMillan et al., 2006; Steele et al., 2006). Increased water uptake associated with greater deep root length and SRL was linked to a large-effect QTL in rice that also contributes to improvements in yield under severe drought stress (Bernier et al.,

2009). More recently Uga et al. (2013) have identified and cloned the *DRO1* gene in rice on chromosome 9 which is associated with rooting depth due to an increased gravitropic response in root tips. After backcross introgression of this gene into the IR64 variety of rice an increase in drought tolerance was seen in drought environments with no apparent reduction in grain yield under well-watered conditions.

In maize, a major constitutive QTL, designated *Root-ABA1*, was associated with crown root branching, diameter, and angle, as well as whole root dry mass (Giuliani et al., 2005b). Being a constitutive QTL, it was detected consistently across different water regimes in both greenhouse and field settings. In the model plant *Arabidopsis thaliana*, researchers have identified QTL for ABA induced reduction in lateral root growth as well as root system plasticity and size (Fitz Gerald et al., 2006; Xiong et al., 2006). Finally, increases in water uptake have also been associated with the up-regulation of aquaporin genes *PIP1* and *RWC-3* in maize, which shows that root physiology, in additional to or concurrent with shifts in root system size, can be associated with increased capacity of root systems to acquire water (Giuliani et al., 2005a).

MARKER ASSISTED SELECTION AND INTROGRESSION IN CEREAL BREEDING PROGRAMS

Root QTL show great potential for marker assisted selection (MAS) when root traits chosen contribute significantly to drought tolerance in the target environment. The selected root morphology or function for use in MAS can vary greatly depending on the targeted environment and the ultimate goal of the researcher (Blum, 2011). Many of the reported markers and QTL for root traits have proven to be confounded by inadequate root phenotyping, inconsistent contribution across populations and environments, or the minor contributions made by the QTL to the variation in the trait of interest (Collins et al., 2008; Blum, 2011). QTL that have been identified in greenhouse or lab conditions must be validated under field conditions and should ultimately relate to improvements in productivity before use in a MAS program. For these reasons, there have been very few reports of the use of MAS for quantitative traits such as root characters in plant breeding programs. One successful example of a cultivar developed through MAS for root traits is the rice line “Birsa Vikas Dhan 111,” which was selected for a larger root system (Steele et al., 2006). The backcrossing selection scheme used in breeding the rice line targeted five donor-parent chromosomal regions, four relating to root traits and one to end-use quality. In addition, multiple markers were selected for maintenance of the recurrent parent background. Work conducted by Mace et al. (2012) on nodal root angle QTL in sorghum (*Sorghum bicolor*) is an example of relating root QTL to grain yield. These authors tested a subset of the QTL mapping population in yield trials where they identified an association between grain yield and three out of the four lab-identified nodal root angle QTL.

Utilization of molecular markers that improve productivity under drought has been, and will continue to be, a daunting challenge in crop improvement. Because root variation is difficult to phenotype in a breeding population of hundreds of genotypes, MAS offers breeders the option to select for favorable combinations of traits both above and below ground. However, in order

for MAS to be successfully adopted by plant breeding programs, either molecular markers must be identified that are in strong linkage disequilibrium with the QTL for desired root traits or the gene itself must be identified. The major obstacle for the use of MAS then becomes accurate phenotyping that can lead to greater accuracy of QTL locations in high density molecular maps (Francia et al., 2005).

RESOURCES FOR GENETIC DIVERSITY

A reduction in diversity of crop species due to domestication or subsequent selection has been described as a genetic bottleneck that may have contributed to a loss in useful alleles (Tanksley and McCouch, 1997). Root traits are no exception as the importance of developing improved root systems has often been overlooked (Herder et al., 2010). In recent years, improvements in genotyping procedures and knowledge of root architecture have made significant advances due to research in model species such as *Arabidopsis* (Benfey et al., 2010), rice (Hochholdinger and Tuberrosa, 2009), and purple false brome (*Brachypodium distachyon*; Draper et al., 2001). The use of model systems offers several advantages. First, comparative mapping of QTL identified to the locations of those QTL in related species is a starting point for candidate gene identification and potential future use in MAS programs (Edwards and Batley, 2010). Second, the use of cloned genes from model systems may be used in altering trait expression in the species of interest through transgenic breeding approaches (Keller et al., 2007; Blum, 2011).

With a better understanding of root traits and their genetics, improvements in root systems can be made by utilizing the diversity currently found within modern cultivated germplasm (Blum, 2011). For example, a comparable amount of unexploited genetic variation contributing to stress tolerance can be found in modern cultivars as in landraces (primitive varieties) of wheat (Trethowan and Mujeeb-Kazi, 2008). Moreover, alleles contributing to more extensive root growth and distribution may be present in cultivated varieties of rice rather than in wild species if observations from container-grown plants hold (Liu et al., 2004). Introgression of alleles from modern varieties reduces the negative effects of linkage drag from the use of wild species and landraces (Hübner et al., 2013). Nonetheless, landrace varieties for certain species may also show potential for introgression of genetic diversity into modern varieties. Not all landrace varieties or wild accessions should be expected to show abiotic stress tolerance, but successful use of this approach can be seen in crops such as barley (Ceccarelli and Grando, 1991), wheat (Trethowan and Mujeeb-Kazi, 2008), and pearl millet (Yadav, 2008).

PATTERNS OF ROOT TRAITS AND RESPONSES OBSERVED FROM SCREENING STUDIES – CASE STUDIES IN LESQUERELLA AND RICE

We will summarize advances made in two contrasting crops, lesquerella and rice. In the first case, screening studies are just beginning on the emerging oilseed crop, lesquerella, for which improvement is an initiative of the U.S. Department of Agriculture. In the second case, screening studies are quite advanced on rice, a dietary staple for many people. Root trait screening in wheat,

which is also advanced, is not reviewed here because it is well covered in recent reviews (Richards, 2006; Wasson et al., 2012).

LESQUERELLA

Lesquerella [*Physaria fendleri* (A. Gray) O’Kane & Al-Shehbaz] is a C₃ dicot and member of the Brassicaceae family. Herbaceous lesquerella plants have yellow flowers and are commonly found on calcareous soil in hot arid environments in the U.S. Southwest (Rollins and Shaw, 1973; Al-Shehbaz and O’Kane, 2002). Since the early 1980s, lesquerella has been domesticated and bred as a new oilseed crop in the U.S. because its unique seed oil contain hydroxy fatty acids that have practical applications in industrial manufacturing and added utility as an additive to biofuels (Hinman, 1984; Thompson and Dierig, 1994; Isbell and Cermak, 2002). The target environments for growing lesquerella are Arizona, New Mexico, and Texas where it can be grown as a winter annual crop. Water management involves keeping the field moist until seedling emergence and ensuring that the plants receive about 635–762 mm of water during the growing season for optimal yields, similar to winter wheat (Wang et al., 2010).

Lesquerella has a well-developed tap root system (Rollins, 1981) which has not been well characterized to date. Past screening studies were not designed to focus solely on roots but were conducted simultaneously with observations on the crop for other agronomic traits, seed yield and total biomass in particular.

Although large root biomass allocation has been associated with drought tolerance in many plant species, this characteristic allocation pattern is also associated with a perennial growth form (Chapin et al., 1993). The perennial *Physaria* species *P. mendozina* and *P. pinetorum* were found to generally accumulate greater root biomass than annual forms (González-Paleo and Ravetta, 2011). However, seed yield (biomass) of *P. mendozina* was similar to that of annual *P. fendleri* when both species were grown under water limited conditions (Ploschuk et al., 2001, 2005).

Planting density and stature influence lesquerella’s taproot length, which was reported to grow deeper with increased planting density (110 mm at 250,000 plants ha⁻¹ and 180 mm at 750,000 plants ha⁻¹; Brahim et al., 1998). Brahim et al. (1998) suggested that deeper rooting in response to increased planting density enabled deeper water and nutrient acquisition to ameliorate increased interplant competition for soil resources.

Various environmental factors affect *Physaria* root traits. In the perennial species *P. ludoviciana*, total root length and branching was greater when plants were grown in growth chambers under medium light intensity (584 $\mu\text{mol m}^{-2} \text{s}^{-1}$) than low light intensity (174 $\mu\text{mol m}^{-2} \text{s}^{-1}$), which matches its seasonal cycle (Grant, 2009). In *P. fendleri*, genotypes respond differently to growth temperatures, with a number of accessions producing larger root systems under higher temperatures (Cruz et al., 2012). Although individual environmental factors were found to affect root traits, interactions among environmental factors affecting root systems have not been fully studied in *Physaria*. In maize, for example, plant performance under water-limited plus high temperature conditions was different than that under water-limited conditions alone (Cairns et al., 2013).

Characterization of lesquerella germplasm accessions in the U.S. National Plant Germplasm System is underway to determine

non-adaptive (constitutive) root traits correlated with increased productivity under drought conditions in improved cultivars of other crops. The methodologies being utilized involve analyzing roots from seedlings in growth pouches, as well as samples grown in the greenhouse and in experimental fields in Maricopa, AZ, USA. Preliminary results of phenotypic evaluation indicate that the relative root size of young plants is maintained through crop maturity (Cruz et al., unpublished). More focused analysis of *lesquerella* root responses to varying environmental and cultural management conditions will determine if *lesquerella* has unique responses to abiotic stress compared to major commodity crops, potentially associated with the origin of *lesquerella* from hot and arid environments.

RICE

Rice, a monocot and a member of the Poaceae (or Gramineae) family, grows in a wide range of environments and cropping systems have been adapted for deep-water, rain-fed lowland, upland, and irrigated conditions (De Datta, 1981). The genetic and genomic resources on rice are tremendous with the species studied as a model organism for monocot crops, similar to *Arabidopsis* for dicots as mentioned earlier (Rensink and Buell, 2004; Coudert et al., 2010). The drought environments of rice are classified based on the duration of the wet season, as well as the severity of water stress at different growth stages (e.g., early in the season during planting, at the tillering to flowering stages, which is typically intermittent, and during the late season from flowering to grain filling; Fukai and Cooper, 1995).

Studies have been conducted on the influence of rice roots on crop productivity. Research is already in advanced stages compared to *lesquerella* and most other crops (Henry, 2012). Rice has a well-described fibrous root system characteristic of monocots and exhibits seminal, nodal, and lateral roots which have been subjected to substantial morphometric, anatomical, and genetic studies (Yoshida and Hasegawa, 1981; Morita and Nemoto, 1995; Rebouillat et al., 2009). Regardless of the ecosystem where rice breeding is aimed, researchers look toward understanding the role of roots for improving nutrient and water acquisition and increasing grain yield.

Tropical japonica types have been known to have fewer tillers and deeper root systems than other rice ecotypes (i.e., indica, aus, rayada; Lafitte et al., 2006). There are significant differences reported in root thickness, depth, and root mass among rice cultivars and there is documented genetic variation for root morphological traits in response to drought (Kondo et al., 2003; Gowda et al., 2011). However, this variation and how it influences the crop's root function for water uptake under drought remains to be fully understood (Gowda et al., 2011). Breeding activities toward a rice plant ideotype and direct selection for yield under drought are underway, supported by physiological studies on rice root function (e.g., root hydraulic conductance, anatomy, and aquaporin expression; Henry, 2012). So far broad examinations of traits show that traits do not appear inherently different between upland and lowland types. Indica types (mostly lowland) have thinner, shallow roots while aus types (often grown upland) exhibit intermediate diameter with length similar to japonicas (which include upland Asian and temperate cultivars; Henry et al., 2012).

Environmental factors and water management practices strongly affect rice root systems. Intermittent irrigation was found to positively affect RLD and total root mass (Shi et al., 2002; Mishra, 2012; Cruz et al., unpublished). Additionally, root size is highly correlated to available growing space, root impedance, and type of existing competitor plants (Fang et al., 2013). Upland rice develops a longer root system compared to lowland counterparts due to environmental factors in these ecosystems (Yong et al., 2007; Fageria, 2013). Well-drained soils in upland areas do not restrict water movement and allow better oxygen diffusion to favor rice root elongation (Yoshida and Hasegawa, 1981; Fageria et al., 2003). Anaerobic flooded field conditions of lowland ecosystems on the other hand can impair root elongation as well as the formation of root hairs (Kawata and Ishihara, 1959; Kawata et al., 1964).

The structure and development of rice root systems largely determines crop functioning under drought (Morita and Nemoto, 1995). Rice improvement programs have determined that deep rooting is a target trait (Gowda et al., 2011). Among upland varieties, cultivars with thicker coarse roots that create an overall deeper root system are generally viewed as desirable under drought conditions along with varieties that have greater RLD in deeper soil layers (Passioura, 1982; Kondo et al., 1999; Steele et al., 2006). Studies of lowland varieties are likewise ongoing to screen for thicker coarse roots to penetrate hardpan soil layers (Gregorio and Cabuslay, 2004; Allah et al., 2010a; Gowda et al., 2011). Greater fine root (lateral) growth has also been found to increase water uptake and rice yield under drought but the mechanism is being further investigated (Henry, 2012).

Various screening methods used to identify root traits associated with drought tolerance in rice germplasm. Root dry mass and length, commonly assessed by direct evaluation, is a good predictor of yield in rice (Beyrouty, 2002; Fageria and Moreira, 2011). Root pulling resistance is also a trait that is highly correlated with root length, thickness, branching number, and dry mass in rice (Price et al., 1989). Root pulling resistance is recommended as an indirect screen to select genotypes that achieve drought tolerance via producing a large root system (Ekanayake et al., 1985; Lafitte et al., 2006). Additionally, researchers used the number of root xylem vessels to gage drought resistance of rice lines (Allah et al., 2010b). However, there is substantial variation in the distribution of xylem vessels across rice roots with lowland rice generally reported to have fewer root xylem vessels than upland rice at the middle and tip sections of the root (Bashar, 1990).

Rice root traits are currently characterized using greenhouse container methods or field sampling techniques, both high-throughput but labor intensive (Gregorio and Cabuslay, 2004; Shashidhar et al., 2012; Cruz and Dierig, unpublished). Root imaging technologies are allowing a closer look at the dynamic nature of rice root system architecture and these present opportunities to fast track understanding the genetic control of root traits, specifically lateral branch formation. Non-invasive imaging techniques provide important insight on spatial distribution of rice roots and might allow the identification of genetic control over rice root system architecture. However, most imaging studies require plants to be grown in artificial media. Further testing is needed to

determine if rice root systems traits observed in artificial media are found under actual field conditions (Clark et al., 2011; Feng et al., 2012). Several mutant lines of rice are being used in studies of the molecular control of lateral root branching (Smith and De Smet, 2012). Molecular studies are also examining genes and signaling pathways that control morphological response to drought (Fukao and Xiong, 2013). Ultimately, further advances in phenotyping methodologies and field validation are needed to link traits identified in these studies to drought resistant in rice varieties (Virmani and Ilyas-Ahmed, 2007).

Several rice germplasm collections in genetic resources centers have been screened for root traits associated with drought tolerance and promising accessions have been identified as useful in breeding programs (Chang and Loresto, 1986; Henry et al., 2011). Examples of germplasm selected for drought related studies include those with (1) high levels of drought tolerance with deep and thicker root systems (e.g., OS4, Salumpikit, Azucena), (2) moderate drought tolerance and early maturity (e.g., Dular, Black Gora, Bala), and (3) improved drought tolerance and ability to produce new tillers after soil water replenishment (e.g., IR43, IET1444, UPLR-5; Virmani and Ilyas-Ahmed, 2007). In addition to cultivated forms, root systems of wild rice germplasm have been characterized for contributions to drought resistance with *O. longistaminata* and *O. rufipogon* identified as potential sources of novel alleles for drought tolerance (Liu et al., 2004). Superior performance under water stressed conditions in the greenhouse was correlated with the production of greater root system length and greater root to shoot ratios when exposed to drought conditions (Feng et al., 2012).

A small set of rice root QTL have been identified and were found to result in increased root penetration, thickness, nodal root apex stiffness and length when introgressed into rice lines (Steele et al., 2006; Clark et al., 2008). These QTL contribute positively in different test environments and the different combinations of the QTL all exhibited advantages in water uptake making them important in crop improvement activities in rice (MacMillan et al., 2006; de Dorlodot et al., 2007). Field testing of upland rice in India with four introgressed QTL were found to produce plants with longer root lengths and a yield advantage of 1 t ha^{-1} compared to controls (Steele et al., 2006). Additionally, transgenic rice plants with increased root diameter, developed by overexpressing *OsNAC5*, were found to increase yield by 9–26% (Jeong et al., 2013).

These practical applications from decades of root research in rice and in other model systems will enable further understanding of important traits that might influence crop yield and productivity under abiotic stress and ensure gains toward global food security.

CONCLUSION

There is maturing promise for breeding plants with root traits to enhance productivity under water deficit. Although much is known about root traits and functioning, there is a need for better understanding of traits in the context of plant strategies for growth under water deficits. A better understanding of tradeoffs in root traits is also needed to guide breeding efforts. Although breeding different crops for specific forms of drought needs to be carefully considered with particulars of

different systems in mind, certain generalities for root traits may hold. Smaller diameter roots, greater SRL, and increased root hair density or length should improve plant acquisition of water under water scarcity and reduce plant carbon investment required for that acquisition. Additionally, crop hydraulic functioning under water scarcity may be improved through increased capacity for nocturnal refilling of embolized xylem and changes in inter-vessel pit anatomy to reduce cavitation, which may not carry negative repercussions under well-watered conditions. The ability of plants to access water from deep depths in the soil profile has been documented and found to benefit crop productivity under water scarcity. Deep water acquisition, however, does not necessarily fully ameliorate crop water requirements during hot dry conditions, even when deep soil water is available (Sun et al., 2011), suggesting that more information is needed on root–shoot interactions governing hydraulic conductance, especially under high temperatures and vapor pressure deficits (e.g., Yang et al., 2012b). Basic information on seasonal growth patterns, essential to understand effective plant capacity for and control over root hydraulic conductance with plant development over the season, especially for woody plants, is frequently missing or incorrectly assumed and is needed to guide breeding efforts (Comas et al., 2005; Eissenstat et al., 2006). While water uptake capacity declines with root aging and exposure to dry soil (Lo Gullo et al., 1998), it is unclear if new root production is required to maintain root hydraulic conductivity or if enhanced aquaporin activity can ameliorate uptake capacity. Abundant progress has been made in understanding root traits and functioning in plant water acquisition with several root QTL identified. There continue to be promising prospects for increasing communication between plant ecophysicists, geneticists, and breeders to learn more about root traits that have the potential to improve plant productivity under drought and put this understanding into practice to improve the performance of crops under water shortages.

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AUTHOR CONTRIBUTIONS

LHC wrote sections pertaining to root traits, growth, and distribution. SRB wrote sections on genetic analyses of root traits with assistance from PFB. VMVC wrote sections on *lesquerella* and rice trait screening with assistance from DAD.

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Rhizosphere wettability decreases with root age: a problem or a strategy to increase water uptake of young roots?

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As plant roots take up water and the soil dries, water depletion is expected to occur in the vicinity of roots, the so called *rhizosphere*. However, recent experiments showed that the rhizosphere of lupines was wetter than the bulk soil during the drying period. Surprisingly, the rhizosphere remained temporarily dry after irrigation. Such water dynamics in the rhizosphere can be explained by the drying/wetting dynamics of mucilage exuded by roots. The capacity of mucilage to hold large volumes of water at negative water potential may favor root water uptake. However, mucilage hydrophobicity after drying may temporarily limit the local water uptake after irrigation. The effects of such rhizosphere dynamics are not yet understood. In particular, it is not known how the rhizosphere dynamics vary along roots and as a function of soil water content. My hypothesis was that the rewetting rate of the rhizosphere is primarily function of root age. Neutron radiography was used to monitor how the rhizosphere water dynamics vary along the root systems of lupines during drying/wetting cycles of different duration. The radiographs showed a fast and almost immediate rewetting of the rhizosphere of the distal root segments, in contrast to a slow rewetting of the rhizosphere of the proximal segments. The rewetting rate of the rhizosphere was not function of the water content before irrigation, but it was function of time. It is concluded that rhizosphere hydrophobicity is not uniform along roots, but it covers only the older and proximal root segments, while the young root segments are hydraulically well-connected to the soil. I included these rhizosphere dynamics in a microscopic model of root water uptake. In the model, the relation between water content and water potential in the rhizosphere is not unique and it varies over time, and the rewetting rate of the rhizosphere decreases with time. The rhizosphere variability seems an optimal adaptation strategy to increase the water uptake of young root segments, which possibly reached new available water, and partly disconnect the old root segments from the already depleted soil.

Keywords: drying/wetting cycles, mucilage, neutron radiography, rhizosphere, root water uptake

INTRODUCTION

Root water uptake depends on the relative importance of root and soil properties. When the soil is wet, rate and location of root water uptake are controlled by root traits. When the soil becomes dry, the soil hydraulic properties affect and, ultimately, limit the water availability to plant roots (Passioura, 1980; Draye et al., 2010).

In addition to the soil and plant hydraulic conductivity, the resistance of the root-soil interface has been supposed to affect root water uptake. Huck et al. (1970) and Carminati et al. (2009) showed that as roots take up water and the soil dries, roots shrink, and air-filled gaps form at the root-soil interface. Nobel and Cui (1992) estimated that in the intermediate dry range gaps are the limiting factor for root water uptake. In a classic paper, Passioura (1980) measured the total hydraulic conductance of soil and roots of an intact plant at controlled transpiration rates. After raising and decreasing the transpiration rate, he measured a decrease in total conductance of the system after the peak in transpiration. He interpreted this result as an increased resistance at the root-soil interface.

Increased resistance of the root-soil interface could be induced by root exudates. Hallett et al. (2003) measured a decrease in water sorptivity in the rhizosphere of barley, which is an indication of increased water repellency of the rhizosphere. Read et al. (2003) showed that lipids present in mucilage of maize, lupin and wheat decreased the surface tension of the soil solution, with a consequent reduction in the water holding capacity of the rhizosphere. Lipids may be responsible of the hydrophobicity of the rhizosphere of lupins measured by Moradi et al. (2012).

It is well-accepted that mucilage favors root penetration by lowering the soil mechanical stress (Iijima et al., 2004). Instead, the direct effects of mucilage on root water uptake are still matter of debate. Carminati et al. (2010) observed higher water content in the rhizosphere than in the bulk soil during a drying period. The increase of water content in the rhizosphere was around $0.05 \text{ [cm}^3 \text{ cm}^{-3}\text{]}$. Higher water content in the rhizosphere than in the bulk soil was observed also by Young (1995) for wheat. Young (1995) and Carminati et al. (2010) explained the higher water content in the rhizosphere with mucilage exudation.

Indeed, McCully and Boyer (1997) showed that mucilage can hold large volume of water. However, they observed that mucilage lost most of the retained water at relatively high water potential. They concluded that mucilage should not play a major role in water storage in soils.

A successive experiment of Carminati et al. (2010) showed that after a cycle of drying and rewetting, the rhizosphere remained temporarily dry. Carminati (2012) hypothesized that the hysteretic and time-dependent behavior of the rhizosphere is explained by drying and wetting of mucilage exuded by roots. After drying, mucilage becomes hydrophobic and it rewets slowly. To include the specific behavior of mucilage, the Richards equation (the classic equation of water flow in soil) was modified by including a non-equilibrium term. It has not yet been proven that such a dynamic behavior of the rhizosphere is actually caused by mucilage.

Higher water content in the rhizosphere during drying and water repellency after rewetting have variable effects on root water uptake. A wet rhizosphere is expected to maintain the rhizosphere at a relatively high hydraulic conductivity also when the bulk soil dries. In fact, when the soil dries, large gradients in water potential are expected to occur in the rhizosphere. A rhizosphere with a high water holding capacity would attenuate this drop in water potential, facilitating root water uptake in dry soils (Carminati et al., 2011). On the other hand, the slow rewetting of the rhizosphere may limit root water uptake after drying and subsequent rewetting.

This picture of water dynamics in the rhizosphere lacks important information: the variation of the rhizosphere properties along roots. Carminati and Vetterlein (2013) suggested that as roots grow, the rhizosphere at a given location becomes old and its hydraulic properties change. The authors suggested that young roots are covered with fresh and hydrated mucilage that helps the uptake of scarce resources. Old roots are instead more isolated from the bulk soil because of gaps and/or water repellent and partly decomposed mucilage and are mainly responsible of long-distance transport. This concept is illustrated in Figure 1, in which the drying/wetting dynamics of the rhizosphere of a growing roots are simplified.

Objective of the present manuscript is to test the following hypotheses:

1. Does the rhizosphere of young roots rewet more quickly than the rhizosphere of old root? In other words: does the rewetting rate of the rhizosphere decrease with root age?
2. Is the rewetting rate of the rhizosphere function of the soil water content? In this case, is there a threshold water content below which the rhizosphere becomes hydrophobic?

To answer these questions we used neutron radiography to monitor the dynamics of water content in the rhizosphere of lupines during several drying/wetting cycles of variable length. The rhizosphere of young (distal) segments and that of older (proximal) segments were compared.

Finally, I implemented these results in the model of Carminati (2012). The model is modified to include temporal changes in the

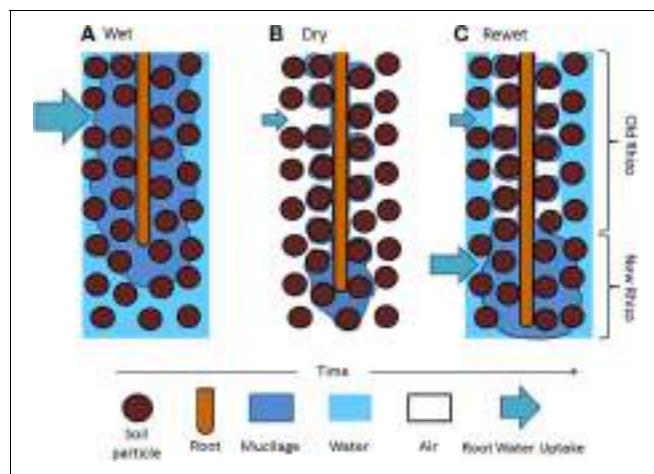


FIGURE 1 | Hypothetical distribution of mucilage and water in the rhizosphere during a drying and wetting cycle. (A) Mucilage is exuded at the root tips and diffuses through the soil matrix. **(B)** As roots take up water and the soil becomes dry, mucilage dehydrates and shrinks around the root and the soil particle near the root (the rhizosphere). As the soil dries and mucilage becomes old, mucilage becomes water repellent and stiff. **(C)** After irrigation, old mucilage rewets slowly and the rhizosphere remains temporarily dry, possibly limiting the local water uptake. Freshly exuded mucilage covering the root tip is expected to rewet quickly. Water uptake might increase at the root apical segments.

rewetting rate of the rhizosphere. This model is ready to be implemented in architectural models of root water uptake (Roose and Fowler, 2004; Doussan et al., 2006; Javaux et al., 2008; Schneider et al., 2010).

MATERIALS AND METHODS

SAMPLE PREPARATION

Six lupins (*Lupinus albus*) were grown in quasi-2D aluminum boxes filled with sandy soil. The aluminum boxes were 30 cm high, 15 cm large, and 1 cm thick. Sandy soil was collected from the catchment of Chicken Creek located near Cottbus, Germany. The soil (sieved to a particle sizes smaller than 2 mm) consisted of 92% sand, 5% silt, and 3% clay. The boxes were placed horizontally with one of the large side open and the sand was slowly and continuously poured into the aluminum box through a 2 mm sieve to achieve a uniform sand packing and minimize layering. The large side was then closed, the samples were turned vertically, and they were gently shaken to achieve a stable sand packing. The samples had holes at the bottom that allowed irrigation from the bottom. The samples were irrigated by slowly immersing the samples into a water reservoir until the water table reached 5 cm above the bottom of the sample. The capillary rise was enough to wet the sample fill the soil surface.

Lupins were germinated on filter paper soaked with CaSO_4 . After 24 h they were planted into the soil at ~ 0.5 cm depth. During the first 7 days, the samples were daily irrigated from the bottom with tap water. During the second week, the samples were irrigated every second day with a nutrient solution composed of (in mM): K_2SO_4 , 0.35; KCl , 0.1; KH_2PO_4 , 0.1; $\text{Ca}(\text{NO}_3)_2$, 0.1; and MgSO_4 , 0.5; and (in μM): H_3BO_3 , 10; MnSO_4 , 0.5; ZnSO_4 , 0.5;

CuSO_4 , 0.2; $(\text{NH}_4)\text{Mo}_7\text{O}_{24}$, 0.01; Fe-EDTA, 20. Plants have been grown for 20 days in a climate chamber with a daily light cycle of 16 h light: 8 h darkness, light intensity of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperature of $24^\circ\text{C}/19^\circ\text{C}$, and relative humidity of 60%.

NEUTRON RADIOPHGRAPHY

Neutron radiography is an excellent method to image root and water distribution in soil samples thinner than 1–2 cm (Moradi et al., 2009). Neutron radiography consists in guiding a parallel neutron beam through a sample and detecting the intensity of the beam transmitted behind the sample. The transmitted beam is detected by a CCD camera and the information is converted into a digital image. The detected image carries the information on the thickness and composition of the sample.

Because of the high sensitivity of neutrons to hydrous materials, water is efficiently detected in neutron radiography. The relation between water content and neutron attenuation is given by:

$$-\log \left(\frac{I(x, z, t) - dc}{I_{\text{dry}}(x, z) - dc} \right) = L_w(x, z, t) \Sigma_w \quad (1)$$

where x, z are the space coordinates of the field of view, t is time, $I(x, z, t)$ is the transmitted beam intensity, $I_{\text{dry}}(x, z)$ is the transmitted beam intensity when the sample is dry (only container and dry soil), dc is the dark current (signal when there is no beam), $\Sigma_w [\text{cm}^{-1}]$ is the neutron attenuation coefficient of water, and L_w is the thickness of water in the beam direction. $I_{\text{dry}}(x, z)$ was measured before the samples were irrigated and lupines planted.

In pixels where there are no roots, the volumetric soil water content, $\theta [-]$, is given by $\theta = L_w/L_{\text{tot}}$, where L_{tot} is inner thickness of the sample in the beam direction. In pixels including roots, θ is the average of the water content in the root and in the soil in front and behind it (Carminati et al., 2010). The water content θ estimated from the radiographs was compared to that directly measured with a balance (the weight of the dry sample was known). The two values matched well and confirmed the image analysis already validated in Carminati et al. (2010).

Roots were segmented (technical word that describes the classification of pixels belonging to roots) using the algorithm *Roottracker2D* developed by Anders Kaestner and described in Menon et al. (2007). After root segmentation, the soil water content was calculated as a function of distance from the roots. The water content of the rhizosphere was calculated as the average water content in the first 1.5 mm near the roots. The image processing was identical to that described in more details in Carminati et al. (2010). To compare roots of different age, rhizosphere and bulk water contents were averaged in regions of size 5 cm by 5 cm, which included at least four roots of similar age. In specific, we calculated rhizosphere and bulk water contents in the upper 3–8 cm and in the lower 23–28 cm. The upper region contained roots that were approximately 5–10 days old at the beginning of the experiment. The roots in the lower regions were 1–4 days old.

Neutron radiography was performed at the NEUTRA facility at the Paul Scherrer Institute (PSI), Villigen, Switzerland. The field of view was $18.3 \times 18.3 \text{ cm}$, with a pixel size of 0.0179 cm .

Two radiographs of the upper and lower part of the samples were needed to image the whole sample. Exposure time was 30 s.

DRYING AND WETTING CYCLES

On day 20 after seed germination, the measurements with neutron radiography started. Two samples (L1 and L2) were irrigated every second day from the bottom. The other four samples were irrigated after 3 days (L3), 4 days (L4), 5 days (L6), and 7 days (L6). The average water content in the 6 samples, as measured from weighing the samples, is plotted in Figure 2. Time zero corresponds to the beginning of the neutron radiography experiment.

The samples were radiographed during day and night at intervals of 6 h. During irrigation, the samples were scanned before and 30 min after irrigation. The samples were weighed at the beginning and at the end of the photoperiod to measure the water consumption.

The neutron radiography experiment lasted 8 days.

MODEL

Carminati (2012) proposed a new model that describes the changes in water content in the rhizosphere during a drying/wetting cycle. Because of the hydrophobicity of the rhizosphere after drying and its consequent slow rewetting, the water content in the rhizosphere, $\theta_{rh} [\text{cm}^3 \text{ cm}^{-3}]$, does not increase as quickly as the soil matric potential $h_{rh} [\text{cm}]$, here expressed in meter heads. In other words, although the matric potential increases, the rhizosphere remains dry for a long period. To describe this process, the assumption of a unique relation between θ_{rh} and h_{rh} has to be abandoned. Carminati (2012) suggested to describe the rewetting of the rhizosphere as:

$$\frac{\partial \theta_{rh}}{\partial t} = C_{rh}(\theta_{rh}) \frac{\partial h_{rh}}{\partial t} + \Gamma_{rh}(\theta_{rh}) (h_{rh} - h_{rh}^{eq}) \quad (2)$$

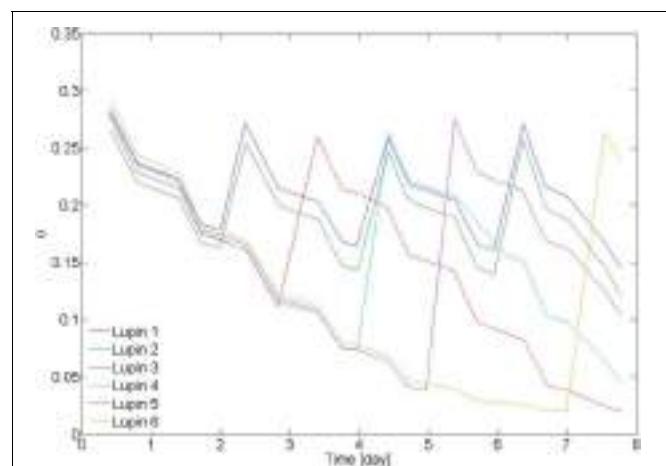


FIGURE 2 | Average water content in the samples. Lupin 1 and 2 were irrigated from the bottom every 2nd day. Lupin 3–6 were irrigated once after a drying period of increasing duration. Water content was measured by weighing the samples. The decrease of water content became slower as the soil became dry, showing a decrease of transpiration. The samples were irrigated by capillary rise from the bottom, setting a water table at 5 cm above the bottom of the samples.

where $C_{rh}(\theta_{rh}) [\text{cm}^{-1}]$ is the specific soil capacity, t is time [s], $\Gamma_{rh}(\theta_{rh}) [\text{m}^{-1} \text{s}^{-1}]$ is a function that describes the rewetting rate of the rhizosphere, and $h_{rh}^{\text{eq}} [\text{cm}]$ is the matric potential that the rhizosphere would have if it was in equilibrium—i.e., $h_{rh}^{\text{eq}} = h_{rh}(\theta_{rh})$.

In Carminati (2012), $\Gamma_{rh} = \Gamma_{rh}(\theta_{rh}) [\text{m}^{-1} \text{s}^{-1}]$ was not function of time. Here I implement the model by considering that Γ_{rh} is also function of the root age. I define t_i [s] as the time when the root reached a given point in the space. The value of $t - t_i$ [s] gives the age of the root at time t . In this way, I implicitly assume that mucilage is exuded only at the root tip. We parameterize $\Gamma_{rh} = \Gamma_{rh}(\theta_{rh}, t, t_i)$ as:

$$\Gamma_{rh} = \Gamma^{\text{sat}}(t) \Theta_{rh}^{\beta} \quad (3)$$

where $\Theta_{rh} = \frac{\theta_{rh} - \theta_{rh}^{\text{res}}}{\theta_{rh}^{\text{sat}} - \theta_{rh}^{\text{res}}} [\text{cm}^3 \text{cm}^{-3}]$ is the rhizosphere water saturation, $\theta_{rh}^{\text{sat}} [\text{cm}^3 \text{cm}^{-3}]$ and $\theta_{rh}^{\text{res}} [\text{cm}^3 \text{cm}^{-3}]$ are the residual and saturated water content of the rhizosphere, and $\Gamma^{\text{sat}}(t) [\text{m}^{-1} \text{s}^{-1}]$ is the rewetting rate of mucilage at saturation which varies with root age according to:

$$\Gamma^{\text{sat}} = (\Gamma_{rh}^M e^{-\gamma(t-t_i)} + \Gamma_{rh}^m) \quad (4)$$

where $\Gamma_{rh}^M [\text{m}^{-1} \text{s}^{-1}]$ is the maximum rewetting rate of new mucilage and $\Gamma_{rh}^m [\text{m}^{-1} \text{s}^{-1}]$ is the minimum rewetting rate of old mucilage. $\beta [-]$ and $\gamma [-]$ are two fitting parameters.

Equation (2) is combined with the Richards equation, the classical equation describing the water flow in soils:

$$\frac{\partial \theta}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left[rk(h) \frac{\partial h}{\partial r} \right] \quad (5)$$

where r is the radial coordinate and $k(h)$ is the soil unsaturated hydraulic conductivity [cm s^{-1}]. Equation (4) is solved with an analytical approach under the *steady-rate approximation*, i.e., $\frac{\partial \theta}{\partial t} = \text{const}$ (Carminati, 2012). The solution is calculated in the two domains, bulk soil ($r_1 < r < r_2$) and rhizosphere ($r_0 < r < r_1$):

$$\theta(r, t) = \begin{cases} \theta_{rh}(r, t) & \text{for } r_0 < r < r_1 \\ \theta_b(r, t) & \text{for } r_1 < r < r_2 \end{cases} \quad (6)$$

$$h(r, t) = \begin{cases} h_{rh}(r, t) & \text{for } r_0 < r < r_1 \\ h_b(r, t) & \text{for } r_1 < r < r_2 \end{cases} \quad (7)$$

The radii of root, rhizosphere, and bulk soil were set equal to those used in Carminati et al. (2011): $r_0 = 0.05 \text{ cm}$, $r_1 = 0.25 \text{ cm}$, and $r_2 = 1 \text{ cm}$. The boundary conditions were no flux at r_2 and constant flux at r_0 , $q(r_0) = -0.5 \text{ cm day}^{-1}$. Initial condition was $h(r_2) = -20 \text{ cm}$. The water retention curves of rhizosphere and bulk were parameterized according to Brooks and Corey (1964):

$$\Theta = (h/h_0)^{-\lambda} \quad (8)$$

$$k = k^{\text{sat}} (h/h_0)^{-\tau} \quad (9)$$

where $k^{\text{sat}} [\text{cm s}^{-1}]$ is the saturated hydraulic conductivity, h_0 is the air-entry value [cm], and $\lambda [-]$ and $\tau [-]$ are fitting parameters.

The parameters for $\theta(h)$ and $k(\theta)$ of bulk soil and rhizosphere were taken from Carminati et al. (2011). The parameters were set to satisfy the following conditions: (1) at equilibrium, the rhizosphere is wetter than the bulk soil at any soil matric potentials. (2) The saturated hydraulic conductivity of the rhizosphere is 100 times smaller than that of the bulk soil. (3) At unsaturated conditions, the rhizosphere conductivity is higher than that of the bulk soil. The parameters for the rhizosphere rewetting [Equations (3, 4)] were estimated by matching the observed water content in the rhizosphere during the drying/wetting cycles.

RESULTS

The average water contents θ of the samples L1–6 during the drying/wetting cycles are plotted in Figure 2. The samples L1 and L2 were irrigated every second day and their average θ was between 0.15 and 0.3. L3 was irrigated at $\theta = 0.12$, L4 at $\theta = 0.07$, L5 at $\theta = 0.04$, and L6 at $\theta = 0.02$. After being rewetted, all samples reached the same water content of 0.26 ± 0.01 . There was no visible effect of the drying/wetting cycles on the water repellency of the bulk soil. Transpiration rate started to decrease at $\sim \theta = 0.05$.

The water content distribution in L4 is shown in Figure 3. Figure 3 shows the images obtained after calibration of the neutron radiographs according to Equation (1) and after division by the neutron attenuation of water \sum_w and the sample thickness L_{tot} . The images show the water content $\theta(x, z)$ and resulted from the superimposition of the neutron radiographs of the upper and lower halves of the sample. Figure 3 shows $\theta(x, z)$ during the drying period (day 1), just before irrigation (day 3 at 23:30), and 30 min after irrigation. On day 1 the rhizosphere of some of the upper roots, in particular in the vicinity of the cluster roots, appeared wetter than the bulk soil. On day 3 at 23:30, θ at the top of the sample is ~ 0.06 and it increased to 0.09 in the lower 5 cm

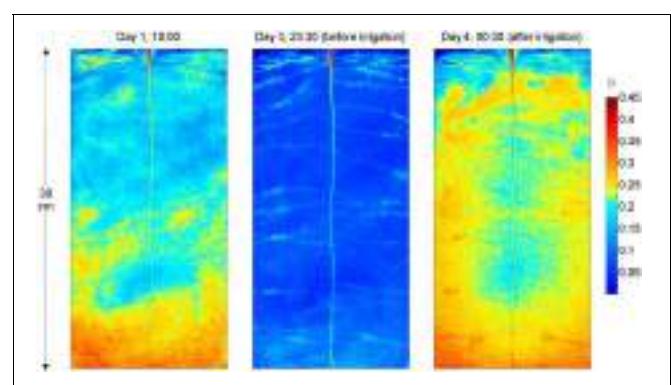


FIGURE 3 | Neutron radiography of L4, during the drying period and after irrigation. The colormap is proportional to the water content. Note the higher water content near the upper roots at day 1. After irrigation (day 4), the rhizosphere of the upper roots, of the tap root, and that of the proximal parts of the lower roots remained markedly drier. On the other hand, the rhizosphere of the root tips in the lower parts of the sample rewetted and a region with high water content is visible around the root tips.

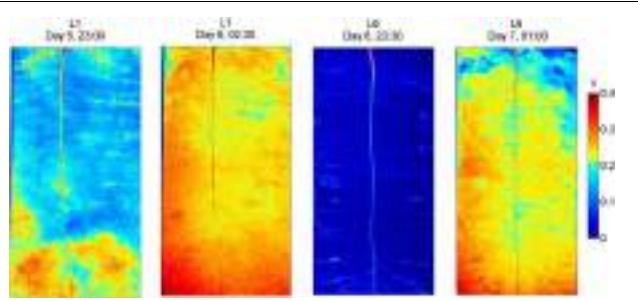


FIGURE 4 | Neutron radiography of L1 and L6 before and after irrigation. L1 was irrigated every second day. Before irrigation its average water content was 0.17. After irrigation, the rhizosphere of the upper and middle part was not fully rewetted. Low and young roots were rewetted. L6 experienced a longer and more pronounced drying and was irrigated when its water content was 0.02 and the plant showed wilting symptoms. After irrigation the rhizosphere of most roots was not rewetted, except at the root tips, which appeared being covered with a blob of wet material.

of the sample. As the soil dried, roots became very well-visible. L4 had a long tap root that grew till bottom of the sample and laterals growing horizontally. The upper laterals showed several root clusters. All samples had a similar root architecture. The radiograph after irrigation shows that the rhizosphere of the upper lateral roots, of the tap root, and that of the proximal parts of the lower lateral roots remained markedly drier. Oppositely, the rhizosphere of the root tips in the lower parts of the sample quickly rewetted and a region with high water content appeared around the root tips.

The radiographs of L1 and L6 before and after irrigation are shown in **Figure 4**. As in L4, also in L1 some wet regions are visible around some of the lateral roots. After the 3rd wetting (day 6), the rhizosphere of the upper lateral roots of L1 remained drier than the bulk soil, indicating that a certain degree of water repellency occurred. The sample L6 was irrigated when the water content was uniformly low along the soil profile. After irrigation, the rhizosphere of the upper lateral roots, of the tap root, and of the proximal segments of the lower laterals remained dry, while the rhizosphere of the young segments of the lower laterals quickly rewetted. As in L4, a wet region appeared around the root tips of the lower laterals. These wet regions that I interpret as the wetting of freshly exuded mucilage, were larger in L6 than in L1.

To quantify the differences between the water content in the rhizosphere and in the bulk soil, I processed the images as in Carminati et al. (2010). Roots were segmented using the algorithm Roottracker2D (Menon et al., 2007). Then I calculated the water content as a function of distance to the root surface. This water content is still an average along the sample thickness—the radiographs are 2D, while the water distribution around roots is 3D. To calculate the actual average water content, I assumed that water content distribution around roots had a radial geometry and we fixed a rhizosphere extension of 1.5 mm. For more details see Carminati et al. (2010).

In Carminati et al. (2010), the rhizosphere water content was averaged along the entire root system. Here, the rhizosphere

water contents were averaged at different locations of the root system. In particular, we focused on two regions, the upper laterals and the lower laterals. The regions used for the calculation were $\sim 5 \times 5$ cm and included around 5 roots. **Figure 5** shows the average water content in bulk soil θ_b and rhizosphere θ_{rh} calculated for different samples and at different soil depth. **Figure 5A**, shows θ_b and θ_{rh} in the upper 5 cm of L4. During drying, θ_b decreased more rapidly than θ_{rh} . Before irrigation, $\theta_{rh} > \theta_b$. After irrigation, θ_{rh} increased much more slowly than the bulk soil. The average values of θ_b and θ_{rh} in the upper 5 cm of L1 are shown in **Figure 5B**. **Figure 5B** shows that the increase of θ_{rh} after rewetting became slower with the increasing number of cycles. The average values of θ_b and θ_{rh} in the upper and lower 5 cm of L6 are shown in **Figures 5C,D**, respectively. θ_{rh} in the upper region did not increase after rewetting. On the other hand, θ_{rh} increased very quickly in the lower region. The root segments in the lower region used for calculating θ_{rh} were ~ 5 days old at the time of rewetting, as estimated from the radiographs at the beginning of the experiment. Based on my previous experiments, I expect that the root segments in the upper region were approximately 2–3 weeks old. In fact, in lupines laterals in the upper region emerge approximately 1–2 weeks after planting.

DISCUSSION

Calculations of the water content in the rhizosphere are likely to be affected by root segmentation, by the contribution of root hairs and fine roots not resolved with neutron radiography, and by the image processing to go from the 2D pictures to the actual water contents in the rhizosphere. Therefore, some errors in the absolute values of the rhizosphere water content cannot be excluded. Instead, the relative difference between θ_b and θ_{rh} and its variation during the drying/wetting cycles are less prone to artifacts. In particular, the observed dryness of the rhizosphere compared to the bulk soil after rewetting and its slow rehydration are not affected by artifacts in the image analysis.

The experiments showed that:

1. Rhizosphere dynamics were not uniform along the root system. The rhizosphere rewetted slowly for the roots of the upper soil region and for the proximal segments of the roots of the lower soil region. On the other hand, the rhizosphere of the young segments of the roots in the lower soil region quickly rewetted. As the water content at the bottom was nearly as dry as at the top (**Figures 5A,B**), I conclude that the rewetting of the rhizosphere is related to the root age. In other words, young rhizosphere rewets quickly and old rhizosphere rewets slowly.
2. The slow rewetting of the rhizosphere occurred also at moist conditions ($\theta > 0.15$, L1–2) and not only below a critical water content. Water repellency in the rhizosphere seems not to be function of the initial water content.

The slow rewetting of the rhizosphere after severe drying is caused by the high water repellency of the rhizosphere (Moradi et al., 2012). This water repellency is likely to be caused by mucilage exuded by roots. In fact, mucilage collected from lupine seeds and

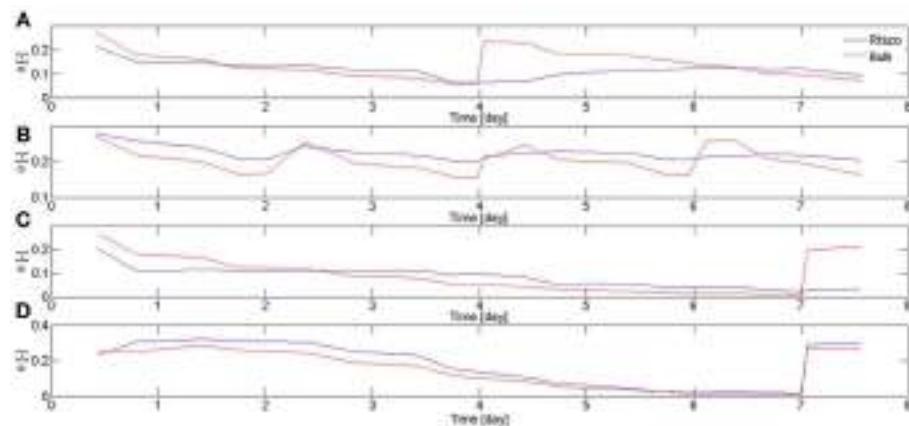


FIGURE 5 | Water content in bulk soil θ_b and rhizosphere θ_{rh} during drying and rewetting calculated for different samples and at different soil depth. Time is calculated from the start of 1st drying cycle when the plants were 2 weeks old. **(A)** Average θ_b and θ_{rh} calculated in the top 5 cm of L4. During the drying period (day 2–4), the rhizosphere was wetter than the bulk soil. The situation reversed after irrigation, with the rhizosphere remaining temporarily dry. The rhizosphere slowly rewetted during the next 2 days. **(B)** θ_b and θ_{rh} at the top 5 cm of L1. The

rhizosphere partly rewetted after the first irrigation. After the following irrigations the rhizosphere rewetted more slowly. **(C)** θ_b and θ_{rh} at the top 5 cm of L6. After severe drying and subsequent irrigation, the rhizosphere did not rewet as quickly as the bulk soil. **(D)** θ_b and θ_{rh} at the bottom 5 cm of L6, where only roots younger than 1 week were present (**Figure 4**). Although the bulk soil became very dry, the rhizosphere rewetted quickly and there was no sign of hydrophobicity. θ_{rh} was calculated for the most apical 4 cm of the roots.

let dry on a thin glass turned hydrophobic (unpublished data). Such hydrophobicity can be caused by lipids present in mucilage (Read et al., 2003).

The radiographs of the samples after irrigation show a wet region around the root tips (**Figures 3, 4**). I interpret these wet regions as highly hydrated mucilage. This observation supports the results of McCully and Boyer (1997) that show that at high water potentials mucilage can hold large volumes of water and should appear as a blob around the root tips. The radiographs show also that freshly exuded mucilage rehydrates fast. On the other hand, mucilage that covers older root segments and that is likely to be as old as them, rehydrates more slowly. Rehydration time of mucilage seems therefore to increase with time. This increase can be the consequence of several factors. After that mucilage is exuded in soils, it reaches an equilibrium with the water potential in the soil. According to the relation between water content and water potential of mucilage measured by McCully and Boyer (1997), equilibration with a dry soil would cause a large dehydration of mucilage. During consequent shrinkage, mucilage is likely to become stiffer and therefore slower in rehydration. Additionally, as xylem vessels develop, root segments covered by mucilage become more and more active in root water uptake (Watt et al., 1994), which would cause additional suction and mucilage dehydration. Furthermore, interactions between mucilage and solutes present in the soil solution, ad example Ca^{2+} , stabilize mucilage and make it stiffer (Carminati and Vetterlein, 2013). It cannot be excluded that microorganisms contribute to the mucilage stiffening as well.

Interaction between microorganisms and mucilage deserve a short discussion. Decomposition rates of mucilage-C by microorganisms may vary between 3 days (Nguyen et al., 2008) and 11 days (Mary et al., 1993). However, gel-like substances are not only decomposed but are also produced by microorganisms.

Bacterial exopolysaccharides (EPS) has physical properties similar to those of mucilage (Chenu, 1993; Or et al., 2007). The mixture of plant derived mucilage and bacterial EPS is often called mucigel. It is commonly accepted that the rhizosphere properties are the result of plant-microorganisms interactions.

The fact that immediately after irrigation the rhizosphere does not rewet, suggests that the outer layer of mucilage in contact with the air-phase has a high water repellency, independently from the mucilage hydration state.

Another interesting observation is that the blob around the root tips after irrigation that I explain as mucilage, is larger in the samples that were exposed to drier conditions. If my hypothesis is right, this shows that mucilage exudation increases with drought stress. Mucilage can be a strategy of plants to increase the rhizosphere hydraulic conductivity when the soil dries, as proposed by Carminati et al. (2011).

These observations about the rewetting rate of mucilage are summarized in the model. Equation (3) means that the rewetting rate of the rhizosphere is a function of root age and water content. The dependence on the water content describes the fact that as mucilage dries it becomes stiffer and more viscous. I used these equations, coupled with the Richards equation in radial coordinates, to calculate the radial flow of water to a single root. Objective of the modeling was not to fit all the experimental data, but rather to find the parameters that qualitatively fit well with the overall results of the experiment. The function describing the dependence of Γ_{rh} on time, Equation (4), is plotted in **Figure 6A**. The parameter β describing the relation with the water content is set to $\beta = 1.8$. I simulated two scenarios, one in which the soil was rewetted every 4th day (as in L4), and one in which the soil was irrigated every 2nd day (as in L1–2). The calculated values of θ_b and θ_{rh} are plotted in **Figures 6B,C**. In the simulations the time corresponds to the root age. The parameters were chosen to

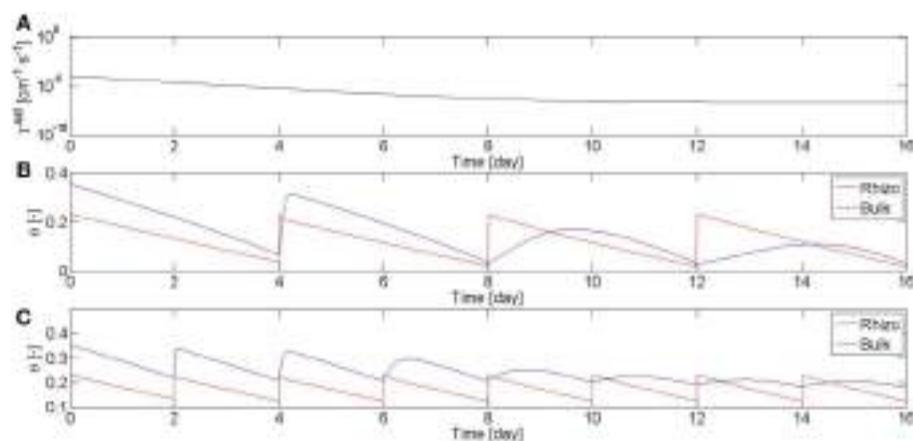


FIGURE 6 | Water content in rhizosphere (blue) and bulk soil (red) during drying and wetting cycles simulated using the model in Equation (2–5).

(A) Coefficient of wetting rate of water saturated mucilage, Γ^{sat} , as a function of mucilage age according to Equation (4). (B) Simulation of the water content

in rhizosphere and bulk soil during drying periods of 4 days and subsequent rewetting to a matric potential of $h = -20$ cm. During the first days, mucilage is fresh and rhizosphere is quickly rewetted. As mucilage ages, rhizosphere rewetting becomes slower. (C) Simulation with drying periods of 2 days.

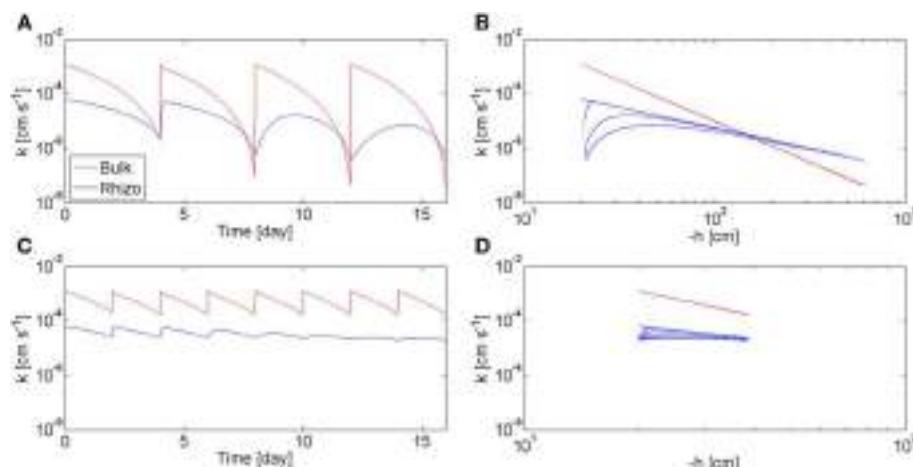


FIGURE 7 | Hydraulic conductivity of bulk soil k_b and rhizosphere k_{rh} during varying drying/wetting as predicted by the model. (A) k_b and k_{rh} as a function of time during the 4 days drying cycles of Figure 6B. When the soil was wet, $k_b > k_{\text{rh}}$. As the soil became dry, the two curves crossed each other and $k_b < k_{\text{rh}}$. As the number of drying/wetting cycles increased, the

recovery of k_{rh} after wetting became slower and slower. (B) k_b and k_{rh} as a function of the soil matric potential h during the 4 days drying cycles. (C) k_b and k_{rh} during to the 2 days drying cycles of Figure 6C. The soil was kept wet ($\theta_b > 0.1$) and $k_b > k_{\text{rh}}$ at all times. (D) k_b and k_{rh} as a function h during the 2 days drying cycles.

match the experimental observations that the rewetting rates are fast for roots younger than 4–6 days, and are slow for roots older than 14 days. The model is capable of reproducing the general behavior of the observations.

The model calculates also the values of the hydraulic conductivity of the bulk soil, K_b , and of the rhizosphere, K_{rh} , during the drying/wetting cycles. In Figure 7, K_b and K_{rh} , calculated at the rhizosphere-bulk soil interface ($r = r_1$), are plotted as a function of time (a, c) and as a function of the matric potential (b, d). The calculations are plotted for the long drying cycles (a, b) and for the short cycles (c, d). At the beginning of the long cycles, $K_{\text{rh}} < K_b$. However, as the soil dried, K_b decreased more rapidly than K_{rh} and there was a period before irrigation when $K_{\text{rh}} > K_b$. During this

period the rhizosphere favors root water uptake. Oppositely to K_b , K_{rh} did not respond immediately to irrigation and its increase became slower with time. In the case of short cycles, the bulk soil remained always relatively wet ($\theta_b > 0.1$), and $K_{\text{rh}} < K_b$ during all time. The relation between hydraulic conductivities and soil matric potential is plotted in Figures 7B,D. In the bulk soil, the relation between K_b and h is a unique relation, i.e., $K_b = K_b(h)$ and at each h corresponds only one value of K_b . This is a classic situation in soil physics when no dynamics and no hysteresis are assumed. Instead, there is no unique relation between k_{rh} and h . The straight blue line in Figure 7B shows the values of K_{rh} at equilibrium. The figure shows that at equilibrium the hydraulic conductivity curves of rhizosphere and bulk soil cross at

$h = -150$ cm. During the 2nd, 3th, and 4th cycle, K_{rh} deviated from the equilibrium values. During the short-cycle scenario, deviation from equilibrium occurred after some cycles.

Figure 7 shows that rhizosphere is an advantage for root water uptake when the soil dries to matric potentials smaller than -150 cm. Below this matric potential, the rhizosphere is more conductive than the bulk soil and it is expected to favor root water uptake by limiting the drop in water potential toward the root (Carminati et al., 2011). Instead, when the soil remains relatively wet, as in the short-cycle scenario, the rhizosphere is less conductive than the bulk soil. Under this condition, the rhizosphere has no apparent advantages for root water uptake; actually, it may even be a limit to root water uptake. However, if we take a typical root hydraulic conductivity of $10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ (Dray et al., 2010) and we convert it into a rhizosphere hydraulic conductivity (assuming a rhizosphere of 1 mm thickness) we would obtain a conductivity of $10^{-10} \text{ cm s}^{-1}$. The rhizosphere would be a limit of root water uptake only after severe drying, when the hydraulic conductivity decreased to $10^{-10} \text{ cm s}^{-1}$. If rewetted after such a severe drying, the rhizosphere would remain dry for some period of time and it would be a limit to root water uptake.

These considerations indicate that, when the soil is relatively wet, plant roots have no reason to modify the rhizosphere properties in order to take up water more easily. Instead, when the soil becomes dry, mucilage exudation maintains the rhizosphere wet, facilitating root water uptake. However, mucilage exudation has a drawback, as it slows down the rhizosphere rewetting after sever cycles of drying. Mucilage exudation would then help the uptake of young, distal root segments covered with fresh mucilage, but over time it would limit the uptake of old, proximal root segments.

Of course, it has to be kept in mind that mucilage exudation has a carbon cost. It is likely that it is a short-term response to water shortage, while on the long-term, if water shortage persists, exudation will decrease. Beside the carbon costs, the pro and contra of mucilage exudation depend on the capacity of roots to uptake water from distal roots and transport it to the shoot. Possibly, the rhizosphere hydrophobicity of the old root segments would help to avoid water loss from roots to soil and it would be beneficial for water transport to the shoot. Hydraulic lift would be reduced by such rhizosphere dynamics.

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The effects of mucilage dynamics on the overall soil-plant water relations are expected to be plant specific. The effects depend on several factors. I focus on two of them: (1) the amount of mucilage exuded and its chemical composition; and (2) on the root architecture. Mucilage exudation is function of plant species. Read and Gregory (1997) reported that maize seedlings produce more mucilage than lupines, and that mucilage produced by lupines rehydrates more slowly after drying. The slow rehydration of lupine mucilage is possible the reason of the slow rewetting of the rhizosphere shown in my study. Considering the tap-rooted architecture of lupines and the high axial conductivity of the tap root, we expect that water uptake in lupines after a drying and wetting cycle shifts to lower soil layers, where lateral roots are relatively younger and can compensate the reduced uptake from the upper soil layers (where roots have a temporarily hydrophobic rhizosphere). However, the situation may be different in plants with fibrous root systems, like maize and wheat, in which the capacity of taking up water from distal root segments may be limited by a low xylem conductivity. In this case, the slow rewetting of the rhizosphere of the old root segments, might be a limit for plant recovery after drying and subsequent irrigation.

However, to quantitatively describe the effects of such rhizosphere dynamics on the overall plant-soil water relations, the single-root model here introduced should be implemented into three-dimensional root water uptake models that explicitly account for root architecture (Roose and Fowler, 2004; Doussan et al., 2006; Javaux et al., 2008; Schneider et al., 2010).

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Integration of root phenes for soil resource acquisition

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Suboptimal availability of water and nutrients is a primary limitation to plant growth in terrestrial ecosystems. The acquisition of soil resources by plant roots is therefore an important component of plant fitness and agricultural productivity. Plant root systems comprise a set of phenes, or traits, that interact. Phenes are the units of the plant phenotype, and phene states represent the variation in form and function a particular phene may take. Root phenes can be classified as affecting resource acquisition or utilization, influencing acquisition through exploration or exploitation, and in being metabolically influential or neutral. These classifications determine how one phene will interact with another phene, whether through foraging mechanisms or metabolic economics. Phenes that influence one another through foraging mechanisms are likely to operate within a phene module, a group of interacting phenes, that may be co-selected. Examples of root phene interactions discussed are: (1) root hair length × root hair density, (2) lateral branching × root cortical aerenchyma (RCA), (3) adventitious root number × adventitious root respiration and basal root growth angle (BRGA), (4) nodal root number × RCA, and (5) BRGA × root hair length and density. Progress in the study of phenes and phene interactions will be facilitated by employing simulation modeling and near-isophenic lines that allow the study of specific phenes and phene combinations within a common phenotypic background. Developing a robust understanding of the phenome at the organismal level will require new lines of inquiry into how phenotypic integration influences plant function in diverse environments. A better understanding of how root phenes interact to affect soil resource acquisition will be an important tool in the breeding of crops with superior stress tolerance and reduced dependence on intensive use of inputs.

Keywords: root architecture, phenomics, functional traits, ideotype, soil resources

INTRODUCTION

Global food security is a serious challenge (Funk and Brown, 2009), with approximately 870 million people experiencing chronic undernourishment (FAO et al., 2012). In much of the developing world, use of nitrogen (N) and phosphorus (P) fertilizers is relatively low, leading to substantial reductions in crop yields (FAO, 2008). In developed nations intensive use of fertilizers is associated with greater crop yields (Roberts, 2009). However, crop plants in these agricultural systems take up only a portion of the applied nitrogen fertilizer (Goulding, 2000), and the remainder pollutes water and the atmosphere (Jenkinson, 2001). Furthermore, phosphorus fertilizers are a non-renewable resource, and global production of phosphorus is expected to peak around the year 2033 (Cordell et al., 2009). Increasing crop acquisition of both nitrogen and phosphorus is therefore a desirable goal for both subsistence and commercial agriculture. Belowground properties of natural ecosystems are also receiving attention because of their influence on important processes including carbon sequestration (Eissenstat et al., 2000) and community structure (Craine et al., 2002).

Root architecture, the spatial arrangement of a root system, has been shown to be important in agricultural systems (Lynch, 1995; Ho and Lynch, 2004; Hirel et al., 2007) and natural systems (Mahall and Callaway, 1992; Comas and Eissenstat, 2009)

for nutrient acquisition, plant interactions, and nutrient cycling. Understanding the contribution of specific root traits, or phenes, to root system function is critical for crop improvement because it allows identification of traits that contribute desired functions (Kell, 2011; Lynch and Brown, 2012). High-throughput root phenotyping is an important tool in this context as it permits the profiling of the extent, magnitude, and distribution of root traits in crop germplasm, and because phenotyping is limiting progress in crop breeding (Furbank and Tester, 2011). Advances in high-throughput phenotyping of roots (Grift et al., 2011; Trachsel et al., 2011; Zhu et al., 2011) will enable focused efforts to improve crop nutrient acquisition by selection for root ideotypes and to understand the influence of inter-and-intraspecific root system variation on community structure and ecosystem function.

Ideotype, or trait-based, breeding was proposed by Donald (1968) as a way to combine traits that would each contribute to increased yield. He identified a flaw in “deficit elimination” or “selection for yield” approaches in that they do not seek to answer *how* increased yield is created (Donald, 1962). Instead, he proposed studying traits in isolation to understand how they contribute to yield then combining such yield improving traits through traditional breeding. Crop breeding programs commonly combine traits, especially in the pyramiding of traits associated with disease resistance (Shen et al., 2001; Singh et al.,

2001; Steele et al., 2006). This approach has contributed substantially to yield gains in several crops, including maize, wheat, and common bean (Mock and Pearce, 1975; Kelly and Adams, 1987; Reynolds et al., 1994; McClean et al., 2011). The trait-based approach inherent in the concept of ideotype breeding forced researchers to not only consider traits of interest in isolation, but also to consider relationships among traits. This is illustrated by the work of Rasmusson (1987), demonstrating that compensation among plant organs can lead to tradeoffs, such as increasing head numbers being associated with fewer, smaller kernels in barley. The integration of traits determines how the whole plant functions and remains an underutilized aspect of ideotype breeding.

A body of work on phenotypic integration in the field of evolutionary biology and ecology has also considered some aspects of the relationships among traits (Murren, 2002; Pigliucci, 2003). In this context phenotypic integration has been defined as the “pattern of functional, developmental, and/or genetic correlation (however measured) among different traits in a given organism” (Pigliucci, 2003). In plants, this area of research originated with the work of Berg (1960) who identified clusters of correlated traits. Strong correlations between traits could imply shared functions, with correlations among traits possibly maintained by stabilizing selection. In some cases researchers have focused on how groups of correlated traits affect plant function in specific ecological contexts (Lechowicz and Pierre, 1988). Economic spectrums that relate traits by their costs and functions have been identified in leaves (Wright et al., 2004), and proposed for roots, though evidence for a root economic spectrum remains inconclusive (Chen et al., 2013). In this research, phenotypic diversity within species or populations has typically been viewed as noise rather than as an important response to heterogeneous and unpredictable environments, competition, and phenotypic plasticity. Both ecological and agricultural research have converged upon concepts of integration through genetic, physiological, and developmental correlation (Graefius, 1978), though researchers in both areas seem to be largely unaware of the other.

Trait “stacking” in genetically modified crops (GMCs) is another form of ideotype breeding and trait integration. Traits of interest here are usually of the “deficit elimination” type, such as reducing susceptibility to insects or herbicides. First-generation stacks included *Bt* toxin-producing and glycophosphate-resistant GMCs that were introduced in 1998 (James, 2000). In order to decrease the selection for *Bt* toxin resistance in agricultural insect pests, 2nd-generation stacks combine several modes of actions for the same trait, which also reduces requirements for non-GMC refuge areas (Que et al., 2010). Stacking technologies have rapidly developed to higher numbers of combined traits, such as the nine foreign proteins combined in *Smart-StaxTM* (Marra et al., 2010). Gene stacking does lead to trait interactions in that most GM traits enhance growth in some situations, and combining modes of action decreases the ability of pests to adapt. Trait synergisms have been considered by biotechnology companies (Then, 2011), but only in terms of multiple modes of action for pest control, similar to the pyramiding of genes for disease resistance through introgression breeding.

Traditional plant breeding has attempted to combine traits that are helpful in isolation, and transgenic crops have also made progress in the stacking of particular traits. Ecologists have observed correlations among traits and between traits and plant function. However, our understanding of non-additive trait interactions is limited, and this is particularly true in root biology. Here we propose a theoretical framework for evaluating root system phenes and their functional interactions in the context of soil resource acquisition. We will show that the combining of traits does not always lead to a simple accumulation of additive effects, so plant biologists and breeders must take into account trait synergisms.

THEORETICAL FRAMEWORK

WHAT IS A PHENE?

“Phene” was used as early as 1925 in animal genetics to describe phenotypic traits under genetic control (Serebrovsky, 1925), and has been used extensively in European and Russian agricultural literature (e.g., Gustafsson et al., 1977). Phene can be defined concisely: *phene* is to *phenotype* as *gene* is to *genotype* (Lynch, 2011; Pieruschka and Poorter, 2012). Just as genes have variants called alleles, phenes have variants we will refer to as *phene states* (*phene* is to *trait* as *phene state* is to *attribute*). The particular combination of states for all phenes constitutes the phenotype of an individual organism. We will use *phenome* as the totality of all possible phene states of a taxon, i.e., phenotypic potential (Figure 1). Alternative more generic terms such as *traits*, *characters*, and *attributes* have been used with ambiguity that can lead to confusion (Vielle et al., 2007), such as by referring to properties at several levels of biological organization or by using trait to refer to either phenes or phene states. Lynch and Brown (2012) proposed that the most useful and meaningful phenes are *elementary* and *unique* at their level of biological organization (e.g., organ, tissue, cell). For example, an elementary root architectural phene

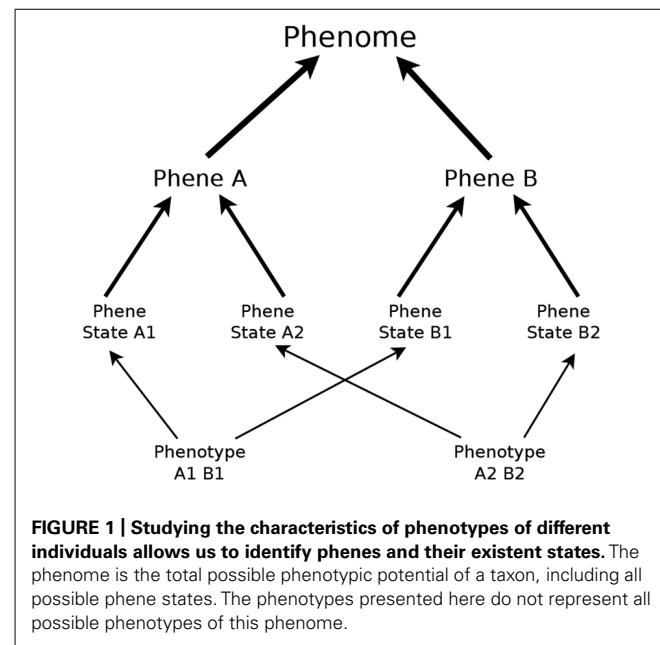
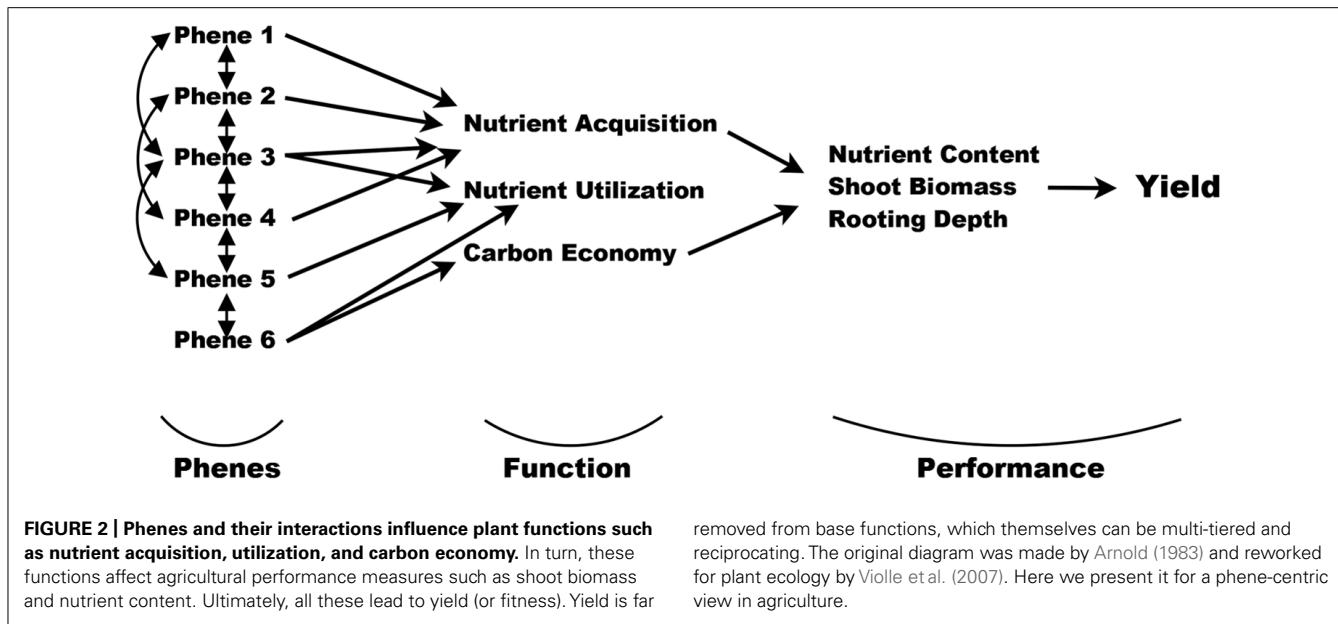
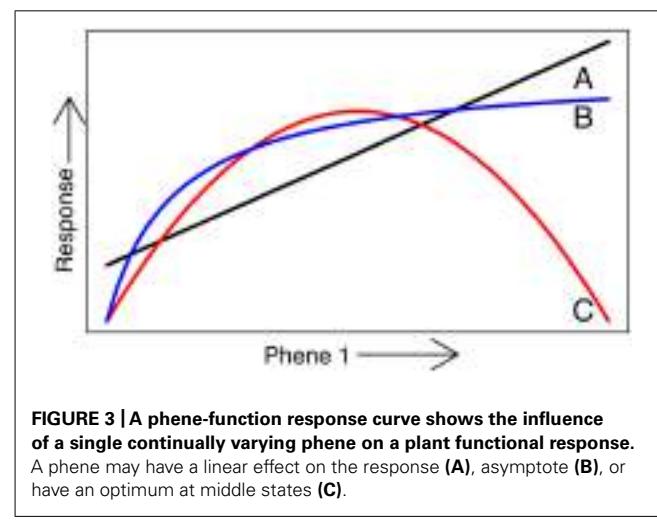


FIGURE 1 | Studying the characteristics of phenotypes of different individuals allows us to identify phenes and their existent states. The phenome is the total possible phenotypic potential of a taxon, including all possible phene states. The phenotypes presented here do not represent all possible phenotypes of this phenome.



should not be the product or aggregation of other more basic architectural phenes. The genetic and developmental processes giving rise to phenes should be unique, i.e., a phene is elemental because it has a unique developmental pathway. Some phenes may be under single gene control, and have phene states that are discrete. Many phenes are probably quantitative traits resulting from the interaction of many genes and the environment, and will show a continuous distribution of phene states. Many measurements of plant phenotypes are aggregates of multiple elemental phenes. For example, rooting depth has been shown to be influenced by separate phenes, such as root growth angle (Trachsel et al., 2013) and aerenchyma (Zhu et al., 2010a). Such plant characteristics may be referred to as *phene aggregates*. Plant measurements similar to yield, plant mass, or nutrient content will not be referred to as phenes or as phene aggregates. Rather, they are functional responses dependent on the state of many components of the plant phenotype.

Phene states make up phenotypes, which are individual manifestations of the phenome of a species. The root phenes of interest to us here have functional utility for resource acquisition (Lynch, 2011), and are components of root architecture, morphology, anatomy, or physiology. In turn, these functions influence agricultural performance such as biomass and yield, or plant fitness in natural systems (Figure 2), *sensu* Arnold (1983) and Violette et al. (2007). Functional utility can be assessed by comparing the functional responses of different phene states. For example, it has been shown that plants with longer root hairs acquire more phosphorus than plants with shorter root hairs or none at all (Bates and Lynch, 2000; Yan et al., 2004; Zhu et al., 2010b). The comparison of the phosphorus acquisition responses of these two root hair phene states demonstrates that the root hair length phene is important for P acquisition, with longer root hairs leading to greater P acquisition. A phene-function response curve shows the influence of a single continually varying phene on a plant function (Figure 3).



ROOT PHENE CLASSIFICATION

Root phenes classified by function, foraging strategy, and metabolic influence

Phenes can be classified in numerous ways. A mechanistic classification of root phenes can be made on the basis of whether they primarily affect resource *acquisition* or resource *utilization*. Phenotypes that affect soil resource acquisition generally affect the coincidence of root foraging and soil resource availability in time and space. Phenotypes that affect resource utilization influence how efficiently resources are used for plant functions including growth, further resource acquisition, and reproduction. Phenotypes that affect resource acquisition can be further classified based on foraging strategy. Foraging strategies exist along a continuum from phenes that influence soil exploration to those that influence soil exploitation. Exploration phenes influence the spatial and temporal exploration of soil domains by roots and root symbionts. Exploitation of soil resources describes how thoroughly resources

are acquired within a given soil domain, i.e., with no further soil exploration. Fitter proposed a measurement of acquisition efficiency to be the quotient of soil volume depleted to total root system volume (Fitter et al., 1991). This volume depends on the mobility of nutrients. Phosphorus depletion zones are only a few millimeters in diameter, while those for nitrate may be 10–100 times larger due to the 1000-fold difference between phosphate and nitrate in effective diffusion coefficients (Barber, 1984). A phene state can increase exploration for an immobile resource by entering new soil domains, while also increasing the exploitation of a domain for a more mobile resource by increasing the intensity of its acquisition (**Figure 4**). The differences in mobility between mobile and immobile nutrients give rise to the *root system depletion zone* and *root surface depletion zones* (lighter gray vs. dark gray in **Figure 4**), respectively (Bray, 1954). The growth angle of axial roots (e.g., nodal roots in maize, basal roots in common bean) influences the relative exploration of shallow and deep soil domains. Topsoil foraging has been shown to be important for phosphorus acquisition in both maize and common bean (Lynch and Brown, 2008), while deep soil foraging has been proposed to be important for the acquisition of water and nitrate (Lynch, 2013). Exploitation phenes affect the rate of nutrient uptake by increasing root density (number or length of roots in a volume) through greater numbers of axial roots, lateral branching, or root hairs and rhizosphere modification, for example. Rhizosphere modification includes decreasing the pH by releasing protons, organic acids, and by exudation of enzymes that release phosphorus from organic compounds (Lambers et al., 2006). Mycorrhizal symbioses can affect both exploration and exploitation, depending on the spatial scale and resource. Mycorrhizal fungi increase soil exploration by the growth of their hyphae, and exchange phosphorus for carbon with their host plant (Harley, 1989). Resource acquisition phenes not only differ in foraging strategies but in how they influence plant metabolism, and effects on metabolism are the mechanism for utilization phenes.

The functional utility of root phenes for soil resource acquisition is strongly influenced by rhizoeconomics (Lynch and

Ho, 2005; Lynch, 2007a), i.e., their relative costs and benefits. One of the major costs of roots is their metabolic demand. Several economic currencies can be used to estimate cost/benefit relationships, such as carbon, nitrogen, and phosphorus (Lynch and Rodriguez, 1994; Lynch and Beebe, 1995). Metabolic costs can be partitioned into construction and maintenance costs (Chapin et al., 1987). Root construction costs are generally strongly influenced by root volume which is proportional to length and diameter, so phenes which determine these (e.g., elongation rate, branching, number of roots formed, and root diameters) will influence construction costs. Roots, like all plant tissues, require not only carbon, but also mineral nutrients for construction and maintenance. Phenomes have been identified that alter root metabolic demand. “Root etiolation,” or decreasing diameter in order to increase length, has been proposed as an adaptive trait for nutrient acquisition (Lynch and Brown, 2008), with empirical support provided in maize (Zhu and Lynch, 2004). Root cortical aerenchyma (RCA) converts living cortical tissue to air space via programmed cell death. This lowers the respiration of root segments (Fan et al., 2003), and has the additional benefit of mobilizing nutrients for other uses (Postma and Lynch, 2010). An economic classification of root phenes is based on how they influence metabolism. **Table 1** presents a number of root phenes and their classification according to these three schemes (acquisition vs. utilization, exploration vs. exploitation, and metabolic influence vs. no metabolic influence).

Not all root measurements are root phenes

An array of root measurements are commonly made in both agricultural and natural systems that do not meet the definition of an elemental phene. Rather, most of these root measurements represent phene aggregates that are influenced by the states of several root phenes (**Table 2**). Others, such as total root length, are *functional responses* that are influenced by states of phenes through their influence on soil resource acquisition and eventual photosynthate allocation to the root system. Unexplained variation in these measurements may be resolved by more thorough documentation of constituent root architectural, anatomical,

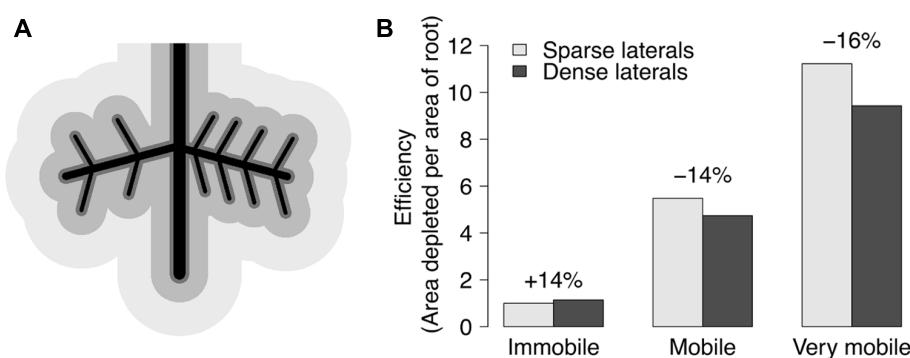


FIGURE 4 | (A) Black lines depict a simplified root system with a lateral root on each side of a tap root. The left side has 4 second order laterals, while the right side has 8 second order laterals. The darkest gray area around roots depicts the depletion zone of immobile resources (like P), while the medium gray depicts the depletion zone of mobile resources (like N), and the lightest gray represents very mobile

resources (like water). **(B)** Efficiency is shown by the quotient of the area (pixel counts) of a respective resource's depletion zone divided by the area of the roots for each half of the root system with sparse or dense second order laterals. Dense laterals increase the efficiency for an immobile resource, but decrease efficiency for mobile resources. Differences would be inflated if areas were converted to volumes.

Table 1 | Classification of root phenes.

Root phene	Mechanism	Foraging	Economy
Axial root growth angle	Acquisition	Exploration	Neutral
Root growth rate	Acquisition	Exploration	Influencing
Number of axial roots	Acquisition	Exploration	Influencing
		Exploitation	
Lateral root branching	Acquisition	Exploitation (N)	Influencing
		Exploration (P)	
Root hair density	Acquisition	Exploitation (P)	Neutral
Root hair length	Acquisition	Exploitation (P)	Neutral
Rhizosphere modification	Acquisition	Exploitation (P)	Influencing
Aerenchyma	Utilization		Influencing
Root etiolation	Utilization		Influencing

Classification of a particular root phene begins by determining its mechanism affecting resource uses, acquisition or utilization. Resource acquisition phenes are classified based on their foraging strategy, exploration or exploitation for a particular resource, with nitrogen (N) representing mobile and phosphorus (P) representing immobile resources. All root phenes are classified by whether they influence metabolic economy or are neutral.

and physiological phenes. These measurements may often be referred to as traits, which highlights the difference between the common usage of “trait” and the biological definition of “phene”.

HOW DO PHENES INTERACT?

Functional response interactions

The utility of a phene can be assessed by comparing the functional responses of varying states of the phene. Similarly, the interaction of two phenes can be assessed by combining at least two phenes states of two different phenes and measuring the functional response of each combination. In such a situation, the null hypothesis is that the functional response of two phene states from two different phenes will be additive. The particular phene state combination is synergistic when the functional response exceeds the sum of the responses of the phene states in isolation. Antagonistic interactions occur when the functional response of phene states in combination is worse than that expected from the sum of their responses in isolation. We can describe the mechanistic basis of the interaction based on the classifications of the component phenes. A phene-function response landscape graphically demonstrates how the simultaneous changes of two or more phenes affect a function (**Figure 5**).

Foraging strategy interactions

Phenes interact through their effects on *foraging* when the mechanism through which one phene affects foraging directly interacts with the mechanism of another phene affecting foraging. For example, axial roots with shallow growth angles will increase the exploration of soil with greater amounts of phosphorus, while increased root hairs will increase the exploitation of the explored

soil. The combined states of shallow growth angles and increased root hairs may have a synergistic interaction beyond what would be expected based on their additive effects on phosphorus acquisition (see Case Study 2).

Economic interactions

The *economic* interaction of two phenes is mediated by the metabolic budget of the plant. Two metabolism influencing phenes will exhibit tradeoffs when occupying more metabolically demanding states. These tradeoffs are expected between root classes, or even between number and length within a class (Walk et al., 2006; Rubio and Lynch, 2007). Building more of one type of root will necessarily limit the metabolic resources available for building other types, or decrease the resources available for elongation of existing roots. However, feedbacks between nutrient acquisition and increased photosynthesis that allow further root growth are possible. Conversely, a metabolically neutral phene will have no economic interaction with a metabolism influencing phene.

Phene modules

Combinations of specific phene states may be more likely to be found together in individuals of a taxon when they act as a functional module through foraging and economic interactions. Modules are aggregates of components that are related, such as in the context of molecular pathways (Hartwell et al., 1999), architectural modules such as leaves, flowers, and roots, and even entire plants as modules in an ecosystem (Prusinkiewicz, 2004). One useful definition for module in the context of phene interactions is a group of phenes that behave synergistically. In roots, such functional module components probably belong to the same parent root class, similar to the “modular unit” suggested by Pregitzer et al. (2002) as lateral branches of tree roots consisting of several orders of the finest roots. In crops such as common bean and maize, these modules are initiated from and include the major axes, i.e., basal roots in bean, nodal roots in maize. Foraging interactions are more likely to occur in modules composed of phenes that are close together because their likelihood of coinciding with a soil resource increases.

Environmental interactions

It is well known that the abiotic and biotic environments can affect the phene states of an organism through the phenomenon of phenotypic plasticity (West-Eberhard, 1989; Callaway et al., 2003). For example, roots have been observed to proliferate in patches of nutrients (Drew and Saker, 1975; Granato and Raper, 1989), change rooting angle (Bonser et al., 1996), change root hair density (Ma et al., 2001a), and alter axial elongation and lateral root density in response to phosphorus availability (Borch et al., 1999). Root phenotypic plasticity constitutes one type of phene-environment interaction. Another type is based on tradeoffs and synergies that may exist between root phenes and particular soil resources, i.e., phene \times environment \times functional response interactions. For example, in low phosphorus soils, phenotypes with shallow root growth angles perform better than phenotypes with steep root growth angles, but in high phosphorus conditions both perform equally well. Steep-angled phenotypes are better at acquiring water

Table 2 | Relation of root measurements to root phenes.

Root measurement	Definition	Influential phenes	Reference
Total root length	The cumulative length of an entire plant root system (m) that is partially a <i>functional response</i> .	Axial root length, number of axial roots, lateral branching, lateral length	Zhu et al. (2006), Brun et al. (2010)
Root length density	The cumulative length of roots per some volume, often from soil cores or monoliths (m cm^{-3}) that is a <i>phene aggregate</i> dependent on the states of constituent phenes.	Axial root length, number of axial roots, lateral branching, lateral length	Ho et al. (2005), Miguel et al. (2013)
Specific root length	The root length per unit mass (m g^{-1}) that is a <i>phene aggregate</i> dependent on the states of constituent phenes.	Xylem area, phloem area, number of cortical cells, cortical cell size, aerenchyma area, secondary development	Fan et al. (2003), Jaramillo et al. (2013)
Root tissue density	The mass of roots per unit root volume (g cm^{-3}) that is a <i>phene aggregate</i> dependent on the states of constituent phenes.	Xylem area, phloem area, number of cortical cells, aerenchyma area, secondary development	Fan et al. (2003), Jaramillo et al. (2013)
Rooting depth	The deepest depth at which roots from a plant are observed (m). Alternatively, the depth at which 95% of root length is at or above may be used. Both are <i>phene aggregates</i> dependent on the states of constituent phenes, and will be influenced by total root length.	Axial root angles, axial root length, number of axial roots, lateral branching, lateral length	Ho et al. (2005), Miguel et al. (2013)
Root respiration	The rate of CO_2 production due to root metabolism ($\text{mmol CO}_2 \text{ m}^{-1} \text{ root s}^{-1}$) that is a <i>phene aggregate</i> dependent on the states of influencing phenes and their contributions to total root segment respiration.	Number of cortical cells, cortical cell size, aerenchyma area, N content, secondary development	Fan et al. (2003), Zhu et al. (2010a), Jaramillo et al. (2013)
Root longevity	The length of time between the formation and loss of a root (s) that is a <i>functional response</i> dependent on root defenses and stress physiology.	Phenolic concentrations, lignin concentration, number of cortical cells, cortical cell size, aerenchyma area	Eissenstat et al. (2000)
Topological index and fractal dimension	Ratios of different measures summarizing the complexity of a network (unitless) that are <i>phene aggregates</i> dependent on the states of constituent phenes.	Axial root length, number of axial roots, lateral branching, lateral length	Fitter and Stickland (1992), Walk et al. (2004)

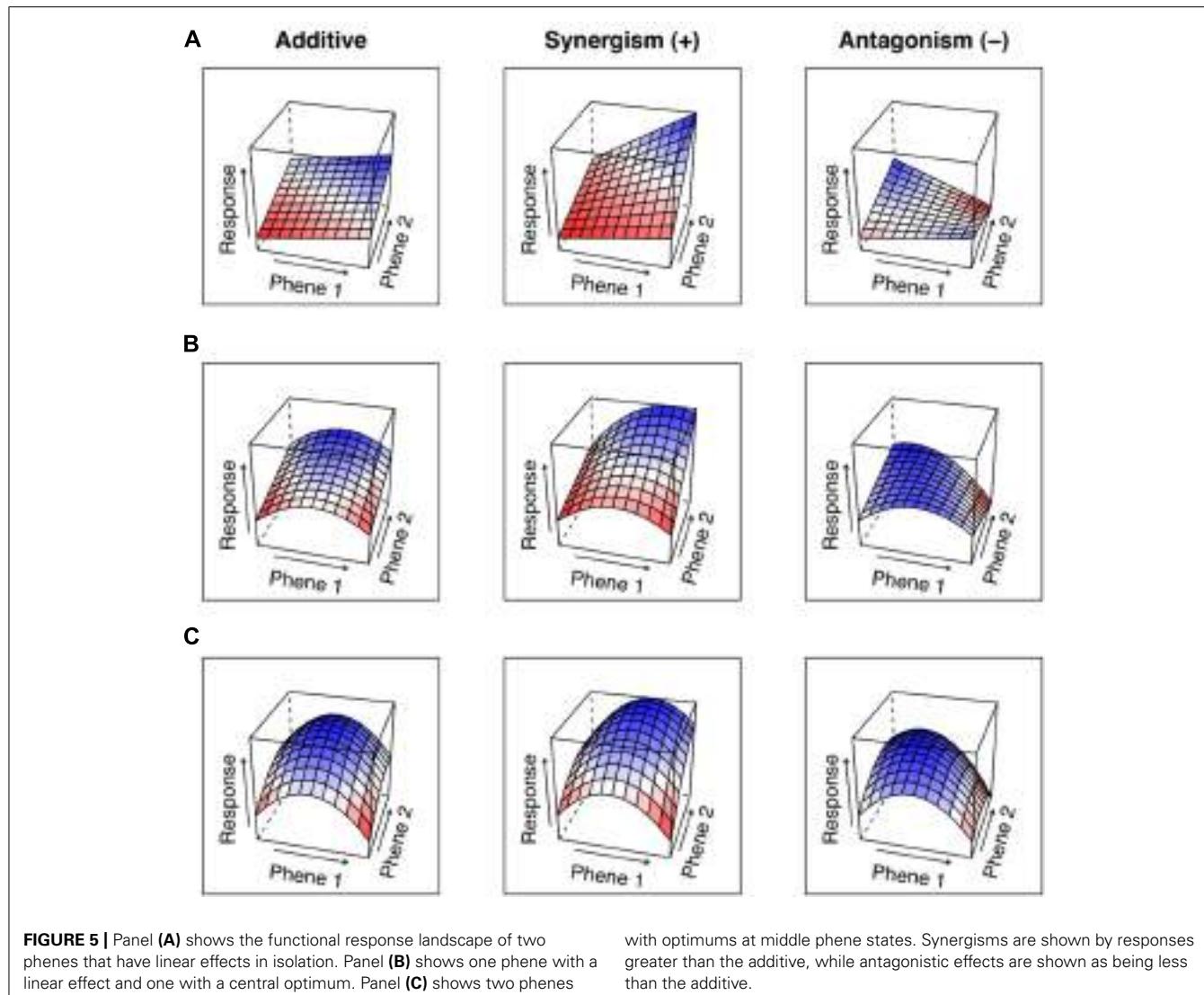
Many common measures of root system and individual root properties are examples of *phene aggregates* that are influenced by several more elemental root phenes, and some are partially *functional responses* dependent on plant performance. These root measures are defined and the phenes that influence the measure are listed. Here, lateral branching includes the branching of successive orders of laterals, i.e., including laterals of axial roots, laterals of laterals, etc.

during terminal drought (Ho et al., 2005), so there is an architectural tradeoff for root growth angle for acquiring resources at different depths in the soil. When both phosphorus stress and terminal drought occur together, shallow-rooted phenotypes performed better because early P uptake allowed the growth of more extensive root systems that then conferred greater tolerance to the terminal drought. Phene \times phene \times environment interactions are more complicated than single phene \times environment interactions, but must be studied in order to understand how plants cope with multiple stresses, and how suites of traits influence fitness.

Interplant interactions

Root competition among plants of different species plays an important role in shaping plant communities (Schenk, 2006) and in the performance of interspecific polycultures in agriculture

(Wilson, 1988; Postma and Lynch, 2012). Competition is expected to be greater for mobile nutrients than relatively immobile nutrients (Postma and Lynch, 2012; Wilberts et al., 2013). Little is known about how specific root traits affect competition and facilitation, but there are a few examples. *Arabidopsis* wildtypes with root hairs were shown to have a competitive advantage over root hairless *rhd2* mutants in low phosphorus media (Bates and Lynch, 2001). Similarly, *Arabidopsis* wildtypes out-competed *axr4* mutants with decreased numbers of lateral roots in low phosphorus, but not in low nitrogen (Fitter et al., 2002). Architectural multilines of common bean composed of equal portions of plants with shallow and steep basal root angles had Land Equivalent Ratios greater than unity (Henry et al., 2010), which means more area must be planted of the monocultures in order to achieve the same levels of yield as the multilines. This implies a competitive release of the dominant shallow-rooted plants when grown with



steep-rooted plants in low phosphorus soils. Common beans were shown to alter root architecture in the presence of neighboring plants due to localized phosphorus depletion (Nord et al., 2011). Clearly, understanding phenes requires an understanding of how phenes will react to other phenes, the environment, and other plants.

Phene integration

Foraging, economic, environmental, and interplant interactions of phenes create an integrated phenotype. The integrated phenotype is more than simply a collection of isolated traits, but rather is a suite of interacting phenes that affect plant functions. These interactions cannot simply be assumed to be additive and will depend on the environmental context. Phene integration occurs at all levels of phenotypic organization, from cells, to modules, to the whole plant.

Phenes may interact via resource partitioning and signaling, even between roots and shoots. Typically, shoots provide photosynthates to the roots, while roots supply soil resources to the

shoot. Thornley (1972) developed a mathematical model with two pools, shoot and roots, and two substrates, carbon and nitrogen, which are supplied by the shoot and roots, respectively. This simple source–sink model demonstrated that plants should balance shoot and root activity and invest in the organs that produce the most limiting resource, and continues to guide whole plant modeling. Empirical work demonstrates that aboveground and belowground organs communicate their internal and environmental status to each other in order to integrate plant function in dynamic environments. For example, root ABA signals induce stomatal closure in leaves which decreases transpiration (Davies and Zhang, 1991). The plant shoot is partially responsible for perceiving the internal nitrogen status and uses reduced nitrogen compounds and auxin to signal roots to form lateral roots (Ruffel et al., 2011). Interestingly, roots can also influence shoot branching through auxin signaling (Bennett et al., 2006), which might suggest root perception of the soil environment informs the regulation of shoot growth. These interactions suggest that another form of phene interaction may be information exchange, which

may apply within the root system as well. The global leaf economic spectrum demonstrates that leaves from a variety of species representing diverse functional groups are constrained by development and natural selection to fall along a single spectrum for a variety of traits (Wright et al., 2004). A direct interaction between a shoot phene such as leaf morphology and an RSA phene like lateral branching is unlikely. Rather, the shoot and root organs integrate information processing and metabolism, and balance production of photosynthates with acquisition of soil resources (**Figure 6**).

Hypotheses regarding the integration of root phenes

We propose the following hypotheses regarding the integration of root phenes:

- (1) Functional synergisms will occur among foraging phenes that act within a module including the axial root and its subordinate roots.

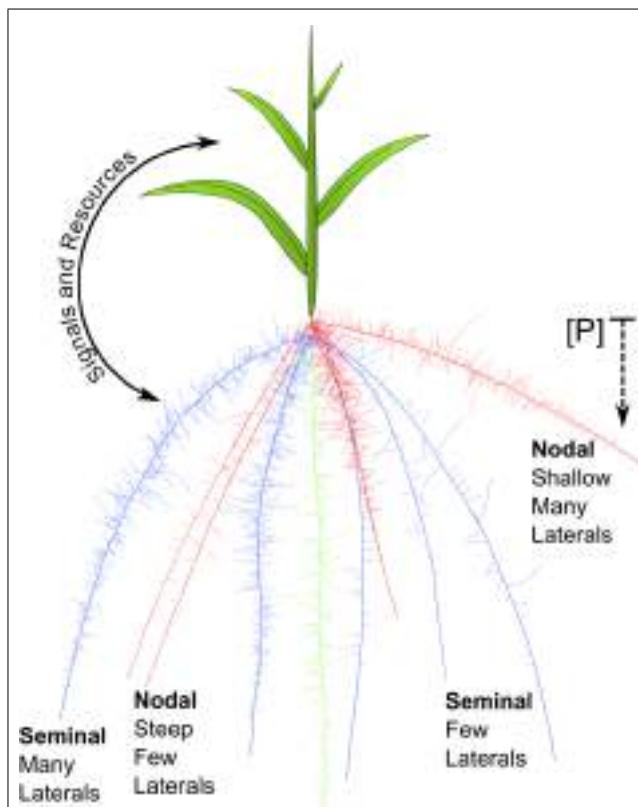


FIGURE 6 | A maize seedling is depicted. Seminal roots (blue) and primary root (green) emerge from the seed. One whorl of nodal roots (red) is shown emerging from belowground stem tissue. The nodal roots on the left have steep growth angles, while those on the right are shallow. The shallow nodal roots on the right also have dense laterals, while the steep nodal roots on the left have sparse laterals. In the context of phosphorus acquisition from the epipedon, shallow nodal roots with many laterals will have a synergistic interaction because they are acting within the same module. Though the seminal roots on the left have many laterals they will not interact synergistically for foraging with nodal root traits because they are in a different root class module. The whole plant is integrated by reciprocal signaling between shoot and roots and by balancing the production of photosynthates with soil resource acquisition.

- (2) Functional synergism will increase as the number of positively acting phene states combined is increased.
- (3) Metabolic tradeoffs will limit synergy created by combining foraging phene states that demand more metabolic resources, except when alleviated by phenes in states that relieve metabolic constraints.
- (4) Synergisms will be more likely to occur when combining metabolically neutral phenes in positively acting states.
- (5) The large diversity of root system phenotypes, i.e., the particular combination of phene states of an individual, is partially explained by the interactions of phenes within plants, between plants, and between phenes and the environment.

CASE STUDIES

Research on phene interactions is nascent, and this is especially true in the case of roots. Much of the evidence for root phene integration comes from research with *SimRoot*, a functional-structural plant model focusing on root system architecture and nutrient acquisition (Lynch et al., 1997; Postma and Lynch, 2010), though we will also discuss empirical evidence and experimental approaches for studying phene interactions.

ROOT HAIR LENGTH × ROOT HAIR DENSITY

Root hairs are subcellular extensions of root epidermal cells that are particularly important for the acquisition of immobile nutrients such as phosphorus. Root hairs can vary in density (i.e., number of root hairs per unit root surface area) and in length. Diversity for both of these traits is evident in several species including common bean, soybean, and maize (Wang et al., 2004; Yan et al., 2004; Zhu et al., 2005a). *SimRoot* was employed to test interactions among root hair length, root hair density, proximity of root hair appearance to the apical meristem, and the spatial patterning of hair-bearing cells (trichoblasts) and non-hair-bearing cells (atrichoblasts) in *Arabidopsis* (Ma et al., 2001b). The synergistic effect of increased root hair length and density (RHLD) phene states was 272% greater than their expected additive effects. Root hair formation nearer the root tip increases P acquisition, while number of files had positive effects when more numerous. All positive phene states were compared to their expected additive function response in two-way, three-way, and four-way combinations. On average, synergistic effects increased with the number of positive interactions: two-way, 168%; three-way, 232%; and four-way, 371% greater than additive effects (new calculations from original data). Changing RHLD in *Arabidopsis* had no direct effect on root respiration (Bates and Lynch, 2000). We hypothesize that metabolically neutral phenes will have the greatest synergisms because of the lack of economic tradeoffs. As this example shows, the magnitude of phene synergisms may increase with the number of positively interacting phene states (Hypothesis 2).

LATERAL BRANCHING × ROOT CORTICAL AERENCHYMA

Variation for lateral root length and density has been observed in both the primary root and nodal roots of maize (Zhu et al., 2005b; Trachsel et al., 2011). Greater lateral root length and density would permit greater soil exploration, and so would improve

acquisition of soil resources. However, increased lateral branching has high metabolic demand, and due to competing sinks it could influence the growth of other root classes. This trade-off could be alleviated by decreasing metabolic demand in other ways. *SimRoot* was used to test the hypotheses that increased lateral root branching would increase N and P acquisition and that this phene would be affected by the formation of aerenchyma (Postma and Lynch, 2011). At the lowest level of nitrogen, there was a 42% reduction in shoot dry weight compared to the expected additive effects of increasing lateral root branching and forming aerenchyma, which constitutes a functional antagonism. However, at the intermediate level of nitrogen a synergetic interaction 220% greater than the expected additive effects was observed. In the low phosphorus condition, the synergetic interaction was 33% greater than the expected additive effects. This broad range of interaction demonstrates the importance of environmental context.

ADVENTITIOUS ROOT NUMBER × ADVENTITIOUS ROOT RESPIRATION AND BASAL ROOT GROWTH ANGLE

Adventitious roots emerge from the hypocotyl in common bean (*Phaseolus vulgaris*) and have less construction and maintenance costs than basal roots (Miller et al., 2003). Adventitious roots emerge in the topsoil and typically have extremely shallow growth angles, so they were hypothesized to be an adaptive trait for topsoil foraging. Basal roots are the principal axial roots in common bean, and a shallow growth angle for basal roots has been shown to be important for topsoil foraging (Bonser et al., 1996; Liao et al., 2004; Ho et al., 2005; Henry et al., 2010). Adventitious roots were found to have a range of respiration rates from the same as tap roots, to 400% greater than tap roots (Bouma et al., 1997; Walk et al., 2006). Because phosphorus has low soil mobility, it accumulates in the topsoil from the deposition of senesced plant tissue (Anderson, 1988). Both functional response and economic interactions were expected between adventitious root number (ARN) and adventitious root respiration (ARR), and between ARN and basal root growth angle (BRGA), which was tested in *SimRoot* (Walk et al., 2006). Increasing ARN greatly increased phosphorus acquisition when ARR was the same as tap root respiration, and marginally benefited phosphorus acquisition when ARR was two times tap root respiration. When ARR was four times greater than tap root respiration, there was a negative relation between increasing ARN and phosphorus acquisition. At the highest level of ARR, not enough metabolic resources were available for the construction of root length adequate for phosphorus acquisition. This shows a functional response antagonism between greater states of ARN and ARR that is mediated through an economic interaction. ARN was also expected to interact with BRGA. However, only additive effects were observed between greater ARN and more shallow BRGA, which suggests adventitious roots and basal roots function as independent modules (Hypothesis 1).

NODAL ROOT NUMBER × ROOT CORTICAL AERENCHYMA

Unpublished results from *SimRoot* show interaction between RCA and number of nodal roots in maize (Figure 7). Across a range of N and P availability, root length and total biomass were strongly affected by nodal root number. RCA had little to no effect on

biomass or root length when there were fewer than optimal crown roots, but increased root length and biomass with optimal or greater than optimal numbers of nodal roots, especially with suboptimal N or P. Because optimal nodal root number differed between N deficient and P deficient conditions, the range of nodal root numbers where RCA increased biomass depended on the environment. At medium levels of nitrogen and phosphorus, the synergetic effects of greater numbers of crown roots and RCA were 31.6% and 132% greater than the expected additive effects, respectively.

BASAL ROOT GROWTH ANGLE × ROOT HAIR LENGTH AND DENSITY

In common bean, BRGA is a soil exploration phene and was hypothesized to influence the utility of the root hair phene, which affects exploitation, by determining the placement of root hairs in the soil profile. A field study was conducted in Mozambique, comparing three recombinant inbred lines (RILs) for each of four phenotypes representing all combinations of shallow and deep BRGA and low and high RHLD; Miguel, 2012). In low P soil, shallow BRGA increased shoot growth by 57.7%, and greater RHLD increased shoot growth by 89.3% (Figure 8). Shoot mass of the combined positive states (shallow angle and greater RHLD) was 298% greater than the base line (steep angle and lower RHLD), which is twice the expected additive effect. Root hairs along with the basal roots or basal root laterals on which they form constitute a functional module which gives rise to high levels of synergism (Hypotheses 1 and 4).

EVIDENCE FOR ROOT PHENE FUNCTION AND INTERACTION IN NATURAL DOMAINS

Variation in root phenes has been observed among wild species along with correlation between phenes, such as between specific root length and lateral branching (Comas and Eissenstat, 2009). Differences in rooting depth among grassland species has been proposed as one contribution to the relationship between biodiversity and ecosystem productivity by allowing plants to exploit particular soil niches (Fargione and Tilman, 2005). As noted above, rooting depth is a phene aggregate influenced by rooting angle, number, and total metabolic allocation to the root system, so diversity for rooting depth among species influencing productivity represents phene × phene × species interactions. A suite of functional traits associated with acquiring nitrogen in nitrogen-limited grassland plants was proposed which included high carbon:nitrogen tissue, slow metabolic rates, and large root length (Craine et al., 2002). McCormack et al. (2012) found relationships across 12 tree species among root morphology, root chemistry, root lifespan, and whole plant traits, though in another study no clear relationship between root traits such as root diameter and nitrogen concentration was identified (Chen et al., 2013). These studies in natural systems demonstrate a growing awareness of the identification of a root economic spectrum that would be a useful tool for understanding variation in root systems. However, to our knowledge, examples are lacking demonstrating the interactions of specific root phenes for specific functions in natural systems. Most studies rely on interspecific diversity to create root phene variation, which confounds specific phenes with many other

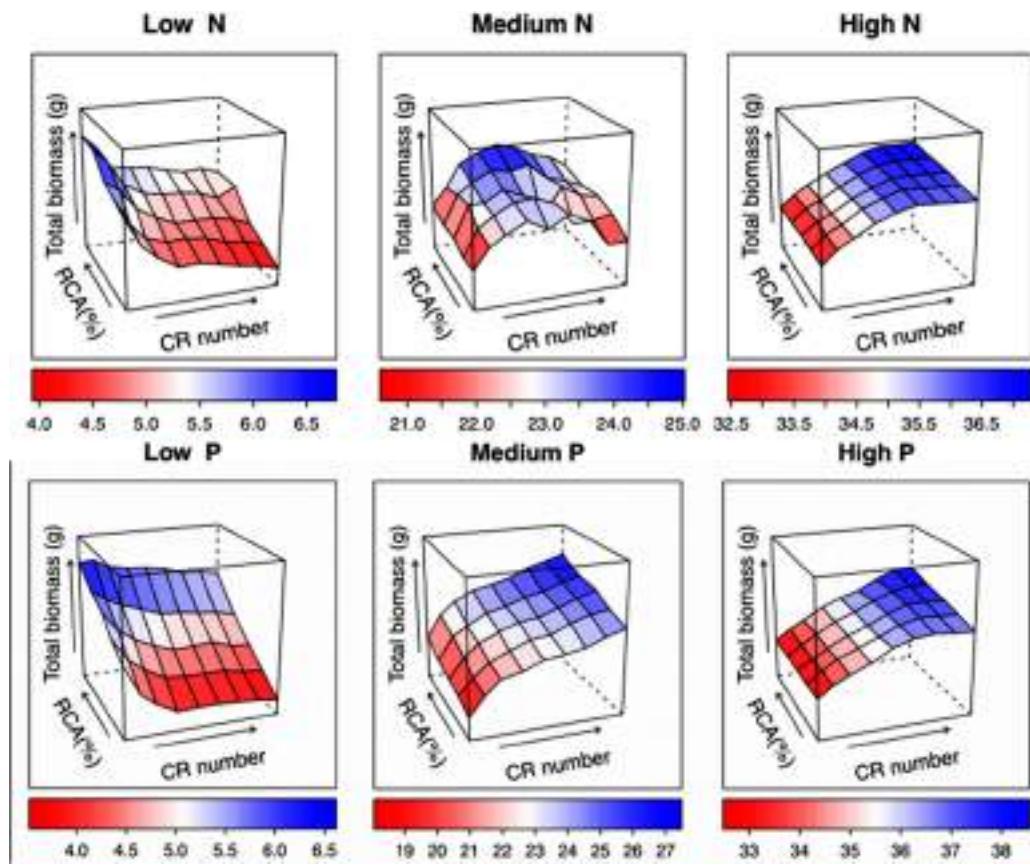
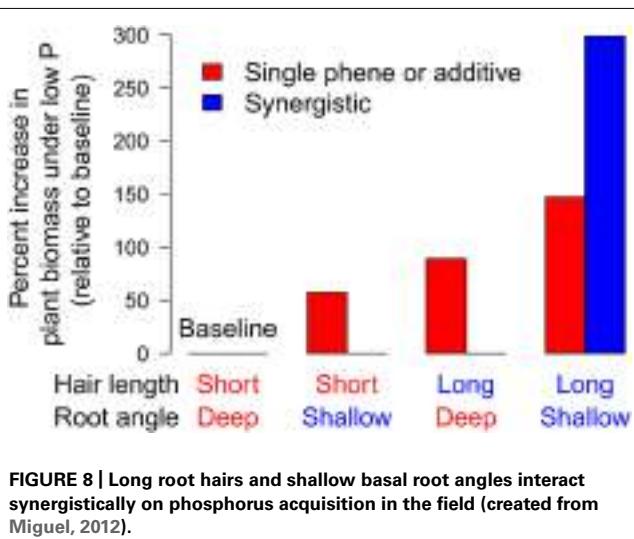


FIGURE 7 | Phene integration of root cortical aerenchyma (RCA) and crown root (CR) number was studied in maize using SimRoot across a range of nitrogen (N) and phosphorus (P) levels. These simulation results demonstrate linear, asymptotic, and optimum single phene responses and their interactions.



covarying factors. Below, we will discuss general approaches to study root phenes and root phene integration that can be extended to any study system.

GAPS IDENTIFIED BY COMPARING KNOWN INTERACTIONS TO POSSIBLE INTERACTIONS

These case studies demonstrate progress in understanding root phene integration. Most of the studies have been conducted with simulation modeling so must be confirmed empirically, but the work of Miguel (2012) with basal root angle and root hairs is a notable exception where root phene state synergisms were demonstrated in agricultural fields. There are no examples of interactions where resource acquisition phenes affecting metabolic economy, such as axial root number and lateral branching, have been simultaneously manipulated, though Walk et al. (2006) showed an interaction between ARN and respiration mediated through architectural tradeoffs with lateral roots of basal and tap roots. Foraging phenes that influence metabolism may have only additive, or even antagonistic, interactions because of tradeoffs in metabolic economy (Hypothesis 3). Further work is also needed to understand how phenes integrate within and between functional modules.

APPROACHES FOR STUDYING PHENE INTEGRATION

Many studies analyzing plant traits have relied on comparisons between species for phene state variation and in natural environmental gradients for differences in abiotic conditions. However, such comparisons are confounded by the multitude of differences

that exist among species and environments. The use of structured genetic populations that vary for specific phenes but share a common genetic background, evaluated in environments in which specific stresses are imposed, is a more powerful approach when possible (Lynch, 2011). This strategy has the advantage of allowing the comparison of different phene states within a common genetic and phenotypic background, which is especially important given our lack of understanding of phene integration. Populations of RILs have been used both for genetic mapping and for near-isophenic comparisons in common bean and maize (Yan et al., 2004; Ho et al., 2005; Zhu et al., 2005a,b; Ochoa et al., 2006; Zhu et al., 2006; Henry et al., 2010). Near-isophenic lines refer to lines that differ primarily in the state of a single phene, or at least a small number of phenes. Populations of near-isophenic lines may also contain plants with combinations of phene states that allow the study of phene integration. Single gene mutants may not always be useful for studies of phenes because many phenes of interest are controlled by several QTL or genes (Lynch, 2011). While biparental RIL populations are useful for these phenotypic contrasts, their limited diversity (descending from two parents) may not allow the measurement of the breadth of the root phenoome. Diversity panels representing broader variation in crops are now being used to probe the breadth of the root phenoome. High-throughput phenotyping must increase in extent and intensity (Houle et al., 2010). Extensive phenotyping is accomplished through the sampling of larger numbers of plants of greater diversity. Intensive phenotyping is the measurement of more traits for each sample. Extensive and intensive phenotyping are benefitting from the application of remote sensing, image analysis, and robotics (Fiorani and Schurr, 2013), including with roots (Galkovskyi et al., 2012). Intensity will be further increased by the inclusion of function-valued traits, or phenes that are best described as mathematic functions rather than single values (Kingsolver et al., 2001). Both extensive and intensive phenotyping will contribute to plant phenomics and the study of root phene integration.

Plant phenomics is generating vast amounts of data, and increases in the extent and intensity of phenotyping will accelerate the pace of data collection. The creation and use of data repositories by teams of scientists is imperative. In order for this data to be useful, it must include metadata (higher level information that describes the data and its context). Metadata has the benefits of increasing data longevity and recycling by the creator and others (Michener, 2006). Metadata for functional-structural phenomics must include ontologies for identifying plant structures and research context (Ilic et al., 2007; Madin et al., 2008). Root functional phenomics should include ontologies for roots that represent their phylogeny, genetics, and development (Zobel, 2011), but also their function. Root phenomics will not mature without thorough documentation and sharing of data, especially due to the significant financial costs of root phenotyping.

Rasmussen (1987) proposed developing a “germplasm bank of ideotype traits” where breeders would agree to cooperate to introgress phenes of interest into elite genetic backgrounds. Diversity in crop species traits is often found in landraces or other unimproved varieties (Bayuelo-Jiménez et al., 2011). Recently,

Burton et al. (2013a,b) reported substantial variation among RILs, maize landraces and teosintes for both root architectural and root anatomical phenes that could be of use in maize breeding. However, these unimproved genetic backgrounds act as barriers to the inclusion of phenes that comprise a desired ideotype for breeding programs. A collaborative network of plant physiologists and breeders working to identify and understand phenes useful for crop performance would benefit from germplasm banks containing phene states in common genetic backgrounds. In order for researchers and breeders to be able to choose appropriate material for their programs, integration of phenomic and germplasm bank databases will be required. Greater collections of such plant material and relevant genetic resources are available for crop species than for wild plants, but model systems such as *Arabidopsis* and *Populus* may act as bridges for the induction of similar studies in other wild species.

Functional-structural plant modeling is an invaluable tool for the study of root phene integration. *SimRoot* will continue to be of great utility in this endeavor, as will other root simulations such as RootMap (Diggle, 1988; Dunbabin, 2007) and R-SWMS (Javaux et al., 2008). Simulations allow the exploration of trait function beyond what is possible in greenhouse and field studies. Genetic and physiological constraints may make it difficult or impossible to study some phene state combinations, but they can still be modeled. Simulations also allow many different climates, soil types, and nutrient levels to be studied. While only contrasting and extreme phene states may be combined factorially for study in the field or greenhouse due to space and labor limitations, modeling allows a greater phenotypic range and phene combinations to be studied. In an iterative fashion, simulations help focus empirical experimentation on the most interesting phenes and phene interactions, while data from empirical studies parameterize and refine root models (Wullschleger et al., 1994). A recent review of three-dimensional root models highlights the various models’ strengths and weaknesses, and proposes how to advance the field by encouraging wider adoption of root models and by making models more realistic through the inclusion of more explicit plant regulatory networks and soil microorganisms (Dunbabin et al., 2013). Simulations should be integrated with phenomic databases to predict functional implications of phenotypic variation, just as models of predicted gene function and subcellular protein targeting augment genomic databases.

FUTURE PROSPECTS

The understanding of phenotypic integration requires research comparing multiple states of single phenes in isolation and in combination, generating phene-function landscapes for multiple environments. Understanding the interaction of phenes is particularly important because there may be emergent properties that cannot be predicted from their function in a single phenotypic background. The phenoome is the interface of the genome and the environment. Phenes and phenotypes arise through plant development under genetic control as influenced by the environment, so genetic information is useful in understanding phenotypic variation. At the same time, we need to know how phenes influence plant function in specific environments, which

will require the collaboration of plant biologists, soil scientists, and climatologists. Many phenes will not be under single gene control, so the use of single gene mutants for phene studies may limit inquiry to the presence or absence of a particular phene, but we also need to know how variation in phene states contributes to different aspects of plant function. The use of emerging technologies in plant genetics, such as RNA interference, may allow more complex developmental manipulation through changes in expression levels of several genes that could possibly give rise to ranges of phene states in common genetic and phenotypic backgrounds (Katoch and Thakur, 2013).

Phenes are properties of the organism that have been neglected in the genomic era. The organism is the fundamental biological unit of organization for studies of phenes and phene interaction. It is surprising how little research focuses on organisms *per se*, in contrast to the organism being treated primarily as a tool to understand genes or ecosystems. Organisms are the entities on which natural and artificial selection act, which genes influence, and of which ecosystems are composed (Lewontin, 1970). The variation in phenes embodied within a taxon cannot simply be averaged to generate an ideal individual because this variation has functional and evolutionary importance. Progress in understanding the plant genome is stunning, and currently far outstrips our understanding of the plant phenotype, despite the fact that the plant phenotype is at least as complex as the genome and arguably more important for human welfare.

The study of phenes is hindered by the lack of relevant conceptual frameworks. Here we have discussed phenes in the traditional context as building blocks of an organism's phenotype. In some cases it may not be clear whether a phene is truly elemental, as it may be influenced by other traits at lower levels of organization. For example, basal root number in common bean was found to be influenced by basal root whorl number (Miguel, 2012). However, the discovery of even more elemental phenes is a useful outcome of applying the phene perspective. The ambiguity of the phene might be necessary for it to be applied in diverse fields and research programs, but the science of the phenotype, phenes and phene interactions will be aided by the development of more precise and informative theoretical frameworks. A better understanding of integrated phenotypes would have benefits

for other fields of biology and agriculture, such as how natural selection has led to the diversity of forms observed within and among species, and how improved crop varieties can be designed and developed. Trait-based, or ideotype, breeding is an important avenue for crop improvement, and has been shown to be more efficient than yield-based selection in some situations (Annichiarico and Pecetti, 1998). Yield and metrics closely associated with yield, such as number of grains, may obscure the advantages of phene states that happen to be in otherwise poor backgrounds. Genetic and developmental pathways may overlap among quantitative traits such as root phenes, so genetic associations with yield or other functional responses are also of limited use. Phene utility should be measured in the field, and for specific environmental stresses, because the advantages of some phene states may only reveal themselves when resources are limiting. Understanding the functional utility of specific root phenes and their interactions requires the employment of near-isogenic plant material in the field and simulation modeling. The opportunities created by the ability to understand the fitness landscape of integrated ideotypes will eventually lead to greater understanding of ecosystem structure and function, and to superior crop lines bred for specific agricultural contexts.

Alleviation of world hunger despite a burgeoning human population, continually degrading natural resources, and global climate change is a primary human challenge for the 21st century. New crop lines with superior soil resource acquisition will be a valuable tool to that end (Lynch, 2007b; Lynch and Brown, 2012). In natural systems, understanding how root phenes influence community structure and ecosystem function will inform policies to manage anthropogenic effects on the climate and environment. Clarification and refinement of phene integration theory, simulation and field studies of phenes and phene interactions, and the distribution of results and plant materials are all essential for the success of this unprecedented opportunity to deploy phenes to provide solutions for pressing world problems.

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Root traits for infertile soils

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Crop production is often restricted by the availability of essential mineral elements. For example, the availability of N, P, K, and S limits low-input agriculture, the phytoavailability of Fe, Zn, and Cu limits crop production on alkaline and calcareous soils, and P, Mo, Mg, Ca, and K deficiencies, together with proton, Al and Mn toxicities, limit crop production on acid soils. Since essential mineral elements are acquired by the root system, the development of crop genotypes with root traits increasing their acquisition should increase yields on infertile soils. This paper examines root traits likely to improve the acquisition of these elements and observes that, although the efficient acquisition of a particular element requires a specific set of root traits, suites of traits can be identified that benefit the acquisition of a group of mineral elements. Elements can be divided into three Groups based on common trait requirements. Group 1 comprises N, S, K, B, and P. Group 2 comprises Fe, Zn, Cu, Mn, and Ni. Group 3 contains mineral elements that rarely affect crop production. It is argued that breeding for a limited number of distinct root ideotypes, addressing particular combinations of mineral imbalances, should be pursued.

Keywords: root architecture, mineral nutrition, rhizosphere, soil solution, uptake

INTRODUCTION

Crop production, worldwide, is restricted by the concentrations and chemical forms of mineral elements present in the soil solution (Table 1). Adequate supplies of the essential mineral elements nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sulphur (S), boron (B), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), nickel (Ni), molybdenum (Mo), and chlorine (Cl) are required for maximal crop production (White and Brown, 2010). Phytoavailability of N, K, P, or S often limits low-input agriculture (Fageria et al., 2011; Mueller et al., 2012), the phytoavailability of Fe, Zn, and Cu limits crop production on alkaline and calcareous soils, which comprise 25–30% of agricultural land (White and Broadley, 2009), and the phytoavailability of K, P, Mg, Ca, or Mo can limit crop production on acid soils, which comprise >40% of agricultural land (von Uexküll and Mutert, 1995; Sumner and Noble, 2003) and additionally suffer from excessive concentrations of protons, aluminium and Mn (Fageria et al., 2011; White and Greenwood, 2013). Since mineral elements are acquired by the root system, it has been suggested that the development of genotypes with appropriate root traits might increase crop yields on infertile soils (White et al., 2005, in press; Lynch, 2007, 2011, in press; Hawkesford, 2011). This paper examines the root traits likely to improve the acquisition of essential mineral elements. It deals with these traits in a general way that is not intended to provide parameter values for sophisticated mathematical models of resource capture (cf. Barber, 1995; Jungk and Claassen, 1997; Dunbabin et al., 2003; Roose and Fowler, 2004; Postma and Lynch, 2012). It is observed that, although efficient acquisition of a particular element requires a specific set of root traits, suites of traits can be identified that benefit the acquisition of several

mineral elements. Breeding for a limited number of distinct root ideotypes, benefitting the acquisition of several elements coincidentally, would optimize the use of research resources.

THE PHYTOAVAILABILITY OF ESSENTIAL MINERAL ELEMENTS

If essential mineral elements are not present in the soil, they must be provided to enable crop production. Various agronomic strategies can be employed to increase the efficiency with which inorganic and organic fertilizers are used. In principle, these optimize the chemistry, quantity, placement, and timing of fertilizer applications (Fageria et al., 2011; Simpson et al., 2011; Mueller et al., 2012; White et al., 2012, in press; White and Greenwood, 2013). These agronomic strategies can be complemented by cultivating genotypes with appropriate root traits. When mineral elements are present in the soil, strategies can be developed to increase their acquisition by roots, thereby improving the mineral nutrition of crops and, ultimately, crop yields (Lynch, 2007, 2011, in press; White and Broadley, 2009; Richardson et al., 2011; White et al., 2012, in press; White and Greenwood, 2013).

Root traits improving the acquisition of essential mineral elements, and tolerance of potentially toxic mineral elements in the soil, have been the focus of many theoretical studies and field, glasshouse and laboratory investigations (White et al., 2005, in press; Lynch, 2007, 2011, in press). The availability of mineral elements for acquisition by roots is determined by (1) interception through root growth, (2) local diffusion in the rhizosphere, and (3) mass flow in the soil solution to the root surface (Table 1; Barber, 1995; Chapin et al., 2002; Fageria et al., 2011). Direct root interception is not considered to be important for the acquisition of mineral elements because the amounts required for

Table 1 | Physical processes likely to supply essential mineral elements to the root surface of plants growing in the field, and the occurrence of deficiency disorders and mineral toxicity symptoms in agricultural systems.

Physical process		Deficiency disorders		Toxicity symptoms	
N	Mass flow \geq Diffusion >> Interception	Frequent	Insufficient N supply	Occasional	Overapplication of N fertilizer
K	Diffusion > Mass flow >> Interception	Frequent	Low phytoavailability, especially in acid soils	Rare	
P	Diffusion >> Mass flow >> Interception	Frequent	Low phytoavailability, especially in acid soils	Rare	
Ca	Mass flow >> Diffusion \approx Interception	Rare	Insufficient Ca in highly weathered tropical soils; low phytoavailability in strongly acidic, sodic or saline soils; low phytoavailability to horticultural crops	Occasional	Calcareous soils
Mg	Mass flow \geq Diffusion >> Interception	Occasional	Insufficient Mg in shallow, coarse soils; low phytoavailability in calcareous, strongly acidic, saline or sodic soils	Rare	
S	Mass flow \geq Diffusion >> Interception	Frequent	Insufficient S supply and low phytoavailability of S in organic fractions	Rare	
B	Mass flow >> Diffusion \approx Interception	Frequent	Insufficient B in sandy, alkaline and heavily limed soils in high rainfall environments	Frequent	Sodic soils
Fe	Mass flow > Diffusion \approx Interception	Frequent	Low phytoavailability in well aerated alkaline and calcareous soils	Occasional	Waterlogged soils
Mn	Mass flow > Diffusion \approx Interception	Occasional	Insufficient Mn in coarse-textured, sandy soils; low phytoavailability in organic, alkaline and calcareous soils	Frequent	Acid mineral soils and waterlogged soils
Zn	Mass flow > Diffusion \approx Interception	Frequent	Low phytoavailability in alkaline and calcareous soils	Occasional	Anthropogenically contaminated soils
Cu	Mass flow > Diffusion \approx Interception	Frequent	Low phytoavailability in organic, alkaline and calcareous soils	Occasional	Anthropogenically contaminated soils
Ni	Mass flow >> Diffusion \approx Interception	Rare	Low phytoavailability in alkaline and mineral soils	Occasional	Soils overlying serpentine or ultrabasic rocks; Anthropogenically contaminated soils
Mo	Mass flow >> Diffusion \approx Interception	Rare	Low phytoavailability in acid soils	Rare	
Cl	Mass flow > Diffusion >> Interception	Rare	Leached soils with low Cl deposition rates	Frequent	Saline soils
Al			Not essential	Frequent	Acid soils
Na			Not essential	Frequent	Saline and sodic soils

It should be noted that the application of mineral fertilizers and soil amendments, soil type, chemistry and microbiology, and the prevailing environmental conditions will all influence the dominant physical process supplying essential mineral elements to the root surface. References: Barber (1995), White and Broadley (2001, 2003, 2009), Chapin et al. (2002), Oliveira et al. (2010), White and Brown (2010), Moradi et al. (2010), Brown and Bassil (2011), Fageria et al. (2011), Marschner and Rengel (2012), White and Greenwood (2013).

plant nutrition are generally far greater than those intercepted. However, root proliferation and elongation are important in exploiting the soil volume, reducing the path length for diffusion and mass flow, and providing an extensive surface area for

the uptake of mineral elements. Diffusion of mineral elements is determined by the concentration gradient between the soil solution and the root surface (White and Greenwood, 2013). It operates over short distances, such as the width of the rhizosphere, and

is especially important for the acquisition of P and K (**Table 1**). It is facilitated by agricultural practices and crop genotypes that increase the concentrations of mineral elements in the soil solution and high-capacity systems for their uptake by root cells (Lynch, 2011; White, 2013). The mass flow of a mineral element to the root surface is determined by its concentration in the soil solution and the transpiration-driven movement of water to the root (White and Greenwood, 2013). It is important for the acquisition of mineral elements with high concentrations in the soil solution, such as N, K, S, Ca, Mg and Cl in agricultural soils, and for mineral elements that are required in relatively small quantities by plants, such as Fe, Mn, Zn, Cu, Ni, B, and Mo (**Table 1**).

Root traits influencing the acquisition of mineral elements include: (1) root elongation rate, lateral root production, root hair characteristics, root length density (root length/soil volume) and soil penetration, all of which increase the volume of soil explored by the root system and the surface area for the uptake of mineral elements, (2) the gravitropism of root growth, which influences the ability to exploit different soil horizons, (3) the proliferation of roots in patches of soil containing high concentrations of mineral elements that are immobile in the soil, which reduces the carbon and energy requirement for their acquisition, (4) the turnover of fine roots, which redistributes carbon following the capture of localized resources, (5) specific root length (length/mass quotient) and formation of aerenchyma, which affect the carbon and energy requirement for resource capture by influencing root respiration, (6) high-capacity systems for the uptake of elements whose delivery to the root surface is determined by diffusion in the rhizosphere, (7) modification of rhizosphere pH and the exudation of organic solutes and enzymes, which affect the concentrations of mineral elements in the soil solution either directly through soil chemistry or indirectly through the culture of appropriate microbial communities, and (8) interactions with microbes either intimately, through mycorrhizal associations or nodulation, or remotely, through the culture of beneficial microbes or exclusion of pathogenic ones in the rhizosphere (**Figure 1A**).

ROOT IDEOTYPES FOR IMPROVING THE ACQUISITION OF ESSENTIAL MINERAL ELEMENTS

The acquisition of different mineral elements, and often different chemical forms of mineral elements, requires different root traits (**Figure 1A**). Nevertheless, suites of traits can be identified that benefit the acquisition of groups of mineral elements (**Figure 1B**). Since different chemical forms of mineral elements are interconverted in the soil, and crop mineral nutrition requires only the acquisition of an essential mineral element, root traits that benefit the acquisition of an element, rather than a particular chemical form of that element, have been identified. For example, the choice of traits for N acquisition is predicated on the supposition that nitrate is often the dominant chemical form of N in agricultural systems, but can be immobilized in the rhizosphere as ammonium, or converted to organic compounds through biological activities. Group 1 comprises elements that are required by crops in large amounts and are often in short supply in low-input agricultural systems. Group 1A comprises elements that are readily soluble and, therefore, can be present

at high concentrations in the soil solution and follow the movement of water in the soil profile. This group includes N, when present as nitrate, and S, when present as sulphate, which are both delivered to the root surface primarily by mass flow, and K, which reaches the root surface both by mass flow and diffusion in the rhizosphere. Boron is also included in Group 1A. The only element in Group 1B is P, which is required in large quantities by plants, can be present in high amounts in the soil, but reaches the surface of the root primarily by diffusion. Group 2 comprises elements required in smaller amounts by crops that are often present in adequate amounts in soils, but whose phytoavailability is constrained by soil chemical properties, such as high soil pH. This group includes Fe, Zn, Ni, Mn, and Cu. Group 3 comprises elements whose supply rarely limits crop production, namely Ca, Mg, Cl, and Mo (White and Broadley, 2001, 2003; Brown and Bassil, 2011; White and Greenwood, 2013). Designing root ideotypes for Group 3 elements is not an immediate priority, and is not discussed further.

ROOT IDEOTYPES FOR THE ACQUISITION OF NITROGEN, SULPHUR, POTASSIUM, AND BORON

The Group 1A root architectural ideotype can be illustrated by the “steep, cheap, and deep” ideotype that facilitates nitrate acquisition, and N-fertilizer use efficiency, in crops (Lynch, in press; White et al., in press). Nitrate is present in high concentrations in the soil solution and follows the movements of water in the soil profile. Deeper roots are considered beneficial for nitrate acquisition and restricting the movement of nitrate to watercourses through leaching (Dunbabin et al., 2003; Lynch, in press; White et al., in press). Nitrate is delivered to plant roots predominantly by mass flow of the soil solution and nitrate acquisition can be accelerated by increasing root length and surface area, and by increasing transpiration (Garnett et al., 2009; Lynch, in press; White et al., in press). Increasing root nitrate uptake capacity can also improve N-acquisition (Lynch, in press). Lynch (in press) has argued that the proliferation of roots in patches of local abundance might be maladaptive for the acquisition of mineral elements that are readily soluble in the soil solution, such as nitrate. However, the proliferation of roots in N-rich patches can improve the acquisition of ammonium (NH_4^+) and organic N-compounds (see below). Increasing the abundance of cortical aerenchyma can reduce the carbon and energy costs associated with a large root system (Lynch, in press). Relationships with N₂-fixing bacteria, whether symbiotic or associative, can improve the N-nutrition of crops and genotypes of both legumes and non-legumes fostering greater biological N₂-fixation often have higher yields in N-limited environments (Kraiser et al., 2011; James and Baldani, 2012; Kumar et al., 2012). The N nutrition of plants growing on unfertilized soils can also be improved through mycorrhizal associations (Fitter and Moyersoen, 1996; Bennett et al., 2013).

Although nitrate is the form of N taken up by roots of most crops, they can also take up NH_4^+ , urea, amino acids, and peptides (Miller and Cramer, 2005; Gojon et al., 2009; Kraiser et al., 2011). Ammonium is the main form of N taken up by plants adapted to acidic and anaerobic soils (Miller and Cramer, 2005). The root architectural ideotype for the acquisition of NH_4^+ , which is relatively immobile in the soil, resembles that for the acquisition

A

Trait	N	S	K	B	P	Fe	Mg	Zn	Mn	Cu	Ca	Mg	Cl	Mo
Early root vigour / faster growth	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Greater root:shoot biomass quotient	1	1	1	1	1	1	1	1	1	1	0	0	0	0
More root branching	1	1	1	1	1	1	1	1	1	1	1	0	0	0
More / longer root hairs	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Greater root length density	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Improved soil penetration	1	0	1	1	0	0	0	0	0	1	0	0	0	0
Deeper roots	1	1	1	1	0	0	0	0	1	0	1	1	0	0
Topsoil foraging (agrvitriopism)	0	1	0	0	1	0	1	0	1	1	0	0	0	0
Lower root turnover	0	0	2	0	0	0	0	0	0	0	1	0	0	0
Root proliferation in organic patches	1	1	0	0	1	1	1	1	1	1	0	0	0	0
Root proliferation in N-rich patches	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Root proliferation in K-rich patches	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Root proliferation in P-rich patches	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Thinner roots	1	1	1	1	1	1	1	1	1	1	0	0	0	0
More aerenchyma (reduced respiratory load)	1	1	1	1	1	1	1	1	1	1	0	0	0	0
Greater cell wall CEC	0	0	2	0	0	0	0	1	1	1	1	1	0	0
Less Casparyan band	0	0	0	1	0	1	1	1	1	1	1	0	1	0
Greater transpiration	1	1	1	1	0	1	1	1	1	1	1	1	1	1
Greater N uptake capacity	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Greater K uptake capacity	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Greater B uptake capacity	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Greater P uptake capacity	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Greater Fe ²⁺ uptake / PM redox capacity	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Greater siderophore uptake capacity	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Secretion of Biological Nitification Inhibitor	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Greater secretion of mucilage	1	1	1	0	1	1	1	1	1	1	0	0	0	0
Increased rhizosphere acidification	0	0	1	1	1	1	1	1	1	1	0	1	0	0
Greater secretion of organic acids	0	1	1	0	1	1	0	1	1	1	0	0	0	0
Greater secretion of siderophores	0	0	0	0	1	1	1	1	1	1	0	0	0	0
Greater secretion of phenolics	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Greater secretion of phosphatases	0	0	0	0	1	1	1	1	1	1	0	0	0	0
Greater secretion of sulphatases	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Modulation	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Mycorrhizal associations	1	1	1	1	1	1	1	1	1	1	0	0	1	0
Culturing N-fixing microbes	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Culturing S-mineralising microbes	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Culturing P-solubilising microbes	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Culturing microbes mobilising micronutrients	0	0	0	0	0	1	1	1	1	1	0	0	0	0
Pathogen resistance	1	1	1	1	1	1	1	1	1	1	1	1	1	1

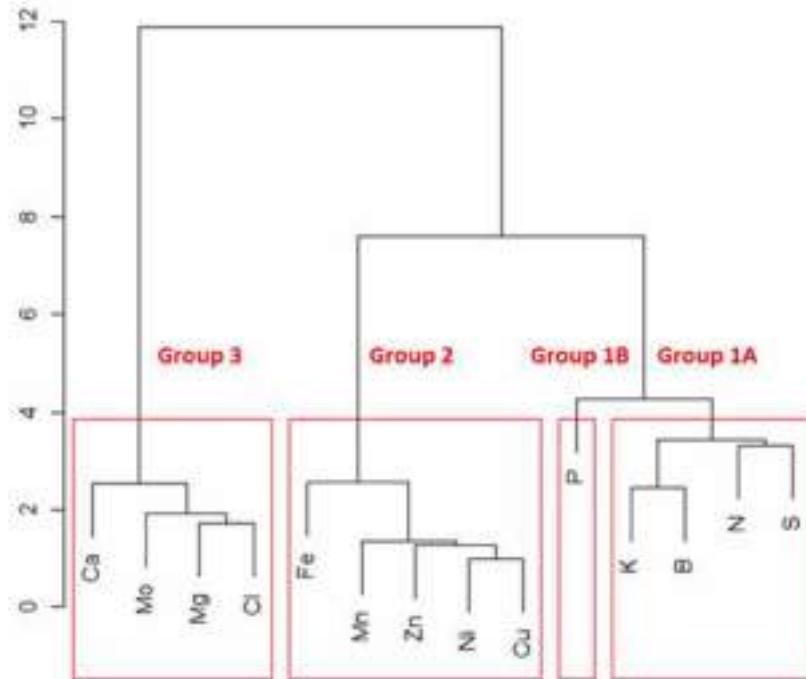
B

FIGURE 1 | (A) Matrix of root traits likely to improve the acquisition of essential mineral elements. Traits are scored as being likely (1) or unlikely (0) to improve the acquisition of an essential mineral element in reduced-input

agricultural systems. **(B)** Clustering of essential mineral elements requiring similar root traits to improve their acquisition. Relationships were calculated from the data presented in the matrix.

of K^+ . This ideotype incorporates high root length densities, which reduce the distance NH_4^+ must diffuse in the rhizosphere to reach the root surface, and the proliferation of roots in NH_4^+ -rich patches. Plants releasing inhibitors of biological nitrification into the rhizosphere limit the conversion of NH_4^+ to nitrate and, thereby, retain N in the vicinity of the root (Subbarao et al., in press). This can improve the efficiency of N-fertilizer use by minimizing nitrate leaching.

Crops take up most of their S as sulphate (De Kok et al., 2011). Effective acquisition of sulphate, which is readily soluble and follows the movement of water in the soil profile, requires similar root architecture to the acquisition of nitrate (White et al., in press). In response to S-deficiency, plants generally increase their capacity for sulphate uptake and alter their root architecture to increase the volume of soil explored at depth, for example by the development of lateral roots close to root apices (López-Bucio et al., 2003; Gojon et al., 2009; De Kok et al., 2011). Increasing transpiration accelerates the delivery of S to the root surface. About 75–90% of the S in soils is present in the topsoil as organic compounds, and S phytoavailability can be enhanced by secreting enzymes degrading soil organic-S, either from plant roots themselves or from their associated microbial communities (Canfield and Farquhar, 2012; Lamers et al., 2012).

Potassium acquisition can be increased by accelerating K^+ delivery to the root surface by increasing mass flow of the soil solution or K^+ diffusion in the rhizosphere. Although K^+ concentrations in soil solutions generally lie between 0.1 and 1 mM, this is not sufficient to supply a rapidly growing crop with enough K by mass flow alone. Root traits that improve K acquisition include: (1) early root vigor and the preferential partitioning of biomass to roots, (2) greater lateral rooting and production of root hairs, increased root length/mass quotient and increased root length density, all of which increase the surface area for K^+ uptake by roots and reduce the distance required for K^+ diffusion and water flow, (3) greater root penetration of strong soils, to improve access to soil resources, (4) the release of organic acids that solubilize “non-exchangeable” K in soils and increase the K^+ concentration in the soil solution, (5) greater K^+ uptake by root cells, which reduces the K^+ concentration at the root surface and accelerates the delivery of K^+ by diffusion, and (6) increasing transpiration, which increases the delivery of K^+ to the root surface through mass flow of the soil solution (Jungk and Claassen, 1997; Rengel and Damon, 2008; White, 2013).

Boron is also included in Group 1A. Boron deficiency in crops is prevalent across a wide range of climates, cropping systems, and soils (Brown and Bassil, 2011). Like other Group 1A elements, B is readily soluble in water and is delivered to the root surface by mass flow. It is easily leached from soils and its distribution in the soil profile is determined by water movements. Thus, root architectural traits required for efficient B acquisition resemble those required for the efficient acquisition of N, S, and K. The phytoavailability of B is restricted in acid and mildly alkaline soils.

ROOT IDEOTYPE FOR PHOSPHORUS ACQUISITION

A “topsoil foraging” root architectural ideotype has been proposed for the acquisition of P, which is relatively immobile in the

soil and concentrated in the topsoil. This ideotype incorporates: (1) early root vigor and the preferential production of roots in the topsoil, (2) greater root branching and the production of long root hairs, (3) high root length density in the topsoil and the proliferation of lateral roots in P-rich patches, (4) greater root length/mass quotient, either through the development of thinner roots or the formation of root aerenchyma, and (5) the partitioning of a greater proportion of plant biomass to the root system (White et al., 2005, in press; Lynch, 2007, 2011; White and Hammond, 2008; Richardson et al., 2011; Brown et al., in press). This architectural ideotype can be complemented by accelerating the diffusion of P to the root surface by increasing (1) the phosphate uptake capacity of root cells, and (2) the phosphate concentration in the rhizosphere solution through the secretion of protons and organic acids to solubilize P-salts and phytases and phosphatases to degrade organic P-compounds, which can be effected either by roots themselves or through the activities of beneficial microbes (Barea et al., 2005; White and Hammond, 2008; Richardson et al., 2011). Associations with mycorrhizal fungi are also known to improve plant P nutrition and increase crop yields (Smith and Read, 2008).

ROOT IDEOTYPES FOR ELEMENTS WITH RESTRICTED PHYTOAVAILABILITY IN ALKALINE SOILS

Topsoil foraging is an appropriate root ideotype for the acquisition Mn, Cu, and Ni, which are relatively immobile in the soil and concentrated in the topsoil, but not necessarily for Fe and Zn, which are distributed more evenly and likely to be phytoavailable throughout the soil profile (White and Greenwood, 2013). An even spread of roots throughout the soil is more suitable for the acquisition of Fe and Zn. Since Fe, Zn, Mn, Cu, and Ni all have restricted mobility in the soil, their acquisition can be improved by investing more biomass in the root system, and by developing a more extensive root system, and by proliferating lateral roots in mineral-rich patches (White and Broadley, 2009; White and Greenwood, 2013). Since the delivery of these elements to the root surface is largely determined by mass flow of the soil solution, increasing transpiration will accelerate their acquisition (White and Greenwood, 2013). Root traits increasing the phytoavailability of these elements in the rhizosphere, such as the secretion of protons, phytosiderophores, and organic acids, increases their acquisition by crops (White and Broadley, 2009, 2011; White and Greenwood, 2013). Similarly, the secretion of enzymes, such as phosphatases, able to degrade organic compounds that chelate cations can increase their phytoavailability and acquisition by crops (White and Broadley, 2009; White and Greenwood, 2013). Associations with mycorrhizal fungi, and the cultivation of microbes that increase the phytoavailability of these elements in the rhizosphere are also beneficial (Barea et al., 2005; White and Broadley, 2009; White and Greenwood, 2013). The diffusion of these elements in the rhizosphere to the root surface can be accelerated by increasing the capacity for their uptake by root cells, either as cations or as phytosiderophore complexes (White and Broadley, 2009; White and Greenwood, 2013). Increasing Fe(III) reductase activity in the rhizosphere increases Fe acquisition (White and Broadley, 2009) and the presence

of microbes oxidizing Mn to Mn²⁺ increases Mn acquisition (Nogueira et al., 2007).

CONCLUSIONS

Although the efficient acquisition of a particular mineral element requires a specific set of root traits, suites of traits can be identified that benefit the acquisition of groups of mineral elements (**Figure 1B**). Elements can be divided into three groups. The elements in Group 1 are nutrients that are often deficient in low-input agriculture. Group 1A comprises N, S, K, and B, whose inorganic forms are readily soluble in the soil solution and large amounts of which reach the root surface by mass flow. Group 1B includes only P, which reaches the root surface primarily by diffusion. The elements in Group 2 are the micronutrients Fe, Zn, Cu, Mn, and Ni that are often present in adequate amounts in soils, but whose phytoavailability is restricted in alkaline soils.

Several root traits will improve the acquisition of mineral elements generally (**Figure 1**). Breeding for crops with improved resource acquisition might target these traits for maximum effect. These traits include early root vigor and greater biomass allocation to roots, architectural traits that increase root exploration of the soil, and anatomical traits that reduce the respiratory burden. Mycorrhizal associations and resistance to root pathogens also appear to benefit the acquisition of most essential mineral elements. Increasing transpiration will improve the acquisition of all elements delivered to the root surface primarily by mass flow of the soil solution. The acquisition of a smaller set of elements is

targeted by breeding for root proliferation in the topsoil (P, Mn, Cu, and Ni) or at depth (N, S, K, and B). Breeding for root proliferation in patches of organic matter will also improve the acquisition of several elements and benefits from the secretion of enzymes that degrade organic compounds into these areas. The secretion of mucilage, organic acids, and phytosiderophores will also improve the acquisition of several mineral elements. By contrast, breeding for root traits that increase the acquisition of either a single or a limited number of element(s) will have utility only in environments in which agricultural production is restricted by lack of a single or few key elements. Such traits include root proliferation in patches of soil with high phytoavailability of a specific element, increasing the phytoavailability of a single element either through the release of specific exudates or by culturing a microbial population with an exclusive function, and increasing the root uptake capacity for a single element.

We believe that a rational way to apportion resources for harnessing root traits for sustainable agriculture is to prioritize traits benefitting the acquisition of several essential mineral elements and pay less attention to those benefitting the acquisition of only one. In this manner root ideotypes for multiple environments can be produced.

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Biomass and morphology of fine roots of sugi (*Cryptomeria japonica*) after 3 years of nitrogen fertilization

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Increasing nitrogen (N) deposition may affect carbon and nutrient dynamics in forest ecosystems. To better understand the effects of N deposition, we need to improve our knowledge of N effects on fine roots (roots <2 mm in diameter), as they are a key factor in carbon and nutrient dynamics. In this study, we fertilized 1 × 2 m plots in a sugi (*Cryptomeria japonica*) stand (336 kg ha⁻¹ y⁻¹) for 3 years and evaluated the responses of the fine roots to high N load. After fertilization, the concentration of NO₃-N in the soil of N-fertilized (NF) plots was five-times as large as that in the control plots and the effect was more remarkable in the subsurface soil than in the surface soil. The biomass of fine roots <2 mm in diameter appeared to be greater in the NF plots (88 ± 19 g m⁻²) than in the control plots (56 ± 14 g m⁻²), but this difference was not statistically significant. In both plots, 76% of the biomass was accounted for by fine roots that were <1 mm in diameter. In the surface soil, the specific root length of fine roots <1 mm in diameter was significantly greater, and the diameter of those fine roots was marginally smaller, in the NF plots than in the control plots. In addition, the concentration of N in fine roots <1 mm in diameter was marginally greater in the NF plots than in the control plots. There may have been increased production of thinner fine roots or increased root branching in the NF plots. This study suggests that, in general, high N load is likely to have positive effects on sugi in terms of fine root characteristics and the effects on fine-root morphology are more evident than the effects on fine-root biomass.

Keywords: fine-root biomass, fine-root length, fine-root diameter, root tissue density, specific root length, soil depth

INTRODUCTION

Fine roots of forest trees are, in general, defined as roots less than 2 mm in diameter. Fine roots are physiologically highly active parts of root systems and play a key role in water and nutrient uptake at the tree–soil interface. In addition, recent studies indicate that fine roots have relatively fast turnover rates and fine root production accounts for a substantial proportion (10–60%) of the net primary production of forests (Hendrick and Pregitzer, 1993; Ostonen et al., 2005). Fine roots are recognized as one of the most important components for tree growth and in the carbon and nutrient dynamics of forest ecosystems.

Nitrogen (N) is quantitatively and functionally the most important nutrient for plants and for other living organisms. Tree roots take up N from the soil in inorganic forms (NH₄⁺ and NO₃⁻; Genenger et al., 2003; Hawkins et al., 2008), while recent studies suggested that organic N compounds, such as amino acids, are also used in many ecosystems (Näsholm et al., 2009). In addition, the amount and spatial distribution of inorganic N in the soil and their chemical forms are likely to affect the biomass and dynamics of fine roots in forest ecosystems (Fujimaki et al., 2004; Tateno et al., 2004).

On the other hand, excessive N can have negative effects on tree growth (Aber et al., 1998). In the past decades, deposition of inorganic N has increased, especially in industrialized countries,

and a number of studies have examined the effects of excess N load on forest ecosystems in Europe and the USA. The results of these studies indicate that high N loads can cause the decline of some forests (Boxman et al., 1998; Magill et al., 2004) and increased or unchanged tree growth in other forests (Magill et al., 2004; Nagakura et al., 2006). For example, Magill et al. (2004) showed that 15 years of N addition resulted in a continuous increase in biomass in a hardwood stand, whereas decreased biomass with high mortality was observed in a red pine stand. The varied responses of forest trees to N deposition could be attributed to factors such as the amount of deposition, soil fertility, and tree species characteristics. High levels of N deposition that are related to industrial or other human activity have been observed in East Asia. In Japan, for example, forests close to urban areas received higher levels of N deposition than those in remote areas (Shibata et al., 2001; Yoshinaga et al., 2012). In addition, recent rapid economic growth of some countries, such as China, may cause further increases in N deposition (Liu et al., 2013). Therefore, further studies and monitoring are needed in order to better understand the effects of N deposition on the soil (e.g., the amount and spatial distribution of N, soil acidification, and so on) and tree growth in the forest ecosystems of this region.

Fine roots are physiologically active parts of root systems and their responses to changing environments are relatively quick.

Therefore, fine roots can be used as a sensitive indicator of changes in tree physiological status under changing soil environmental conditions, such as drought and acidification (Hirano et al., 2007; Konôpka et al., 2007). Fine roots respond to changes in environmental conditions with changes in fine-root biomass and spatial distribution (Helmisari et al., 2007; Finér et al., 2011); fine root morphology, including diameter and specific root length (SRL) (Ostonen et al., 2007a,b); and fine root chemistry, such as N content and Al/Ca molar ratio (Hirano et al., 2007; Vanguelova et al., 2007). Improving our knowledge about the responses of fine roots to N fertilization would help us to better understand the effects of the changing N conditions on forest ecosystems.

Recently, a chronic N fertilization experiment was conducted in a 20-year-old sugi (*Cryptomeria japonica*) plantation in eastern Japan (Nagakura et al., 2006). In this experiment, inorganic N was supplied at the rate of $336 \text{ kg N ha}^{-1} \text{ y}^{-1}$ for 7 years. Although the N concentration in leaves was higher in the fertilized plots during first 3 years of the experiment, there was no significant effect on aboveground growth of the sugi trees. However, another study showed that 3-year N fertilization at the same rate to small plots ($1 \text{ m} \times 2 \text{ m}$) significantly affected fine root dynamics (Noguchi et al., 2013). In that study, the elongation rates and residence time of fine roots increased with N fertilization, whereas stem growth was not significantly affected. This suggests that sugi is tolerant of high N loads. Neither of these reports provided information about fine root parameters, such as morphology and N concentration, and further study on these fine root parameters was needed to better understand how the trees could acclimate to high N load. Therefore, the objective of this study was to determine the responses of the fine roots of sugi trees to 3 years of N fertilization in terms of biomass, morphology, and N concentration.

MATERIALS AND METHODS

SUGI (*Cryptomeria japonica*)

Sugi is one of the major coniferous species in Japan. Plantations of this species cover half of the plantation area (nearly 20% of all forested area) in the country (Japan FAO Association, 1997) and they are often established on foot slopes or along streams. Rooting depths of sugi trees are about 2–3 m and vertical changes in fine root biomass are moderate (Karizumi, 1974, 1976). In addition, Fujimaki et al. (2007) reported that sugi stands showed higher coefficients for vertical distribution of fine roots (β ; Gale and Grigal, 1987) than shallow-rooting species, such as *Chamaecyparis obtusa* and *Picea glauca* (0.95–0.99 for sugi vs. 0.91 for *Chamaecyparis obtusa* and 0.89 for *Picea glauca*). Their results suggest that the proportion of fine root biomass in subsoil is larger for sugi than for shallow-rooting species. However, Fujimaki et al. (2007) also found low β values in a young (4-year-old) sugi stand and in a sugi stand that seemed to be N deficient (0.83 and 0.91, respectively), suggesting that the vertical distribution of sugi fine roots are affected by factors such as stand age and soil conditions (Fujimaki et al., 2007). As for symbioses with mycorrhizal fungi, sugi has been reported to form arbuscular mycorrhiza, but not ectomycorrhiza (Matsuda, 1994).

STUDY SITE

This study was conducted in a 28-year-old sugi (*Cryptomeria japonica*) plantation in eastern Japan ($36^{\circ}10'N$, $140^{\circ}13'E$, 41 masl). Mean annual air temperature and mean annual precipitation for 1981–2010 at the Tsuchiura weather station, which is located at 10 km south from the study site (Japan Meteorological Agency, <http://www.jma.go.jp/jma/menu/report.html>), were 14.4°C and 1188 mm, respectively. Stand density at the study site was ca. $4,000 \text{ trees ha}^{-1}$. Mean diameter at breast height (DBH) and mean height of the trees were 15.9 cm and 16.9 m, respectively. The soil type was Andosol, according to FAO soil classification (Sakai et al., 2010) and there was only sparse understory vegetation, due to the closed canopy (limited light) and litter that covered the forest floor (Konôpka et al., 2006).

In August 2003, 12 plots ($1 \text{ m} \times 2 \text{ m}$) were established between planting lines. The plots were fertilized with N from October 2003 to November 2006. Each month, 20 L of 10 mM ammonium nitrate solution was sprayed onto 6 of the 12 plots ($336 \text{ kg N ha}^{-1} \text{ y}^{-1}$). The remaining six plots were supplied with same amount of tap water that was equivalent to 120 mm rainfall y^{-1} and used as control plots. Among the 12 established plots, 10 (five fertilized and five control plots) were used for this study. Further information on the site characteristics and the design of the N-fertilization experiment can be found in Noguchi et al. (2013) and Sakai et al. (2010).

SOIL ANALYSES

Soil coring was conducted in August 2003 (before N fertilization) and November 2006 (19 days after the last N fertilization) using a soil auger with a 4.8 cm inner diameter (Split tube sampler, Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands). For initial sampling, three core samples were taken at corners of the $1 \text{ m} \times 2 \text{ m}$ plots. Each core sample included soil from 0 to 20 cm below the surface. The soil cores were divided into two depth levels (0–10 and 10–20 cm). The soil samples from the three sampling locations in each plot were pooled before air-drying. For final sampling, a soil core sample was taken from the inside of each plot and divided into two depth levels. Thus, one sample of each depth level was obtained for each plot [i.e., $N = 5$ for both the N-fertilized (NF) and the control plots]. Samples were air-dried and sieved through a 2-mm mesh. Roots were removed from the samples. Then, 20 g of each soil sample was suspended in 100 mL of 2 mol L^{-1} KCl solution and shaken for 1 h to extract inorganic N. After extraction, the supernatant was clarified by filtration, and the inorganic N concentration was analyzed using flow-injection N analyzers (TN-30 for $\text{NH}_4\text{-N}$ and TN-50 for $\text{NO}_3\text{-N}$, Mitsubishi Chemical Analytech, Tokyo, Japan).

FINE ROOT ANALYSES

Soil coring for fine root analyses were conducted in November 2006 with the same soil auger used for soil analyses. Two 20-cm soil cores were obtained from each plot. The soil cores were divided into two depth levels (0–10 and 10–20 cm) and the soil samples from the two sampling locations in each plot were pooled before root sample preparation. Thus, one sample of each depth level was obtained for each plot (i.e., $N = 5$ for both the NF and the control plots). The soil samples were washed with tap water on a sieve that

had a 0.5-mm mesh in order to remove the soil. Fine roots were manually collected using tweezers. Live roots were distinguished from dead roots primarily by root color and resilience, i.e., black and/or easily broken fragile fine roots were assumed to be dead roots (Konôpka et al., 2006, 2007).

The fine roots were divided into diameter classes of <1 mm and 1–2 mm and were subjected to image analysis. The image analysis was conducted using a root image analysis system that included image analysis software and an image scanner (WinRHIZO Pro 2005b, Regent Instruments, Quebec, QC, Canada). To obtain the images, fine root samples were placed in tap water in a transparent plastic tray and were scanned at 400 dpi using the scanner and a transparency unit. Root length, diameter, and volume were used in further analyses. Data were analyzed separately for diameter classes of <0.5, 0.5–1.0, and 1.0–2.0 mm. After the scanning, fine root samples were dried at approximately 70°C for more than 48 h and were weighed.

Specific root length and root tissue density (RTD) were calculated by following equations:

$$\text{SRL (m g}^{-1}\text{)} = \text{fine root length (m m}^{-2}\text{)}/\text{fine root biomass (g m}^{-2}\text{)} \quad (1)$$

$$\text{RTD (g cm}^{-3}\text{)} = \text{Fine root biomass (g m}^{-2}\text{)}/\text{fine root volume (cm}^3\text{ m}^{-2}\text{)} \quad (2)$$

After weighing, the N concentration of the fine roots was measured using an NC analyzer (Sumigraph NC-22F, SCAS, Tokyo, Japan). Fine roots 1–2 mm in diameter at soil depths of 10–20 cm were not subjected to this N analysis due to limited sample quantity.

STATISTICS

Split-plot ANOVA was conducted to examine the effects of N fertilization and soil depth on fine root parameters and soil inorganic N contents. Sampling plots were considered as a random effect. Analyses of fine root parameters were performed separately for each fine root diameter class. In addition, one-way ANOVA was conducted to examine effects of N fertilization at each soil depth level. A Wilcoxon rank sum test was performed to examine effects of N fertilization on the proportion of fine root length in different diameter classes. These statistical analyses were performed using JMP 9.0 (SAS Institute, Cary, NC, USA). For the Wilcoxon rank sum test, we referred to a table of critical values due to the small number of replicates.

RESULTS

INORGANIC N IN THE SOIL

In August 2003, prior to fertilization, concentrations of NH₄-N and NO₃-N were similar in the control and NF plots (**Tables 1** and **2**). In November 2006, 19 days after the final fertilization, NO₃-N concentrations were significantly greater in the NF plots than in the control plots, but NH₄-N concentrations did not significantly differ between NF and control plots (**Tables 1** and **2**). The increase in NO₃-N concentration was more evident in subsurface soil than in the surface soil.

BIO MASS AND LENGTH OF FINE ROOTS

Mean biomass of total fine roots (i.e., fine roots <2 mm in diameter at soil depths of 0–20 cm) was 56 and 88 g m⁻² in the control

Table 1 | Concentration of inorganic nitrogen in the soil before and after N fertilization.

	Soil depth (cm)	August 2003		November 2006	
		Control	NF	Control	NF
NH ₄ -N (mg kg ⁻¹)	0–10	30 ± 1	31 ± 1	23 ± 3	17 ± 1
	10–20	16 ± 1	15 ± 1	8 ± 0	8 ± 1
NO ₃ -N (mg kg ⁻¹)	0–10	12 ± 1	10 ± 2	22 ± 3	39 ± 6
	10–20	16 ± 3	14 ± 3	9 ± 3	127 ± 31

Data shown are means ± SE (N = 5).

Table 2 | Results of split-plot ANOVA examined for effects of N fertilization (treatment) and soil depth (depth) on concentrations of inorganic nitrogen (NH₄-N and NO₃-N).

Parameter	Effect	August 2003		November 2006	
		F-value	P-value	F-value	P-value
NH ₄ -N	Treatment	0.6	0.48	2.3	0.17
	Depth	390	<0.01	66.8	<0.01
	Treatment × depth	1.5	0.25	4.3	0.07
NO ₃ -N	Treatment	0.9	0.36	14.3	<0.01
	Depth	5.1	0.05	7.2	0.02
	Treatment × depth	0.0	0.92	12.8	<0.01

and the NF plots, respectively. Fine roots <1 mm in diameter accounted for 76% of the total fine root biomass in both plots (**Table 3**). Although mean biomass of the fine roots <1 mm in diameter in the NF plots was 1.6-times as large as that in the control plots, the difference was not statistically significant (**Table 4**). On the other hand, the effect of soil depths on the biomass of fine roots <1 mm in diameter was significant: fine root biomasses in the surface soil were four-times and 2.5-times as large as those in subsurface soil of the control and the NF plots, respectively (**Tables 3** and **4**). The biomass of fine roots 1–2 mm in diameter was not affected significantly by either N fertilization or soil depth (**Table 4**).

The mean length of total fine roots (i.e., fine roots <2 mm in diameter at soil depths of 0–20 cm) was 0.71 in control plots and 1.27 km m⁻² in NF plots. Fine roots <1 mm in diameter accounted for 96% of the total fine root length in both plots (**Table 3**). The length of fine roots <1 mm in diameter was significantly larger in the surface soil than in subsurface soil, but the effect of N fertilization on fine root length was not significant (**Table 4**). For fine roots <1 mm in diameter in the surface soil, the proportion of “very” fine roots (diameter <0.5 mm) was significantly greater in the NF plots (65%) than in the control plots (56%; Wilcoxon rank sum test, $\alpha = 0.05$; **Figure 1**). The length of fine roots 1–2 mm in diameter was not significantly affected by either N fertilization or soil depth (**Table 4**).

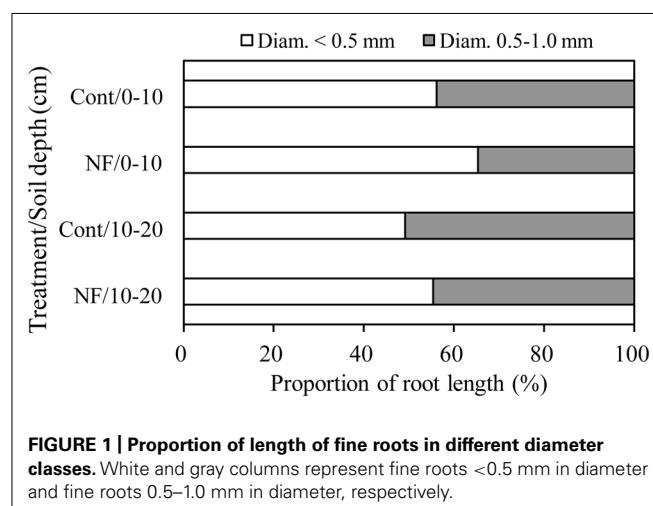
Table 3 | Biomass and length of fine roots of sugi at control and nitrogen-fertilized (NF) plots.

Diameter class	Soil depth (cm)	Biomass (g m^{-2})		Length (m m^{-2})	
		Control	NF	Control	NF
Diameter <1 mm	0–10	34.1 ± 8.0	47.8 ± 10.1	557 ± 146	912 ± 188
<1 mm	10–20	8.6 ± 5.6	19.4 ± 6.3	125 ± 76	311 ± 100
Diameter 1–2 mm	0–10	12.2 ± 4.1	10.5 ± 3.1	24 ± 7	24 ± 7
1–2 mm	10–20	1.1 ± 0.7	10.8 ± 8.3	6 ± 4	27 ± 17

Data shown are means ± SE ($N = 5$).

Table 4 | Results of split-plot ANOVA examined for effects of N fertilization (treatment) and soil depth (depth) on biomass and length of fine roots.

Parameter	Diameter class	Effect	F-value	P-value
Biomass	Diameter <1 mm	Treatment	1.8	0.21
		Depth	19.7	<0.01
		Treatment × depth	0.1	0.82
	Diameter 1–2 mm	Treatment	0.4	0.52
		Depth	2.3	0.17
		Treatment × depth	2.5	0.15
Length	Diameter <1 mm	Treatment	3.0	0.12
		Depth	23.1	<0.01
		Treatment × depth	0.6	0.45
	Diameter 1–2 mm	Treatment	0.8	0.39
		Depth	0.8	0.41
		Treatment × depth	1.8	0.22

**FIGURE 1 | Proportion of length of fine roots in different diameter classes.** White and gray columns represent fine roots <0.5 mm in diameter and fine roots 0.5–1.0 mm in diameter, respectively.

MORPHOLOGY OF FINE ROOTS

The mean diameter of fine roots <1 mm in diameter ranged from 0.52 to 0.57 mm and was significantly smaller in the surface soil than in subsurface soil (Tables 5 and 6). In the surface soil, root diameter was marginally smaller in the NF plots than in the control plots (Table 5; One-way ANOVA, $P = 0.06$). Mean RTD of fine roots <1 mm in diameter ranged from 0.23 to 0.26 g cm $^{-3}$ and was not significantly affected by either soil depth or N fertilization (Tables 5 and 6). Mean SRL of fine roots <1 mm in diameter ranged from 16.0 to 19.2 m g $^{-1}$ (Table 5). Although the effects of soil depth and N fertilization on SRL were not significant, their interactive effect was marginally significant ($P = 0.05$; Table 6), indicating that N fertilization affected SRL differently at different soil depths. At soil depths of 0–10 cm, SRL was significantly greater in NF plots than in control plots (One-way ANOVA, $P = 0.01$). SRL did not differ significantly between NF and control plots at soil depths of 10–20 cm.

The diameter, RTD, and SRL of fine roots 1–2 mm in diameter in the surface soil were similar in control and NF plots and were not significantly different. Statistical analyses were not performed for the fine roots 1–2 mm in diameter in the subsurface soil, because there were no fine roots in this diameter class in the three of five control samples.

N CONCENTRATION IN FINE ROOTS

Nitrogen concentration ranged from 16.2 to 18.2 g kg $^{-1}$ in fine roots <1 mm in diameter, whereas it ranged from 7.7 to 9.0 in roots that were 1–2 mm in diameter (Table 7). The N concentration of fine roots <1 mm in diameter in the surface soil was marginally higher in NF plots than in control plots (One-way ANOVA, $P = 0.06$). However, there was no significant difference between control and NF plots in the N concentration of roots in the subsurface soil or the N concentration of roots 1–2 mm in diameter (data not shown). The effects of soil depth on N concentrations in fine roots were also not significant (data not shown).

DISCUSSION

EFFECTS OF N FERTILIZATION ON THE SOIL

After the 3 years of N fertilization, there was a greater concentration of NO $_3$ -N in the soil of NF plots than in control plots, whereas NH $_4$ -N concentration was not significantly different between the plots. Soil pH decreased in NF plots (from 5.5 to 4.5; Noguchi et al., 2013) and this, when considered together with the results of the present study, suggests that fertilization facilitated nitrification. In addition, the differences in NO $_3$ -N concentration between NF and control plots were more evident in the subsurface soil than in the surface soil. This vertical gradient of NO $_3$ -N in the soil can be attributed to downward movement of NO $_3^-$ ions due to their high mobility, which was often linked to N export from forested watersheds (Ohrui and Mitchell, 1997; Shibata et al., 2001).

Nagakura et al. (2006) conducted a 7-year N-fertilization experiment in an adjacent sugi stand. In that study, NH $_4$ NO $_3$ solution was supplied at the same rate as in the present study (336 kg N ha $^{-1}$ y $^{-1}$). They found that N fertilization increased NO $_3^-$ concentration and decreased pH in the soil solution, which is consistent with our results. In addition, they reported that concentrations of Ca $^{2+}$ and Mg $^{2+}$ in the soil solution also increased

Table 5 | Morphological parameters of fine roots of sugi at control and nitrogen-fertilized (NF) plots.

Diameter class	Soil depth (cm)	Diameter (mm)		RTD (g cm ⁻³)		SRL (m g ⁻¹)	
		Control	NF	Control	NF	Control	NF
Diameter <1 mm	0–10	0.55 ± 0.02	0.52 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	16.0 ± 0.9	19.2 ± 0.3
	10–20	0.57 ± 0.03	0.57 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	17.9 ± 2.1	16.1 ± 0.5
Diameter 1–2 mm	0–10	1.4 ± 0.1	1.3 ± 0.1	0.33 ± 0.03	0.33 ± 0.03	2.2 ± 0.4	2.3 ± 0.3
	10–20	1.3 ^a	1.2 ± 0.1	0.17 ^a	0.20 ± 0.07	5.1 ^a	7.0 ± 3.0

Data shown are means ± SE ($N = 2\text{--}5$).

^aStandard error (SE) was not shown because the number of replication was 2 for these data.

Table 6 | Results of split-plot ANOVA examined for effects of the N fertilization (treatment) and soil depth (depth) on diameter, root tissue density (RTD) and specific root length (SRL) of fine roots <1 mm in diameter.

Parameter	Effect	F-value	P-value
Diameter	Treatment	0.8	0.41
	Depth	11.5	0.01
	Treatment × depth	3.7	0.09
RTD	Treatment	0.0	0.90
	Depth	3.4	0.10
	Treatment × depth	1.7	0.23
SRL	Treatment	0.3	0.62
	Depth	0.3	0.60
	Treatment × depth	5.2	0.05

Table 7 | N concentrations in fine roots of sugi in control and nitrogen-fertilized (NF) plots.

Diameter class	Soil depth (cm)	N concentration (g kg ⁻¹)	
		Control	NF
Diameter <1 mm	0–10	16.6 ± 0.5	18.2 ± 0.5
	10–20	17.9 ± 1.1	16.2 ± 1.1
Diameter 1–2 mm	0–10	7.7 ± 0.6	9.0 ± 0.6
	10–20	N.A.	N.A.

Data shown are means ± SE ($N = 4\text{--}5$).

N.A., not analyzed.

after 2–3 years of fertilization. Thus, cations might also have been released in the NF plots during the present study.

EFFECTS OF N FERTILIZATION ON FINE ROOTS

A number of studies have indicated that soil fertility affects fine root biomass (Yuan and Chen, 2010). In natural conditions, for example, fine root biomass or biomass allocation to fine roots decreased with increasing N availability along slopes (Tateno et al., 2004) or latitudinal gradients (Helmisaari et al., 2007). On the

other hand, N fertilization may increase, decrease, or have no effect on fine root biomass. In Norway spruce (*Picea abies*) stands in Finland and Sweden, for example, N fertilization increased fine root biomass, except at a site that had a fertile soil type (Helmisaari and Hallbäcken, 1999). In contrast, N fertilization combined with wood ash application tended to decrease fine root biomass (Helmisaari et al., 2009). These results suggest that soil fertility together with other chemical conditions in the soil affect the responses of fine roots to N fertilization. The scale of fertilization also affects the pattern of changes in fine root biomass. For example, N fertilization of micro-sites often results in the proliferation of fine roots (Pregitzer et al., 1993; Hodge, 2004).

In the present study, we showed that the biomass and length of fine roots appeared to be larger in NF plots than in control plots, although the difference between the plots was not significant (Tables 3 and 4). A previous study at the same study site indicated that the elongation rate of fine roots <1 mm in diameter was higher and the residence time of the fine roots was longer in NF plots than in control plots (Noguchi et al., 2013). Thus, in the present study, the sugi trees likely responded positively to N fertilization by increasing the biomass and length of fine roots.

The effects of N fertilization were more evident in fine root morphology than in fine root biomass. We found that the 3 years of N fertilization significantly increased the SRL of fine roots <1 mm in diameter in the surface soil (Table 5). The SRL can be described as a function of the diameter and RTD of fine roots (Ostonen et al., 2007b). Our results showed that diameter of fine roots <1 mm in diameter in the surface soil was marginally smaller in NF plots than in the control plots (0.52 vs. 0.55 mm, Table 5), whereas there was no effect of N fertilization on the RTD of these fine roots (Tables 5 and 6). Furthermore, in the surface soil, the proportion (by length) of “very” fine roots (diameter <0.5 mm) was significantly greater in the NF plots than in the control plots (Figure 1). These results suggest that the N fertilization in this study increased the amount of thinner fine roots relative to the amount of thicker fine roots or increased branching of fine roots. Branching of fine roots can result from proliferation of lower-order roots with small diameter (Pregitzer et al., 2002).

The SRL of fine roots has been previously reported to vary with soil N conditions. A meta-analysis by Ostonen et al. (2007b) indicated that the SRL of fine roots generally decreases with N fertilization. This was also supported by a recent study, in which N fertilization decreased SRL of *Pinus tabuliformis* roots, especially

for 1st- and 2nd-order roots (Wang et al., 2013). On the other hand, under heterogeneous soil nutrient conditions, root proliferation often occurs in nutrient rich patches and rapid root proliferation is linked to high SRL (Eissenstat, 1991; Hodge, 2004). SRL has often been used as an index of the cost and benefit of fine roots, assuming that root length is proportional to resource acquisition (benefit) and root mass is proportional to construction and maintenance (cost; Eissenstat and Yanai, 1997). In addition, SRL is reported to be positively correlated with root respiration and N uptake (Reich et al., 1998; Makita et al., 2009). Therefore, the increased SRL of fine roots <1 mm in diameter that was observed in the present study may be a cost-effective response of sugi to increase acquisition of soil resources from N-rich sites.

It is, however, still difficult to explain why SRL increased in the NF plots only in the surface soil and not in the subsurface soil (**Table 5**). Increased N availability is not the only reason because the concentration of inorganic N was much greater in the subsurface soil than in the surface soil (**Table 1**). This vertical pattern of N concentration in the soil is also not consistent with the vertical pattern of N concentrations in fine roots (**Table 7**). In the NF plots, most of inorganic N in the subsurface soil was present as NO_3^- that was highly mobile in the soil solution (**Table 1**). In this condition, roots might not need to proliferate to increase the N acquisition (Hodge, 2004). A recent study of four Norway spruce stands suggested that vertical patterns of SRL might be associated with a base-saturation gradient (Borken et al., 2007). In our study site, N fertilization likely increased cation release into the soil solution,

as occurred in an adjacent sugi stand (Nagakura et al., 2006). Soil physical properties, such as porosity and bulk density, might also affect SRL, as mentioned by Borken et al. (2007). These chemical and physical characteristics of the soil may also affect the morphological responses of fine roots to N fertilization observed in this study.

In conclusion, this study suggests that the effect of high N load was more evident in fine root morphology than in fine root biomass. Fine root biomass tended to increase under high N load, although the effect was not statistically significant. The increase in SRL in the surface soil may reflect a cost-effective means of acquiring soil resources from N-rich patches. However, the observed differences in SRL could not be fully explained by increased N availability, given their vertical patterns. A better understanding of sugi responses to high N load may be gained from future studies in which chemical and physical characteristics of the soil, such as cation leaching or porosity, are examined together with fine root characteristics.

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Interspecific coordination and intraspecific plasticity of fine root traits in North American temperate tree species

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Fine roots play an important role in nutrient and water absorption and hence overall tree performance. However, current understanding of the ecological role of belowground traits lags considerably behind those of aboveground traits. In this study, we used data on specific root length (SRL), fine root diameter (D) and branching intensity (BI) of two datasets to examine interspecific trait coordination as well as intraspecific trait variation across ontogenetic stage and soil conditions (i.e., plasticity). The first dataset included saplings of 12 North American temperate tree species grown in monocultures in a common garden experiment to examine interspecific trait coordination. The second dataset included adult and juvenile individuals of four species (present in both datasets) co-occurring in natural forests on contrasting soils (i.e., humid organic, mesic, and xeric podzolic). The three fine root traits investigated were strongly coordinated, with high SRL being related to low D and high BI. Fine root traits and aboveground life-strategies (i.e., relative growth rate) were weakly coordinated and never significant. Intraspecific responses to changes in ontogenetic stage or soil conditions were trait dependent. SRL was significantly higher in juveniles compared to adults for *Abies balsamea* and *Acer rubrum*, but did not vary with soil condition. BI did not vary significantly with either ontogeny or soil conditions, while D was generally significantly lower in juveniles and higher in humid organic soils. D also had the least total variability most of which was due to changes in the environment (plasticity). This study brings support for the emerging evidence for interspecific root trait coordination in trees. It also indicates that intraspecific responses to both ontogeny and soil conditions are trait dependent and less concerted. D appears to be a better indicator of environmental change than SRL and BI.

Keywords: specific root length, fine root diameter, branching intensity, tree fine roots, phenotypic plasticity, functional traits

INTRODUCTION

The search to understand the effects of species on ecosystem functioning has brought forward the functional role of various traits. Functional traits have been shown to link species to the roles they play in the ecosystem. Through changes at the organismal level they not only influence individual performance but also higher organizational levels and hence drive ecosystem processes and services (Diaz et al., 2004; Garnier et al., 2004). However, we know much more about aboveground traits, their coordination, phenotypic plasticity and linkages to ecosystem functioning than we know about belowground traits.

Although the physiological and ecological importance of roots is well established, the great variability of root systems, the small and varied size of fine roots and the relative inaccessibility of the belowground realm have all hampered exhaustive root research. In addition, the lack of consensus about how to classify and measure fine roots has constrained the development of a unified framework toward a root economics spectrum as was achieved for both leaves (Wright et al., 2004) and wood (Chave et al., 2009) traits. Fine roots have traditionally been distinguished from coarser roots using various diameter classes of arbitrary width,

with 2 mm being the most common threshold (Pregitzer, 2002; Hishi, 2007; Guo et al., 2008). Consequently, fine root samples of different or even the same species may include varying numbers of root orders. Fine root traits such as specific root length (SRL), diameter, root length density as well as nitrogen, lignin, non-structural carbohydrate, and cellulose concentrations have been found to systematically change with root order (Pregitzer et al., 2002; Guo et al., 2004; Wang et al., 2006). Such morphological and physical changes with root order translate into potentially large differences in functional properties such as water uptake (Rewald et al., 2011), respiration (Jia et al., 2011) or fine root mortality (Wells et al., 2002). More recently, a functional classification approach based on root orders has been applied (Guo et al., 2008; Rewald et al., 2011). In tree roots, a first order root would usually be the smallest (i.e., shortest) segment, which would be attached to a second order branch and so forth (Fitter, 2002). Although this approach attempts to control for confounding factors, comparisons across studies are restricted due to varying numbers of root orders included (see for example Yu et al., 2007; Comas and Eissenstat, 2009; Alvarez-Uria and Körner, 2011; Chen et al., 2013).

Above- and below-ground organs share many functions, such as nutrient acquisition and transfer. Some functional coordination between above and belowground traits is therefore expected (Westoby and Wright, 2006). Despite examples of strong coordination in some traits and ecosystems (Reich et al., 1998; Craine et al., 2001; Tjoelker et al., 2005), results remain inconsistent (Westoby and Wright, 2006; Freschet et al., 2010; Chen et al., 2013).

Apart from mean trait values used to coordinate and characterize species, trait plasticity has gained momentum as a driver of individual fitness and consequently, community dynamics. Evidence is accumulating that through changes in realized niches, trait plasticity can be linked to a species' competitive ability and hence overall fitness (Berg and Ellers, 2010). Due to higher spatial and temporal variability of resources belowground, phenotypic plasticity (i.e., plasticity due to environmental changes) is expected to be greater for below- than aboveground traits. There is also evidence of drastic ontogenetic changes in trait values (Cornelissen et al., 2003) that should be more pronounced in long living organisms such as trees. However, only little information about root acclimations to changes in the environment or in ontogeny is available, especially for trees. In addition, much of our knowledge about plant root function is based on seedling responses (Zobel et al., 2007) and on experiments conducted in pots or containers.

Probably the most studied fine root trait is SRL, the ratio between root length and weight (Zobel et al., 2007). Much like the well-known specific leaf area (SLA) for leaves, SRL is thought to describe the economical aspect of a root by weighing the costs (weight) per potential return (length) (Ryser, 2006). Under the assumption that investment in carbon per unit length should be minimized to exploit a larger volume of soil, SRL is expected to be highly plastic and increase under nutrient limitation. Despite examples confirming the assumption (see Ostonen et al., 2007 for a meta-analysis), increases in SRL with increasing nutrient supply as well as no response to changes in nutrient supply have been reported (see Ryser, 2006 for a summary), with equally variable responses to changes in soil water (Ostonen et al., 2007; Cortina et al., 2008; Bakker et al., 2009).

Through its link to surface area and volume, fine root diameter (D) is an important trait directly linked to nutrient and water absorption. Although D has been shown to be plastic and strongly dependent on nutrient supply (Eissenstat et al., 2000), it is rarely a focus of fine root research except as average diameter (Zobel et al., 2007). Research on the response of D to nutrient concentrations showed species specific responses with increases and decreases possibly depending on nutrient, species and their interaction (Zobel et al., 2007).

Lastly, branching intensity (BI, also called root tip density) is a fine root trait describing the topology of fine roots by counting the number of tips per unit root length. Changes in BI to environmental factors have been assessed in only a handful of studies, with contrasting results (Ahlström et al., 1988; George et al., 1997; Kakei and Clifford, 2002).

In the present study, we examined interspecific (coordination) and intraspecific variation across contrasting soil conditions (i.e., plasticity) as well as with ontogenetic stages (i.e., adults versus

juveniles) for SRL, D and BI. A first dataset ("common garden," CG), including 12 North American temperate tree species grown in a common garden experiment was used to examine trait variation across species. We tested the hypotheses that under uniform controlled conditions:

1. SRL, BI and D are strongly coordinated across species of wide variation in root morphology; and
2. Belowground fine root traits are correlated to whole-plant life-strategies, such as relative growth rate.

A second dataset ("natural forest", NF) of four tree species (also present in the CG dataset) that included adults and juveniles co-occurring on contrasting soil conditions in natural forests was employed to examine trait variation in relation to species, ontogeny and soil conditions. More specifically, we tested the hypotheses that:

1. SRL and BI are greater and D smaller in juvenile compared to adult trees;
2. SRL and BI generally increase while D decreases with decreasing soil moisture and nutrient content;
3. Phenotypic plasticity is greater in fine root traits that are more strongly associated with resource uptake (i.e., SRL and D).

MATERIALS AND METHODS

COMMON GARDEN DATASET—CG

Study site

The study site for the first dataset was located at Ste-Anne-de-Bellevue, near Montreal, Québec, Canada (45°26'N, Long 73°56'W, 39 m.s.l.). Mean annual temperature is 6.2°C with a mean annual precipitation of 963 mm (climate.weatheroffice.gc.ca). On this former agricultural field that has been managed for several decades (Marc Samoisette, personal communication, October 2011), monocultures of twelve North American temperate forest species were established in spring 2009 with seedlings of 1 (broadleaf) or 2 (conifer) years of age. These monocultures are part of an ongoing experiment on biodiversity and ecosystem functioning with trees (Tobner et al., submitted). Within the objectives of this biodiversity experiment, the 12 species were selected to cover a wide range of functional traits, including angio- and gymnosperms, and early and late successional species: *Acer saccharum* Marsh., *Acer rubrum* L., *Betula alleghaniensis* Britton, *Betula papyrifera* Marsh, and *Quercus rubra* L. as well as seven conifers: *Abies balsamea* (L.) Mill., *Larix laricina* (Du Roi) K. Koch, *Pinus strobus* L., *Pinus resinosa* Aiton, *Picea glauca* (Moench) Voss, *Picea rubens* Sarg., and *Thuja occidentalis* L.

Each species was planted in a square plot of eight by eight individuals (50 × 50 cm). Plots were replicated four times within an area of ~0.6 ha. Plots were weeded manually and a fence was installed to protect against ungulate herbivory.

Common garden trait measurements

Traits were measured in September 2011. From each plot, two individuals were selected that were growing in the outer rows (to minimize impacts on the ongoing experiment). This was repeated

for each of the four replicate blocks resulting in eight individuals sampled per species. Following the main axis (i.e., stem), a root that grew toward the inside of the plot was detected and followed until it branched off into roots <2 mm. Roots were excavated and placed in a cooler for transport. Roots were then stored at 4°C until processing that occurred no later than 2 weeks after sampling.

Roots were carefully washed and separated into segments of the first three orders. This classification approach (i.e., 1st to 3rd order roots) was chosen following Guo et al. (2008). Root samples were then scanned for subsequent image analysis (Winrhizo, Regent software, Québec). Total root length, average diameter and number of root tips were measured for each sample. Finally, root samples were oven-dried at 65°C and weighed to calculate SRL (m g^{-1}). Relative growth rate (RGR) was calculated based on volume ($([\text{trunk diameter at } 5 \text{ cm}]^2 \times \text{total tree height})$): $\text{RGR} = (\log \text{vol fall 2011} - \log \text{vol spring 2009})/3$ growth periods (i.e., vegetation periods 2009 through 2011).

NATURAL FOREST DATASET—NF

Study site

The study site for the second dataset was situated at the Station de biologie des Laurentides of Université de Montréal in St-Hippolyte, Québec, Canada (Lat 45°59'N, Long 73°59'W, 366 m.s.l.). The research station consists of an area of about 16 km² of forest and lakes dedicated to research and has been protected from other human activities since 1963. Birch (*Betula papyrifera* and *Betula alleghaniensis*) and maple (*Acer saccharum* and *Acer rubrum*) communities are the dominating forest types covering more than 60% of the land surface in terms of canopy cover (Savage, 2001). Mean annual temperature is 3.9°C with a mean annual precipitation of 1164 mm (climate.weatheroffice.gc.ca).

Four forest species, also present in the CG dataset, co-occur in the forests of the research station on contrasting soil conditions: *Acer rubrum*, *Betula papyrifera*, *Abies balsamea*, and *Thuja occidentalis*. Species were selected to include a broad spectrum of phylogeny and different life strategies (growth rate, life span, type of mycorrhization, etc.). We identified three different soil conditions where the studied species occur:

- Humisols with standing water level between 10 to 20 cm below-ground and *T. occidentalis* as the dominant species, hereafter referred to as “humid organic”;

- Orthic humoferric podzols (Courchesne and Hendershot, 1989, personal communication Courchesne, March 2011) on slopes of 28–46° and strong water runoff with *T. occidentalis* as the dominant species, hereafter referred to as “xeric podzol” and
- Orthic humoferric podzols with good drainage, nil to very gentle slope and *B. papyrifera* as the dominant species, hereafter referred to as “mesic podzol.”

For each soil type, three plots covering at least 200 m² were established. Plots were located under closed canopy, with no recent sign of perturbation and at least four adult and four juvenile individuals of the target species. Exceptions were *T. occidentalis* that never occurred on mesic podzols and *B. papyrifera*, for which no juvenile individuals were found, as this species does not regenerate under closed canopies. Juveniles were defined as tree saplings between 25 and 100 cm in height and adult trees were defined as trees with a diameter at 1.3 m (DBH) >10 cm.

Soil characterization

At the center of each plot, one soil sample was taken at 20 cm depth on August 22, 2011. The average daily temperature in the 2 weeks preceding soil sampling was 17.5°C. Precipitation for the same period amounted to 46 mm distributed over 6 days with 15 mm being the strongest precipitation event for 1 day.

Soil samples were placed in resealable plastic bags and immediately stored at –18°C before further processing that occurred no later than 1 week after collection. Samples were then oven-dried at 65°C until they reached constant weight and sieved through a 2 mm mesh prior to soil analyses. Soil moisture was the difference in sample weight before and after drying. Soil pH was measured in water in a ratio of one part soil (10 mg) to two parts water for mineral soil and one part soil (4 mg) to five parts water for organic soils (Canadian Society of Soil Sciences, 2007). Cation exchange capacity (CEC) and base saturation (BS%) were assessed through dissolving soil samples in barium chloride solution and atomic spectroscopy (Canadian Society of Soil Sciences, 2007) (Table 1).

Natural forest trait measurements

On each plot, species and DBH of all adult trees (i.e., DBH >10 cm) were recorded to calculate basal area (Table 1). Adult trees of the site are usually not older than 90 years as the

Table 1 | Soil and stand characteristics of the three soil conditions (means ± sd) for the Natural Forest dataset.

	Soil moisture (%)	pH	CEC (cmol kg ⁻¹)	BS%	Basal area (m ² ha ⁻¹)				
					<i>Abies balsamea</i>	<i>Thuja occidentalis</i>	<i>Acer rubrum</i>	<i>Betula papyrifera</i>	others
HO	85.2 ± 1.8	4.88 ± 1.1	1.9 ± 1.1	95.9 ± 3.4	5.9 ± 2	14.85 ± 2.6	7.1 ± 0.4	6.3 ± 3.5	8.0 ± 4.2
MP	30.7 ± 3.0	5.05 ± 0.0	0.6 ± 0.2	29.9 ± 16.2	7.2 ± 3		4.7 ± 0.4	23 ± 14.1	9.5 ± 5.6
XP	19.2 ± 7.2	4.70 ± 0.3	0.5 ± 0.1	19.1 ± 4.7	6.0 ± 3.4	10.1 ± 5.6	4.0 ± 2.9	6.7 ± 3.0	11.6 ± 14.0

HO, humid organic; MP, mesic podzol; XP, xeric podzol; CEC, cation exchange capacity; BS, base saturation.

last high-intensity fire passed through the research area around 1923 (Savage, 2001).

For the four target species, at least four adult and four juvenile individuals were sampled (i.e., total of 12 adults and 12 juveniles per soil condition). For each adult tree, two root samples were collected in opposite directions from each other. From the stem, roots were excavated and followed until they branched off into fine roots (<2 mm diameter). Roots of adult individuals were excavated from the mineral or organic soil horizons, never from the humus or litter layers. Furthermore, for each adult individual, at least three of the highest branches were harvested with the help of a professional tree climber to obtain sun leaves. For juveniles, the entire plant was excavated for root samples and at least three leaves or 20 needles were collected.

Leaf and root samples were immediately put into sealed plastic bags, labeled and stored at about 4°C until further processing, occurring no later than 6 weeks after sampling. For each individual, 3–5 leaves were punched with a hollow metal pin, yielding leaf samples of a standard surface area. A minimum of 20 needles of the previous year of growth were plucked off the branch and scanned. Samples were then oven-dried to constant weight to calculate SLA (foliage area/foliage weight, mm mg⁻¹).

Root samples (<2 mm) of each individual were carefully washed and scanned and analyzed in an identical fashion to the CG dataset. Once the complete sample was scanned, parts of the image containing first to third order roots were selected and re-analyzed. For these subsamples, average diameter, total length and number of tips were calculated. In addition, root diameter was assessed following the handbook of trait measurements (Cornelissen et al., 2003), on first order roots, after the root hair zone (i.e., after tapering).

Hereafter for both datasets, traits measured on complete root samples (roots <2 mm) are noted using the subscript “c” (e.g., D_c), while results for fine roots defined as first to third order roots are noted with subscript “3” (e.g., D₃). Diameter measured on first order roots is noted as “D₁”.

Phenotypic plasticity

The total phenotypic variability of a population is the result of genetic and environmental sources and their interaction (Hartl and Clark, 1997; Whitman and Agrawal, 2009). To quantify the total variability of a trait we employed the coefficient of variance (CV), i.e., the standard deviation divided by the mean.

In a second step, for each trait and species we calculated an index of the variability which is due solely to variation in the environment, the phenotypic plasticity index (PI). Determining the contribution of the environmental source of variability is essential in assessing a population’s potential to adapt to heterogeneous or changing environments (Byers, 2008). The ability of a genotype to express different phenotypic values for a given trait under different environmental conditions, the phenotypic plasticity (Valladares et al., 2006), is strongly linked to individual fitness (Bell and Galloway, 2007; Nicotra and Davidson, 2010) and hence population demographics as it can generate novelty

and facilitate evolution (Draghi and Whitlock, 2012). Phenotypic plasticity has gained increasing interest with the necessity to predict species responses to global change (Matesanz et al., 2010; Richter et al., 2012). Several metrics have been proposed to assess this environmental source of variability (Valladares et al., 2006). In the present study, we employed the phenotypic plasticity index (PI), a metric recommended to explore functionally related traits. PI is based on maximum and minimum trait means across environmental conditions and was calculated for every trait and species as:

$$\text{[max(trait mean among soil conditions) - min(trait mean among soil conditions)]/max[trait mean among soil conditions]}$$

(Valladares et al., 2006).

Finally, to compare the phenotypic plasticity with the overall phenotypic variability, we computed a ratio of PI to CV (PI:CV) as an expression of how much of the overall phenotypic variability is due to plastic responses to the environment. Both CV and PI vary between zero and one. Hence, a PI:CV of zero would indicate no environmental source of variability, whereas a PI:CV of one would indicate that the overall phenotypic variability is completely due to acclimations to the environment. Although the literature on trait variation and plasticity is rich, we are not aware of other studies using PI:CV to explore differences in relative plasticity between species and traits.

DATA ANALYSIS

For both datasets, traits were tested for normality with the Shapiro test and transformations were applied where needed to correct for deviations. To test for species differences within the CG dataset, a One-Way ANOVA with subsequent Tukey HSD test was performed. Trait correlations were assessed using the Pearson correlation coefficient.

To test for effects of soil condition and ontogenetic stage on fine root traits in the NF dataset, linear mixed effect models (REML) with site (random effect) as well as the interaction of plot and ontogenetic stage nested within soil condition were applied for each species. The asymptotic inference test for coefficients of variation as described in Miller and Feltz (1997) was used to test for differences in CV as well as PI:CV between traits and species. Subsequent Dunn-Sidak corrections (Šidák, 1967) were applied to correct alpha levels for multiple comparisons. To test for differences in PI, resampling methods were applied to create populations per species, ontogenetic stage and trait ($N = 999$). Data were then analyzed using ANOVA models to test for effects of trait and species.

RESULTS

INTERSPECIFIC TRAIT COORDINATION (CG)

In the common garden, fine root traits were highly coordinated across species, especially SRL₃ and D₃ (Table 2). SRL₃ increased with BI₃ and decreased with D₃. Consequently, BI₃ was negatively correlated with D₃. Correlations between fine root traits and whole plant strategies such as RGR were much weaker and never significant (Table 2). In general, conifers showed greater D₃, lower SRL₃, and BI₃ (Table 3).

Table 2 | Correlation matrix for functional traits of 12 North American temperate forest species grown in a common garden.

	D₃	SRL₃	BI₃
SRL ₃	-0.83		
BI ₃	-0.64	0.66	
RGR	0.05	0.07	0.07

Traits include belowground specific root length (SRL), diameter (D) and branching intensity (BI) as well as whole-plant life-strategy measures (i.e., relative growth rate – RGR). Fine root traits were measured on first three root orders (subscript “3”). Significant correlations appear in bold type ($P < 0.001$ in all cases).

Table 3 | Mean trait values for 12 North-American temperate forest species grown in a common garden.

Species	D₃	SRL₃	BI₃	RGR
<i>Thuja occidentalis</i>	0.57 ^A	13.9 ^F	1.2 ^F	0.79 ^{BC}
<i>Pinus strobus</i>	0.56 ^{AB}	16.1 ^F	3.2 ^{BCD}	0.70 ^{CD}
<i>Abies balsamea</i>	0.45 ^{BC}	23.9 ^{EF}	1.9 ^{EF}	0.59 ^{DE}
<i>Larix laricina</i>	0.38 ^{CD}	41.3 ^{DE}	2.8 ^{DE}	0.88 ^{AB}
<i>Pinus resinosa</i>	0.37 ^{CD}	39.5 ^{DE}	3.9 ^D	0.69 ^{CD}
<i>Acer rubrum</i>	0.35 ^{DE}	64.5 ^{ABC}	3.1 ^{CD}	0.75 ^{BC}
<i>Acer saccharum</i>	0.33 ^{DEF}	57.8 ^{BCD}	2.7 ^{DE}	0.67 ^{CD}
<i>Picea glauca</i>	0.33 ^{DEFG}	48.3 ^{CD}	3.1 ^{CD}	0.59 ^{DE}
<i>Betula alleghaniensis</i>	0.28 ^{EFG}	90.3 ^A	4.0 ^{AB}	0.74 ^C
<i>Quercus rubra</i>	0.27 ^{FG}	71.9 ^{ABC}	4.6 ^A	0.68 ^{CD}
<i>Picea rubens</i>	0.27 ^{FG}	68.3 ^{ABC}	2.9 ^{ABC}	0.49 ^E
<i>Betula papyrifera</i>	0.26 ^G	74.0 ^{AB}	4.5 ^A	0.94 ^A

Traits include belowground specific root length (SRL), diameter (D) and branching intensity (BI) as well as whole-plant life-strategy measures (i.e., relative growth rate—RGR). Fine root traits were measured on first three root orders (subscript “3”). Different letters indicate significant differences between species. Angiosperms are underlined in gray.

INTRASPECIFIC TRAIT VARIATION ACROSS ONTOGENETIC STAGES AND CONTRASTING SOIL CONDITIONS (NF)

In the natural forest, fine root diameter in woody (i.e., D_c and D₃) as well as non-woody roots (i.e., D₁) was generally greater in humid organic than in mesic and xeric podzol conditions. However, differences were only significant for *A. balsamea* and *T. occidentalis* (Tables 4, 5). D was also significantly lower for juveniles compared to adults in all three species (Tables 4, 5 and Figure 1). While differences for *A. rubrum* were consistent across fine root classification (i.e., size versus functional) for *T. occidentalis* differences were only significant for the two functional classifications of fine roots (i.e., D₃ and D₁), and for *A. balsamea* there only were significant differences in non-woody roots (i.e., D₁, Tables 4, 5).

SRL_c never varied significantly across soil conditions but was significantly greater for juveniles compared to adults in *A. balsamea* and *A. rubrum*. For juveniles of *T. occidentalis*, SRL_c was smaller as well, but did not vary significantly (Tables 4, 5 and Figure 1). Conversely, BI_c never varied significantly across soil conditions or ontogenetic stage (Tables 4, 5).

PI was greatest in D_c except for *B. papyrifera* adults and *A. rubrum* juveniles. PI for SRL_c and BI_c was more variable and depended on species (Figure 2). The amount of total trait variability (CV), tended to be significantly higher in SRL_c and BI_c, compared to D_c (Figure 2). Consequently, D_c was also the trait with the highest PI:CV.

As expected, SLA was significantly higher in shade-grown leaves of juveniles compared to sun leaves of adults (Table 4). SLA did not vary significantly with soil conditions. The significant interaction term of soil condition and ontogenetic stage for *A. rubrum* is due to a slightly higher SLA for juveniles in mesic conditions (Table 4). When analyzed by species and ontogenetic stage, no significant correlation was found between SLA and SRL (data not shown).

Although fine root classification based on root orders did not uniformly reduce variation (i.e., CV) compared to fine root classification based on size (Table 5), in some cases, it helped detect treatment differences (e.g., D_c to D₃ for *T. occidentalis*, Tables 4, 5).

DISCUSSION

INTERSPECIFIC TRAIT COORDINATION

The observed belowground trait correlations across various taxa indicate strong coordination among fine root morphological traits supporting the idea of a generalized tree root syndrome (Holdaway et al., 2011).

As root diameter and root mass density constitute the two components of SRL, the strong negative correlation between SRL and D was expected (Fahey and Hughes, 1994; Comas and Eissenstat, 2009; Chen et al., 2013). Branching patterns were found to negatively correlate with D when measured as BI (i.e., number of root tips divided by root length, Comas and Eissenstat, 2009) or as branching ratio (number of root tips divided by number of second order roots, Chen et al., 2013) and positively with SRL (Comas and Eissenstat, 2009). As shown by Comas and Eissenstat (2009), there is a possible link between BI and mycorrhization that may in turn determine internal cell structure (e.g., layers of root cortex) and hence D and SRL.

Although evidence is still sketchy, root syndromes are based on a trade-off between life-history strategies (e.g., RGR) and tissue longevity. Thus, roots with high SRL, thin D and low tissue density are generally associated with greater root proliferation, greater RGR and shorter overall longevity (Eissenstat, 1992; Wright and Westoby, 1999). In previous studies, growth rates of juvenile and adult trees have been linked to root traits with fast-growing species showing higher SRL (Reich et al., 1998; Comas et al., 2002; Comas and Eissenstat, 2004), smaller root diameter and greater degree of branching (Comas et al., 2002; Comas and Eissenstat, 2004, note that for these papers, results are for phylogenetically constrained contrasts). Other studies documented no or even negative relationships between SRL and SLA or RGR in grasslands (Poorter and Remkes, 1990; Laughlin et al., 2010; Kembel and Cahill, 2011) and trees (with phylogenetic independent contrasts, Chen et al., 2013).

In the present study, no significant relationships were found between fine root traits and RGR based on volume, height or diameter (only volume is reported). Here, the two species with

Table 4 | P-values for fixed effects (soil condition and ontogenetic stage—OS) of linear mixed models (REML) and their interactions on functional traits of four North-American temperate forest species (NF dataset).

		Diameter			SRL _c	Branching intensity		SLA
		D _c	D ₃	D ₁		BI _c	BI ₃	
Abies balsamea	Soil	0.03*	0.07*	<0.01**	0.95	0.67	0.17	0.47
	OS	0.12	0.30	0.03*	0.01*	0.58	0.44	<0.01**
	Soil+OS	0.72	0.43	0.20	0.71	0.98	0.96	0.34
Thuja occidentalis	Soil	0.09*	0.02*	0.03*	0.22	0.77	0.60	0.66
	OS	0.09*	0.02*	<0.01**	0.72	0.21	0.71	<0.01**
	Soil+OS	0.71	0.95	0.67	0.51	0.66	0.59	0.42
Acer rubrum	Soil	0.13	0.76	0.14	0.55	0.11	0.10	0.09*
	OS	0.04*	0.04*	<0.01**	0.02*	0.63	0.13	<0.01**
	Soil+OS	0.99	0.33	0.53	0.75	0.33	0.47	0.04*
Betula papyrifera ¹	Soil	0.15	0.54	0.10	0.15	0.50	0.65	0.77

Traits include belowground specific root length (SRL), diameter (D) and branching intensity (BI) as well as aboveground specific leaf area (SLA). Fine root traits were measured on roots <2 mm (subscript "c"), first three root orders (subscript "3") or first order roots only (subscript "1").

Significant effects are annotated as **P < 0.01, *P < 0.05, and •P < 0.1.

¹No *B. papyrifera* juveniles were found in the NF plots.

highest SRL were also the species with the highest and lowest RGR (*B. papyrifera* and *P. rubens*, respectively). The study site for the common garden experiment has been intensively cultivated for decades. Nutrient availability can be assumed to be abundant. Interestingly, the four species occurring in both datasets have markedly higher SRL (less so for *T. occidentalis*) in the common garden site, compared to the nutrient poorer natural forest, confuting the often-assumed increase in SRL with nutrient limitation. This indicates that in nutrient abundant habitat, SRL may not be a trait of primary importance for plant growth.

TRAIT VARIATION BETWEEN ONTOGENETIC STAGES

Trait responses to ontogenetic stage were trait dependent. Similar trends of decreasing SRL with age as shown in our study have been reported in the literature for Japanese cedar (*C. japonica*) (Fujimaki et al., 2007), silver birch (*B. pendula*) (Rosenvald et al., 2012), European spruce (*P. abies*) and Turkey oak (*Q. cerris*) (Claus and George, 2005) or in a comparison of laboratory-grown seedlings to field-grown adult trees of six temperate North American tree species (Comas and Eissenstat, 2004). D was also found to increase with tree age (Jagodziński and Kaucka, 2010; Rosenvald et al., 2012).

Two possible mechanisms may explain differences in root morphology with age. On the one hand, higher SRL and lower D in juveniles could be an artifact of differences in root orders measured as it is likely that juvenile root samples <2 mm contain fewer root orders than their conspecific adults. For a multitude of species, SRL and D have been shown to significantly change with root order (Pregitzer et al., 2002; Wang et al., 2006). However, when controlling for root orders in both adults and juveniles, SRL was still higher in juveniles compared to adult trees (Comas and Eissenstat, 2004; Rosenvald et al., 2012).

It appears thus more likely, that the observed changes in root morphology with ontogenetic stage may be an adaptation to rooting depth. In most of the above-mentioned studies examining the effect of tree age on root morphology, including the present study, soil depth was not accounted for. However, changes in SRL and diameter with soil depth have been reported in other studies (Wang et al., 2006; Makita et al., 2011). In the present study, root samples for adult trees were collected in the mineral horizons (often below 10 cm soil depth) while the entire root system of juveniles often did not exceed 10 cm soil depth. Furthermore, juveniles were frequently found on or near rotting logs. Increased SRL and lower D of juveniles could thus be an acclimation to shallow soil depth and possible higher nutrient availability. This is congruent with the assumption that species experiencing large shifts in height and therefore environmental conditions while maturing should experience corresponding shifts in traits (Grime, 2001; Smilauerova and Smilauer, 2007).

It was surprising that BI never changed significantly with ontogenetic stage. In fact, BI also never changed significantly with soil condition, pointing toward a rather conservative trait and fine root topology.

TRAIT PLASTICITY ACROSS SOIL CONDITIONS

As shown above with ontogenetic stages, fine root responses to soil conditions were also trait specific. Despite the large gradient in soil nutrients and water (Table 1), SRL and BI never varied significantly across soil conditions for the four target tree species; only D tended to be greater in humid organic soils.

SRL has been studied extensively and it was often associated with root proliferation in response to nutrient heterogeneity (Hodge, 2004). For trees, SRL has even been described as a

Table 5 | Mean/coefficient of variance (CV) for three fine root traits measured on the same root samples but following different fine root classification approaches.

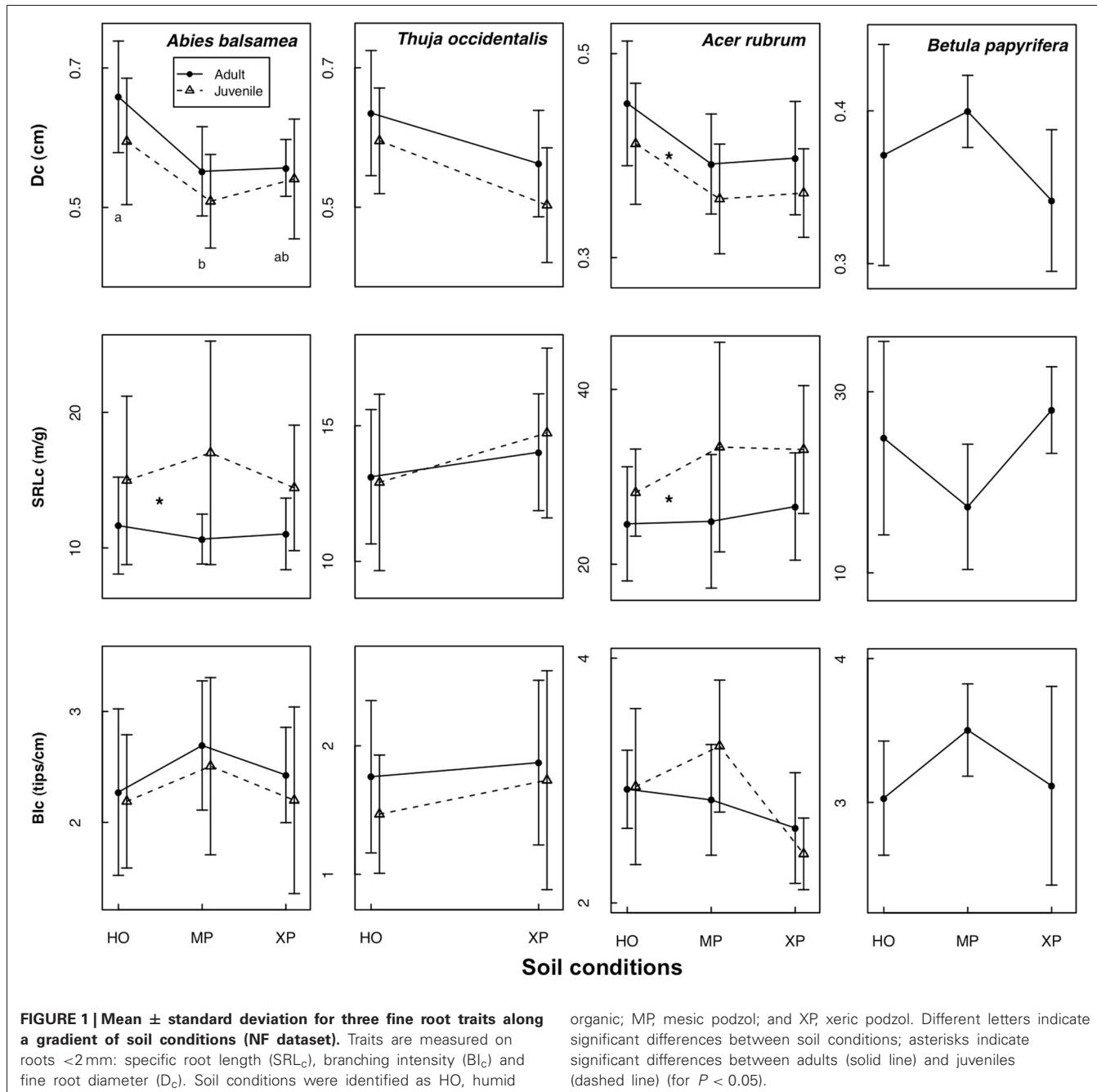
Species	OS	Soil condition	Diameter			SRL _c	Branching Intensity	
			D _c	D ₃	D ₁		BI _c	BI ₃
<i>Abies balsamea</i>	A	HO	a 0.66/0.12	0.62/0.11	a 0.53/0.18	11.7/0.31	2.3/0.33	2.1/0.39
		MP	b 0.55/0.12	0.46/0.23	b 0.39/0.14	10.7/0.17	2.7/0.22	2.7/0.23
		XP	b 0.56/0.07	0.55/0.15	a 0.48/0.16	11.0/0.24	2.4/0.18	2.0/0.30
		All sites	0.59/0.14	0.55/0.20	0.47/0.20	11.1/0.25 AB	2.5/0.25 AB	2.3/0.32
	J	HO	a 0.60/0.15	0.55/0.19	a 0.45/0.12	15.0/0.41	2.2/0.27	2.2/0.34
		MP	b 0.51/0.13	0.47/0.15	ab 0.40/0.15	17.0/0.49	2.5/0.32	2.8/0.22
		XP	ab 0.54/0.16	0.50/0.13	b 0.39/0.12	14.4/0.32	2.2/0.38	2.2/0.28
		All sites	0.55/0.16	0.51/0.17	0.42/0.14	15.5/0.42 a	2.3/0.32 ab	2.4/0.29
<i>Thuja occidentalis</i>	A	HO	0.64/0.14	a 0.65/0.10	a 0.60/0.05	13.1/0.19	1.8/0.34	1.4/0.24
		XP	0.56/0.14	b 0.55/0.21	b 0.51/0.13	14.0/0.15	1.9/0.34	1.3/0.24
		All sites	0.60/0.15	0.60/0.18	0.55/0.14	13.6/0.17 B	1.8/0.33 A	1.3/0.24
	J	HO	0.59/0.13	a 0.57/0.13	a 0.51/0.09	12.9/0.25	1.5/0.31	1.6/0.37
		XP	0.50/0.16	b 0.46/0.13	b 0.40/0.18	14.7/0.21	1.7/0.49	1.4/0.42
		All sites	0.55/0.17	0.52/0.16	0.46/0.18	13.8/0.24 b	1.6/0.43 a	1.5/0.39
<i>Acer rubrum</i>	A	HO	0.45/0.14	0.40/0.14	0.42/0.13	24.6/0.27	2.9/0.11	3.6/0.21
		MP	0.39/0.13	0.36/0.17	0.36/0.19	24.9/0.31	2.8/0.16	3.6/0.24
		XP	0.40/0.14	0.39/0.17	0.36/0.14	26.6/0.23	2.6/0.17	3.1/0.28
		All sites	0.41/0.15	0.39/0.16	0.38/0.17	25.4/0.26 AB	2.8/0.15 b	3.4/0.25
	J	HO	0.41/0.14	0.32/0.18	0.36/0.11	28.2/0.18	3.0/0.22	3.0/0.23
		MP	0.36/0.15	0.33/0.14	0.31/0.15	33.4/0.36	3.3/0.16	3.5/0.21
		XP	0.36/0.12	0.35/0.13	0.34/0.14	33.1/0.22	2.4/0.12	2.7/0.25
		All sites	0.38/0.15	0.33/0.15	0.34/0.14	31.3/0.28 b	3.0/0.21 BC	3.1/0.24
<i>Betula papyrifera</i>	A	HO	0.37/0.20	0.29/0.32	0.30/0.16 b	24.9/0.43	3.1/0.13	3.7/0.29
		MP	0.40/0.06	0.26/0.35	0.22/0.26 a	17.3/0.40	3.5/0.09	3.8/0.20
		XP	0.34/0.14	0.26/0.21	0.23/0.17 b	28.0/0.17	3.1/0.22	4.1/0.23
		All sites	0.36/0.16	0.27/0.29	0.25/0.24	23.2/0.38 A	3.2/0.17 B	3.9/0.24

Subscript "c" indicates a trait measured on roots <2 mm, subscript "3" indicates a trait measured on first to third order roots and subscript "1" indicates a trait measured on first order roots (diameter only). Data shown separately according to ontogenetic stage (OS): A, Adults; J, Juveniles and soil conditions; HO, Humid organic; MP, Mesic podzol and XP, Xeric podzol soil conditions. Different letters to the left of a column indicate significant differences in mean; different letters to the right of a column indicate significant differences in CV between soil conditions. Letters for all sites indicate significant differences between species (upper case for adults, lower case for juveniles).

successful indicator of nutrient availability (Ostonen et al., 2007). Empirical responses of SRL to increases in nutrients have been mixed, however, (Ryser, 2006). Initially, it was proposed that under growth limiting conditions, SRL should be greater (and D smaller) in order to decrease construction costs and invest in greater soil exploitation (Ryser, 2006). And indeed, decreases in SRL with nutrients have been documented (Trubat et al., 2006; Ostonen et al., 2007). However, positive (Majdi and Viebke, 2004; Yu et al., 2007) or non-significant (George et al., 1997; Mei et al., 2010) responses of SRL to nutrients have been documented as well. Despite advances in root research, responses of SRL to nutrient availability still appear somewhat "mysterious" (Ryser, 2006) and SRL has been shown to vary significantly with type of fertilizer, sampling method (i.e., pot, soil coring or ingrowth core)

and root diameter class sampled (i.e., 0–1 mm, <2 mm, etc.) (Ostonen et al., 2007).

As mentioned earlier, SRL has two components: diameter and root mass density. While SRL did not change significantly with soil conditions, D was higher in humid organic conditions compared to mesic and xeric podzolic conditions implying a possible inverse response of root mass density that could explain the lost signal in SRL. In grasses, decreases in nitrogen and phosphorus have been shown to decrease root diameter and increase tissue mass density (Ryser and Lambers, 1995). If the same applied to temperate tree species, then humid organic conditions with their greater water and nutrient content (**Table 1**) would constitute an improvement in plant nutrition. Tissue density in roots has been related to the proportion of stele and of cell wall in the stele,



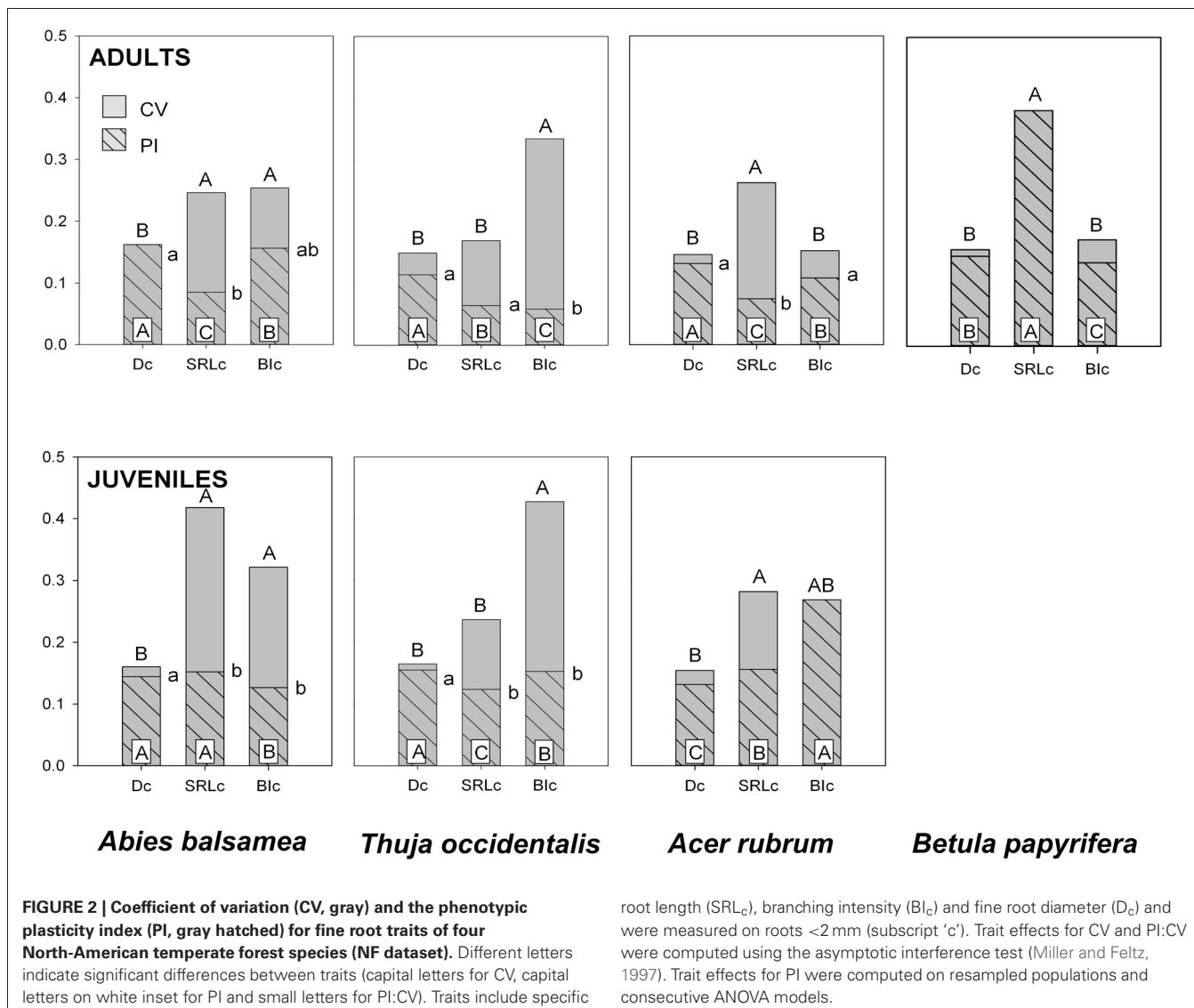
and to characteristics of the tracheary system (Wahl and Ryser, 2000). A reduced percentage of stele in fine roots with decreasing tissue mass density could indicate a reduced importance of conductive tissue in an environment of good plant nutrition as in humid organic soil conditions. Although some studies have reported increases in D with nutrients (Holdaway et al., 2011) and water (Peek et al., 2005; Cortina et al., 2008), its potential as environmental indicator may have been underestimated so far.

A limited number of studies have examined responses of BI to soil nutrition, reporting mostly non-significant changes (George et al., 1997; Bakker et al., 2000). Interestingly, among these few

studies on BI, contrasting results were reported within species (i.e., *Pinus sylvestris*) (Ahlström et al., 1988; George et al., 1997). In the present study, BI proved to be the least variable and least plastic fine root trait responding to neither ontogenetic stage nor soil conditions.

TRAIT PLASTICITY

From the three fine root traits assessed in the present study, D clearly showed the greatest plasticity (PI) and was also the trait where phenotypic plasticity contributed the most to total phenotypic variability (highest PI:CV). This coincides with it



being the most responsive trait to soil conditions (Tables 4, 5). Although more often used to assess acclimations to changes in the environment, SRL_c had significantly greater CV and a lower PI:CV than D_c in most cases. Interestingly, the species with the greatest CV within SRL_c are the two ectomycorrhizal species, *A. balsamea* (juvenile) and *B. papyrifera* (Table 5 and Figure 2), indicating that this greater variability may be due in part to methodological challenges of hyphenated root samples.

Variability of BI was highly species specific. In adults and juveniles, CV for Blc was similar to those of D_c for the two angiosperm species and significantly higher for the two gymnosperm species. In addition, CV was generally higher in juveniles compared to adults. This trend is reversed in many cases when measured on D₃, D₁, or Bl₃ (Table 5), indicating a possible effect of greater variation in root orders comprised in samples <2 mm for juveniles.

CONCLUSION

Fine root morphological traits were found to be strongly coordinated across species, but further work is needed to test for general patterns across ecosystems and biomes. Above- and below-ground traits and whole-plant-strategies may not be as coordinated as previously thought once other factors such as site productivity are accounted for or controlled as we have done in this study for the common garden experiment. For the natural forest experiment, fine root traits responded differently to soil conditions within species, with fine root diameter being the most responsive. Diameter showed the least total variation yet much of it was explained by changes in the environment. Consequently, D may be the most suitable trait for evaluating plasticity to soil nutrition for the rhizosphere.

Lastly, the present study underscores the need for a unified framework of fine root classification and stronger control for the many possible confounding factors in root studies. Although a

functional classification of fine roots managed to reduce variance in a limited number of cases, it improved estimator evaluation in at least one species. Most importantly, a unified framework would greatly facilitate the comparison of studies and therefore increase current understanding of the functional ecology of roots.

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Intraspecific variation in root and leaf traits and leaf-root trait linkages in eight aspen demes (*Populus tremula* and *P. tremuloides*)

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Leaf and fine root morphology and physiology have been found to vary considerably among tree species, but not much is known about intraspecific variation in root traits and their relatedness to leaf traits. Various aspen progenies (*Populus tremula* and *P. tremuloides*) with different growth performance are used in short-rotation forestry. Hence, a better understanding of the link between root trait syndromes and the adaptation of a deme to a particular environment is essential in order to improve the match between planted varieties and their growth conditions. We examined the between-deme (genetic) and within-deme (mostly environmental) variation in important fine root traits [mean root diameter, specific root area (SRA) and specific root length (SRL), root tissue density (RTD), root tip abundance, root N concentration] and their co-variation with leaf traits [specific leaf area (SLA), leaf size, leaf N concentration] in eight genetically distinct *P. tremula* and *P. tremuloides* demes. Five of the six root traits varied significantly between the demes with largest genotypic variation in root tip abundance and lowest in mean root diameter and RTD (no significant difference). Within-deme variation in root morphology was as large as between-deme variation suggesting a relatively low genetic control. Significant relationships existed neither between SLA and SRA nor between leaf N and root N concentration in a plant. Contrary to expectation, high aboveground relative growth rates (RGR) were associated with large, and not small, fine root diameters with low SRA and SRL. Compared to leaf traits, the influence of root traits on RGR was generally low. We conclude that aspen exhibits large intraspecific variation in leaf and also in root morphological traits which is only partly explained by genetic distances. A root order-related analysis might give deeper insights into intraspecific root trait variation.

Keywords: fine root morphology, genetic variation, intraspecific variation, relative growth rate, root tissue density, specific root area

INTRODUCTION

Leaf morphology and foliar nitrogen (N) content are easy to measure plant traits that have widely been used for characterizing plant growth and resource use strategies (e.g., Reich et al., 1997; Diaz et al., 2004). The analysis of large data bases has revealed general patterns of leaf trait syndromes (e.g., Reich et al., 2003) which reflect trade-offs in terms of energy requirements (Wright et al., 2004) and physical constraints of plant growth. Much less information exists about root traits, in particular traits of fine roots (<2 mm in diameter), and their indicative value for recognizing strategies of soil resource exploitation and belowground competitive ability (Bauhus and Messier, 1999). Besides total root biomass and maximum rooting depth (Schenk and Jackson, 2002), important fine root morphological traits are specific root area (SRA, root surface area per mass), specific root length (SRL, root length per mass), root tissue density (RTD, mass per root volume), and fine root tip abundance (no. of tips per root mass) which may have a large influence on the rates of resource uptake (Jackson et al., 1997), root respiration (Pregitzer et al., 1998; Reich et al., 1998b) and rhizodeposition (Nadelhoffer and Raich, 1992;

Jackson et al., 1997). Other functionally important traits are root N concentration and fine root lifespan that influence a root's economy of resource capture (Ryan et al., 1996; Pregitzer et al., 1998; Volder et al., 2005). Roots with greater length and surface development per biomass (high SRL and SRA) can explore larger soil volumes more efficiently and typically have higher resource uptake rates per unit root mass produced than roots with lower SRL and/or SRA. A higher surface (or length) per mass can be achieved either by reducing RTD or/and by decreasing root diameters (Eissenstat, 1991; Reich et al., 1998a; Ryser, 1998; Wright and Westoby, 1999). It has been found that root life span increases with growing RTD, decreased SRA and lowered root N concentration (Withington et al., 2006) in a similar manner as it is characteristic for leaf life span, specific leaf area (SLA) and foliar N concentration.

Despite their small contribution to overall tree biomass (Vogt et al., 1995), fine roots are functionally highly important tree organs that form the plant's interface with the soil and thus may sensitively reflect belowground responses to the environment. While basic knowledge exists about tree species differences in the

structure and dynamics of fine roots (Leuschner and Hertel, 2003; Withington et al., 2006), root traits might also differ among the different genotypes of a species. However, information on the genetic background of intraspecific variation in fine root system structure and its architectural, morphological, and physiological properties is very scarce. This is also true for the linkage between root and leaf traits within the genotypes of a species (Ryser and Eek, 2000). A notable exception with respect to woody plants is the study by Withington et al. (2006) who compared various root traits among 11 temperate tree species and investigated root-shoot relationships on the species level.

The maintenance of intraspecific diversity (i.e., genetic diversity) is an important component of adaptive evolution, driving the ability of plants to colonize habitats of wide ecological amplitudes and to tolerate environmental change (Gregorius and Kleinschmit, 1999; Albert et al., 2011). Early-successional tree species such as *Betula*, *Populus*, and *Salix* taxa with broad ecological niches and large distribution ranges should reveal a particularly large intraspecific diversity with respect to leaf and root traits. Trembling aspen with the European species *Populus tremula* L. (European Common Aspen) and its close North American relative *Populus tremuloides* Michx. (American Quaking Aspen) belong to the most widespread woody species in the world (Hultén, 1986; Dickmann and Kuzovkina, 2008). Due to their large genotypic and also phenotypic variability, aspen may achieve a higher adaptability to future climatic changes than species with less intraspecific variation in leaf and root traits (Hamrick, 2004). Examining this variability particularly for root-related functional traits should substantially improve our understanding of the potential of trees to respond to different environmental conditions.

The present study investigates genotypic variation in fine root traits of aspen populations that originate from a broad range of sites in Central Europe and eastern North America with different climatic conditions. Aspen (*P. tremula* and *P. tremuloides*) as fast-growing pioneer trees with considerable drought tolerance and relatively low nutrient demand are one of the species being considered in short-rotation forestry for producing fiber, wood, and energy (Bradshaw et al., 2000; Taylor, 2002). Due to the continent-wide distribution, aspen may represent a promising study object for investigating genotypic and phenotypic variation in root traits and their linkage to variation in leaf traits. In plantation forestry, it is increasingly important to select genotypes which combine maximum wood production with broad tolerance of stresses associated with climate change. While the intraspecific variation in aboveground morphological, phenological, and physiological traits in aspen and their relation to growth have been investigated in much detail (e.g., Barnes, 1975; Calagari et al., 2006; Müller et al., 2012a), it is not known whether this variation is similarly reflected in root morphology. However, a better understanding of intraspecific trait variation in the root system and its dependence on the genetic relatedness between demes could improve the match between sown varieties and their growth conditions, hence improve growth performance under altered environmental conditions.

The overall goal of this study was to investigate how aspen demes of different geographic origin vary in important root and

leaf morphological traits and biomass N contents when grown at a common site. Following the definition of Gilmour and Gregorius (1939), we use the term “deme”, i.e., an assemblage of taxonomically closely related individuals, for identifying the progeny arrays. These poplar demes do not necessarily represent a specific taxonomic category (e.g., species, subspecies or varieties) or a specific origin of a species in the sense of a locally interbreeding population (Zhang, 2012). More specifically, we aimed to examine whether (1) the within-deme and between-deme variation in leaf morphological traits matches with similar patterns in root morphological trait variation, (2) the intraspecific variation in root and leaf morphology is related to genetic differences between the demes, and (3) how root and leaf morphological traits relate to aboveground productivity.

MATERIALS AND METHODS

STUDY SITE DESCRIPTION

The study was conducted in the framework of the multidisciplinary experiment POPDIV at the University of Göttingen which investigates the role of intraspecific diversity in aspen for productivity and selected ecosystem functions. The common garden experiment was established on the Relliehausen Experimental Farm near Silberborn ($51^{\circ} 44'56''N$, $09^{\circ}32'28''E$) in the Solling Mountains, about 60 km west of Göttingen (Lower Saxony, Germany). The study area is located at 485 m a.s.l. in the uplands of Central Germany with a sub-oceanic, cool-temperate climate (mean annual temperature of $6.6^{\circ}C$; annual mean precipitation of 1110 mm). The soil is unfertilized relatively nutrient-poor haplic Cambisol on Triassic sandstone (Middle Bunter) of sandy-loamy texture (Keuter et al., 2013). The site was previously used as extensive cattle pasture. A coring campaign prior to the experiment's start showed that the soil is homogenous across the site, thus effects of soil heterogeneity can be excluded throughout all 14 investigated blocks. Some soil characteristics are given in **Table 1**.

PLANT MATERIAL

For the study, we used saplings of seven demes of *P. tremula* and one deme of the closely related *P. tremuloides*. The American taxon *P. tremuloides* and its close Eurasian relative *P. tremula* are either considered as sister species (Cervera et al., 2005; Pakull et al., 2009; Grant and Mitton, 2010) or as conspecific subspecies (Eckenwalder, 1996), depending on the criteria of relatedness used. Both taxa are assumed to have split in the late Miocene

Table 1 | Soil characteristics of the experimental site (0–10 cm, total contents).

soil pH (H_2O)	5.32 ± 0.21
C (%)	4.36 ± 0.03
N (%)	0.33 ± 0.01
K ($mg\ g^{-1}$)	3.70 ± 0.02
Ca ($mg\ g^{-1}$)	1.58 ± 0.02
Mg ($mg\ g^{-1}$)	1.52 ± 0.01
Mn ($mg\ g^{-1}$)	0.67 ± 0.01
Fe ($mg\ g^{-1}$)	12.01 ± 0.08

Given are means \pm se across 14 blocks (after Kleemann, 2010).

about 5–10 Ma ago (Schoell et al., 1994; Shevenell et al., 2004). The data on genetic differentiation among the demes, i.e., the analysis of simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) markers, was kindly provided by the Department of Forest Genetics and Forest Tree Breeding at the University of Göttingen (Zhang, 2012). The places of origin cover a broad range of moderately warm to cool and oceanic to continental temperate climates and include gradients in mean annual temperature (5.4–10.7°C) and annual precipitation (592–1112 mm, **Table 2**). Saplings were raised from seeds or provided as wildlings and out-planted according to a randomized block design comprising 20 blocks (18.0 × 25.5 m) each consisting of six plots. All blocks were surrounded by an additional single tree row serving as buffer zone to avoid edge effects. In each plot, 25 3-y-old poplar plants of a deme were arranged in a rectangular grid with a plant distance of 1.5 m.

ROOT COLLECTION AND ROOT TRAIT ANALYSIS

For the root study, 44 of the 120 plots (in 14 of the 20 blocks) were chosen by random. Between June and early September 2010, fine root (<2 mm in diameter) samples were collected from 18–20 tree individuals per deme in the 44 plots; the sampled individuals were chosen by random from the each 25 plants per plot. With a spade, root samples were collected from the upper 30 cm of the mineral soil at a stem distance of 15–30 cm. To ensure that the root samples taken consisted indeed of fine roots of the nearby target tree, coarse roots from the respective stem were traced toward the terminal root endings and root coring was carried out at this location. We excavated soil monoliths of ~4000 cm³ volume containing coarse and fine roots of the respective plant individual, transported them to the laboratory and cleaned it with tap water from adherent soil. Fine roots of herbaceous plants were separated from the aspen fine roots and discarded. One aspen fine root branch of ~10 cm length was

extracted from each monolith and used for subsequent analyses of root morphological traits and C and N concentrations in the dry mass. Thus, 18–20 replicate root samples per deme were analysed.

The fine root branches were spread out in a water bath and scanned for their surface area with a transmitting scanner system (Epson Expression 1680 1.0, Japan). Image analysis for determining the surface area, length and mean diameter of the root segments with a maximum diameter of 2 mm was conducted with WinRhizo software (Régent, Quebec, Canada). Additionally, the number of root tips per fine root individual was counted under a stereo-microscope and related to root dry mass. The analysed rootlets were oven-dried at 70°C for 48 h until constant weight. SRL (cm g⁻¹) was calculated from root length divided by dry mass, SRA (cm² g⁻¹) and RTD (g cm⁻³) were obtained from surface area divided by dry mass or dry mass divided by fine root volume, respectively. The dried root material was ground and the C and N concentrations determined with an elemental analyser (Vario III EL, elementar Analysensysteme GmbH, Hanau, Germany).

LEAF COLLECTION AND LEAF TRAIT ANALYSIS

Simultaneously with root sampling, leaf samples were collected from the same 18–20 individuals per deme chosen for root sampling. Four leaves of the first-order twig on the main terminal shoot of a plant were collected from each tree. Digital images of the leaves were taken using a flatbed scanner (Epson Expression 1680 1.0, Japan). The images were analysed with the software WinFolia 2005b (Régent, Quebec, Canada) for their leaf area. The leaves were dried until constant weight at 70°C for 48 h and SLA calculated. The leaves were ground and the leaf material analysed for the C and N contents with an elemental analyser (Vario III EL elementar Analysensysteme GmbH, Hanau, Germany).

Table 2 | The eight aspen demes used in the study and their origin.

Acronym	Country, location	Type of culture	Coordinates	Elevation (m)	Mean annual precipitation (mm)	Mean annual temperature (°C)	Climate characteristics
AU	Austria, Vienna	Seeds	48°16'N 16°19'E	390	600	9.9	Moderately cold winters, warm summers
CH	Switzerland, Birmensdorf	Seeds	47°21'N 08°24'E	692	1101	8.5	Moderately cold winters, moder. warm summers
G1	Germany, Ahrensböck	Seeds	53°59'N 10°38'E	25	664	8.8	Maritime winters, mild summers
G2	Germany, Göttingen	Seeds	51°32'N 09°56'E	315	645	8.7	Mild winters, moder. warm summers
G8	Germany, Göttingen	Seedlings	51°32'N 09°56'E	315	645	8.7	Mild winters, moder. warm summers
PL	Poland, Białystok	Seedlings	53°08'N 23°09'E	160	592	6.7	Cold winters, moder. warm summers
S	Sweden, Edsvalla	Wildlings	59°26'N 13°12'E	101	635	5.4	Cold winters, cool summers
USA	U.S.A.: Mass., Boston/Sandwich	Seeds	42°14'N 71°23'W	80	1112	10.7	Relatively cold winters, warm summers

RELATIVE GROWTH RATE AND ABOVEGROUND BIOMASS

Because the poplar plants were part of a long-term experiment, destructive harvests for determining biomass data and relative growth rate (RGR) directly were not possible. Alternatively, we estimated aboveground biomass (AGB) from root collar diameter (D_0) and tree height (h) applying an allometric equation (Equation 1) established empirically by Heinrichs (2010) in a nearby young *P. tremula* stand on a forest clear-cut with similar site conditions.

$$\text{AGB} = 0.038 \times D_0^{1.270} \times h^{1.388} \quad (1)$$

The calculation of aboveground productivity and aboveground RGR ($\text{g g}^{-1} \text{ d}^{-1}$) based on two sequential measurements of tree height and root collar diameter done for 4–15 plants per deme in April 2010 and April 2011.

STATISTICAL ANALYSES

Statistical analyses were carried out with the software R, version 2.13.2 (R Development Core Team, 2011). The dataset was tested for normal distribution by the Shapiro & Wilk test. In case of non-gaussian distribution, the parameters were log-transformed to meet the assumptions of parametric tests. To test for heteroscedasticity, the fitted values were plotted against the residuals and inspected graphically. We used one-way ANOVA to analyse the influence of deme identity on the investigated morphological trait interactions. The General Linear Hypotheses (glht) procedure with Tukey's *post-hoc* test (contained in the "multcomp" -package) was applied to detect significant differences in the analysed trait means among the eight demes. Pearson correlation analysis was used to test for relationships between different root traits of the plants and for investigating interrelationships between above- and belowground traits. To test for the relatedness of morphological trait variation and genetic variation across the eight demes, a Mantel test was performed (5000 permuted data sets) using the software Past (Hammer et al., 2001). The information on genetic differentiation among the demes, which bases on the analysis of SSR and AFLP markers, was kindly provided by the Department of Forest Genetics and Forest Tree Breeding at the University of Göttingen (Zhang, 2012). We calculated coefficients of within-deme variation (CV_{intra}) and of between-deme variation (CV_{inter}) using Equation 2:

$$\text{CV} (\text{in percent}) = \text{SD}/\text{mean} \times 100 \quad (2)$$

for allocating total measured trait variation to a genetic component (CV_{inter}) and a predominantly environmental component (CV_{intra}).

A Principal Components Analysis (PCA) was performed on leaf and root morphological and growth-related traits using the software Canoco for Windows 4.5. Means of all investigated parameters were standardized and constructed on the two main axes (PC1 and PC2) in the orthogonal plane in addition to the allocation of the eight demes.

All traits that were found in the PCA to be most closely related to RGR were used as explanatory variables in a multiple linear regression to identify their relative importance for plant productivity; traits with close interrelationship or derived from each

other were excluded (except for leaf size and SLA). Multiple linear regressions were calculated by stepwise backward model selection using the "stepAIC"-function from the "MASS"-package (Venables and Ripley, 2002) for model simplification.

RESULTS

BETWEEN- AND WITHIN-DEME VARIATION IN ROOT MORPHOLOGY AND ROOT N CONCENTRATION

Five of the six investigated root traits (diameter, SRA, SRL, root tip abundance, root N concentration) differed significantly between the demes while one (RTD) did not (Table 3). Mean fine root diameter was very uniform across the seven *P. tremula* demes (means: 0.23–0.27 mm), while the American *P. tremuloides* deme had a significantly larger mean diameter (0.30 mm; Table 4). The relatively large diameter of this deme corresponded to a particularly small SRA and SRL, while the G1, G2 and G8 demes (*P. tremula*) had the highest SRA and SRL means in correspondence with low diameters (0.23–0.25 mm; however, the difference between these two deme groups mark only a non-significant trend Table 4). The highest between-deme variation was observed for root tip abundance (means ranging from 22.5–39.1 n mg⁻¹; between-deme variation 47.7%; Table 4). The root N concentration mean ranged between 1.39 and 1.75% among the demes and between-deme variation was relatively small (21.3%). RTD was not significantly different between the demes (Table 4). The three demes Austria (AU), Germany (G1) and Poland (PL) showed a particularly high within-deme variation that exceeded for most of the seven root traits the between-deme variation. In the other five demes, CV_{intra} was mostly smaller than CV_{inter} . Between-deme (genetically-determined) variation was largest in root tip abundance and SRL, intermediate in SRA and root N concentration, and lowest in root diameter (Table 4).

BETWEEN- AND WITHIN-DEME VARIATION IN LEAF MORPHOLOGICAL AND CHEMICAL TRAITS

Leaf N concentration showed a similarly small variation between the demes (means of 2.21–2.65%; $\text{CV}_{\text{inter}} = 16.9\%$) as root N

Table 3 | Results of ANOVA on leaf and root trait differences between the eight demes.

Trait	F	df	P
Relative growth rate	4.34	73	<0.001
Leaf size	29.80	146	<0.001
SLA	3.49	146	<0.001
Leaf N concentration	3.26	146	<0.01
Fine root diameter	5.80	146	<0.001
SRA	2.73	146	<0.01
SRL	3.84	146	<0.001
Tip abundance	5.33	145	<0.001
RTD	2.04	146	n.s.
Root N concentration	3.25	146	<0.01

Parameters with significant variation across the demes are printed in bold.

Table 4 | Morphological and chemical traits of leaves (fully expanded leaves on terminal twigs) and fine roots (<2 mm in diameter) of the eight aspen demes and their mean relative growth rates (RGR, only aboveground biomass; in mg g⁻¹d⁻¹ for the period April 2010 to April 2011).

	AU	CV intra (%)	CH	CV intra (%)	G1	CV intra (%)	G2	CV intra (%)	G8	CV intra (%)	PL	CV intra (%)	S	CV intra (%)	USA	CV intra (%)	CV inter (%)	CV intra (%)
RGR (mg g ⁻¹ d ⁻¹)	3.20 ± 0.55ab	48.44	2.46 ± 0.42b	64.5	3.67 ± 0.68ab	42.7	1.68 ± 0.24b		2.42 ± 0.96b	88.1	1.59 ± 0.90ab	—	5.25 ± 0.69a	49.2	5.55 ± 0.98a	43.15	76.55	
LEAF TRAITS																		
Leaf size (cm ²)	10.24 ± 0.84a	36.6	6.4 ± 1.02b	69.7	5.55 ± 0.50b	39.6	5.15 ± 0.40b	34.5	4.68 ± 0.50b	48.3	8.19 ± 0.93ab	48.0	6.31 ± 0.63b	44.3	19.11 ± 1.58c	35.2	70.5	
SLA (cm ² g ⁻¹)	105.48 ± 3.25ab	13.8	102.68 ± 2.57b	10.9	119.51 ± 5.19a	18.9	102.96 ± 2.56b	11.1	109.47 ± 3.31ab	13.5	111.33 ± 3.62ab	13.8	110.15 ± 2.23ab	9.0	100.05 ± 3.03b	12.9	14.2	
Leaf N (%)	2.65 ± 0.09a	15.9	2.45 ± 0.11ab	19.5	2.52 ± 0.07ab	12.1	2.22 ± 0.06b	12.8	2.24 ± 0.10b	20.0	2.41 ± 0.10ab	18.1	2.21 ± 0.08b	15.4	2.42 ± 0.07ab	12.6	16.9	
ROOT TRAITS																		
Diameter (mm)	0.27 ± 0.01ab	11.7	0.25 ± 0.01bc	9.5	0.23 ± 0.01c	16.4	0.25 ± 0.01bc	8.9	0.25 ± 0.01bc	9.2	0.26 ± 0.01bc	18.7	0.26 ± 0.01bc	15.7	0.30 ± 0.01a	18.9	15.8	
SRA (cm ² g ⁻¹)	392.77 ± 31.83a	36.2	456.13 ± 22.28ab	21.3	501.09 ± 35.21ab	30.6	515.73 ± 27.28ab	23.7	530.91 ± 23.95b	20.2	454.80 ± 37.61ab	35.1	471.07 ± 29.01ab	27.5	415.64 ± 23.78ab	24.3	28.5	
SRL (m g ⁻¹)	58.66 ± 6.10a	46.5	74.74 ± 4.60ab	26.8	90.59 ± 8.97b	43.2	84.83 ± 5.99ab	31.6	88.82 ± 4.72b	23.7	74.73 ± 8.22ab	46.6	75.14 ± 5.31ab	31.6	58.61 ± 5.31a	38.4	38.6	
RTA (n mg ⁻¹)	22.48 ± 2.47ab	49.2	30.37 ± 2.28bc	32.8	38.09 ± 3.68c	41.0	33.83 ± 2.46ac	32.5	36.08 ± 2.56c	31.0	31.13 ± 3.43bc	46.7	33.3 ± 2.55ac	34.2	20.88 ± 2.37b	48.8	42.7	
RTD (g cm ⁻³)	0.31 ± 0.01a	n.s.	0.32 ± 0.02a	n.s.	0.34 ± 0.02a	n.s.	0.28 ± 0.01a	n.s.	0.29 ± 0.01a	n.s.	0.31 ± 0.02a	n.s.	0.27 ± 0.01a	n.s.	n.s.	n.s.	n.s.	
Root N (%)	1.45 ± 0.07ab	21.1	1.73 ± 0.07b	18.1	1.54 ± 0.09ab	24.7	1.68 ± 0.06ab	16.6	1.75 ± 0.07b	19.2	1.39 ± 0.07a	21.0	1.52 ± 0.06ab	16.3	1.53 ± 0.09ab	25.4	21.3	

For deme acronyms see **Table 2**. The eight aspen demes used in the study and their origin. Given are means ± se, morphological and chemical traits: n = 18 – 20, relative growth rate n = 4 – 15; p < 0.05. The coefficient of variation within the demes (CV_{intra}, %) and between the eight demes (CV_{inter}, %; last column) are given. Different letters indicate significant differences in the means between the demes. RTA – root tip abundance.

concentration. A low between-deme variation (14.2%) was also found for SLA (relatively high SLA means in the *P. tremula* deme G1, particularly low SLA in the *P. tremuloides* deme USA; **Table 4**). In contrast, leaf size was the trait with by far largest between-deme variability (70.5%; **Table 4**). The *P. tremuloides* deme had a four times greater mean leaf size than the deme with smallest leaves (G8) and it exceeded the deme with second largest leaves (AU) nearly twofold (**Table 4**). In contrast to all other investigated leaf or root traits, leaf size showed a much smaller within-deme than between-deme variation (34.5–69.7 vs. 70.5%). A larger leaf size was associated with a higher foliar N concentration; leaf N also increased with increases in SLA (**Table 5**). SLA itself was not related to leaf size in our sample.

THE INFLUENCE OF GENETIC VARIATION ON LEAF AND ROOT TRAIT VARIATION

The results of the Mantel test revealed a close relation between the genetic variation among the demes as visible in the AFLP markers and the variation in aboveground plant biomass recorded for the demes in the year 2011 ($r = 0.87$, $p = 0.04$). Significant relations were also observed for the parameters leaf size and SLA, whereas aboveground growth rate (RGR) and leaf N concentration revealed no correspondence in the distances between the molecular and the trait datasets (**Table 6**). From all investigated root traits, only RTD and root tip abundance showed a significant correspondence between the two data matrices, while the other root traits (root diameter, SRA, SRL, root N concentration) varied independently from genetic variation across the demes. None of the morphological parameters revealed significant relations to SSR markers (data not shown). When the leaf or root traits are pooled in the Mantel test analysis, e.g., all investigated leaf traits or all root traits were merged together, the relations remained significant for the aboveground parameters ($r = 0.77$, $p = 0.001$), while this was not the case for the root traits ($r = 0.71$, $p = 0.07$). When all measured above- and belowground traits were investigated together, the relation was significant ($r = 0.84$, $p = 0.05$).

RELATIONSHIPS AMONG LEAF TRAITS, ROOT TRAITS AND RGR

As expected, SRA and SRL showed highly significant negative correlations to root diameter across the sample ($p < 0.001$, $r = -0.52$ and -0.70 , respectively; **Table 5**). Further, we found inverse relations of RTD to SRA, SRL, and root diameter, i.e., higher tissue densities in thinner roots. Root tip abundance increased with SRA and SRL and decreased with increasing diameter (**Table 5**). Roots with smaller diameter but relatively high SRA and SRL had higher root N concentrations; low tissue density was also linked to higher N concentrations.

Of the 18 tested relationships between root and leaf traits, only five were significant. Demes with higher SLA had smaller fine root diameters, and large-leaved demes had larger root diameters but lower SRA, SRL and tip numbers than demes with smaller leaves (**Table 5**). No significant relationships were found between leaf N concentration and root N concentration, and between SLA and SRA or SRL. While root N concentration and leaf N concentration showed similar variation among the eight demes (CV_{inter} values of 21.3 and 16.9%), SRA and SRL were more variable than

Table 5 | Pearson correlation coefficients for linear relationships between three leaf and six root traits across the eight demes (*n* = 154).

	Leaf size	SLA	Leaf N	Root diam.	SRA	SRL	Tip abund.	RTD	Root N
Leaf size	-								
SLA	-0.11	-							
Leaf N	0.24**	0.32***	-						
Root diameter	0.22**	-0.23**	-0.02	-					
SRA	-0.18*	0.09	-0.03	-0.52***	-				
SRL	-0.21**	0.15	-0.01	-0.70***	0.95***	-			
Tip abundance	-0.26**	0.15	-0.02	-0.63***	0.80***	0.84***	-		
RTD	-0.06	0.07	0.07	-0.27***	-0.54***	-0.29***	-0.22**	-	
Root N	-0.12	0.01	0.10	-0.29***	0.46***	0.46***	0.36***	-0.13	-

Significant correlations are marked by *(*p* < 0.05), **(*p* < 0.01) or ***(*p* < 0.001) and are printed in bold.

Table 6 | Results of a Mantel test conducted to analyse the relationship between morphological trait variance (first matrix) and genetic variance according to AFLP markers (second matrix) in the sample of eight demes.

	Mantel's <i>r</i>	Probability <i>P</i>
Aboveground RGR	0.416	0.082
Aboveground biomass 2010	0.310	0.025
Aboveground biomass 2011	0.870	0.041
Leaf size	0.916	0.040
SLA	0.362	0.002
Leaf N concentration	-0.165	0.773
Fine root diameter	0.855	0.087
SRA	0.280	0.196
SRL	0.478	0.065
Root tip abundance	0.493	0.047
RTD	0.518	0.046
Root N concentration	0.516	0.129
All leaf morphological traits	0.767	0.009
Biomass and growth traits	0.784	0.055
Leaf morphological traits	0.852	0.009
All root traits	0.711	0.074
All root morphological traits	0.567	0.067
All traits	0.840	0.047

Significantly correlating leaf or root traits are printed in bold (*p* < 0.05).

SLA (CV_{inter}: 28.5 and 38.6% vs. 14.2%). Mean leaf size varied up to twofold among the demes and showed a higher total variation (CV_{inter}: 70.5%) than any root trait.

The estimate of mean aboveground RGR for the period April 2010 to April 2011 revealed large differences between the eight demes. The demes with highest RGR (USA: 5.55 and S: 5.25 mg g⁻¹ d⁻¹) grew more than three times faster than the two demes with lowest growth rate (PL: 1.59 and G2: 1.68 mg g⁻¹ d⁻¹) (Table 4). The other four demes reached intermediate rates (2.42–3.67 mg g⁻¹ d⁻¹). The two main axes of the PCA explained 81% of the variability in the ten investigated above- and below-ground variables including RGR (Table 7, Figure 1). Axis 1 with

Table 7 | Principal Components Analysis of the eight poplar demes with respect to relative growth rate and leaf and root morphological properties.

Variables	Axis 1 EV (0.636)	Axis 2 EV (0.176)	Axis 3 EV (0.105)
GROWTH-RELATED VARIABLE			
Aboveground RGR	0.484	-0.035	0.858
LEAF-RELATED VARIABLES			
Leaf size	0.925	-0.167	0.264
SLA	-0.680	0.434	0.364
Leaf N concentration	0.504	0.773	-0.159
ROOT-RELATED VARIABLES			
Fine root diameter	0.944	-0.253	-0.018
SRA	-0.927	-0.324	0.102
SRL	-0.979	-0.129	0.103
Root tip abundance	-0.963	-0.013	0.212
RTD	-0.299	0.859	0.077
Root N concentration	-0.908	-0.146	-0.120

Given are the loadings of the selected variables along the three most important explanatory axes. Eigenvalues are given in brackets in the headline. Numbers in bold mark the variables with the closest correlation to the respective axis (*n* = 4–15 individuals per deme).

an eigenvalue of 0.64 was strongly positively correlated with leaf size and fine root diameter but negatively with the fine root morphological traits SRA, SRL, the number of root tips and root N concentration. However, none of these root traits were significantly related to RGR indicating that the studied aspen genotypes do not achieve faster aboveground growth through alteration of root morphological characteristics in the range of trait variability investigated here. The second axis (eigenvalue 0.18) was primarily associated with leaf N concentration and RTD. Axis 3 contributed with only 11% to the variance and was strongly related to RGR, with no other trait being significantly related to this axis.

A multiple regression analysis with backward variable selection of the possible growth-influencing factors leaf size, SLA, SRL and root tip abundance as predictor variables identified none of the belowground traits as influencing RGR, while leaf size (as a proxy of total leaf area) was detected as the single most

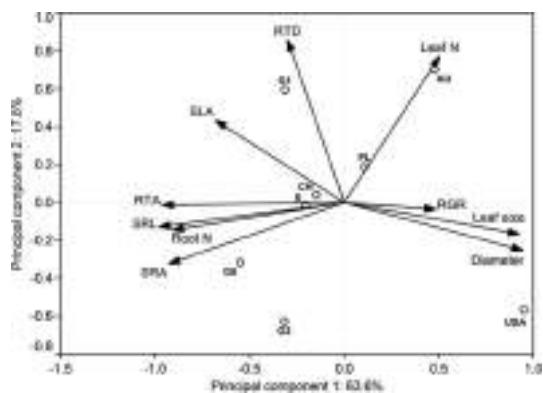


FIGURE 1 | Distribution of relative growth rate and root and leaf morphological properties in the orthogonal plane of the Principal Components Analysis for the eight poplar demes and the percentage contribution of the respective axis to total variability, $n = 4-15$ individuals per deme. G1, G2, G8, CH, S, PL, AU and USA stand for the eight demes.

important trait. However, the model fitted for the whole data set (eight demes) explained only 18% of the RGR variation (data not shown).

DISCUSSION

THE ASPEN FINE ROOT SYSTEM: GENOTYPIC VARIATION vs. PHENOTYPIC PLASTICITY

Across the eight demes and the 18–20 plants investigated per deme, fine root morphology showed a high variability in all parameters except in fine root diameter. Despite identical climatic conditions and uniform soil across the experimental site, within-deme variation was considerable which may be explained either by genetic variation within the deme or by small-scale soil heterogeneity (e.g., variable stone content at the plant scale). The 18–20 plants of a deme varied in their genetic constitution to a certain degree because they were reared after natural pollination on the same parent tree or represent the offspring of a few trees of a population. This genetic variation should add to the phenotypic plasticity due to small-scale environmental variation at the experimental site. An experiment with clonal plants instead of plants reared from seed would allow differentiating between the effects of genetic variability and those of phenotypic plasticity on root morphology. Measuring errors are another likely source of variation. The remarkably small variation in root diameter found across the ~160 aspen plants has to be interpreted with care. It is well recognized that mean fine root diameter is not a good descriptor for the large variation in root morphology and function occurring along the branching hierarchy from the root ending to higher root orders (Pregitzer et al., 2002; Goebel et al., 2010; Rewald et al., 2011; Beyer et al., 2013). Inherent trait variation within the fine root system has also been found in other root traits and it should determine the uptake capacity for water and nutrients through alteration in root surface area or specific root length. For example, even though the means of SRL and root N concentration were similar to our data, these traits varied by a factor of two among

the different fine root orders in the *Populus balsamifera* plants examined by Pregitzer et al. (2002). A more detailed analysis of aspen root systems based on root orders might well have detected morphological differences between the demes that were not visible in our analysis. All five investigated root morphological traits revealed a within-deme variation that was in the same magnitude or higher than between-deme variation. Addressing our second study objective, these findings indicate that the studied traits do not underlie strong genetic control. High phenotypic plasticity represents an adaptive advantage when resource availability varies rapidly in time and space as is the case in soils where alternating periods of infiltration and soil drying and pulsed nutrient release from mineralization require a high flexibility in the placing of roots and in root uptake activity. In contrast to root morphological traits, genotype had a strong influence on leaf morphology and aboveground plant biomass what is in line with a former study by Müller et al. (2012a).

Highly variable environmental conditions such as N and water availability exert a large influence on the structure and morphology of plant root systems; this may often mask the genotypic influence (e.g., Lohmus et al., 1989; Ostonen et al., 2007). Strategies for capturing belowground resources at minimal costs include the production of fine roots with high SRL and SRA allowing to achieve high root length densities in large parts of the soil at relatively low cost, or growing roots selectively into nutrient hotspots and moist patches as observed in two grass species (Mommer et al., 2011). *Populus* species produce very thin roots and can reach much higher SRL than other North American tree species (Pregitzer et al., 2002), what is in line with the observed fast spread of the mainly lateral-distributed root systems of poplars (Pregitzer and Friend, 1996). Intensive lateral root growth indicates that poplars seem to follow strategies of short-term reaction to nutrient hotspots rather than maintaining active root systems in large soil volumes. Such a strategy would fit the adaptation to unstable habitats such as bare sandy soils or flooded alluvial soils where many poplars thrive.

CO-VARIATION BETWEEN ROOT AND LEAF TRAITS

In grassland plants, quite a number of studies have examined the interrelation between leaf and root traits for characterizing resource economic trade-offs, mostly with a focus on SLA and SRA or SRL, or leaf and root N concentrations (e.g., Craine and Lee, 2003; Craine et al., 2005; Tjoelker et al., 2005). As far as we know, our study is the first to search for co-variation in leaf and root traits among different genotypes of a single tree species or species aggregate. Across the eight aspen demes, SLA was inversely correlated with fine root diameter in a similar manner as it was found by Withington et al. (2006) in 11 Central European tree species. In contrast, the SLA–SRL relation was not significant in our study, even though we investigated a total of ~160 plants. The missing SLA–SRL relation in aspen matches with results obtained from the comparison of different grass species (Reich et al., 2003; Tjoelker et al., 2005), but contrasts the tighter SLA–SRL relation detected when comparing the seedlings of different tree species (Reich et al., 1998a; Wright and Westoby,

1999; Withington et al., 2006). A significant relation between root and leaf N concentrations was also lacking in our aspen deme sample which contrasts with the close inter-relationships detected in grass species by Craine and Lee (2003), Craine et al. (2005), Tjoelker et al. (2005) but is in accordance with findings from 11 temperate tree species by Comas and Eissenstat (2009). We also tested for deme differences in the relationship between root and shoot traits using linear models with deme and the respective root trait as explanatory factors and SLA or leaf size as dependent variables, but similarly did not find a significant deme effect on the root-leaf trait linkage. It appears that the significance of inter-relationships between leaf and root properties in a plant is dependent on the variation in plant architectural types and life forms covered by the analysis. The range of trait variation is typically smaller in intraspecific than interspecific comparisons (Comas and Eissenstat, 2009) with the consequence that possible relationships between root and leaf traits may well be masked when the within-deme variation in root traits is high as in our study. Again, a root order-related analysis of root traits might have revealed clearer relations between root and leaf traits even at the intraspecific level. However, applying a more sophisticated root order-related approach would result in a reduced number of replicate root samples that can be processed in due time.

ROOT TRAIT VARIATION AND PLANT GROWTH

Only few studies have examined how root traits are related to plant productivity and growth strategies. Most of the relevant research was carried out with tree seedlings (Reich et al., 1998b; Wright and Westoby, 1999; Comas et al., 2002) or herbaceous plants in greenhouse experiments. Comas and Eissenstat (2004) studied the relation between fine root morphology and chemistry, and growth rate in six-year-old fast- or slow-growing deciduous tree species and found that trees with high potential growth rates constructed roots with smaller diameter, higher SRL, more root tips per unit length and higher root N concentration. In contrast, the recent results of Tobner et al. (2013) did not confirm significant coordination of fine root traits and RGR across North American temperate tree species. Observations in our study hint to the better studied aboveground trait syndromes where high RGR is typically associated with high SLA (Poorter and Garnier, 1999) and a high leaf mass ratio (leaf mass per plant mass) (Poorter and Remkes, 1990; Walters et al., 1993), high shoot N contents and a relatively short leaf longevity (Wright and Westoby, 2000). Müller et al. (2012a,b) conducted a detailed growth analysis in four of the eight aspen demes of this study searching for growth-determining leaf and shoot traits. They concluded that aboveground RGR was primarily determined by total leaf area which itself was largely dependent on the onset of leaf abscission in early autumn in the aspen plants with their continuous leaf production throughout the growing season. Leaf assimilation rate was of minor importance; root traits were not investigated. The results of our regression analysis, which included the aboveground variables leaf size (as a proxy of total leaf area) and SLA and the belowground parameters SRL and root tip abundance, also showed leaf size to be the principal determinant of RGR in the eight-deme sample.

Both the PCA and the multiple regression analysis revealed that root traits in general had only a weak or even no influence on aboveground RGR.

We had assumed that the aspen demes with highest SRA and SRL would grow fastest because high growth rates are generally linked to high rates of water and nutrient consumption (Van den Driessche et al., 2003) requiring root systems with high uptake capacity as indicated in the study of Comas and Eissenstat (2004). Long thin roots with high SRL and SRA should be more effective in the exploration of water and nutrient reserves in a given soil volume (e.g., Bauhus and Messier, 1999). However, they may be more costly in terms of plant resources needed for building them as compared to roots with smaller surface per mass ratios because the former are typically turned over faster and often contain more N per dry mass (Reich et al., 1998b). Surprisingly, we found in the aspen demes a tendency for a negative relation between (aboveground) RGR and SRA, SRL, and root tip abundance, while growth rate seemed to increase with growing fine root diameter. Even though this relation was not significant, it suggests that these root characteristics are not important for aboveground productivity.

The lack of a linkage between fast growth and a high specific fine root surface area (and root traits in general) may have several reasons. First, we investigated only aboveground, but not belowground productivity. Rapid growth requires a high leaf mass ratio which could lead to simultaneous resource limitation for root growth, demanding for the production of less costly thicker roots with higher longevity. Second, fast-growing trees with higher demand for soil resources can achieve the required uptake capacity either by producing thinner more active fine roots, which explore the space more intensively, or by extending their root system if sufficient unexplored soil space is available. The three-year-old aspen plants were still in the stage of expansive root system growth when root sampling took place. Thus, it is possible that the fast-growing demes achieved the assumed higher uptake rate mainly through root system extension and not by forming thinner, more uptake-efficient roots. Unfortunately, we have no information on total root mass and root system size in the eight demes. Finally, genotypic differences in root growth phenology could be as influential, or even more important, for RGR than root morphological traits. Pregitzer and Friend (1996) showed that fast growth in young *Populus* trees was associated with early root growth. Müller et al. (2012a,b) identified phenological traits (the timing of bud burst and the onset of leaf abscission in late summer) as key factors determining aboveground productivity in *P. tremula*. While we found bud burst to differ by two weeks among the demes, we have no data on root phenology.

The aboveground phenological traits of aspen seem to be largely under genetic control but they showed no simple relation to the latitude or temperature at the place of origin (Kleemann, 2010; Müller et al., 2012a). Monitoring of root growth and death by direct observation techniques has to show whether root phenology is indeed a factor influencing aboveground productivity, and how it depends on genetic or environmental control.

CONCLUSIONS

The fine root system of three-year-old aspen progenies (demes) from origins with broadly contrasting climate differed significantly in several morphological traits indicating that SRA, SRL, RTD, tip abundance and mean root diameter are at least to some extent determined by the genetic constitution. However, within-deme variation in the each 18–20 plants was of similar magnitude as between-deme variation, demonstrating a high intraspecific morphological plasticity of the fine root system probably in response to small-scale soil heterogeneity. We did not find a significant relationship between morphological trait variance and genetic variance suggesting that genetic distance is not an important determinant of root trait divergence. The relation between analogous above- and belowground traits was not very tight at the intraspecific level, probably due to masking by high within-deme variation. The large differences in aboveground RGR among the eight demes were tightly linked to genetically determined leaf morphological and phenological traits but were only to a small extent explained by variation in fine root morphology. Even though the studied fine root traits seem not to be good predictors of aspen growth performance, we need more information on genotypic differences in

root morphology and function for aspen progenies and other fast-growing tree species used in short-rotation forestry. The limitations of a simple categorization of fine root biomass into diameter classes suggest applying a morphometric approach based on the separation of root orders for coping with the hierarchical heterogeneity in anatomy, chemistry and function of the branching structure of the fine root system. This may allow characterizing specific belowground resource acquisition and allocation strategies among different provenances of a tree species.

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Intraspecific functional trait variation and performance of *Populus tremuloides*

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A commentary on

Intraspecific variation in root and leaf traits and leaf-root trait linkages in eight aspen demes (*Populus tremula* and *P. tremuloides*)

by Hajek, P., Hertel, D., and Leuschner, C. (2013). *Front. Plant Sci.* 4:415. doi: 10.3389/fpls.2013.00415

The fact that plant-trait and functional-trait syndromes can describe plant growth strategies, and as a result their ecological requirements, has gained considerable importance in ecology (Grime et al., 1997; Westoby et al., 2002). At the global scale, the existence of a major axis of specialization among plants has been determined by measuring leaf functional traits; this axis highlights a fundamental trade-off between rapid acquisition of resources and conservation of resources within well-protected tissues (Díaz et al., 2004; Wright et al., 2004). Díaz et al. (2004) demonstrated that water and nutrient stresses lead to trait-syndrome convergence, with conservative species most present in stressful growth conditions, while acquisitive ones most present in non-stressful growth conditions. This result highlights the functional traits capacity to explain species adaptation to different growth conditions. Moreover, despite the evidence of a large functional traits differentiation within species and the importance of trait variability within a species to establish itself in different habitats (Violle et al., 2012), functional strategies generally have been identified at the species level and did not incorporate within species traits variability.

The fact that functional traits measurement allowed the definition of plant

strategies, and as a result their ability to develop biomass in a variety of growth conditions, led Garnier and Navas (2011) to recommend this approach for agricultural and forestry systems to maximize services of these types of production, and to reduce their environmental drawbacks. However, several questions remain: (i) Do the species used in forestry and agro-systems display different trait syndromes among genotypes?; (ii) Is trait-value variation among genotypes related to genetic differences? and (iii) Are the strategies related to performance of the genotype?

To answer these questions Hajek et al. (2013) test if leaf as well as root functional traits are related to *Populus* varieties performance. In order to do that, they grew seven demes, i.e. an assemblage of closely related individuals (Gilmor and Gregor, 1939), of two conspecific subspecies, the European *Populus tremula* and one deme of the North American *P. tremuloides* in monoculture stands in a common garden in the Solling Mountains (Germany). For each deme, roots were collected in the upper 30 cm of the mineral soil at a stem distance of 15–30 cm from 18 to 20 randomly chosen juvenile trees. Root functional traits, including specific root length (SRL, m.g^{-1}), specific root area (SRA, $\text{cm}^2 \text{g}^{-1}$), root tissue density (RTD, g cm^{-3}), root tip abundance, root diameter, and N content were measured on fine roots (<2 mm) according to Cornelissen et al. (2003). Simultaneously with root sampling, leaves were collected from the same trees to measure leaf area (cm^2), specific leaf area (SLA, $\text{cm}^2 \text{g}^{-1}$), and N and C contents.

Hajek et al. (2013) showed strong intraspecific variation in nearly all functional traits; only RTD did not vary

among demes. These results highlight that although root traits can describe species' strategies well, they may also allow populations within a species to be differentiated. This interpretation is strengthened by the fact that despite the small number of demes considered, two roots traits (root tip abundance and RTD) and two leafs traits (leaf size and SLA) appeared to be linked to genetic variation among demes. Moreover, some demes showed particularly high within-deme trait variation, suggesting the existence of strong within-deme genetic diversity. This result challenge the idea that a species should be described by one trait value and opens interesting perspectives in plant breeding by highlighting the existence of strong within-species trait diversity linked to genetic differentiation, which is a prerequisite for breeding based on functional traits. The authors did not highlight links between above-ground relative growth rates (RGR) and root traits, while leaf traits appeared to be linked to the RGR but are poor predictors of it. They hypothesized that this was due to a stronger relation between root traits and below-ground RGR than above-ground RGR, showing the need for more whole-plant holistic studies to better understand plant functioning. These links were tested in a single growth environment. Root functional traits should be linked more to species performance under conditions where water and nutrient availability limits plant growth and thus where different root strategies should result in contrasting performance (Zhu et al., 2010).

In summary, study by Hajek et al. (2013) demonstrated the existence of significant intraspecific trait variation

linked to genetic differences among demes. They also demonstrated that our knowledge about the links between traits and plant performance must be improved. It is hoped that the Hajek et al. (2013) study will stimulate more research efforts to clarify the relations among trait values, plant relatedness, plant performance and environmental factors, in order to let the concept of functional traits be functional in forestry and agronomy.

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Aluminum exclusion and aluminum tolerance in woody plants

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The aluminum (Al) cation Al^{3+} is highly rhizotoxic and is a major stress factor to plants on acid soils, which cover large areas of tropical and boreal regions. Many woody plant species are native to acid soils and are well adapted to high Al^{3+} conditions. In tropical regions, both woody Al accumulator and non-Al accumulator plants occur, whereas in boreal regions woody plants are non-Al accumulators. The mechanisms of these adaptations can be divided into those that facilitate the exclusion of Al^{3+} from root cells (exclusion mechanisms) and those that enable plants to tolerate Al^{3+} once it has entered the root and shoot symplast (internal tolerance mechanisms). The biochemical and molecular basis of these mechanisms have been intensively studied in several crop plants and the model plant *Arabidopsis*. In this review, we examine the current understanding of Al^{3+} exclusion and tolerance mechanisms from woody plants. In addition, we discuss the ecology of woody non-Al accumulator and Al accumulator plants, and present examples of Al^{3+} adaptations in woody plant populations. This paper complements previous reviews focusing on crop plants and provides insights into evolutionary processes operating in plant communities that are widespread on acid soils.

Keywords: acid soils, adaptation, aluminum, organic acids, tolerance, resistance, toxicity

INTRODUCTION

Aluminum (Al) is a prevalent constituent of most soils and is one of the major stresses to plants in acid soils. Most of the Al in soils is incorporated into aluminosilicates and other precipitated forms, which are harmless to plants. Under acid soil conditions, these minerals solubilize to a limited extent, and the toxic ion Al^{3+} is released into the soil solution (Kinrade, 1997). This form of Al is capable of inhibiting root growth and damaging cells at the root apex, which is the most sensitive part of the root to Al^{3+} (Ryan et al., 1993; Kochian, 1995). However, the mechanism underlying Al^{3+} toxicity is not clearly understood. Because Al^{3+} can interact with a number of extracellular and intracellular structures, many different mechanisms of Al^{3+} toxicity have been proposed. These mechanisms include modification of the cell wall, disruption of the plasma membrane and transport processes, interruption of signaling pathways, and Al^{3+} binding to the DNA (Kochian et al., 2005).

Al^{3+} toxicity is an important research topic, because many crop plants are susceptible in acid soils, and their growth and yield are limited by high Al^{3+} conditions. Less attention is paid to native plant communities, which tolerate acid soil conditions over large areas in different biomes. Acid soils occupy about 30% of the ice-free land area in the world and primarily occur in the humid tropics and the boreal region. Large parts of these soils (about 67%) are covered by forests and woodland (von Uexküll and Mutert, 1995; Figure 1). The biodiversity and high biomass production of both tropical and boreal forests suggest that their plants are not affected by Al^{3+} toxicity.

Al^{3+} toxicity may occur in forests that are exposed to acid deposition derived from air pollutants (Cronan and Schofield, 1979;

Ulrich et al., 1980; Larssen et al., 2006). On sensitive sites, acid deposition accelerates soil acidification and leads to increased Al^{3+} concentrations in the soil solution (Blaser et al., 1999; Fowler et al., 1999). Recently, it was found that soils, affected by acid deposition, showed signs of recovery due to the reduction in sulfate deposition (Stoddard et al., 1999; Evans et al., 2001). However, inputs of nitric acid and ammonia continue to alter the chemistry of forest soils and are likely to promote acidification (Graf Pannatier et al., 2011; Zang et al., 2011). Acid soils characteristically contain high amounts of Al^{3+} and low amounts of the base cations (BC) Ca^{2+} , Mg^{2+} , and K^+ , which are important plant nutrients. Since Al^{3+} and BC interact at the plasma membrane surface, it is not the soil Al^{3+} concentration alone that determines the plant responses to Al^{3+} exposure (Sverdrup and Warfvinge, 1993; Cronan and Grigal, 1995; Kinrade, 2003). A ratio of $\text{Ca}^{2+}/\text{Al}^{3+}$ or BC/Al^{3+} in the soil solution lower than 1 is widely used as an ecological indicator for potentially adverse effects of Al^{3+} stress and nutrient imbalance on tree growth. Alternative indicators are based on the Al and Ca concentrations in fine roots and provide information on the availability of toxic Al^{3+} in the soils (e.g., Brunner et al., 2004; Richter et al., 2007; Vanguelova et al., 2007).

Al^{3+} EXCLUSION AND Al^{3+} TOLERANCE MECHANISMS

The mechanisms conferring resistance to Al^{3+} have been the focus of intensive research in crop plants and in the model plant *Arabidopsis*. Many different mechanisms have been suggested, but for most of them, the supporting genetic and physiological evidence is not provided. Therefore, these mechanisms have to remain speculation or hypotheses until supporting data is provided. One exception is the Al^{3+} -induced efflux of organic acids

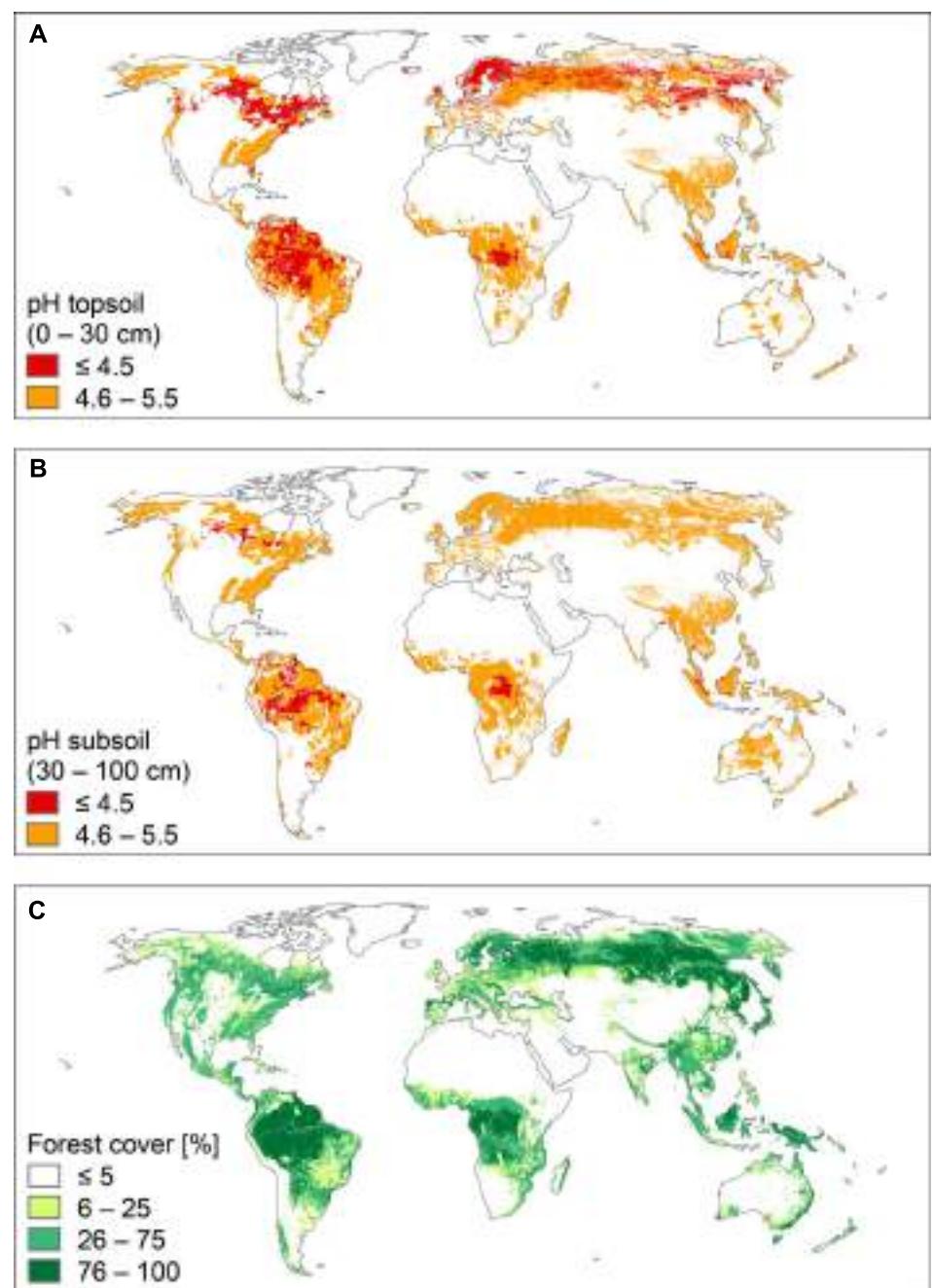


FIGURE 1 | World acid soils and world forests. **(A)** pH of topsoil (0–30 cm), **(B)** pH of subsoil (30–100 cm), and **(C)** forest cover. Soil pH is presented in two classes: pH ≤ 4.5 (strongly acid soils) and pH 4.6–5.5 (moderately acid soils). Data were retrieved from the Harmonized World Soil Data Base

(FAO/IIASA/ISRIC/ISS-CAS/JRC, 2012). Forest cover is presented in four classes: ≤ 5 , 6–25, 26–75, and 76–100%. Data were retrieved from the Food Insecurity, Poverty and Environment Global GIS Database (FGGD; FAO and IIASA, 2007).

from roots, which has been demonstrated to be a major Al^{3+} resistance mechanism in several plant species (Delhaize et al., 1993, 2007).

Following Ryan and Delhaize (2010) and Horst et al. (2010), the term “ Al^{3+} resistance” is used here as a plant property that allows a plant to grow with little or no injury under elevated Al^{3+} conditions. The potential mechanisms conferring resistance

to Al^{3+} can be broadly divided into those that exclude Al^{3+} from the root symplast (exclusion mechanisms) and those that enable plants to cope with Al^{3+} safely, once it enters the symplast (internal tolerance mechanisms; e.g., Kochian, 1995). Exclusion mechanisms depend on the release of ligands which chelate and detoxify Al^{3+} externally and limit its uptake in the cytosol. Tolerance mechanisms include those that chelate the Al^{3+} entering

the root cells, with subsequent transport and sequestration into less sensitive parts of the plant and subcellular compartments. The physiology, biochemistry, and molecular biology of these mechanisms have been thoroughly discussed in several review articles (e.g., Jones and Ryan, 2003; Kochian et al., 2004; Ma, 2007; Poschenrieder et al., 2008; Horst et al., 2010; Ryan et al., 2011; Delhaize et al., 2012; Inostroza-Blancheteau et al., 2012). The proposed principles of these mechanisms are summarized in **Figure 2**.

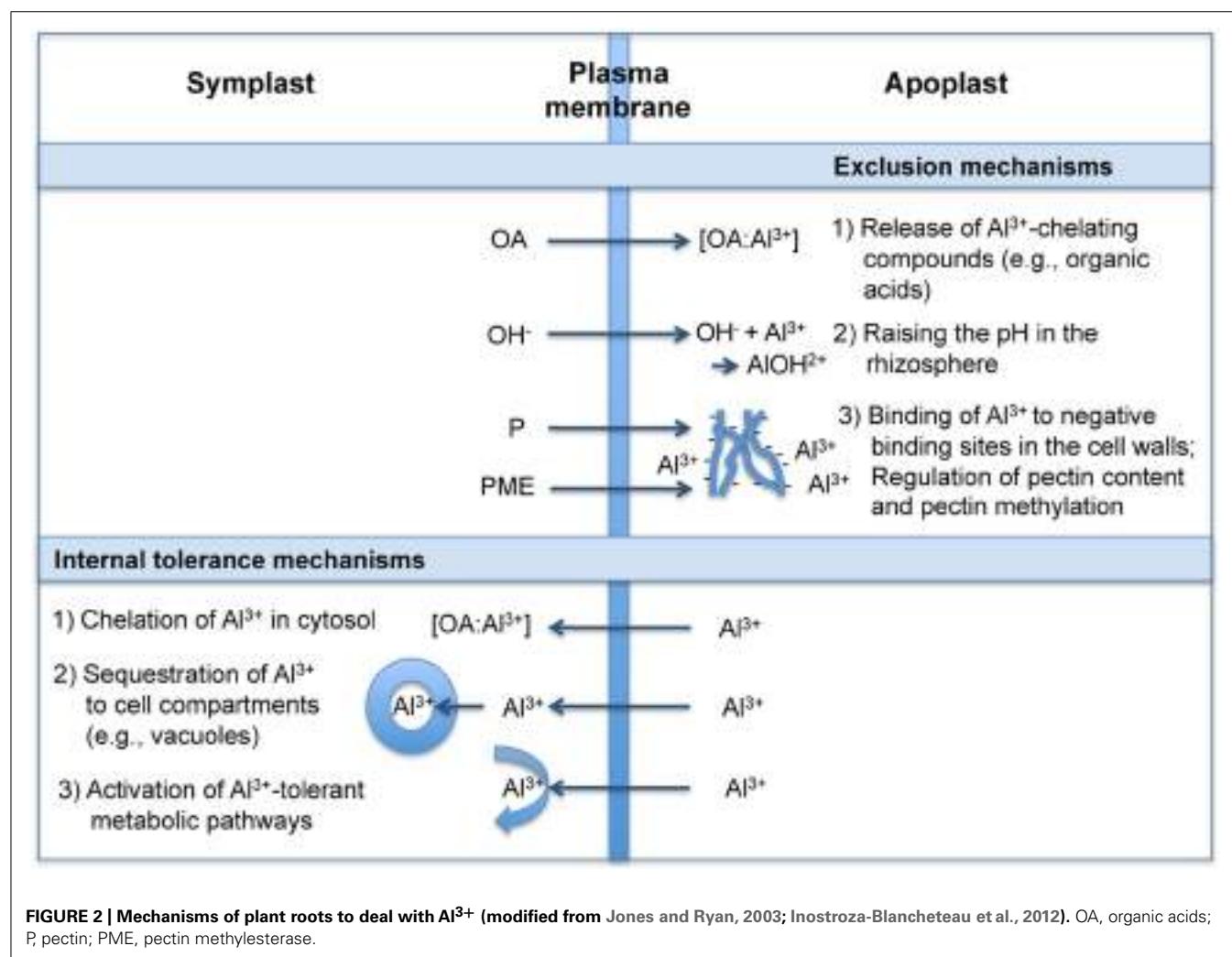
In this paper, we review the literature on proposed Al^{3+} resistance mechanisms of woody plants. We summarize information obtained from crops and *Arabidopsis*, and then review relevant results from similar studies in woody plants. In addition, we discuss the occurrence of Al accumulators and Al excluders in different forest biomes of the world.

Al^{3+} EXCLUSION

RELEASE OF SUBSTANCES THAT CHELATE AND DETOXIFY Al^{3+}

The best-documented mechanism of Al^{3+} exclusion is the Al^{3+} -activated efflux of organic acids from roots. Typical organic acids released by plants are citrate, malate, and oxalate. These organic

acids are deprotonated anions at the pH found in the cytosol, and once transported out of the root, they chelate the toxic Al^{3+} in the rhizosphere, forming stable and non-toxic complexes. Citrate and malate are present in all plant cells because they are involved in the mitochondrial respiratory cycle. Oxalate is a common cellular constituent involved in Ca^{2+} regulation, ion balance, and metal detoxification (Franceschi and Nakata, 2005; Rahman and Kawamura, 2011). The three organic acid anions form complexes with Al^{3+} with the following order of strength: citrate > oxalate > malate (Libert and Franceschi, 1987; Jones and Ryan, 2003). Physiological and genetic evidence from several plant species shows that the Al^{3+} -activated efflux of organic acid anions indeed confers resistance to Al^{3+} . The most convincing support comes from genotypes within a species that show contrasting levels of Al^{3+} resistance. In wheat (*Triticum aestivum*), for example, a pair of near-isogenic lines that differ in resistance at a single locus was used to show that the Al^{3+} -activated efflux of malate from roots was greater in the resistant genotype than in the sensitive genotype (Delhaize et al., 1993). The same wheat genotypes were used to clone the Al^{3+} -activated malate transporter (*TaALMT1*) gene, the first Al^{3+} resistance gene isolated from plants (Sasaki



et al., 2004). This gene encodes a plasma membrane-bound protein responsible for the efflux of malate from roots. Subsequently, *TaALMT1*-like genes were isolated from several additional plant species, including *Arabidopsis* and rape (*Brassica napus*; Hoekenga et al., 2006; Ligaba et al., 2006; see also Delhaize et al., 2007). Citrate efflux, on the other hand, was found to be mediated by members of another protein family, the multidrug and toxic compound extrusion (MATE) family (Furukawa et al., 2007; Magalhaes et al., 2007). The molecular background of oxalate exudation has not yet been identified.

Numerous studies, some of which are described here, have found that woody plants release organic acid anions following Al³⁺ exposure. In the model tree poplar (*Populus*), the Al³⁺-activated release of organic acid anions has been studied both at the physiological and molecular level. In young rooted cuttings of *Populus tremula*, Al³⁺ induces the release of citrate and oxalate (Qin et al., 2007). In a follow-up study with the same poplar clone, Grisel et al. (2010) identified a MATE gene with a 60% amino acid sequence identity to the *AtMATE1* gene of *Arabidopsis*. The poplar gene is induced by Al³⁺ in both root and stem tissue, but not in the leaves, consistent with a function of this gene in the efflux of citrate from roots. In seedlings of two other poplar species, *Populus tremuloides* and *Populus trichocarpa*, Al³⁺ induced the exudation of citrate, malate, and oxalate from roots (Naik et al., 2009). In these species, organic acids accounted for 20–64% of the total C released upon Al³⁺ exposure (Naik et al., 2009). The minimal concentrations of Al³⁺ required to induce organic acid exudation in *Populus tremula* are between 50 and 100 μM Al³⁺ (Table 1; Qin et al., 2007). Using the same solution culture medium, similar threshold values were found in the two coniferous trees *Cryptomeria japonica* and *Pinus thunbergii* (Hirano et al., 2012; Table 1). Although these studies clearly demonstrate that Al³⁺ induces the release of organic acid anions from roots, direct physiological and genetic evidence for their role in Al³⁺ resistance has not been established. For example, it is not known whether the organic acid anions are released primarily from the root tip, which would be indicative for a role in Al³⁺ resistance.

Woody plant species vary considerably in the organic acid compounds they release in response to Al³⁺ exposure. Many species exude more than one organic acid anion, with various combinations of malate, oxalate, and succinate. In the broad-leaved deciduous and evergreen trees and shrubs assayed so far, citrate is the most common organic acid anion identified (Table 2 and references therein). Of the five coniferous tree species analyzed, three released oxalate, and the two remaining citrate and succinate, respectively (Table 2).

Little is known about other substances that may be released by roots to chelate Al³⁺. Proposed compounds include polypeptides, phenolic compounds, cyclic hydroxamates, and rhizodepositions in the form of mucilage (Jones and Ryan, 2003; Poschenrieder et al., 2008). In the tea plant, *Camellia sinensis*, Morita et al. (2011) observed beside of oxalate an increase of the release of caffeine, a phenolic compound, in response to Al³⁺ exposure. Phenolic compounds were also exuded in Al³⁺-treated *Eucalyptus camaldulensis* and two *Melaleuca* species (Nguyen et al., 2003).

Table 1 | Organic acid release from roots of *Cryptomeria japonica* (μmol g⁻¹ day⁻¹ FW), *Pinus thunbergii* (μmol g⁻¹ day⁻¹ FW), and *Populus tremula* (μmol g⁻¹ DW) after exposition to Al³⁺ (according to Qin et al., 2007; Hirano et al., 2012).

Tree species	Al ³⁺ concentration (μM)	Citrate	Malate	Oxalate
<i>Cryptomeria japonica</i>	0	0.08 ^a	0.10 ^a	0.14 ^a
	100	0.39	0.14	0.49
	500	0.41	0.14	0.75
	1000	0.38	0.13	0.70
	P value ^b	ns	ns	*
<i>Pinus thunbergii</i>	0	0.03 ^a	0.03 ^a	0.47
	100	0.10	0.03 ^a	1.08
	500	0.10	0.03 ^a	2.93
	1000	0.11	0.03 ^a	4.04
	P value	*	ns	**
<i>Populus tremula</i>	0	0.0 ^a	0.0 ^a	0.1
	50	0.8	0.0 ^a	4.5
	100	1.9	0.0 ^a	3.9
	200	18.4	0.0 ^a	5.6
	500	20.5	0.0 ^a	25.7
	1000	20.3	0.0 ^a	18.8
	P value	***	ns	***

^aBelow detection limit.

^bANOVA: ***P < 0.001, **P < 0.01, *P < 0.05; ns, not significant.

RAISING THE pH IN THE RHIZOSPHERE

According to Kochian et al. (2005), only one study to date has unequivocally demonstrated that raising the pH in the rhizosphere can protect plants from Al³⁺. For two distinct classes of Al³⁺-tolerant *Arabidopsis* mutants, it was shown that Al³⁺ resistance is mediated by the exclusion of Al³⁺ from the root either by exudation of malate and citrate (Larsen et al., 1998) or by H⁺ influx at the root apex (Degenhardt et al., 1998). The H⁺ influx resulted in an increase in the rhizosphere pH, and subsequently in a significant decrease in the Al³⁺ activity around the root tip. However, there is currently no evidence to support that this mechanism operates in *Arabidopsis* ecotypes. Whether the roots of woody plants make use of such a mechanism remains elusive.

MODIFICATION OF Al³⁺ BINDING SITES IN THE CELL WALL OF ROOT CELLS

The cell wall of root cells has been suggested to be a site of both Al³⁺ toxicity and Al³⁺ exclusion (Horst et al., 2010). It has been determined that up to 90% of the Al³⁺ absorbed by roots can be localized to the apoplast (Kochian, 1995). The primary site of Al³⁺ binding is probably the pectin matrix, which is largely composed of homopolymers of galacturonic acid (Mohnen, 2008; Horst et al., 2010). Al³⁺ is known to bind far more strongly to pectin than Ca²⁺, whose binding to the cell wall is required for proper cell wall functioning (Franco et al., 2004). It has been proposed that Al³⁺ binds to the cell wall through a replacement of Ca²⁺, making

Table 2 | Al³⁺-activated release of organic acids from roots of woody plants.

Plant species	Al ³⁺ -activated organic acids	Reference
Non-mycorrhizal roots; coniferous trees		
<i>Cryptomeria japonica</i>	Citrate, oxalate	Hirano et al. (2012)
<i>Picea abies</i>	Oxalate	Heim et al. (2001)
<i>Picea abies</i>	–	Eldhuset et al. (2007)
<i>Pinus sylvestris</i>	Oxalate	Ahonen-Jonnarth et al. (2000)
<i>Pinus thunbergii</i>	Citrate, oxalate	Hirano et al. (2012)
Non-mycorrhizal roots; broad-leaved trees (deciduous)		
<i>Populus tremula</i>	Citrate, oxalate	Qin et al. (2007)
<i>Populus tremuloides</i>	Citrate, malate, oxalate, succinate	Naik et al. (2009)
<i>Populus trichocarpa</i>	Citrate, malate, oxalate, succinate	Naik et al. (2009)
Non-mycorrhizal roots; broad-leaved trees and shrubs (evergreen)		
<i>Acacia auriculiformis</i>	Citrate, oxalate	Nguyen et al. (2003)
<i>Camellia sinensis</i>	Oxalate	Morita et al. (2011)
<i>Camellia sinensis</i>	–	Ishikawa et al. (2000)
<i>Cinnamomum camphora</i>	Citrate	Osawa et al. (2011)
<i>Citrus grandis</i>	Citrate, malate	Yang et al. (2011)
<i>Citrus junos</i>	Citrate	Deng et al. (2009)
<i>Citrus sinensis</i>	Citrate, malate	Yang et al. (2011)
<i>Eucalyptus camaldulensis</i>	Citrate, oxalate	Tahara et al. (2008)
<i>Eucalyptus camaldulensis</i>	Citrate, oxalate	Nguyen et al. (2003)
<i>Eucalyptus cloeziana</i>	Citrate	Silva et al. (2004)
<i>Eucalyptus dunnii</i>	Citrate, malate, oxalate	Silva et al. (2004)
<i>Eucalyptus globulus</i>	Citrate, malate	Silva et al. (2004)
<i>Eucalyptus grandis</i>	Citrate	Silva et al. (2004)
<i>Eucalyptus saligna</i>	Citrate	Silva et al. (2004)
<i>Eucalyptus urophylla</i>	Citrate, malate, oxalate	Silva et al. (2004)
<i>Melaleuca bracteata</i>	Citrate	Tahara et al. (2008)
<i>Melaleuca cajuputi</i>	Citrate, malate	Tahara et al. (2008)
<i>Melaleuca cajuputi</i>	Citrate, oxalate	Nguyen et al. (2003)
<i>Melaleuca leucadendra</i>	Citrate	Nguyen et al. (2003)
Mycorrhizal roots		
<i>Picea abies</i>	Succinate	Heim et al. (2003)
<i>Picea abies</i>	–	Eldhuset et al. (2007)
<i>Pinus densiflora</i>	Citrate	Tahara et al. (2005)
<i>Pinus sylvestris</i>	Oxalate	Ahonen-Jonnarth et al. (2000)

The references are divided into studies dealing either with non-mycorrhizal roots or with mycorrhizal roots.

the cell wall more rigid, and thus reducing its extensibility which is required for normal cell elongation (Tabuchi and Matsumoto, 2001).

Several studies have suggested that the pectin content and the degree of pectin methylation are important determinants of the amount of Al³⁺ that can bind to the cell wall of root cells. In maize (*Zea mays*), rice (*Oryza sativa*), and common bean (*Phaseolus vulgaris*), differences in the pectin content and/or the degree of pectin methylation were linked with Al³⁺ sensitivity/resistance (Eticha et al., 2005; Yang et al., 2008; Rangel et al., 2009). Al³⁺-resistant lines of all three plant species were found to have a higher degree of pectin methylation, and a lower cell wall Al content when compared to Al³⁺-sensitive lines, supporting a role for pectin methylation in Al³⁺ exclusion. A modulating role for the degree of pectin methylation is further supported by the finding that the expression of pectin methylesterase (PME), the enzyme responsible for the demethylation of pectin, was lower in Al³⁺-resistant lines than in Al³⁺-sensitive lines (Maron et al., 2008; Yang et al., 2008).

Current evidence, based on X-ray microanalyses, indicates that the apoplast is a major site of Al accumulation also in woody plants. In Al³⁺-treated seedlings of the conifer *Picea abies*, Al was found in both epidermal and cortical cells of the root tip (Heim et al., 1999). In both cell types, more than 88% of the total Al localized to the cell wall. In addition, it was observed that the amount of Ca in the cell wall of both cell types was much lower in Al³⁺-treated seedlings than in control plants, suggesting that Al³⁺ replaced Ca²⁺ at the exchange sites of the cell wall. These findings are further substantiated by results of a study conducted in *Picea abies* and *Populus tremula*, cultivated in a model ecosystem for 3 years (Brunner et al., 2008). In *Picea abies*, Al accumulated continuously over time in the cell wall of root epidermal cells, whereas in *Populus tremula*, Al accumulated in the cell wall of both root epidermal and cortical cells. In both species, Al did not accumulate intracellularly (Table 3).

In root cells of woody plants, little evidence exists about the relationship between cell wall polysaccharides and Al³⁺ sensitivity/resistance. In a set of poplar clones, representing several interspecific crosses, it was found that the Al content of the root symplast was higher in Al³⁺-resistant clones than in Al³⁺-sensitive clones (Smith et al., 2011). The Al content of the root symplast, on the other hand, was lower in Al³⁺-resistant clones than in Al³⁺-sensitive clones. This pattern of cellular Al distribution suggests that the cell wall of root cells prevented Al³⁺ from entering the root symplast. Additional parameters investigated were pectin and callose, the latter of which is a widely used indicator of early Al³⁺ toxicity symptoms (Hirano et al., 2004, 2012; Kochian et al., 2005). Treatment with Al³⁺ increased pectin and callose levels in all clones, but more prominently in Al³⁺-sensitive clones. A clear conclusion about the impact of pectin could not be drawn because the degree of pectin methylation was not assessed.

Al³⁺ TOLERANCE

CHELATION OF Al³⁺ WITH ORGANIC SUBSTANCES IN THE CYTOSOL

Organic acid anions and phenolic compounds have also been implicated in internal Al³⁺ tolerance. Once Al³⁺ enters the cell, the concentration of free Al³⁺ cations in the cytosol will be very

low, but even at these concentrations, Al^{3+} remains a hazard. The very high affinity of Al^{3+} for oxygen ligands allows it to compete with other ions for metabolically important sites despite a large disparity in their concentrations (Jones and Ryan, 2003).

Indeed, studies of several woody plant species demonstrate that intracellular Al^{3+} is chelated by organic acid anions. In the small shrub *Melastoma malabathricum*, upon entering the root, Al^{3+} binds to citrate, and the Al–citrate complex itself is transported from the root to the shoot (Watanabe and Osaki, 2001). In the leaves, the Al–citrate complex is transformed into Al–oxalate 1:1, 1:2, and 1:3 complexes. The former two complexes are potentially toxic to the plant. A similar transformation of Al–organic acid complexes is described for the tea plant. Upon entering the root cell, Al^{3+} binds to oxalate and then is transported from the root to the shoot in the form of Al–citrate and Al–malate complexes (Morita et al., 2004, 2008). In a comparison of several *Eucalyptus* species, the concentration of root tip malate was found to correlate positively with the degree of Al^{3+} resistance in the presence of Al^{3+} (Silva et al., 2004). In contrast, in the poplar clones of the above-mentioned study, the concentrations of symplastic citrate and formate correlated closely with Al^{3+} sensitivity (Smith et al., 2011).

Besides organic acids, there are other complex forming compounds, e.g., phenolic substances, that bind Al^{3+} in the cytosol. For example, in the tea plant, Al–catechin complexes were described (Nagata et al., 1992). In the sepals of the small shrub *Hydrangea macrophylla*, Al^{3+} is bound to both 3-caffeoquinic acid and delphinidin 3-glucoside, where Al^{3+} is thought to play a role in stabilizing the two organic compounds, and thus causing the color to change from red to blue (Ma et al., 2001). In the root apices of the camphor tree (*Cinnamomum camphora*), an accumulation of proanthocyanidin, which is composed of flavan-3-ols (e.g., catechin), has been demonstrated (Osawa et al., 2011). An increase in root phenolics has been observed by Ofei-Manu et al. (2001) in a series of woody plants upon Al^{3+} exposure, including *Camellia sinensis*, *Cryptomeria japonica*, *E.*

viminalis, *Gleditsia triacanthos*, *Picea abies*, *Pinus densiflora*, *Pinus thunbergii*, *Populus tremuloides*, *Robinia pseudoacacia*, and *Rhus succedanea*.

SEQUESTRATION OF Al^{3+} TO METABOLICALLY LESS SENSITIVE COMPARTMENTS

The uptake and storage of high Al^{3+} concentrations in aerial parts of the plant is a trait common to many plant species of tropical regions, where the ability to cope with Al^{3+} stress is a strong prerequisite for survival (Ryan and Delhaize, 2010). Plants that accumulate $>1 \text{ mg g}^{-1}$ DW Al are considered Al-hyperaccumulators (Jansen et al., 2002). Plant families with woody Al-hyperaccumulators storing very large amounts of Al ($>10 \text{ mg g}^{-1}$ DW) in their leaves include Melastomataceae, Rubiaceae, and Theaceae (Matsumoto et al., 1976; Watanabe et al., 1997; Masunaga et al., 1998; Jansen et al., 2003; Olivares et al., 2010; Gonzalez-Santana et al., 2012).

A typical example of a woody plant capable of accumulating large amounts of Al ($>15 \text{ mg g}^{-1}$) in its leaves is the tree species *Richeria grandis* from the Venezuelan cloud forest. Using X-ray microanalysis, Cuenca et al. (1991) showed that Al is stored extracellularly in the cell wall of mature leaves. Further extracellular cell wall storage of Al was demonstrated in the woody Al-hyperaccumulators *Camellia sinensis*, *Conostegia xalapensis*, *Faramea marginata*, and *Melastoma malabathricum*. The cell wall of the epidermal and the mesophyll cells of the leaves were the main sites for Al accumulation (Watanabe and Osaki, 2001; Britez et al., 2002; Tolra et al., 2011; Gonzalez-Santana et al., 2012). Interestingly, the chloroplasts of *Qualea grandiflora* and *Callisthene major*, two Al-hyperaccumulating woody plant species from the Vochysiaceae family, which grow in the Brazilian Cerrado, have been suggested as a primary compartment for Al sequestration (De Andrade et al., 2011). Al $^{3+}$ can also be sequestered into cells specialized for storage functions, e.g., idioblasts containing Ca-oxalate crystals have been considered as a location for Al $^{3+}$ detoxification in the leaves of *Corchorus olitorius* (Mazen, 2004).

Table 3 | Al accumulation in fine roots of *Picea abies* and *Populus tremula* [Al concentrations of bulk material; Al net counts of compartments using energy-dispersive X-ray spectroscopy (EDX)-analyses] after growth in weakly acidic soil (pH 6.5) with different length of exposition time (according to Brunner et al., 2008).

Tree species	Time (year)	Al concentration (mg g $^{-1}$)	Al counts in epidermal cells		Al counts in cortical cells	
			Cell wall	Intracellular	Cell wall	Intracellular
<i>Picea abies</i>	0.5	2.12	213	83	126	94
	1.5	8.23	284	73	122	72
	2.5	9.50	355	101	140	80
	P value ^a	**	***	ns	ns	ns
<i>Populus tremula</i>	0.5	1.81	168	67	96	65
	1.5	16.40	359	50	152	51
	2.5	5.36	338	57	163	63
	P value ^b	— ^b	***	ns	**	ns

^aRepeated measures ANOVA: ***P < 0.001, **P < 0.01, *P < 0.05, ns, not significant.

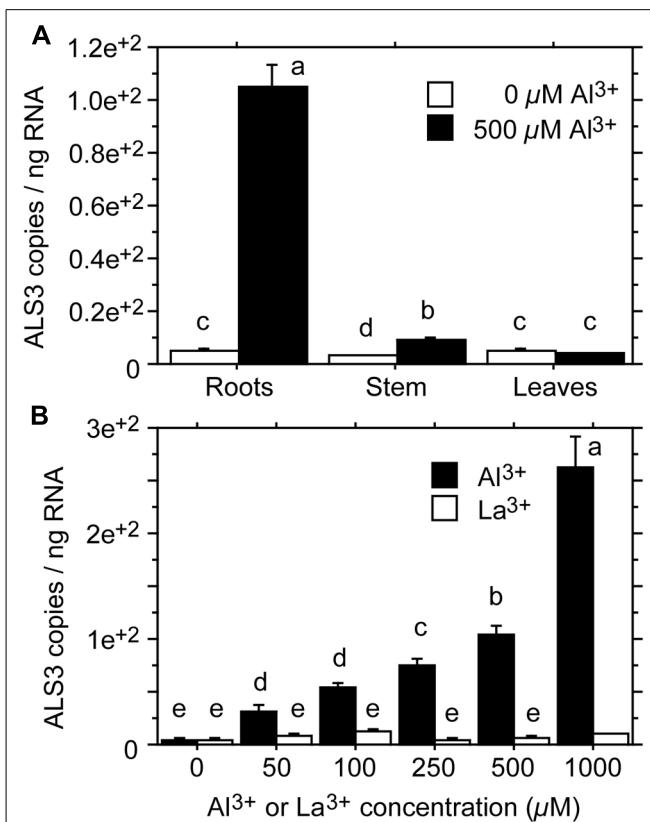
^bNo statistical analysis possible because of missing replicates.

Transport of Al from the root to the shoot is likely to involve complexes of Al with organic acids (see above). Little is known about the transport of Al across the plasma membrane and further sequestration into subcellular compartments. Transport across membranes requires transport proteins. Candidates for such proteins have been identified in the Al^{3+} -sensitive mutants *als1* and *als3* of *Arabidopsis*. Both mutants are mutated in genes encoding proteins that belong to the ATP-binding cassette (ABC) transporter superfamily. ALS1 (Al^{3+} -sensitive) is a half type ABC transporter, whereas ALS3 is an ABC transporter-like protein, lacking the ABC domain. The functions and substrates of ALS1 and ALS3 are not known, but the mutant phenotypes, the subcellular localization of the proteins, and tissue-specific gene expression have led to the assumption that the two proteins sequester and transport Al^{3+} to overcome Al^{3+} toxicity. ALS1 is likely to be involved in the intracellular transport of Al^{3+} to vacuoles of root tip cells and cells of the plant vasculature (Larsen et al., 2007). ALS3 is mainly localized to the plasma membrane of root cortex cells and phloem cells throughout the plant, suggesting that it mediates the intercellular redistribution of accumulated Al away from sensitive tissues (Larsen et al., 2005). In *Populus tremula*, Grisel et al. (2010) identified an ALS3-like gene with a 79% amino acid sequence identity with the *Arabidopsis* ALS3 gene. The poplar gene was found to be expressed in the root, stem, and leaves, and was strongly induced by Al^{3+} in the root (44-fold; Figure 3A). In addition, the poplar gene was inducible by Al^{3+} , but not by La^{3+} (lanthanum; Figure 3B), consistent with the finding that the *Arabidopsis* mutant *als3* is not affected by La^{3+} (Larsen et al., 1997).

ACTIVATION OF METABOLIC PATHWAYS TO OVERCOME THE TOXIC EFFECTS OF Al^{3+}

Moderate Al^{3+} concentrations are not fatal, and roots may at least partially recover (see also Matsumoto and Motoda, 2012). This is well documented in *Populus tremula* treated with either no Al^{3+} or increasing concentrations of Al^{3+} up to 1,000 μM . Two phases of root growth could be distinguished: a rapid Al^{3+} -induced growth inhibition (within 6 h at Al^{3+} concentrations $> 250 \mu\text{M}$) and a subsequent phase of growth recovery (within 2 days at Al^{3+} concentrations $\leq 500 \mu\text{M}$; Grisel et al., 2010). The root growth of plants treated with 1,000 μM Al^{3+} further decreased. This pattern of root growth recovery may reflect the success of the roots in activating metabolic pathways to overcome the toxic effects of Al^{3+} and/or Al^{3+} resistance mechanisms. Matsumoto and Motoda (2013) suggested that the recovery of roots exposed to Al^{3+} is associated with the reduction of Al^{3+} -induced oxidative stress. In *Populus tremula*, two genes of the oxidative stress pathway (a peroxidase gene and an alternative oxidase gene) were strongly induced upon Al^{3+} exposition after 6 h (>8 -fold), while their expression decreased to control levels after 2 days (Grisel et al., 2010).

Two additional genes of *Populus tremula* that may play a role in root growth recovery encode CorA-like Mg^{2+} transporters (Grisel et al., 2010). These genes were induced up to fivefold by Al^{3+} . The activity of a homologous CorA-like Mg^{2+} transporter from *Arabidopsis* was shown to be blocked by micromolar concentrations of Al^{3+} , when expressed in bacteria (Li et al., 2001). In



addition, the same CorA-like Mg^{2+} transporter alleviated Al^{3+} toxicity when overexpressed *in planta* (Deng et al., 2006). Mg^{2+} has been reported to be able to alleviate Al^{3+} toxicity in a number of crop plants (see reviews of Bose et al., 2011; Chen and Ma, 2013). Various mechanisms have been put forward to explain how Mg^{2+} can alleviate Al^{3+} toxicity. These mechanisms include increased ionic strength of the solutions, reduction in Al^{3+} saturation at the apoplastic exchange sites, and decreased Al^{3+} activity at the root cell plasma membrane surface (Bose et al., 2011). However, the identified Mg^{2+} transporters indicate that, besides of electrostatic interactions, biochemical processes may be involved in the rescue of Al^{3+} toxicity.

ADAPTATIONS

The tropical forests and the forests of boreal and temperate regions have evolved on geological timescales under very different conditions. The forests of boreal and temperate regions were repeatedly affected by the Pleistocene glaciations, while the impact of the Pleistocene climatic fluctuations was certainly much less severe in tropical regions. Most tropical forests have not been disturbed for hundred thousands of years, and thus typically grow on highly

weathered soils, which are strongly acidic, both in the topsoil and the subsoil (**Figures 1A,B**).

Accumulating evidence shows that in tropical regions both Al excluders and Al accumulators occur. Examples of woody species that are strong excluders are *Melaleuca cajuputi*, *Acacia mangium*, and *Leucaena leucocephala* (Osaki et al., 1997), which are all capable of exuding organic acid anions from their roots (see also **Table 2**). Examples of woody species that store high amounts of Al in leaves are *Melastoma malabathricum* and *H. macrophylla*. Other woody species, such as *Vaccinium macrocarpon*, store high amounts of Al in their roots (Osaki et al., 1997). Most Al-hyperaccumulator plants are shrub-type broad-leaved woody plants.

Phylogenetic analyses indicate that Al hyperaccumulation is a trait that has arisen a number of times, and this trait is scattered over more than 20 orders across about 45 families belonging to magnoliids (e.g., Laurales), eudicots (e.g., Proteales), rosids (e.g., Malpighiales, Myrtales), and asterids (e.g., Gentianales, Ericales; **Table 4**; Jansen et al., 2002). To date, Al accumulation in tall trees has only been found in a few tree species of the Euphorbiaceae (Osawa et al., 2013). The predominance of Al accumulators within non-flowering plants suggests that Al accumulation evolved early in the evolution of land-plants and is probably a primitive characteristic associated with survival in ancient Al³⁺-rich environments (Jansen et al., 2002).

An analysis of the variation in foliar Al and macronutrient concentrations in a global dataset of plant species in a phylogenetic framework showed that the frequency distribution of foliar Al concentration in tropical regions clearly had two peaks (“bimodal”), whereas in temperate regions it showed only one peak (“unimodal”; Metali et al., 2012). This conclusion supports the hypothesis that Al accumulators and non-Al accumulators exist as distinct, but overlapping, groups of species. The estimated threshold value of foliar Al concentrations that distinguishes Al accumulators from non-Al accumulators varies geographically. The foliar threshold of tropical plants is higher (2.3–3.9 mg g⁻¹) than that of temperate plants (1.1 mg g⁻¹; Metali et al., 2012). Among angiosperm species, there was a significant phylogenetic signal in foliar Al concentrations, substantiating results of previous studies, suggesting a greater prevalence of Al

accumulators in some families than in others (e.g., Jansen et al., 2002, 2003). A phylogenetical signal may also arise when related species occupy relatively similar habitats that differentially influence nutrient uptake and accumulation (Thompson et al., 1997). For example, Schreeg et al. (2010) have provided evidence for significant associations between high soil Al and Mn concentrations and the distributions of trees in the Vochysiaceae and Myrtaceae on a 50-ha plot in a semideciduous moist forest in Panama.

Compared to soils of tropical regions, soils of boreal and temperate regions are generally younger, although large areas, such as Siberia and Beringia, were ice-free during the Last Glacial Maximum (26,500–18,000 year before present; Clark et al., 2009). However, soils of these ice-free areas were severely affected by cold climate and permafrost, strongly limiting soil chemical and soil biological processes. As a consequence, soils in boreal and temperate regions are generally less weathered and thus less acidic than tropical soils, particularly in the subsoil (**Figure 1B**). In contrast, topsoils of the boreal regions are highly acid due to the strong acidifying effect of the coniferous litter during the incomplete decomposition process and the formation of humic acids (Schachtschabel et al., 1992).

Aluminum concentrations measured in roots from temperate or boreal woody species suggest, that the immobilization of Al³⁺ in the cell wall is most likely an important adaptation. Al concentrations in the fine roots of common temperate and boreal woody species usually exceed the limit for being hyperaccumulators (>1 mg g⁻¹). For example fine roots of *Abies alba*, *Castanea sativa*, *Fagus sylvatica*, *Picea abies*, *Pinus cembra*, and *Pinus montana* all have values of 1–10 mg g⁻¹ DW (Zyss et al., 1996; Brunner et al., 2002; Genenger et al., 2003; Hirano et al., 2006; Richter et al., 2011; see also **Table 3**). All these woody species are ectomycorrhizal, suggesting that ectomycorrhizal structures, such as fungal mantle and Hartig net, further contribute to the accumulation of Al in roots by immobilizing Al in the cell wall of the fungal hyphae (Brunner and Frey, 2000; Heim et al., 2003) or in the fungal vacuoles (Martin et al., 1994). Ectomycorrhizas in temperate and boreal regions have, we assume, the function not only to take up water and nutrients but also to immobilize toxic Al³⁺. Because ectomycorrhizal trees and ericoid-mycorrhizal Ericales dominate in temperate and boreal regions, we propose that this mechanism to immobilize Al³⁺ is a major mechanism for excluding Al³⁺ from the roots of woody plants in these regions, a suggestion first proposed by Jansen et al. (2002). Moreover, mycorrhizal fungi may well contribute significantly to the cycling of Al in forest ecosystem because high concentrations of Al can be found in their fruiting bodies (Smits and Hoffland, 2009). An additional adaptation of tree roots from temperate or boreal forests to acid soils is the observation that the roots have a shorter lifespan, which means that the turnover rate of roots exposed to elevated Al³⁺ concentrations is higher (Godbold et al., 2003; Leuschner et al., 2004; Richter et al., 2013). Reasons for a shorter lifespan could be that Al accumulation has reached its saturation point faster compared to roots from non-acid soils, causing the root to die earlier.

Some woody plants in temperate regions have not evolved strategies to deal with high levels of Al³⁺ and thus are not adapted to acid soils. Examples are the two species *Fraxinus excelsior* and

Table 4 | Families of woody plants with strong and/or numerous Al-hyperaccumulators (according to Jansen et al., 2002).

Clade	Order	Family
Magnoliids	Laurales	Lauraceae, Monimiaceae, Siparunaceae
Eudicots	Proteales	Proteaceae
Eurosids I	Malpighiales	Euphorbiaceae
Eurosids II	Myrtales	Crypteroniaceae, Melastomataceae, Vochysiaceae
Asterids	Ericales	Diapensiaceae, Symplocaceae, Ternstroemiaciae, Theaceae
Euasterids I	Gentianales	Rubiaceae

Acer pseudoplatanus, which both are not ectomycorrhizal. They grow mainly on forest sites with high pH and high base saturation, and are unlikely to occur on very acid soils (Weber-Blaschke et al., 2002). In a recent study, Walthert et al. (2013) showed that *Fraxinus excelsior* and *Acer pseudoplatanus* respond much more sensitively to soil properties than *Fagus sylvatica* does. The soil properties limiting their distribution are Al in the case of *Fraxinus excelsior*, and Al together with nutrient availability (C/N ratio) in the case of *Acer pseudoplatanus*. On the contrary, no soil-induced distribution limits were detectable for *Fagus sylvatica* (Walthert et al., 2013).

Differences in Al^{3+} resistance not only exist among different tree species but also among local populations of the same species. Kidd and Proctor (2000) assessed the level of Al^{3+} resistance in four populations of *Betula pendula* growing on soils that differ in soil acidity and levels of Al^{3+} . Using a solution culture system which simulates natural soil solutions, the authors found that seedlings originating from populations from acid mineral soils ($\text{pH } 4.3$, Al^{3+} concentration 21.1 mg l^{-1}), had a significant higher level of Al^{3+} resistance (measured with the root elongation rate) compared to seedlings from populations from non-acid soils ($\text{pH } > 4.8$, $\text{Al}^{3+} < 5.3 \text{ mg l}^{-1}$). A similar study was performed by Wilkins and Hodson (1989) who analyzed two populations of *Picea abies* growing on an acid mineral soil and a calcareous soil, respectively. The growth of seedlings from the acid soil was slightly stimulated by the Al^{3+} treatment, whereas the growth of that from the calcareous soil was greatly reduced. The results of these studies suggest that high Al^{3+} soil conditions are a significant force for population divergence due to strong selective pressure associated with adaptation.

Further evidence for population adaptation to acid soil conditions comes from a study of *Pinus contorta* growing along a steep gradient of soil acidity at the northern coast of California (Eckert et al., 2012). In this area, five well-developed marine terraces exist, whose soils represent a chronosequence ranging from fertile soils close to the coast to podzolic soils with low pH and high Al^{3+} concentrations about 5 km inland (Westman, 1975). *Pinus contorta* is one of three conifers that have colonized and diversified on the extreme podzolic soils. Shore pine (*Pinus contorta* ssp. *contorta*) is found along the lower terraces, while Bolander pine (*Pinus contorta* ssp. *bolanderi*), which has a pygmy growth habit, is endemic to the upper marine terraces. Using a candidate gene approach, Eckert et al. (2012) investigated the molecular basis for the colonization of the podzolic soils by *Pinus contorta* populations. Patterns of nucleotide diversity were analyzed in genes that are related to growth or response to several soil parameters,

such as Al^{3+} , BC, and phosphate. The majority of the 21 genes analyzed did not or only weakly deviate from neutrality in patterns of nucleotide diversity. However, two of the genes carried clear signatures of positive selection: one was a putative homolog of the *Arabidopsis ALS3* gene and the other an inorganic phosphate transporter gene. The *ALS3* gene was characterized by a derived non-synonymous mutation, which is extremely rare in populations of the lower terraces and almost or completely fixed in those of the higher terraces. The phosphate transporter gene included two highly divergent haplotypes, whose frequency differed among lower and higher terraces. The results of this study shed light on some of the genetic components underlying adaptation to local soil conditions along this unique environmental gradient.

CONCLUSION

Current evidence indicates that woody plants native to acid soils have evolved various strategies to overcome Al^{3+} stress. These strategies include exclusion of Al^{3+} from the root tip, probably through the release of organic acid anions. The formation of ectomycorrhizal structures, which are capable of accumulating Al in the cell wall of hyphal cells, may also be regarded as an exclusion strategy, reducing the degree of contact of root cells to Al^{3+} . Internal strategies rely on the transport and sequestration of Al in aerial parts of the plant, where the Al may be stored in the cell wall of different leaf tissues, or in subcellular compartments, like chloroplasts or possibly vacuoles. Some woody plant species may also store high levels of Al in the cell wall of root cells. The biochemical and molecular mechanisms underlying these strategies, however, remain largely to be determined. The few studies performed so far in woody plants provide hints in which direction research may focus. With the exception of poplar, forest trees are generally not amenable to genetic engineering for testing the impact of candidate genes. Similarly, progenies of controlled crosses, which are valuable for testing segregation of specific traits and genes, exist only for a few tree species. An alternative approach would involve the identification of genes that play a role in adaptation through association of genetic variation with particular soil Al^{3+} conditions. This has already been initiated and has provided insights into the mechanisms of population adaptation to Al^{3+} -rich environments.

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Effect of lead on root growth

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Lead (Pb) is one of the most widespread heavy metal contaminant in soils. It is highly toxic to living organisms. Pb has no biological function but can cause morphological, physiological, and biochemical dysfunctions in plants. Plants have developed a wide range of tolerance mechanisms that are activated in response to Pb exposure. Pb affects plants primarily through their root systems. Plant roots rapidly respond either (i) by the synthesis and deposition of callose, creating a barrier that stops Pb entering (ii) through the uptake of large amounts of Pb and its sequestration in the vacuole accompanied by changes in root growth and branching pattern or (iii) by its translocation to the aboveground parts of plant in the case of hyperaccumulators plants. Here we review the interactions of roots with the presence of Pb in the rhizosphere and the effect of Pb on the physiological and biochemical mechanisms of root development.

Keywords: root, lead, tolerance, uptake, root development

INTRODUCTION

Lead (Pb) is a heavy metal of anthropogenic origin (Sharma and Dubey, 2005). Pb is a pollutant that accumulates in soils, sediments, and water and is extremely persistent in the environment (Traunfeld and Clement, 2001). Pb has no biological function and it is toxic to living organisms even at low concentrations. Although Pb is not an essential element, some plant species proliferate in Pb-contaminated area and accumulate it in different parts. Roots are the first organ in contact with the various components of rhizosphere (Lynch and Whipps, 1990). Roots have evolved various mechanisms to reduce Pb uptake and transfer to the aboveground parts of the plant, and limit its deleterious effects. This article reviews the origins of Pb contamination, availability, and uptake of Pb, and recent knowledge on physiological, biochemical, and ultrastructural changes in roots due to the presence of Pb in the rhizosphere.

SOURCES OF LEAD

Lead is one of the most widely distributed trace metals. It is ranked second of all hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR, 2007). Because of natural deposits and increasing human activities, Pb has become ubiquitous in the soil and in the environment. Natural inputs include weathering and erosion of parent rocks that Pb to the transfer of large quantities of metals to water bodies and land (Gadd, 2010). Volcanic eruptions also contribute to natural inputs. For instance, the atmospheric emission from volcanoes was estimated

at 540–6,000 tons for 1983 (Nriagu and Pacyna, 1988), and 1,000–10,000 tons for 2001 (Richardson et al., 2001). Pb has been used by humans for centuries but anthropogenic activities related to this metal have increased significantly in recent decades. These activities include mining, smelting, fuel combustion, synthetic fertilizers, and various industrial processes: building construction, Pb-acid batteries, bullets and shot, solder, pewter, and fusible alloys (Mukai et al., 2001). Human activities significantly influence the global cycles of Pb. In 2004, 3,150,000 tons of Pb were extracted from the earth's crust and brought into circulation in society (USGS, 2006). In 1983, a total of 400,000–1,000,000 tons of mobilized Pb were disposed of with waste from metal extraction (Nriagu and Pacyna, 1988).

Lead is not biodegradable and is extremely persistent in both water and soil. Pb can be retained in the environment for 150–5000 years (Saxena et al., 1999). Most of Pb accumulates in the top 8" of the soil where it has a very low mobility. Without remedial action, high soil Pb levels will never return to normal (Traunfeld and Clement, 2001). When Pb enters the soil matrix, it is very difficult to remove it. The capacity of soil to adsorb Pb increases with increasing pH, cation exchange capacity, redox potential, organic carbon content, and chelates (phosphate) levels (United States Environmental Protection Agency, 1992).

The main part of the extracted Pb will not contribute to long-range environmental transport or be in a form that is readily bioavailable, but may later in the life cycle Pb to local impacts if not managed properly.

AVAILABILITY AND UPTAKE OF LEAD BY ROOTS

The rhizosphere is where interactions take place between roots and soils constituents (Lynch and Whipps, 1990). When a root absorbs water or nutrients from soil, ions and molecules move toward this organ both by mass flow with soil water and by diffusion (Robinson, 1991). Pb may be present in different fractions in the soils. It was previously thought that Pb had low solubility and availability for plant uptake because it forms precipitates with phosphates, sulfates, and chemicals in the rhizosphere (Blaylock and Huang, 2000). These geo-chemical forms of Pb in soils affect its solubility, which directly influences its mobility. However, roots produce and excrete protons, exudates and several metabolites, which can modify the soil pH and thus interfere with the dissolution processes and formation of soluble metal–organic complexes (Leyval and Berthelin, 1991). Citric, fumaric, and uronic acids as well as many polysaccharides are able to form complexes and to chelate metal ions including Pb (Mench et al., 1987). Indeed, in *Vicia faba* and *Typha angustifolia*, Pb uptake by roots was shown to increase significantly in the first hour after adding organic ligands [ethylenediaminetetraacetic acid (EDTA), citric acid; Muhammad et al., 2009; Shahid et al., 2012]. However, Quartacci et al. (2006) reported that citric acid supplied to a metal contaminated soil did not cause any change in metal uptake in *Brassica juncea*.

Lead uptake is greatly affected by rhizospheric processes. Lin et al. (2004) explained the ability of *Oryza sativa* L. to absorb high levels of Pb from soil by a decrease in soil pH due to root exudates, solubilization of Pb by rhizosphere microorganisms and complexation of Pb with organic matter at the soil–root interface. These authors also found larger amounts of NH₄OAc extractable Pb in the rhizosphere than in bulk soil, pointing to the involvement of root activities in changes in Pb availability (Lin et al., 2004).

Uptake of and tolerance to Pb depends on root system conditions. In sunflower, Pb accumulation and cell response was shown to differ between seedlings with a primary root system (PRS) and seedlings with adventitious root systems (ARS) only (in which the primary roots were cut off). The ARS was found to be more tolerant to Pb than the PRS in *Helianthus annuus* L. and *Allium cepa* (Michalak and Wierzbicka, 1998; Strubińska and Hanaka, 2011). This suggests that ARS have additional mechanisms that protect them against Pb penetration and Pb-induced oxidative stress. However, these mechanisms are still unknown.

Rhizosphere organisms also affect the metals availability and speciation. In *Lantana camara*, Pb accumulation in roots increases in the presence of earthworms (*Pontoscolex corethrurus*; Jusselme et al., 2012). Similar trends were observed in the *Thlaspi caerulescens* rhizosphere of Pb-contaminated soil (Epelde et al., 2008). These authors found that microbial activity was stimulated by interaction between microorganisms and macroorganisms. The effect of earthworms on Pb uptake may be due to their impact on the distribution of soil microorganisms by providing suitable conditions for microbial growth (Brown, 1995) but the mechanisms involved are not clear.

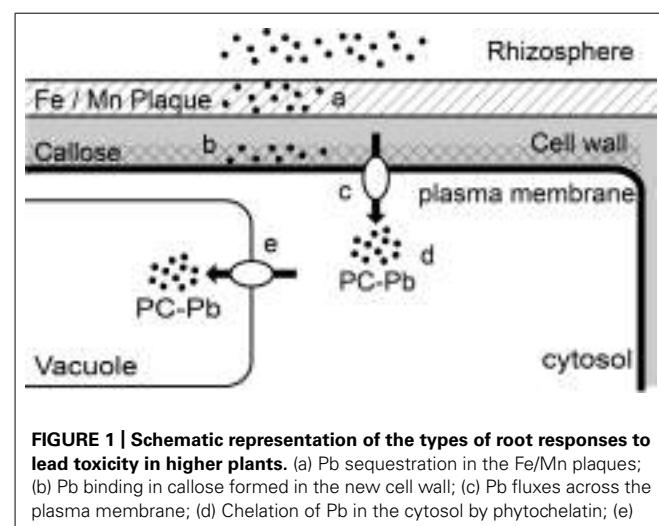
Lead availability is also affected by the presence of other heavy metals. Orroñoa et al. (2012) reported that Pb availability was reduced when it was supplied with five heavy metals (Cd, Zn, Cr, Cu, and Ni) that have an antagonist effect. These authors

also reported that, when Pb was supplied alone or in ternary combination (with Zn and Cu), its availability increased due to the antagonistic interaction between Cu and Zn, which made Pb more available for plant uptake.

The uptake of Pb is based mainly on the plant species and the interaction between roots (structures and synthesized exudates) and the rhizosphere (biochemical properties). Indeed, several factors must be taken into account when developing strategies for phytoremediation of Pb. Besides the organic and mineral composition of the soil and rhizopsheric organisms and microorganisms, the ability of roots to modify the mobility and the bioavailability of Pb by changing rhizospheric conditions can significantly contributes to a successful phytoremediation program.

ROOT DEFENSE AGAINST LEAD STRESS

In response to Pb exposure, plants have developed a variety of tolerance mechanisms (Figure 1). Roots are the first organs, exposed to Pb ions (Piechalak et al., 2002). The first defense strategy is to stop the metal entering the root tissues by excluding it (Mishra et al., 2006). Roots rapidly respond to the presence of Pb by forming mechanical barrier. In some plants, there is synthesis and deposition of callose between the plasma membrane and the cell wall. This newly formed structure functions as a barrier against stress factors including metals (Bacic et al., 2009; Krzesłowska, 2011). Samardakiewicz et al. (2012) examined whether callose forms an efficient barrier against Pb penetration in the roots of *Lemna minor* L. exposed to 15 μM of Pb for 6 h. This treatment resulted in the synthesis and deposition of callose in the newly formed cell wall in the protoderm in the center of the root tip. After callose deposition the Pb concentration was restricted in these superficial cells. Similar observations have been made in other species exposed to Pb including *Arabidopsis thaliana* (Lummerzheim et al., 1995) and *Funaria hygrometrica* (Krzesłowska et al., 2009). Pb-induced callose deposition has been detected in the rhizodermis and in the center of the stele of Pb-treated soybean *Glycine max* roots tips (Samardakiewicz et al., 1996). Under metal stress, the synthesized callose inhibits cell-to-cell transport.



This may result in the prevention of a wide incursion of Pb ions, but it can simultaneously inhibit the transport of other molecules. However, the synthesis of callose is not a general pattern in plants in response to Pb, in *Zea mays* and *G. max*, low level Pb treatment did not result in any callose deposition in root tissue. Although, these species synthesized callose in response to cadmium or arsenic (Pirselova et al., 2012). It seems that the formation of callose was closely related to the amount of Pb entering the cell, and subsequently the level of stress.

In some plants, the formation of Fe and Mn plaques on roots surface may provide a means of attenuation and external exclusion of metals. These plaques increase the sequestration of Pb on the root surface and in the rhizosphere, providing a means of external exclusion of soil Pb (Hansel et al., 2002). In some rice cultivars, Fe plaques were shown to affect patterns of Pb uptake and accumulation. Lower concentrations of Pb were found in the root tissues of rice plants with plaque compared to concentrations found in the plants without plaque. But the functions of plaque are limited as they are only efficient in relatively low or moderately Pb-contaminated soil (Liu et al., 2011). Fe plaque and organic matrix with high Pb affinity were found in root epidermis of *Typha latifolia*, and were shown to prevent the accumulation and the translocation of Pb within the root (Qian et al., 2012; Feng et al., 2013).

In most plants, 90% of the total Pb is accumulated in roots (Kumar et al., 1995). Most Pb in roots is localized in the insoluble fraction of cell walls and nuclei, which is linked with the detoxification mechanism (Piechalak et al., 2002). After exposure to Pb, cell mechanisms that minimize the potential for toxicity are rapidly activated. In the roots of several species including *Pisum sativum* (Malecka et al., 2009); *Allium sativum* (Jiang and Liu, 2010), and *Athyrium yokoscense* (Nishizono et al., 1987), the cell walls, the first barrier against Pb stress, can immobilize and accumulate some or even most Pb ions. The important role of the cell wall in the defense response of plants to trace metals was recently reviewed by Krzeslowska (2011). The capacity of cell walls to bind divalent metal cations mainly depends on the amount of polysaccharides with many carboxyl groups (Inoue et al., 2013). In *Arabidopsis thaliana*, Pb-galacturonic acid fragments were detected in root treated with Pb (Polec-Pawlak et al., 2007). Brunet et al. (2008) showed that root of *Lathyrus sativus* L. exposed to Pb contained much less calcium than control plants, and explained the reduction in Ca content by the replacement of Ca ions by Pb ions, which have a high affinity for pectin in cell walls. In *Raphanus sativus*, Pb²⁺ was also shown to bind to carboxyl groups of pectin in cell walls (Inoue et al., 2013). All the examples described above clearly show that the cell wall is one of the preferred and essential compartments for Pb accumulation, deposition, and sequestration. Therefore, these results shed a new light on the functioning of the cell walls in plant cell defense strategy against Pb. Heavy metals including Pb are likely to enter plant cells via essential cations transporters. *AtCNGC*, homologous to a non-selective cation channel, was suggested to enable Pb²⁺ entry since over-expression of the truncated gene resulted in tolerance to Pb²⁺ (Sunkar et al., 2000). Ca²⁺ was also reported to compete with Pb²⁺ for entry into rice root cells. When Ca²⁺ was supplied in the medium, it reduced Pb uptake and toxicity (Kim et al., 2002).

This suggests that Pb enters the root cells via Ca²⁺/Mg²⁺ gated channel (Kim et al., 2002).

In *Allium sativum*, as soon as excessive Pb ions enter the cytoplasm, a defense mechanism is activated, protecting the cells against Pb toxicity. Endocytotic and exocytotic processes are involved in these phenomena. The plasma membrane represents a “living” barrier of the cell to free inward diffusion of Pb ions. Invaginations of plasmalemma and some vesicles from dictyosomes and endoplasmic reticulum (ER) could prevent the free circulation of Pb ions in the cytoplasm. The vacuole is ultimately one of the main storage sites for metal sequestration (reviewed by Sharma and Dubey, 2005; Clemens, 2006). In *Allium sativum* roots, cysteine-rich peptides commonly referred as phytochelatins (PCs) were detected only after 2 h of Pb exposure (Jiang and Liu, 2010). This indicates that Pb ions can induce synthesis of PCs. Piechalak et al. (2002) demonstrated that the synthesis of PCs takes place under the influence of Pb ions in root cells of three tested plant species of the Fabaceae family: *Pisum sativum*, *V. faba*, and *Phaseolus vulgaris*. The complex PC–Pb formed is then transported through the cytosol into the vacuoles (Piechalak et al., 2002). *AtHMA3*, encoding a *P_{1B}-2-ATPase*, a heavy metal transporter, is localized in the vacuolar membrane of roots cells in *Arabidopsis thaliana* (Talke et al., 2006; Morel et al., 2009). This transporter is involved in the transfer of complexed heavy metals, including Pb, from the cytoplasm to the vacuole (Morel et al., 2009). Root length was less affected by Pb in *Arabidopsis thaliana* plants overexpressing *AtHMA3* than in wild-type plants (Morel et al., 2009). *B. juncea* appears to tolerate high concentrations of Pb thanks to its efficient cell roots vacuolar storage mechanisms. In this species, Pb sequestration was restricted to vacuoles (Meyers et al., 2008). In addition, it was suggested that exposure to Pb causes the production of additional vacuole specifically for Pb storage in the root tips of *B. juncea* (Meyers et al., 2008). The increase in the production of vacuoles could be regarded as a defense and adaptation strategy to elevated levels of Pb in the root cells. This roots potential storage can be used in phytoremediation processes. **Table 1** shows a list of plant species effective in the accumulation of Pb in roots that could be used in rhizoremediation.

Table 1 | Plant species proposed for lead rhizoremediation.

Plant species	Area of application	Reference
<i>Carex pendula</i>	Wastewater	Yadav et al. (2011)
<i>Pistia stratiotes</i>	Contaminated water	Baharudin (2008); Vesely et al. (2012)
<i>Eichhornia crassipes</i>	Aquatic system	Tiwari et al. (2007)
<i>Scirpus americanus</i>	Aquatic system	Santos-Díaz and Barrón-Cruz (2011)
<i>Phaseolus vulgaris</i>	Contaminated water	Piechalak et al. (2002)
<i>Typha latifolia</i>	Aquatic system	Santos-Díaz and Barrón-Cruz (2011)
<i>Cistus libanotis</i>	Contaminated soil	Laplaze et al. (2010)
<i>Hirschfeldia incana</i>	Contaminated soil	Auguy et al. (2013)

In a metallophilous ecotype of *Elsholtzia argyi*, Pb is found in fine particles dispersed through root cell membranes and cell wall fractions whereas in non-metallophilous roots, most Pb was found as large aggregates deposited in the cell wall fractions. These differences in localization explained why non-metallophilous roots were not able to transfer Pb to above ground parts via the apoplast (Islam et al., 2007). In some plants, Pb can be transported via vascular tissues to aerial parts (Hanc et al., 2009). In *Sesbania drummondii*, Pb is transported to leaves after complexation with acetate, nitrate, and sulfide (Sharma et al., 2004). In tobacco, a *cyclic nucleotide gated channel* (*NtCBP4*) was suggested to be involved in Pb transport (Sunkar et al., 2000).

To sum up, Pb pathway may include the following stages in roots: Pb can bind with physical barrier (callose, Fe/Mn plaques, cell wall...). At high concentration, this barrier is broken and the flux of Pb enters the cell through the plasma membrane using the ions transporters. In cytoplasm, Pb is chelated with PCs. The complexe formed is then sequestered in the vacuoles. In accumulator plants, Pb can be transported via phloem to aerial parts (Figure 1).

Compared to Zn and Cd, very little is known about the molecular mechanisms of acquisition, transport, and accumulation of Pb. This is due first to the characteristics of Pb which precipitates with some components of the culture media making difficult to study its bioavailability to the roots. On the other hand, the lack of model plant for studying the mechanisms of tolerance to this metal. Among the 450 species known as metal hyperaccumulator and tolerant plants, Pb accumulating species are rather exceptional. Recently, Auguy et al. (2013) identified *Hirschfeldia incana*, a member of the Brassicaceae family, as a Pb accumulator plant. They demonstrated that this species, owing to its close genetic proximity to *Arabidopsis*, is a good model to identify genes involved in Pb tolerance and accumulation. This can open up new possibilities for understanding the molecular mechanisms of Pb tolerance in plants.

EFFECT OF LEAD ON ROOT DEVELOPMENT AND PHYSIOLOGY

PHYSIOLOGY AND ULTRASTRUCTURAL EFFECTS OF LEAD

The primary effect of Pb toxicity in plants is a rapid inhibition of root growth, probably due to the inhibition of cell division in the root tip (Eun et al., 2000). It was demonstrated that Pb caused inhibition of cell division in *Lemna minor* roots (Samarakiewicz and Wozny, 2005). In several plant species, including *Triticum aestivum* (Dey et al., 2007; Kaur et al., 2013), *Z. mays* L. (Kozhevnikova et al., 2009), *Pisum sativum* (Malecka et al., 2009), and *Sedum alfredii* (Gupta et al., 2010), a decrease in the length and in root dry mass under Pb toxicity have been reported (Munzuroglu and Geckil, 2002). Verma and Dubey (2003) showed that growth of rice roots was significantly inhibited at 0.5–1 mM Pb²⁺; up to 40% reduction in root length was observed in 20-day-old rice seedlings. In Pb-treated *Elsholtzia argyi* and *Elsholtzia splendens*, the length and surface area of roots were strongly affected (Peng et al., 2005).

In response to Pb exposure, roots can also respond via changes in volume and diameter, with the production or inhibition of lateral roots. Root cells viability in rice is affected by Pb²⁺ ions and cell death increased at different Pb concentrations (Huang and Huang, 2008). Furthermore, cell wall distention, formation of

folds, protuberances, and nicks were observed in response to different Pb concentrations in *Triticum aestivum* (Kaur et al., 2013), *Elsholtzia argyi* (Islam et al., 2007), and *Allium cepa* (Wierzbicka, 1998). Pb has been reported to disrupt microfibrils and microtubules, resulting in the formation of folds (Liu et al., 2009). In addition, Kaur et al. (2013) observed distentions and lesions in cell wall of *Triticum aestivum* roots as a result of activation of certain wall-degrading enzymes in response to Pb exposure. In *Z. mays* roots, Pb treatment resulted in Pb accumulation in the meristem in both apoplastic and symplastic pathways, associated with changes in microtubule organization (Eun et al., 2000).

Lead also has an impact on mineral homeostasis. Brunet et al. (2008) found that roots of *Lathyrus sativus* exposed to Pb showed an increase in Pb content along with an increase in Na levels, which is absorbed to compensate the loss in K. A reduction in Ca contents in Pb-exposed plants has also been observed in other species, such as maize, tomato, and mustard varieties (White and Broadley, 2003; Sharma and Dubey, 2005) and could result from the inhibition of Ca transporters by Pb ions (Wojas et al., 2007) and/or replacement of Ca ions with Pb ions due to its high affinity for Ca binding-sites on biological structures (Habermaann et al., 1983). A reduction in Zn, Cu, and K contents in response to Pb exposure was observed in *Cucumis sativus* and *Z. mays* plants, as a result of a possible blockage of the transporter proteins by Pb (Sharma and Dubey, 2005).

Finally, Pb induces genotoxicity in plants (Rucińska et al., 2004). The comet assay evaluating the DNA-damaging effect of Pb showed an increase in DNA damage in root nuclei of tobacco and lupin (Rucińska et al., 2004; Gichner et al., 2008).

BIOCHEMICAL EFFECTS OF LEAD

The cytotoxic mechanisms of Pb in plants are not entirely understood. It has been reported that Pb leads to the overproduction of reactive oxygen species (ROS) such as superoxide radicals (radical O²⁻) and hydrogen peroxide (H₂O₂) in plant cells (Reddy et al., 2005; Liu et al., 2010). These can cause lipid peroxidation, membrane damages, and oxidative stress (Sharma and Dietz, 2009). When pea (*Pisum sativum*) roots were exposed to 0.1 and 0.5 mM of Pb(NO₃)₂, a rapid increase in superoxide anion (O₂⁻) and H₂O₂ levels occurs after 2 and 8 h of Pb treatment, respectively (Malecka et al., 2009). Liu et al. (2012) reported that after Pb treatment, roots of *Ficus microcarpa* produced high concentrations of H₂O₂ along with an increase in O₂⁻ accumulation. O₂⁻ is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the plasma membrane, and is converted to H₂O₂ through non-enzymatic pathways or by superoxide dismutase (SOD; Passardi et al., 2004). Some ROS can alter gene expression and modulate the activity of specific proteins in the plant defense system (Sharma and Dubey, 2005). To protect cells and tissues from injury and dysfunction, plants have developed various strategies, such as over expression of SOD, catalase (CAT), peroxidase (POX), and ascorbate POX genes. In addition, non-enzymatic antioxidants with low molecular weights, such as proline, cysteine, non-protein thiol, ascorbic acid, and glutathione, which can reduce oxidative stress by scavenging ROS are synthesized (Choudhury and Panda, 2005; Singh et al., 2006; Malecka et al., 2009). Responses to metal toxicity involving these enzymes and non-enzymatic

antioxidants differ depending on the plant species, type of tissue, and metal concerned. Huang and Huang (2008) showed that in rice roots, Pb²⁺-induced ROS production and Ca²⁺ accumulation and activated MAP (mitogen-activated protein) kinases (proteins kinase cascade and major pathways by which extracellular stimuli are transduced into intracellular responses in all eukaryotic cells; Jonak et al., 2002) which are located in the apical region in rice roots. They demonstrated that treatment with glutathione, a powerful antioxidant, decreased Pb²⁺-induced root cells death and reduced MAP kinases activity. An increase in H₂O₂ content upon Pb exposure was observed in response to Pb²⁺, with an increase in CAT activity in *Triticum aestivum* (Kaur et al., 2013), *Elsholtzia argyi* (Islam et al., 2007), and *Pisum sativum* (Malecka et al., 2009). Pb-induced lipid peroxidation and enhanced H₂O₂ content in roots of *Allium sativum* (Liu et al., 2009), *Z. mays* (Gupta et al., 2009), and *B. campestris* (Singh et al., 2011). However, Islam et al. (2007) and Kaur et al. (2013) reported a decline in the activity of POXs in *Elsholtzia argyi* and *Triticum aestivum* roots upon Pb exposure. Therefore, a higher concentration of Pb or longer treatment inhibit cell metabolism and H₂O₂ production, resulting in a decrease in the activity of some antioxidant enzymes (CAT; Verma and Dubey, 2003; Malecka et al., 2009). Plant enzymatic antioxidant defense systems vary with the plant species and with the intensity of Pb treatment. Production of ROS is common to different plant species. Some of these produced ROS may function as important signaling molecules by altering gene expression and modulating activity of specific defense proteins. However, all ROS can be very harmful to organisms at high concentrations.

Lead also increased protein and proline contents in roots of two varieties of *B. napus* with an increase in the concentration of Pb in the nutrient solution (Gohari et al., 2012). Proline plays an essential role in reducing environmental stress, including that caused by heavy metals. Piechalak et al. (2002) found that *V. faba* and

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Pisum sativum roots produced high amount of thiol peptides and PCs after Pb exposure. Although, the high level of these proteins allows tolerance to Pb for these species roots.

CONCLUSION

Root system is the first organ in contact with the different components of the soil and water. By their exudates and their effects on rhizosphere activities (proliferation of microorganisms, metal chelation, acidification, etc.) plant roots can tolerate and in some cases accumulate high levels of Pb. An overall higher rate of accumulation was observed in roots rather than leaves in several species. Almost 90% of Pb accumulated in a number of species of the Brassicaceae family (Kumar et al., 1995) and some crops species such as *Z. mays* (Małkowski et al., 2002) and *Pistia stratiotes* (Vesely et al., 2012) was located in roots. This accumulator potential can be used in phytoremediation process. Rhizofiltration is a subset technique that uses both terrestrial and aquatic plants roots to absorb, concentrate, and precipitate metals from polluted water to their biomass (Dushenkov et al., 1995). This technique is cost-effective, and can be used for site restoration including maintenance of the biological activities of the polluted site. In this context, several plants have been identified whose roots could be used to clean up land contaminated by Pb. Therefore, improvement of the capacity of plant roots to tolerate and accumulate Pb by genetic engineering should open up new opportunities for rhizoremediation.

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How to study deep roots—and why it matters

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The drivers underlying the development of deep root systems, whether genetic or environmental, are poorly understood but evidence has accumulated that deep rooting could be a more widespread and important trait among plants than commonly anticipated from their share of root biomass. Even though a distinct classification of “deep roots” is missing to date, deep roots provide important functions for individual plants such as nutrient and water uptake but can also shape plant communities by hydraulic lift (HL). Subterranean fauna and microbial communities are highly influenced by resources provided in the deep rhizosphere and deep roots can influence soil pedogenesis and carbon storage. Despite recent technological advances, the study of deep roots and their rhizosphere remains inherently time-consuming, technically demanding and costly, which explains why deep roots have yet to be given the attention they deserve. While state-of-the-art technologies are promising for laboratory studies involving relatively small soil volumes, they remain of limited use for the *in situ* observation of deep roots. Thus, basic techniques such as destructive sampling or observations at transparent interfaces with the soil (e.g., root windows) which have been known and used for decades to observe roots near the soil surface, must be adapted to the specific requirements of deep root observation. In this review, we successively address major physical, biogeochemical and ecological functions of deep roots to emphasize the significance of deep roots and to illustrate the yet limited knowledge. In the second part we describe the main methodological options to observe and measure deep roots, providing researchers interested in the field of deep root/rhizosphere studies with a comprehensive overview. Addressed methodologies are: excavations, trenches and soil coring approaches, minirhizotrons (MR), access shafts, caves and mines, and indirect approaches such as tracer-based techniques.

Keywords: deep roots, biogeochemical and ecological functions, root measure

INTRODUCTION

Studies on below-ground ecosystem processes are relatively rare compared to those dealing with above-ground traits of plants; roots and the rhizosphere being “hidden” in the soil (Smit et al., 2000), their observation and study relies on deploying special methodologies that are generally time-consuming and often costly. Even though methodologies to study belowground processes have significantly improved and the number of studies addressing roots has increased in recent decades, studies on roots remain mostly confined to the uppermost soil horizons. While Canadell and colleagues (1996) highlighted the potential influence of “deep roots” on many ecosystem processes nearly two decades ago, information about the actual importance of deep roots in terms of plant and ecosystem functioning, (global) water cycles and biogeochemistry remains scarce. This situation appears to be related to two major factors: (i) technological and economical limitations, i.e., the absence of tools to measure roots with sufficient throughput and standardization at affordable costs (Böhm, 1979; Vogt et al., 1996; Smit et al., 2000), and (ii) the widespread assumption that deep roots are a rather marginal component of plants. Even though deep roots may, in most cases,

represent a relatively small fraction of the overall root system biomass, they likely fulfill much more essential functions than commonly accepted; an increasing number of studies clearly indicate that “looking deeper” is essential to increase our understanding of plant ecophysiology, but also of community ecology and geochemical cycles (Harper and Tibbett, 2013; see below). This review highlights the increasing importance and impact of deep roots in environmental research and provide some guidance to future research.

In this context, this review elaborates on the physiological and ecological significance of deep roots before providing a detailed overview on methods to study deep roots. Addressed methodologies are (i) excavations, trenches and soil coring approaches, (ii) minirhizotrons (MRs), (iii) access shafts, (iv) caves and mines, and (v) indirect approaches such as tracer-based techniques.

THE CHALLENGE OF DEFINING DEEP ROOTS AND MEASURING ROOTING DEPTHS

Factors that drive root growth and root system expansion are known from a diversity of field and laboratory observations. Previous publications have described the genetic control of root

traits such as length, branching and root hair formation [see references in Kell (2011)]; however, the mechanistic details, resulting in different root system phenotypes, are often unknown [but see e.g., Kato et al. (2006) for “root growth angle”]. With regard to genetic control, some root systems were found to develop rapidly: *Pinus radiata* and *Robinia pseudoacacia* roots reached a depth of 2.5 and 3.7 m after 4 years respectively (Stone and Kalisz, 1991). Similarly, Christina et al. (2011) reported that roots of Eucalypt trees could progress downward at rates of 0.55 m month^{-1} , 9–10 months after planting. Beside genetics, root architecture is controlled by hormonal influences from the plant (e.g., Santner et al., 2009) and soil organisms, and by the environment.

Due to the fact that soils are the most complex of all environments (Fitter et al., 2000) and nutrients are often strongly bound to the soil matrix (Strong et al., 1999), soil resources are inherently patchy and poorly available to organisms. In turn, plants have evolved complex strategies to forage for soil resources; root growth and root system development correspond to the allocation of assimilates to individual root apices capable of independent, yet coordinated at the plant level, morphological and physiological responses to their immediate environment. In view of the major influence of soil patchiness on root growth, it is not unexpected that spatial rooting patterns are highly variable. Indeed, one major confounding factor that often precludes accurate estimation of rooting depth is the inherent variability of root distributions (e.g., Nicoullaud et al., 1995). Further, even when this variability is taken into account, sampling depths are often decided arbitrarily and set to values that are too shallow to allow reliable estimates of rooting depth (Schenk and Jackson, 2002).

However, studies focusing on rooting depth have clearly shown that woody plants are, on average, more deeply rooted than herbaceous ones (e.g., Shalyt, 1952; Baitulin, 1979; Kutschera and Lichtenegger, 1997; Schenk and Jackson, 2002). According to Canadell et al. (1996), the rooting depths of herbaceous plants, shrubs and trees are globally in the magnitude order of $2.6 \pm 0.1\text{ m}$, $5.1 \pm 0.8\text{ m}$, and $7.0 \pm 1.2\text{ m}$, respectively. Many trees (*Eucalyptus spp*) and shrubs in arid areas are very deep rooted, with woody legumes such as *Acacia*, and *Prosopis* reaching depths of 20 m and even extremes such as 50–60 m (Stone and Kalisz, 1991). Canadell et al. (1996) have pointed out that tropical savannah is the biome with the deepest mean rooting depth ($15 \pm 5\text{ m}$) and also has the deepest recorded root system (i.e., 68 m; Jennings, 1974). However, even in evergreen tropical forests a number of tree species have deep root systems ($>8\text{ m}$), which enable them, e.g., to survive periodic droughts (see below).

Thus, aside from genetic control and the physiological needs of each single species, external physical or biochemical factors influence the root development. Indeed, Harper et al. (1991) proposed to define root system architecture (RSA) as an evolutionary response to the spatio-temporal variability of resource availability and the corresponding constraints to growth. Some studies suggested that maximum rooting depth is mostly limited by water tables or by subsoil characteristics that prevent rooting (Cannon, 1949; Stone and Kalisz, 1991; Stone and Comerford, 1994) while others demonstrated that trees can grow roots well beyond the subsoil into the weathered bedrock (Schwinning, 2010) and/or maintain active roots below the mean water table

(Wardle et al., 2004; Laio et al., 2009), e.g., by carrying and releasing oxygen under water-logged conditions (Justin and Armstrong, 1987; Shimamura et al., 2007). Thus at least some plants can modify the soil properties in their immediate vicinity (Hodge et al., 2009) to allow for deeper root system placement. However, according to Schenk (2008), roots grow as shallow as possible and as deep as necessary in response to the required water supply. Despite providing a rational explanation for the development of deep roots under a range of environmental conditions, this approach overlooks other major root functions such as nutrient foraging (see below) and plant anchorage. In addition, some experiments conducted under favorable environments (with no water or nutrient constraints, no anchoring hindrances) evidenced substantial root systems (Passioura and Wetselaar, 1972), which contradicts the former statement and confirms the generic value of the concept of a plastic root growth (Hodge, 2006).

Given this inherent plastic nature of root system development and the resulting variability of rooting patterns, there is currently no consensus on the definition of “deep root.” Based on a global review of 565 root profiles, Schenk and Jackson (2002) derived average rooting profiles for 15 terrestrial biomes including all latitudes; the average of these 15 profiles indicates that soil depths of 1.1, 0.7, and 0.4 m correspond to cumulated root proportions of 95, 90, and 80%, respectively. Schenk and Jackson (2002) also found that the median sampling depth for root profiles was 0.88 m. Based on these figures, and notwithstanding species-specific or functional definitions, we therefore propose here to qualify “deep roots” in general as roots growing at soil depths of at least 1 m.

PHYSICAL, BIO-CHEMICAL, AND ECOLOGICAL FUNCTIONS OF DEEP ROOTS

While it is impossible to attribute most traits and functions exclusively to shallow or deep roots, some distinctions can be made in their specialization and their impact on the environment. The main ecological and geochemical impacts of deep roots are highlighted in this first part of the review and key processes are visually summarized in **Figure 1**.

THE ROLE OF DEEP ROOTS IN WATER UPTAKE AND REDISTRIBUTION

Water uptake is one of the key functions of deep root systems, especially in the driest and rockiest environments. Stone and Kalisz (1991) identified more than 30 species of trees that develop roots over long distances and can access deep water tables. Water storage in bedrock may also be of global importance: plants that experience soil moisture deficits might keep expanding their root systems in the weathered bedrock (Schwinning, 2010), an hypothesis supported by findings that shallow-soil endemic plants developed the special ability to explore large rock surface areas, which increases their chance to locate and explore cracks in the underlying rock (Poot and Lambers, 2008; Schenk, 2008). For example, evergreen forests in Northeastern Pará state in Brazilian Amazonia maintain transpiration during the up to 5-month dry periods by absorbing water from the soil to depths $>8\text{ m}$ (Nepstad et al., 1994). Similar, most deciduous species in dry monsoon forests of South and Southeast Asia form new leaves 1–2 months

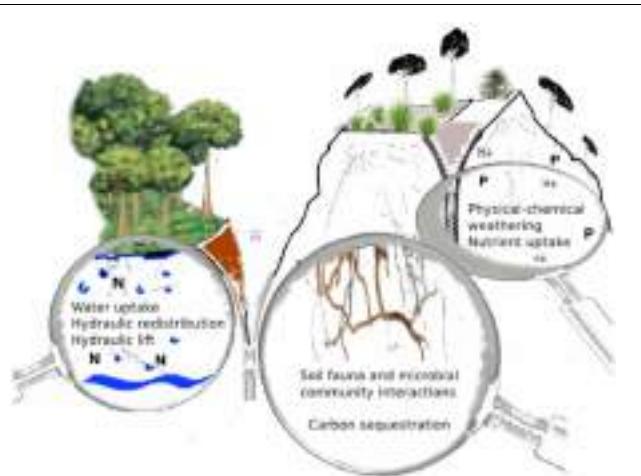


FIGURE 1 | Summary of major impacts of deep roots on the subsoil and deep roots' functions, i.e., water uptake and hydraulic redistribution, nutrient uptake, physical–chemical weathering and C sequestration, and deep root-fauna and -microbial interactions.
See text for further information.

before the first monsoon rains, during the hottest and driest part of the year, which indicates that climate is not the principal determinant of their vegetative phenology which most likely depends on deep rooting (Elliott et al., 2006). More surprisingly, significant contributions of deep root to plant water uptake appears not to be restricted to water-limited environments; for example, Dawson and Ehleringer (1991) found that mature riparian trees hardly used readily available stream water and derived most of their water supply from ground water at much greater depth.

It has been argued that under pronounced seasonal arid climates deep roots favor hydraulic lift (HL), also termed hydraulic redistribution (HR; Burgess et al., 1998; Burgess, 2000), i.e., the nocturnal transfer of water by roots from moist to dry regions of the soil profile. In addition to the effects on water uptake, HL and HR can indirectly influence the availability of some nutrients (Snyder et al., 2008; see below). The process of HL was probably first described by Breazeale (1930) and received much attention since the late 1980's (Richards and Caldwell, 1987; Caldwell and Richards, 1998). HL is known to predominantly—while not exclusively—occur in deep rooted vegetation of biomes such as savannahs and shrublands, mobilizing water resources down to depths of 20 m (Bleby et al., 2010). HL and HR have been reported to provide benefits for mixed species stands/intercrops in many different biomes (Peñuelas and Filella, 2003; Goldstein et al., 2008; Zapater et al., 2011) and as a consequence, to have an impact on ecosystem functioning (Horton and Hart, 1998; Oliveira et al., 2005). With regard to agro-ecosystems, HL could contribute to develop more efficient intercropping systems (Mulia and Dupraz, 2006; Malézieux et al., 2009) with positive plant-plant interactions at best acting as a “water-safety net” (Sekiya et al., 2010). Thus, it has been proposed that breeding and engineering efforts aimed at facilitating water redistribution could eventually be used to boost yields in intercropping/agroforestry systems (Burgess, 2010).

In a wider perspective, the impact of deep roots on hydrological cycles could indirectly influence regional climates; Kleidon and Heimann (2000) concluded that deep-rooted vegetation is an important part of the tropical climate system and that without considering deep roots, the present-day surface climate cannot be simulated adequately. As many tree species of tropical forests establish a link between groundwater and the atmosphere, the presence or absence of un-degraded tropical forest reportedly influences regional climate (Brujinzeel, 2004). In summary, there is diverse, yet consistent evidence that deep roots play a major role in plant water uptake, soil water availability and the water cycle at various scales from the rhizosphere to whole catchments (Bengough, 2012).

DEEP ROOTS AND NUTRIENT UPTAKE

RSA, i.e., the spatial distribution and morphology of roots, root physiology and symbiotic interactions affect the ability of plants to access nutrients. The occurrence of deep-rooted plants, especially in (semi-) arid ecosystems, is classically explained in regard to water uptake (see above). However, McCulley et al. (2004) collected evidence suggesting that water uptake at depth can be limited, even under arid conditions. Furthermore, they found that some nutrients had comparable if not larger plant available pools in deeper soil layers; for example, P weathering (see below) is usually greater in deep soil layers than in the topsoil (Sverdrup et al., 2002). These results, in addition to data on strontium (Sr) uptake from deep soil horizons, suggest that deep soils in (semi-) arid regions may be more significant nutrient sources than commonly believed (He et al., 2012). In addition, HR could mobilize nutrients within the soil and supply those to roots through mass flow or diffusion (McCulley et al., 2004; Lambers et al., 2006; Da Silva et al., 2011). While data on the contribution of deep roots on nutrient uptake in other ecosystems such as highly weathered tropical soils is still scarce (Hinsinger et al., 2011), it is generally believed that deep(er) root systems are important for the uptake of mobile nutrients such as potassium (K) but also nitrogen (N). While an increase in roots length in the topsoil will not increase uptake due to overlapping depletion zones (Andrews and Newman, 1970), deep roots can significantly expand the soil volume accessible for uptake and thus, e.g., increase the N-uptake fraction (McMurtrie et al., 2012). Differences in N depletion due to differences in rooting depth are of special interest for environmental protection; N in deep soil layers is more prone to leaching than N in shallow soil horizons (Thorup-Kristensen and Nielsen, 1998; Thorup-Kristensen, 2001). While, due to the high mobility of nitrate, high root densities may not be needed to enable plants to deplete specific soil areas (Robinson, 1991; Robinson et al., 1996), a linear relationship was found between root density and ¹⁵N uptake from different depths (Kristensen and Thorup-Kristensen, 2004). In addition, early root growth to deeper soil horizons has been found to be important because N depletion of deep soil can be slower than N uptake in shallow soil horizons (Strebel et al., 1989), cited after (Thorup-Kristensen, 2001). For trees, Laclau et al. (2010) demonstrated that 6 m-deep roots of *Eucalyptus* spp. limited nutrient losses through deep drainage, following clear-cutting of previous tropical vegetation. While Kristensen and Thorup-Kristensen (2004, 2007)

indicate that different N use efficiencies of crops depend more on species-specific differences in root development over time and space than on differences in N uptake physiology of roots, Göransson et al. (2006, 2007, 2008) found differences in the nutrient uptake capacities, i.e. root physiology, between shallow-(5 cm) and deeper-growing (50 cm) oak roots. While such differences were not found for beech and spruce, and P uptake of oak, estimates of fine root distribution alone may thus not reflect the uptake capacity of all nutrients and all tree species with sufficient accuracy (Göransson et al., 2008). Similar differences in root uptake potentials between shallow and deep roots under tropical conditions have been found for *Eucalyptus* spp. (Da Silva et al., 2011; Laclau et al., 2013). Interestingly, Pregitzer et al. (1998) found declining root respiration rates with increasing soil depth in Sugar maple. In summary, the previous studies indicate that deep rooting species such as oak, Sugar maple and *Eucalyptus* may have evolved different physiological uptake strategies in deep and shallow soil horizons, possibly optimizing uptake efficiency in terms of carbon costs by functional specialization [see also discussion in Da Silva et al. (2011)] under reduced competition. Future studies on the physiological properties of deep roots are imperative for a better understanding of the functional specialization of nutrient uptake by fine roots in general and the development of improved nutrient uptake models in specific.

PHYSICAL-CHEMICAL WEATHERING BY DEEP ROOTS

Growing roots tend to follow pores, channels and preferentially explore soil less dense than the bulk soil (Moran et al., 2000); as woody roots grow radially, they expand in volume and exert enormous pressure on the surrounding soil (Misra et al., 1986). In contrast to roots in uppermost soil horizons, growth pressure by deep roots cannot be relieved by upward displacement but by soil compaction, reducing for example, porosity and subsequently hydraulic conductivity and aeration and thus biogeochemical functioning. Even relatively consolidated, un-weathered rocks are susceptible to the physical effects of deep roots: rock wedging results when growing roots expand at joints or fractures and the pressure can accelerate chemical dissolution of minerals (Richter and Markewitz, 1995; Richter and Walther, 2007). It has been known for decades that roots exert physical–chemical weathering actions on their environment (Meyer and Anderson, 1939), and that such processes are decisive for the mobilization of nutrients. Roots influence the ionic concentrations in their immediate environment and are also involved in other interactions due to the root exudates in the rhizosphere (Hinsinger, 1998). While such processes have almost exclusively been studied in top soils, it is certainly valid to consider that they also prevail in deep soil layers (Richter and Markewitz, 1995). Indeed, it was shown that fine roots at a soil depth of 1 m could balance chemical adversity in natural soil (Richter and Walther, 2007). Carboxylate exudation by deep roots can contribute accessing poorly soluble iron phosphate in arid zones (He et al., 2012). As deep roots directly influence the depth distribution of soil carbon dioxide and acidity, there is no doubt that they play an active role in the physical–chemical weathering of mineral material and thus contribute to pedogenesis, but the precise biogenic effects of deep roots remain to be clarified (Richter and Markewitz, 1995).

INFLUENCE OF ROOTING DEPTH ON C BIOGEOCHEMISTRY

Despite their low carbon (C) content, subsoil horizons contribute to more than half of the total soil C stocks, and therefore need to be considered in the global C cycle (Harrison et al., 2011; Koarashi et al., 2012; Harper and Tibbett, 2013). Soil organic carbon (SOC) has three main origins: plant root growth including exudates, dissolved organic carbon (DOC) transport and bioturbation (Rumpel and Kögel-Knabner, 2011). While the relative importance of each source is dependent on, for example, climate, soil and vegetation types, the general importance of roots for soil C sequestration (Kell, 2011) is underlined by the fact that the root-derived C has a high potential to be stabilized long-term. Beside other stabilizing factors (Rumpel and Kögel-Knabner, 2011), roots are often more recalcitrant than topsoil litter (Abiven et al., 2005; Rasse et al., 2005). The deposition and fate of C from deep roots (and their associated biota, see below) has rarely been examined in detail (Clemmensen et al., 2013; Harper and Tibbett, 2013). Furthermore, root C fluxes to deep soil layers are poorly understood mainly due to uncertainties associated with the measurement of total root C input, i.e., sloughing of root cells during growth, root exudates and root turnover. Because subsoil horizons with low C concentrations may not yet be saturated in SOC, it has been suggested that they may have the potential to sequester SOC through increasing C input by turnover of deep roots and DOC following preferential flow pathways such as root pores (Lorenz and Lal, 2005; Rumpel and Kögel-Knabner, 2011). The dynamics of deep SOC is largely controlled by interactions with soil minerals (Koarashi et al., 2012), and as both processes are highly influenced by deep roots (see above), future studies are urgently needed, including estimates on C changes in deep soil profiles in response to land-use changes such as de-/reforestation or the disappearance of specific deep-rooted plant species. Further studies on deep roots will significantly improve information on root-derived C, which is needed to accurately describe critical processes like net primary production and carbon storage from ecosystem to global scales and under recent and future climates (McCormack et al., 2013).

IMPACT OF DEEP ROOTS ON SOIL FAUNA AND MICROBIAL COMMUNITIES

Fauna diversity was described as declining from the shallow toward the deep subterranean habitats (Culver and Pipan, 2009), however it is still widely unknown how deep roots influence the vertical distribution of soil fauna. While it is well known that fauna in the uppermost soil horizons and litter layers utilize roots for feed, it was also shown that deep plant roots are the major energy source, and provide shelter and cocoon-building material for troglobionts, i.e., invertebrates restricted to subterranean environments (Howarth et al., 2007; Silva et al., 2011; Novak and Perc, 2012). Both living and dead roots are used, providing resources for a wide diversity of cave organisms, including root-feeders, scavengers, and predators (Howarth, 1983). Freckman and Virginia (1989) showed that in some ecosystems the majority of nematodes, and thus herbivory, may occur at soil depths rarely studied. Because deep roots can directly or indirectly support the fauna, the loss of deep-rooted plants in general or of specific species will affect subterranean animals—as far as eliminating

host root-specific animal (Reboleira et al., 2011). Knowledge on deep root-fauna interactions is thus decisive for development of conservation strategies in ecosystems and to understand root herbivory. While Silva et al. (1989) claimed that deep-rhizosphere micro-arthropod fauna is a reduced subset of the fauna of shallow soil horizons, Novak and Perc (2012) stated that the division of soil fauna into shallow and deep communities is a global pattern, at least in karst ecosystems with deep-rooted vegetation. While caves might represent very special ecosystems, the concentrations of organic matter and bioavailable nutrients usually decrease with soil depth; thus, in deep soil horizons the rhizosphere is “an oasis of resources compared with the [bulk soil]” (Richter and Walther, 2007). For example, the fungal biomass in forest bulk soil decreased steadily by three orders of magnitude from the soil surface to 2.5 m depth whereas the fungal biomass in the rhizosphere remained relatively constant between depths of 0.4–2.5 m and was higher than in bulk soil (Richter and Walther, 2007), illustrating the impact of roots on the depth distribution of fungal biomass. Furthermore, fungal species community compositions can change with depth too, i.e., different species or fungal functional groups form mycorrhizal symbioses with deep roots than with shallow roots (e.g., Rosling et al., 2003; Clemmensen et al., 2013). While it is known that the diversity of microorganisms is typically decreasing with depth and the community composition is changing (Eilers et al., 2012), high levels of bacterial biomass were found to remain down to 8 m depth in prairie soils (Dodds et al., 1996); it is thus currently unknown which roles

deep roots play for soil microbial communities in detail. However, because deeper occurring microbes may have a greater influence on soil formation processes than their counterparts in shallow soil horizons, due to their proximity to soil parent material (Buss et al., 2005) and a critical influence on longer-term soil carbon sequestration (Rumpel and Kögel-Knabner, 2011), further studies including the rhizosphere of deep roots are imperative. A first indication of the importance of deep roots on bacterial communities is given by Snider et al. (2009), who observed complex interaction between deep roots and bacterial communities, some bacteria from the soil overlaying the cave being introduced by the roots while deep roots could acquire bacteria from the cave walls.

In general, the distributions of root-associated biota through the soil profile remains poorly understood, as most studies focus on communities in shallow soil horizons. This emphasizes the importance of future research into faunal, fungal and microbial communities adapted to the deep root zone, enhancing understanding of subterranean ecology and ecosystem functioning (Cardon and Whitbeck, 2007).

DIRECT AND INDIRECT METHODS TO STUDY DEEP ROOTS

In this second part of the review we highlight the most important methods to access and to study deep roots directly and visually (**Figures 2–6**) and discuss their main advantages and shortcomings (**Table 1**). More precisely, we present four methodological groups: (i) excavations, trenches and soil coring

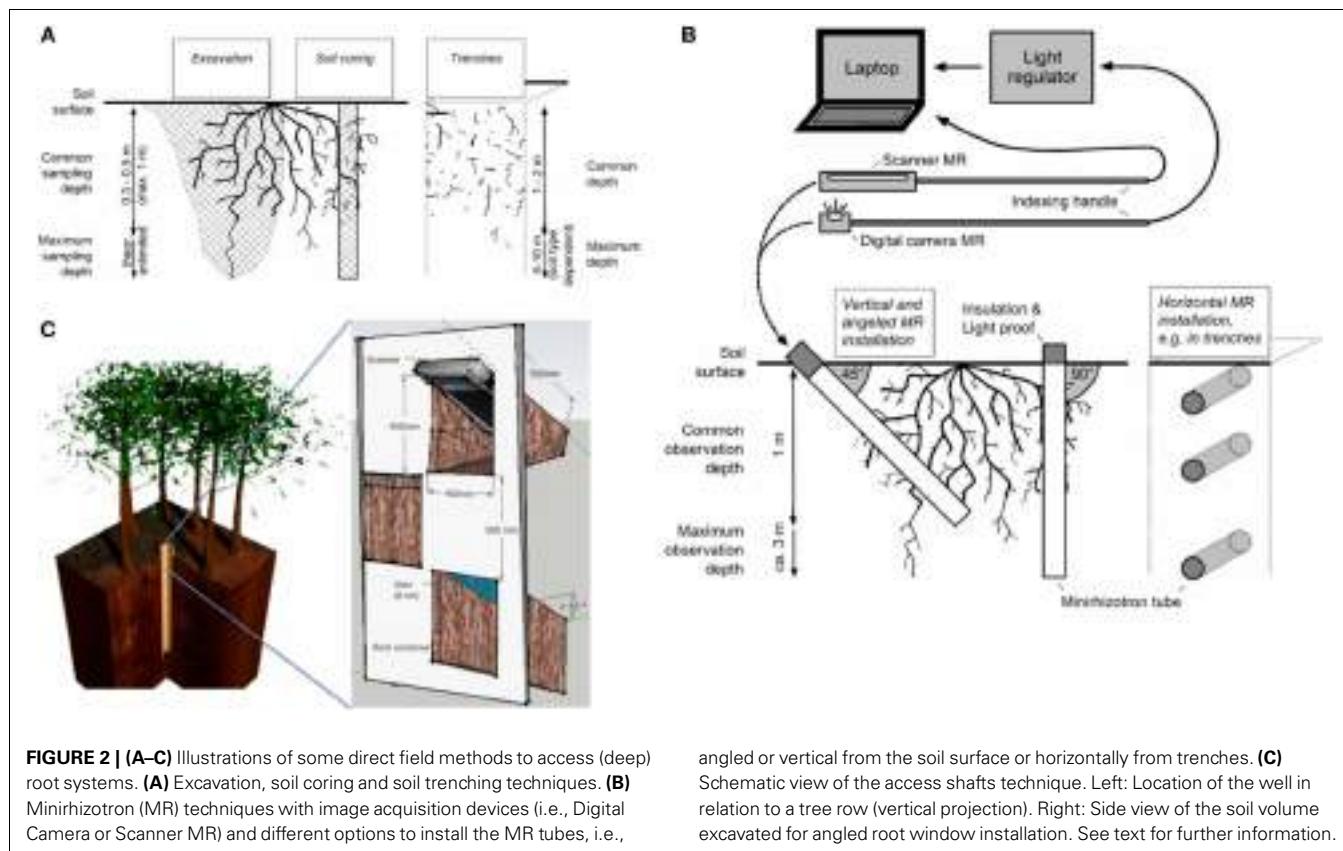




FIGURE 3 | Root mapping and collection in a trench (4 m deep) in Thailand (Maeght, 2009).



FIGURE 5 | Root scanning in access shaft (5 m deep) in Lao PDR (Maeght, 2012).



FIGURE 4 | Root sampling from an excavation (7 m deep) in Lao PDR (Maeght, 2009).

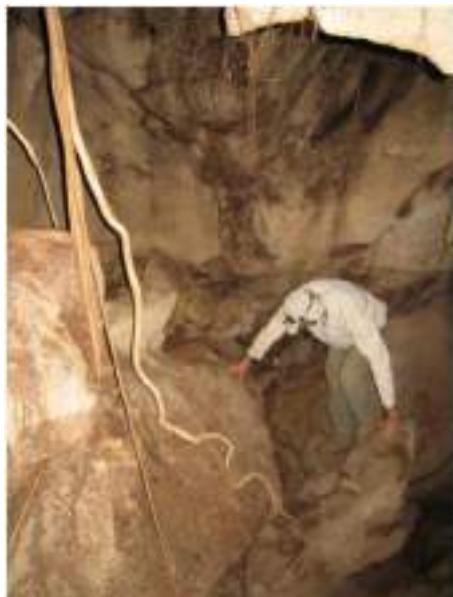


FIGURE 6 | Cave prospection (12 m deep) for root studies in Lao PDR (Pierret, 2010).

approaches, (ii) MRs, (iii) access shafts, and (iv) caves and mines. In addition, a short overview on (v) indirect approaches such as tracer studies is given.

EXCAVATION, TRENCHES, AND CORING APPROACHES

Despite advances in root studies in the last five decades, the most common methods used to obtain data on root distribution and structure have not changed substantially: excavation

and coring techniques are still and by far the preferred methods. Recently, the term “shovelomics” was established (Trachsel et al., 2010) to qualify simple but effective approaches to determine root phenotypes including maximum rooting depth. Excavation methods include manual digging and up-rooting, the use of various mechanical devices, explosives, and high pressure water or air (Weaver, 1919; Stoeckeler and Kluender, 1938; Mitchell and Black, 1968; Newton and Zedaker, 1981; Rizzo and Gross, 2000). Coring can be conducted manually by pushing or hammering sampling equipment into the soil using various devices from simple, sharpened steel augers to advanced cryogenic devices for sampling wetland soil (Cahoon

Table 1 | Main advantages and disadvantages of direct (i.e. mechanical, visual) methods to access and to study deep roots.

Method	Realistic replication per plot	Key benefits	Disadvantages
Excavation	Very few (~1–2)	3D information, possibility of mapping root systems layer by layer (root biomass). Root samples can be analysed further (e.g. for root morphology, to digitize the coarse root system)	Fine roots are often omitted. Very destructive and very labor intensive for bigger plants
Trenching	Few (<3)	Vertical and horizontal information (2D, root counting). Possibility to take root and soil samples and to install MR tubes and other measurement gear	Difficulty to establish deep trenches without reinforcements. Limited time of usability. Destructive and labor intensive
Soil coring	Many (>10–20)	Vertical information (fine root biomass). Root samples can be analysed further (e.g. for root morphology). Easy to replicate in stone-free soils. Minor plot disturbance	Requires a large number of samples. Moderate destructive and labor intensive rinsing. Logistically difficult if machine drilled
Minirhizotrons	Average (5–8)	Continuous, vertical information (fine root length density, root dynamics). Relatively easy to replicate in stone-free soils. Minor plot disturbance	Difficult set-up into deep soil layers ("gap formation"). Time lag before first measurement. Limited length of commercial tubes (<3 m). Expensive imaging equipment. Very labor intensive analysis and logically difficult if machine drilled
Access shafts	Few (<3)	Continuous, vertical information (fine root length density, root dynamics). Possibility to manipulate/sample roots and soil at different depths. Sufficient space for additional measurements/devices	Adaptation depends on soil type and local geography. Moderate plot disturbance and very labor intensive. Logistically difficult for enforcement delivery
Mines and caves	Not controllable	Can provide cost-efficient access to the greatest depth. Intrinsic potential to study root-cave animal/microbe interactions. Sufficient space to install (sap-flow) sensors	Not a "normal" soil environment. Difficulties in identifying the parent plant taxa/individual from the root. Replication not controllable. Often difficult to enter

Description of key benefits is based on one replicate per method.

et al., 1996; Rewald and Leuschner, 2009). In addition, vehicle-mounted or hand-held mechanical devices have been developed to take soil cores in the field, especially to greater depth or with larger diameters (see Kornecki et al., 2008 and references within). An overview on the historical use of coring and excavation methods for root studies can be found in Böhm (1979).

While commonly used, most excavation and trenching approaches (**Figures 2A, 3, 4**) are limited to the first meter and reach only occasionally soil depths of two meters and below (Wearver, 1915; Eamus et al., 2002; Silva and Rego, 2003; Dauer et al., 2009; De Azevedo et al., 2011). While commercial trench diggers, e.g., for sewer placement, can easily be used to excavate at greater soil depth (e.g., 5 m), the stability of unsupported side walls, which depends on soil type and moisture levels (Vanapalli and Oh, 2012), is the major obstacle limiting pit/trench depth. However, occasionally several meters deep trenches can be established (**Figure 3**). The cost of establishing deep trenches lead many researchers to use available soil profile-walls, created by road cuts, exposed at stream cut-banks or after landslides, to determine vertical rooting pattern (Canadell et al., 1999; Silva and Rego, 2003). Common analyses at all profile-walls are root counts and estimations of the root length density RLD; "trench profile"

technique (Van Noordwijk et al., 2000) and the determination of maximum rooting depth. While some innovations such as radiotracers (Abbott and Fraley, 1991; see below) or digital imaging (Dauer et al., 2009) have been introduced, overall profile-walls are used to quantify roots by soil location in a similar manner since the end of the 19th century (Weaver and Bruner, 1927 and references within). In contrast, excavations (**Figure 2A**) give full biomass per individual and often allow taking photographs/3D-scans of whole (coarse) root systems (Wagner et al., 2010)—providing valuable data on the vertical and horizontal root system distribution. However, because excavations, especially of larger plants, are particularly labor intensive, they are frequently restricted to the analysis of the upper soil layers, omitting deep roots of mature plants, and/or to low sample numbers (Cameron, 1963; Silva and Rego, 2003; Fang et al., 2012).

Soil coring approaches (**Figure 2A**) are suitable to obtain estimates of root length and mass, and root morphology beside data on root distribution. However, root coring is also often restricted to the uppermost soil layers because the majority of fine roots can be found in the first 0.3–0.5 m of soil. In addition, the occurrence of stones or boulders or high soil densities can prevent the use of simple and cheap manual coring tools for sampling of deep roots. However, corers have occasionally been taken to a much

greater soil depth with technical help; for example Virginia et al. (1986) took samples down to the water table at 5–6 m depth in the Sonoran Desert, and Ritson and Sochacki (2003) sampled roots down to six meters with a motor driven corer to determine the root biomass of *Pinus pinaster* in Australia. Rarely much greater soil depths are explored by machine drilling of cores (<20 m, Carbon et al., 1980; <34 m, Dalpé et al., 2000). At moderate depths, soil coring was found to be a more efficient option for fine root distribution mapping than trenching (Dauer et al., 2009) but this advantage might not hold for deeper soil horizons. Upscaling from core data to stand level root biomasses is in general only possible if sample numbers are sufficiently high due to heterogeneous root distribution (see above). For deep roots this might be especially problematic because of the low biomass of deep roots and their even more heterogeneous distribution; thus, high sample numbers are essential for deep root sampling by soil coring (Bengough et al., 2000).

MINIRHIZOTRON TECHNIQUES

Non-destructive methods for studying root systems, rhizotrons, “root windows” and MRs have the advantage of allowing the repeated observation of particular locations in the soil profile. The techniques also permits visualization of very small roots, and occasionally hyphae, through the transparent observation windows/tubes. The MR method was probably first used by Bates (1937); Bates, and described again later (Waddington, 1971; Vos and Groenwold, 1983; see Rewald and Ephrath, 2013 for a recent review). This method is now widely used in multiple fields of root research, such as studies on root distribution and root demography, and interaction between roots and root-soil (organisms) (Poelman et al., 1996; Majdi et al., 2005). Setting up MR tubes in the field requires the use of a soil corer (Hummel et al., 1989) or manual auger (Kage et al., 2000), and can be technically complex depending on the nature of the soil (e.g., smearing of walls with high clay content, presence of gravels preventing progress, lack of cohesiveness in sandy soils or in water saturated soils, etc.). Nevertheless, some researchers have successfully installed MR tubes in rocky soil (Phillips et al., 2000) and in wetlands (Iversen et al., 2011). MR tube installation from the soil surface (vertical or angled; **Figure 2B**) rarely occurs beyond the first meter of the soil profile, due to the above-mentioned difficulties encountered during installation (Rewald and Ephrath, 2013). For soil with higher bulk densities and to access greater depths, researchers need to use portable mechanical drilling devices or tractor-mounted auger systems (Brown and Upchurch, 1987; Kloeppe and Gower, 1995). Furthermore, the length of commercially available transparent observation tubes (norm: 2 m long, max. length: approx. 3 m) presents a constraint for continuous tube installation to greater depth. This problem is partially circumvented by researchers by installing MR tubes horizontally in rhizo-lysimeters or from trenches (**Figure 2B**). However, because of the workload such attempts have been extremely rare; examples are the field-based rhizo-lysimeter complex of Charles Sturt University, Australia (Eberbach et al., 2013) and MR tube installation in 8 m deep trenches in a plantation of eucalypt trees in Brazil (Hinsinger et al., 2012).

The MR method permits calculation of fine-root length production, mortality and turnover (Trumbore and Gaudinski, 2003); the same fine-root segments can be monitored over their lifetime and pictures are stored in a database for processing (Rewald and Ephrath, 2013). However, the conversion of MR data, i.e., RLD, to root biomass requires the simultaneous collection of root cores to develop correlations. Compared to excavated roots and repeated coring approaches, the MR technique allows relatively continuous segregation of live and dead root since image sequences that span the life-time of roots are acquired (but see Rewald and Ephrath, 2013). However, it has been documented that one major limitation of MR studies with regard to the assessment of root turnover is that they over-sample the smaller and more dynamic lower-order roots (Guo et al., 2008).

A common limitation of the MR technique (Johnson et al., 2001) is the difficulty in obtaining good contact between the tube and the soil; in many soil types gaps form in some places along the tube, creating artificial conditions for root growth. This problem is suggested to aggravate with increasing drilling depth (“off-centered”) and the use of machine drilling which creates less precisely sized holes than manual hammering. In conclusion, MR tubes installed from the soil surface rarely reach much more down than one meter because this is the depth to which manual installation is often possible. The installation of deep horizontal MR tubes, e.g., in trench profiles, is difficult due the limited space for using an auger and inserting tubes, and laborious due to the additional trenching. However, the most serious limitations to the MR technique seem to be the initial costs of hard- and software and the time lag until soil and root dynamics come back to steady state conditions after tube installation. Furthermore, while labor costs for tube installation and picture capturing are relatively moderate, image analysis can become very time consuming and sufficient resources have to be scheduled for these purposes.

ACCESS SHAFTS

The access shaft (or access well) observation technique (**Figures 2C, 5**) is a recent evolution and combination of the different techniques for root observation described in Böhm (1979) and in the two previous method sections of this review. The access well method provides safe access to deep soil observation locations, by means of ladders affixed to the well’s wall. Depths of several meters, typically between 5 and 10 m depending on soil conditions, can be investigated. Building the well can take about a week and the walls are reinforced with concrete tubes or other materials (Maeght et al., 2012), distinguishing this techniques from trenches. Importantly, wells maximize the accessible soil depth: volume of displaced soil ratio, compared to other types of excavations.

Similar to MR techniques, access shafts allow direct observation of root growth dynamics using adapted “root windows” through which roots can be observed at regular time intervals. Using an access-well and a window scanner technique, following a procedure similar to that described by Maeght et al. (2007), root growth dynamics and root turnover could be monitored at 0.5 m soil depths increments down to 4.5 m in a rubber tree plantation in NE Thailand (Gonkhamdee et al., 2009). The number

of root windows should be adapted to the well depth; windows should be geometrically arranged to allow for complete observation of the profile without compromising the strength of the reinforcing structure. Each root window includes a specifically designed glass frame supporting, on its upper side, a piece of 10 mm thick glass ($\sim 25 \times 30$ cm) pressed against the soil at a 45° angle (Figure 2C; Maeght et al., 2012). On the frame's lower side, two guide rails allow the insertion of a standard flatbed scanner; the images can be analysed analogue to pictures from MR tubes and similar constraints to data analysis apply (see above). However, the advantage of the access shafts method is that it provides physical access to deep soil horizons for (manipulative) research, e.g., to measure microbiological activities, and nutrient and water uptake *in situ*. Access shafts also allow the installation of various sensors at soil depths that have not been investigated in greater detail, examples are special devices for imaging the dynamics of soil pH as influenced by roots (e.g., optodes, Blossfeld et al., 2011) or NIR/VIS portable spectrometry analysis (Nakaji et al., 2008).

CAVES AND MINES

Deep roots of trees and shrubs are regularly found in caves and mine shafts (Cannon, 1960, cited after Stone and Kalisz, 1991; Stone, 2010). However, such observations have most often been mentioned in the literature as curiosities. Only in the last decade caves have been used more systematically for studies on roots. In 1999, Jackson et al. used 21 different deep caves (5–65 m deep) in the Edwards Plateau, USA to study the community composition below ground and maximum fine root depth of six dominant tree species (Jackson et al., 1999). They linked deep roots to each species and individual DNA sequence variation of the internal transcribed spacer (ITS) and inter-simple sequence repeats (ISSR) (Rewald et al., 2012), and found that all six tree species grew roots below 5 m, and at least four of the six reached a depth of 18 m. Similarly, Howarth et al. (2007) determined species composition of deep roots in Hawaiian lava tube caves with DNA sequence variation and related root taxa to cave arthropod fauna. In more recent years, the caves utilized by Jackson et al. (1999) were frequently used for further studies, e.g., to compare the hydraulic parameters of deep vs. shallow roots and to determine the water flux thru deep roots (McElrone et al., 2004, 2007; Bleby et al., 2010). In Europe, Filella and Peñuelas (2003) studied tree access to deep water sources and the possibility of HL from the deep roots of one *Pinus nigra* tree. They enriched the deep roots with deuterium by accessing them from a cave at 8 m depth, showing that, in this Mediterranean forest and during the dry summer, *P. nigra* trees accessed a deep water source and recycled it via HL. In Australia, Doody and Benyon (2011) installed sap-flow sensors on *P. radiata* roots, extending through a limestone cave to an unconfined aquifer 14 m below the surface, to quantify the contribution of deep roots to whole plant water uptake (>22%). Thus, caves can provide access to intact, functioning deep roots and several research groups have taken advantage of these natural access tunnels to deep roots in the past. While research in caves of mesic areas has been conducted (e.g., McElrone et al., 2004; Novak and Perc, 2012), results of root-specific studies are overwhelmingly available for deep roots

in (karst) caves of (semi-) arid ecosystems. Aside from questions of maximum rooting depth and species community composition below ground, research mainly addressed root hydraulics and water flux patterns *in situ*. The abundance of caves and the unique environment of caves are two factors limiting the broad use of this technique, especially for studies of deep root functioning in "normal" soil environments and for quantifying deep roots.

INDIRECT APPROACHES FOR THE OBSERVATION DEEP ROOTS

Quite a few indirect approaches have been used to study and quantify the role of deep roots in plant species and on the environment; while this is outside the focus of this review we will give an overview on some of them in the following.

To assess differences in uptake capacity between different soil depths, *tracers* can be injected at different depths for later recovery in the biomass; the amount of tracer in plant biomass is related to the uptake from each depth (Lewis and Burgoy, 1964). Tracer element can be either radioactive or stable isotopes, or analogous elements. Analogous are chemical elements, which are similar to specific nutrient ions, thus uptake, and integration into biomass works the same way as the nutrient (e.g., Sr^{2+} instead of Ca^{2+}). Some factors must be considered to successfully use tracers: (i) the application method must label the respective soil horizon uniformly and dilution effects must be predictable, (ii) the root-available amount of tracer must be predictable with respect to competing processes such as microbial immobilization and soil adsorption, and (iii) the uptake capacity of the tracer by roots, compared to (other) nutrients (i.e., discrimination factor), should be known under different soil properties (after Göransson et al., 2006, modified).

Electrical capacitance has been proposed as a means to estimate root mass based on the premise that the equivalent parallel resistance-capacitance of the electrical circuit formed by the interface between soil water and plant root surfaces is proportional to the overall amount of active roots present. Good correlations between root capacitance and root mass were obtained for young plants (Chloupek et al., 2006). However, the relative influence of deep vs. shallow roots on root electrical capacitance remains unclear (Herrera et al., 2012).

Electrical resistivity tomography (ERT) can be used to monitor soil water movement in large volumes of soil. A field study with 3-month-old maize showed that this technique could be used to non-destructively quantify in 2-D, root water uptake as well as preferential infiltration and drainage under plant rows (Michot, 2003). More recently, ERT was used as part of an experiment set up in a mature tropical forest in eastern Amazonia to demonstrate greater depletion of soil water in the 11–18 m depth increment of a throughfall exclusion plot compared with a control in the experiment (Davidson et al., 2011). These authors used a soil water content measure obtained with a TDR probe to convert soil apparent electrical resistivity values to soil water contents. Despite its sensitivity to soil characteristics, which can affect its performance, ERT is an effective means to obtain, non-destructive, indirect information about root functioning at considerable soil depths.

Soil moisture measurements, assessing soil moisture changes over time, represent an indirect way to detect signs of root activity namely water uptake. For example, based on soil moisture measurements, Calder et al. (1997) found clear evidence of water uptake down to a soil depth of 7.5 m under three species of plantation trees. Based on an analysis of water balance changes in a crop sequence with lucerne, Dunin et al. (2001) estimated an apparent root extension for lucerne 2–2.5 m beyond that of annual crops. Similar, simple rainfall and groundwater monitoring can be used to relate the survivorship/transpiration of some species in arid systems to the plant's ability to tap water from permanent water tables, which are sometimes located at depths of 18 m or more (Rawitscher, 1948).

CONCLUSION AND OUTLOOK

Although the literature does not include, by far, as many references on deep roots as it does on shallow roots, the available information has clearly demonstrated that deep roots are common and of pivotal importance for plant functioning, subterranean biocenosis and many biogeochemical cycles and associated ecosystem services such as pedogenesis, soil carbon sequestration and moisture regulation in the lower troposphere. We hope that this review will lead to a sustained interest on deep roots and the deep rhizosphere in the future; while it remains difficult to define “deep roots” in an absolute manner, there is

a pressing need to reassess current root sampling and monitoring schemes, to avoid introducing bias in future assessments of root system traits. Because no methodologies exist today to characterize the entire RSA of mature plants at once, particularly not for large-sized organisms such as trees, the methods presented in this review need to be improved further. Clever combinations of techniques, such as access shafts, must be developed toward reaching deeper soil horizons at lower costs—allowing for more frequent “deep-root”-studies. While we predict that research on deep roots and the deep rhizosphere will remain laborious in the years to come, the crucial knowledge gained in regard to plant and ecosystem functioning by “looking deeper” will leave us no choice, especially not in times of increasing climate change.

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Impact of tapping and soil water status on fine root dynamics in a rubber tree plantation in Thailand

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Fine roots (FR) play a major role in the water and nutrient uptake of plants and contribute significantly to the carbon and nutrient cycles of ecosystems through their annual production and turnover. FR growth dynamics were studied to understand the endogenous and exogenous factors driving these processes in a 14-year-old plantation of rubber trees located in eastern Thailand. FR dynamics were observed using field rhizotrons from October 2007 to October 2009. This period covered two complete dry seasons (November to March) and two complete rainy seasons (April to October), allowing us to study the effect of rainfall seasonality on FR dynamics. Rainfall and its distribution during the two successive years showed strong differences with 1500 and 950 mm in 2008 and 2009, respectively. FR production (FRP) completely stopped during the dry seasons and resumed quickly after the first rains. During the rainy seasons, FRP and the daily root elongation rate (RER) were highly variable and exhibited strong annual variations with a total FRP of 139.8 and 40.4 mm⁻² and an average RER of 0.16 and 0.12 cm day⁻¹ in 2008 and 2009, respectively. The significant positive correlations found between FRP, RER, the appearance of new roots, and rainfall at monthly intervals revealed the impact of rainfall seasonality on FR dynamics. However, the rainfall patterns failed to explain the weekly variations of FR dynamics observed particularly during the rainy seasons. At this time step, FRP, RER, and the appearance of new FR were negatively correlated to the average soil matric potential measured at a depth of between 30 and 60 cm. In addition, our study revealed a significant negative correlation between FR dynamics and the monthly production of dry rubber. Consequently, latex harvesting might disturb carbon dynamics in the whole tree, far beyond the trunk where the tapping was performed. These results exhibit the impact of climatic conditions and tapping system in the carbon budget of rubber plantations.

Keywords: *Hevea brasiliensis*, fine root dynamics, root elongation rate, fine root production, soil water content, field rhizotrons, seasonal climatic variations, Thailand

INTRODUCTION

Changes in terrestrial carbon stocks have significantly contributed to the increase of greenhouse gases (GHGs) in the atmosphere (Houghton, 1999). Land use changes (deforestation–afforestation) are important drivers of the global carbon balance. Forest conversion can have a profound effect on the carbon cycle (Lal, 2005; Jandl et al., 2007; Li et al., 2008) and large areas of the remaining tropical rainforests are being logged and converted to agricultural systems at high rates (Nepstad et al., 1999; Achard et al., 2002). In the tropical belt, and more particularly in southeast Asia, the rapid expansion of tree plantations (mainly oil palm, rubber, and coffee) has been among the main causes of deforestation in the last 20 years (Ziegler et al., 2012; Chiti et al., 2013; Ramdani and Hino, 2013). Conversely, tree plantations have expanded also onto degraded or marginal lands where they could contribute to

the rehabilitation of those lands (Sang et al., 2013). A benefit of tree plantations is that in addition to timber and agricultural products (such as fruits and latex), they are forest-like ecosystems that can improve some ecosystem services like water regulation, soil fertility, and carbon sequestration in the soil (Vihervaara et al., 2012). However, the appropriate plantation management should be applied to optimize those ecosystem services.

Due to the increasing world demand for natural rubber, most of the countries producing natural rubber (*Hevea brasiliensis*) have supported the expansion of rubber plantations in “non-traditional” environments particularly in Thailand and China (Fox and Castella, 2013). In southern China, rubber plantations have been set up at the expense of secondary forests causing a significant loss in the soil carbon stock (de Blécourt et al., 2013). In Thailand, the top producer of natural rubber in the world, rubber

plantations have expanded to the north-eastern part of the country where they have replaced mainly cash crops like sugar cane. In these new planting areas, the sustainability of rubber plantations is challenged by sub-optimal weather conditions (drought, low temperature), the low fertility of most of the soils (Boithias et al., 2011; Isarangkool Na Ayutthaya et al., 2011; Clermont-Dauphin et al., 2013), and the variability of the typical monsoon climate prevailing in mainland southeast Asia (Bridhikitti, 2013). Little is known about the carbon balance of rubber plantations under these particular conditions or about the best management practices for the optimum carbon sequestration in the soil. Wauters et al. (2008) found a 46% decrease in the carbon stock of the standing biomass of rubber plantations grown in sub-optimal conditions in Brazil compared to plantations in Ghana. Satakhun et al. (2013) showed that the soil water content (SWC) is the main driver of soil respiration in a rubber plantation under a sub-optimal rainfall regime with higher rates of soil CO₂ efflux during the rainy season and lower rates during the dry season. The dynamics of fine roots (FRs) in a rubber plantation have not been studied in details yet despite the fact that the belowground C allocation is a major component of the carbon balance, depending largely on FR production (FRP), mortality and turnover (Jackson et al., 1997; Matamala et al., 2003). In addition, FRs play an essential role in the acquisition of water and essential nutrients, while at the ecosystem level, they make a significant contribution to biogeochemical cycling (Pregitzer et al., 2002). A better understanding of FR dynamics is therefore important in the design of an appropriate management plan for the plantations (timing of fertilization, control of understorey, etc.). Previous studies on the root system of rubber trees were mainly conducted on seedlings either in field or in greenhouse conditions (Le Roux and Pagès, 1994; Thaler and Pagès, 1996a,b). To our knowledge, only two papers reported studies about root dynamics in mature plantations. George et al. (2008) determined the active root distribution pattern of rubber trees by the radioassay of latex serum which revealed that 55% of the root activity was confined to the top 10 cm of the soil layer and that root activity declined with increasing soil depth and the concentration of physiologically active roots at 90 cm depth was only 6%. Gonkhamdee et al. (2009) used a permanent access well 4.5 m deep equipped with rhizotrons to monitor FR appearance/disappearance during 17 months in a rubber plantation in Thailand. They showed how FR dynamics changed with time at different depths but their study did not provide any quantitative analysis of the relationships between FR dynamics and the environmental conditions or the stand characteristics.

A number of studies have demonstrated that FR growth was influenced by both exogenous and endogenous parameters (Moroni et al., 2003; Tierney et al., 2003). For exogenous factors, Konópka et al. (2007) reported that drought stress was most intense for FRs in the topsoil and weakest for FRs in the deepest soil layers. Studies of forest in which the rainfall is highly seasonal have shown that the roots grow mostly in the rainy season (Kavanagh and Kellman, 1992; Lopez et al., 1998) and die during the dry season (Srivastava et al., 1986; Kummerow et al., 1990). Although these patterns suggest direct control by soil water availability, growth also coincides with the leaf flush in the canopy and a very sharp increase in the soil nutrient availability as the rains begin

(Singh et al., 1989; Roy and Singh, 1995). The endogenous factors controlling the root growth are mainly linked to the development of the aerial part and the partitioning of carbon resources between the aboveground and belowground parts of the tree. Research by Thaler and Pagès (1996a,b) on rubber tree seedlings showed that both the apical diameter and the elongation rate of roots were depressed during the period of shoot growth. This may be related to carbon availability, as Singh and Srivastava (1986) found that the total non-structural carbohydrate content of teak (*Tectona grandis*) FRs was highest during the dry summer and lowest in the early part of the rainy season. In this regard, the carbon dynamics in the rubber tree present two specific features. First, the trees completely shed their leaves and produce new leaves every year over a period of 4–5 weeks, called wintering (Sabu and Vinod, 2009). In the marginal areas of Thailand, this wintering period happens in the middle of the dry season when the SWC, leaf gas exchange, and radial growth are at their lowest (Carr, 2011). Secondly, the carbon allocation in the trunk is strongly modified when the tree is tapped to harvest latex; radial growth is depressed (Silpi et al., 2006) and non-structural carbohydrates are diverted to build up trunk reserves (Silpi et al., 2007). Several methods have been used to estimate the FR biomass, production, and turnover (Santantonio and Grace, 1987; Hendrick and Pregitzer, 1992; Nadelhoffer and Raich, 1992). The sequential soil core method has been used widely (Persson, 1980, 1983; Ahlström et al., 1988; Comeau and Kimmins, 1989; Yin et al., 1989), but this method provides only a momentary representation of the FR biomass; the actual growth of FRs cannot be followed (Makkonen and Helmisaari, 1999). Direct observation methods of root dynamics are now commonly applied on a field scale using transparent acrylic tubes (mini-rhizotrons) or transparent panes of glass (rhizotrons) inserted in the soil (Jourdan and Rey, 1997; Hendricks et al., 2006; Jourdan et al., 2008). They allow the direct measurement of the appearance, disappearance, speed of growth, mortality, and lifespan of individual roots (Keyes and Grier, 1981) at a high temporal frequency (Metcalfe et al., 2007). Rhizotrons have several advantages over most of the other root study methods (Taylor et al., 1990; Box, 1996) as they allow the determination of the seasonal pattern of root growth and periods of minimal and maximal root growth (Vogt et al., 1998). Such non-destructive techniques are also important when dynamic changes of the roots in response to the environment are to be studied (McMichael et al., 1992). The disadvantages of rhizotrons are: the difficulty of precisely measuring very small roots, especially in the upper few millimeters of the soil (Vos and Groenwold, 1987); and the root growth disturbance effects of the window installation (Joslin and Wolfe, 1999; Coleman et al., 2000). However, no technique has been accepted universally as the best (Jourdan et al., 2008).

The current study presents the dynamics of FRs observed with flat rhizotrons during two successive years in a mature rubber plantation grown in a non-traditional area of Thailand, with sub-optimal annual rainfall for rubber cultivation (i.e., below 1500 mm), a 4- to 5-month dry season and intermittent spells of drought during the rainy seasons. This study was conducted in the framework of the Rubberflux project which aims to quantify the carbon, water, and energy budget of a rubber plantation (Thaler and Kasemsap, 2007). In this regard, this study had two main

objectives. The first objective was to study the relations between FR dynamics and other components of the net primary productivity (NPP) of the stand such as leaf phenology, stand growth, and latex harvesting. According to previous soil respiration studies conducted on the same site (Satakhun et al., 2013), we assumed that the FR growth would be lower in the dry season than in the rainy season as suggested by the soil CO₂ efflux dynamics. The second objective was to assess the impact of climatic factors on FR dynamics, particularly the inter- and intra-annual variability of the rainfall regimes.

MATERIALS AND METHODS

SITE DESCRIPTION

The experimental site was located at the Chachoengsao Rubber Research Center, Chachoengsao province (13°41'N, 101°04'E, and 69 m elevation), eastern Thailand. The observation plot was a monoclonal stand of rubber trees (*Hevea brasiliensis* Müll. Arg.) planted with the clone RRIM 600 in 1994 after cassava cultivation, with a tree spacing of 7 m × 2.5 m. The clone RRIM 600 is the most extensively planted in Thailand (78% of the planted area). Tapping for latex production began when the trees were 9 years old in 2003. Since then, the trees have been tapped each year during the 9 months from late April/early May to the end of January. During this period, tapping was performed every two or three days with a half-spiral downward cut [(1/2) S d/2, (1/2) S d/3]. The average diameter of the trees at 1.70 m from the ground was 20.04 cm (3.95 cm standard deviation) at the beginning of the study in November 2007.

The soils in the plot belong to the Kabin Buri series with 50% sand, 15% silt, and 35% clay. The soil depth is limited at 1–1.5 m by a compact layer of ferrallitic concretions that strongly limits root growth. The mean annual air temperature and cumulative rainfall were 28.1°C and 1328 mm, respectively, with a strict dry season between November and April (sourced from the Thai Meteorological Department).

MONITORING OF FINE ROOT DYNAMICS

Fine root dynamics were monitored from November 2007 to October 2009 using flat rhizotrons (Jourdan and Rey, 1997) installed in the vicinity of three trees. The selected trees had a girth at breast height in the range of the average girth of the plot and had no dead trees in their immediate surroundings. Two types of rhizotron were installed for each tree at a distance of about 1.5 m from the base of the trunk in the inter-row: one near-horizontal rhizotron with an inclination of 20° from the horizontal and one near-vertical rhizotron with an inclination of 20° from the vertical. Each rhizotron was made of a square-shaped piece of Plexiglas pane (0.8 m × 0.8 m) reinforced with a metal frame. The depth of soil explored by the rhizotrons was 27 cm for the near-horizontal one and 75 cm for the near-vertical one to characterize the shallow- and “deep”-FR dynamics, respectively.

The six rhizotrons were set up in September 2007 on the soil wall toward the tree in trenches 1 m wide by 1 m long and 30 cm deep for the near-horizontal and 100 cm deep for the near-vertical units. A 2–3 cm layer of 2-mm sieved soil prepared when the trenches were dug was inserted between the soil wall of the trench and the Plexiglas pane and compacted as much as possible to reach

the former soil compaction and to provide a good contact between the transparent pane of glass and the sieved soil. Each transparent screen was covered by double-layer black plastic sheets to prevent light from hindering the root growth. The trenches were covered by a metal roof to protect the rhizotrons from direct sunlight, rainfall, insects, and rodents.

Observation of the appearance, growth, and development of the roots started 3 weeks after the installation of the rhizotrons. Every week from November 6, 2007 to October 19, 2009, we traced the new segments of roots, linked to the growth of the existing roots or the apparition of a new root in the rhizotron, using permanent colored markers on a transparent plastic sheet fixed on the Plexiglas of each screen. A different color marker was used on each sampling date.

Every transparent plastic sheet filled with root drawings was digitized manually using a 61 cm × 91 cm format digitizer (Summagrid V, GTCO CalComp Inc., Columbia, MD, USA) and the RhizoDigit software (CIRAD, Montpellier, France). The RhizoDigit software facilitated the generation and management of the database including the date of apparition of each root segment and its length at each observation date and for each root diameter class.

ENVIRONMENTAL CONDITIONS OF THE STUDY

Daily data of the average air temperature, cumulative rainfall, and cumulative photosynthetic active radiation (PAR) were computed from 30-min data continuously measured at the top of a 25-m-high tower set up in the center of the observation plot (Thaler and Kasemsap, 2007). Every month during the observation period, soil samples at 20, 40, and 60 cm were collected in three locations near each rhizotron; the samples were used to determine the water content after oven drying for 24 h at 105°C. In June 2008, manual tensiometers (Raindrop, Eastern Agritek Co., Rayong, Thailand) were installed at soil depths of 30 and 60 cm at three locations close to each selected tree, that is, near each pair of horizontal and vertical rhizotrons. The soil matric potential (SMP) was recorded once every 2 days from July 3, 2008 to October 21, 2009 except between January 1, 2009 and June 14, 2009 because the soil was too dry during this period to measure the SMP with the tensiometers.

STAND CHARACTERISTICS: PAI, GIRTH INCREMENT, LEAF LITTER, AND LATEX YIELD

The plant area index (PAI; i.e., leaf plus branch area index) of the stand was measured from hemispherical pictures of the canopy taken in the vicinity of the rhizotrons with a Nikon Coolpix 995 camera and a Nikon FC-E8 fish-eye lens. All pictures were analyzed using the GLA software (Institute of Ecosystem Studies, Simon Fraser University, Burnaby, Canada).

Leaf litter samples were collected every 2 weeks in twenty 1-m² litter traps randomly positioned in the stand. The dry biomass of the litter was measured after drying the samples at 60°C until constant weight. The girth of the trees at 1.7 m above the ground was measured once a year at the time of leaf shedding. The latex yield was determined monthly by weighing the rubber coagulum. The total solid content was measured on a sub-sample in order to convert the fresh weight into grams of dry rubber. Those measurements were used to calculate the components of the aboveground

NPP of the plantation, namely, the annual increment of tree girth as a proxy of the annual increment in standing biomass, the annual latex production and litter biomass ($t\text{ ha}^{-1}$). We calculated those variables for the physiological cycle of the rubber trees, i.e., the period between the annual wintering period marked by the complete shedding of the leaves and the quick regrowth that follows (between January 23, 2008 and January 31, 2009 and between February 4, 2009 and January 16, 2010).

DATA ANALYSIS

The root elongation rate (RER, cm day^{-1}) was computed from the database generated by the RhizoDigit software as shown in Eq. 1:

$$\text{RER} = (\text{RL}_{d_2} - \text{RL}_{d_1}) / (d_2 - d_1) \quad (1)$$

where RL_{d_1} and RL_{d_2} are the length (cm) of a root segment between the two dates of observation (d_1 and d_2). In addition, the RhizoDigit database was used to count the total number of roots in each rhizotron and among all these roots, the number of new roots, the number of growing roots, and the number of paused roots (Thaler and Pagès, 1996a,b; Nodichao et al., 2011) for each observation date. Because the mortality of roots is difficult to estimate through a transparent screen, we have defined “paused roots” as the roots that exhibited no elongation in length and diameter between two or more successive observation dates. The paused roots turned into the “dead roots” category when the absence of growth was persistent over 2 months along with morphological and color changes. The FRP (mm^{-2}) on a seasonal or annual pattern was assessed by summing the total root length produced between two successive dates in the corresponding period divided by the related observation screen area (m^2) of the rhizotron. Next, the average value at each date of observation (i.e., every week) of the RER, FRP, total number of roots, and number of roots of different categories (new, growing, paused), was computed for the six rhizotrons. The monthly average RER and FRP, or the sum of root numbers, were also calculated when the SWC was measured. These weekly or monthly data were analyzed against climatic, soil water status, and PAI data using an LSD test for comparison of mean values, the Pearson multiple correlation test, and non-linear regression performed with the Xlstat software (Addinsoft, Paris, France).

RESULTS

CLIMATIC CONDITIONS DURING THE MEASUREMENT PERIOD

The daily rainfall pattern during the measurement period showed the succession of rainy and dry seasons (Figure 1A). The dry seasons extended from early November to mid-March in both years. These dry seasons were characterized by only five rainy days (days with more than 1 mm of rain), and a total cumulative rainfall of 53 mm for the dry season in 2007–2008 and 44 mm for the dry season in 2008–2009 (Table 1). The rainy seasons, though extending over the same months in both years, showed contrasting figures; the cumulative rainfall and the number of rainy days were 1500 mm and 88 days in 2008 and 952 mm and 76 days in 2009, respectively. The daily mean air temperature and cumulative PAR did not show marked seasonal trends. The temperature varied between 20 and 30°C, and PAR varied between 13.8 and 50.1 $\text{mol m}^{-2} \text{ day}^{-1}$.

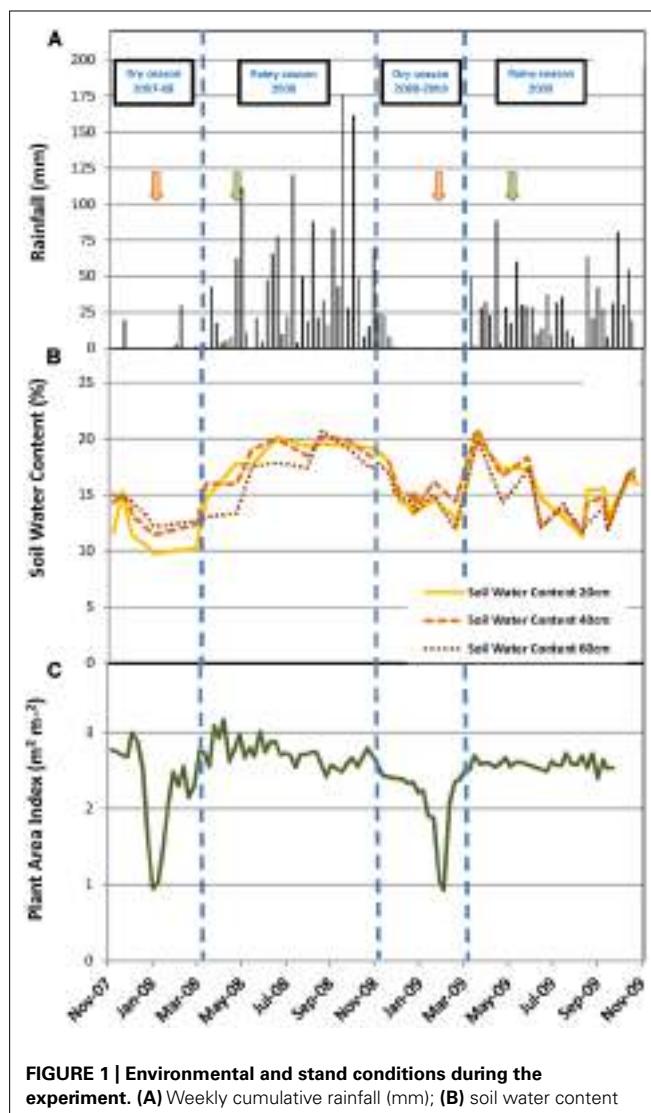


FIGURE 1 | Environmental and stand conditions during the experiment. (A) Weekly cumulative rainfall (mm); **(B)** soil water content (%) measured every month at soil depths of 20, 40, and 60 cm; **(C)** plant area index ($\text{m}^2 \text{ m}^{-2}$) measured weekly. Vertical dashed lines indicate the limits of dry and rainy seasons. Green and orange arrows mark the beginning and the end of the tapping season, respectively.

SOIL WATER STATUS

The SWC at 20, 40, and 60 cm soil depth varied from 9.8 to 20.1% during the measurement period (Figure 1B). The highest values of the SWC were reached during the rainy seasons and the lowest during the dry seasons. However, the dynamics of the SWC were different in the two years, particularly during the rainy season. In 2008, the SWC increased progressively from 12% in February to 18% in May and then varied a little between 18 and 20% until the end of the rainy season in October. Conversely in 2009, the SWC increased rapidly from 13% in February to 20% in March, then decreased to 11.5% in August and varied between 11 and 17% until the end of the rainy season (Figure 1B). Consequently, the average SWC during the rainy season was significantly higher in 2008 than in 2009 (18.5 versus 15.7%; Table 1). The same results were observed for the average SMP measured at 30 and 60 cm soil depth during the rainy season (Table 1).

Table 1 | Statistics for variables describing the environmental conditions of the study and fine root dynamics for each of the four seasons identified based on the rainfall regime during the observation period.

	Dry season (November 2007 to March 2008)	Rainy season (April to October 2008)	Dry season (November 2008 to March 2009)	Rainy season (April to October 2009)
Cumulative rainfall (mm)	53.0	1499.8	43.8	952.3
Rainy days (day)	5	88	5	76
Air temperature (°C)	25.7 (b)	26.1 (ab)	24.8 (c)	26.2 (a)
PAR ($\text{mol m}^{-2} \text{ day}^{-1}$)	34.6 (ab)	35.2 (ab)	33.8 (b)	36.1 (a)
Soil water content (%)	13.0 (c)	18.5 (a)	15.0 (b)	15.7 (b)
Soil matric potential (MPa)	NA	0.0137 (a)	NA	0.0395 (b)
PAI ($\text{m}^2 \text{ m}^{-2}$)	2.3 (b)	2.7 (a)	2.2 (b)	2.6 (a)
Fine root production ($\text{cm m}^{-2} \text{ week}^{-1}$)	122.1 (b)	395.8 (a)	34.8 (c)	116.2 (b)
Root elongation rate (cm day^{-1})	0.08 (bc)	0.16 (a)	0.04 (c)	0.12 (b)
Number of roots (week^{-1})	119.8 (b)	168.6 (a)	36.2 (d)	67.4 (c)
Number of growing roots (week^{-1})	23.7 (b)	57.5 (a)	6.3 (c)	22.7 (b)
Number paused roots (week^{-1})	74.1 (a)	52.8 (b)	23.2 (c)	24.7 (c)
Number of new roots (week^{-1})	16.9 (bc)	51.0 (a)	5.1 (c)	19.9 (b)
% of growing roots (week^{-1})	19 (b)	31 (a)	13 (b)	33 (a)
% of paused roots (week^{-1})	63 (a)	36 (b)	75 (a)	38 (b)
% of new roots (week^{-1})	13 (b)	28 (a)	9 (b)	29 (a)

Each number is the seasonal average of weekly or daily data except for cumulative rainfall and rainy days which are sums. Numbers with different letters in parentheses on the same line are significantly different at $p < 0.05$ (LSD test). NA, data not available.

STAND CHARACTERISTICS

The PAI varied between 1.0 and $3.2 \text{ m}^2 \text{ m}^{-2}$ (Figure 1C). The PAI values were significantly higher during the rainy seasons compared to the dry seasons (Table 1). In both years, the PAI dropped sharply to a value of $1 \text{ m}^2 \text{ m}^{-2}$ during the dry season and increased to $2.5 \text{ m}^2 \text{ m}^{-2}$ within the following 3–4 weeks (Figure 1C). These data illustrate the wintering period of the rubber trees with complete leaf shedding followed by quick leaf regrowth that occurs annually in clone RRIM 600 rubber tree plantations. The increase in the average girth of the trees at 1.7 m over the physiological cycle of the trees was $+2.1\%$ in 2008 and $+2.5\%$ in 2009. Similarly, dry rubber production was higher in 2009 (1.38 t ha^{-1}) than in 2008 (1.16 t ha^{-1}) while the aboveground litter production was lower in 2009 (1.21 t ha^{-1}) than in 2008 (1.31 t ha^{-1}).

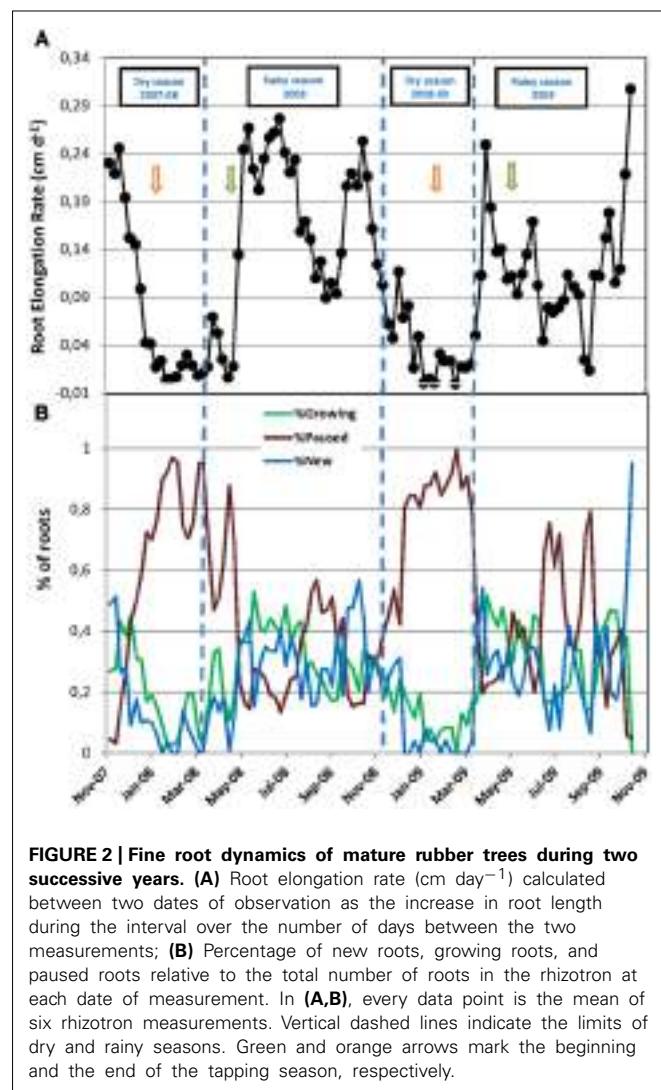
FINE ROOT DYNAMICS

The root dynamics observed through horizontal and vertical rhizotrons did not exhibit any significant differences either when compared to the same soil horizon prospected by roots nor to different soil depths. Consequently, results presented hereafter were issued from both horizontal and vertical rhizotrons.

The total number of roots and the numbers of new, growing, and pause roots were significantly higher in the first year of the experiment, from November 2007 to October 2008, compared to the second year, from November 2008 to October 2009 (Table 1). Consequently, we calculated the numbers of new, growing, and paused roots as a percentage of the total root number at each observation in order to account for these differences when comparing the root dynamics between the two years. The data transformed

this way showed a remarkably similar pattern over the two years, with a sharp decrease in the number of new and growing roots at the beginning of the dry seasons along with a sharp increase in the number of paused roots up to 100% (Figure 2B). Root growth and production resumed when the rainy seasons started and then varied between 0 and 52–56%. The average percentage of each category of roots was not significantly different between the two rainy seasons and the two dry seasons (Table 1).

The RER and FRP were also strongly affected by the alternation of the dry and rainy seasons (Figure 2A; Table 1). In both dry seasons, the RER decreased quickly from values above 0.25 cm day^{-1} in early November to less than 0.05 cm day^{-1} at the end of December in each observed year. Then, the RER remained below 0.05 cm day^{-1} until the onset of the rainy season at the end of March. FRP in the same dry periods remained low with 27.1 and 8.0 m of cumulated FR length per m^{-2} of observation screen area for 2008 and 2009, respectively (Table 1). During the rainy seasons, the RER varied from 0.01 to 0.31 cm day^{-1} (Figure 2A) with an average of 0.16 and 0.12 cm day^{-1} in 2008 and 2009, respectively (Table 1). The average RER and FRP values during the rainy season were significantly higher in 2008 than in 2009 (Table 1). This resulted in a total FRP of 139.8 m m^{-2} over the tree physiological cycle from the end on January 2008 to the end of January 2009. FRP was not measured over the whole cycle from January 2009 to January 2010. However, data from 2008 showed that FRP between October 2008 and January 2009 accounted for only 4% of FRP in the 2008–2009 cycle. The total FRP for the 2009–2010 cycle could thereby be estimated as 40.4 m m^{-2} , that is, 71% lower than in 2008–2009.



CORRELATION BETWEEN FINE ROOT DYNAMICS, ENVIRONMENTAL CONDITIONS, AND STAND CHARACTERISTICS

Table 2 shows the Pearson coefficients of correlation between the variables describing the environmental conditions during the study, PAI and the variables related to the FR dynamics, namely the RER, FRP, and the percentage of growing roots, paused roots, and new roots. The test was performed first on the data computed with a monthly time step over the entire study period in order to include the data on the SWC. Using this time step, we found a significant positive correlation between FR dynamics and rainfall, the number of rainy days and the PAI. On the other hand, we found a significant negative correlation between FR dynamics and dry rubber production.

Secondly, the Pearson correlation test was performed on weekly data during the rainy seasons only to study the short-term dynamics of FRs with information on the short-term dynamics of the soil water status provided by the measurement of the SMP. Using this time step, the RER was not correlated with rainfall but was negatively correlated with the air temperature and strongly negatively correlated with the SMP. Figure 3 shows that the relationship

between the RER and the SMP is well fitted by a negative exponential model. The percentages of the FRP and the new and paused roots were also correlated to the SMP. The percentage of growing roots did not show any correlation with the measured environmental conditions.

DISCUSSION

This study is the first detailed assessment of FR dynamics (the RER, FRP, and FR status) in a rubber tree plantation. There was a strong decrease in every measured parameter (number of the different types of roots, FR elongation rate, and FRP) between the first (2008) and the second (2009) year of the experiment. This may have been due to the rhizotron methodology. Disturbance of roots and of the rooting environment during the rhizotron installation may have been offset by an overproduction of roots during the weeks or months after the installation (de Ruijter et al., 1996; Vogt et al., 1998). Consequently, it is generally recommended to wait a certain period after the installation of a rhizotron before starting any measurement of FR dynamics along the glass surface. In some species, this lag time could be up to 3–8 months according to the stabilization of the FR standing length (Green et al., 2005; Hendricks et al., 2006; Metcalfe et al., 2007). Other potential sources of error with rhizotron approaches may be the effect of the observation window on root longevity (Withington et al., 2003) and the difficulty to distinguish the senescent process of FRs leading to many biases in the estimation of the amount of dead roots (Stevens et al., 2002) and globally the mortality process. Wang et al. (2005) also showed that a nutrient depletion zone at the root–rhizotron interface could be observed after several months and could lead to a decrease in the occurrence of new roots in the rhizotron (Mao et al., 2013). It is difficult to say if the growth of FRs was affected by any offset growth or the depletion of nutrient at the soil–rhizotron interface in our study. Nevertheless, our results clearly showed that the development pattern of the FRs was remarkably similar for the two years. Therefore, we can conclude that despite a possible impact on the number of roots, the rhizotrons used in our study provided reliable data on the dynamics of the FRs of rubber trees.

The average RER of the rubber trees in the 2008 wet season was 0.16 cm day^{-1} and only 0.12 cm day^{-1} in October 2009, with a maximum value of 0.30 cm day^{-1} in both years. These rates are lower than for common tree roots ($0.3\text{--}0.5 \text{ cm day}^{-1}$; Kramer and Boyer, 1995) and lower than for other tropical trees such as eucalypts (from 0.6 to 1.5 cm day^{-1} ; Misra, 1999; Thongo M'Bou et al., 2008) or oil palm grown in the Côte d'Ivoire (0.3 cm day^{-1} ; Jourdan and Rey, 1997). The lower rate of root elongation in the rubber trees in the current study might have been due to the depressing effect on tree growth of the tapping for latex production as was shown by the negative correlation found between FR dynamics and the dry rubber yield using the monthly time step. The negative impact of tapping (i.e., severing a thin slice of bark on a regular basis to collect the latex contained in the laticifer vessels of the phloem) on the aboveground rubber tree biomass, growth, and carbohydrates allocation at the trunk scale has been well studied for decades (from Pyke, 1941 to Silpi et al., 2007). The results of Silpi et al. (2006) showed a sharp decline in the radial growth of tapped trees compared to untapped trees within 2 weeks from

Table 2 | Correlation coefficients (Pearson test) between variables describing the environmental conditions of the study, the stand characteristics and the variables related to the fine root dynamics.

	Root elongation rate (cm day⁻¹)	%Growing roots	% Paused roots	% New roots	Fine root production (cm m⁻² week⁻¹)
Monthly data (all seasons)					
Air temperature	−0.070	0.148	0.002	−0.053	0.083
Rainfall	0.605**	0.457*	−0.591**	0.656**	0.566**
Rainy days	0.557**	0.539**	−0.617**	0.668**	0.468*
PAR	0.108	0.282	−0.147	0.067	0.115
Plant area index	0.545**	0.551**	−0.584**	0.550**	0.459*
Dry rubber production	−0.582*	−0.618**	0.591*	−0.582*	−0.678**
Soil water content	0.439*	0.270	−0.370	0.391	0.456*
Soil matric potential	NA	NA	NA	NA	NA
Weekly data (rainy seasons only)					
Air temperature	−0.309*	0.045	0.319**	−0.179	0.022
Rainfall	0.115	−0.010	−0.084	0.068	0.052
Rainy days	0.100	0.044	−0.115	−0.001	−0.053
PAR	0.001	0.160	−0.047	0.096	0.125
Plant area index	0.138	0.103	−0.107	0.022	0.337
Dry rubber production	NA	NA	NA	NA	NA
Soil water content	NA	NA	NA	NA	NA
Soil matric potential	−0.770***	−0.154	0.627***	−0.383*	−0.548***

Upper half of the table shows the correlation between data computed monthly for both years of the study. Lower half of the table shows the correlation between the data computed weekly during the two rainy seasons only. The soil water content and soil matric potential data used for these tests were the average of the measurements done at three and two different depths, respectively. Bold numbers indicate significant correlations at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NA, data not available.

the beginning of the tapping season. It illustrates the strength of the carbon sink created by tapping and the competition between this new sink and the primary growth. More surprisingly, Silpi et al. (2007) showed that tapping increased the storage of carbohydrates as reserves in the trunk, thereby increasing the strength of this sink and the overall competition for carbohydrates at the trunk level. Besides competition for carbon resources, tapping may also result in a limitation of carbohydrates transportation below the tapping cut due to the disruption of the phloem tissues on this part of the trunk (Silpi et al., 2007). FR dynamics are likely to be affected by these important changes in the carbon dynamics at the trunk level. Both the FRP and life span are indeed very sensitive to changes in the sink strength of the above-ground parts of trees, either due to the phenology of the shoots and leaves or due to the management of the trees for example by pruning (Comas et al., 2000; Pregitzer, 2003; Steinaker and Wilson, 2008). In rubber trees, Thaler and Pagès (1996b) showed that root growth was depressed every time a new flush of leaves was produced. Interestingly, they found that the number of paused roots increased during periods of leaf growth. We also found a positive correlation between the percentage of paused roots and dry rubber production. Comparing the FR dynamics of the tapped and untapped trees would be relevant to confirm the impact of tapping on the root system and to investigate the underlying mechanisms. In this regard, it would be interesting to test several

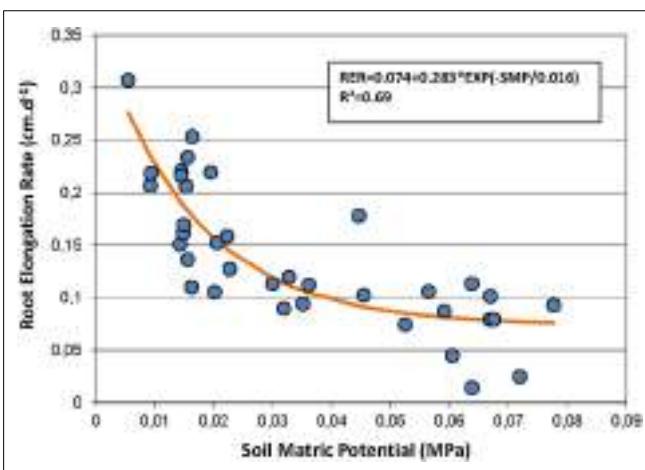


FIGURE 3 | Non-linear regression between the soil matric potential (SMP, MPa) and the root elongation rate (RER, cm day⁻¹). Symbols represent the measured data, and the line shows the non-linear model fitting the data. The model is $RER = 0.074 + 0.283 \times \exp(-SMP/0.016)$.

tapping systems corresponding to a gradient in tapping intensity (Lacote et al., 2010), and thereby to a gradient in the strength of the latex sink to establish a response curve between FR and latex production.

Root growth is not only influenced by endogenous factors linked to carbohydrates availability but also by exogenous factors related to environmental conditions (Moroni et al., 2003; Tierney et al., 2003). In our study, rainfall and the soil water status clearly appeared as the main environmental drivers of FR dynamics, whereas other climatic factors had less effect. This is consistent with previous works on tree plantations (Thongo M'Bou et al., 2008), or forest stands in tropical conditions (Green et al., 2005). First, we observed a similar seasonal trend in FR growth and development during the two different years which is consistent with the succession of the dry and rainy seasons. This observation was confirmed by the good correlations between the root parameters and the rainfall characteristics using a monthly time step. Root growth almost stopped during the dry season and quickly resumed at the onset of the rainy season. This was linked to the proportion of growing roots and the production of new roots, in a similar manner to the results from FR dynamics in a tropical forest (Green et al., 2005). However, it is noteworthy that during the dry season, a large proportion of roots (up to 100%) stopped growing but did not die, as they resumed growing in the next rainy season. Secondly, we also observed significant differences in root growth between the two rainy seasons, with a 25% reduction in the average RER of the 2009 rainy season compared to the 2008 rainy season. The 2009 rainy season was remarkably drier than in 2008 with 36% less rainfall (952 mm in 2009 versus 1500 mm in 2008), resulting in a 3% reduction in the average SWC. These results are consistent with those of Meier and Leuschner (2008) who found a 30% decrease in the FR biomass when the rainfall was reduced by 40%. However, the weekly variations in the RER during the rainy seasons, characterized particularly by a sharp decrease in the RER in August of both years, were more surprising. These variations could be explained neither by the rainfall events used with this time step nor by the evolution of the SWC, which remained rather high during all of the second half of the 2008 rainy season. A closer assessment showed that there was a clear negative relationship between the SMP and the FR elongation rate. This showed that FR growth was closely dependent on the soil water availability, in ranges of the SMP (below -0.05 MPa) that would hardly result in measurable changes in the soil volumetric content. To our knowledge, such a relationship between the SMP and the FR growth of field-grown trees has not been shown before. Previously, Kuhns et al. (1985) using black walnut trees and Bengough et al. (2011) using several annual crops showed a sharp decrease of FR growth at much lower SMPs (between -0.5 and -1.0 MPa). The values of the SMP reported in these papers were taken at or close to the root surface. In our study, it is likely that the SMP at the surface of the roots growing in the rhizotron was lower than the readings of the tensiometers installed a few meters away from the rhizotrons. However, this relationship suggests that the FR growth of rubber trees was very sensitive to water stress in this study as was shown previously by Chiatante et al. (1999) using pine saplings and by Konôpka et al. (2007) using Japanese cedar.

These contrasted conditions for rainfall and the soil water status between the two years resulted in a reduction of 71% in the total FRP in the rhizotrons in 2009 compared to 2008. The other components of the NPP did not show such a big variation. The

aboveground litter production was only reduced by 8% in 2009 compared to 2008 while the girth increment and latex production were higher in 2009 than in 2008. This would suggest that the aboveground parts of the trees were less sensitive than the FRs to the seasonal drought and the water stress events during the rainy seasons. Those differences in the sensitivity of NPP parameters to a variation in water supply could be partly explained by the timing and duration of the processes of trunk and leaf growth in the interaction with latex production. On the one hand, our data showed that rubber trees were characterized by a decoupling of the leaf and root phenology. Most of the leaves were produced within 2–4 weeks after the complete shedding of the trees in the middle of the dry season, when the SWC in the soil layer explored by the rhizotrons was at its lowest and the FR growth had almost ceased. Moreover, the dynamics of the PAI in our plantation showed that the duration of leaf growth was only 2 months while the duration of FR growth was about 10 months. However, on the other hand, Silpi et al. (2006) showed that the radial growth of the trunk was strongly reduced within 2 weeks from the beginning of the tapping season. Consequently, we could assume that most of the annual radial trunk growth occurred during the period when the trees were not being tapped, that is from the end of January to late April/early May. Therefore, most of the leaf and trunk growth was not exposed to the intermittent water stress events during the rainy season that greatly impacted on the FRP. Besides, a proper estimation of the contribution of the root system to the NPP should take into account deep roots (Maeght et al., 2013). Our data do not tell us anything about the behavior of the FRs below the maximum depth explored by the rhizotrons used in this study (75 cm). Meier and Leuschner (2008) found a shift with decreasing precipitation of the FR growth from the top soil to deeper layers in European beech stands. Under soil and climatic conditions similar to those in the current study, Gonkhamdee et al. (2009) showed that the growth of FRs below a soil depth of 75 cm in a 12-year-old rubber stand occurred mostly between July and November after the FRs had stopped growing in the upper layers. Thereby, the FR growth at deeper layers could have compensated for the water-stress-limited growth in the upper layers.

CONCLUSION

The FR elongation rate and the FRP of field-grown rubber trees showed marked seasonal and inter-annual variations. The seasonal changes clearly relate to rainfall and soil water availability with the appearance of new roots and root growth being highly sensitive to slight decreases in the soil water potential during the rainy season. We also found that FR dynamics were also depressed by the tapping of the trunk for latex harvesting. This result demonstrates that tapping disturbed the carbon dynamics in the whole tree far beyond the area of the trunk where it was performed. In this regard, we recommend that greater attention be paid to the diversity of existing tapping systems in further studies on the carbon balance of rubber plantations.

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Dynamics of soil exploration by fine roots down to a depth of 10 m throughout the entire rotation in *Eucalyptus grandis* plantations

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Although highly weathered soils cover considerable areas in tropical regions, little is known about exploration by roots in deep soil layers. Intensively managed *Eucalyptus* plantations are simple forest ecosystems that can provide an insight into the belowground growth strategy of fast-growing tropical trees. Fast exploration of deep soil layers by eucalypt fine roots may contribute to achieving a gross primary production that is among the highest in the world for forests. Soil exploration by fine roots down to a depth of 10 m was studied throughout the complete cycle in *Eucalyptus grandis* plantations managed in short rotation. Intersects of fine roots, less than 1 mm in diameter, and medium-sized roots, 1–3 mm in diameter, were counted on trench walls in a chronosequence of 1-, 2-, 3.5-, and 6-year-old plantations on a sandy soil, as well as in an adjacent 6-year-old stand growing in a clayey soil. Two soil profiles were studied down to a depth of 10 m in each stand (down to 6 m at ages 1 and 2 years) and 4 soil profiles down to 1.5–3.0 m deep. The root intersects were counted on 224 m² of trench walls in 15 pits. Monitoring the soil water content showed that, after clear-cutting, almost all the available water stored down to a depth of 7 m was taken up by tree roots within 1.1 year of planting. The soil space was explored intensively by fine roots down to a depth of 3 m from 1 year after planting, with an increase in anisotropy in the upper layers throughout the rotation. About 60% of fine root intersects were found at a depth of more than 1 m, irrespective of stand age. The root distribution was isotropic in deep soil layers and kriged maps showed fine root clumping. A considerable volume of soil was explored by fine roots in eucalypt plantations on deep tropical soils, which might prevent water and nutrient losses by deep drainage after canopy closure and contribute to maximizing resource uses.

Keywords: root front, root growth, root density, *Eucalyptus*, forest, oxisol, tropical tree, Brazil

INTRODUCTION

Rooting depth is an important functional trait in terrestrial ecosystems. Meta-analyses have shown that the rooting depth for trees tends to be greater than for shrubs and grasses and that the maximum rooting depth in forest ecosystems is greater in equatorial regions than in boreal regions (Jackson et al., 1997; Schenk and Jackson, 2002a). Deep-rooted trees can have a strong influence on ecosystem services in tropical regions, both locally and globally. At a local scale, stream flows can be reduced after afforestation in grasslands and deep-rooted trees are important drivers of water cycling in dry ecosystems that can have a significant effect on landscape hydrology (Jackson et al., 2005; Bleby et al., 2010; Dye, 2012; Brown et al., 2013). At a global scale,

modeling studies have shown that the current Amazonian climate is dependent on considerable amounts of water being extracted by trees from very deep soil layers and transpired back into the atmosphere during dry periods (Kleidon and Heimann, 2000; Saleska et al., 2007). A rainfall manipulation experiment showed that total carbon (C) stocks were strongly influenced by the availability of water in Amazonian forests (Brando et al., 2008) and the capacity of trees to take up water from deep soil layers during droughts (Bruno et al., 2006) can, therefore, influence C sequestration in rainforests. Although the major role of deep roots on C and water cycling has been described for several decades in tropical forest ecosystems (Nepstad et al., 1994), there are still few studies dealing with fine root development at depths greater

than 5 m (Schenk and Jackson, 2002a,b, 2005; Christina et al., 2011).

Eucalyptus plantations cover about 20 million hectares and are expanding in tropical regions (Booth, 2013). Although considerable areas are concerned, there is still little information on the consequences of the afforestation of grasslands with *Eucalyptus* plantations on the storage of water, carbon, and nutrients in deep soil layers. The gross primary production (GPP) of commercial *Eucalyptus* plantations in Brazil is more than $3500 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Ryan et al., 2010; Cabral et al., 2011; Nouvellon et al., 2012), among the highest in the world for forests (Luyssaert et al., 2007). This simple agro-ecosystem (with only 1 plant species growing in highly weathered soils without root growth barriers) provides an opportunity to investigate the belowground growth strategy of fast-growing trees in tropical regions. Most of the current information on tropical forests comes from indirect estimates of root activity from soil moisture monitoring (Calder et al., 1997; Robinson et al., 2006; Mendham et al., 2011) or tracer uptake (Lehmann, 2003; McCulley et al., 2004; da Silva et al., 2011). Spatial patterns of soil water depletion by *Eucalyptus* trees in Australian agroforests showed that *Eucalyptus* roots can take up water from the top soil up to 20 m from the tree belts and down to at least 8–10 m within 7 years after planting (Robinson et al., 2006). A recent study showed water uptake at a depth of 10 m 3.5 years after planting *Eucalyptus grandis* W. Hill ex Maiden trees in Brazil and a synchrony in vertical growth aboveground and belowground in very deep soils (Christina et al., 2011). Maps of fine root intersects counted in grids on vertical trench walls have been used to study the spatial distribution of roots in forest ecosystems (e.g., Laclau et al., 2001; Sudmeyer et al., 2004; Schmid and Kazda, 2005). This approach showed a tendency toward homogeneous soil exploration down to a depth of 3 m at 1 and 2 years after afforestation of a savanna with *Eucalyptus* trees in the Congo, followed by a concentration of fine roots in the upper soil layers at the end of the rotation period (Bouillet et al., 2002). However, fine roots were not observed beyond a depth of 3 m.

Our study aimed to gain an insight into the soil exploration strategy throughout the growth of *Eucalyptus* trees that enabled them to achieve the highest GPP in the world for forests. The study was based on the hypotheses that: (i) the root front velocity was at the uppermost range reported for tree species, as observed for eucalypt height growth, and (ii) most of the soil volume was explored by fine roots in the upper 3 meters from 1 year after planting onwards, which might explain the very low losses of

mobile ions applied with fertilizers in these plantations (Laclau et al., 2010; Silva et al., 2013).

MATERIALS AND METHODS

STUDY SITE

The study was carried out in *E. grandis* plantations established at Itatinga, State of São Paulo ($23^{\circ}02'S$, $48^{\circ}38'W$). The mean annual rainfall over the 15 years prior to this study was 1360 mm and the mean annual temperature was 20°C , with a seasonal cold period from June to September. The elevation was 850 m with a gently undulating topography typical of the São Paulo Western Plateau.

A chronosequence of *E. grandis* plantations covering an entire rotation cycle for pulpwood production (6 years) was studied on sandy soil (Table 1). The soils were deep Ferralsols ($>10 \text{ m}$), developed on Cretaceous sandstone, with a clay content ranging from about 15% in the A_1 horizon to 20–25% in deep soil layers. The mineralogy was dominated by quartz, kaolinite, and oxyhydroxides, with acidic soil layers containing very small amounts of available nutrients (see Campoe et al., 2012, for soil analyses). After harvesting a 10-ha *Eucalyptus* plot located on a hill top (slope $<3\%$), plots (about 0.25 ha each) were planted every year with the same silvicultural practices, representative of commercial plantations.

Fine roots were studied within a radius of 300 m in 1-, 2-, and 3.5-year-old stands planted with *E. grandis* seedlings selected by the Suzano forest company. The youngest stand in the chronosequence was planted 1 year after the previous stand had been harvested. The area was kept free of other plants by successive glyphosate applications during the period between harvesting and planting. Management practices in Brazilian *Eucalyptus* plantations commonly use herbicide the first two years after planting to support tree growth through an efficient weed control (Gonçalves et al., 2008). Soil sampling in an adjacent unplanted area showed that the period of 2 years between clear cutting the previous stand and root sampling was sufficient to distinguish, without any doubt, between the living roots of the current stand and the dead roots of the previous stand. The roots from the previous rotation in the youngest stand of our chronosequence were already decomposed or in an advanced stage of decomposition, whatever the soil layer. All seedlings received standard commercial plantation fertilization, which was non-limiting for tree growth in this soil (120 kg N ha^{-1} , 33 kg P ha^{-1} , 100 kg K ha^{-1} , 2 t ha^{-1} of dolomitic lime and micronutrients). Fertilizer was only applied on planting, except KCl and $(\text{NH}_4)_2\text{SO}_4$ fertilizer, a quarter of the

Table 1 | Main characteristics of the stands sampled on sandy soil (chronosequence) and clayey soil.

Stand age (months)	Planting date	Soil type	# Soil profiles; (maximum depth)	Mean height (m)	LAI ($\text{m}^2 \text{ m}^{-2}$)
12	July 2006	Sandy soil	6 (1.5); 2 (6.0)	4.4	2.8
22	July 2005	Sandy soil	6 (1.5); 2 (6.0)	10.2	4.8
42	April 2004	Sandy soil	6 (3.0); 2 (10.0)	17.8	3.2
68	December 2002	Sandy soil	6 (3.0); 2 (10.0)	23.6	2.2
72	December 2002	Clayey soil	6 (3.0); 2 (10.0)	25.8	3.0

The numbers of soil profiles and the maximum depth studied in each stand are indicated, as well as mean stand height and leaf area index (LAI). A complete description of the sandy soil (20% clay content) and the clayey soil (40% clay content) is given in Campoe et al. (2012).

total amount being applied on planting with further applications at 6, 12, and 18 months of age. Experiments conducted over an entire rotation in Brazil showed that large amounts of fertilizers applied before canopy closure (as in our study) meet the nutritional demand of *Eucalyptus* trees up to the harvest age (Stape et al., 2010). The oldest stand of the chronosequence was sampled 6 years after planting. This stand was located 13 km away on the same type of soil and in a similar topographic position. Seedlings came from the same source with a similar planting strategy, except that the spacing was 1.6×3.8 m as opposed to 2×3 m in the other stands of the chronosequence. The growth curves were similar for all the stands and no biotic or abiotic factors severely affected their growth.

The oldest stand of the chronosequence was in a 50-ha plot. The downhill corner of this stand was growing in a clayey soil (from 37–40% clay in the A₁ horizon to 42–45% down to a depth of 10 m). This corner was also studied 6 years after planting. The clayey soil was developed on basaltic material. Although weathered stones were found at a depth below 8 m (no stones were found down to a depth of 10 m in the chronosequence), fine roots were found between the stones down to a depth of 10 m. The stem biomass in the 6-year-old stand on clayey soil was 28% higher than in the 6-year-old stand on sandy soil (Campoe et al., 2012).

FINE ROOT SAMPLING METHODOLOGY

Three pits were dug in each stand close to three trees of mean basal area (no weeds or missing trees within a radius of 10 m).

Root intersects were counted on two vertical trench walls at right-angles to the planting row in each pit: profile P0, from the bottom of the studied tree to the middle of the inter-row, and profile P1, from midway between two adjacent trees in the planting row to the middle of the inter-row (**Figure 1**). Three replicates of the P0 and P1 soil profiles (a total of 6 profiles observed for each stand age) were studied down to a depth of 1.5 m during the early growth phase (at 1 and 2 years after planting), and down to a depth of 3.0 m from mid-rotation onward (at 3.5 and 6 years after planting, on both sandy and clayey soils). Deep soil layers were sampled in 2 trench walls selected to represent extreme distances relative to trees (1 P0 and 1 P1 at each age) from 1.5 to 6 m deep at 1 and 2 years after planting, and from 3.0 to 10.0 m deep at 3.5 and 6 years after planting. All vertical soil profiles were divided into 5×5 cm grid cells and roots were exposed using a small knife to remove surrounding soil. The number of intersections of roots with the vertical plane was counted in each grid cell of 25 cm^2 , distinguishing three sizes of diameter (fine roots less than 1 mm, medium-sized roots between 1 and 3 mm and coarse roots over 3 mm). Root classes were chosen as in previous studies carried out in Brazilian *Eucalyptus* plantations (e.g., da Silva et al., 2009; Laclau et al., 2013). Only living roots were counted on trench walls (as far as we could distinguish between living roots and dead roots from their color and flexibility). Root intersects were counted in 89,440 grid cells on 27 m^2 of trench walls in 1- and 2-year-old stands, 48 m^2 in 3.5-year-old stands, and 61 m^2 in the 6-year-old stands on both sandy and clayey soils.

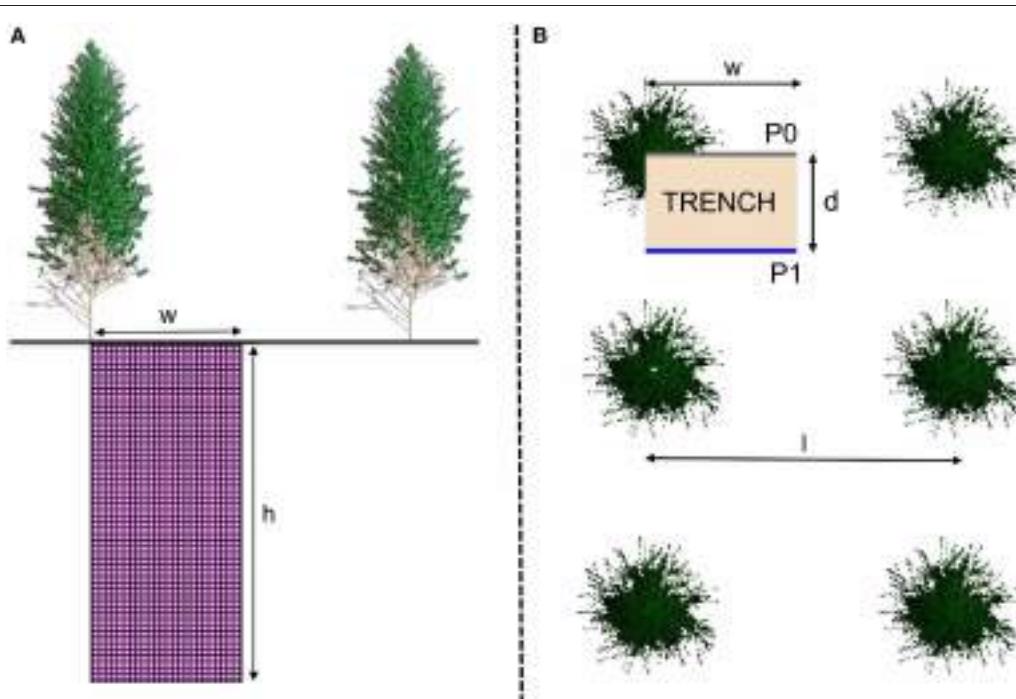


FIGURE 1 | Layout of soil profiles P0 (from the bottom of a trunk to the middle of the inter-row) and P1 (from midway between 2 trees in the planting row to the middle of the inter-row) in each stand showed in a side (A) and top view (B). The distance between rows (l) was 300 cm and the distance between trees in the planting row ($2d$) was 200 cm, in the

stands sampled 1, 2, and 3.5 years after planting. Spacing was slightly different in the 6-year-old stands ($l = 380$ cm and $2d = 160$ cm) for a similar planting density. The maximum depth sampled (h) was 6 m in 1- and 2-year-old stands and 10 m in 3.5–6-year-old stands. The width of the soil profiles was $l/2$.

Root growth is very fast in *Eucalyptus* plantations (Jourdan et al., 2008; Christina et al., 2011) and the roots counted in stands from 1 year after planting onwards belonged to several trees. The root characteristics were, therefore, representative of the stand and not only influenced by the nearest tree. The 3 pits studied at each age were more than 10 m from each other.

Fine root distribution was also studied by taking soil cores, on January 2012, down to a depth of 13 m at 2.1 years after replanting the oldest stand of the chronosequence. The methodology described by Christina et al. (2011) was used. Soils were sampled by drilling at a distance of 0.4, 0.9, and 1.5 m from 3 trees with the same basal area as the mean of the stand, along a diagonal between trees in adjacent rows. Only soil blocks from the central part of the auger were considered (the upper and lower parts were discarded), thus avoiding contamination from upper soil layers. Easily identifiable fine roots were separated by hand picking in the field. The soil samples were taken to the laboratory for thorough quantification of extremely fine roots. The root front was defined at each sampling position as the depth where the deepest root was observed.

WATER WITHDRAWAL FROM DEEP SOIL LAYERS

The volumetric soil water content (SWC) was monitored at 30 min intervals in the oldest stand of the chronosequence established on sandy soil, before (March 2008–October 2009) and after (November 2009–October 2012) replanting. Forty-two CS616 probes (Campbell Scientific, Shepshed, England, UK) were installed: 5 probes in 5 pits at depths of 0.15, 0.50, and 1.00 m, 3 probes in 3 pits at depths of 2.00 and 3.00 m and 3 probes at different distances from trees in the same pit at depths of 4, 5, 6, 7, 8, 9, and 10 m. The pits were dug manually and the CS616 probes were buried horizontally in an undisturbed area from the vertical wall of each trench. The trenches were then back filled, keeping the soil horizons in their original positions. The probes were calibrated using gravimetric SWC and bulk density measurements.

The first occurrence of water withdrawal from deep soil layers after planting crops or trees has been used as an indicator of the root front displacement (Calder et al., 1997; Dardanelli et al., 1997; Battie-Laclau and Laclau, 2009). It was estimated that the age of the stand when the root front reached the soil moisture probes was shown by the first sharp decline in SWC. However, disruption of the supply of gravitational water to a given depth (resulting from water withdrawal by trees in the upper soil layers) could lead to a decrease in SWC, even though the roots may not have yet reached this depth. Therefore, only the depths where the SWC dropped to the lowest values observed before harvesting were taken into account in our study. It was considered that the root front reached the soil moisture probes at a given depth when an initial decrease in SWC due to the interruption of drainage from upper soil layers was followed by a sudden change in the slope of the SWC curve. In addition, the tree height was measured every 3 months in 4 plots (336 trees measured within a radius of 200 m from the soil moisture probes) to compare the vertical growth above- and belowground. The tree height was linearly interpolated to

estimate the mean stand height each month throughout the study period.

DATA ANALYSES

The numbers of intersects of fine and medium-sized roots per area of 25 cm² of soil are presented as fine root density (FRD) and medium-sized root density (MRD). Coarse roots were not taken into account because they were only found close to the stump. The model proposed by Bouillet et al. (2002) was used to predict root intersects in trench walls throughout the development of eucalypt plantations:

$$\text{FRD}_z = a_0 - a_1 \times D_z + b \times \exp(-c \times D_z) + \varepsilon_z, \quad (1)$$

where FRD_z is the mean FRD at depth z, D_z is depth z, (a₀ + b) is the FRD at D_z = 0, (a₀ - a₁D_z) tends to 0 when D_z increases, c controls the shape of the curve and ε is the residual error. As the relationship between the MRD and the soil depth was weak for most stand ages, only the means and standard deviations of MRD in each soil layer are shown.

Local and global fits of FRD models were compared between stand ages, soil profiles, and soil types. Models 1 (local models for each situation) and model 2 (global model for the whole data set) were fitted using SAS PROC NLIN. Differences in local and global models were evaluated using F-tests calculated on the residuals. This test is based on the error sum of squares (SSE) and the total number of parameters involved in the models. It compares F_{obs} and F_{tab} calculated as:

$$F_{\text{obs}} = \frac{(\text{SSE}_2 - \text{SSE}_1)/(p_1 - p_2)}{\text{SSE}_1/(n - p_1)}, \quad (2)$$

where p₁ is the number of parameters for the local model, p₂ is the number of parameters for the global model (p₂ < p₁), SSE₁ is the error sum of squares for the local model, SSE₂ is the error sum of squares for the global model and n is the number of measurements. F_{tab} is the theoretical value given in Fischer's table: F_{tab} = F_(p1-p2, n-p1). If F_{obs} > F_{tab} then the local model described the data set better than the global model and the factor studied had a significant effect (Brown and Rothery, 1993). All differences were considered significant at a 5% threshold.

The spatial distribution of roots was analyzed using classical univariate geostatistical methods including semivariogram analysis and interpolation (kriging) to describe spatial patterns (Isaaks and Srivastava, 1989). Semivariogram analyses were performed for omni-directional semivariograms. However, as the vertical gradient might have an effect on the spatial distribution, anisotropy was also studied by calculating directional semivariograms for the horizontal (X) and the vertical (Z) axes. Standardized semivariograms (standard semivariogram divided by the experimental sample variance for all spatial locations) were also calculated to compare results from different datasets. As the horizontal dimension was smaller (1.5 m) than the vertical dimension (6–10 m) in the soil profiles studied, spatial analyses were performed for 1.5 × 2.0 m areas in the top (0–2 m) layer, the middle (2–4 m) layer, and the bottom (4–6 m) layer of each soil profile. All geostatistical analyses were run using GS+ (Gamma

Design Software, 2004). Semivariograms were modeled by fitting the parameters using the least-squares method (autofit facility of GS+). For each analysis, an average ratio of anisotropy (R) was calculated:

$$R = \frac{1}{p} \sum_{i=1}^{i=n} \frac{\gamma_X(h_i)}{\gamma_Z(h_i)} \quad (3)$$

where p was the number of experimental semivariograms values calculated using an active lag distance set to 1 m and a lag class interval of 0.05 m ($p = 19$), h_i was the separation distance used to calculate the semivariograms, and γ_X and γ_Z were the directional semivariograms for the X and Z directions. R -values close to 1 indicated an isotropic spatial structure.

RESULTS

ROOT FRONT DISPLACEMENT

The SWC time series showed a fast displacement of the root front in deep soil layers (Figure 2). Gravitational drainage at

the end of the first rainy season after planting led to a slow decline in SWC, at all depths between 3 and 10 m. There was a sharp acceleration in the decrease in SWC during the first dry season, reflecting the uptake of substantial amounts of water by tree roots (Figure 2). The SWC time series showed a displacement of the root front down to a depth of 7 m within 1.1 years after planting, which indicated a mean root growth rate downwards of approximately 1.8 cm day^{-1} . The SWC down to a depth of 6–7 m at the end of the first dry period after planting (at about 1 year of age) was similar to values at the end of the dry season before clear cutting (Figures 2B, 3A). This pattern indicated that all the available water stored down to a depth of 6–7 m after clear-cutting the previous stand was already taken up 1 year after re-planting. The soil down to more than 10 m was replenished during the second rainy season after planting but gravitational water did not reach more than 5 m down during the third year after planting (Figure 2). Soil cores showed that the deepest roots reached a depth of 11.4 ± 1.6 m at 2.1 years after planting (Figure 3B). SWC monitoring at the depths of 6, and 7 m as well as soil coring at 2.1 years of age suggested a roughly symmetrical vertical extension of trees aboveground and belowground over the early growth of this *E. grandis* stand.

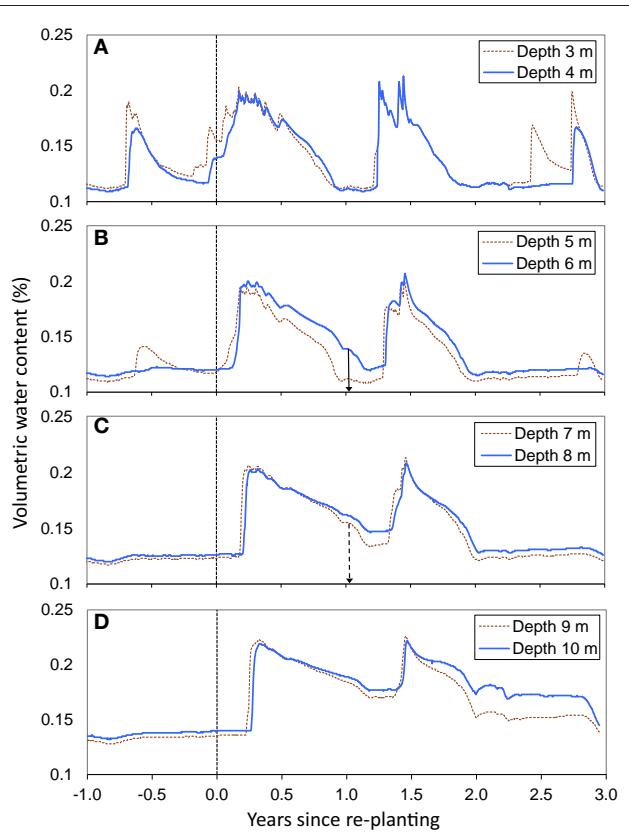


FIGURE 2 | Time series of volumetric soil water content at depths of 3 and 4 m (A), 5 and 6 m (B), 7 and 8 m (C), and 9 and 10 m (D). Vertical arrows at depths of 6 and 7 m indicate the approximate age of the stand when water withdrawal at the root front led to a sharp decline in soil water content after stabilization resulting from a disruption of drainage from upper soil layers. The timecourse of soil water content at the other depths (3, 4, 5, 8, 9, and 10 m) did not make it possible to estimate the stand age at the arrival of the root front. Trees were harvested ~1 month before re-planting.

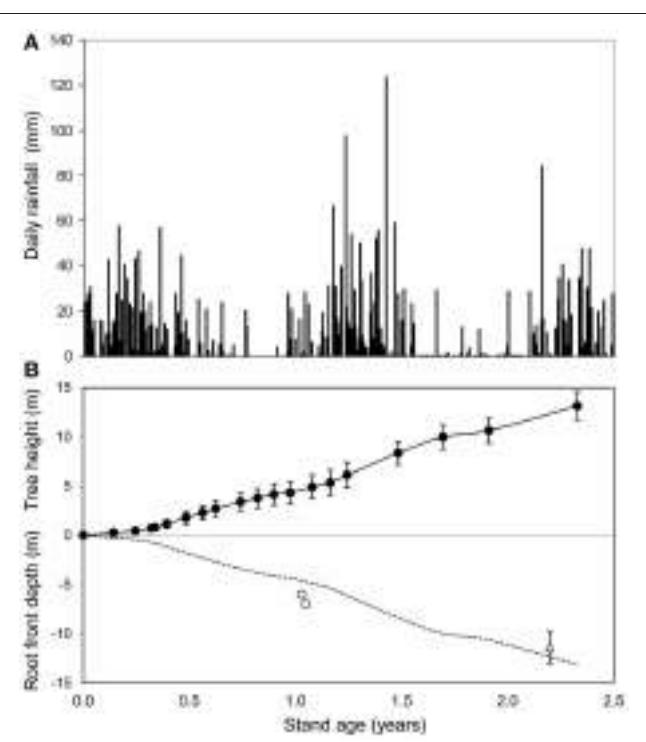


FIGURE 3 | Time series of daily rainfall (A) and mean stand height (filled circles) as well as root front depth (empty circles) estimated from soil water content monitoring and soil coring at 2.1 years (empty triangles) after replanting the oldest stand of the chronosequence (B). Standard deviations of tree height ($n = 336$) and root front depth at age 2.1 years ($n = 3$) are shown. The dotted line shows the symmetrical belowground of mean height.

DYNAMICS OF SOIL EXPLORATION BY ROOTS

Maps of fine root densities confirmed fast exploration of deep soil layers throughout the development of *E. grandis* plantations with some fine roots observed at a depth of 6 m after only 12 months of growth (**Figure 4**). 1 and 2 years after planting, local models predicting the FRD for each soil profile (P0 and P1) were significantly different to global models based on both soil profiles, with a higher FRD in the soil profile at the bottom of a tree (P0) than in the profile further from the trees (P1) (**Tables 2, 3**). However, local and global models of FRD were not significantly different for the P0 and P1 profiles at 3.5 and 6 years after planting. This pattern suggested that soil exploration by roots was not greatly influenced by the distance from the nearest tree during the second half of the rotation cycle. Highly significant differences between

local models predicting FRD at each stand age and a global model including all the ages showed a strong effect of stand development on the distribution of fine roots down to a depth of 10 m (**Table 2**). There was great spatial variability in FRD and MRD in deep soil layers. The coefficients of variation of FRD and MRD in 2 m thick soil layers below a depth of 2 m were >100 and >600%, respectively, for all stand ages and soil profiles (**Table 3**). Large changes in FRD distribution depending on tree age showed that the FRD tended to increase in the 0–5 cm soil layer at the end of the rotation cycle (**Figure 4** and **Table 3**). The soil texture also had a significant effect on fine root distribution at 6 years of age (**Table 2**). The mean FRD down to a depth of 10 m was 40% higher in the clayey soil than in sandy soil (**Figure 4**). The MRD distribution was similar to the FRD distribution

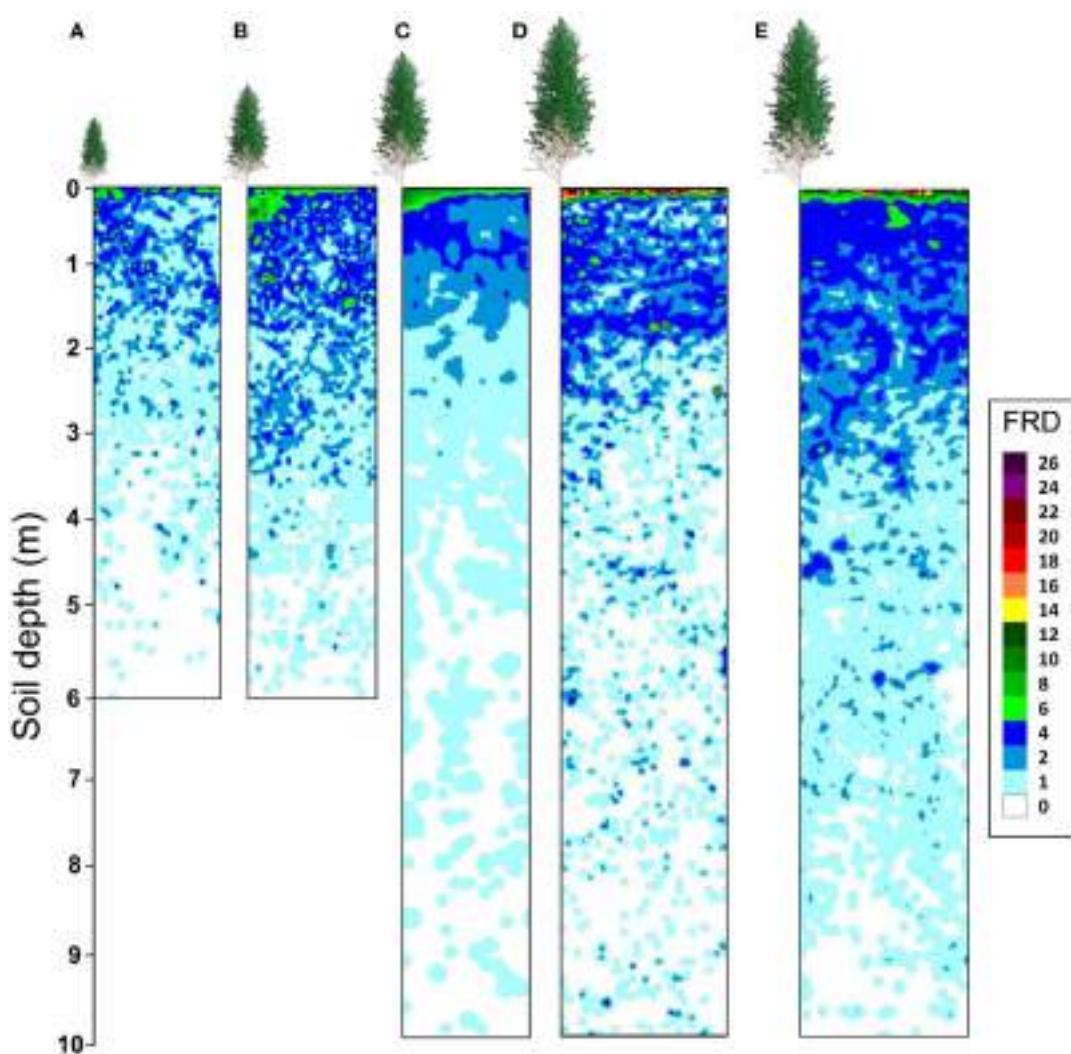


FIGURE 4 | Kriged maps of roots less than 1 mm in diameter (FRD, number of root intersects counted in a 25 cm² area of trench wall) on the P0 soil profile. Pits were at right angles to planting rows in a chronosequence of *Eucalyptus grandis* plantations down to a depth of 6 m at 1 and 2 years after planting (**A** and **B**, respectively) and down

to a depth of 10 m in 3.5-year old (**C**) and 6-year-old (**D**) stands on a sandy soil (20% clay). Fine root densities down to a depth of 10 m in a 6-year-old stand on a clayey soil were also studied (**E**). Eucalypts indicate the position of the nearest tree (not at the scale of soil profiles).

Table 2 | Comparison of local and global models predicting fine root intersect densities (number of fine root intersects per 25 cm² grid cell) across soil profiles, stand ages, and soil types.

Soil profile	Stand age (yr)	Soil type	Local models	R ²	RMSE	Global models	R ²	RMSE	F _{obs}
P0	1	20% clay	FRD = 1.4677 - 0.2960D + 6.7027exp(-19.0512D)	0.91	0.23	FRD = 1.3307 - 0.2587D + 5.3375exp(-19.3729D)	0.83	0.28	15.4***
			FRD = 1.1931 - 0.2213D + 3.9528exp(-19.7684D)	0.78	0.26				
P0	2	20% clay	FRD = 1.9000 - 0.3504D + 3.7492exp(-4.0025D)	0.83	0.24	FRD = 1.6917 - 0.3118D + 3.6078exp(-3.4614D)	0.94	0.25	9.4***
			FRD = 1.4520 - 0.9407D - 0.1275exp(-0.6502D)	0.95	0.22				
P0	3.5	20% clay	FRD = 2.7974exp(-0.7111D)	0.90	0.21	FRD = 2.7168exp(-0.7178D)	0.88	0.22	2.7 NS
			FRD = 2.6389exp(-0.7262D)	0.87	0.22				
P0	6	20% clay	FRD = 1.4419 - 0.1743D + 18.1805exp(-14.7543D)	0.92	0.36	FRD = 1.4221 - 0.1726D + 17.1942exp(-13.1137D)	0.92	0.36	2.2 NS
			FRD = 1.4023 - 0.1709D + 16.4777exp(-11.8440D)	0.93	0.35				
All	1	20% clay	FRD = 1.3307 - 0.2587D + 5.3375exp(-19.3729D)	0.83	0.17	FRD = 1.2318 - 0.1649D + 6.9213exp(-9.0045D)	0.69	0.54	330.0****
			FRD = 1.6917 - 0.3118D + 3.6078exp(-3.4614D)	0.94	0.25				
			FRD = 2.7168exp(-0.7178D)	0.88	0.22				
			FRD = 1.4221 - 0.1726D + 17.1942exp(-13.1137D)	0.92	0.36				
All	6	40% clay 20% clay	FRD = 1.8160 - 0.2120D + 11.4810exp(-8.0817D)	0.91	0.38	FRD = 1.6472 - 0.1964D + 14.7334exp(-11.3966D)	0.90	0.40	39.9***
			FRD = 1.4221 - 0.1726D + 17.1942exp(-13.1137D)	0.92	0.36				

F-tests compare local models with a global model.

Only parameters significantly different from 0 ($P < 0.05$) are shown. D is soil depth in meters. NS, *, **, and **** indicate non-significant differences at $P > 0.05$ and significant differences at the thresholds of 0.05, 0.01, 0.001, and 0.0001, respectively.

Table 3 | Mean, standard deviation (Std), and coefficient of variation (CV expressed in %) of fine and medium-sized root intersects counted in 25 cm² grid cells (number of roots per 25 cm²) on the P0 (close to the stump) and the P1 (at mid distance between two trees in the planting row) soil profiles.

Age(yrs)	Soil layer (m)	Fine roots (diameter < 1 mm)						Medium-sized roots (diameter 1–3 mm)					
		P0			P1			P0			P1		
		Mean	Std	C.V.	Mean	Std	C.V.	Mean	Std	C.V.	Mean	Std	C.V.
1	0.0–0.1	4.38	3.11	71	2.91	1.79	61	0.08	0.27	345	0.12	0.33	269
	0.1–0.3	1.68	1.44	85	1.11	1.20	108	0.03	0.16	625	0.02	0.15	664
	0.3–0.5	1.58	1.45	92	0.66	0.97	145	0.03	0.16	592	0.04	0.21	513
	0.5–1.0	1.48	1.37	92	0.91	1.03	113	0.04	0.20	526	0.03	0.16	618
	1.0–2.0	1.19	1.12	95	1.12	1.12	101	0.03	0.18	576	0.03	0.17	586
	2.0–4.0	0.38	0.67	176	0.46	0.84	183	0.02	0.16	747	0.02	0.15	684
	4.0–6.0	0.08	0.34	425	0.07	0.28	402	0.00	0.06	1732	0.00	0.05	1998
2	0.0–0.1	4.88	2.11	43	4.59	2.30	50	0.37	0.68	184	0.51	0.74	145
	0.1–0.3	3.72	2.02	54	3.37	1.95	58	0.14	0.36	249	0.18	0.46	263
	0.3–0.5	2.30	1.71	74	2.12	1.45	69	0.10	0.30	296	0.08	0.30	355
	0.5–1.0	1.89	1.42	75	1.75	1.49	85	0.07	0.26	398	0.07	0.25	378
	1.0–2.0	1.56	1.52	97	1.34	1.39	103	0.05	0.23	435	0.05	0.24	457
	2.0–4.0	0.76	0.86	113	0.57	0.78	136	0.00	0.06	1732	0.01	0.07	1411
	4.0–6.0	0.18	0.43	241	0.13	0.35	272	0.00	0.03	3478	0.00	0.00	
3.5	0.0–0.1	3.69	3.49	94	3.89	4.40	113	0.12	0.40	342	0.12	0.35	304
	0.1–0.3	2.05	1.97	96	1.67	1.21	73	0.03	0.18	650	0.06	0.23	413
	0.3–0.5	1.53	1.05	69	1.55	1.13	73	0.04	0.20	480	0.03	0.18	539
	0.5–1.0	1.75	1.08	62	1.70	1.13	67	0.05	0.24	435	0.04	0.21	483
	1.0–2.0	1.06	0.91	86	1.00	0.95	95	0.04	0.20	511	0.03	0.17	577
	2.0–4.0	0.31	0.62	202	0.32	0.56	175	0.01	0.10	956	0.01	0.12	947
	4.0–6.0	0.08	0.29	347	0.13	0.36	284	0.00	0.06	1545	0.00	0.03	3478
	6.0–8.0	0.07	0.28	402	0.09	0.30	343	0.00	0.04	2444	0.00	0.04	2444
	8.0–10.0	0.03	0.21	641	0.05	0.23	447	0.00	0.00	0.00	0.00	0.06	1545
6	0.0–0.1	10.34	5.62	54	11.24	6.20	55	0.55	0.97	178	0.50	0.95	191
	0.1–0.3	2.75	2.69	98	2.83	2.39	84	0.09	0.43	506	0.11	0.50	452
	0.3–0.5	1.63	1.27	78	1.82	1.62	89	0.07	0.27	410	0.08	0.78	954
	0.5–1.0	1.97	1.35	68	1.83	1.17	64	0.07	0.27	408	0.07	0.32	484
	1.0–2.0	1.62	1.23	76	1.57	1.09	69	0.05	0.23	449	0.05	0.24	479
	2.0–4.0	0.73	1.01	138	0.73	0.89	122	0.02	0.14	774	0.02	0.15	733
	4.0–6.0	0.28	0.60	211	0.26	0.61	233	0.01	0.10	1187	0.01	0.08	1202
	6.0–8.0	0.18	0.51	278	0.15	0.45	294	0.01	0.08	1261	0.01	0.09	1151
	8.0–10.0	0.11	0.37	336	0.09	0.34	383	0.00	0.06	1630	0.00	0.06	1630

Root length densities (RLD, expressed in cm cm⁻³) can be estimated for fine roots using the formula fitted by Maurice et al. (2010) in the same stands: RLD = 1.89 LAI N_t; where N_t is the root intersect density expressed as number of fine roots cm⁻².

but the values were much lower and the variability was higher (**Table 3**).

Although the highest FRD was found in the top soil, less than 20% of the total fine root intersects down to a depth of 10 m were counted in the 0–50 cm soil layer in the 1-, 3.5-, and 6-year-old stands of the chronosequence (**Figure 5A**). Half of the total amounts of fine and medium-sized root intersects were found below a depth of 1.0–1.5 m in all the sampled stands (except for medium-sized roots at age 1 year). The proportion of fine roots below a depth of 4 m increased with

stand age. They represented 5% of the total fine root intersects in the 1-year old stand, 10% in the 3.5-year old stand, 15% in the 6-year-old stand of the chronosequence and 20% in the 6-year-old stand on clayey soil. Medium-sized roots tended to accumulate in the upper soil layers throughout the stand development: about 30% of the cumulated medium-sized root intersects down to a depth of 10 m were found in the 0–1 m soil layer in the 1-year-old stand, 45% in the 3.5-year-old stand and 50% in the 6-year-old stand of the chronosequence (**Figure 5B**).

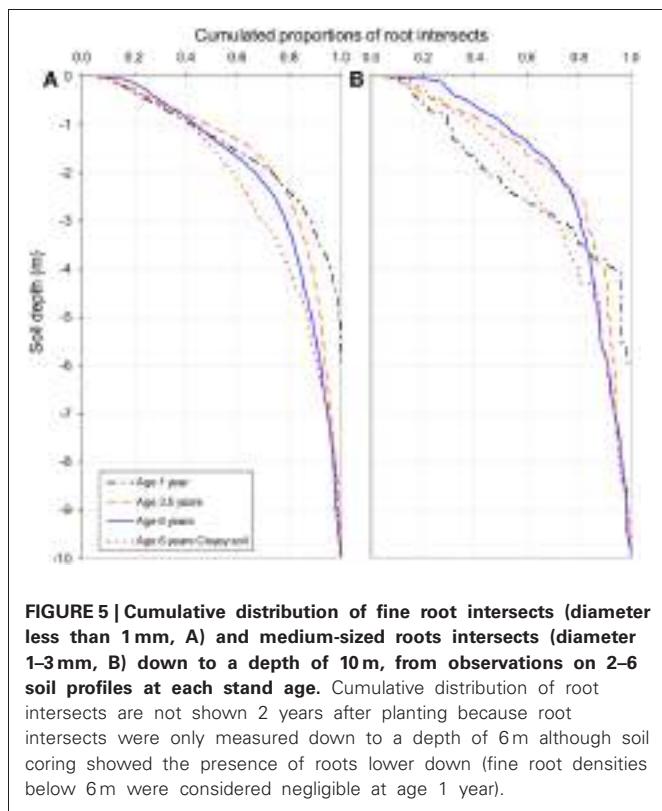


FIGURE 5 | Cumulative distribution of fine root intersects (diameter less than 1 mm, A) and medium-sized roots intersects (diameter 1–3 mm, B) down to a depth of 10 m, from observations on 2–6 soil profiles at each stand age. Cumulative distribution of root intersects are not shown 2 years after planting because root intersects were only measured down to a depth of 6 m although soil coring showed the presence of roots lower down (fine root densities below 6 m were considered negligible at age 1 year).

SPATIAL EXPLORATION OF SOIL BY FINE ROOTS

Standardized variograms showed a spatial dependence of fine roots in the upper layer (0–2 m) with an anisotropy increasing with stand age (Figure 6). Below a depth of 2 m, the spatial dependence of fine roots was weak, being limited to short distances (standardized variograms were horizontal for distances greater than 30 cm). Ratios of anisotropy close to 1.0 for all stand ages in the 2–4 and 4–6 m soil layers showed an isotropic exploration of deep soil layers by fine roots. Similar variograms at each age in the P0 and P1 soil profiles (data not shown) indicated that the spatial structure of fine root exploration was not strongly influenced by the distance from the nearest trees from 1 year after planting onwards. No spatial dependence was observed for medium-sized roots, whatever the stand age and the soil layer.

DISCUSSION

DOWNDWARD ROOT GROWTH RATES

In accordance with the first hypothesis, the displacement of the root front in *E. grandis* plantations was fast in comparison to other plant species. Soil coring and SWC monitoring provided consistent estimates of root elongation rates (RER) downwards close to 2 cm day^{-1} , which were similar to the mean height growth rates over the first two years after planting. A slight decrease in root front velocity with stand age in *E. grandis* plantations (as observed for height growth rates) might explain why the deepest roots were found at a depth of 9.5 m at 1.5 year after planting in Brazil (Christina et al., 2011) and at a depth of 28 m at 9 years of age in South Africa (Dye, 1996). High root front velocities have been reported for some herbaceous species growing in deep

soils with no impediment to root growth. The SWC time series after an induced drought in a field experiment showed that the root front velocity peaked at 4.4 cm day^{-1} for sunflowers, 3.4 cm day^{-1} for soybeans, 3.0 cm day^{-1} for maize, and 2.3 cm day^{-1} for peanuts (Dardanelli et al., 1997). Sequential soil coring showed that the root front depth increased by 2.5 cm day^{-1} for sorghum and 4.1 cm day^{-1} for sunflowers, from 20 to 60 days after emergence (Stone et al., 2001). For sugarcane crops in Brazil, the mean root front velocity from 4 months after planting to harvesting was 1.9 cm day^{-1} (Battie-Laclau and Laclau, 2009). So far as we are aware, the root front velocity has never been measured for woody species growing in very deep tropical soils and the highest RER for tree species have been measured in pot experiments. The maximum values for rhizotron-grown *E. nitens* and *E. globulus* seedlings were about 2.5 cm day^{-1} (Misra, 1999). It has been shown for seedlings in rhizopods that lowering the water table encourages root elongation downwards for phreatophytic species (Stave et al., 2005; Canham, 2011). The maximum RER in rhizopods filled with a medium to coarse sand was 3.7 cm day^{-1} for *Banksia attenuata* seedlings and 1.8 cm day^{-1} for *Banksia littoralis* seedlings (Canham, 2011). RER reached 2.1 and 1.4 cm day^{-1} for seedlings of *Acacia tortilis* and *Faidherbia albida* in another experiment carried out in rhizopods (Stave et al., 2005). The mean root front velocity the first year after planting in our study was, therefore, close to the highest values reported for phreatophytic species in response to lowering the water table.

Although other studies have estimated the root front displacement from SWC time series (e.g., Calder et al., 1997; Dardanelli et al., 1997), it has only been possible to distinguish between the effects of a disruption of drainage from upper soil layers and water uptake by tree roots at depths of 6 and 7 m. The fact that SWC down to a depth of 6–7 m at ~ 1 year after planting was similar to the values at the end of the dry season before clear cutting could only be explained by substantial water withdrawal. The estimation of the root front depths from several approaches in our study (SWC time series at depths of 6 and 7 m, fine root distributions on trench walls 1 and 2 years after planting, and soil coring at age 2.2 years) were consistent and confirmed a synchrony between the vertical extension of shoots and roots already shown by Christina et al. (2011) in *E. grandis* plantations. The drop in SWC shown in the time series for depths of 8, 9, and 10 m, that was thought to have resulted only from the disruption of drainage from the upper soil layers, might also be caused by water withdrawal by tree roots (Figure 2). The water table was at a depth of 14 m from 1 to 1.3 years after planting (data not shown) and capillary rises in the soil (70% sand) could not account for the dynamics of SWC observed (Fan and Miguez-Macho, 2010). Further studies based on successive soil coring are needed to assess whether root front velocity in very deep soil layers increases during dry periods. In addition, stand evapotranspiration measured accurately by eddy-covariance at this study site could be used with a modeling approach to estimate the amounts of water stored in deep soil layers that are taken up throughout the rotation cycle.

High RER in deep soil layers in this study may be explained by a combination of favorable factors, including high water requirements in Brazilian *Eucalyptus* plantations (Cabral et al., 2010) for a GPP of about $4000 \text{ g C m}^{-2} \text{ yr}^{-1}$ at the study site (Campoe

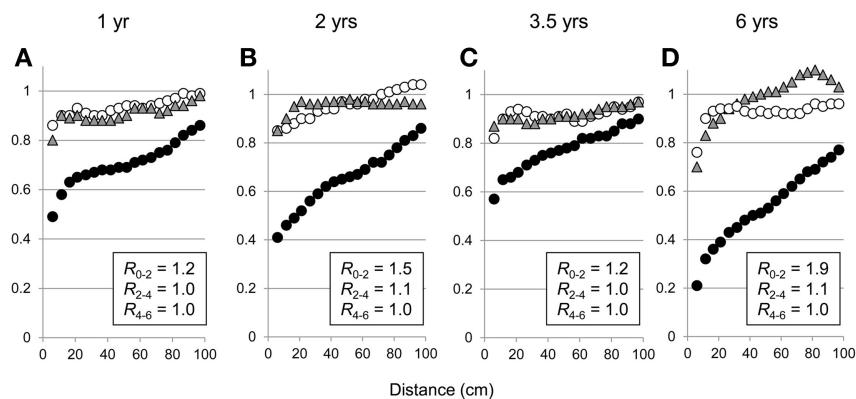


FIGURE 6 | Standardized variograms of fine root intersects in soil layers 0–2 m (filled circles), 2–4 m (filled gray triangles), and 4–6 m (empty circles) for the P0 soil profile in 1-, 2-, 3.5-, and 6-year-old stands on a

sandy soil (A, B, C, D, respectively). R_{0-2} , R_{2-4} , and R_{4-6} indicate the average ratios of anisotropy in soil layers 0–2, 2–4, and 4–6 m, respectively (see equation 3).

et al., 2012), favorable soil temperature and SWC for root growth in deep soil layers (Iijima et al., 1998; Thongo M'bou et al., 2008) and the lack of physical or chemical limitations on root growth in the soil. Recent studies in *Arabidopsis* plants showed that water potential gradients and/or moisture sensors are likely to trigger ABA and cytokinin signaling to modulate hydrotropism gene networks (Cassab et al., 2013). Stimulating the genes involved in root hydrotropism in response to the development of a gradient of soil water potential may account for high RER of *Eucalyptus* trees planted on land previously used for agriculture in Australia. A strong relationship was found between the mean tree height and lateral extent of roots of four commonly planted tree species (*E. globulus*, *Pinus radiata*, *Pinus pinaster*, and *E. kochii*) at 12 sites in Australian agro-forests. In particular, fine roots were found in the top soil up to a distance of 37 m from 15 m tall *Eucalyptus globulus* trees 6 years after planting (Sudmeyer et al., 2004). Horizontal RER in Australian agro-forests were, therefore, close to the values estimated for vertical RER in our study. Contrary to the pattern observed in Australian agro-forests, excavation of *E. grandis* superficial roots at our study site showed that the root lateral extension was less than 7 m, from 1 year after planting to harvesting (Christina et al., 2011). High inter-tree competition for water resources in the top soil in monospecific eucalypt plantations while large amounts of water are stored in deep soil layers after clear-cutting lead to the development of a vertical SWC gradient. It would be worthwhile studying the role of root hydrotropism in explaining high RER along gradients of soil water potential, horizontally in agro-forests and vertically in monospecific plantations.

SPATIAL EXPLORATION OF CONSIDERABLE SOIL VOLUMES

In agreement with the second hypothesis, most of the soil volume was explored by fine roots in the upper 3 meters from 1 year after planting onwards. Counting root intersects on 3 faces of more than 1000 soil cubes (1 dm³ in volume) in the same *Eucalyptus* stands as used for this study, Maurice et al. (2010) showed that soil space occupation by fine roots was isotropic below a depth of 60 cm, while both isotropy and anisotropy could be found

in the upper soil layers depending on stand age. This study indicated that fine root length densities were strongly correlated to root intersect densities on vertical soil profiles, even though the relationships depended on stand age and soil fertility. The relative FRDs between soil layers estimated from root intersect counts in our study were, therefore, probably similar for fine root length densities. Kriged maps showed that the development of anisotropic soil exploration in the upper layers led to fine root clustering, as shown by Bouillet et al. (2002) in *Eucalyptus* plantations and Schmid and Kazda (2005) in *Fagus sylvatica* and *Picea abies* forests. Severe soil hydrophobicity in the Congo led to root clumping in preferential drainage channels under *Eucalyptus* plantations, which helped to explain a rapid nutrient uptake from soil solutions (Laclau et al., 2001). In the present study, clustered fine roots in the top soil probably reflected a concentration of resources throughout stand growth. Whilst gravitational water reached depths of 10 m in the first 2 years after planting, it did not reach 6 m deep thereafter. Although fertilizers are applied to the top soil in *Eucalyptus* plantations, significant amounts of potassium and nitrate are leached to a depth below 1 m in sandy soils (Silva et al., 2013), and taken up by tree roots between depths of 1 and 3 m (Laclau et al., 2010). From 2 years after planting onwards, the biological cycle leads to an accumulation of nutrients in the top soil (Laclau et al., 2003) and water availability is low between the depths of 5 and 10 m (Figure 2). The spatial variation in FRD throughout the rotation in tropical *Eucalyptus* plantations is, therefore, well-suited to prevent water and nutrient losses by deep drainage. A functional specialization of fine roots, with a higher capacity to take up Sr²⁺ and Rb⁺ (analogs of Ca²⁺ and K⁺, respectively) in deep soil layers rather than in top soil layers, also helps to prevent nutrient losses in *E. grandis* stands (da Silva et al., 2011). The functional role of deep roots in these plantations has been confirmed by modeling approaches, which show that the predictions of production are greatly improved when water storage in very deep soil layers is taken into account (Mendham et al., 2011; Marsden et al., 2012).

E. grandis trees explored a considerable volume of soil with limited carbon cost. Fine roots below a depth of 4 m

accounted for less than 20% of the total fine root intersects down to 10 m, for all stand ages. Despite a tendency toward fine root clumping in deep soil layers (spatial dependence less than 30 cm below a depth of 2 m), the SWC time series showed that low fine root densities had the capacity to withdraw large amounts of water. Fine root clumping in soil areas of preferential infiltration of gravitational water through the top soil, as well as the development of a superficial root mat in the forest floor (Laclau et al., 2004) may also help to prevent water and nutrient losses after canopy closure in tropical *Eucalyptus* plantations. The plasticity in soil exploration by fine roots throughout tree growth probably plays a major role in maximizing resource use in these fast-growing plantations.

To conclude, this study shows very fast soil exploration by fine roots down to a depth of 10 m in *E. grandis* plantations. All the water available for trees that was stored down to a depth of 6–7 m after clear cutting was withdrawn during the first year after planting. High FRD in the upper 3 m of soil and sparse clustered fine roots in very deep soil layers made it possible to prevent water loss

by deep drainage after canopy closure. These results suggest that the functional role of deep roots has not been sufficiently taken into account by forest managers. The soil water holding capacity down to depths greater than 10 m is an important criterion to select the most suitable land for afforestation and to improve the predictions of biomass production by process-based models. Further studies of the anatomical, architectural and functional characteristics of fine roots along very deep soil profiles should be carried out to gain an insight into their potential impact on C, water and nutrient cycles in tropical regions.

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Root-microbe systems: the effect and mode of interaction of Stress Protecting Agent (SPA) *Stenotrophomonas rhizophila* DSM14405^T

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Stenotrophomonas rhizophila has great potential for applications in biotechnology and biological control due to its ability to both promote plant growth and protect roots against biotic and a-biotic stresses, yet little is known about the mode of interactions in the root-environment system. We studied mechanisms associated with osmotic stress using transcriptomic and microscopic approaches. In response to salt or root extracts, the transcriptome of *S. rhizophila* DSM14405^T changed drastically. We found a notably similar response for several functional gene groups responsible for general stress protection, energy production, and cell motility. However, unique changes in the transcriptome were also observed: the negative regulation of flagella-coding genes together with the up-regulation of the genes responsible for biofilm formation and alginate biosynthesis were identified as a single mechanism of *S. rhizophila* DSM14405^T against salt shock. However, production and excretion of glucosylglycerol (GG) were found as a remarkable mechanism for the stress protection of this *Stenotrophomonas* strain. For *S. rhizophila* treated with root exudates, the shift from the planktonic lifestyle to a sessile one was measured as expressed in the down-regulation of flagellar-driven motility. These findings fit well with the observed positive regulation of host colonization genes and microscopic images that show different colonization patterns of oilseed rape roots. Spermidine, described as a plant growth regulator, was also newly identified as a protector against stress. Overall, we identified mechanisms of *Stenotrophomonas* to protect roots against osmotic stress in the environment. In addition to both the changes in life style and energy metabolism, phytohormones, and osmoprotectants were also found to play a key role in stress protection.

Keywords: plant-microbe interaction, oilseed rape, PGPR, SPA, transcriptomics, root exudates, FISH-CLSM

INTRODUCTION

Crop cultivation in salinated soils is one of the major challenges facing agriculture today. Salinated areas are increasing world-wide and plants growing under saline or water-imbalance stress are more vulnerable to diseases caused by soil-borne pathogens (FAO, 2005). Biocontrol using salt-tolerant, plant growth-promoting rhizobacteria (PGPR) to protect plant roots against high salinity and pathogens offers sustainable solutions for plant protection, and *Stenotrophomonas rhizophila* is a model bacterium for a rhizosphere- and phylloplane-competent, salt-tolerant PGPR (Ryan et al., 2009; Berg et al., 2010, 2013). While the species *S. maltophilia* has become important as a nosocomial human pathogen, no pathogenic potential for humans has ever been observed in the related species *S. rhizophila* (Wolf et al., 2002). Moreover, both species can be easily distinguished by the production of the osmoprotective substance glucosylglycerol (GG) (only present in *S. rhizophila*) and the occurrence of specific multidrug-efflux pumps (only present in *S. maltophilia*) (Ribbeck-Busch et al., 2005).

Plant growth promotion by *S. rhizophila* strain DSM14405^T (syn. strain e-p10) was observed under greenhouse conditions (Schmidt et al., 2012) and in the highly salinated soils of Uzbekistan at levels up to 180% (Egamberdieva et al., 2011). Use of classical physiological and biochemical methods unveiled the mechanisms of plant growth promotion and biocontrol against soil-borne pathogens (Berg and Ballin, 1994; Kobayashi et al., 1995; Jacobi et al., 1996; Dunne et al., 2000; Suckstorf and Berg, 2003) as well as the production of high amounts of osmolytes trehalose and GG in response to salt stress (Roder et al., 2005). Next generation sequencing techniques have allowed for new possibilities to study plant-microbe interaction. For example, genome sequencing has given new insight into the genetic sources that provide beneficial plant-associated bacteria with traits such as plant growth promotion, protection against phytopathogens, and osmoprotection. In general, the described mode of action could be confirmed due to the presence of genes possibly responsible in the genome of *S. rhizophila* DSM14405^T (Berg et al., 2013). For example, *S. rhizophila* possesses genes

responsible for the synthesis and transport of osmoprotective molecules out of the cell. In addition, it contains a number of genes involved in the biocontrol of soil-borne pathogens and important genes that aid in the competition for nutrients and niches as well. Additionally, *S. rhizophila* is equipped with several genes which may play a role in root colonization, such as those that encode the O-antigen, capsule polysaccharide biosynthesis pathways, hemagglutinin, and outer membrane adhesion proteins. However, despite this knowledge, there is still no evidence that these genes are involved in successful root-microbe interactions under salinized conditions. In addition to the high salinity, the role of root exudates for this interaction was pointed out in other studies (González-Pasayo and Martínez-Romero, 2000; rev. in Bais et al., 2006). The ability of cells to respond appropriately to changing environmental conditions can be investigated using a transcriptomic approach. This technique offers a new and powerful tool to evaluate these hypothetical mechanisms *in situ*, as shown already by van de Mortel et al. (2012) for the *Pseudomonas-Arabidopsis* and by López-Guerrero et al. (2012) for the *Rhizobium-Phaseolus* interaction.

The objective of our study was to investigate the response to changing environmental conditions associated with osmotic stress (1) salt stress and (2) root exudates to understand stress protection against changing osmotic conditions of roots by the endophytic bacterium *S. rhizophila* DSM14405^T in more detail. We hypothesized that there is a general response to changing osmolarities, but also a specific answer to each other of the two parameters which are important for colonizing the root system of plants.

MATERIALS AND METHODS

TREATMENT WITH OILSEED RAPE EXUDATES

Root exudates were collected from oilseed rape cultivar Californium (Kwizda, Austria) and grown for 14 days in gnotobiotic systems of 50 ml of sterilized vermiculite packaged in pots and covered with lids (Metro, Austria). Prior to sowing the seeds, about 50 ml of tap water was amended with 1/10 [v/v] of minimal medium (Gamborgs B5 basal salt mixture; Duchefa), and the seeds were surface-sterilized in sodium hypochlorite (10% wt/wt) for 10 min and washed successively with sterile water under sterile conditions. No seeds were sown in the control system. Plant and control systems were arranged in a replicate randomized block design and maintained at 20°C under 16-h light and 8-h dark conditions. After 14 days, plants were removed and the root exudates and liquid from the control system were collected in sterile bags and squeezed. To corroborate sterility, both root material and exudates were plated on nutrient agar. Root exudates were centrifuged (10 min, 5000 × g), and the supernatant was collected, filter-sterilized (first 0.45 µm, second 0.22 µm filter, Millipore), and stored at -20°C in the dark until use. *S. rhizophila* DSM14405^T was cultivated under agitation in 40 ml CAA (per liter: 5.0 g casamino acids, 1.54 g K₂HPO₄·3H₂O, 0.25 g MgSO₄·7H₂O) and supplemented with 10 ml of the root exudates and the control liquid, respectively, at 30°C for 48 h. Cells were harvested using centrifugation at 2500 × g for 1 min for RNA extraction.

SALT SHOCK

S. rhizophila DSM14405^T was cultivated in 50 ml CAA under agitation at 30°C for 13 h (per liter: 5.0 g casamino acids, 1.54 g K₂HPO₄·3H₂O, 0.25 g MgSO₄·7H₂O) until an optical density of OD₆₀₀ 0.8 was reached. A final salt (NaCl) concentration of 3% in the medium was reached by using a sterile concentrated sodium chloride stock solution (0.3 g l⁻¹). After 2.7 h cultivation in the medium containing 3% salt, the *S. rhizophila* DSM14405^T culture (OD₆₀₀ = 0.9) was used for RNA extraction. Two independent replicates were performed as described above.

RNA EXTRACTION AND TRANSCRIPTOMIC ANALYSES

RNA was extracted using the RNAProtect® Bacteria Reagent (Qiagen, Hilden, Germany). Total rRNA was removed and mRNA was enriched using the MICROBExpress kit, according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). The mRNA was sequenced using LGC Genomics (Berlin, Germany), and data collection was performed using MicroDiscovery (Berlin, Germany). The data used for assessing the changes in gene transcription correspond to normalized values for the number of reads that uniquely mapped to each CDS. Transcription fold change for each CDS was assessed by dividing the corresponding value from the cells that were either treated with root exudates or exposed to salt shock by those from the control group. Of the total genes either up or down-regulated, only those showing fold changes greater than or equal to 1.5 and less than or equal to 0.6 were considered significantly impacted.

GERMINATION POUCH COLONIZATION ASSAY

A batch of 200 oilseed rape seeds were surface-sterilized with 40 ml of 3% NaOCl for 1 min and subsequently washed twice with 40 ml of water for 1 min each time. Surface-sterilized seeds were inoculated with *S. rhizophila* DSM14405^T by incubating in a 2 ml cell suspension containing 10⁷ CFU ml⁻¹. The control included seeds treated with 0.85% NaCl. Twelve seeds per treatment were placed into 2 (6 seeds per pouch) sterile Cyg™ germination pouches (Mega International, West St. Paul, MN, USA) wetted with 10 ml of sterilized deionized water or 1.25% NaCl solution. Germination pouches were then placed in sterile, aseptically sealed containers and placed in a growing chamber for 9 days with controlled day and night settings (12 h of light at 25°C and 12 h of dark at 20°C). After 9 days of growth, roots of 3 seedlings were combined for determination of cell counts resulting in 4 replicates per individual treatment. All root material was cut and transferred to Whirlpak® bags (Carl Roth, Karlsruhe, Germany) containing 2 ml of 0.85% NaCl solution. The roots in the bags were then crushed using a pestle to form a homogenous suspension, which was subsequently serially diluted and drop-streaked onto LB Petri dishes. The plates were then incubated at 30°C for 24 h.

FLUORESCENT *in situ* HYBRIDIZATION (FISH)

To study the oilseed rape colonization ability of *S. rhizophila* DSM14405^T using confocal microscopy, the oilseed rape roots grown in seed germination pouches were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) (3:1 vol/vol). The control group contained roots without bacterial treatment.

The fixed samples were then stored in PBS/ 96% ethanol (1:1) at -20°C . The FISH probes were purchased from genXpress® (Wiener Neudorf, Austria), and the in-tube FISH was performed as described by Cardinale et al. (2008). The FISH probes used for the hybridization step were labeled with the fluorescent dye Cy3 and included EUB338 (Amman et al., 1990), EUB338 II, and EUB338 III (Daims et al., 1999), all directing eubacteria. An equimolar ratio of the FISH probes was used for the hybridization step to detect *S. rhizophila* DSM14405^T. In this step, 30% formamide was added to the samples which were then subsequently incubated in a water bath (43°C) for 90 min. After hybridization, the samples were washed at 44°C for 15 min. Microscopy and image capturing were performed using a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) with the Leica ACS APO 63X OIL CS objective (NA: 1.30). A z-step of 0.4–0.8 μm was applied to acquire confocal stacks.

RESULTS

TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO SALT STRESS

Under salt stress of 3% NaCl, a total number of 912 and 1521 genes of *S. rhizophila* DSM14405^T were significantly up and down-regulated, respectively. The impact of salt shock on the transcription of *S. rhizophila* DSM14405^T with respect to various functional gene groups is shown in **Figure 1**. The majority of functional groups were strongly affected, such as up-regulated genes involved in translation, synthesis of the cell wall, outer or cytoplasm membrane, nucleotide and amino acid transport and metabolism, and the production and conversion of energy. In contrast, genes involved in cell motility, secretion, intracellular trafficking, defense mechanisms, and the transport and metabolism of carbohydrates and inorganic ions are down-regulated. Moreover, genes responsible for lipid metabolism and hypothetical genes are somewhat ambiguously affected by salt stress as some are up while others down-regulated.

Of the genes that are significantly impacted by salt stress in *S. rhizophila* DSM14405^T (**Table 1**, **Tables S1A, S1B**), a number of those responsible for general and specific stress responses are up-regulated. While *surA* and *dnaJ* code for general stress chaperones, *ggpS* and *ycaD* build a well-known salt stress response mechanism in *S. rhizophila* DSM14405^T through the synthesis of the osmolyte GG and show a fold change of 8.3, and 7.7, respectively (Hagemann et al., 2008). Moreover, two genes responsible for cold shock, *deAD* and *cspA*, are also strongly up-regulated under salt stress. In addition, the transcription of *ousA* that codes for an osmotic stress protein is positively affected as well. Cellular ion exchange mechanisms and some iron uptake genes are also up-regulated in *S. rhizophila* DSM14405^T as the result of salt stress.

Although unable to synthesize xanthan, *S. rhizophila* DSM14405^T possesses some of the up-regulated xanthan-coding genes including *xanA*, *xanB*, and *rmlAC*. These genes are involved in biofilm formation in addition to their role in xanthan biosynthesis (Huang et al., 2006). Likewise, the alginate coding gene *algJ* shows a fold change of 3.2 as a result of salt shock. Alginate is an exopolysaccharide involved in the development and architecture of biofilms that protect bacteria from antibiotics

and other harmful environmental factors (Monday and Schiller, 1996; Stapper et al., 2004). Furthermore, specific secretion and transport systems such as those that code for the type VI secretion system (TVISS) are strongly up-regulated in *S. rhizophila* DSM14405^T under salt shock, however, it should be noted that closely related plant-associated *Stenotrophomonas* strains such as *S. maltophilia* R551-3 lack the TVISS. Moreover, genes involved in the conversion and transport of substances through the cell wall and those responsible for cell division are also up-regulated.

Genes responsible for flagellar apparatus and fimbriae-biosynthesis genes are comparatively down-regulated in *S. rhizophila* DSM14405^T under salt shock. Similarly, salt shock also negatively impacted the predicted capsule biosynthesis genes.

TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO ROOT EXUDATES

A total of 763 and 246 genes were significantly up and down-regulated, respectively, as a result of the addition of oilseed-rape root exudates (**Figure 2**). In general, the effect of root exudates on the functional groups is indeterminate, as some genes of a particular group are up-regulated while others are transcribed in lesser numbers. However, some functional groups are equally affected by plant root exudates, as the transcription of almost all corresponding gene sequences is either up or down-regulated. For instance, as shown in **Figure 2**, root exudates have only a positive effect on the transcription of genes responsible for amino acid, nucleotide, and carbohydrate transport and metabolism, as well as biogenesis of cell membranes, transport of substances through the cell, and the genes responsible for the transport of secondary metabolites and coenzymes. Conversely, genes involved in the secretion, transport, and metabolism of inorganic ions as well as in cell motility are mainly down regulated in response to root exudate stress.

Of those genes with a significant transcription fold change discussed above, some code for products with a known physiological function and are presented in **Table 2**. The complete list of *S. rhizophila* DSM14405^T genes with a significant transcription fold change is presented in **Tables S2A, S2B**. Cell wall breakdown and cell adherence are early and crucial steps in host-plant colonization. As presented in **Table 2**, the treatment of *S. rhizophila* DSM14405^T cells with oilseed rape seedling exudates resulted in enhanced expression of *cbg-1* and *xynB* that code for beta-glucosidase and xylanase B, respectively, and are involved in cell wall breakdown. Furthermore, both these genes are conserved among plant-associated *Stenotrophomonas* strains as they are present in both *S. rhizophila* DSM14405^T and the plant-benefiting *S. maltophilia* R551-3, but absent from the human-pathogenic *S. maltophilia* K279a. In addition, *Sr14405_2818*, which is also up-regulated by 2.4 folds, codes for an adhesin protein and is homologous to the haemagglutinin-like protein coding gene from the human-pathogenic *S. maltophilia* K279a (**Table 2**). Other up-regulated genes include two adjacent genes, *mdtI* and *mdtJ* that both code for spermidine export proteins. Spermidine is a plant growth regulator and has been recently shown to strongly promote the growth of arugula plants (Al-Whaibi et al., 2012). Moreover, several genes that code for multidrug resistance pumps, efflux transporters, heavy metal transport systems, and

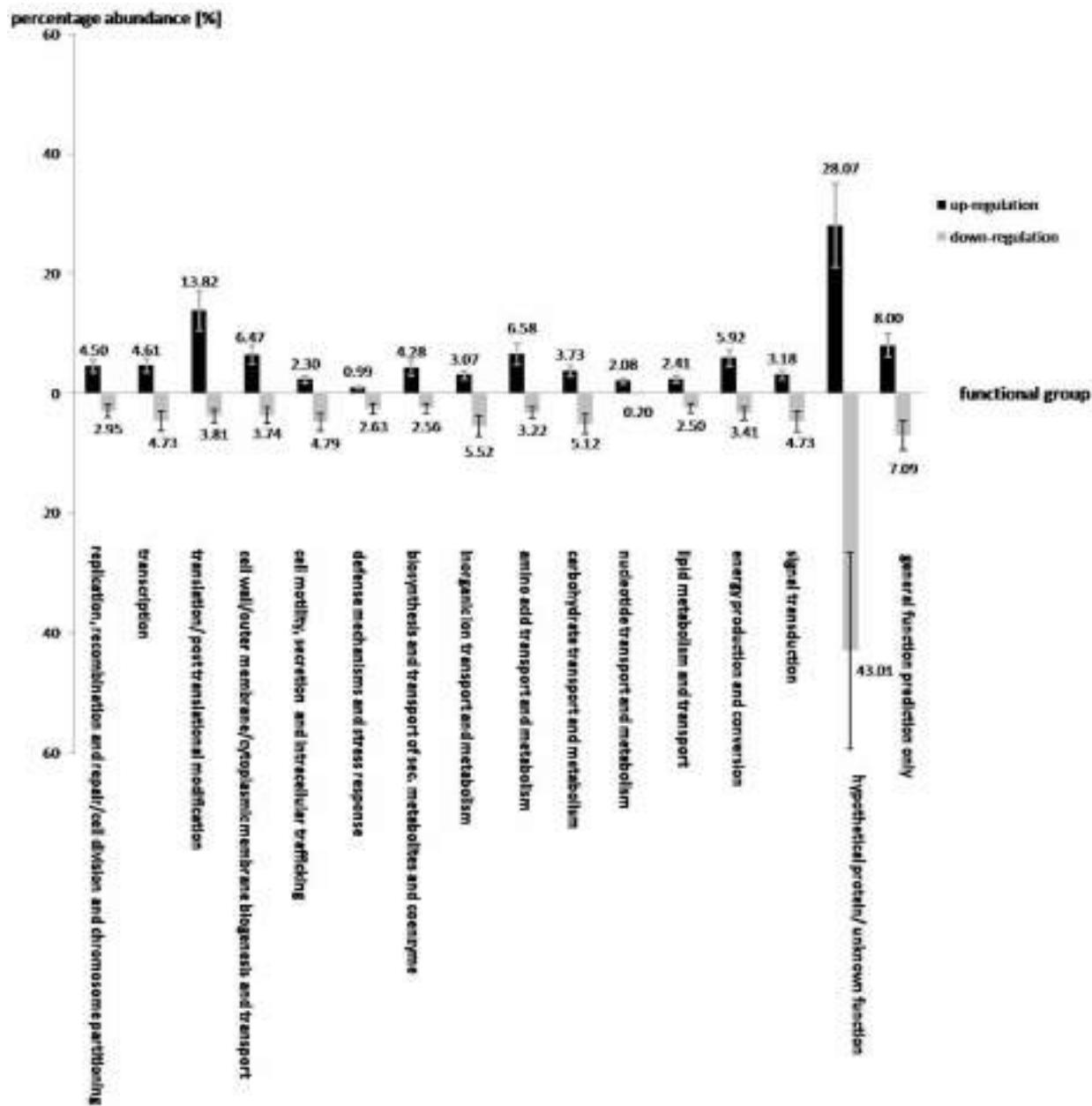


FIGURE 1 | The effect of salt shock on the gene expression of various functional gene groups in *S. rhizophila* DSM14405^T. A total of 912 and 1521 genes were significantly up and down-regulated, respectively. The impact of salt stress on most functional gene groups is clearly pronounced, as a given functional group shows either an increase or decrease in the transcription of genes belonging to that group. Genes involved in translation, synthesis of the cell wall, outer or cytoplasm membrane, nucleotide and amino acid transport and metabolism, energy production and conversion are up-regulated. In contrast, genes involved in cell motility, secretion, and intracellular trafficking, defense mechanisms, and transport and metabolism

of carbohydrates and inorganic ions are down-regulated. Genes involved in lipid metabolism, and the hypothetical genes are rather ambiguously affected by salt stress, as some of these are up and others down-regulated. The values above each column correspond to the percentage abundance of the corresponding functional group relative to the total count of the up and down-regulated genes. The transcription fold change for each CDS corresponds to the ratio calculated for *S. rhizophila* under salt shock compared with the control. Data are presented as the mean value of two independent replicates. The error bar shown on each functional group corresponds to the mean value of errors for all genes belonging to that functional group.

resistance against antibiotics are positively affected by seedling exudates.

S. rhizophila DSM14405^T contains two flagella-encoding gene blocks that are almost entirely negatively affected

by the addition of oilseed rape seedling exudates. The complete list of the flagellar apparatus-coding genes that are down-regulated is not confined to those noted in Table 2, and is presented in Tables S2A, S2B. Likewise, the

Table 1 | Selected *S. rhizophila* DSM14405^T genes with known biological roles impacted by salt shock.

Gene	(Putative) Product	Transcription fold change	Biological function
<i>ggpS</i>	Glucosylglycerol-phosphate synthase	8.3	Salt shock response protein
<i>ycaD</i>	MFS-type transporter	7.7	Salt shock response protein transporter
<i>xanA</i>	Hosphohexane mutases	3.0	Xanthan biosynthesis; biofilm formation
<i>xanB</i>	Xanthan biosynthesis protein xanB	2.9	Xanthan biosynthesis; biofilm formation
<i>rmlC</i>	dTDP-4-dehydrorhamnose 3,5-epimerase	2.3	Xanthan biosynthesis; biofilm formation
<i>algJ</i>	Alginate biosynthesis protein	3.2	Alginate biosynthesis
Sr14405 2749	TVISS effector, Hcp1 family protein	2.6	Type VI secretion system
Sr14405 2755	Rhs element Vgr protein	4.2	Type VI secretion system
Sr14405 2761	Rhs element Vgr protein	3.4	Type VI secretion system
<i>icmF</i>	TVISS protein	5.0	Type VI secretion system
Sr14405 2781	TVISS-associated protein, ImpA family	2.4	Type VI secretion system
Sr14405 2791	Rhs element Vgr protein	6.8	Type VI secretion system
<i>deaD</i>	Cold-shock DEAD box protein A homolog	8.4	Cell shock response
<i>cspA</i>	Major cold shock protein	4.3	Cell shock response
Sr14405 1916	Beta-lactamase L2 protein	6.3	Antibiotic resistance
<i>tetA</i>	Tetracycline resistance protein	3.8	Antibiotic resistance
Sr14405 1293	Bacterioferritin-associated ferredoxin	2.1	Iron uptake and transport
<i>bfr</i>	Bacterioferritin	5.9	Iron uptake and transport
<i>hisl</i>	Histidine biosynthesis bifunctional protein	4.6	Histidine biosynthesis
<i>ousA</i>	Osmoprotectant uptake system protein	4.2	Osmotic stress response
<i>surA</i>	Chaperone protein	3.8	Cellular stress response
<i>dnaJ</i>	Chaperone	3.1	Stress response
<i>ompW</i>	Outer membrane protein	3.6	Transport
<i>oprF</i>	Outer membrane protein	2.6	Transport
<i>ftsQ</i>	Cell division protein	3.4	Cell division
<i>ftsA</i>	Cell division protein	2.3	Cell division
<i>ftsY</i>	Cell division protein	2.6	Cell division
<i>ftsZ</i>	Cell division protein	2.0	Cell division
<i>lptF</i>	Lipopolysaccharide export system permease protein	4.5	Cell wall transport
<i>lptG</i>	Lipopolysaccharide export system permease protein	3.3	Cell wall transport
Sr14405 2454	Peptidoglycan-associated outer membrane lipoprotein	2.5	Cell wall protein
<i>mltD</i>	Muramidase	3.2	Bacterial cell wall biodegradation
Sr14405 1936	Peptidoglycan-associated lipoprotein	2.8	Cell wall structure protein
Sr14405 4324	Cell morphology protein	2.7	Unknown
<i>clcA</i>	H(+)/Cl(−) exchange transporter	2.7	Ion regulation
<i>kefA</i>	Potassium efflux system	2.3	Ion regulation
<i>flgA</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>flgC</i>	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
<i>flgG</i>	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
<i>flgF</i>	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
<i>fliF</i>	Flagellar basal body P-ring biosynthesis	0.3	Flagellar-driven motility
<i>flhA</i>	Flagellar biosynthesis	0.3	Flagellar-driven motility
<i>flhB</i>	Flagellar biosynthesis	0.3	Flagellar-driven motility
<i>cfaB</i>	CFA/I fimbrial subunit B	0.3	Fimbriae synthesis
<i>cfaC</i>	CFA/I fimbrial subunit C	0.4	Fimbriae synthesis
<i>csoB</i>	Fimbrial subunit B	0.2	Fimbriae synthesis
Sr14405 3215	Capsule polysaccharide biosynthesis protein	0.1	Capsule biosynthesis
Sr14405 3217	Putative UDP-glucose 4-epimerase	0.2	Capsule biosynthesis
<i>wzc</i>	Tyrosine-protein kinase	0.2	Capsule biosynthesis

The values for fold changes correspond to the *S. rhizophila* DSM14405^T subjected to the 3% NaCl shock compared to the control.

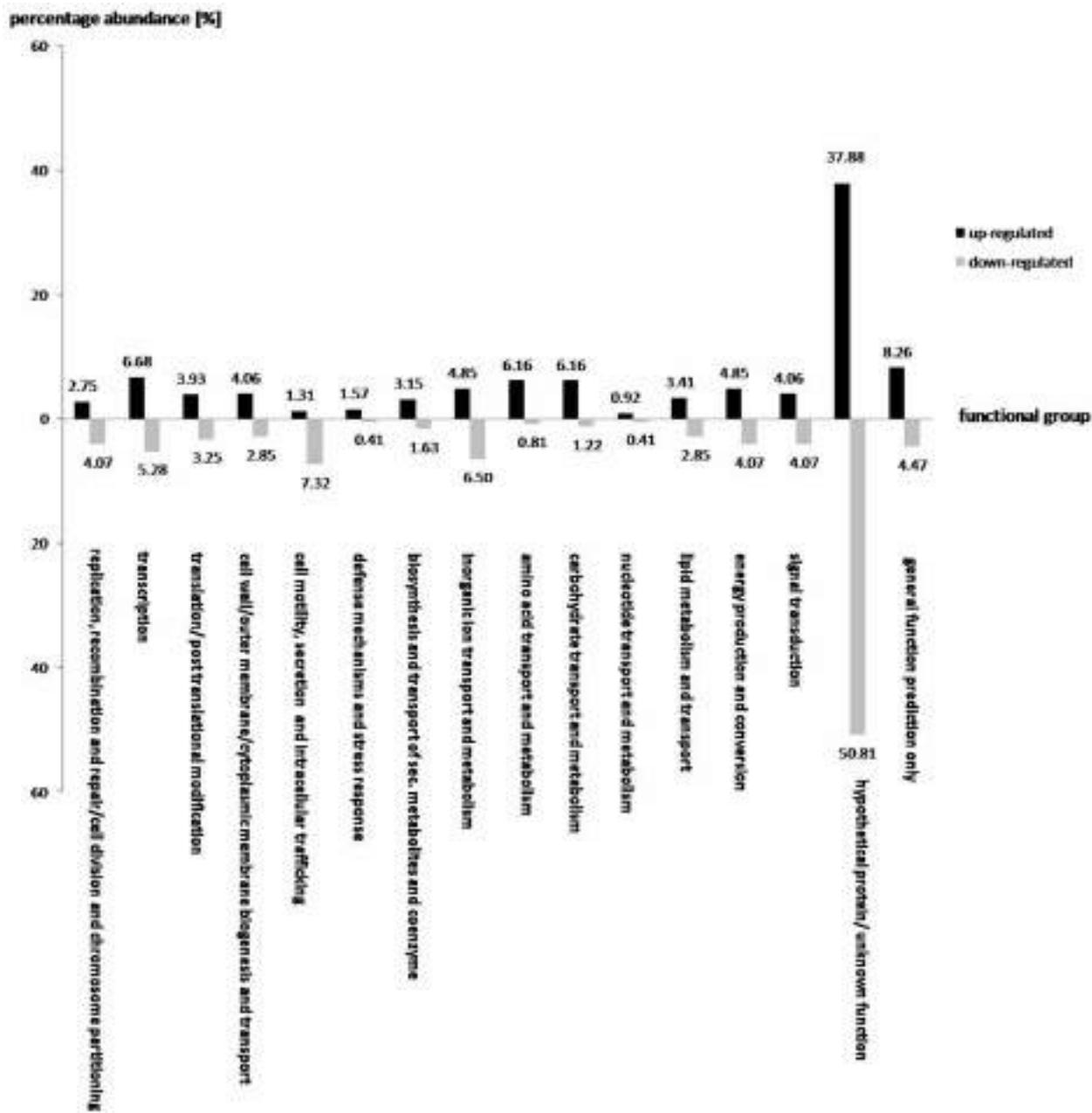


FIGURE 2 | The effect of oilseed rape seedling exudates on gene expression of various functional gene groups in *S. rhizophila*

DSM14405^T. A total of 763 and 246 genes were significantly up and down-regulated, respectively. While some functional groups are both positively and negatively regulated by root exudates, others show a clear and pronounced alteration, as the majority of the corresponding genes are either up or down-regulated. For example, genes responsible for amino acid, nucleotide, and carbohydrate transport and metabolism, and those

involved in cell wall, outer-membrane or cytoplasmic membrane biogenesis and transport as well as genes responsible for the transport of secondary metabolites and coenzymes are mainly up-regulated. In contrast, genes involved in cell motility and secretion, and those responsible for the transport and metabolism of inorganic ions are mainly down-regulated. The value above each column corresponds to percentage abundance of the corresponding functional group in the total count of the up or down-regulated genes.

expression of the genes responsible for fimbriae-driven cell motility, such as *cfaB* and *csoB* is negatively impacted by seedling exudates. Moreover, genes involved in the uptake, transport, and bioavailability of iron are also down-regulated.

SIMILARITIES IN THE TRANSCRIPTIONAL RESPONSE OF *S. rhizophila* DSM14405^T TO SALT AND ROOT EXUDATES

In response to both oilseed rape root exudates and salt shock, *S. rhizophila* DSM14405^T copes with osmotic stress in a surprisingly similar way through the alteration of gene

Table 2 | Selected *S. rhizophila* DSM14405^T genes with known biological roles impacted by plant root exudates.

Gene	(Putative) Product	Transcription fold change	Biological function
<i>mdtI</i>	Spermidine export protein	6.3	Export of the plant growth regulator spermidine
<i>mdtJ</i>	Spermidine export proteins	7.6	Export of the plant growth regulator spermidine
Sr14405_2818	Adhesin	2.4	Host cell surface attachment/colonization
<i>cbg-1</i>	Beta-glucosidase	1.7	Plant cell wall biodegradation/colonization
<i>xynB</i>	Xylanase B	1.6	Plant cell wall biodegradation/colonization
Sr14405_4324	Cell morphology protein	2.2	Unknown
Sr14405_1672	Generally characterized MFS-type transporter	3.0	Antibiotic resistance
Sr14405_1673	Multidrug synthesis protein	8.8	Antibiotic resistance
Sr14405_2718	Multidrug synthesis protein	3.5	Antibiotic resistance
<i>tetX</i>	Tetracycline resistance protein	3.5	Antibiotic resistance
Sr14405_4658	Acriflavin resistance protein	1.6	Antibiotic resistance
Sr14405_2827	Heavy metal transport and detoxification protein	2.0	Heavy metal efflux system
Sr14405_1538	Efflux transporter	1.5	Efflux of unknown target
<i>flgA</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>flgC</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>flgG</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>flgF</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>fliF</i>	Flagellar basal body P-ring biosynthesis	0.5	Flagellar-driven motility
<i>fliA</i>	Flagellar biosynthesis	0.6	Flagellar-driven motility
<i>fliB</i>	Flagellar biosynthesis	0.5	Flagellar-driven motility
<i>cfaB</i>	CFA/I fimbrial subunit B	0.5	Fimbriae synthesis
<i>csoB</i>	Fimbrial subunit B	0.4	Fimbriae synthesis
Sr14405_1293	Bacterioferritin-associated ferredoxin	0.6	Iron uptake and transport
Sr14405_1746	Heme oxygenase	0.4	Iron bioavailability
<i>fpvA</i>	Ferricytochrome c oxidoreductase	0.5	Iron uptake and transport
Sr14405_4245	Outer-membrane hemin receptor	0.4	Iron uptake and transport

The values for fold changes correspond to the *S. rhizophila* DSM14405^T treated with plant root exudates compared to the control.

expression. Numerous functional gene groups are up-regulated in response to osmotic stress factors and include those involved in energy production, as well as those involved in the synthesis and transport of cell wall, outer membrane, and cytoplasmic membrane, and those responsible for the metabolism and transport of amino acids, nucleotides, and secondary metabolites (Figure 3). Conversely, genes responsible for cell motility, secretion, intracellular trafficking, and the transport and metabolism of inorganic ions are down-regulated under both salt stress and treatment with root exudates.

COLONIZATION PATTERNS OF *S. rhizophila* DSM14405^T ON ROOTS UNDER STRESS

S. rhizophila DSM14405^T intensely colonizes oilseed rape plants, as revealed by the cell count of \log_{10} 9.47 CFU g⁻¹ root fresh weight (± 0.08) for seeds treated with deionized water. The treatment of seeds with 1.25% NaCl, however, decreased the colonization ability by nearly half resulting in a cell count of \log_{10} 9.09 CFU g⁻¹ root fresh weight (± 0.18). Furthermore, microscopic images captured using FISH combined with confocal laser scanning microscopy (CLSM) also revealed a significant decrease in the colonization of oilseed rape roots treated with 1.25% NaCl (Figure 4).

DISCUSSION

We studied the response of *S. rhizophila* DSM14405^T to osmotic changes in the form of plant root exudates and salt shock at both the physiological and molecular level. Even though we found a notable similarity in how the cell copes with these stressors, the individual responses included a great deal of specificity at the gene level thus. The response of *S. rhizophila* DSM14405^T to both oilseed rape root exudates and salt corresponds with several functional gene groups including those responsible for the synthesis and transport of cell wall, outer membrane, and cytoplasmic membrane, the metabolism and transport of amino acids, nucleotide, and secondary metabolites, energy production, cell motility, secretion and intracellular trafficking, and the transport and metabolism of inorganic ions. For *S. rhizophila* treated with root exudates, however, the shift from the planktonic lifestyle to a sessile one as expressed in the down-regulation of flagellar-driven motility is targeted to colonize the plant host, and is well in accordance with the observed positive regulation of host colonization genes. In addition to the changes in behavior and lifestyle of the bacterium, several bioactive substances were identified as key factors in stress protection. The first among them is the plant growth regulator, spermidine. Although this substance is known to strongly promote growth, this is the first evidence to show its involvement in stress protection of roots. The second group includes osmoprotective substances which were both produced

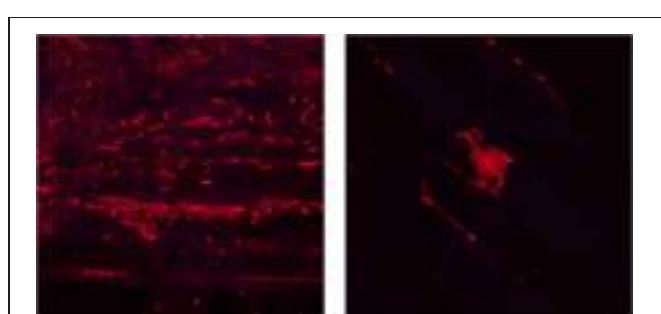
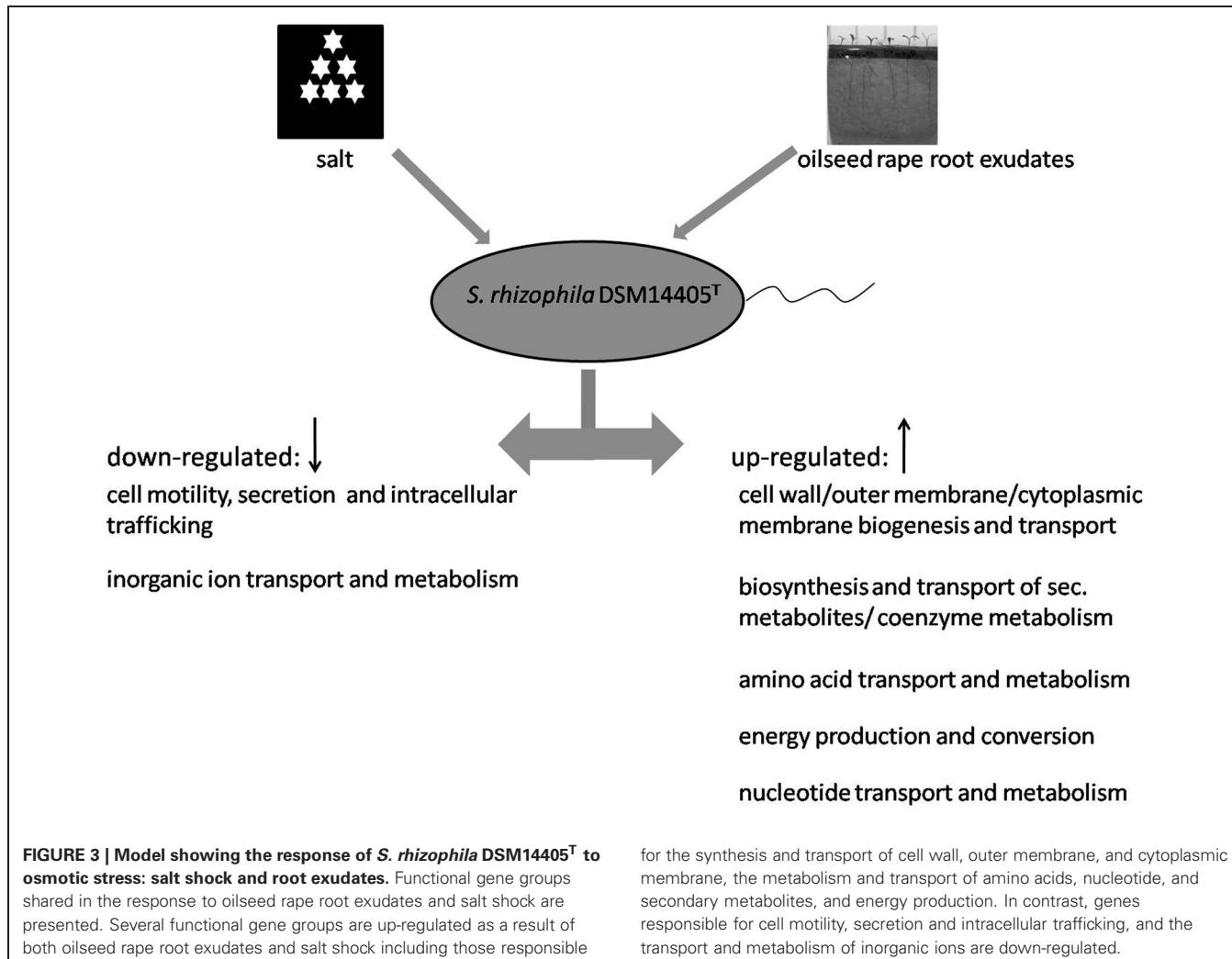


FIGURE 4 | The impact of salt stress on the capability of *S. rhizophila* DSM14405^T to colonize the oilseed rape rhizosphere visualized using FISH-CLSM. *S. rhizophila* DSM14405^T intensely colonizes the oilseed rape rhizosphere (left) while the treatment of seeds with 1.25% NaCl (right) severely decreases the colonization capability. An equimolar ratio of the FISH probes EUB338, EUB338 II, and EUB338 III labeled with the fluorescent dye Cy3 was used in the hybridization step. Microscopic images were captured using a Leica TCS SPE confocal microscope. The Leica ACS APO 63X OIL CS objective (NA: 1.30) was used to acquire confocal stacks by applying a z-step of 0.4–0.8 μm.

and excreted in high volumes as described earlier (Roder et al., 2005).

Spermidine is a well-known plant growth regulator and has been revealed to play a critical role in plant embryo development (Imai et al., 2004). Moreover, it has been recently shown to strongly promote the growth of arugula plants (Al-Whaibi et al., 2012). In addition, spermidine affects biofilm formation in various bacterial species via multiple pathways that involve both transport and signaling networks (McGinnis et al., 2009). As a result, enhanced biofilm formation or possible plant growth regulation resulting from the up-regulation of *S. rhizophila* spermidine export genes would well-serve the lifestyle shift that ultimately leads to efficient colonization of the plant host in the presence of oilseed rape exudates. Spermidine was found to prolong the life span of several eukaryotic model organisms including yeasts, nematodes, flies, and plants as well as significantly reduce age-related oxidative protein damage in mice which could indicate a potential universal anti-aging drug for eukaryotes (Imai et al., 2004; Eisenberg et al., 2009).

GG and trehalose are well-studied general osmoprotective substances that protect cells from high salt

concentrations (Ferjani et al., 2003; Hincha and Hagemann, 2004). While both species produce trehalose, GG is synthesized exclusively in *S. rhizophila* thus distinguishing itself from the pathogenic *S. maltophilia* (Ribbeck-Busch et al., 2005; Roder et al., 2005). In *S. rhizophila* DSM14405^T, *ggsS* and *ycaD* are both strongly up-regulated under 3% salt and are essential for the synthesis and transport of GG. This finding corresponds completely with both the general role of GG as a cell protector and previous findings that the amount of GG excreted into the medium increases substantially in comparison with intracellular GG content resulting from a shift of lower (less than 2%) to higher salt concentrations (Roder et al., 2005). Thus, GG production is the specific mechanism of *S. rhizophila* DSM14405^T to cope with salt stress.

TVISS genes represent a novel key virulence system used by many important pathogenic bacteria in eukaryotic host infection (Bingle et al., 2008; Pieper et al., 2009) and are intensely up-regulated under salt shock. In addition, plant growth promotion increased up to 180% in the highly salinated soils of Uzbekistan in the presence of *S. rhizophila* DSM14405^T (Egamberdieva et al., 2011). Similarly, Schmidt et al. (2012) reported that this plant growth promotion effect was more pronounced in soil than under gnotobiotic conditions suggesting it is due to the control of diseases and deleterious microorganisms. Together with the absence of TVISS genes from the other known plant-beneficial *Stenotrophomonas* strains with no plant growth promoting effect under saline conditions, these findings imply that the salt-stimulated *S. rhizophila* DSM14405^T TVISS is indirectly harnessed to promote plant growth by eliminating harmful and deleterious microorganisms in soil.

The treatment of *S. rhizophila* DSM14405^T with oilseed rape exudates resulted in the down-regulation of iron uptake and transport genes. This change is possibly due to the fact that once treated with oilseed rape exudates, *S. rhizophila* is provided with biologically available iron bound with plant siderophores resulting in less demand for the synthesis of bacterial siderophores to bind and uptake biologically unavailable iron ions present in the

medium. Moreover, treatment with root exudates resulted in the up-regulation of several multidrug resistance pumps thus demonstrating that the role of the multidrug pumps is not confined to the export of antibiotics out of the cell, but also includes a more general function with the transport of other substances once in a hyperosmotic environment.

Several questions still remain, such as the reason for positive regulation of several genes responsible for iron uptake and transport, as well as the reason for cell division under salt shock or the role of other remaining genes that are significantly up or down-regulated by osmotic stress factors. However, this work has shed light on the so far unknown mode of action of *S. rhizophila* DSM14405^T to a-biotic changes by unveiling the mechanisms that are harnessed to establish in highly salinated plant root ecosystems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Functional_Plant_Ecology/10.3389/fpls.2013.00141/abstract

Table S1A | Significantly up-regulated genes in *S. rhizophila* DSM14405^T under salt shock.

Table S1B | Significantly down-regulated genes in *S. rhizophila* DSM14405^T under salt shock.

Table S2A | Significantly up-regulated genes in *S. rhizophila* DSM14405^T under treatment with root exudates.

Table S2B | Significantly down-regulated genes in *S. rhizophila* DSM14405^T under treatment with root exudates.

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Buffet hypothesis for microbial nutrition at the rhizosphere

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An emphasis is made on the diversity of nutrients that rhizosphere bacteria may encounter derived from roots, soil, decaying organic matter, seeds, or the microbial community. This nutrient diversity may be considered analogous to a buffet and is contrasting to the hypothesis of oligotrophy at the rhizosphere. Different rhizosphere bacteria may have preferences for some substrates and this would allow a complex community to be established at the rhizosphere. To profit from diverse nutrients, root-associated bacteria should have large degrading capabilities and many transporters (seemingly inducible) that may be encoded in a significant proportion of the large genomes that root-associated bacteria have. Rhizosphere microbes may have a tendency to evolve toward generalists. We propose that many genes with unknown function may encode enzymes that participate in degrading diverse rhizosphere substrates. Knowledge of bacterial genes required for nutrition at the rhizosphere will help to make better use of bacteria as plant-growth promoters in agriculture.

Keywords: rhizosphere, speciation, root exudates, soil microbiology, bacterial genomes

INTRODUCTION

Ecophysiology of root systems cannot be understood without the microbiota that colonize outside and inside roots. Bacteria and fungi may impact root physiology, produce hormones, stimulate root growth or alter its morphology. Microbes provide protection against pathogens, tolerance to abiotic stresses, resistance to insect or herbivore attack; even allelopathy may be due to root-associated microorganisms. An extensive review on the ecophysiological contributions of microorganisms to plants has been published (Friesen et al., 2011) and reviews on rhizospheric bacteria also highlight their effects on plants (van Loon et al., 1998; Bais et al., 2006; de Bruijn, 2013). Microbial endophytes (meaning residing inside the roots) may contribute to nutrient assimilation and other plant traits, however, they are normally in lower numbers than rhizospheric bacteria (Rosenblueth and Martínez-Romero, 2006; Hirsch and Mauchline, 2012) and we will focus only on the latter. Over the years, studies on root microbiota have addressed several questions such as: How are microbes selected or maintained in roots? What are the sources and resources for root microbes? How do bacteria or fungi affect root physiology? Are there key species that have a larger impact on plants? Is nutrient competition driving bacterial evolution? There are still questions without answer.

The term rhizosphere was proposed by Hiltner (1904) and refers to 1–7 mm of soil from the root surface. The rhizosphere effect is the enrichment of microbial populations at the root–soil interface. Outside roots there is a heavy colonization of bacteria (for example, 10^9 *Rhizobium phaseoli* cells per gram of fresh maize root; Gutiérrez-Zamora and Martínez-Romero, 2001) mainly stimulated by root-derived nutrients. The microbial community itself may modify root nutrients and may contribute with resources by transforming soil material (Baelum et al., 2008), by fixing nitrogen (Fischer et al., 2012) or producing vitamins (Phillips et al.,

1999; Ramírez-Puebla et al., 2013). Rhizosphere nutrients may be very variable depending on the plant (Brown et al., 2008; Haichar et al., 2008; Badri et al., 2013) and the soil biotic and abiotic conditions. There are bacterial species commonly encountered as rhizosphere colonizers but each plant species may harbor particular microbes at the rhizosphere (Lundberg et al., 2012). A complex rhizosphere community may be structured in relation to the microbial specialization for different nutrients. The diversity of nutrients available at the rhizosphere may be equated to a buffet, and distinct microbes may have preferences for some of them. Furthermore, we propose that a large proportion of products from genes highly expressed by bacteria at the rhizosphere are involved in the transport and catabolism of the various buffet entries.

PLANT-DERIVED NUTRIENTS AT THE RHIZOSPHERE

Plants may be considered as a growth media for their microbiota (Brown et al., 2008). Root exudates determine bacterial community structure (Haichar et al., 2008) and rhizodeposits (Dennis et al., 2010) may do the same as well. Root exudates contain a large diversity of molecules (reviewed in Walker et al., 2003; Bais et al., 2006; Dennis et al., 2010; Ramírez-Puebla et al., 2013) and around 10,000 types of flavonoids are known from plants (Ferrer et al., 2008). Additionally, arabinogalactan-proteins (AGPs) that have a large proportion of carbohydrates covalently bound to polypeptides are found abundantly in exudates (Fincher et al., 1983). AGPs are considered the most structurally complex molecules in nature (Majewska-Sawka and Nothnagel, 2000).

Exudates and other plant substances may act to select microorganisms (Walker et al., 2003; Shaw et al., 2006; Badri and Vivanco, 2009; Dennis et al., 2010; Berendsen et al., 2012) as prebiotics do (Ramírez-Puebla et al., 2013); additionally, just adhesion to plant lignocellulose acts to select bacteria from the soil (Bulgarelli et al.,

2012). From root extracts, the phenolic fraction was found to have an important role in conditioning bacterial communities (Badri et al., 2013). Roots have a remarkable ability to synthesize diverse secondary metabolites (Flores et al., 1999) and many complex carbon molecules (Dennis et al., 2010; Mathesius and Watt, 2011). Seeds are also a source of nutrients for plant-associated bacteria and some contain large amounts of phytate (Lott et al., 2000). Germinated seedlings provide enough sulfur in root exudates for bacterial growth (Snoeck et al., 2003).

Plants may control bacterial growth with antimicrobials such as phytoalexins (González-Pasayo and Martínez-Romero, 2000; Shaw et al., 2006), bacterial-quorum plant-produced mimics (Bauer and Robinson, 2002), or other substances yet unknown. Additionally, plant-derived substances may control bacterial metabolism (Shaw et al., 2006; Hassan and Mathesius, 2012), perhaps to the plant own benefit. On roots, bacteria exhibit a differential gene expression that varies depending on the plant (Ramachandran et al., 2011; López-Guerrero et al., 2012). The analysis of known bacterial genes expressed in the root or rhizosphere may help us deduce conditions therein. Based on the large numbers of transporters expressed by rhizospheric bacteria (Ramachandran et al., 2011; López-Guerrero et al., 2012), we propose that each bacterial species can use a wide range of the nutrients that plants provide from roots.

Root-derived nutrients may be modified by the associated microbiota directly by transforming them to new substances (Shaw et al., 2006) or by inducing changes in plant production of exudates from the interaction with the plant. Symbiosis with microbes and fungi can alter the composition of exudates (Bais et al., 2006; Scheffknecht et al., 2006).

SOIL-DERIVED NUTRIENTS

Besides root-derived nutrients, microbes at the rhizosphere may profit from soil-derived substrates. Many soils are substrate rich especially those having high content of organic matter, not even considering man-derived soil contaminants. Soil has perhaps the highest microbial diversity of all habitats. This may be explained by soil structure, diverse soil physical characteristics, differences in pH, minerals, metals, plethora of soil microhabitats but also by an unknown large diversity of natural substances found in soil. Humic acids in soils are very complex and their diverse chemical structure has just started to be determined (Nebbioso and Piccolo, 2001). In the rhizosphere different Amadori compounds (*N*-glycosylamines) may be found that form spontaneously from decomposing plant material or by *Agrobacterium* spp. (Baek et al., 2003).

Soil is not only the depository of plant and animal decay matter but it is also the residence of fungi, nematodes, protozoa, insects and their products, as well as human-derived recalcitrant substances, all of them constitute an enormous array of potential food for most diverse microbes. Their use would benefit not only microbes but also their plant hosts when making nutrients available. Soil bacteria have major roles in nutrient cycles. Phosphorus solubilizing rhizospheric bacteria promote plant growth (Rodríguez and Fraga, 1999) and microorganisms participate in plant mineral acquisition (Hinsinger, 1998).

LIFE AT THE RHIZOSPHERE FROM A NUTRITIONAL PERSPECTIVE

Different rhizosphere bacteria may have preferences for distinct substrates (Shaw et al., 2006) and this would allow a complex community to be established at the rhizosphere. Different parts of the roots are colonized by different microbes and exudation and rhizodeposition varies qualitatively in different parts of the roots (Badri and Vivanco, 2009; Dennis et al., 2010). Some plants may exude more than others (Dennis et al., 2010) and maintain larger microbial populations. Results from a proteomic-based analysis suggested that bacteria may adapt to a new range of nutrients from exudates (Cordeiro et al., 2013).

We documented simultaneous assimilation of different substrates in *Rhizobium* (Romanov and Martínez-Romero, 1994; Romanov et al., 1994). This type of metabolism would be advantageous at the rhizosphere and it has been observed in rhizoremediation (González-Paredes et al., 2013). To nourish on several plant exudated substances at the same time as well as from diverse soil substances could be a characteristic of successful rhizospheric bacteria. Genes encoding enzymes for the utilization of some Amadori compounds that may be found in the rhizosphere are patchily distributed in rhizobia (Baek et al., 2005) indicating that not all bacteria have the same degrading capacities. We have compared rhizospheric bacteria to gut bacteria in the process of digesting and converting food to host usable products (Ramírez-Puebla et al., 2013).

Pseudomonas, *Burkholderia*, *Streptomyces*, and rhizobia have high degrading capabilities (Kontchou and Blondeau, 1992; Juhasz et al., 1996, 2003). All may be found associated to roots and their high degrading capacities may be advantageous in rhizospheres. They have also characteristic large genomes (for examples, Bentley et al., 2002; Kaneko et al., 2002; Paulsen et al., 2005; Yan et al., 2008; Ormeño-Orrillo et al., 2012) that may be in relation to their high degrading capabilities. We suggested that many rhizobial genes of unknown function participate in the catabolism of root, rhizospheric, and soil substances (Ormeño-Orrillo and Martínez-Romero, 2013) and this could apply to other soil and rhizospheric bacteria as well.

Interestingly mutants in single genes involved in nutrient usage at the rhizosphere (Rosenblueth et al., 1998; Ramachandran et al., 2011) normally do not have clear phenotypes indicating that there are other substrates available that may be used by bacteria at the rhizosphere.

In modern times, rhizospheric microorganisms are exposed as well to anthropogenic contaminants (González-Paredes et al., 2013). Rhizoremediation takes advantage of the degrading capabilities of rhizospheric microorganisms. Organic matter in soil strongly influences the fate of contaminants (Li et al., 2011).

CONCLUDING REMARKS

After considering the large diversity of potential nutrients (from rhizodeposits, root exudates, seeds, decaying organic matter, soil, and the rhizosphere community itself) for microbes at the rhizosphere we propose a hypothesis for bacterial nutrition at the rhizosphere: a buffet hypothesis where commensals

choose their food from a diversity of options. This is in contrast to the proposal of oligotrophy at the rhizosphere (Ramachandran et al., 2011). Copiotrophic rhizobia are very successful rhizosphere colonizers (Gutiérrez-Zamora and Martínez-Romero, 2001). Microbial respiration is not carbon limited in the rhizosphere (Cheng et al., 1996). Rhizosphere is a complex environment with substitutable resources. In experimental evolution in complex environments with substitutable resources, *Pseudomonas* lineages evolved as imperfect generalists that differentiate to assimilate a certain range of substrates but not all

(Barrett et al., 2005), this seems to happen with microbes at the rhizosphere.

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Plant growth-promoting rhizobacteria and root system functioning

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The rhizosphere supports the development and activity of a huge and diversified microbial community, including microorganisms capable to promote plant growth. Among the latter, plant growth-promoting rhizobacteria (PGPR) colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead, mostly, to enhanced lateral root branching and development of root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plant. Recent results provided first clues as to how PGPR signals could trigger these plant responses. Whether local and/or systemic, the plant molecular pathways involved remain often unknown. From an ecological point of view, it emerged that PGPR form coherent functional groups, whose rhizosphere ecology is influenced by a myriad of abiotic and biotic factors in natural and agricultural soils, and these factors can in turn modulate PGPR effects on roots. In this paper, we address novel knowledge and gaps on PGPR modes of action and signals, and highlight recent progress on the links between plant morphological and physiological effects induced by PGPR. We also show the importance of taking into account the size, diversity, and gene expression patterns of PGPR assemblages in the rhizosphere to better understand their impact on plant growth and functioning. Integrating mechanistic and ecological knowledge on PGPR populations in soil will be a prerequisite to develop novel management strategies for sustainable agriculture.

Keywords: plant-PGPR cooperation, rhizo-microbiome, rhizosphere, phytohormone, plant nutrition, ISR, functional group

INTRODUCTION

Photosynthetic terrestrial plants play key roles as ecosystem engineers (Wright and Jones, 2006; Hartmann et al., 2009). They contribute, for instance, to the establishment of specific microbial ecological niches in plant-based systems. This is particularly the case in the rhizosphere, i.e., the soil in contact with plant roots. Besides its role in plant anchorage in soil, absorption of water and ions, nutrient storage, and plant vegetative growth, the root system is in close contact with a wide range of soil microbial populations (Berg and Smalla, 2009).

Despite their interactions with the biotic environment, the root system and its rhizosphere have received much less attention by plant physiologists than the rest of the plant. Plant roots exude a huge diversity of organic nutrients (organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage) and signals that attract microbial populations, especially those able to metabolize plant-exuded compounds and proliferate in this microbial habitat (Bais et al., 2006; Pothier et al., 2007; Badri et al., 2009; Shukla et al., 2011; Drogue et al., 2013). Root

exudates being the largest source of carbon supply within soil, the rhizosphere compartment houses a rich microbial community, comprising up to 10^{10} bacteria per gram of soil (Gans et al., 2005; Roesch et al., 2007) and encompassing a large diversity of taxa (Kyselková et al., 2009; Gomes et al., 2010). The corresponding microbial community associated to plant roots can be referred to as the rhizo-microbiome (Chaparro et al., 2013). Its composition is distinct from that of the microbial community of the surrounding soil, a direct consequence of bacterial competition for nutrients liberated in the vicinity of plant roots (Raynaud et al., 2008; Bulgarelli et al., 2013; Chaparro et al., 2013). Since root exudate composition changes along the root system, according to stages of plant development and to plant genotypes, the rhizo-microbiome composition differs accordingly (Berg and Smalla, 2009; Aira et al., 2010; Bouffaud et al., 2012; Bulgarelli et al., 2013; Chaparro et al., 2013). Plant-driven selection of bacteria is an important issue recently discussed in several reviews (Hartmann et al., 2009; Doornbos et al., 2012; Drogue et al., 2012; Bulgarelli et al., 2013).

Within the rhizo-microbiome, some microorganisms can promote plant growth and provide better plant health through several indirect or direct mechanisms (Couillerot et al., 2009; Richardson et al., 2009). Beneficial plant-microbe interactions are symbiotic interactions in which costs and benefits are shared by the plants and the microorganisms (Odum and Barrett, 2005; Bulgarelli et al., 2013) and can be categorized into two main types of interactions (Drogue et al., 2012). First, mutualistic interactions correspond to intimate and mostly obligate interactions between microbes and a restricted range of compatible host plants. They generally lead to the formation of a structure specifically dedicated to the interaction (e.g., nodules during the symbiosis between nodulating rhizobia and Fabaceae, arbuscules in the endomycorrhizal symbiosis; Parniske, 2008; Masson-Boivin et al., 2009). Second, cooperations (also called associative symbioses) correspond to less obligate and specific interactions (Barea et al., 2005; Drogue et al., 2012). They involve soil bacteria able to colonize the surface of the root system (and sometimes root inner tissues) and to stimulate the growth and health of the plant, and are referred to as plant growth-promoting rhizobacteria (PGPR; Barea et al., 2005). Colonization of plant host roots by PGPR is heterogeneous along the root system; their competitiveness regarding this process is a *sine qua non* for plant growth promotion (discussed in Benizri et al., 2001; Compant et al., 2010; Dutta and Podile, 2010; Drogue et al., 2012). In comparison to mutualistic symbionts, PGPR are thought to interact with a large range of host plant species and to encompass a huge taxonomic diversity, especially within the Firmicutes and Proteobacteria phyla (Lugtenberg and Kamilova, 2009; Drogue et al., 2012). PGPR can enhance plant nutrition via associative nitrogen fixation, phosphate solubilization, or phytosiderophore production (Richardson et al., 2009). They can improve root development and growth through the production of phytohormones or enzymatic activities, as well as favor the establishment of rhizobial or mycorrhizal symbioses. Others can protect the plant through inhibition of phytoparásites, based on antagonism or competition mechanisms, and/or by eliciting plant defenses such as induced systemic resistance (ISR; Couillerot et al., 2009; Lugtenberg and Kamilova, 2009). Some PGPR can also help plants withstand abiotic stresses including contamination by heavy metals or other pollutants; certain are even able to increase the capacity of plants to sequester heavy metals (Jing et al., 2007; Saharan and Nehra, 2011; Tak et al., 2013). Therefore, utilizing PGPR is a new and promising approach for improving the success of phytoremediation of contaminated soils (for recent reviews see Zhuang et al., 2007; Shukla et al., 2011; Tak et al., 2013).

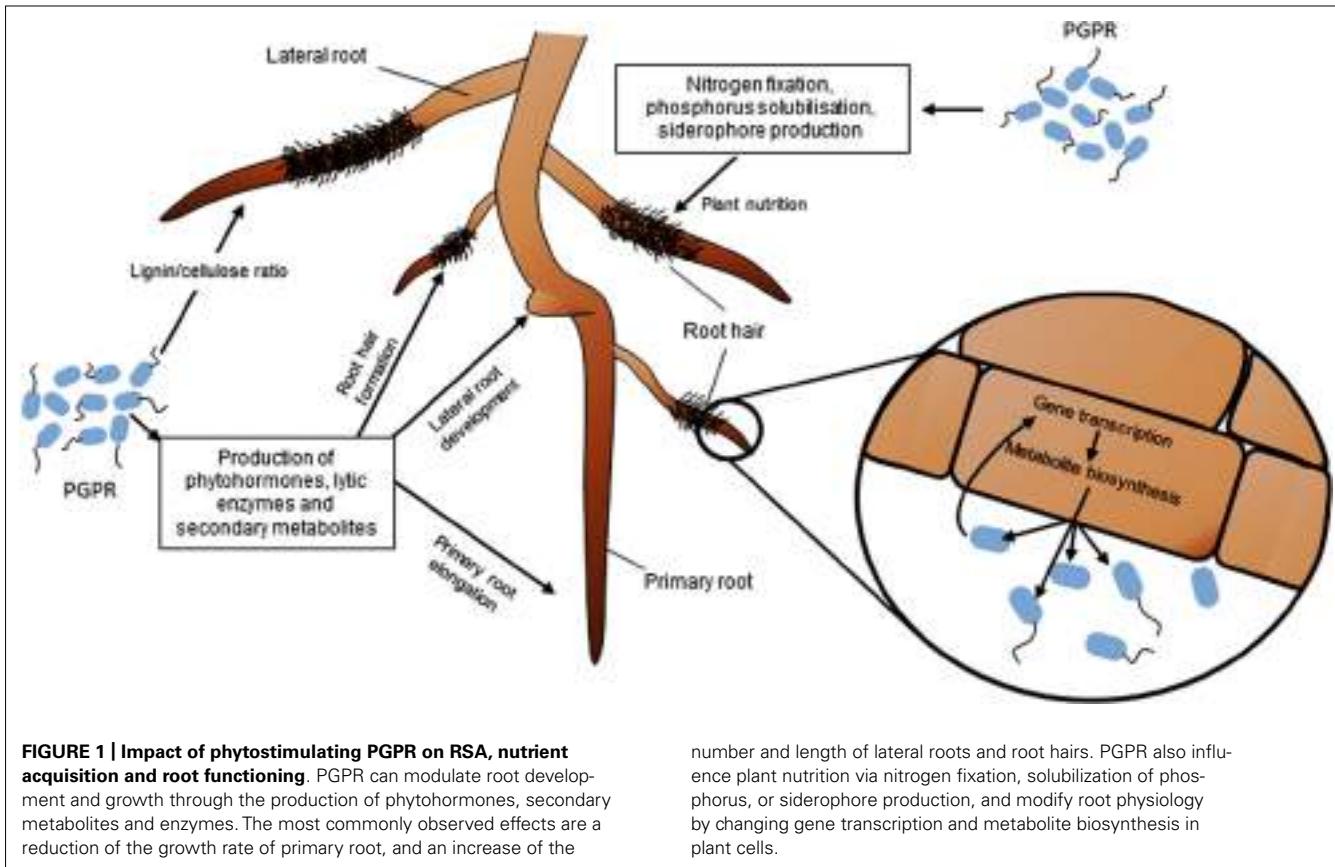
Understanding and quantifying the impact of PGPR on roots and the whole plant remain challenging. One strategy is to inoculate roots with a PGPR *in vitro* and monitor the resulting effects on plant. This showed that many PGPR may reduce the growth rate of the primary root (Dobbelaere et al., 1999), increase the number and/or length of lateral roots (Combes-Meynet et al., 2011; Chamam et al., 2013), and stimulate root hair elongation *in vitro* (Dobbelaere et al., 1999; Contesto et al., 2008). Consequently, the uptake of minerals and water, and thus the growth of the whole plant, can be increased. Some of these effects, including increased root and shoot biomass, are also documented for

PGPR-inoculated plants growing in soil (El Zemrany et al., 2006; Minorsky, 2008; Veresoglou and Menexes, 2010; Walker et al., 2012).

The focus of this paper is to review the main modes of action of PGPR strains, the functioning of PGPR populations, and their ecology in the rhizosphere. Description of plant-beneficial properties of PGPR has been the focus of several reviews (e.g., Vessey, 2003; Richardson et al., 2009; Bashan and de-Bashan, 2010), but without integrating actual PGPR gene expression on roots, the interactions between different PGPR populations in the rhizosphere, or the resulting plant-beneficial effects. This paper is organized into four sections. In the first section, we present the molecular mechanisms through which PGPR may affect the architecture of the root system and interfere with the plant hormonal pathways, and review our current understanding of their impact on the structural properties of the roots. In the second section, recent findings related to the impact of PGPR on the physiology of the whole plant are presented, with a focus on plant nutrient acquisition, plant transcriptome and plant metabolome. The third section shows how expression of plant-beneficial properties can be affected within the rhizosphere by molecules emitted by other microbial populations or by the plant. As PGPR strains are not acting individually in the rhizosphere, the ecology of PGPR populations and notably the complexity of the interactions taking place between PGPR populations is discussed in the fourth section. Finally, we conclude on the importance of integrating molecular investigations on the modes of action and ecology of PGPR strains with high-throughput analyses on the abundance, taxonomic/functional diversity and activity of rhizosphere microbial communities, and with the monitoring of plant molecular responses.

IMPACT OF PGPR ON ROOT SYSTEM ARCHITECTURE AND ROOT STRUCTURE

Most terrestrial plants develop their root system to explore soil and find nutrients to sustain growth. Root is a complex organ made of distinct regions such as the root tip, root meristem, differentiation and elongation zones, and emerging lateral roots (Scheres et al., 2002). These regions have distinct roles. For instance, root hairs are differentiated epidermal cells important for plant mineral nutrition, as inferred from gene expression studies (Lauter et al., 1996; von Wieren et al., 2000) and nutrient accumulation measurements (Ahn et al., 2004). Root functional specificity is also reflected at the level of plant-microbe interactions. In Fabaceae for example, the root tip is the most important region to initiate the rhizobial colonization process leading eventually to the formation of a root nodule (Desbrosses and Stougaard, 2011). In Poaceae, root hairs and lateral roots are preferentially colonized by PGPR, where they may express their plant beneficial properties (Pothier et al., 2007; Combes-Meynet et al., 2011). Root system architecture (RSA) integrates root system topology, the spatial distribution of primary and lateral roots, and the number and length of various types of roots. Several abiotic and biotic factors can influence RSA, including PGPR strains. PGPR modify RSA and the structure of root tissues mainly through their ability to interfere with the plant hormonal balance (**Figure 1**).



PGPR EFFECTS ON RSA VIA MODULATION OF HOST HORMONAL BALANCE

Changes in RSA may result from interferences of PGPR with the main hormonal pathways involved in regulating plant root development: auxin, cytokinin, ethylene, and to a lesser extend gibberellin, and abscisic acid (ABA) (Moubayidin et al., 2009; Stepanova and Alonso, 2009; Dodd et al., 2010; Overvoorde et al., 2011). The balance between auxin and cytokinin is a key regulator of plant organogenesis, and shapes root architecture (Aloni et al., 2006). The auxin to cytokinin ratio can be affected by PGPR because they are able to produce a wide range of phytohormones, including auxins and/or cytokinins, and secondary metabolites that can interfere with these hormonal pathways.

Many PGPR are able to produce phytohormones and secondary metabolites interfering with the plant auxin pathway, such as auxins, 2,4-diacetylphloroglucinol (DAPG), and nitric oxide (NO). Indole-3-acetic acid (IAA) is the best-characterized auxin produced by many plant-associated bacteria, including PGPR (Spaepen et al., 2007a). Exogenous IAA controls a wide variety of processes in plant development and plant growth: low concentrations of IAA can stimulate primary root elongation, whereas high IAA levels stimulate the formation of lateral roots, decrease primary root length and increase root hair formation (Figure 1; Dobbelaere et al., 1999; Patten and Glick, 2002; Perriq et al., 2007; Spaepen et al., 2007b; Remans et al., 2008). IAA is usually synthesized by rhizobacteria from tryptophan, which is found at different concentrations in root exudates according

to plant genotype (Kamilova et al., 2006). In PGPR strains, several IAA biosynthetic pathways have been described depending on the metabolic intermediates (Spaepen et al., 2007a). The indole-3-pyruvate decarboxylase (encoded by the *ipdC/ppdC* bacterial gene) is a key enzyme involved in the indolepyruvic acid pathway. Effects of *ipdC* mutants on plant root morphology are often altered in comparison to those of wild-type strains (Brandl and Lindow, 1998; Dobbelaere et al., 1999; Patten and Glick, 2002; Suzuki et al., 2003; Malhotra and Srivastava, 2008).

Plant growth promotion by PGPR can also result from indirect stimulation of the plant auxin pathway. For example, several PGPR strains like *Azospirillum brasilense* have a nitrite reductase activity and consequently are able to produce NO during root colonization (Creus et al., 2005; Pothier et al., 2007; Molina-Favero et al., 2008). NO is involved in the auxin signaling pathway controlling lateral root formation (Creus et al., 2005; Lanteri et al., 2006, 2008; Molina-Favero et al., 2008). DAPG is a well-known antimicrobial compound produced by biocontrol fluorescent pseudomonads (Couillerot et al., 2009). At lower concentrations, DAPG can also be a signal molecule for plants, inducing systemic resistance (Iavicoli et al., 2003; Bakker et al., 2007), stimulating root exudation (Phillips et al., 2004), and enhancing root branching (Brazelton et al., 2008; Couillerot et al., 2011; Walker et al., 2011). DAPG can interfere with an auxin-dependent signaling pathway and thus modify RSA (Brazelton et al., 2008). Indeed, applications of exogenous DAPG, at a concentration around 10 μ M, inhibited primary root growth and stimulated lateral root production in tomato

seedlings. Furthermore, roots of an auxin-resistant *diageotropica* mutant of tomato displayed reduced DAPG sensitivity (Brazelton et al., 2008).

The growth-promotion effect of auxin or auxin-like compounds by PGPR may require functional signaling pathways in the host plant. To test that hypothesis, one could use a host plant defective at a particular step of the hormone-signaling pathway and assess whether PGPR inoculation complements or not the effect of the mutation. This strategy requires the use of model plant such as *Arabidopsis*, the only biological system that provides to date enough documented mutant plants (Dubrovsky et al., 1994; Alonso et al., 2003). Consistent with that, some *Arabidopsis* auxin-signaling mutants failed to show the typical root architecture changes in response to the beneficial rhizobacterium *Phyllobacterium brassicacearum* STM196 (Contesto et al., 2010). However, auxin content was not increased in roots upon inoculation with *Phyllobacterium brassicacearum* STM196, ruling out the potential implication of auxin of bacterial origin (Contesto et al., 2010). Nevertheless, the use of *Arabidopsis DR5::GUS* reporter line, whose expression is restricted to the root meristem where the auxin maximum is located (Ulmasov et al., 1997; Casimiro et al., 2001), showed a change of expression pattern in response to STM196 inoculation (**Figure 2**). GUS staining appeared more intense on a wider region of the root tip as well as in the vasculature,

suggesting that there was a change of auxin distribution in the root in response to STM196 inoculation, even though this strain is a low auxin producer (Contesto et al., 2010). Interestingly, a similar observation was made when *Arabidopsis* plantlets were inoculated with the PGPR *Bacillus subtilis* GB03 (Zhang et al., 2007), which emits volatile organic compounds (VOCs), or with *Pseudomonas fluorescens* WCS417 (Zamioudis et al., 2013).

Cytokinin production (especially zeatin) has been documented in various PGPR like *Arthrobacter giacomelloi*, *Azospirillum brasiliense*, *Bradyrhizobium japonicum*, *Bacillus licheniformis*, *Pseudomonas fluorescens*, and *Paenibacillus polymyxa* (Cacciari et al., 1989; Timmus et al., 1999; de García Salamone et al., 2001; Perdigó et al., 2007; Cassán et al., 2009; Hussain and Hasnain, 2009). Cytokinins stimulate plant cell division, control root meristem differentiation, induce proliferation of root hairs, but inhibit lateral root formation and primary root elongation (Silverman et al., 1998; Riefler et al., 2006). Inoculation of plants with bacteria producing cytokinin has been shown to stimulate shoot growth and reduce the root to shoot ratio (Arkhipova et al., 2007). Bacterial genes involved in cytokinin biosynthetic pathways have been identified *in silico* but their role has not yet been validated through functional analyses (Frébort et al., 2011). Consequently, the contribution of cytokinin production by PGPR to RSA modifications remains speculative.

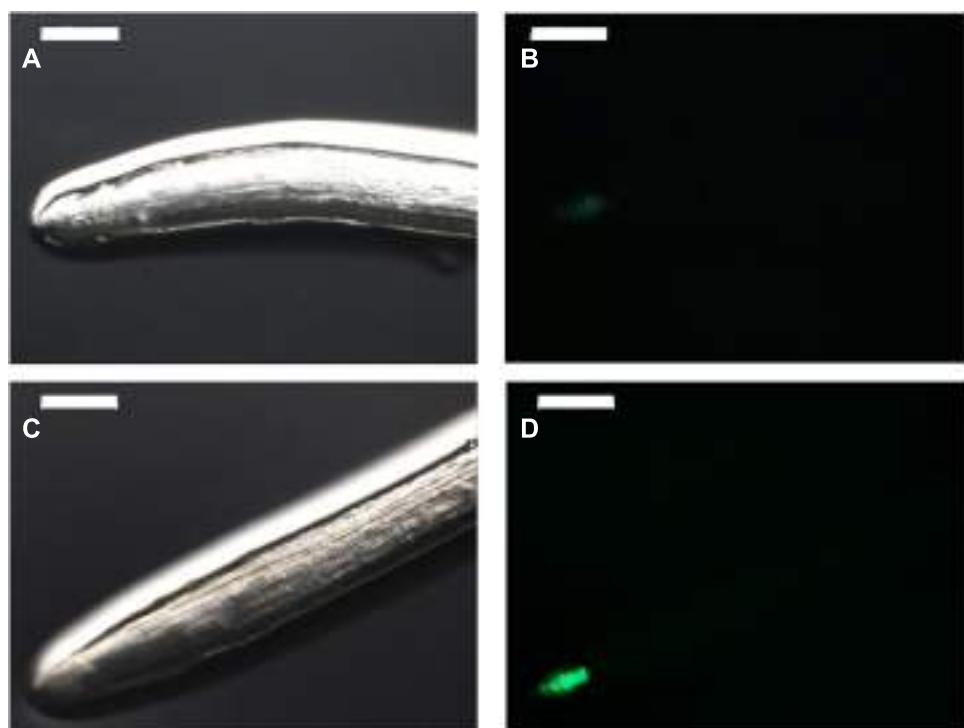


FIGURE 2 | PGPR-mediated changes in RSA may relate to modifications of auxin content in roots. Six-day-old *Arabidopsis* plantlets expressing the *GFP* gene under the control of the auxin-sensitive *DR5* artificial promoter were inoculated (**C, D**) or not (**A, B**) with the PGPR *Phyllobacterium brassicacearum* STM196. Six days later, root tips were observed under normal light (**A, C**) or UV light (**B, D**) with a microscope (Z16APO, Leica,

Bensheim, Germany). Scale bars represent 200 μ m. Inoculation by STM196 modified root traits such as root hair elongation and primary root growth, which coincided with an increase in GFP signal in the root tip in inoculated (**D**) compared with control plants (**B**). These observations confirm previous results with a different *Arabidopsis DR5* reporter line (Contesto et al., 2010).

Ethylene is another key phytohormone, which inhibits root elongation and auxin transport, promotes senescence and abscission of various organs, and leads to fruit ripening (Bleecker and Kende, 2000; Glick et al., 2007). Ethylene is also involved in plant defense pathways (Glick et al., 2007). This phytohormone can be produced in small amounts from the precursor methionine by some PGPR, like *Azospirillum brasilense* (Thuler et al., 2003; Perri et al., 2007). The ability of *Azospirillum brasilense* to produce ethylene presumably promotes root hair development in tomato plants. Indeed, exogenous ethylene supply to the plant mimicked the effect of *Azospirillum brasilense* inoculation, while the addition of an ethylene biosynthesis inhibitor blocked this effect (Ribaudo et al., 2006). Actually, PGPR are more widely able to lower plant ethylene levels through deamination of 1-aminocyclopropane-1-carboxylic acid (ACC). Many genomes of PGPR do contain a gene (*acdS*) coding for an ACC deaminase, which degrades ACC into ammonium and α -ketobutyrate (Blaha et al., 2006; Contesto et al., 2008; Prigent-Combaret et al., 2008). By lowering the abundance of the ethylene precursor ACC, the PGPR AcdS activity is thought to decrease root ethylene production, which can in turn alleviate the repressing effect of ethylene on root growth (Glick, 2005). Despite being widely accepted and supported by experimental data (Penrose et al., 2001; Contesto et al., 2008), the model raises issues that have not been well addressed yet. The first one deals with ethylene production within roots. Light is promoting ethylene biosynthesis, providing there is a sufficient CO₂ supply for shoots (Yang and Hoffman, 1984). Exposition of roots to light was shown to trigger an increase in ethylene production (Lee and Larue, 1992). In soil however, roots are sheltered from light, suggesting that this organ might not be able to synthesize large amounts of ethylene. In agreement with that, Fabaceae roots did produce ethylene in response to rhizobial colonization in presence of light, but less when they were in the dark (Lee and Larue, 1992). Secondly, there is a regulation of ethylene synthesis by a feedback loop (Yang and Hoffman, 1984). This loop should stimulate ethylene biosynthesis when the level of ACC is low. Unless PGPR disconnect that feedback loop, lowering ACC content would eventually result in stimulation of ethylene production. There is no indication yet how the feedback loop would work in presence of a PGPR. Last but not least, if ethylene plays a key role during the plant-PGPR interaction, one would expect that either plant ethylene mutants or impaired AcdS activity in the bacteria would result in clear disturbance of the plant responses to bacteria. However, minor effects on RSA were observed when plants were inoculated with an *acdS* bacterial mutant, or when plants affected in their ethylene signaling pathway were inoculated with wild-type PGPR (Contesto et al., 2008; Galland et al., 2012; Zamioudis et al., 2013). It suggests that ethylene participates to the root architecture response but is not a key player. Taken together, the functional importance of the bacterial ACC deaminase function needs further clarification. One hypothesis could be that AcdS contributes to the fine-tuning of ethylene biosynthesis during the plant-PGPR cooperation.

Several reports have revealed that PGPR are able to produce ABA or gibberellic acid, or to control the level of these hormones in plants (Richardson et al., 2009; Dodd et al., 2010). The first one, ABA, is well known for its involvement in drought stress. During

water stress, increase in ABA levels causes closing of stomata, thereby limiting water loss (Bauer et al., 2013). However, this hormone also plays different roles during lateral root development (De Smet et al., 2006; Dodd et al., 2010). Inoculation with *Azospirillum brasilense* Sp245 led to an increase of ABA content in *Arabidopsis*, especially when grown under osmotic stress (Cohen et al., 2008). Gibberellins promote primary root elongation and lateral root extension (Yaxley et al., 2001). Production of gibberellins has been documented in several PGPR belonging to *Achromobacter xylosoxidans*, *Acinetobacter calcoaceticus*, *Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Herbaspirillum seropedicae*, *Gluconobacter diazotrophicus* and *rhizobia* (Gutiérrez-Mañero et al., 2001; Bottini et al., 2004; Dodd et al., 2010). Application of gibberellic acid on maize, at a concentration similar to that produced by *Azospirillum*, promotes root growth; furthermore, gibberellin content increases in maize root inoculated with *Azospirillum* (Fulchieri et al., 1993). In addition to playing a role in plant RSA, these two hormones are involved in plant defense mechanisms. Thus, PGPR producing these hormones may modulate the hormonal balance involved in plant defense, including the jasmonate and salicylic acid pathways (for a review see Pieterse et al., 2009).

Although the production of hormones by PGPR has been well described, the genetic determinants involved in their biosynthesis remain largely unknown and bacterial mutants affected in hormone biosynthesis are mostly lacking. Consequently, the involvement of hormones of bacterial origin in the modulation of plant hormonal balance has not been fully demonstrated.

MODIFICATION OF ROOT CELL WALL AND ROOT TISSUE STRUCTURAL PROPERTIES BY PGPR

Many PGPR can lead to modifications of the chemical composition and therefore structural properties of root cell walls (Figure 1; El Zemrany et al., 2007; Zhang et al., 2007). For example, the bio-control agent *Bacillus pumilus* INR-7 is able to enhance lignin deposition in pearl millet epidermal tissues, and this plant defense response appears much more rapidly in PGPR-primed plants infected by the pathogen *Sclerospora graminicola* compared to non-primed plants (Niranjan Raj et al., 2012). The sole inoculation of INR-7 led to callose apposition. Although the precise location of these deposited polymers was not investigated, it is possible that their enhanced accumulation may participate to pathogen inhibition and disease suppression. A similar response was also triggered by *Bacillus pumilus* SE34 and *Bacillus subtilis* UMAF6639 when inoculated to respectively pea and melon roots. In both cases, it led to enhanced fungal pathogen tolerance (García-Gutiérrez et al., 2013). Inoculation of *Pseudomonas fluorescens* 63-28R to pea roots induced accumulation of lignin in root cells and inhibited colonization by the oomycete *Pythium ultimum* (Benhamou et al., 1996). The same result was observed with a *Pseudomonas putida* strain inoculated on bean roots (Anderson and Guerra, 1985). These cell wall modifications have been reported in the case of PGPR that protect plants against phytopathogens by activating ISR plant defense responses (Iavolli et al., 2003; Desoignies et al., 2012; Weller et al., 2012; García-Gutiérrez et al., 2013). One of the consequences of ISR is thus the reinforcement of the cell wall through enhanced lignin synthesis and callose apposition (Kovats et al., 1991; Strömberg and Brishammar, 1993), which

restricts the progression of phytopathogens through plant tissues (García-Gutiérrez et al., 2013).

Modifications of the chemical composition of root cell walls are also triggered by PGPR that directly promote plant growth (**Figure 1**). Through the analysis of the infrared spectral characteristics of crude cell wall preparations of maize roots, El Zemrany et al. (2007) concluded that roots inoculated with *Azospirillum lipoferum* CRT1 had lower lignin content than uninoculated ones. This result contrasts with those aforementioned for bio-control agents. Nevertheless, lower lignin content may facilitate cell elongation, and therefore overall root elongation. Similarly, *Azospirillum irakense* produces pectate lyases that are capable of degrading the pectate content of root cell wall and might allow its progression between root cortex cells and its functioning as an endophyte (Bekri et al., 1999). Up to now, the impact on plant lignin content of PGPR that are both inducing ISR and promoting root growth has not been clarified.

Modifications of root cell wall ultrastructure are thought to result mainly from PGPR-triggered changes in plant gene expression. Indeed, *Bacillus subtilis* GB03 promotes *Arabidopsis* growth by producing VOCs that were shown to modulate the expression of 38 genes with known functions associated with cell wall structure (Ryu et al., 2003; Zhang et al., 2007). Among them, 30 were implicated in cell wall expansion or loosening. The endophytic PGPR *Azospirillum irakense* was also shown to stimulate the expression of polygalacturonase genes in inoculated rice roots (Sekar et al., 2000).

Chemical mediators involved in the effects of PGPR on root cell walls have received little attention. A single report has indicated that the exogenous addition of auxins enhanced the induced polygalacturonase activities observed in *Azospirillum irakense* inoculated rice roots (Sekar et al., 2000).

SYSTEMIC EFFECTS OF PGPR ON WHOLE PLANT PHYSIOLOGY AND FUNCTIONING

In addition to their effects on root tissues, PGPR can modify the physiology and functioning of plant tissues located at a substantial distance from the colonized sites, such as shoots. Two types of mechanisms are involved. On the one hand, some PGPR can enhance nutrient availability/uptake for plant roots. Stimulation of plant nutrition will lead to modifications in primary metabolism and consequently will contribute to enhance growth. On the other hand, certain PGPR trigger specific systemic responses, mostly by unknown signaling mechanisms. High-throughput analyses of plant transcriptomic and metabolomic responses have evidenced the effects of PGPR on plant gene expression and metabolite accumulation, respectively. These results highlight the extensive effect of PGPR on whole plant physiology and functioning (**Figure 1**), and provide clues to understand the systemic effect of PGPR.

IMPACT OF PGPR ON PLANT NUTRITION

The impact of PGPR on plant nutrition may result from effects on plant nutrient uptake and/or on plant growth rate (Mantelin and Touraine, 2004). It is indeed commonly hypothesized that nutrient uptake is increased as a consequence of increased root surface area triggered by PGPR. However, root ion transporters are under the

control of regulatory processes that adjust their activity to the plant nutritional demand (Imsande and Touraine, 1994; Lappartient and Touraine, 1996; Lappartient et al., 1999; Nazoa et al., 2003), so that regulations of root development and ion transporter activities are antagonistically coordinated to maintain steady nutrient acquisition rate (Touraine, 2004). Hence, PGPR must interfere with pathways coordinating plant development and plant nutrition to elicit both increased nutrient acquisition rate and plant growth promotion (**Figure 1**).

Plant growth-promoting rhizobacteria can directly increase nutrient supply in the rhizosphere and/or stimulate ion transport systems in root. With regards to increased nutrient supply, two main types of bacterial activities can be considered. Firstly, phosphate solubilization is one key effect of PGPR on plant nutrition. Soils generally contain a large amount of phosphorus, which accumulates in the wake of regular fertilizer applications, but only a small proportion of the latter is available for plants. Plants are able to absorb on their own mono and dibasic phosphate; organic or insoluble forms of phosphate need to be mineralized or solubilized by microorganisms, respectively (Richardson et al., 2009; Ramaekers et al., 2010). Many PGPR – such as *Pseudomonas*, *Bacillus*, *Rhizobium* – are able to dissolve insoluble forms of phosphate (for a review see Richardson et al., 2009). Two main processes exist: acidification of the external medium through the release of low molecular weight organic acids (such as gluconic acid) that chelate the cations bound to phosphate (Miller et al., 2009), and production of phosphatases/phytases that hydrolyse organic forms of phosphate compounds. Secondly, many associated bacteria can fix N₂ so that they could provide nitrogen to the plant. Evidence in favor of the participation of PGPR to the plant N budget has been reported for several plants, especially sugar cane (Boddey et al., 2003). However, the impact of N₂-fixation by PGPR is still debated and is rarely credited for the stimulation of plant growth (for review see Dobbelaere et al., 2003). In addition, non-fixing rhizobacteria can promote plant growth, showing that N provision is not obligatory for plant growth promotion. For instance, *Phyllobacterium brassicacearum* STM196 is unlikely to fix N₂ while it promotes the growth of canola and *Arabidopsis* (Bertrand et al., 2000, 2001; Mantelin et al., 2006).

With regards to the impact of PGPR on nutrient uptake systems, only very few studies have been published so far. Inoculation of canola with *Achromobacter* sp. strain U80417 resulted in an increase of both NO₃⁻ and K⁺ net influx rates per root surface area unit (Bertrand et al., 2000). In this study, the net H⁺ efflux was also enhanced, so that increased NO₃⁻ and K⁺ uptake rates may be part of a general mechanism leading to increased ion uptake rate, similar to energization of nutrient transport by enhanced proton pump activity (Sondergaard et al., 2004). In favor of this hypothesis, acidification of the rhizosphere has also been reported with *Arabidopsis* exposed to the VOC-emitting *Bacillus subtilis* strain GB03 (Zhang et al., 2009).

In *Arabidopsis*, NO₃⁻ uptake measurement in response to PGPR, over time, can lead to contradictory results: NO₃⁻ influx was increased in seedlings, upon 24 h-inoculation with *Phyllobacterium brassicacearum* STM196, while it was reduced 7 days later (Mantelin et al., 2006). However, it is hard to draw a firm conclusion as the net NO₃⁻ uptake rate remained unknown since ion

efflux was not measured in these experiments. Except for the *NRT2.5* and *NRT2.6* genes, the accumulation of transcripts of nitrate and ammonium transporters was very slightly or not significantly changed upon *Phyllobacterium brassicacearum* STM196 inoculation (Mantelin et al., 2006). Interestingly, these two genes have recently been shown to be required in *Arabidopsis* growth promotion by this PGPR (Kechid et al., 2013). Since these two genes code for two plasma membrane-localized NO_3^- transporters (Kotur et al., 2012), this discovery raises the question of the interactions between N nutrition and plant development in PGPR-inoculated plants. The *NRT2.5* and *NRT2.6* genes are predominantly expressed in shoots (Mantelin et al., 2006). Their role in *Phyllobacterium brassicacearum* STM196 plant growth promotion and/or root architecture modification are not linked to changes in NO_3^- uptake rate or NO_3^- distribution between roots and shoots (Kechid et al., 2013), suggesting an involvement in N-signaling rather than a direct role in N-metabolism.

Evidence in favor of a regulation of ion transporters at a transcriptional level by PGPR has been obtained in studies with *Bacillus subtilis* GB03. This strain induces concomitant down- and up-regulation of *HKT1* expression in roots and shoots of *Arabidopsis* seedlings, respectively (Zhang et al., 2008). In the shoots, *HKT1* functions in phloem tissues to retrieve Na^+ from the xylem (Berthomieu et al., 2003) and in the roots it is involved in Na^+ uptake (Rus et al., 2001). The differential regulation of *HKT1* expression in roots and shoots resulted in reduced accumulation of Na^+ and increased accumulation of K^+ in both organs of GB03-inoculated seedlings under salt-stress conditions (Zhang et al., 2008). Consistent with the effect of GB03 on *HKT1*, GB03 failed to rescue salt-stressed *hkt1* mutant seedlings from elevated Na^+ accumulation.

Volatile organic compounds emitted by GB03 also activate the plant's iron acquisition machinery leading to increased iron assimilation (Zhang et al., 2009). Firstly, this PGPR leads to acidification of the rhizosphere, both directly due to chemical effects of some unidentified VOCs and indirectly through increased root proton efflux. Secondly, GB03 up-regulates the expression levels of *FRO2* and *IRT1* genes, coding respectively for a Fe^{3+} chelate reductase and a Fe^{2+} transporter. As a result, GB03-exposed *Arabidopsis* has enhanced ferric chelate reductase activity and increased iron content. Finally, it has been shown that this PGPR induces the expression of the *FIT1* transcription factor that regulates positively *FRO2* and *IRT1* expressions (Zhang et al., 2009). The fact that GB03 fails to increase root ferric reductase activity and plant iron content in *Arabidopsis fit1* mutants shows that PGPR can modify indirectly ion uptake by interfering with plant regulatory processes that control ion transporter expressions and/or activities (Zhang et al., 2009).

IMPACT OF PGPR ON PLANT TRANSCRIPTOME

Targeted or genome-wide analyses of plant gene expression following root inoculation by PGPR were reported with various bacterial models: phytostimulating PGPR, endophytes and PGPR exerting a biocontrol activity. Inoculation of the phytostimulator *Pseudomonas putida* MTCC5279 triggered overexpression of 520 genes and repression of 364 genes (threefold changes) in leaves of *Arabidopsis*; upregulated genes were involved in maintenance of

genome integrity, growth hormone and amino acid syntheses, ABA signaling and ethylene suppression, Ca^{2+} dependent signaling and induction of ISR (Srivastava et al., 2012). On rice, a recent study performed with *Azospirillum* points towards association specificity (Vargas et al., 2012). The targeted expression of ethylene receptors was followed after inoculation of *Azospirillum brasiliense* Sp245 on two rice cultivars of contrasted ability to gain nitrogen from biological nitrogen fixation. Seedlings of cultivar IR42, which enabled higher nitrogen fixation, also displayed higher expression of ethylene receptors compared to cultivar IAC 4440 (Vargas et al., 2012). The transcript accumulation of all ethylene receptors might be necessary for the establishment of a beneficial association between the plant and the bacteria.

As for endophytes, differential colonization of rice roots was observed with an *Azoarcus* PGPR. In a less compatible interaction, a slight defense response occurred and was accompanied by the induction of pathogenesis-related proteins and proteins sharing domains with receptor-like kinases induced by pathogens; those proteins were also induced by a jasmonate treatment (Miché et al., 2006). Inoculation of rice roots with the endophytic PGPR *Herbaspirillum seropedicae* triggered the expression of genes responsive to auxin and ethylene and the repression of the defense-related proteins PBZ1 and thionins (Brusamarello-Santos et al., 2012). These studies suggest that endophytes modulate plant defense responses during colonization.

Plants treated with biocontrol PGPR, usually belonging to the *Pseudomonas* genus, are more resistant to subsequent infections by bacterial or fungal pathogens. In *Arabidopsis*, this rhizobacteria-mediated ISR requires sensitivity to jasmonate and ethylene, and the regulators MYC2 (Pieterse et al., 1996, 2000; Pozo et al., 2008), NPR1 (Pieterse et al., 1998), and MYB72 (Van der Ent et al., 2008) played a central role in this signaling. One of the earliest transcriptomic study performed with *Pseudomonas fluorescens* WCS417r indicated that bacteria elicited a substantial change in the expression of 97 genes in roots whereas none of the approximately 8,000 genes tested showed a consistent change in expression in the leaves (Verhagen et al., 2004). Subsequent studies on *Arabidopsis* reported an increase of defense-related transcripts, including PR-related proteins, in shoots of bacterized plants compared to untreated shoots (Cartieaux et al., 2003; Wang et al., 2005; van de Mortel et al., 2012). Interestingly, the ISR induced by *Pseudomonas fluorescens* SS101 was recently reported to be dependent on salicylic acid signaling and not on jasmonic acid and ethylene signaling (van de Mortel et al., 2012); moreover, a prominent role of camalexin and glucosinolates in the ISR was proposed. In wheat, bacterization with *Pseudomonas fluorescens* Q8r1-96 also triggered the accumulation of defense-related transcripts (Okubara et al., 2010; Maketon et al., 2012) and neither DAPG nor the type three secretion system were key single factors in the expression of these genes (Maketon et al., 2012). The establishment of beneficial associations requires mutual recognition and substantial coordination of plant and microbial responses and consequently beneficial microbes modulate plant immunity.

IMPACT OF PGPR ON PLANT METABOLOME

Several studies have addressed the metabolomic changes triggered by PGPR inoculation, by analyzing metabolite contents of root

exudates, root tissues and shoots under normal or stressful conditions (**Figure 1**). Some studies have shown that PGPR can elicit changes in the activities of root enzymes involved in the production of metabolites, especially flavonoids, leading to changes in the pattern of root exudation (Lavania et al., 2006; Shaw et al., 2006). Some *Azospirillum* PGPR stimulated by up to one-third the level of carbon compounds exuded from roots (Heulin et al., 1987). Moreover, compounds of microbial origin, such as phenazines and DAPG, could enhance total net efflux of amino acids in plant species (Phillips et al., 2004). On soybean roots, the PGPR *Chryseobacterium balustinum* Aur9 influences flavonoids exudation (Dardanelli et al., 2010). PGPR strains from *Chryseobacterium* (Dardanelli et al., 2010) or *Azospirillum* (Burdman et al., 1996) may influence flavonoid exudation by Fabaceae roots. This property can be important for the design of mixed inoculants that will include a PGPR strain promoting flavonoid exudation together with rhizobia that will respond to plant flavonoids (Burdman et al., 1996).

In addition to effects on root exudates, PGPR can trigger modifications of metabolite composition of the whole plant. For instance, rice plants inoculated with *Herbaspirillum seropedicae* showed higher shoot contents in malate and in key amino acids than those of control plants (Curzi et al., 2008). Many more studies focused on modifications of secondary metabolites. Elicitation of isoflavone accumulation was observed on soybean inoculated with various PGPR, either by increasing the total isoflavone content in seedlings or by causing an asymmetric distribution of isoflavones throughout the plant (Ramos-Solano et al., 2010). Increase in the content of several alkaloid and terpenoid compounds of pharmaceutical relevance was demonstrated in medicinal plants following PGPR inoculation (Manero et al., 2003; Jaleel et al., 2007; Bharti et al., 2013). Recent studies investigated the early impact of several *Azospirillum* strains on root and shoot secondary metabolite profiles of maize and rice; analysis of secondary metabolites of two maize cultivars, inoculated by three different *Azospirillum* strains under greenhouse conditions, revealed major qualitative and quantitative modifications of the contents of secondary metabolites, especially benzoxazinoids (Walker et al., 2011). In the same way, a metabolic profiling approach of two rice cultivars inoculated with two different *Azospirillum* strains under gnotobiotic conditions, showed that profiles of secondary metabolites were modified with phenolic compounds such as flavonoids and hydroxycinnamic derivatives being the main metabolites affected (Chamam et al., 2013). Both studies evidenced a specific response, as plant metabolic changes differed according to the *Azospirillum* strain-cultivar combination. Moreover, PGPR applied to the roots can affect the composition of secondary metabolites in shoots, pointing towards systemic effects (Chamam et al., 2013).

Accumulation of secondary compounds was also modified in several plants inoculated with consortia containing arbuscular mycorrhizal fungus and PGPR. Blumenin accumulation triggered by *Rhizophagus irregularis* (formerly *Glomus intraradices*) in barley and wheat roots was increased when a rhizosphere bacterium was applied with the fungus (Fester et al., 1999). Leaf secondary metabolites (total phenols and ortho dihydroxy phenols), as well as leaf mineral content (phosphorus, potassium, zinc, copper, and iron) were maximal when *Begonia malabarica* or *Solanum viarum*

were inoculated with consortia containing two fungi and a *Bacillus coagulans* strain (Selvaraj et al., 2008; Hemashenpagam and Selvaraj, 2011). Field-inoculation of maize with selected strains of *Pseudomonas*, *Azospirillum* or *Rhizophagus/Glomus*, or with these strains combined two by two or all three together, led to qualitative and quantitative modifications of root secondary metabolites, particularly benzoxazinoids and diethylphthalate (Walker et al., 2012). These modifications depended on fertilization level and on the type of microorganisms inoculated. The three selected strains gave distinct results when used alone, but unexpectedly all microbial consortia gave somewhat similar metabolic responses.

Plant growth-promoting rhizobacteria can help plants to withstand saline stress, a feature that may be linked to accumulation of specific metabolites. A higher level of proline content was reported in inoculated *Bacopa monnieri* (Bharti et al., 2013), as well as higher accumulation of glycine betaine-like quaternary compounds in rice inoculated with *Pseudomonas pseudoalcaligenes* (Jha et al., 2011). Similarly, *Arabidopsis* inoculation with the VOC-emitting strain *Bacillus subtilis* GB03 induced strong plant accumulation of glycine betaine and its precursor choline, and GB03-induced drought tolerance was lost in the *xipot* mutant of *Arabidopsis* with reduced choline production (Zhang et al., 2010). Alleviation of cold stress was demonstrated for *Burkholderia phytophaga* PsJN on grapevine; this endophytic strain promotes plant post-chilling recovery by improving acclimation to cold (Ait Barka et al., 2006). This is accompanied by accumulation of stress-related metabolites such as proline, malondialdehyde and aldehydes (known as lipid peroxidation markers), hydrogen peroxide, and by higher expression of defense- and cold-related genes (Theocharis et al., 2012). Bacterization resulted in a 1.2-fold increase in starch content and in a two-fold increase in total soluble sugars, with sugars known to be involved in low-temperature tolerance (glucose, sucrose, and raffinose) displaying higher concentrations in treated plantlets (Fernandez et al., 2012). Independently of temperature, inoculation also enhanced phenolic content (Ait Barka et al., 2006).

EXPRESSION OF PLANT-BENEFICIAL FUNCTIONS OF PGPR IN THE RHIZOSPHERE

One PGPR strain can harbor several plant-beneficial properties, which may be co-regulated or not. Within the rhizosphere, the expression of PGPR's plant-beneficial properties is affected by both abiotic factors (like pH, oxygen, clay mineralogy, heavy metals, etc.) and biotic factors (i.e., compounds produced by plants or the rhizo-microbiome) that can lead to distinct expression patterns in space and time, possibly with different effects on host plant (Piccoli and Bottini, 1994; Pothier et al., 2008; Prigent-Combaret et al., 2008; Dutta and Podile, 2010; Almario et al., 2013b; Drogue et al., 2013). In this section, a focus is put on the regulation of the expression of PGPR plant-beneficial properties by biotic factors occurring in the rhizosphere.

REGULATION OF PGPR FUNCTIONS BY ROOT EXUDATES

Through the release of root exudates, plants can impact bacterial gene expression, especially genes encoding plant-beneficial traits. Composition of root exudates is dependent upon intra and inter-specific genetic variability (Bertin et al., 2003; Czarnota et al.,

2003; Phillips et al., 2004), plant developmental stage (Lynch and Whipps, 1990; Bacilio-Jiménez et al., 2003) and soil abiotic factors (Lipton et al., 1987). One of the major studies aiming at analyzing the impact of root exudates variability on bacterial gene expression was carried out on *phlA*, involved in DAPG biosynthesis, in *Pseudomonas protegens* (formely *Pseudomonas fluorescens*) CHA0 (Notz et al., 2001). The expression of *phlA* was increased four-fold in the rhizosphere of monocots (maize and wheat) compared to the rhizosphere of dicots (bean and cucumber). The analysis of six maize cultivars also revealed that *phl* expression and hence biocontrol activity could be affected by plant genotype (Notz et al., 2001). Specific components of root exudates, notably sugars, were shown to affect the production of antimicrobial compounds, such as DAPG, pyoluteorin and pyrrolnitrin by fluorescent pseudomonads, with some strain-dependent effects (Duffy and Défago, 1999). Among 63 plant compounds related to defense or development, or involved in plant-microbe interactions (flavonoids, phenolic acids, phytohormones, etc.), many could modulate the expression of *phlA* and *pltA* in *Pseudomonas protegens* CHA0 (de Werra et al., 2011). No specific chemical structures were identified that generally induced or repressed *phlA* or *pltA* expression (de Werra et al., 2011). Umbelliferone led to the strongest inhibition of *phlA*; salicylate, jasmonate, and methyl jasmonate, all slightly reduced *phlA* expression, whereas the plant hormone IAA induced *phlA* expression. None of these compounds had an effect on *pltA* expression (de Werra et al., 2011) whereas a previous study reported repression of both DAPG and pyoluteorin biosynthesis genes by salicylate (Baehler et al., 2005).

1-Aminocyclopropane-1-carboxylic acid deamination (encoded by *acdS*) is another bacterial function that may be differentially expressed according to plant genotypes. Indeed, *in vitro* experiments demonstrated that some compounds present in root exudates tightly control *acdS* expression. First, ACC, the precursor of ethylene that is metabolized by AcdS, can positively regulate *acdS* expression (Hontzeas et al., 2004; Prigent-Combaret et al., 2008). Second, leucine, by inhibiting oligomerization of the Lrp-type regulator AcdR, prevents transcription of *acdS* leading to a decrease of ACC deaminase activity in *Pseudomonas putida* UW4 (Li and Glick, 2001) and in *Azospirillum lipoferum* 4B (Prigent-Combaret et al., 2008). Finally, carbon sources can also influence *acdS* transcription (Prigent-Combaret et al., 2008).

As presented above, bacterial IAA biosynthesis mostly depends on tryptophan-related pathways (Spaepen et al., 2007a). The main source of tryptophan for PGPR is root exudates. Measurement of tryptophan bioavailability from graminaceous roots (*Avena barbata*) indicated that this amino acid is abundant at the emergence of secondary roots (Jaeger et al., 1999). In the absence of exogenous tryptophan supply, bacterial IAA biosynthesis is insignificant (Ona et al., 2005; Malhotra and Srivastava, 2006). Next to being an IAA precursor, tryptophan also plays an important role in regulating positively the *ipdC/ppdC* gene (Ona et al., 2005). Other root-exuded amino acids like tyrosine and phenylalanine can also induce *ipdC/ppdC* expression (Rothballer et al., 2005). Besides amino acids, plant roots release compounds like vitamins (e.g., pyridoxine and nicotinic acid) and organic acids (e.g., phenylacetic acid and prephenic acid; Shukla et al., 2011). All these compounds increase significantly IAA production

in *Azospirillum brasiliense* Sp245 (Zakharova et al., 2000; Somers et al., 2005).

Metabolites present in root exudates can thus specifically modulate the expression of key genes involved in plant-beneficial functions. Consequently, specific physiological responses of the plant are dependent on the PGPR strain/plant cultivar combination (Drogue et al., 2012).

REGULATION OF PGPR FUNCTIONS BY MICROBIAL SIGNALS

Plant growth-promoting rhizobacteria exchange several types of cell-to-cell communication signals between each other and with other rhizosphere-inhabiting bacteria and fungi, i.e., quorum-sensing (QS) signals that allow bacteria to monitor their density and to coordinate gene expression only when a quorum of cells is achieved (Fuqua et al., 1994) and other bacterial signals that regulate gene expression independently of the cell density.

Quorum-sensing relies on the synthesis and perception of small diffusible molecules, such as *N*-acyl-homoserine lactones (AHLs). In fluorescent pseudomonads, colonization properties and biosynthesis of antimicrobial metabolites, such as phenazines, is often subjected to an AHL-based QS regulation (Pierson et al., 1994; Chin-A-Woeng et al., 2001; De Maeyer et al., 2011). Production of pyrrolnitrin in *Serratia plymuthica* HRO-C48, a strain isolated from the rhizosphere of oilseed rape and able to protect crops against *Verticillium* wilt, is also under QS regulation (Liu et al., 2007). In *S. plymuthica* G3, an endophytic strain, QS positively regulates antifungal activity, production of exoenzymes, but negatively regulates IAA production (Liu et al., 2011). Among the genus *Azospirillum*, only a few strains belonging to the *lipoferum* species and isolated from rice, display the ability to produce AHL signals (Vial et al., 2006). In the rice endophyte *Azospirillum lipoferum* B518, AHL inactivation abolishes pectinase activity, increases siderophore synthesis and reduces IAA production (in stationary phase) but no effect is observed on cellulase activity and on the phytostimulatory effect (Boyer et al., 2008). Moreover, a proteomic approach indicates that AHL-based QS regulation in *Azospirillum* is rather dedicated to control functions linked to rhizosphere competence and adaptation to plant roots (Boyer et al., 2008).

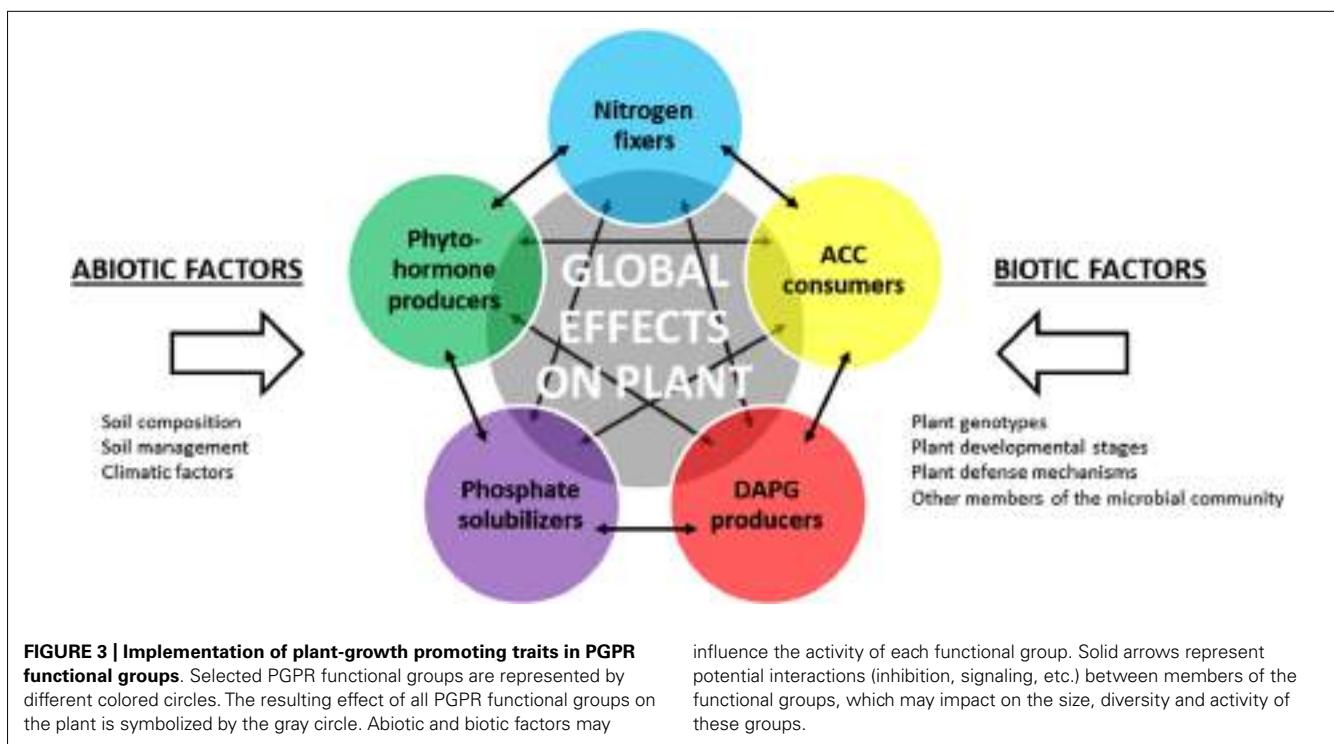
Interestingly, several studies have shown that bacterial communication of a specific bacterial population could be jammed by other microbes; indeed, some soil bacteria can inactivate AHL (notably members of the genus *Bacillus*), whereas others can intercept AHL or can act as a physical barrier preventing their diffusion (Boyer and Wisniewski-Dyé, 2009). Consequently, other members of the bacterial rhizosphere community can compromise expression of biocontrol traits in PGPR. Conversely, cross-talk between species using the same AHL signal or a structurally-related AHL can occur in natural habitats and was evidenced in the rhizosphere of wheat and tomato (Pierson et al., 1998; Steidle et al., 2001). Finally, plant compounds designated as AHL-mimics can also interfere with bacterial QS and may influence the expression of plant-beneficial functions (Teplitski et al., 2000; Vandepitte et al., 2010). Some *Pseudomonas fluorescens* strains unable to synthesize AHLs but possessing the cognate receptor may even recognize a plant compound to trigger expression of genes involved in biocontrol properties (Subramoni et al., 2011).

Exometabolites produced by microbial populations including pathogenic fungal strains can also affect PGPR plant-beneficial properties. For instance, fusaric acid produced by *Fusarium oxysporum* represses the production of DAPG in the biocontrol strain *Pseudomonas protegens* CHA0 (Notz et al., 2002). Next to their antifungal effect, some *Pseudomonas*-produced compounds can influence gene expression of biocontrol traits in pseudomonads. Indeed, in *Pseudomonas protegens* strains CHA0 and Pf-5, DAPG and pyoluteorin productions are influenced by positive autoregulation; moreover, DAPG and pyoluteorin mutually inhibit one another's production (Brodhagen et al., 2004; Baehler et al., 2005). In order to determine if DAPG could act as a signal on other PGPR strains than those of the fluorescent *Pseudomonas* group, a differential fluorescence induction promoter-trapping approach based on flow cytometry was developed on *Azospirillum*. Using this approach DAPG was shown to enhance expression of a wide range of *Azospirillum brasilense* genes, including genes involved in phytostimulation. Four of them (i.e., *ppdC*, *flgE*, *nirK*, and *nifX-nifB*) were upregulated on roots in the presence of *Pseudomonas fluorescens* F113 compared with its DAPG-negative mutant (Combes-Meynet et al., 2011). Accordingly, *Pseudomonas fluorescens* F113 but not its DAPG-negative mutant enhanced the phytostimulatory effect of *Azospirillum brasilense* Sp245 on wheat. Thus, DAPG can act as a signal by which some beneficial pseudomonads may stimulate plant-beneficial activities of *Azospirillum* PGPR (Combes-Meynet et al., 2011). This finding is also relevant in the context of inoculation with microbial consortia, in which different types of PGPR may be combined. The number of studies investigating the efficacy of such combined inoculations is growing, with variations in the number of microorganisms and the nature of the combinations (PGPR strains only, PGPR and fungi,

etc.; Cassán et al., 2009; Couillerot et al., 2012; Kumar et al., 2012; Walker et al., 2012). Field inoculation of sorghum with fluorescent *Pseudomonas* strains alone or in combination with arbuscular mycorrhizal fungi showed a better effect when in presence of the latter (Kumar et al., 2012). Field inoculation of maize with a consortium consisting of two PGPR (*Azospirillum lipoferum* CRT1 and *Pseudomonas fluorescens* F113) and one mycorrhizal strain (*Rhizophagus irregularis/Glomus intraradices* JJ291) showed an increase of root surface, root volume and number of roots, although data were not statistically significant compared to the single *Rhizophagus* inoculation (Walker et al., 2012). Modification of one member of this consortium (three different *Azospirillum* strains were tested) could lead to significant modification of maize growth (Couillerot et al., 2012). Further studies are needed to describe the synergistic effects between beneficial microorganisms at a molecular scale and to analyse the expression of plant-beneficial functions when consortia are used.

ECOLOGY OF PGPR POPULATIONS AND IMPACT ON ROOT SYSTEM FUNCTIONING

Many studies have deciphered the mechanisms of action of PGPR using one individual strain and one host plant. But in reality, as described above, PGPR strains are not acting individually in the rhizosphere but rather as part of bacterial communities, in which cell communication signals may coordinate the activities of all individual strains. Indeed, a vast array of PGPR populations displaying co-occurring plant-beneficial activities and that may share between each other antagonistic or synergistic effects are interacting with a same host plant (Figure 3). When analysing plant growth-promoting effects, it is thus important to integrate the complexity of the interactions between PGPR populations within



the rhizo-microbiome. To do so, functional ecology approaches are needed, in which the relations between the size, diversity and activities of PGPR assemblages in the rhizosphere are taken into account. This is of particular importance when assessing the effect of various environmental factors, including that of plant genotype.

PGPR ECOLOGY IN THE RHIZOSPHERE: FROM INDIVIDUAL STRAINS TO FUNCTIONAL GROUPS

Plant growth-promoting rhizobacteria strains occur in various taxonomic groups, and these different taxonomic groups may be present simultaneously in a given soil (Kyselková et al., 2009; Almario et al., 2013a). This suggests that taxonomically-contrasted PGPR strains may coexist in soil and colonize a same rhizosphere, along with all non-PGPR members of the bacterial community. This possibility has been documented repeatedly, especially when characterizing the taxonomic status of bacterial isolates selected based on their positive effect on plant growth or health, their ability to inhibit phytopathogens, or the occurrence of a particular gene or property of relevance for PGPR effect (Bertrand et al., 2001; Barriuso et al., 2005; Upadhyay et al., 2009). In fact, this possibility seems to be the rule rather than the exception. PGPR populations contributing to a same type of function (i.e., ISR, nitrogen fixation, nutrient solubilization, plant development enhancement, etc.) belong to a same functional group. Functional group approaches can be implemented when specific genes are documented. For instance, nitrogen fixers can be assessed using the *nifH* gene, which encodes the dinitrogenase subunit of the nitrogenase. Its sequence is well conserved within the functional group and it is commonly used as marker to monitor the size and diversity of the diazotrophic community (Poly et al., 2001; Dixon and Kahn, 2004). Some of these PGPR functional groups are taxonomically narrow, such as the *Pseudomonas* DAPG producers (Frapolli et al., 2012). In contrast, others are much more diversified, and certain bacterial functional groups may also comprise both PGPR and non-PGPR strains. For instance, nitrogen fixers include PGPR as well as mutualistic symbionts and even a few pathogens (Herridge et al., 2008).

When considering PGPR-plant relationship in fields, the co-occurrence of genetically contrasted PGPR strains from a same functional group in the rhizosphere has two consequences. First, the activity of a given PGPR functional group corresponds to the resulting contributions of all active individual cells from each type of bacterium within the functional group. If synergistic effects occur between the PGPR populations, the expected performance level for the PGPR function might be higher than if only one type of strain was involved. On this basis, knowing the size of the functional group will help understand the potential importance of the corresponding function. Indeed, for functions leading to enhanced nutrient availability to the plant, such as nitrogen fixation or phosphorus solubilization, the higher the better. For others where optimality matters, such as the production of auxinic signals (Dobbelaere et al., 1999; Spaepen et al., 2007b), the performance level of the functional group will need fine-tuning to avoid production levels too small or too great. How this is ecologically regulated at the scale of the corresponding functional group is unknown, but it raises the possibility of co-evolutionary patterns. To bridge the gap between the potential of a plant-beneficial PGPR function and

its actual implementation by PGPR strains, the regulatory effects need also to be taken into consideration. Some of these regulatory effects will be common to all members of the functional group (Prigent-Combaret et al., 2008). However, other regulatory effects may be relevant for a subset only of the functional group. For instance, zinc sulfate stimulates DAPG production in certain but not all genetic groups of *Pseudomonas* PGPR strains (Duffy and Défago, 1999).

Second, the relationships amongst the different PGPR strains co-occurring in a same rhizosphere are important. Interactions will take place within a functional group, as illustrated above with QS regulation of phenazine production in fluorescent *Pseudomonas* PGPR (Pierson et al., 1994). Interactions may also take place between different PGPR functional groups (Figure 3), integrating competitive and inhibitory effects (Couillerot et al., 2011), signal jamming (Boyer and Wisniewski-Dyé, 2009) and positive signaling (Combes-Meynet et al., 2011), as well as more indirect processes such as root exudation modifications (Phillips et al., 2004; Dardanelli et al., 2010). These interactions have the potential to modulate spatial colonization patterns of PGPR on roots (Couillerot et al., 2011) and to affect PGPR performance (Pierson et al., 1998). This also suggests that members of different PGPR functional groups can function together, as consortia, with the possibility of synergistic effects or, contrariwise, antagonistic effects. These positive effects may be sought by implementing inoculation procedures in which different types of plant-beneficial microorganisms are used in combination, as highlighted above. Even in this context, interactions between the different microbial strains that are inoculated and indigenous microorganisms (including PGPR) probably matter.

IMPACT OF PLANT GENOTYPES ON PGPR FUNCTIONAL GROUPS

Plants at species, sub-species and variety levels exhibit substantial genetic and phenotypic diversity (Salamini et al., 2002; Vaughan et al., 2008). In the rhizosphere, different plant genotypes will have a different impact on the number, diversity and activity of microorganisms (Bais et al., 2006; Micallef et al., 2009). This has been shown when comparing different plant species (Grayston et al., 1998; Costa et al., 2006; Berg and Smalla, 2009) or varieties within species (Germida and Siciliano, 2001; van Overbeek and van Elsas, 2008; İnceoğlu et al., 2010; Bouffaud et al., 2012). It entails differences noticeably in root system structure, root exudation profile, and nutrient acquisition (Czarnota et al., 2003; Comas and Eissenstat, 2009). These effects have also been evidenced when considering microbial functional groups of PGPR or where PGPR predominate.

Nitrogen-fixing bacteria are particularly important for plant nitrogen nutrition (Hsu and Buckley, 2009; Turk et al., 2011). The analysis of functional groups indicated that the size and/or composition of nitrogen-fixing bacteria is influenced by host plant features (Figure 3), both at plant species (Perin et al., 2006) and variety levels (Coelho et al., 2009; Wu et al., 2009). Analysis of *nifH* gene transcripts extracted from the rhizosphere showed that only a fraction of the community expresses *nifH*, and that the corresponding bacterial species differed according to the plant variety, pointing to an influence of plant genotype on the functioning of nitrogen-fixing bacteria (Knauth et al., 2005;

Mårtensson et al., 2009; Orr et al., 2011). Similar findings were made with the functional group of phosphate solubilizers (Richardson and Simpson, 2011). Their selection by roots varies according to host plant species (Kaepller et al., 2000; Chen et al., 2002; Ramaekers et al., 2010).

Other functional groups, such as those involved in plant protection from parasites, act mainly by competition or antagonism, even though direct ISR effects might also take place (Weller et al., 2012). For these microorganisms as well, plant genotype can have a major effect on microbial selection processes, as shown with fluorescent pseudomonads producing DAPG (Picard et al., 2004; Bergsma-Vlami et al., 2005; Picard and Bosco, 2006; Frapolli et al., 2010) or hydrogen cyanide (Jamali et al., 2009; Rochat et al., 2010). Plant-genotype differences in rhizosphere ecology may also matter in terms of plant protection efficiency (Smith and Goodman, 1999; Mazzola and Gu, 2002; Mazzola et al., 2004; Ryan et al. 2004).

CONCLUSION

Plants have evolved different types of biotic interactions with soil microbial populations, ranging from commensalism to mutualism. Within this continuum of interactions, the plant-PGPR cooperation plays a major role by enhancing growth and health of widely diverse plants. Recent progress has helped to understand key features regarding the modes of action and ecology of plant-PGPR interactions, but major knowledge gaps remain. In terms of molecular signaling and functioning, whether PGPR fine-tune plant hormonal pathways similar to those induced by pathogens and symbionts and/or trigger yet-unknown specific pathways requires clarification.

Plant growth-promoting rhizobacteria are able to modulate RSA and *in fine* the vegetative growth and physiology of the whole plant. RSA effects have long been associated with the production of IAA by PGPR. Surprisingly, bacterial modulation of plant auxin distribution and IAA signal transduction pathways, independently of IAA production by PGPR, has also been revealed. It is obviously a step forward in our understanding of plant-PGPR cooperation but it does not fully clarify the bacterial functions

and plant hormonal networks involved. Plant hormones regulate genes for the biosynthesis of other hormones or components of hormonal pathways. Consequently, it is possible that PGPR can affect these cross-talks too. It would explain why PGPR can have such pleiotropic effects on plants. One of the major current scientific challenges lying ahead is to understand how these different signaling pathways are integrated to coordinate plant growth and development, and how PGPR influence the plant hormonal network.

Distinct PGPR populations present in a same soil can express plant-beneficial properties in concert. As aforementioned, the relationships between plants and their rhizo-microbiome are complex and vary both according to plant genotypes and soil inhabiting populations (and thus local soil properties, more generally speaking). Next-generation sequencing technologies have started to reveal their taxonomic and functional diversity. They have begun to bring new knowledge on the ecology of PGPR functional groups. In the near future, it is expected that metatranscriptomics and metaproteomics will develop drastically, and will allow further progress on the understanding of the activity and ecological behavior of natural PGPR populations within the rhizosphere. However, given the heterogeneity in space and time of the rhizosphere habitat, samplings at different times and locations within the plant rhizosphere and within fields will be essential to better understand the ecology and performance of PGPR at plant and field plot scales. Nevertheless, despite being very reductionist, mechanistic functional studies using one PGPR and one plant are still useful to investigate the ways PGPR exert beneficial effects on plants. We think that the most important advances on plant-PGPR cooperation will be brought in the future by combining both ecology and functional biology approaches.

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Spatial patterns of ectomycorrhizal assemblages in a monospecific forest in relation to host tree genotype

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Ectomycorrhizas (EcM) are important for soil exploration and thereby may shape below-ground interactions of roots. We investigated the composition and spatial structures of EcM assemblages in relation to host genotype in an old-growth, monospecific beech (*Fagus sylvatica*) forest. We hypothesized that neighboring roots of different beech individuals are colonized by similar EcM assemblages if host genotype had no influence on the fungal colonization and that the similarity would decrease with increasing distance of the sampling points. The alternative was that the EcM species showed preferences for distinct beech genotypes resulting in intraspecific variation of EcM-host assemblages. EcM species identities, abundance and exploration type as well as the genotypes of the colonized roots were determined in each sampling unit of a 1 L soil core ($r = 0.04$ m, depth 0.2 m). The Morisita-Horn similarity indices (MHSI) based on EcM species abundance and multiple community comparisons were calculated. No pronounced variation of MHSI with increasing distances of the sampling points within a plot was found, but variations between plots. Very high similarities and no between plot variation were found for MHSI based on EcM exploration types suggesting homogenous soil foraging in this ecosystem. The EcM community on different root genotypes in the same soil core exhibited high similarity, whereas the EcM communities on the root of the same tree genotype in different soil cores were significantly dissimilar. This finding suggests that spatial structuring of EcM assemblages occurs within the root system of an individual. This may constitute a novel, yet unknown mechanism ensuring colonization by a diverse EcM community of the roots of a given host individual.

Keywords: belowground interactions, community ecology, ectomycorrhiza, deciduous forest, intraspecific variation, interspecific variation

INTRODUCTION

In Central Europe, beech (*Fagus sylvatica*) is a dominant, ecologically, and economically important tree species (Ellenberg and Strutt, 2009). In mono- and hetero-specific forests roots compete for limited resources of water and nutrients (Bobowski et al., 1999; Jackson et al., 1999; Linder et al., 2000; Brunner et al., 2001; Hölscher et al., 2002; Meinen et al., 2009a,b). In mixtures beech roots were often the superior competitor compared with other tree species (Schmid and Kazda, 2002; Bolte and Villanueva, 2006; Rewald and Leuschner, 2009). With the advent of molecular techniques, genotyping of tree individuals of the same species became possible and was applied to study the intraspecific patterns of root soil occupation (Brunner et al., 2004; Lang et al., 2010). Genotyping of beech roots revealed no evidence for competition of tree individuals for soil exploration (Lang et al., 2010).

However, nutrient uptake by beech roots is primarily achieved by ectomycorrhizal (EcM) fungi, which colonize the root tip and form a new compound organ, the EcM. EcM enwrap the root tip by a mantle-like structure from which hyphae emanate into the soil. Thereby, EcM enlarge the surface for soil exploration and can overcome nutrient depletion zones (Cairney, 2011). Beech trees form EcM with a large number of different fungal species (Buée

et al., 2005; Pena et al., 2010; Lang et al., 2011). Functional traits for nutrient acquisition vary among different EcM species including biochemical and morphological features such as exudation of organic acids for nutrient solubilization, exudation of hydrolytic and oxidative enzymes as well as different hyphal lengths which enable different EcM to forage in different soil volumes (Courty et al., 2010; McGuire et al., 2010; Plassard et al., 2011; Pritsch and Garbaye, 2011; Agerer et al., 2012; Weigt et al., 2012; Pena et al., 2013). If different EcM species provided different benefits, we expect that neutral behavior for resource competition in monospecific beech forests is mediated by mixed EM fungal assemblages with no preference for individual trees.

However, there is now evidence that the ability for mycorrhization with distinct fungal species is under genetic control of the host (Peterson and Bradbury, 1998). For example, greenhouse studies with Scots pines from different seed sources and with different Norway spruces clones showed strong intraspecific host differences in colonization and EcM species composition (Leski et al., 2010; Velmala et al., 2012). Mycorrhizal colonization of poplar hybrids and their parents varied strongly and affected EcM enzymatic activities suggesting a genetic basis for plant-EcM interactions (Tagu et al., 2001;

Courty et al., 2011). Furthermore, in a poplar plantation differences in EcM community composition were found among different transgenic poplars modified in lignification enzymes and also among different *P. x euramericana* clones (Danielsen et al., 2013). Because of the significance of EcM for plant nutrition and ecosystem functioning, it is important to understand the links between inter- and intraspecific plant and mycorrhizal diversity.

The aim of our study was to investigate the relationship between EcM fungal assemblages and the roots of individual beech trees. Since beech propagates typically by seedlings, each tree is usually a distinct genotype. In mono-specific forests roots of a given individual are strongly intermingled with those of the neighboring trees, even close to the stem the individual (Lang et al., 2010). Therefore, analyses of the relationship between roots of distinct trees and their mycorrhizal assemblage require root genotyping and fungal species identification of that specific root. We used this strategy to test our working hypothesis that the EcM species composition of different neighboring root genotypes is more similar than that of the same genotype sampled at different positions. The alternative was that the EcM species showed preferences for distinct beech genotypes resulting in intraspecific variation of host fungal assemblages. For the purpose of this study we defined the roots in our sampling unit of a 1L-soil core ($r = 0.04\text{ m}$, depth 0.2 m) as neighboring roots (small spatial scale) compared with roots in different soil cores collected at distances of $1\text{--}9\text{ m}$ within a plot and those collected in different plots at distances of about 40 m . We analyzed the EcM species abundances and identities on all root tips in each soil core and determined the genotypes of colonized roots. We used these analyses to describe the spatial pattern of EcM diversity and to investigate the similarities of EcM assemblages on roots of different beech genotypes at small and larger spatial scales.

MATERIALS AND METHODS

STUDY SITE AND SAMPLING

The study was conducted in the area of the National Park Hainich (Thuringia, Germany, $51^{\circ}05'28''\text{N}$, $10^{\circ}31'24''\text{E}$), where the long-term annual sum of precipitation is 670 mm and the annual mean temperature 7.5°C (Leuschner et al., 2009). The soil type is Stagnic Luvisol developed from loess on limestone with an acidic pH 5.1, C/N ratio of about 30 in the humus layer and an organic carbon content of $2.9\text{--}3.7\text{ kg m}^{-2}$ (Guckland et al., 2009). An area of $100 \times 100\text{ m}$ was selected in a long-term unmanaged, old-growth beech stand in the northeastern part of the National Park, in which three plots at distances of about 40 m were set up as described before (Lang et al., 2010). Each plot consisted of three trees with a mean stem diameter of $51 \pm 5\text{ cm}$, denominated as A, B, and C respectively. The trees formed a triangle (Figure 1). For sampling the geometric center (M) was determined and three further sampling points were determined at regular distances between M and the stem each of the trees (Figure 1). This design resulted in 10 sampling points per triangle and 30 samples in total. Soil cores of a volume of 1 L (radius of 0.04 m , depth of 0.2 m) were collected in June 2009. Triangle-forming trees A, B, and C and their neighbors were mapped and leaves were collected for microsatellite analyses (Lang et al., 2010).

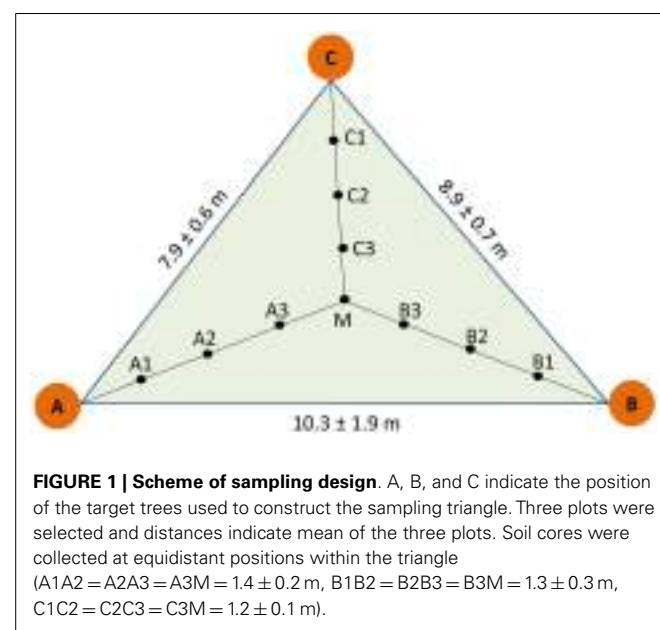


FIGURE 1 | Scheme of sampling design. A, B, and C indicate the position of the target trees used to construct the sampling triangle. Three plots were selected and distances indicate mean of the three plots. Soil cores were collected at equidistant positions within the triangle ($A1A2 = A2A3 = A3M = 1.4 \pm 0.2\text{ m}$, $B1B2 = B2B3 = B3M = 1.3 \pm 0.3\text{ m}$, $C1C2 = C2C3 = C3M = 1.2 \pm 0.1\text{ m}$).

ANALYSES OF ROOTS AND ECTOMYCORRHIZAS

The roots were removed from the soil core by careful washing and stored at 4°C between moist tissue papers. All root fragments of each soil core were used for mycorrhizal analysis. For this purpose the roots were spread under a compound microscope (Leica M205 FA, Wetzlar, Germany) and all root tips were counted and classified as either dead, vital non-mycorrhizal, or vital mycorrhizal root tips. The vital mycorrhizal root tips were morphotyped after a simplified method of Agerer (1987/2006) using color, texture of the EcM mantel, branching, abundance of external hyphae, and rhizomorphs as classification criteria. Exploration types were assigned after Agerer (2001), Courty et al. (2008, Supplementary material S1) and Pena et al. (2010). Pictures were taken and deposited together with the fungal description and molecular information (see below) under <http://www.uni-goettingen.de/de/92389.html>. The abundance of each EcM morphotype on each root fragment was recorded. Aliquots of each fungal morphotype (10–20 root tips) were collected and stored at -80°C . After mycorrhizal analysis, aliquots of the root fragments were also stored (-80°C). Coarse and fine roots ($<2\text{ mm}$ diameter) were separated and weighed.

To determine EcM species identities DNA was extracted from the morphotypes with the DNeasy Mini Plant Kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of the fungal rDNA was amplified by using the primer pair ITS5 and ITS4 (MWG, Biotech, Ebersberg, Germany) after White et al. (1990). The PCR conditions and sequencing procedures have been reported before (Pena et al., 2010). For fungal identification, BLAST searches were carried out against the NCBI¹ and UNITE² public sequence databases. Sequences were assigned matching species names when the BLAST matches showed identities higher than 97% and scores higher than 800 bits. If no

¹<http://www.ncbi.nlm.nih.gov/>

²<http://unite.ut.ee>

appropriate match was found, the sequence was assigned a higher-level taxonomic name or was called an uncultured ectomycorrhizal fungus (UECM) and numbered. The sequences have been deposited in NCBI with the following accession numbers: EU346875, EU816604, EU816608, EU816609, EU816611, EU816616, EU816619, EU81662, EU816623, EU816625, EU816642, EU816643, EU816646, EU816647, EU816653, EU816654, EU816670, EU816679, EU826353, HQ336683, HQ336695, HQ336696, HQ336697, and HQ336701. *C. geophilum* was determined as black morphotype.

Genotyping of roots and leaves has been reported before (Lang et al., 2010). Briefly, individual trees and their roots were identified by sequence analyses of four highly polymorphic microsatellite loci (*sfc0108*, *sfc0161*, *sfc1143*, *sfc1063*), previously developed for *Fagus crenata* (Asuka et al., 2004) and tested for *Fagus sylvatica* (Lang et al., 2010).

DATA ANALYSIS

Mycorrhizal colonization (%) was calculated as: number of vital mycorrhizal root tips $\times 100$ / (number of vital mycorrhizal root tips + number of vital non-mycorrhizal root tips). The vitality index of the root tips was calculated as: (number of vital mycorrhizal root tips + number of vital non-mycorrhizal root tips) $\times 100$ / (number of vital mycorrhizal root tips + number of vital non-mycorrhizal root tips + dead root tips). The Shannon–Wiener index for roots of different tree genotypes in a soil core was calculated on the basis of the relative abundance of fine root biomass per individual tree. The Shannon–Wiener index for EcM species on the root tips in a soil core was calculated on the basis of the abundance of the EcM species per total number of root tips in the soil core. This yielded Shannon–Wiener indices for the diversity of individual trees present in a sample (H' _{tree}) or for the diversity of EcM in a sample (H' _{EM}) with $H' = -\sum p_i \ln p_i$, where p is the relative abundance of the tree genotype i or the relative abundance of the EcM species i (Shannon and Weaver, 1949).

Similarity indices were calculated as generalized Morisita–Horn index C_qN by comparing N communities on species information shared by at most q communities using the procedure developed by Chao et al. (2008) and implemented in the program SPADE by Chao and Shen (2010)³. EcM species abundances per soil core, per tree genotype, or per soil core and tree genotype were used as input parameters and run with a bootstrap value of 200.

Statistical analyses and curve fitting were performed with STATGRAPHICS Centurion (Statistical Graphics Corp., Warrenton, USA) or ORIGIN 7.0 (Origin Lab Corp., Northampton, USA). When the data were not-normal distributed two sample comparisons were conducted with the Mann–Whitney W -test for medians.

RESULTS

INTERSPECIFIC FUNGAL DIVERSITY IN RELATION TO INTRASPECIFIC HOST DIVERSITY

The mean fine root biomass in the top 20 cm of the soil was $2.5 \pm 0.3 \text{ g L}^{-1}$ and not affected by the distance of the soil core

from the next tree (Lang et al., 2010). However, in individual soil cores the amount of fine roots was variable with increasing amounts of fine roots corresponding to increasing numbers of root tips (Figure 2A). Because the number of EcM species detected in ecosystems depends on the sampling effort (Taylor, 2002), we expected that the number of different EcM species would increase with increasing number of root tips. We found between 3 and 10 EcM species per soil core, but these numbers were not related to the number of root tips in that soil core (Figure 2B). The Shannon–Wiener diversity index of the EcM community in a soil core was neither affected by the number of root tips in that soil core ($R = 0.021$, $P = 0.909$, not shown).

We have previously reported that roots of 21 beech genotypes were identified in the three study plots with a mean of 3.3 ± 0.2 individuals per soil core and H' _{tree} genotype ranging from 0.27 to 1.08 (Lang et al., 2010). We plotted the interspecific fungal diversity per soil core H' _{EM} against H' _{tree} in this soil core to find out whether higher intraspecific host diversity was related to higher interspecific diversity of the EcM fungi on the roots (Figure 2C). No significant correlation was observed (Figure 2C).

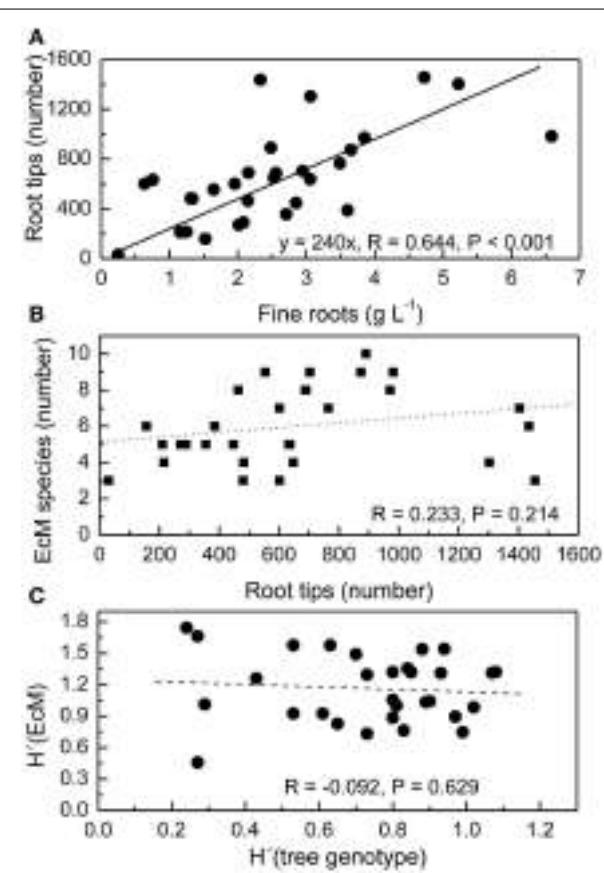


FIGURE 2 | Relationships between the number of root tips and fine root mass per soil core (A), the number of ectomycorrhizal fungal species (EcM) and the number of root tips per soil core (B), and the Shannon–Wiener index H' of EcM diversity and root genotype diversity H' (tree genotype) per soil core (C). The volume of the soil core was 1 L. All root tips were counted and analyzed.

³<http://chao.stat.nthu.edu.tw>

SPATIAL PATTERNS OF ECM SPECIES RICHNESS AND ABUNDANCES IN THE STUDY PLOTS

We recorded 6801, 7248, and 5578 vital mycorrhizal root tips on plot 1, 2, and 3, respectively. Mycorrhizal root tip colonization ($99.3 \pm 1.4\%$, $P = 0.48$) and root vitality ($32.4 \pm 1.6\%$, $P = 0.31$) did not differ between the three plots. We found a total number of 26 ECM species, of which 8 colonized together 90% of the mycorrhizal root tips (Figure 3). *Clavulina christata*, *Russula chloroides*, and *Laccaria subdulcis* were the most abundant species, followed by UECM-125, *Cenococcum geophilum*, *Tomentella subtilacina*, *Corticarius anomalus*, *Genea hispidula* (Figure 3). Analysis of fungal exploration types revealed that about 50% of the root tips were colonized with medium distance fungi and 30% with contact types, whereas short and long distance exploration type fungi colonized only about 10% of the root tips (Figure 3, inset).

The pattern of ECM species abundance in different soil cores revealed large variations in the fungal assemblages (Figure 4, Table 1). While the roots in some soil cores were strongly dominated by one or two fungi, others contained higher species richness (Figure 4). To investigate the similarity between ECM fungi in different soil cores, we used the Morisita–Horn index, which is based on the relative abundance of species, by multiple community comparisons as introduced by Chao et al. (2008). Analysis of the fungal patterns for all sample combinations in a plot revealed that the similarity indices covered the whole range from almost zero (no overlap of ECM) to almost 1 (complete overlap of ECM, Table 1). The mean similarity of all plots was moderate and significantly lower between plot 1 and 2 than in the other combinations (Table 2). The similarities between the plots were much higher

when the ECM were categorized after exploration types than after species (Table 2).

Because the hyphae of ECM fungi can grow several meters and generate large belowground networks connecting trees (Beiler et al., 2010), we compared the overlap of fungal communities by Morisita–Horn similarity indices (MHSI) of ECM assemblages of neighboring soil cores with those of increasing distance. ECM communities in neighboring soil cores (mean distance 1.3 m) were slightly more similar than in cores collected at distances of about 2.6 m, but overall there were no significant differences up the largest distances between the positions of soil cores within a plot (Figure 5). This shows that the similarity of ECM assemblages did not decrease with increasing distances as one might have expected.

FUNGAL ASSEMBLAGES IN RELATION TO HOST GENOTYPE

We combined all roots found for a distinct beech genotype and determined ECM species richness per host genotype. As the number of root tips per host genotype was highly variable, we analyzed the relationship between the number of detected root tips and ECM species richness (Figure 6). A saturation curve was found suggesting that many trees were undersampled and that therefore the comparison of fungal assemblages between all different genotypes would have been biased by differences in sample abundance.

To circumvent this problem, we reasoned that if there were preferences of ECM species for distinct beech individuals, the ECM assemblages on roots of a given genotype should be more similar to those of the same genotype in other soil cores than to the ECM communities of other host genotypes in same soil core. To test

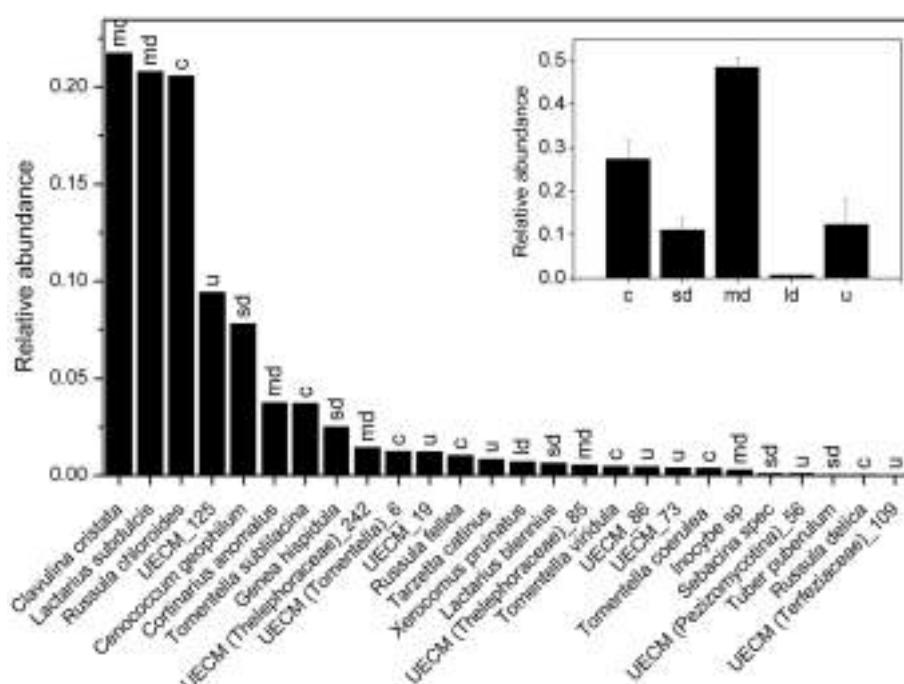


FIGURE 3 | Relative abundance of ectomycorrhizal fungal species on root tips of beech (*Fagus sylvatica*). The sum of all ECM root tips of the three plots analyzed was set as 1. Letters above bars indicate exploration types:

c = contact, sd = short distance, md = medium distance, ld = long distance, u = unknown. The inset shows the relative contribution of different ECM exploration types to root tip colonization.

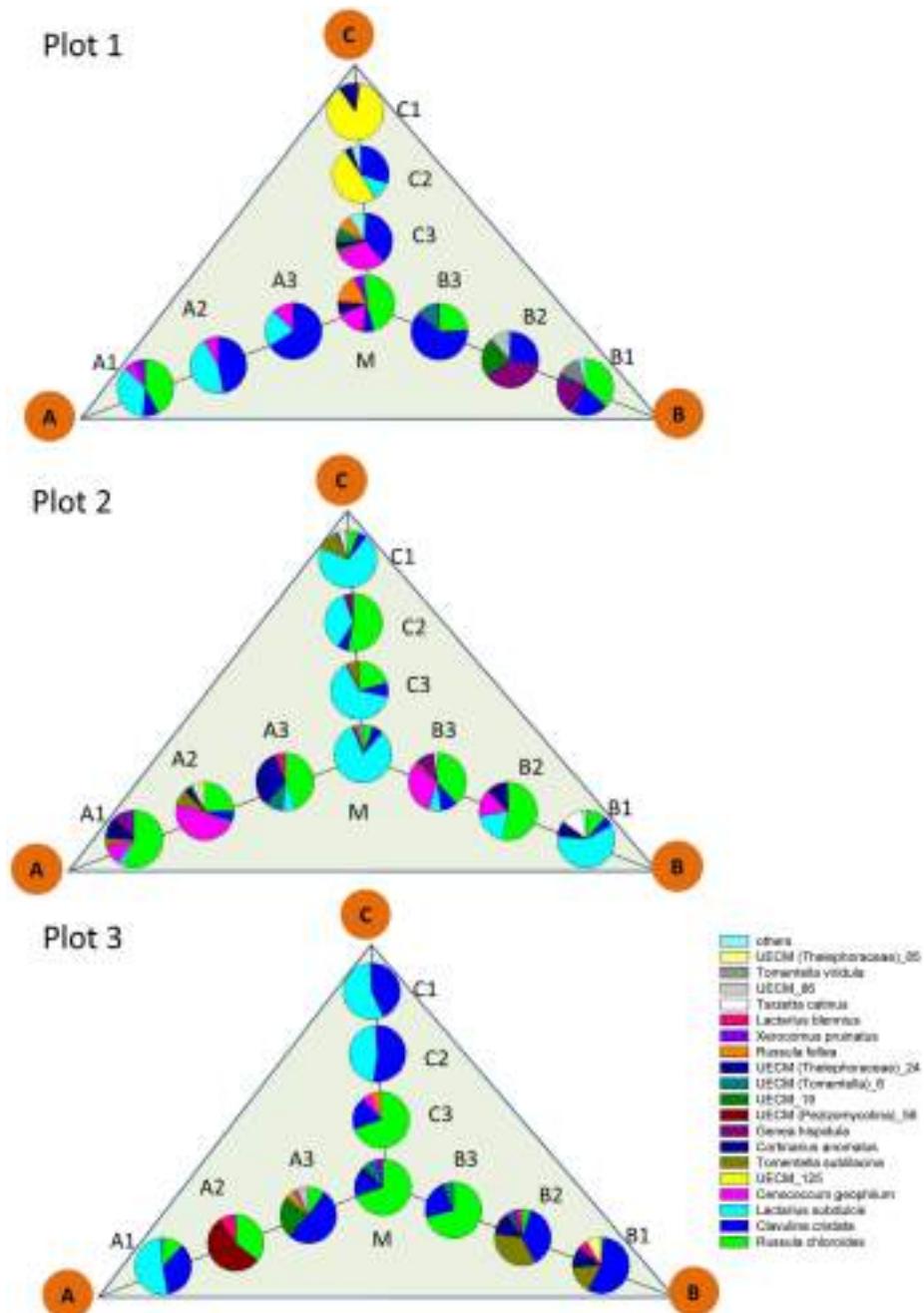


FIGURE 4 | Scheme of localization and relative abundance of EcM species in 1L soil cores. Abbreviations for the position of the soil cores as in **Figure 1**. Others: sum of UECM_73, *T. coerulea*, *Inocybe* sp., *Sebacina* sp., *T. puberulum*, *R. delicia*, UECM (Terfeziaceae)_109, which accounted together for <1% of the total EcM abundance.

this hypothesis we identified trees whose roots were found in two or more soil cores and used only those soil cores which contained also a reasonable number of root tips of other beech genotypes as well (means per sample: 235 ± 70). According to these criteria we identified the following trees on plot 1: A, B, C, on plot 2: 223, 218, C, U1 and on plot 3: B, 310, and 256 (cf. **Figure 6**). We calculated similarity indices for the EcM assemblages of a given

genotype in different soil cores (G_G) and for the given genotype with the EcM community of the roots of the other genotypes in the same soil core (G_R, **Figure 7**). The similarity of the EcM communities in the same soil core was very high regardless of the genotype (G_R), whereas the EcM on the same beech genotype in two adjacent soil cores (G_G) were dissimilar (**Figure 7**). The similarity of the total EcM communities in the soil cores (C_C)

Table 1 | Morisita–Horn similarity indices for the EcM species composition in all combinations of soil cores within a plot (based on shared information between any two communities).

Plot 1	A2	A3	B1	B2	B3	C1	C2	C3	M
A1	0.554	0.350	0.631	0.080	0.408	0.004	0.197	0.198	0.714
A2		0.905	0.306	0.365	0.664	0.013	0.494	0.594	0.124
A3			0.390	0.469	0.861	0.017	0.530	0.755	0.160
B1				0.514	0.644	0.010	0.222	0.372	0.737
B2					0.460	0.007	0.263	0.411	0.070
B3						0.014	0.466	0.686	0.417
C1							0.779	0.025	0.019
C2								0.384	0.072
C3									0.367

Plot 2	A2	A3	B1	B2	B3	C1	C2	C3	M
A1	0.629	0.745	0.263	0.947	0.849	0.158	0.826	0.361	0.130
A2		0.360	0.133	0.649	0.885	0.095	0.397	0.200	0.067
A3			0.263	0.791	0.596	0.186	0.709	0.354	0.171
B1				0.483	0.291	0.953	0.687	0.960	0.932
B2					0.866	0.383	0.920	0.576	0.364
B3						0.208	0.712	0.380	0.191
C1							0.613	0.970	0.977
C2								0.783	0.596
C3									0.949

Plot 3	A2	A3	B1	B2	B3	C1	C2	C3	M
A1	0.102	0.529	0.523	0.408	0.335	0.979	0.955	0.321	0.292
A2		0.089	0.027	0.057	0.520	0.000	0.000	0.524	0.531
A3			0.830	0.648	0.402	0.537	0.653	0.386	0.337
B1				0.882	0.288	0.564	0.685	0.259	0.214
B2					0.283	0.420	0.511	0.258	0.236
B3						0.177	0.215	0.991	0.995
C1							0.985	0.160	0.124
C2								0.194	0.150
C3								1.000	0.986

Analyses were based on EcM species abundance. Increasingly intense colors indicate increasing similarity. Species are shown in **Figure 4**.

Table 2 | Morisita–Horn similarity indices for the comparison of all three plots (based on shared information between any two communities) and for the three plots among each other.

Plot	Level	Similarity index \pm SE	95% Confidence interval
All	Species	0.586 \pm 0.007	(0.573, 0.599)
1_2	Species	0.392 \pm 0.010	(0.373, 0.412)
1_3	Species	0.689 \pm 0.009	(0.671, 0.707)
2_3	Species	0.660 \pm 0.011	(0.638, 0.682)
All	Ex type	0.832 \pm 0.006	(0.821, 0.843)
1_2	Ex type	0.791 \pm 0.008	(0.775, 0.808)
1_3	Ex type	0.770 \pm 0.008	(0.754, 0.786)
2_3	Ex type	0.921 \pm 0.006	(0.910, 0.933)

Analyses were based on species abundance (species) or on exploration type (ex type). SE, standard error.

used for this analysis was intermediate between G_R and G_G (**Figure 7**) and similar to the mean Morisita–Horn index found for the three plots (**Table 2**).

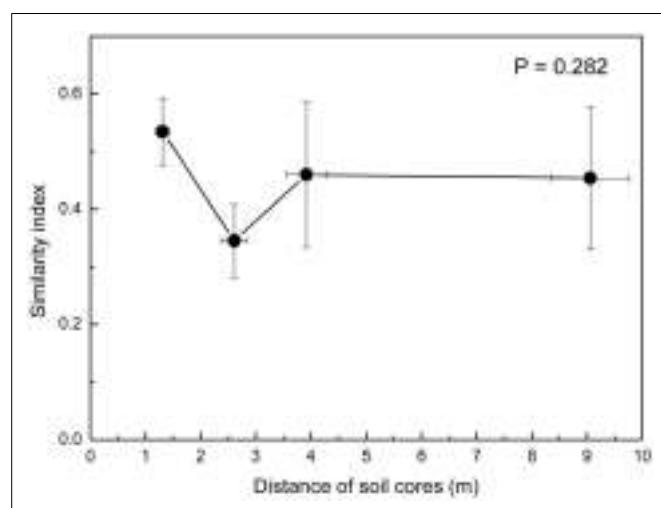


FIGURE 5 | Morisita–Horn similarity indices of ectomycorrhizal assemblages calculated for pairs of soil cores at increasing distance from each other. 1.3 m = neighboring cores, 2.6 m = every second core (e.g., A1_A3, A2_M, B1_B3, etc.), 3.8 m = every third core (e.g., A1_M, B1_M, etc.), and 9 m = largest distances within plots (A1_B1, A1_C1, B1_C1). Data indicate means for the three plots.

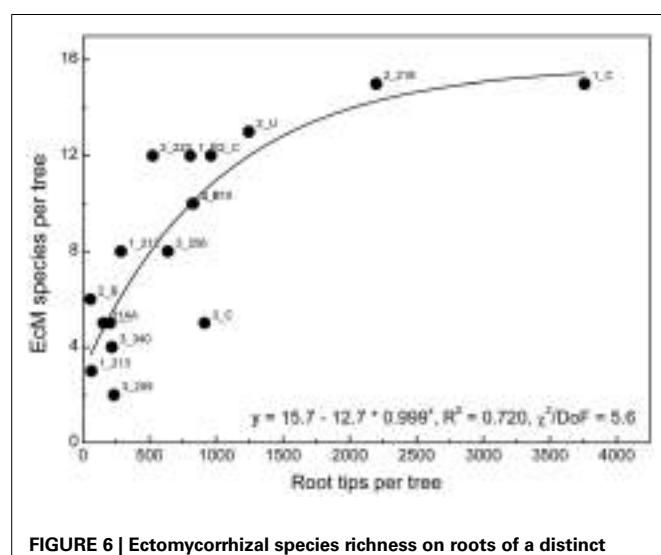
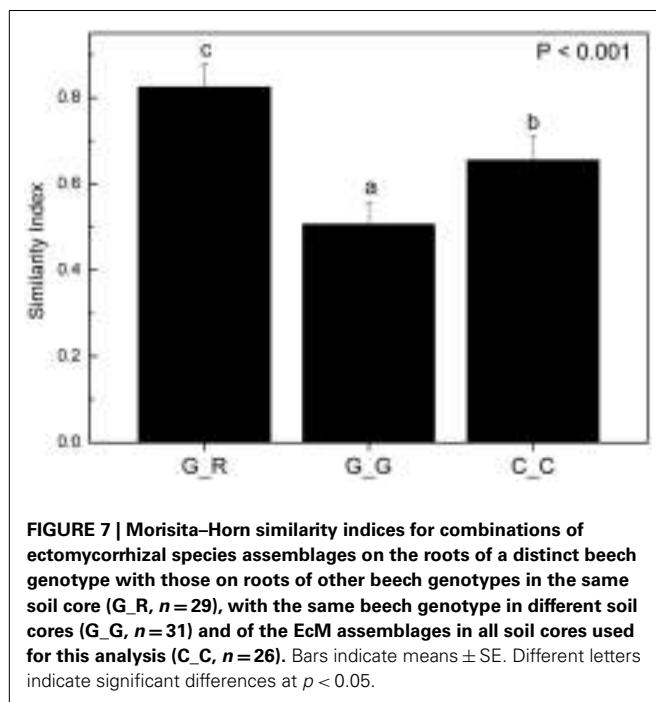


FIGURE 6 | Ectomycorrhizal species richness on roots of a distinct beech genotype in relation to the number of root tips found for this individual in all samples. Labels indicate plot number followed by tree number. The position of all trees in the plots has been shown in Lang et al. (2010). The data were fitted by a Boltzman function.

DISCUSSION

In recent years considerable efforts have been made to describe and interpret the ecological significance of spatial patterns of EcM (O'Hanlon, 2012). A key challenge is to find out whether predictable relationships exist between inter- and intraspecific plant and mycorrhizal fungal diversity, which may be key factors in understanding ecosystem functioning (Johnson et al., 2012). The present study contributes to this question by linking EcM species patterns to beech genotypes with a spatial resolution of



about 0.04–9 m. The fungal community composition on the beech roots of our study and their general structures with few dominant and many scarce species are typical for Fagaceae forests (Buée et al., 2005; Courty et al., 2005; Lang and Polle, 2011; Lang et al., 2011). Because we found almost complete colonization of vital root tips with EcM, all nutrients taken up by a beech tree must have passed the EcM. Therefore, EcM are expected to play an important role in the distribution of nutrients between conspecific neighbor trees and may lead to asymmetric competition, if the EcM species differed in functions and preferences for distinct genotypes.

Data regarding functional classifications of EcM are still incomplete. EcM fungi exude different exoenzymes to mobilize nutrient resources (Cairney, 2011; Plassard et al., 2011; Pritsch and Garbaye, 2011; Hobbie and Höglberg, 2012; Habib et al., 2013), but groupings for these traits are still lacking because of strong variations of the enzyme activities with their biotic and abiotic environment (Courty et al., 2008). Currently, the most frequently used classification system assigns EcM fungi according to their hyphal morphology such as lengths, densities, and surface properties to different soil exploration types, which reflect spatial differences for nutrient absorption (Agerer, 2001). In our study the abundant EcM include contact (*R. chloroides*, *T. sublilacina*), short distance (*C. geophilum*, *Genea hispidula*) and medium distance (*C. christata*, *L. subdulcis*, *C. anomalous*) soil exploration types, with a potential reach of up to 16 cm per cm EcM length (Agerer, 2001; Agerer et al., 2012; Weigt et al., 2012). Thus, the majority of EcM species can forage for nutrients beyond the dimensions of the soil core. A yet larger outreach is achieved by long distance rhizomorph-forming fungi with an exploration potential >400 cm per cm of EcM length (Agerer et al., 2012), which colonized, however, only a small fraction of the root tips in our study (about 1%, *X. pruinatus*). In

other forest communities the abundance of rhizomorphic exploration types was found to be very high (Heinonsalo et al., 2007). Here, the similarity of EcM species among the plots used in our study was only moderate, but the similarity based on exploration types was very high. This finding suggests that there were no major differences between the plots with respect to soil foraging by EcM.

Previous studies in a pine forest have shown that EcM communities were highly similar at scales <3.4 m (Pickles et al., 2012). In our study we also found high similarities of EcM communities within the plots, but no significant differences between adjacent (ca. 1 m) and more distant (ca. 9 m) sampling points. Fine scale analyses of EcM at the cm-scale showed that some fungi, e.g., *Clavulina* sp. and *Cortinarius* sp., which were also present in our study, can form mycelial and EcM patches, whereas this was not the case for *C. geophilum* (Genney et al., 2006; Pickles et al., 2010). Clusters for *Cortinarius* and other fungal species (*Tomentella*, *Piloderma*) were also detected on oak (Gebhardt et al., 2009). The formation of clusters indicates non-random spatial structuring of the EcM communities. It has been suggested that interspecific competition or priority effects could lead to spatial partitioning of fungal species on the root system (Pickles et al., 2012).

Another possibility, which was addressed in our study, is that intraspecific host diversity may lead to structuring of the fungal assemblages. Since strong host preferences of EcM species have been found in mixtures of beech with other deciduous tree species (Lang et al., 2011) and effects of the host genotype were reported under experimental conditions (Tagu et al., 2001; Leski et al., 2010; Courty et al., 2011; Danielsen et al., 2013), it is clear that links exist between the fungal assemblage and the host genotype. However, in the present investigation we found no evidence for discernible EcM communities on distinct beech genotypes. One reason may be that the genetic structure of the genotypes studied in this old-growth unmanaged stand might have been relatively similar because the trees were established by natural regeneration and significant family structures were found in the plot (Rajendra, 2011). To further address the question of interactions between host genotype and fungal assemblages, field studies with different beech ecotypes/populations will be required.

The most striking finding of our study was that the similarity of EcM communities of different beech genotypes within a soil core was almost twice higher than for same genotype in different soil cores. Because the dimensions of the soil core were smaller than the radius of most fungal hyphae, it is possible that the same fungal genotype colonized neighboring roots of different host trees in the same soil core. Although we have not determined fungal genets, this assumption is not unreasonable because others have shown that fungal genets connect hetero- as well as conspecific neighbors (Curlevski et al., 2009; Beiler et al., 2010). The connectivity was especially strong for old, dominant individuals, where one individual was connected with as many as more than 40 other conspecific trees and could cover distances of up to 20 m (Beiler et al., 2010). Mycorrhizal networks may facilitate resource transfer within the fungal web and between, thereby, foster the establishment of seedlings with access to the common mycorrhizal network (Teste and Simard, 2008).

In our study the high dissimilarity of fungal assemblages at roots of the same genotypes at spatial distances of some meters was unexpected because the overall similarities of fungal communities in the soil cores of plot were not significantly different. This is an exciting finding because it suggests that spatial structuring occurs within the root system of an individual. Spatial segregation of different ECM species – mediated by unknown host mechanisms – can ensure colonization by a diverse ECM community on the roots of a given host genotype. Thereby, asymmetric competition between conspecific neighbors can be avoided. We are aware that this suggestion is preliminary because our study includes only few individuals. However,

it opens a new avenue to look at spatial structuring of ECM communities.

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Morphological plasticity of ectomycorrhizal short roots in *Betula* sp and *Picea abies* forests across climate and forest succession gradients: its role in changing environments

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Morphological plasticity of ectomycorrhizal (EcM) short roots (known also as first and second order roots with primary development) allows trees to adjust their water and nutrient uptake to local environmental conditions. The morphological traits (MTs) of short-living EcM roots, such as specific root length (SRL) and area, root tip frequency per mass unit (RTF), root tissue density, as well as mean diameter, length, and mass of the root tips, are good indicators of acclimation. We investigated the role of EcM root morphological plasticity across the climate gradient (48–68°N) in Norway spruce (*Picea abies* (L.) Karst) and (53–66°N) birch (*Betula pendula* Roth., *B. pubescens* Ehrh.) forests, as well as in primary and secondary successional birch forests assuming higher plasticity of a respective root trait to reflect higher relevance of that characteristic in acclimation process. We hypothesized that although the morphological plasticity of EcM roots is subject to the abiotic and biotic environmental conditions in the changing climate; the tools to achieve the appropriate morphological acclimation are tree species-specific. Long-term (1994–2010) measurements of EcM roots morphology strongly imply that tree species have different acclimation-indicative root traits in response to changing environments. Birch EcM roots acclimated along latitude by changing mostly SRL [plasticity index (PI) = 0.60], while spruce EcM roots became adjusted by modifying RTF (PI = 0.68). Silver birch as a pioneer species must have a broader tolerance to environmental conditions across various environments; however, the mean PI of all MTs did not differ between early-successional birch and late-successional spruce. The differences between species in SRL, and RTF, diameter, and length decreased southward, toward temperate forests with more favorable growth conditions. EcM root traits reflected root-rhizosphere succession across forest succession stages.

Keywords: plasticity, ectomycorrhizal roots, morphological characteristics, root acclimation, silver birch, Norway spruce, forest succession, climate gradient

INTRODUCTION

The development of an efficient root system is necessary for trees to ensure sufficient nutrient uptake in various conditions. Hence, trees must acclimate by modifying either the biomass of fine roots or the morphology and physiological activity of nutrient absorbing root tips or both (Löhmus et al., 2006). Morphological plasticity of roots with primary development (generally first (youngest) and second branching order roots) is the fastest mechanism for root acclimation in trees. A clear morphological response of ectomycorrhizal root tips has been shown along latitudinal gradient of Norway spruce forests (Ostonen et al., 2011) as well as in long-term soil temperature and nutrient manipulation experiments (Leppälammi-Kujansuu et al., 2013). Morphological plasticity of roots within species can be defined as the response range of root traits to different environments. The traits of roots with primary

structure are important for optimizing the mineral nutrition of the plant (Curt and Prévosto, 2003; Comas and Eissenstat, 2004) although they may vary considerably within a family, a genus, and even within a species (Ostonen et al., 2007a,b; Francini and Sebastiani, 2010), and also with tree age (Rosenvald et al., 2011a, 2013).

Root trait values of different genotypes within a tree species in changing environment can be considered as reaction norms in wider sense. Plasticity response is higher the further away the stand is from the optimum growth condition (Ghalambor et al., 2007). Since the structure of EcM roots is closely related to root functions, different morphological and functional root parameters are potential indicators of the nutrition of trees in relation to soil conditions. Most commonly used root traits to characterize the morphological response to the local environment are

specific root area (SRA, $\text{m}^2 \text{ kg}^{-1}$), specific root length (SRL, m g^{-1}) and root tissue density (RTD, kg m^{-3}), but also mean diameter and length of root tips (used here as synonym to EcM roots) (Fitter, 1985; Löhman et al., 1989; Ostonen et al., 1999; Comas and Eissenstat, 2004; Guo et al., 2004; Leuschner et al., 2004; Ostonen et al., 2006, 2007a,b).

High SRL and SRA values are believed to be the morphological tool of roots to increase the surface area, which may lead to improvement in nutrient acquisition (Schippers and Olff, 2000). Furthermore, morphological plasticity of the primary root tips enables trees to survive in primary succession stands, for example following oil-shale mining (Ostonen et al., 2006; Kuznetsova et al., 2010; Rosenvall et al., 2011a), where the pedogenesis only started on detritus (Reintam, 2004). Trees show high SRL and SRA of root tips at young age (Löhman et al., 2006; Rosenvall et al., 2011a, 2013), whereby the changes in EcM root morphology of silver birch occur faster at younger age—before the age of 10 years (Rosenvall et al., 2011a, 2013). However, the examples of forests acclimation via plasticity of EcM root tips and general trends in the fine root system over time, e.g., in a chronosequence or in different successional stages of forest are very seldom documented.

Picea abies is one of the dominant conifer species in the late stage of forest succession in boreal forests, while the deciduous *Betula* species tend to occur in the early stages of forest succession in Northern Europe and Russia. The environmental gradient from boreal to temperate spruce and birch forests includes substantial changes in growing conditions—increasing mean annual temperature, length of growing season etc., as well as rise in anthropogenic N deposition, and land use intensity. Climate change induced climatic alterations similar to those taking place along latitudinal gradient within the territory ranging from northern boreal to temperate forests are expected to occur in time in northern Europe.

The birch forests (predominantly *Betula pendula* and some mixed stands of *B. pendula* and *B. pubescens*) investigated in our study, include primary and secondary successional stands growing on reclaimed mining areas, afforested abandoned fields and in forest areas. The spruce forests, on the other hand, range from poor to the most fertile sites replacing the broadleaved trees from mid-successional stages in the boreal and temperate zone.

We show morphological plasticity of ectomycorrhizal root tips in Norway spruce and birch to be an active mechanism in root acclimation to site conditions; larger plasticity may enhance root acclimation capacity to local environmental conditions in short-term and adaptation capacity to climatic changes in long-term scale. We considered EcM morphological plasticity as the response of certain morphological parameters to different site conditions and stand age. To examine the greatest possible extent of the morphological plasticity, stands of very different growth conditions were included in our meta-study.

We hypothesized that:

1. Late-successional and pioneer tree species have different root tip morphological plasticity and strategies to cope with colder climate with shorter vegetation period, and show distinctive

acclimation patterns along the latitudinal gradient and forest zones.

2. High level of environmental stress, as exists in northern boreal forests and initially in primary successional birch forests, leads to extensive alteration in EcM root morphology.

The specific aims were to find out the tree species-specific EcM root traits being most indicative and responsive in morphological acclimation across environmental gradients concurrent in forest succession.

MATERIALS AND METHODS

In order to analyse the morphological plasticity of EcM roots of an early-successional and a late-successional tree species, we compiled a database of morphological characteristics measured for EcM roots originating from 14 Norway spruce (*Picea abies*) and 30 birch (*Betula pendula* and *B. pubescens*) stands (all silver birch stands except two mixed stands in Finland: Olkilouto and Punkaharju), partly published earlier (Supplementary Tables 1, 2). Data of control plots were used in case of manipulation experiments (Leppälämmi-Kujansuu et al., 2013; Parts et al., in preparation).

The spruce stands covered a latitudinal range from 48 to 68°N including 5 temperate, 3 hemi-boreal, and 6 boreal stands in age from 30 to 140 years. The birch stands covered a latitudinal range from 53 to 66°N and a longitudinal range from 2°W to 51°E and included 6 native forest stands in boreal, 8 in boreo-nemoral, and 1 in the temperate zone (Figure 1). Six birch stands of different ages were growing in reclaimed mining areas with stony and alkaline soil, and 9 young birch stands in abandoned agricultural fields. To analyse changes in root morphological traits (MTs) across forest succession gradient, we divided birch stands into three groups: primary successional stands on alkaline quarry detritus exposed by mining (mine forest), secondary successional stands in abandoned agricultural fields (field forest) and in long-term forests on fertile

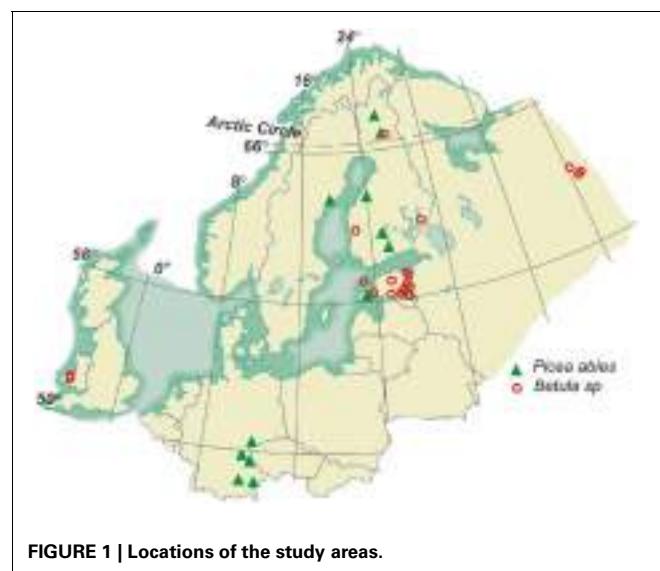


FIGURE 1 | Locations of the study areas.

soil (native forest). Age-driven morphological plasticity was analyzed in two silver birch chronosequences growing in mine area and on native forest land in Estonia (included stands are indicated by asterisks in Supplementary table 2). Stands included in native forest chronosequence belong to the *Oxalis* forest site type (Paal, 1997), characterized by high productivity (C/N = 12–23) and acidic soil, and are naturally regenerated; whereas stands growing in a mining area were plantations. To calculate PI-s of root traits for young (<10 years) and all older silver birch, trait means of all 30 birch stands and 46 measurements were used.

In order to be able to compare the plasticity of EcM roots between the tree species, the effect of previous land use was excluded by using only native forest stands, which were older than 10 for birch and 30 years for spruce i.e., have already undergone fast age-induced changes in EcM root morphology (Rosenvald et al., 2013). The EcM root traits were measured in several consecutive years for 11 birch and 7 spruce stands. In the same areas repeatedly measured morphological root traits were used to analyse the effect of study year in both tree species. However, to assess reaction norm of EcM root parameters in silver birch and Norway spruce forests across an environmental gradient, the mean of all years for a site was used.

The study sites for both tree species between latitudes 68 and 48°N display gradients in climate (e.g., mean annual temperature and precipitation, growing season length) as well as in N deposition (for some spruce stands see Ostonen et al., 2011). The different fertility of studied spruce sites was reflected by soil C/N ratio. In general, the southern stands displayed higher fertility. Silver birch stands on native forest land belonged all to the fertile forest site types.

Assuming the increase of RTD and mass (W, mg) of EcM root tips through aging, we analyzed the frequency distributions of RTD and W of root tips in two most northern (Kivalo and Pallasjärvi) and two most southern (Höglwald and Altötting) sites collected in 2008 for spruce (Ostonen et al., 2011). For birch, we compared the frequency distributions of RTD and root tip mass for EcM roots from two northern (Kivalo and two older sites in Syktykvar) and from two southern (Risley Moss, Erastvere) sites, samples were collected in 2009.

MORPHOLOGICAL CHARACTERISTICS AND PI CALCULATION

EcM roots were sampled at the end of the growing season (during September and October) by the same methodology throughout different years (Supplementary Table 1). Ten root samples per stand were collected with a spade from the organic layer and up to 20-cm-deep mineral soil layers at random locations. Two or three random EcM root subsamples (20–30 EcM root tips) were taken from each sample to measure the length, diameter, and projection area of root tips using WinRHIZO™ Pro 2003b (Regent Instruments Inc. 2003). The number of short root tips per sample collection of a stand ranged from 234 to 949 for spruce and from 239 to over 1000 for birch. The EcM root tips were washed cleaning with a small soft brush, to remove all soil particles, and counted under a microscope after separation from the long roots. The presence of ectomycorrhizal infection was recorded, >97% of the analyzed root tips were ectomycorrhizal. As the EcM root

tips were taken randomly from the subsamples, the mean values of root MTs per stand reflected the site-specific proportion of different morphotypes.

The air-dry root tips were dried at 70°C for 2–3 h to constant weight and weighed to 0.01 mg. Root tissue density (kg m^{-3}), SRA, ($\text{m}^2 \text{kg}^{-1}$) and SRL (m g^{-1}) were calculated as M/V , S/M , and L/M , where S , M , and L are mean root tip surface area, dry mass and length, respectively. Root tip frequency was expressed as the number of root tips per 1 mg of dry mass (RTF, No/mg). The methods for determining root MTs are given in detail by Ostonen et al. (1999).

Plasticity index (PI) was calculated as follows:

$$\text{PI} = (X_{\max} - X_{\min}) / X_{\max}$$

where X_{\max} —maximum stand mean, X_{\min} —minimum stand mean in the sample of the analyzed stands.

The reaction norms were expressed as the functional relations between values of a root trait and an environmental parameter. Plasticity of mean EcM root MT of the i -th stand in response to particular environmental conditions expressed through latitude (L) was defined as the absolute value of the slope of the reaction norm between MT and L (e.g., Scheiner, 1993; Pigliucci and Schlichting, 1998; Lepik et al., 2005). Such a plasticity estimate is comparable across different traits and species (Lepik et al., 2005).

To diminish age and land type effect species-specific plasticity was evaluated for older (>10 years) stands growing on native forest land.

STATISTICS

Repeated measures ANOVA was used on repeatedly measured data of native forest stands to evaluate the influence of measurement year on EcM root morphology. General Linear Models analyses (GLM) were applied to prove the impact of tree age and succession type (mine, field, and native forest) on EcM root tip parameters.

Redundancy analysis (RDA) (CANOCO program; ter Braak and Smilauer, 2002) was used to detect and visualize relationships between the root morphological characteristics (7) and sites (44) and between latitude, C/N, pH and stand age. The significance of RDA results was tested with a permutation test ($P < 0.01$).

T-test was applied to control the differences in trait means and PI values between of spruce and birch, and between birch successional types. Simple regression model was used to estimate the relationships between means of SRL and RTF and the location of stands. Forward stepwise regression analysis was used to reveal which parameters explain best the variation of SRL and RTF.

RESULTS

PLASTICITY OF EcM ROOTS IN SILVER BIRCH, THE INFLUENCE OF FOREST SUCCESSIONAL TYPE AND STAND AGE

As the collected birch data were obtained from stands with different age, site, and climate conditions, the results show the variation range of EcM root tip morphology of silver birch (Table 1). Plasticity indices were higher for functional EcM root parameters (SRA, SRL and root tissue density) of birch; the lowest plasticity showed EcM root diameter.

The effect of tree age and stand successional type (mine forest as primary successional forests, field and native forests as secondary successional forests) on EcM root morphology of silver birch was illustrated in an ordination biplot based on RDA of EcM root morphological parameters, where only Estonian stands were included in order to exclude the influence of climate (**Figure 2**). Tree age and forest successional type both affected significantly EcM root tip traits of silver birch (GLM, $p < 0.01$). Native forest stands formed a separate group also in the RDA ordination plot (**Figure 2**). Mine site forests had the biggest between-site

Table 1 | The means of stand means and variation between stand means of morphological characteristics of EcM roots of silver birch.

EcM root parameter	Mean	Min	Max	CV	PI
SRL (m/g)	91.2	44.6	313.9	0.61	0.86
SRA (m ² /kg)	82.5	45.1	274.6	0.50	0.84
RTD (kg/m ³)	186	54	279	0.31	0.81
Diameter (mm)	0.310	0.212	0.413	0.15	0.49
Length (mm)	1.24	0.76	2.65	0.27	0.71
Mass (mg)	0.0165	0.0077	0.0279	0.31	0.72
RTF (No/mg)	73.4	39.4	143.1	0.32	0.73

All 31 silver birch stands (characteristics are shown in Supplementary table 2) and 46 measurements are included. Abbreviations: CV, coefficient of variation; PI, plasticity index of stand means.

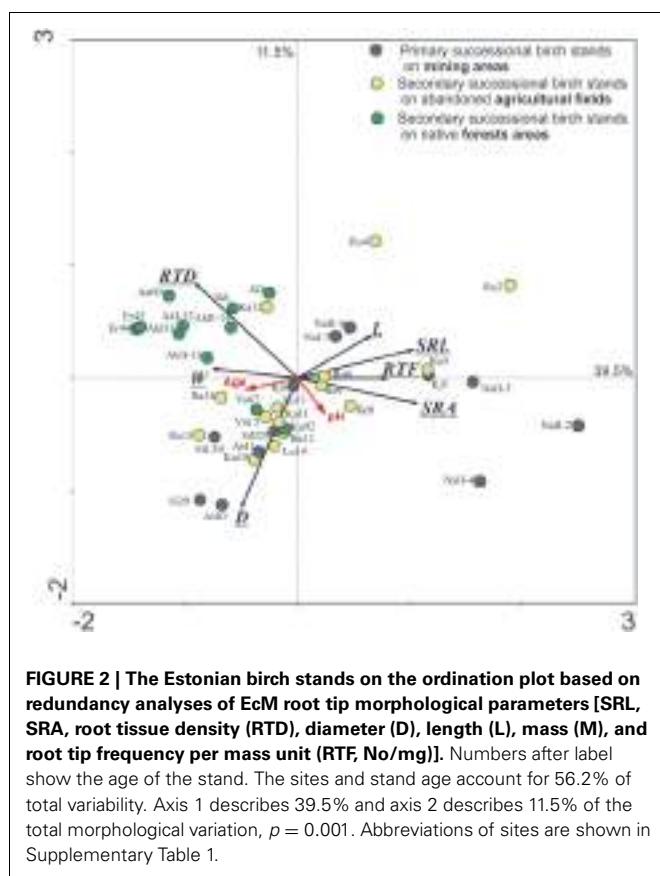


FIGURE 2 | The Estonian birch stands on the ordination plot based on redundancy analyses of EcM root tip morphological parameters [SRL, SRA, root tissue density (RTD), diameter (D), length (L), mass (M), and root tip frequency per mass unit (RTF, No/mg)]. Numbers after label show the age of the stand. The sites and stand age account for 56.2% of total variability. Axis 1 describes 39.5% and axis 2 describes 11.5% of the total morphological variation, $p = 0.001$. Abbreviations of sites are shown in Supplementary Table 1.

variation because great changes occurred in soil conditions during stand development on very stony and alkaline mine spoil in primary succession (Rosenvald et al., 2011a). In the ordination plot, stand age increased along the first axis for all three forest succession types. EcM root SRL, SRA, length, and RTF decreased with tree age, whereas mean diameter, mass, and RTD increased with stand age (**Figure 2**). Changes in root traits across sites in the ordination plot reflect also soil and rhizosphere succession—mine and field stands are likely to develop toward the fertile native forest stands. Plasticity indices of root traits of stands belonging to the fertile silver birch chronosequence of native forest (0.21–0.56, mean PI = 0.37) is lower than that of the mine chronosequence (PI = 0.34–0.85, mean PI = 0.65), where young birch stands have harsh soil conditions (**Table 2**).

In the mine area, 3-years-old (119 ± 7 m/g) and 6–7-years old (113 ± 9) birches have both higher (*t*-test, $p < 0.01$) SRL compared to the same age birches growing in fertile native forest (82 ± 4 and 70 ± 5 , respectively). Mean SRL values of EcM roots of silver birch chronosequences in fertile natural forest and in mine area with stony alkaline soil became more similar in older forests (**Figure 3A**). However, the difference in mean diameter between two chronosequences increases by tree ageing (**Figure 3B**). Young birches have similar mean D of EcM roots (0.26–0.28 mm) in both successional type, but root D of birches belonging to the three older age groups is thicker in native forest compared to respective age groups of mine area (*t*-test, $p < 0.01$).

Morphological response to different environmental conditions of young (≤ 10 years) birches was generally higher compared to older (> 10 years) birch trees (**Figure 2, Table 3**) except PI of EcM root D. PI-s of functional parameters of young stands were especially high (> 0.8). PI values did not vary much between parameters inside the group of older birch

Table 2 | Plasticity indices (PI) of root parameters of stands belonging to two silver birch chronosequences: a chronosequence of fertile stands on native forest land belonging to the same forest type (*Oxalis*) (3–60 years old stands) and recultivated mine area stands (1–41 years old).

EcM root parameters	Silver birch chronosequences	
	PI Native forest	PI Mining area
SRL (m/g)	0.317	0.777
SRA (m ² /kg)	0.456	0.845
RTD (kg/m ³)	0.215	0.676
Diameter (mm)	0.206	0.344
Length (mm)	0.295	0.712
Mass (mg)	0.546	0.570
RTF (No/mg)	0.563	0.600
Mean	0.371	0.646

$N = 9$ in the forest chronosequence, and $N = 9$ in the mine area chronosequence.

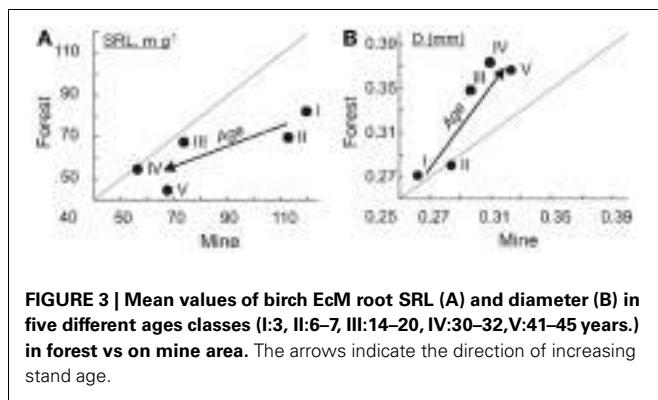


Table 3 | Plasticity indices (PI) of EcM root parameters for the young ($N = 17$) and older ($N = 29$) age groups of silver birch.

	Young birches (≤10 yrs.) PI	Older birches (11–92 yrs.) PI
SRL (m/g)	0.829	0.598
SRA (m ² /kg)	0.820	0.442
Density (kg/m ³)	0.806	0.478
Diameter (mm)	0.425	0.474
Length (mm)	0.684	0.483
Mass (mg)	0.684	0.530
RTF (No/mg)	0.660	0.536
Mean	0.699	0.517

stands (0.44–0.60). However, SRL had again the highest PI there.

SPECIES-SPECIFIC PLASTICITY OF EcM ROOTS: COMPARISON OF SILVER BIRCH AND NORWAY SPRUCE

Variation of EcM root traits

The greatest difference between birch and spruce EcM root morphology was in mean mass of root tip, which was more than two times higher for spruce (Table 4). The reason of that is that EcM roots of spruce are longer and thicker, because EcM root tissue density did not differ between species. Birch has higher EcM root SRL and SRA values and lower diameter.

Mean PI of EcM root traits did not differ (pairwise t -test, $p > 0.05$) between birch and spruce, 0.46 and 0.50, respectively. However, root traits with highest PI values differed between tree species: birch had highest PI values for SRL and spruce for root tip frequency (Table 4). PI and also variation of diameter of EcM root tips was small for both species. The biggest difference between species appeared in EcM root length, which had for Norway spruce roughly twice higher PI and variation coefficient than for silver birch. PI indexes and variation coefficients were well-correlated ($r = 0.96$ for birch and $r = 0.98$ for spruce, $p < 0.01$ in both cases) (Tables 3, 4).

The influence of forest zone on EcM traits

The morphology of EcM roots across the climate gradient from subarctic to temperate forests measured in different study years

varied greatly for both tree species (Figures 4A,B). Based on the RDA, the sites, geographical location explained 33 and 24% of the variation in EcM root tips morphology in spruce and birch, respectively. The northern boreal forests grouped separately from hemi-boreal and temperate sites on the ordination biplot. Spruce sites located along the first axis, which correlated best with the root tip length, SRA and RTF, and explained about 18% of the total morphological variation (Figure 4A). SRL and D of EcM roots of spruce correlated best with the second axis, which seems to be related to the study year—the stands studied in 2008 locate below the first axis toward the decrease in D, the stands studied in 2007 located above the first axis (Figure 4A). Birch forests located along second axis, which correlated with D and again with the length of root tips (Figure 4B). SRA, RTD, root tip mass and RTF correlated best with the first axis explaining 12% of the total variation in EcM root morphology.

In both tree species, EcM root tips are longer in northern sites, and in the case of birch also thinner. If in the spruce forests, we have clear gradient in edaphic conditions, from low fertility soils in northern stands to high fertility soils in southern stands—the C/N ratio in soil decreased more than two times (Supplementary Table 1) and correlated with latitude ($r = 0.67$, $p < 0.01$), then birch forests were all of high fertility.

We estimated the plasticity using reaction norms across forest zones for SRL and root tip frequency as most highly responsive traits on the basis of root traits PI values (Table 4) and RDA analysis (Figures 4A,B). When subjected to harsher conditions in higher latitudes, the birch increases the SRL of EcM roots, while spruce EcM roots showed no plasticity for this particular trait (Figure 5A). Although RTF expressed considerable plasticity across the latitudinal gradient in both spruce and birch, the tree species had reversed slopes of the regression line (i.e., a different response to environment). The spruce EcM roots showed decreasing and the birch EcM roots increasing RTF toward the northern forests (Figures 5A,B). Though in the case of birch, the regression was not significant due to shorter latitudinal gradient.

When the mean values of a root trait of birch are plotted against the values of spruce in boreal, hemi-boreal, and temperate native forests (Figures 6A–D), a remarkable trend appears—the morphology of the studied tree species becomes more similar toward southern forests. Although the values of EcM root tip SRL, RTF, D and L differed between species more in boreal forests, the mean RTD of root tips did not show clear trend in terms of forest zones.

The functional traits SRL and RTF both depend on root D, L and RTD. Forward stepwise regression analysis showed that the root tip frequency was mostly explained by root tip length for spruce ($r^2 = 0.80$, $p < 0.001$) and SRL by variation of D for birch ($r^2 = 0.61$, $p < 0.001$).

The study year had significant effect on RTD and SRA of spruce EcM roots (Repeated measures ANOVA; $n = 6$ boreal spruce forests, studied in 2007 and 2008; $p < 0.001$). However, the general stands consecution of different forest zones remains unchanged in all years for both tree species (Figures 4A,B) in spite of the annual variability of these root traits in some repeatedly studied sites.

Table 4 | The means and variation between stand means of morphological characteristics of EcM root tips and their PI values in silver birch and Norway spruce forests.

EcM root tip parameter	Species	Mean	Minimum	Maximum	Variation coefficient	PI
SRL (m/g)	birch	65.5	44.6	111.1	25.2	0.60
	spruce	42.1	34.6	57.2	13.5	0.40
SRA (m ² /kg)	birch	59.8	45.1	80.8	17.7	0.44
	spruce	45.3	37.8	55.3	10.0	0.32
Root tissue density (kg/m ³)	birch	235	151	279	17.4	0.46
	spruce	261	179	353	17.8	0.49
Diameter (mm)	birch	0.306	0.217	0.354	11.4	0.39
	spruce	0.357	0.279	0.436	11.2	0.36
Length (mm)	birch	1.22	0.99	1.48	12.6	0.33
	spruce	1.85	1.10	2.79	24.5	0.60
Mass (mg)	birch	0.0207	0.0133	0.0279	21.3	0.52
	spruce	0.047	0.0284	0.0766	30.6	0.63
RTF (No/mg)	birch	56.7	39.4	80.8	22.0	0.51
	spruce	25.4	13.1	41.2	28.7	0.68

Only native forests older than 10 years were included in calculations. N = 16 for silver birch, and N = 23 for Norway spruce; 12 and 14 different forest sites were included respectively. Differences (t-test, p < 0.05) between means of birch and spruce EcM root parameters are indicated in bold.

SPECIES-SPECIFIC DISTRIBUTION OF RTD AND ROOT TIP MASS IN FOREST ZONES

The distributions of root tip mass and root tissue density of EcM roots differed between northern boreal and temperate Norway spruce stands (**Figures 7C,D**), while we could not find any difference for birch EcM roots tissue density (**Figure 7A**). EcM roots of spruce gain higher mass and RTD in northern boreal forests, which coincides with relatively longer roots in north (**Figure 4A**). Although the EcM root tips of birch were also longer in the north, the root tip mass was significantly higher in the southern stands (**Figure 7B**). The birch root tips were heavier in south due to 1.4 times thicker root tips, while the diameter of spruce EcM roots did not differ between southern and northern stands (**Figure 6A**).

DISCUSSION

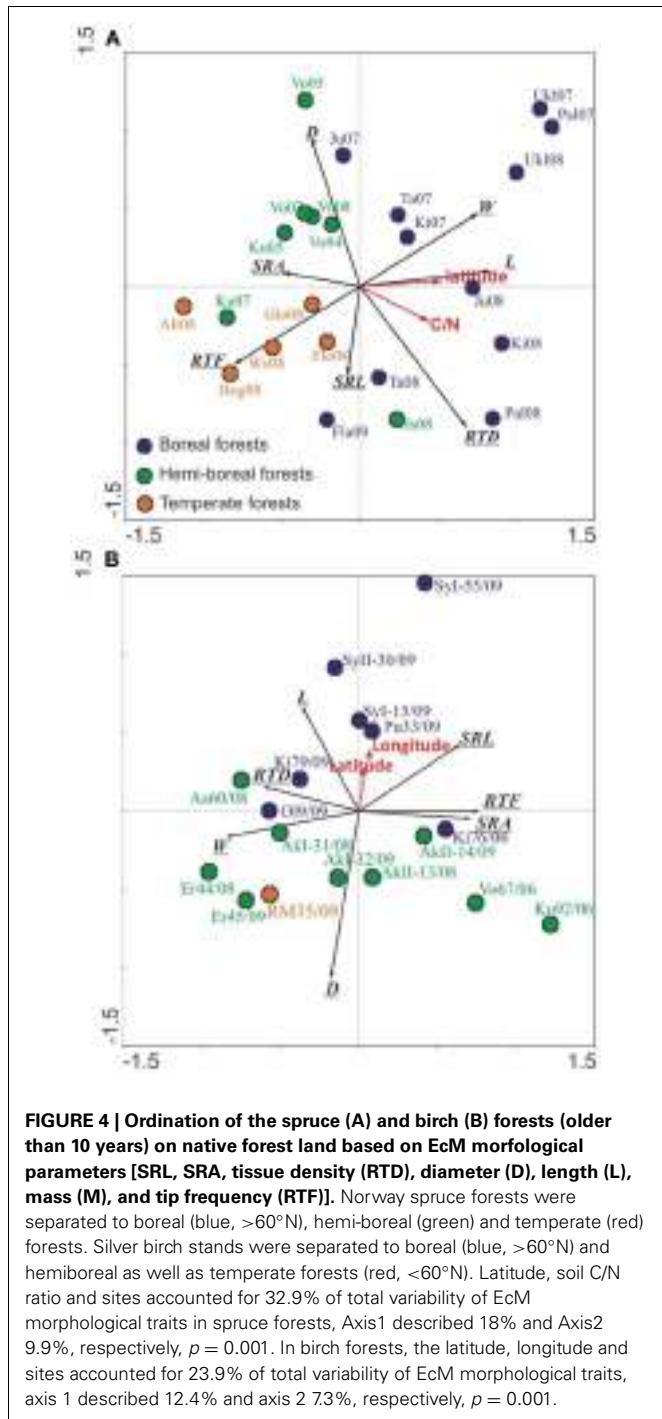
Our results strongly imply that tree species may have different acclimation-indicative root traits in response to changing environments. Birch ensures the morphological acclimation across forest zones by changing SRL of the EcM roots, which is mainly (61%) determined by the variation of diameter. The acclimation of spruce EcM roots is based mainly on the variation of root tip frequency per mass unit, which, in turn is essentially (41%) determined by the variation of root tip length. Silver birch as a pioneer species must have a broader tolerance to environmental conditions across multiple environments; however, the mean PI of all MTs did not differ between early-successional birch and late-successional spruce. Duan et al. (2013) also found that deciduous *Populus yunnanensis* and evergreen *Abies faxoniana* differed in PI-s of their key traits but not in their overall plasticity. The difference between birch and spruce in the plasticity response of EcM roots and in their most indicative root trait across climate gradient shows the existence of equivalent morphological acclimation strategies in terms of optimal foraging. The clearly higher similarity in root traits of birch and spruce in temperate forest

compared to boreal forests can be explained by more fertile and closer to optimal growing conditions.

Although, we considered the MTs of EcM short root tips in general, without specifying the morphotype, the species of fungal symbionts may have significant impact to shape of root tips (Ostonen et al., 2009; Makita et al., 2012). In our previous study, we have reported a shift in dominating colonizers of root tips in spruce forests across a latitudinal gradient (Ostonen et al., 2011), this might be one of the reasons behind changes in EcM root traits, such as root tip frequency in spruce or SRL in birch. However, the increase in root tip frequency may occur also due to differences in host tree genotype in northern compared to southern forests, as Korkama et al. (2006) have shown higher root tip frequency per fine root biomass for fast-growing spruce clones, which might be the case of southern Norway spruce forests. Furthermore, the increase in fine root RTF of fast growing clones coincided with changes in EcM community structure (Korkama et al., 2006). Thus, the shifts in EcM communities may play an important role in morphological acclimation and mineral nutrition of trees and should be studied in the future. Nevertheless, the alterations in EcM root morphology is one of the important mechanisms in forest trees root system acclimation to changing environments and the elucidation of general patterns of different tree species is important for predicting the ability of different species to acclimate to global change.

CHANGES IN EcM MORPHOLOGY ACROSS FOREST SUCCESSION STAGES REFLECT ROOT-RHIZOSPHERE LEVEL SUCCESSION

The biggest part of the morphological variation of birch EcM roots was described by SRL, which has been used as an indicator of environmental change, characterizing also the economic aspects of the root system and being sensitive to nutrient availability of trees (Ostonen et al., 2007a,b; Rosenvald et al., 2011b). Morphological plasticity includes the possibility to



induce changes in physiology or activity (e.g., the metabolism of root tips (Useche and Shipley, 2010; Makita et al., 2011). Also our findings about birch indicate that change in SRL of EcM roots gives probably the biggest physiological effect in water and nutrient uptake, because it's the highest phenotypically plastic trait among studied traits. SRL was high in conditions of resource deficiency, when trees have limited amount of assimilates to invest in EcM roots, but they need to increase the absorbing surface—that is to

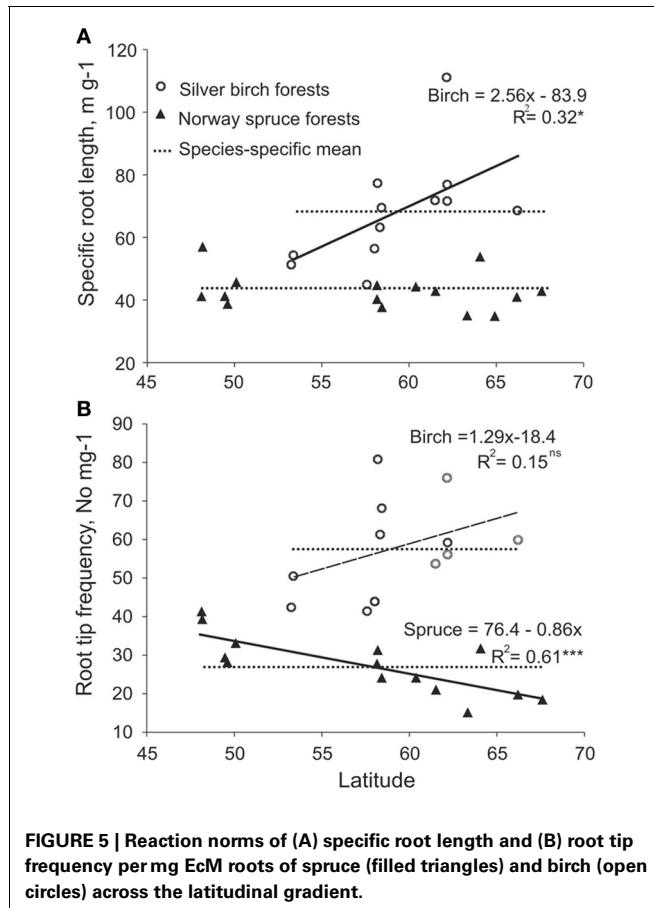


FIGURE 5 | Reaction norms of (A) specific root length and (B) root tip frequency per mg EcM roots of spruce (filled triangles) and birch (open circles) across the latitudinal gradient.

grow thin and long roots—to acquire sufficient amounts of nutrients and water. In line with our hypothesis, achieving high SRL of EcM roots is an acclimation strategy of silver birch in stress and unfavorable growing conditions (e.g., in subarctic forests) and at younger age. Our results are in good accordance by Kalliokoski et al. (2010), who showed the increase of SRL from the most fertile sites to the least fertile sites even for fine roots (<2 mm) of *Betula pendula*.

Plasticity indices of the functional parameters (SRA, SRL, RTD) were considerably higher also in the younger age group (≤ 10 years) compared to older silver birches. High plasticity and high SRL as well as SRA at younger age are essential, because trees have to acclimate and grow quickly to survive in competition for light, nutrients, and other resources.

Plasticity indices of root traits of stands belonging to the naturally regenerated fertile silver birch native forest chronosequence reflect mainly age-driven plasticity, because growing conditions remain relatively stable along the chronosequence (Rosenvald et al., 2013). In the case of primary succession as occurs in the chronosequence of mine site forests, soil conditions improve tremendously in time, and plasticity of root parameters is the sum of the age-related and site-conditions-related plasticity. However, mean SRL values of EcM roots of silver birch in native forest and in mine forest are more similar in older stands. As EcM root SRL of silver birch is related to soil fertility (Rosenvald et al.,

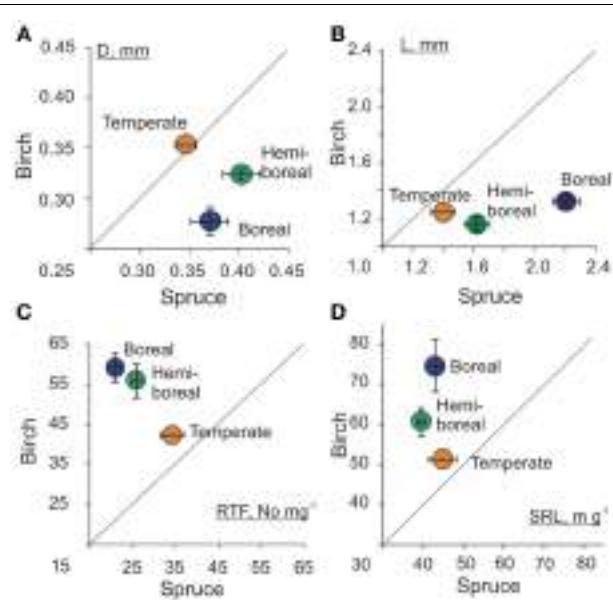


FIGURE 6 | Mean values of morphological traits (A) diameter (D, mm), (B) length (L, mm), (C) root tip frequency per mass unit (RTF No mg^{-1}) and (D) specific root length (SRL, m g^{-1}) of birch and spruce in three forest zones: boreal, hemi-boreal, and temperate. Shown are average values and standard errors of the sample. For birch forests, only older than 10 years stands growing on native forest land were included and only one stand from temperate zone was included.

because of the differences in soil, as the soil formation in mining areas has just started. Furthermore, the difference between EcM root diameters in mine and native forest stands increases with age. Our results indicate that for sufficient mineral nutrition of birch the optimal SRL values are achieved by changing EcM root diameter and to lesser extent also the root tissue density. Our RDA analysis showed increase in the mean stand age across forest succession gradient toward fertile native forest sites. Considering forest successional stages across mine, field and native birch forests, the stands on afforested agricultural land locate between mine and native forests. The root morphology in mine and field forests become more similar with native forests over time.

Thus, we can say that changes in EcM root morphology reflect the root-rhizosphere level succession as the root traits seems to be tightly associated with development of soil and the rhizosphere in a wider sense.

CAN ROOT MORPHOLOGY BE USED AS AN INDICATOR OF EcM ROOT LIFESPAN?

Root traits, such as root tissue density, diameter and SRL of EcM root tips are hypothesized to be correlated with root tips lifespan (Withington et al., 2006). The differences in the distribution of mean mass and tissue density of EcM roots show distinct EcM root populations in north and south for spruce, but not for birch, though there was tendency for higher average RTD of EcM roots in northern birch stands. Higher amount of EcM roots with bigger root tissue density might indicate longer lifespan of EcM roots in northern forests. The median longevity for roots in the <0.5 mm diameter group, consisting in majority of EcM roots (Withington et al., 2006), has been reported to be more than two times longer in southern Sweden (Hansson et al., in press) compared to south-east Germany (Gaul et al., 2009). Our morphological analysis of EcM roots shows that spruce trees in northern boreal forests increase the persistence of existing EcM roots instead of forming new root tips, which also leads to the increase in root tip mass and tissue density. This might be related to fine root foraging strategies of trees, which is expressed through higher biomass of EcM roots in nutrient-poor soils of northern boreal spruce forests (Helmisaari et al., 2009; Ostonen et al., 2011) and lower turnover rate of fine roots as reported at higher latitudes (Yuan and Chen, 2010; Finér et al., 2011). In grasses, the high root tissue density has been associated with stressed environments (Craine et al., 2001). Nevertheless, the absence of differences in EcM root tissue density distributions between northern and southern birch forests and even higher average EcM root tip mass in southern stands might show either the species-specific differences in root morphology between the pioneer- and late-successional tree species and/or no difference in longevity of EcM root tips across the latitudinal gradient of the birch stands.

In conclusion, we can say that morphological parameters of EcM roots in Norway spruce and silver and downy birch, particularly the SRL, root tip frequency per mass unit as well as the mean diameter and length of the root tips, become more similar toward southern forests, which can be considered as more fertile and closer to optimal growing conditions compared to boreal

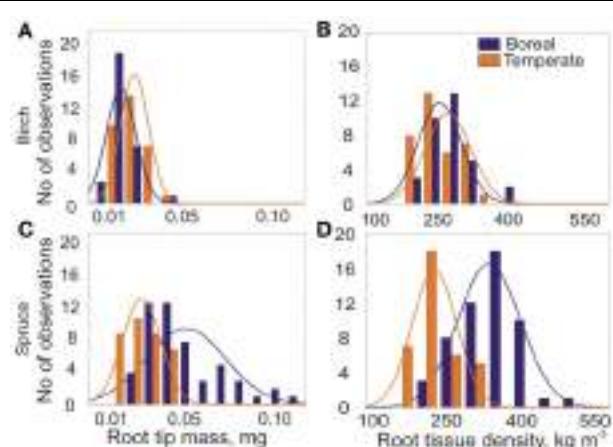


FIGURE 7 | Frequency distribution of (A and C) the mean root tip mass (W, mg) and (B and D) root tissue density of EcM roots (RTD, kg m^{-3}) for two northern birch stands in Siberia (Syktykvar55, Syktykvar30) and for two southern birch stands in UK and Estonia (Risley Moss, Erastvere45) and for two most northern (Kivalo and Pallasjärvi in Finland) and two most southern (Höglwald and Altötting in Germany) spruce sites collected in 2008 (Ostonen et al., 2011).

2011b), more similar soil conditions in older stands determine also more similar SRL values there. Majority of the studied native birch forests still form a separate group from mine site forests in the ordination plot based on all EcM root traits probably

forests. Furthermore, the alteration of EcM roots morphology is part of the acclimation process of birch trees, which spread in the first stage of primary and secondary successional forest areas, reflecting probably the simultaneous succession in soil, at root-rhizosphere level.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Functional_Plant_Ecology/10.3389/fpls.2013.00335/abstract

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Earthworm effects on native grassland root system dynamics under natural and increased rainfall

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Earthworms (EWs) can modify soil structure and nutrient availability, and hence alter conditions for plant growth through their burrowing and casting activities. However, few studies have specifically quantified EW effects by experimentally manipulating earthworm densities (EWDs). In an earlier field study in native grassland ecosystems exposed to ambient and experimentally elevated rainfall (+280 mm year⁻¹, projected under some climate change scenarios), we found no effects of EWDs (37, 114, 169 EW m⁻²) and corresponding EW activity on aboveground net primary productivity (ANPP), even though soil nutrient availability likely increased with increasing EWDs. The lack of effects of EWDs on ANPP suggested that EWs may have adversely affected root systems in that study in some way. The objective of the present study was to quantify responses of root length density (RLD), using data collected from the same grassland plots during the earlier study. RLDs were highest in plots with low EWDs and decreased in plots with higher EWDs. Elevated rainfall primarily increased RLDs in the low EWD treatment (by almost +40%). Reductions in RLDs resulting from increased EWDs did not affect ANPP. Our results indicate that elevating EWDs above ambient levels may limit root growth through large increases in soil bioturbation, but concurrent increases in cast production and nutrient availability may compensate for the suppression of root nutrient absorbing surface area leaving ANPP unchanged, but with shifts in growth (biomass) allocation toward shoots. Similarly, reductions in EWDs appeared to promote higher RLDs that increased soil nutrient foraging in soil with lower amounts of nutrients because of reduced casting activity. Amplified responses observed when rainfall during the growing season was increased suggest that EWs may mainly affect RLDs and above- vs. belowground growth (biomass) allocation under climate changes that include more frequent wetter-than-average growing seasons.

Keywords: belowground–aboveground interactions, grassland ecology, plant–animal interactions, root ecology, soil ecology, root growth, plant growth (biomass) allocation

INTRODUCTION

Ever since the late 1800s, with the pioneering work of Hensen (1877) and Darwin (1881), earthworms (EWs) have been known for their large “engineering” effects (Jones et al., 1994) on the chemistry and physical structure of soils. These effects include stimulation of litter and soil organic matter decomposition and soil nutrient mineralization that can enhance soil nutrient availability (e.g., Lee, 1995; Edwards and Bohlen, 1996) and plant productivity (Curry, 1987; Scheu, 2003). Anthropogenic global change is presently modifying environmental factors that can impact the engineering activity of EWs (tunneling, cast production) – either indirectly via bottom-up plant responses to rising atmospheric CO₂ (Zaller and Arnone, 1997) or by changes in plant species diversity (Zaller and Arnone, 1999b; Arnone et al., 2013) or directly via changes in amounts of precipitation (Zaller and Arnone, 1999a). Thus, a mechanistic understanding of how earthworm density (EWD, or community size) itself may influence aboveground net primary productivity (ANPP) under changing climatic conditions – especially

altered amounts of growing season rainfall (IPCC, 2007) – is important.

While many greenhouse pot studies have shown that the presence of EWs can stimulate plant growth in the short term [79% of the 67 studies reviewed by Scheu (2003); with the remaining studies reporting zero or slightly negative EW effects on plant growth], only two studies have specifically quantified EW effects by experimentally manipulating their densities in field plots (Blair et al., 1997 – showing moderate stimulatory effects on ANPP; Zaller and Arnone, 1999a – showing no effects on ANPP). Many fewer studies have quantified EW effects on root growth. These studies have reported enhancement of root growth (e.g., Wurst et al., 2008; Zaller et al., 2011), reductions in root growth (cf. Scheu, 2003), or no effect on root growth (e.g., Eisenhauer et al., 2009). In cases where increased shoot growth was found, some of this increase may have resulted from a general increase in soil nutrient availability or from a stimulation of root growth into nutrient-rich EW casts (Hirth et al., 1997, 2005; Zaller and Arnone, 1999c; Decaens et al., 2001; Zaller et al., 2013). Alternatively, increased shoot

growth may have resulted from shifts in plant growth (biomass) allocation toward shoots of all or some species present in plant communities in response to increases in soil nutrient availability (e.g., Lambers et al., 1998) that resulted from EW casting and soil bioturbation. However, in cases where no stimulation of shoot biomass production was observed, the extent to which EWs may have caused these effects by somehow impeding root growth (e.g., Baylis et al., 1986; Cortez and Bouché, 1992; Gunn and Cherrett, 1993; Fisk et al., 2004; Birkhofer et al., 2011) is unclear. No studies appear to have specifically quantified how EWs affects root system size (e.g., root length density, RLD) and temporal dynamics in natural plant communities. Yet, a quantitative understanding of how EWs affect root systems of native grasslands is necessary as a basis for assessing how global anthropogenic change will alter the function of these ecosystems (e.g., Zaller and Arnone, 1997, 1999c; Arnone et al., 2013).

In an earlier study (Zaller and Arnone, 1999a) in which we manipulated EWDs in field plots in a native plant species-rich calcareous grassland in NW Switzerland, we found that experimentally increasing EWDs also increased EW activity (measured in surface cast production) but did not change ANPP, even when the period of seasonal EW activity and plant growth was extended through application of artificial rains. While additional rainfall stimulated ANPP by 30% in that study (mean of $440 \text{ g m}^{-2} \text{ year}^{-1}$ in plots with natural rainfall and mean of $580 \text{ g m}^{-2} \text{ year}^{-1}$ in plots with additional rain) primarily by enhancing the growth of graminoid species (Zaller and Arnone, 1999a), the lack of effects of EWDs on ANPP in that study was surprising because previous studies in these calcareous grasslands have shown that increases in EW activity increased soil nutrient availability (Zaller and Arnone, 1997) and stimulated shoot growth (Zaller and Arnone, 1999c) and ANPP (Arnone et al., 2013). Thus, the results from our earlier study (Zaller and Arnone, 1999a) showing no EWD effects on ANPP, suggested that elevated EWDs may have adversely affected root systems in some way, while reduced EWDs may have somehow benefitted root systems.

Therefore, the objectives of the present study were to quantify the effects of EWD and rainfall treatments imposed by Zaller and Arnone (1999a) on plant community RLD to evaluate whether possible earthworm-induced changes to the root systems of these intact native grassland plant communities can mechanistically explain the lack of ANPP response reported by Zaller and Arnone (1999a).

MATERIALS AND METHODS

Because the results presented here represent the analysis of a second data set generated during the Zaller and Arnone (1999a) study (which focused on aboveground ANPP responses to EWD and rainfall), the material and methods described in that paper apply here, as well. However, for the sake of completeness and convenience, we summarize critical elements of the methods here, and provide data from Zaller and Arnone (1999a) that describe the effectiveness of the EWD and rain treatments.

SITE DESCRIPTION

The calcareous grassland we studied is located on a 20° southwest-facing slope near the village of Nenzlingen (canton Basel-Land),

NW-Switzerland (500 m a.s.l., $47^\circ 27' \text{N}$, $7^\circ 34' \text{E}$). Mean annual precipitation is about 920 mm and mean air temperatures of about 8.5°C (Ogermann et al., 1994). Up to 1993 this grassland had been used for extensive cattle grazing and since 1993 the area has been fenced and mown twice a year in spring and autumn. Among the 100 vascular plant species found on this site, the grass *Bromus erectus* L. is dominant (Huovinen-Hufschmid and Körner, 1998). Soils are classified as a transition Rendzina (pH is about 6.5, bulk density of the top soil 1.1 g cm^{-3} , C-to-N ratio about 12), with a well developed, stone-free, loamy topsoil and a rapid transition at 15–25 cm depth to the underlying calcareous scree material (Ogermann et al., 1994).

EXPERIMENTAL DESIGN

The design used in our study was identical to the one described in Zaller and Arnone (1999a). To control EWD, $30 \text{ } 1 \times 1 \text{ m}$ plots were trenched to a depth of 45 cm with 1-mm-mesh nylon window screen in spring 1995. The screen extended 15 cm above the soil surface to create an aboveground EW barrier. Trenching to 45 cm in these shallow soils would be expected to strongly limit subsurface lateral movement of EWs into or out of the plots. Plots were arranged in a randomized complete block design (five blocks), with three EW densities (low, ambient, and high) and two amounts of rainfall (ambient and 280 mm year^{-1} additional rain). These amounts of added rain were applied to the appropriate plots during dry periods in the growing season to maintain sufficient soil moisture for EWs to stay active in all but the driest periods. Volumetric soil water content was continuously monitored over the topmost 10 cm of the topsoil using time-domain-reflectrometry (one measurement every 20 min). We also continuously recorded soil temperature in each plot with thermistors placed at depths of 5 and 15 cm (one reading h^{-1}). Time courses of soil water content and soil temperature over the experimental period are presented in Zaller and Arnone (1999a).

Earthworm density treatments were established in May 1996 by first extracting EWs from each plot by applying an electrical current to moist soil (Thielemann, 1986; Ketterings et al., 1997). This non-destructive method has been shown to provide comparable estimates of EW community size and composition to other more conventional sampling methods, as long as EWs are sampled at times when they are active and when soil moisture is sufficient (Schmidt, 2001). A total of nine EW species were collected (nomenclature follows Bouché, 1972) representing three ecological groups (Bouché, 1977): anecics (*Nicodrilus longus* Ude, *N. nocturnus* Ev., *Lumbricus terrestris* L.), endogeics (*N. caliginosus* Sav., *Allolobophora chlorotica* Sav., *A. rosea* Sav., *Octolasion cyanum* Sav.) and epigeics (*L. castaneus* Sav., *Dendrobaena mammalis* Ger.).

We created field plots with three levels of experimental EW densities (low, ambient, and high) using the following procedure. All of the EWs collected from each plot in each experimental block (six plots per block) was temporarily placed in pale containing cool water (see above). Worms in the pale were then sorted into one of three ecological groups, with worms from each ecological group placed temporarily in a smaller polyethylene beaker filled with cool water. One-sixth of the worms from each beaker were placed on the mowed surface of each of the two ambient density

plots in that block. One-third of the worms from each beaker were placed on the surface of the two high density plots in that block, and no worms were placed in the plots assigned to the low EWD treatment. This procedure was repeated for each of the five blocks in May 1996 (start of density treatment) and again in September 1996 and May 1997 to maintain density treatments. All worms reentered the soil immediately after being placed on the surface of the plots. We were unable to achieve complete EW removal from low density plots because not all worms are able to exit the soil during application of electrical stimulation. Details of aboveground plant biomass sampling and harvesting procedures are described in Zaller and Arnone (1999a). The total amount of plant biomass harvested in May and September 1997 was used to estimate ANPP.

Cumulative surface cast production (dry mass) was measured biweekly during periods of highest EW activity (Zaller and Arnone, 1997) from October 1996 to May 1997 on one of the two 25×25 cm sub-plots in each plot as an indicator of EW activity. After weighing cast fresh mass in the field on a portable balance, it was returned to its original position and deformed slightly to facilitate the identification of newly produced casts at the next sampling date. Cast subsamples from each plot were taken at each sampling date to calculate fresh mass-to-dry mass ratios (80°C , 24 h).

Results published Zaller and Arnone (1999a) showed that our manipulation of EW community size was effective, although EW populations fluctuated between sampling dates with community size (number and biomass) tending to increase slightly in low density plots, tending to decrease slightly in high density plots, but remaining largely unchanged in ambient density plots. Averaged across the experimental year, Zaller and Arnone (1999a) found that low density plots contained the fewest EWs (37 ± 5 worms m^{-2}) and least biomass (26.7 ± 3.3 g m^{-2}). The ambient density plots contained about twice the number and biomass of the low density plots. The high density plots contained about 50% more worms and 50% more biomass than the ambient plots contained. Zaller and Arnone (1999a) further demonstrated that increasing EWD also resulted in significant increases in EW activity measured as cumulative surface cast production. However, additional rain had no detectable effect on the size of EW communities in any EWD treatment even though soil water content was consistently greater in these plots compared to plots receiving no additional water (Zaller and Arnone, 1999a). Daily mean soil temperature in all plots fluctuated in a normal fashion with season, but did not differ significantly among worm density or rain treatments, or between 5 and 15-cm soil depths, at any time during the study (Zaller and Arnone, 1999a).

MEASURING ROOT LENGTH DENSITY USING MINIRHIZOTRONS

In late April 1995 we installed one transparent minirhizotron tube (5 cm in diameter, 100 cm long) in each experimental plot at an angle of about 35° to the plane of the soil surface. The tubes were inserted through the A horizon and into the upper 3 cm of the rocky subsoil allowing us to observe roots in the top 18 cm of soil, the layer in which 80% of all roots occur (Arnone et al., 2000). Before installing the tubes we etched an observation track (18 mm wide, 54 cm long) on the outside upper surface of each

tube. We then divided each track into 45 frames, each 12 mm high and 18 mm wide. The tube bottoms were capped before insertion into pre-cored cylindrical holes, and the top 10 cm of each tube wrapped in opaque tape and stoppered to prevent light penetration and entry of debris and insects.

We were unable to distinguish among roots of the more than 30 species growing in each experimental plot and thus only considered responses of the root system of the entire plant community. In April 1996, we recorded video images of roots in all 45 frames in each minirhizotron using a Bartz BCT-2 Minirhizotron Camera (Bartz Technology Co., Santa Barbara, CA, USA) attached to a Hi-8 Sony Camcorder (all mounted on a backpack). We repeated this on 11 more dates up to April 1997. All 45 frames along the tubes were used to quantify (RLD, cm root cm^{-2} minirhizotron tube surfaces) through the soil profile. This was accomplished by viewing undigitized video tapes and counting intersects with gridlines drawn on an overhead transparency and placed over the video monitor (Tennant, 1975). Average RLD during the first year (April 1996–April 1997) was calculated for each minirhizotron observation frame using the Tennant (1975) method and expressed as cm of root length per cm^2 of minirhizotron observation area (Smit et al., 2000).

CALCULATIONS AND STATISTICAL ANALYSIS

In all analyses we used the plot as the experimental unit. First, we tested the effects of EWD, additional rain, sampling date, and their interactions, on RLD using a three-way analysis of variance (ANOVA) model. In these ANOVAs, the EWD effect was tested against the EWD \times Block term, the Rain effect was tested against the Rain \times Block term, the Sampling date effect was tested against the residual term, and the EWD \times Rain effect was tested against the EWD \times Rain \times Block term. We also used two-way ANOVAs to explore EWD effects within each Rain treatment to further elucidate the possible occurrence of significant ($P < 0.05$) EWD \times Rain interactions. In these ANOVAs, the EWD effect was tested against the EWD \times Block term, and the Sampling date effect was tested against the residual term, and the EWD \times Sampling date effect was tested against the EWD \times Sampling date \times Block term. Because the block effect was never statistically significant ($P > 0.05$), this factor was removed from all ANOVAs. Second, the effect of additional rain on RLD over time was tested using repeated measures ANOVA (von Ende, 1993) for each soil depth, and the sum of all depths, and EWD. Third, we used Pearson correlations (e.g., Zar, 1998) to test *a priori* linear relationships between RLD and EW activity (cast production), RLD and EWD, RLD and EW biomass, and RLD and annual net aboveground (shoot) plant biomass production. We used a 3-parameter asymptotic regression fitting procedure for non-linear exponential relationships (Stata-Corp, College Station, TX, USA). Data were transformed before ANOVA as necessary to ensure homogeneity of variance and normal distributions. All statistical analyses were performed using Stata version 11.1. Values given throughout the manuscript are means \pm SEs.

RESULTS

Root length densities averaged across all depths (0–20 cm) increased during the growing season starting in April reaching

peak values in August 1995 in all plot regardless of EWD treatment (**Figure 1**; **Table 1**). These maximum RLDs were maintained throughout the cold season into mid-March 1996, at which point RLDs in all EWD treatments decreased by mid-May 1996 to densities measured in May of the previous year that in all EWD

treatments corresponded to RLDs measured in plots kept at natural EWD densities. This temporal pattern was discernable at all of the depths in the upper soil layer (data not shown).

Overall, reducing EWD below natural levels increased RLDs when viewed across all depths, while increasing EWD above

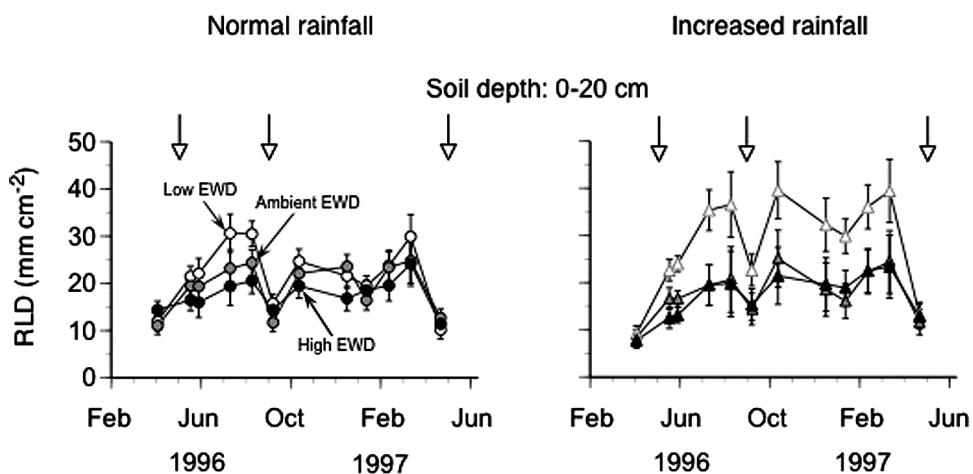


FIGURE 1 | Seasonal dynamics of root length density (RLD) measured using minirhizotrons (0–20 cm topsoil, one 60 cm long tube per plot) in trenched grassland plots with manipulated earthworm densities (EWDs) under ambient and increased rainfall (means \pm SEs, $n = 5$ plots per

experimental treatment; treatment effects analyzed using repeated measures ANOVAs – see **Table 1**). Open symbols represent low EWD; gray symbols: ambient EWD; and black symbols: high EWD. Circles indicate ambient rainfall means, and triangles increased rainfall means.

Table 1 | Results of analyses of variance (ANOVA) quantifying treatment effects of earthworm density (EWD) and additional simulated rainfall (Rain) on root length density (RLD) measured in the topsoil (0–20 cm) of 1×1 m experimental plots in native calcareous grassland in the Jura hills of northwestern Switzerland.

Reference figure	Factor	F-value	df	P-value	Note
1	EWD	5.06	2,12	0.0254	All data
	Rain	0.41	1,4	0.5569	
	Date	2.75	11,319	0.0809	
	EWD \times Rain	1.69	2,8	0.2446	
1	EWD	0.80	2,12	0.4727	Natural rain
	Date	16.44	11,132	<0.0001	only
	EWD \times Date	1.34	22,132	0.1640	
1	EWD	5.65	2,12	0.0187	Added rain
	Date	27.22	11,132	<0.0001	only
	EWD \times Date	2.43	22,132	0.0010	
2	EWD	5.06	2,12	0.0254	All data
	Rain [R]	0.41	1,4	0.5569	
	Soil depth [D]	4.31	2,8	0.0437	
	EWD \times Rain	1.69	2,8	0.2446	
	EWD \times Depth	0.66	6,36	0.6801	
	Rain \times Depth	3.92	3,12	0.0365	
	EWD \times R \times D	0.28	6,24	0.9392	

Repeated measure ANOVA was used to analyze treatment effects in the seasonal time courses of RLD (Reference **Figure 1**). A three-way ANOVA was used to analyze treatment effects on RLD at different depths in the topsoil (Reference **Figure 2**).

natural levels had no detectable effects (**Figure 1**; **Table 1**). These patterns were also apparent when comparing mean annual RLDs measured at different soil depths (**Figure 2**; **Table 1**). However, the statistically significant EWD effects detected in ANOVAs were primarily due to the stimulation of RLDs in low EWD plots that received additional simulated rain (**Table 1**).

Mean annual RLD viewed across all depths and treatment combinations appeared to be unrelated to mean annual EWD or mean annual EW biomass (**Figure 3A**: $P_{\text{slope}} = 0.1566$, **Figure 3B**: $P_{\text{slope}} = 0.4290$). However, mean annual RLD was highly related to mean annual surface cast production, with RLD decreasing exponentially with increasing EW surface cast production (**Figure 3C**: $P_{\text{slope}} = 0.0055$, $r^2 = 0.88$).

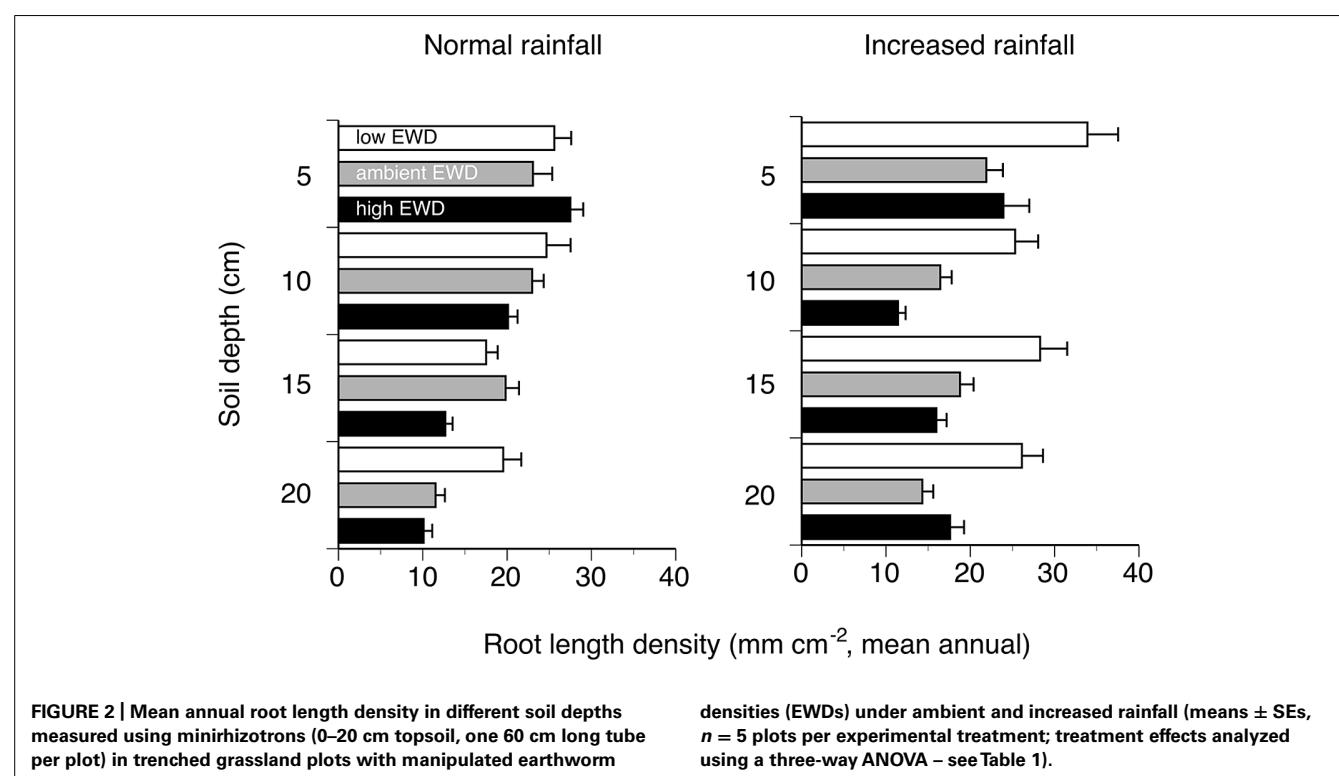
The scatter diagram (**Figure 4**) of treatment mean annual ANPP measured during the study year plotted on corresponding treatment mean annual RLD, calculated across all depths, indicated a lack of significant relationship between ANPP and RLD. However, when we removed from the scatter diagram the point in the upper right (outlier), we observed a significant negative exponential relationship between the two variables, with ANPP declining precipitously with increasing RLD ($P = 0.0070$, $r^2 = 0.94$; **Figure 4**). This point represented the treatment mean from the low EWD plots that received additional rain.

DISCUSSION

This study for the first time shows that during the course of 1 year, EW densities significantly affected the size (length density) of native grassland root systems, particularly when EWDs were kept low and when sufficient soil moisture was present (**Figure 1**).

Increased RLDs observed under low EWDs (especially under increased rainfall), relative to RLDs measured under ambient and high EWDs, may have occurred for a number of possible reasons. These possibilities include: (i) an increased need for plants in these communities to invest in root production (e.g., plant functional equilibrium adjustment; e.g., Lambers et al., 1998) to forage for lower levels of available nutrients (Fitter, 1994; Hutchings et al., 2000) relative to plants in ambient and high EWD plots as EWs increase soil nutrient availability (Zaller and Arnone, 1997; Arnone et al., 2013); (ii) lower root nutrient uptake efficiency as suggested by results of Fisk et al. (2004) with more plant carbon invested in root tissue growth per unit of nutrient taken up; (iii) reduced physical disturbance by EWs of newly formed root tips (which has not yet been experimentally addressed); or (iv) a reduction in possible root herbivory under lower EWDs (suggestive indirect evidence: Baylis et al., 1986; Gunn and Cherrett, 1993; Birkhofer et al., 2011).

The lack of an increase in RLDs under ambient and high EWDs (observed under both ambient and increased rainfall), relative to RLDs measured under low EWDs, also may have occurred for a number of reasons. These possibilities include: (i) a reduced need for plants to invest in root production where EWs increased soil nutrient availability; (ii) greater root nutrient uptake efficiency; (iii) increased physical disturbance by EWs of newly formed root tips; or (iv) an increase in possible root herbivory. Potential facilitation of root growth in high EWD plots through enhanced creation of EW channels observed in other studies (Springett and Gray, 1997; Pitkänen and Nuutinen, 1997) did not seem to be operative in our ecosystems where we actively manipulated EWDs [as Carpenter (1985) also found].



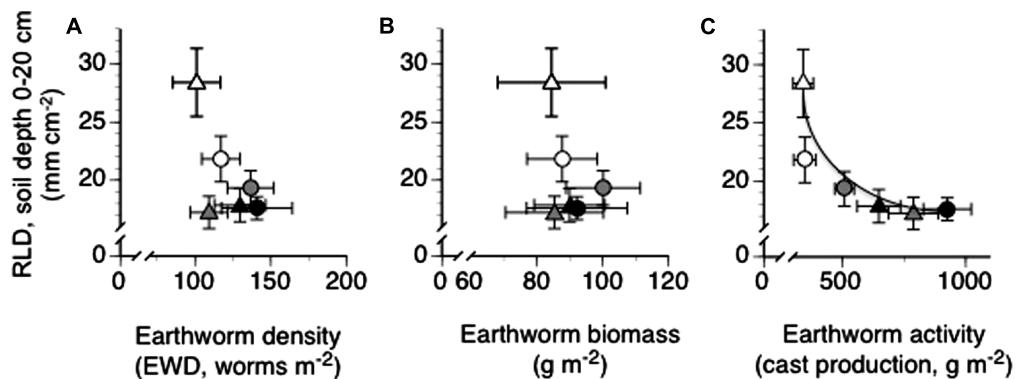


FIGURE 3 | Relationships between mean annual root length density (RLD) and mean annual earthworm density (A), mean annual earthworm biomass (B) and mean cumulative annual earthworm activity – cast production (C) in trenched grassland plots with manipulated earthworm densities (EWDs) under ambient and increased rainfall (means \pm SEs, $n = 5$ plots per experimental treatment) analyzed using both simple linear regression and exponential best fit algorithms (see Materials and Methods) of mean treatment values. Scatter plots with no best fit lines shown indicate a lack of a statistically significant ($P < 0.05$) slope or curve. Symbology for treatment given in legend to Figure 1.

$n = 5$ plots per experimental treatment) analyzed using both simple linear regression and exponential best fit algorithms (see Materials and Methods) of mean treatment values. Scatter plots with no best fit lines shown indicate a lack of a statistically significant ($P < 0.05$) slope or curve. Symbology for treatment given in legend to Figure 1.

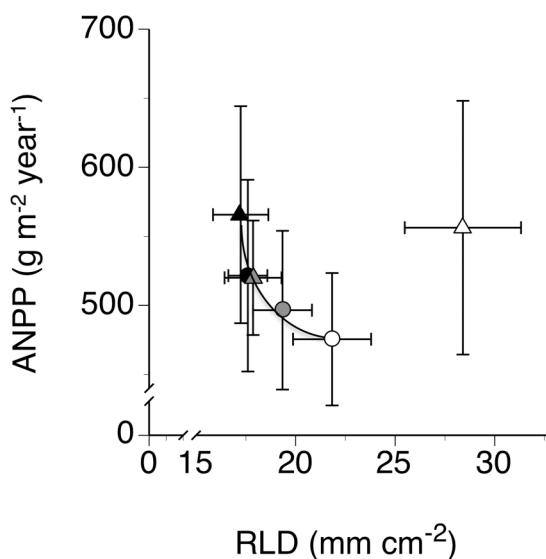


FIGURE 4 | Relationships between mean annual aboveground plant biomass production (ANPP) and mean annual root length density (RLD) in trenched grassland plots with manipulated earthworm densities (EWDs) under ambient and increased rainfall (means \pm SEs, $n = 5$ plots per experimental treatment) analyzed using both simple linear regression and exponential best fit algorithms (see Materials and Methods) of mean treatment values but excluding the treatment mean for the low EWD and increased rainfall (upper rightmost point). Symbology for treatments given in the legend to Figure 1.

Our finding that RLD was substantially lower during the vegetation period (growing season) than during winter, when EWs were less active, suggests a possible wintertime reduction in “negative” EW-induced effects on RLD. Observed temporal fluctuations in RLDs (Figure 1) in all treatments indicate that neither EWD treatments nor rainfall additions altered normal seasonal behavior of root systems of these native plant communities. Not surprisingly, supplemental rain caused deeper infiltration of water into

soils of these plots than occurred in plots receiving only ambient rain. Higher moisture at depth promoted root growth that resulted in higher RLDs at depth in plots receiving additional rain.

The presence of a significant negative relationship between RLD and EW activity (Figure 3C), and the absence of significant relationships between RLD and EWD (Figure 3A) or EW biomass (Figure 3B), indicate that EW bioturbation may be primarily responsible for reductions in RLD under high EWDs (Figure 1). However, the mechanism(s) by which increases (high EWDs) and decreases (low EWDs) in bioturbation may have acted to reduce (high EWDs) or enhance (low EWDs), respectively, RLDs is unclear.

Physical disruption of root growth through bioturbation under high EWDs, and release from disruption under low EWDs, could explain the patterns we observed in RLD. However, according to the principles of shoot:root functional equilibrium (e.g., Brouwer, 1963; Thornley, 1972; Iwasa and Roughgarden, 1984; Lambers et al., 1998), if RLDs were reduced under high EWD because of physical damage to roots, then growth (biomass) should be allocated to roots away from shoots leading to lower ANPP and higher root mass fractions (RMFs). However, our data do not show that this occurred.

A more likely explanation of bioturbation effects supported by our results (Figure 4) involves functional equilibrium growth shifts in response to changes in soil nutrient mineralization and soil nutrient availability (cf., Lee, 1985; Edwards and Bohlen, 1996; Willems et al., 1996; Zaller and Arnone, 1997; Görres et al., 2001; Whalen et al., 2001; Araujo et al., 2004). These results showed (a) no change in ANPP (Zaller and Arnone, 1999a) but reductions in RLDs under high EWDs, and (b) no change in ANPP and increases in RLDs under low EWDs (Figure 1). Thus, our data indicate that the following two treatment response paths likely occurred in our study. (1) High EWDs led to high bioturbation, high microbial and EW (via casting) nutrient mineralization, high plant nutrient availability, low RLDs (reduced need for plants to invest in nutrient foraging organs, – e.g., Fitter, 1994; Hutchings et al., 2000), and low RMFs. (2) Low EWDs led to low

bioturbation, low microbial, and EW nutrient mineralization, low plant nutrient availability, high RLDs (increased need for plants to invest in nutrient foraging organs), and high RMFs (**Figure 4**).

Together, the results of our study conclusively show that increasing EW activity can reduce the size of native grassland root systems in the field that, in the short term, do not appear to affect ANPP. In the longer term, however, it is unclear whether (a) bioturbation from large EW populations could lead to greater nutrient leaching from soils (Bohlen et al., 2004) that lead to reductions of ANPP; or (b) the absence of any increase in ANPP in grassland ecosystems under high EW populations (Zaller and Arnone, 1999a) would continue to provide sufficient carbon inputs to support such large EW populations. Finally, our results suggest that EWDs may mainly affect RLDs and plant community aboveground vs. belowground growth (biomass) allocation under climate changes that include more frequent wetter-than-average growing seasons.

AUTHOR CONTRIBUTIONS

Both authors jointly developed the concept of this experiment, conducted the measurements, analysed the data, and wrote the manuscript.

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Belowground neighbor perception in *Arabidopsis thaliana* studied by transcriptome analysis: roots of *Hieracium pilosella* cause biotic stress

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Root-root interactions are much more sophisticated than previously thought, yet the mechanisms of belowground neighbor perception remain largely obscure. Genome-wide transcriptome analyses allow detailed insight into plant reactions to environmental cues. A root interaction trial was set up to explore both morphological and whole genome transcriptional responses in roots of *Arabidopsis thaliana* in the presence or absence of an inferior competitor, *Hieracium pilosella*. Neighbor perception was indicated by *Arabidopsis* roots predominantly growing away from the neighbor (segregation), while solitary plants placed more roots toward the middle of the pot. Total biomass remained unaffected. Database comparisons in transcriptome analysis revealed considerable similarity between *Arabidopsis* root reactions to neighbors and reactions to pathogens. Detailed analyses of the functional category "biotic stress" using MapMan tools found the sub-category "pathogenesis-related proteins" highly significantly induced. A comparison to a study on intraspecific competition brought forward a core of genes consistently involved in reactions to neighbor roots. We conclude that beyond resource depletion roots perceive neighboring roots or their associated microorganisms by a relatively uniform mechanism that involves the strong induction of pathogenesis-related proteins. In an ecological context the findings reveal that belowground neighbor detection may occur independently of resource depletion, allowing for a time advantage for the root to prepare for potential interactions.

Keywords: *Arabidopsis thaliana*, belowground, biotic interaction, *Hieracium pilosella*, interspecific interaction, microarray, pathogenesis-related proteins, root distribution

INTRODUCTION

Information on neighboring organisms is crucial to a plant, because neighbors are potential interaction partners both in competition and facilitation (Cahill et al., 2010; Faget et al., 2013). Aboveground mechanisms of plant neighbor detection are well understood. The ratio of red to far-red wavelength bands is altered by neighboring plants through light transmission or reflection, and can therefore be perceived as a signal (Ballaré et al., 1990). By contrast, belowground mechanisms of neighbor perception are by far less understood and they are likely to be more complicated. Gersani et al. (2001) demonstrated that plants with root contact engaged in a "Tragedy of the Commons" even when resources per plant are kept constant. Hence, it became clear that roots can perceive each other and as a consequence the plants increase allocation to roots. This finding triggered intense research on root-root interactions that comprises several related ideas and research topics namely (i) distinction of kin and stranger roots (stranger recognition) (ii) distinction of own and foreign roots (self/non-self recognition) (iii) perception of the presence/absence of neighbor roots (neighbor perception). In all of these research directions there are reports on plastic responses of interacting roots fostering those attributes that can

increase belowground competitive ability. Yet, there are also many reports that do not confirm such findings, so that there are several connected topics that need to be followed up for functional mechanisms:

1. Stranger recognition, i.e., differentiation of close or distant genetic relations, was demonstrated for a number of different species. *Cakile edentula* var. *lacustris* increased allocation to roots when sharing a pot with strangers as opposed to siblings (Dudley and File, 2007; Bhatt et al., 2011). *Impatiens pallida* was found to vary in above-ground traits depending on neighbor identity, but only when root contact was given (Murphy and Dudley, 2009). *Arabidopsis thaliana* responded differentially to root exudates from siblings and strangers by forming more lateral roots when treated with exudates from stranger genotypes (Biedrzycki et al., 2010). Likewise, root exuded proteins and metabolites were found to differ depending on neighbor identity (Badri et al., 2012). Root exudates are therefore seen as possible mediators of stranger recognition (Bais et al., 2006; Badri and Vivanco, 2009; Biedrzycki and Bais, 2010). Indeed some components of plant exudates have already been found to be perceived by some specialized

plant species (e.g., Strigolactones perceived by parasitic plants, Koltai et al., 2012). Likewise, Arabionogalactan proteins from root exudates are involved in many interactions of plant roots (e.g., with microbes), though any role of these in plant-plant interactions has as yet not been proven (Nguema-Ona et al., 2013). However, other studies reported that stranger recognition is a rather uncommon phenomenon (Milla et al., 2012; Lepik et al., 2012). Furthermore, *A. thaliana* plants in soil culture were not affected by relatedness to their neighbor (own or stranger genotype) and not even whole genome transcriptome analysis found differences related to neighbor genotypes (Masclaux et al., 2010).

2. In self/non-self recognition there are variable findings concerning reactions and proposed mechanisms: Falik et al. (2003) found that split root peas produced less root biomass when interacting with own roots than with roots from other plants. Only part of this reaction could be attributed to physiological co-ordination, the other part was possibly due to allorecognition. In contrast to this, Semchenko et al. (2007) found that neighbor identity (same clone, different clone) did not influence root reactions neither in *Fragaria vesca* nor in *Glechoma hederacea*. Biedrzycki et al. (2010) found that *A. thaliana* developed shorter roots in hydroponic solution which previously contained a different genotype than in its own hydroponic solution. Since the application of a secretion inhibitor had no effect on this response, it was followed that this case of self/non-self recognition was not mediated by root exudates but must have been due to other mechanisms (Biedrzycki et al., 2010).

Neighbor perception in general is a well-known phenomenon (Callaway, 2002). Altered root placement due to the presence of neighboring roots are well-known indicators of belowground neighbor perception and usually feature avoidance reactions (root segregation, Schenk et al., 1999; Cahill et al., 2010). Bartelheimer et al. (2006) varied presence/absence and species identity of neighbors in a controlled field experiment and found that in the presence of a neighbor, horizontal root distribution was altered and roots were placed toward rather than away from the neighbor (root aggregation). It was followed that such root reactions increase competitive ability and would be triggered by cues other than resource depletion. A recent study by Masclaux et al. (2012) analyzed the transcriptomic outcome of a competition setup with *A. thaliana* allowing for both, intraspecific interaction and intense resource depletion. A number of differentially expressed genes were found enriched in gene networks involved in nutrient deficiency and biotic stress. In detail the experiment revealed that in competing roots especially genes involved in cation transport, sulphur compound metabolic processes, transport processes, and secondary metabolism were affected, as well as many genes responsive to plant hormones. From a list of gene sets responsive to various stresses, the same experiment found enrichments in sets responsive to nitrogen-, phosphorus-, or potassium-starvation, cold-, salt- and wounding-stress, as well as to interaction with different pathogens (Masclaux et al., 2012). On the other hand, Nord et al. (2011) found no evidence for altered root placement due to the presence/absence of neighbors

in common bean and showed that all observed root reactions were mediated by resource availability.

Following the above considerations it is clear that root-root interactions are as yet unpredictable, and the observed modes of reactions are highly diverse. Especially the mechanisms of neighbor perception and distinction are unclear (De Kroon, 2007) and current explanations range from the perception of resource depletion over physiological integration, if present, to mediation by root exudates.

In this paper, we address the topic of interspecific neighbor perception by a presence/absence approach. In order to identify the root-morphological and gene-transcriptional outcome of root interactions, we combined a root interaction experiment and a transcriptome analysis. Single plants of *A. thaliana* were either grown solitarily (control) or in the presence of *Hieracium pilosella*, which was chosen for its competitive inferiority to *A. thaliana*, thus minimizing effects and intensity of resource depletion. Different to the setup chosen by Masclaux et al. (2012), we vary the presence of a heterospecific instead of a conspecific neighbor.

The underlying hypotheses are

1. Heterospecific neighbor roots (represented by the weak competitor *H. pilosella*) induce characteristic modifications in the *A. thaliana*-transcriptome, which go beyond effects attributable to resource depletion.
2. The alterations in transcript levels between roots of solitary plants and those grown with a neighbor provide information on how *A. thaliana* reacts to the presence of heterospecific neighbor roots.

METHODS

EXPERIMENTAL STRATEGY

We used *A. thaliana* as target species to examine impacts of neighbors on both root morphological traits and genome-wide gene transcription. While intraspecific approaches have been reported before (Masclaux et al., 2012), *Hieracium pilosella* was used as an interspecific neighbor species. The species was chosen for the following reasons: gene transcription is strongly dependent on plant size, ontological stage and on environmental factors (von Tienderen et al., 1996). We minimized such factors co-varying with the presence/absence of a neighbor by using the weak competitor *Hieracium pilosella* challenging *A. thaliana*. The reasoning of this is that a weak competitor has little impact on the biomass of the target plant and produces a low degree of resource depletion (cf. Müller and Bartelheimer, 2013). In addition, *H. pilosella* is a rosette plant, so shading did not take place in this experiment. Both species naturally co-occur in European dry sandy grasslands [Sedo-Scleranthetalia, Corynephoretum (Hegi, 1986)]. The two treatments (with/without neighbor) were cultivated with an $n = 18$ to give an $n = 9$ for morphological traits and an $n = 9$ for transcriptional traits.

PLANT CULTURE

The experiment was set up in a climate chamber (short day conditions: 8/16 h; 20/15°C; 50% relative humidity), where photon flux density was $132 \pm 3 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (mean \pm SE measured

at 18 evenly distributed points directly above the pots). Both *A. thaliana* (Col-0) and *H. pilosella* (wild collection near the village of Bad Laer, Lower Saxony, Germany) were germinated on potting soil (Einheitserde Classic, Pikiererde CL T, Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Simtal-Jossa, Germany), pricked out as seedlings and grown for 10 days on a sand/potting soil substrate (2 parts sand/1 part potting soil). Equally developed plants with four leaves were used for the experiment, where plants were cultured in rectangular pots ($13 \times 10 \times 10$ cm l/w/h, compare **Figure 1A**) filled with quartz sand (1.7 kg dry weight; max. grain size 0.7 mm) as substrate. *A. thaliana* seedlings were planted at defined positions either as control or with a plant of *H. pilosella* 3 cm apart (interaction treatment) (**Figure 1A**). 100 ml of nutrient solution were added to the cache pots on a weekly basis ($875 \mu\text{M NO}_3^-$; $125 \mu\text{M H}_2\text{PO}_4^-$; $125 \mu\text{M K}^+$, $250 \mu\text{M Ca}^{2+}$; $65 \mu\text{M Mg}^{2+}$; $65 \mu\text{M SO}_4^{2-}$; $7.5 \mu\text{M Fe}^{3+}$; $22.5 \mu\text{M Cl}^-$; adjusted to pH = 6.0) and additional deionized water (50–100 ml) was supplied according to consumption. By this fertilizer regime we kept resources constant per pot, though not per plant (compare Bartelheimer et al., 2006; Fang et al., 2013 for similar setups with additive designs on interspecific or intergenotypic interactions), taking into account the competitive inferiority of *H. pilosella* to *A. thaliana* (compare **Figure 2** and Müller and Bartelheimer, 2013) to minimize effects of resource depletion. Plants were harvested after 48–50 days after planting and before shoot buds were visible.

We measured horizontal root placement in two adjacent cubes ($3 \times 6 \times 8$ cm l/w/h, compare **Figure 1B**) by cutting the soil beneath the *Arabidopsis* plants in two equal halves. These halves (termed inner and outer cube in the following, **Figure 1**), represented the soil sphere close to the neighbor plant, if present, and the soil sphere on the distant side, respectively. For cutting we used a sharpened metal frame (dimensions according to **Figure 1B**), which was pushed into the soil after cutting off the leaf rosettes. Roots from the cubes as well as from the remainder

in the pot were washed out of the sand by use of a 1 mm sieve. Roots were assigned to species on the basis of differences in color, general morphology, and diameter. *H. pilosella* roots are silverish to yellowish, somewhat wrinkled and comparatively thick and can be easily distinguished from the white, unwrinkled and very fine roots of *Arabidopsis* by visual inspection. Validation of accuracy of the method by repetition examples as well as a photograph of both species' roots are available as supplemental material (**Figure A1**). Roots were spread on a glass recording tray and scanned with 300 dpi in gray shades with an EPSON Perfection V700 Photo scanner with transparency lighting system (Seiko Epson Corporation; Suwa, Nagano; Japan). Scans were analyzed with WinRhizo (V. 2008a Pro; Regent Instruments Canada Inc.; Ottawa; Canada) with a threshold value of 230 for background distinction and filters for objects smaller than 0.001 cm^2 and with a length to width relation smaller than 6.0. All plant parts were oven-dried at 70°C to constant weight.

Horizontal root distribution was expressed as the log₂-transformed ratio of roots in the outer and inner cube (compare **Figure 1**) with

$$\log 2RR = \log_2(R_{\text{inner}}/R_{\text{outer}})$$

with $\log 2RR$: log₂-transformed root ratio; R_{inner} : root parameter in the inner cube (toward the neighbor); R_{outer} : root parameter in the outer cube (away from the neighbor).

The $\log 2RR$ was applied as it is symmetric around zero, meaning that during calculation of mean values, a particular ratio makes the same numerical contribution as its reciprocal value, which would not be the case for untransformed ratios.

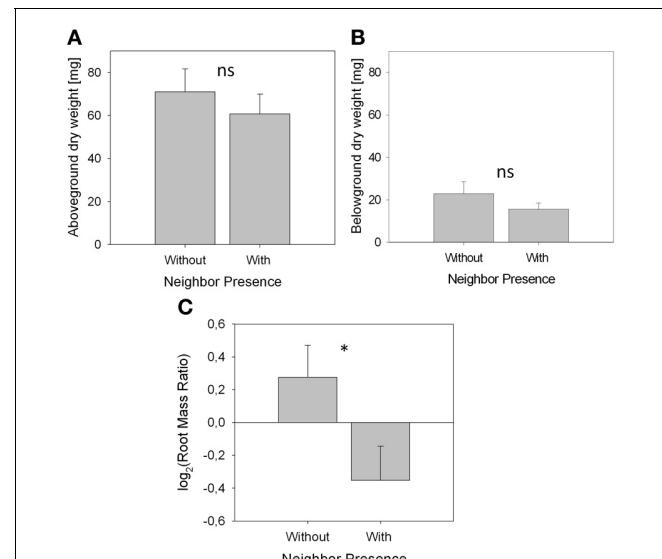
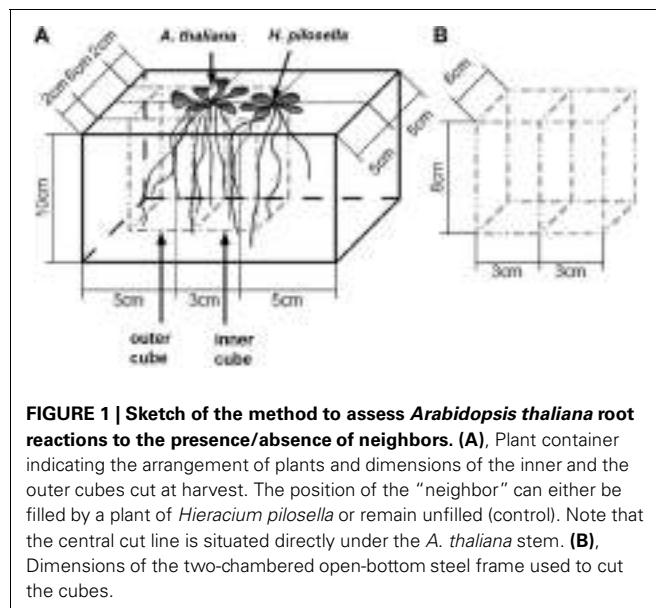


FIGURE 2 | Biomass parameters and root distribution (means \pm SE) of *A. thaliana* without neighbor (control, $n = 11$) and with neighbor present ($n = 9$). Asterisks denote statistically significant differences in t-tests with $*p < 0.05$; ns, not significant. (A), Above-ground biomass. **(B)**, Belowground biomass. **(C)**. Root distribution pattern represented by $\log_2(\text{RR})$ based on root dry weight.

ROOT SAMPLING FOR RNA EXTRACTION

To minimize the circadian impacts on gene activity sampling started 3 h after the climate chamber switched to “day-conditions.” From the nine individual plants per treatment, samples of three plants each were pooled to minimize effects of individual variation and to give a total $n = 3$ of independent biological replicates for molecular analyses. Soil cubes were cut as described above, but only the inner cube (position close to the neighbor if present, compare **Figure 1**) was used further. Cubes of the three respective plants were pooled in a 1 mm sieve and washed out of the sand. Roots were suspended in water and thoroughly assorted by species. *Arabidopsis* roots were cut from the tap-root and stored at -80°C for further use. Processing time per pooled sample was exactly 9 min each. Samples requiring less time, especially controls, where root sorting did not apply, were left to stand until the 9 min were up, to prevent effects of different harvest duration on transcription.

PCR-BASED SCREENING FOR *PHYTOPHTHORA* CONTAMINATIONS

During data analysis (see below) a high degree of resemblance in transcriptome response to roots challenged by *Phytophtthora* spec. made a screen for contamination necessary. Absence of any *Phytophtthora*-contamination was tested by PCR using genus-specific primers FMPH-8b: AAAAGAGAAAGGTGT TTTTATGGA and FMPH-10b: GCAAAAGCACTAAAATTAAATATAA.

GENECHIP MICROARRAY ANALYSIS

Tissues were homogenized with 1.4 mm ceramic beads for 30 s at 6,000 rpm using the Precellys Homogenizer (PEQLAB, Erlangen, Germany), followed by total RNA purification with RNeasy Mini columns (Qiagen, Hilden, Germany) and an Agilent 2100 bio-analyzer quality assessment (Agilent Technologies, Palo Alto, USA). Gene expression profiles were determined by *Arabidopsis* ATH1 Genome Arrays according to the GeneChip 3' IVT Express Kit Manual (Affymetrix, Santa Clara, USA). Two hundred and fifty nanogram of total RNA were used to generate double-stranded cDNA and subsequently Biotin-labeled aRNA. Following fragmentation, aRNA products were hybridized to the array for 16 h at 45°C in a rotating chamber. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000-7G. Tissue homogenization, RNA purification and sample processing were performed at an Affymetrix Service Provider and Core Facility, “KFB—Center of Excellence for Fluorescent Bioanalytics” (Regensburg, Germany).

MICROARRAY DATA ANALYSIS

The MAS5 algorithm of the Affymetrix Command Console Software (AGCC) was used for Single Array Analysis. A global scaling strategy was employed by setting the average signal intensities of all arrays to a target value of 500. All detectable expressed genes were defined using P-, M- or A-calls. Baseline comparison and significance analysis (unpaired *t*-test) were performed in Microsoft Excel.

Significantly regulated genes had to meet the following criteria: (a) a *p*-value smaller than 0.05 and (b) expressed (P-call) in at least two of the six samples.

Microarray data was deposited at the ArrayExpress repository under accession number E-MTAB-1582.

DATA PROCESSING

Relative expression values were calculated and expressed as log₂-transformed mean signal ratios. Functional category scoring (**Table 2**, **Figure 6**) was implemented using MapMan software (Usadel et al., 2005), where all non-significant log₂(mean signal ratios) were set to zero and Wilcoxon Rank Sum tests with Benjamini Hochberg correction were applied.

A signature analysis using Genevestigator software (Hruz et al., 2008) was carried out to depict microarray studies resembling the microarray at hand (termed neighbor-perception microarray in the following). From the list of significantly altered gene-products with at least two P-calls, the 20 genes with the highest as well as the 20 genes with the most negative log₂(mean signal ratios) were used to represent the neighbor-perception-array. The Genevestigator database was narrowed down to include solely studies in lateral roots of *Arabidopsis* wildtype, resulting in a total of 537 transcriptome studies. Manhattan Distance based on the 40 named transcripts was used as a measure to determine the relative similarity of particular arrays from this database to the neighbor-perception-array. For this, Genevestigator software relates an absolute similarity value (in our case based on the 40 mentioned genes) to an average similarity gained over all included experiments. More precisely, if the similarity S_i is defined as $1/d_i$ with d_i the distance of category i to the signature then the relative similarity RS of a category c is calculated following the formula

$$RS_c = S_c / N \sum_{i \neq c} S_i$$

with RS, relative similarity; S , absolute similarity; c , considered category.

Higher values in relative similarity thus indicate higher similarity relative to average similarity (according to documentation on www.genevestigator.com; accessed Feb. 26, 2013).

All other statistics were carried out using SPSS 19 software (SPSS, Chicago, IL, USA).

RESULTS

THE PRESENCE OF *H. pilosella* ROOTS INDUCES ROOT SEGREGATION IN *ARABIDOPSIS*

Biomass of both above- and belowground plant parts as well as total biomass were slightly but not significantly reduced when a neighbor was present (**Figures 2A,B; Table 1**). Root distribution varied significantly between treatments with control plants placing more of their root biomass in the center of the pot [$\log_2(\text{Root Mass Ratio}) > 0$] while plants exposed to neighbors placed more root biomass toward the margin of the pot [$\log_2(\text{Root Mass Ratio}) < 0$; **Figure 2C**]. Measures of root length and root surface area reacted in accordance with root biomass distribution (**Table 1**), indicating that *A. thaliana* placed its roots preferably away from the neighbor (root segregation). Root diameter was overall higher in the neighbor treatment, which was especially

Table 1 | Biomass and root morphological traits (means \pm SE) of *A. thaliana* in control ($n = 11$) and neighbor contact treatment ($n = 9$).

	Root fraction	Control	With neighbor	p-Value	Effect size r
Total biomass [g]	–	94.04 \pm 16.01	76.31 \pm 11.92	>0.05	0.20
Root / shoot ratio	–	0.29 \pm 0.03	0.24 \pm 0.03	>0.05	0.21
Root length [cm]	inner cuboid	548.72 \pm 49.71	389.99 \pm 74.13	>0.05	0.40
	outer cuboid	459.78 \pm 62.06	515.37 \pm 87.01	>0.05	0.12
	Σ	1008.50 \pm 95.43	905.37 \pm 154.91	>0.05	0.14
	log₂(ratio)	0.33 \pm 0.20	-0.46 \pm 0.19	0.013*	0.55
Root surface area [cm ²]	inner cuboid	37.93 \pm 3.58	27.96 \pm 5.41	>0.05	0.35
	outer cuboid	30.43 \pm 3.98	37.51 \pm 6.41	>0.05	0.22
	Σ	68.36 \pm 6.10	65.47 \pm 11.35	>0.05	0.06
	log₂(ratio)	0.38 \pm 0.23	-0.50 \pm 0.20	0.010*	0.56
Root diameter [mm]	inner cuboid	0.2193 \pm 0.0047	0.2285 \pm 0.0048	>0.05	0.30
	outer cuboid	0.2114 \pm 0.0022	0.2354 \pm 0.0066	0.006**	0.74
	mean	0.2153 \pm 0.0029	0.2320 \pm 0.0056	0.021*	0.63

'Inner and outer cube' are soil volumes cut from positions below the plants according to **Figure 1**. Asterisks denote statistically significant differences in T-tests with * $p < 0.05$ and ** $p < 0.01$. Effect sizes were calculated as Pearson's correlation coefficient r with $r \geq 0.1$ showing a small, $r \geq 0.3$ a medium and $r \geq 0.5$ a large effect of the treatment on corresponding variable (Cohen, 1988).

the case in roots that were placed away from the neighbor (outer cube) but less so in roots that were close to the neighbor (**Table 1**).

The part experiment that was sampled for transcriptome analysis (nine plants per treatment) could not be analyzed for root dry weight data, but above-ground biomass was assessed. As in the part experiment used to infer biomass and root distribution data, above-ground biomass was slightly but not significantly reduced when a neighbor was present (control: 80.86 ± 5.49 mg; neighbor treatment: 67.79 ± 3.95 mg; mean \pm SE for $n = 9$; ns in t-test with $p = 0.071$; data not shown).

THE TRANSCRIPTOME ANALYSIS REVEALS "BIOTIC INTERACTIONS" AS A MAJOR MECHANISM OF NEIGHBOR PERCEPTION

Amounts of gene transcripts as measure of gene activity were examined using ATH1-microarrays of *Arabidopsis* roots exposed to roots of *H. pilosella* and of controls without neighbor roots. In a total of 22,810 expressed genes, we found 797 and 652 significantly induced and repressed transcripts, respectively, cf. Table S1, meaning that neighbor contact affected 6.35% of examined genes.

A signature analysis (**Figure 3**) between the present neighbor-perception microarray, represented by the 20 most strongly induced plus the 20 most strongly repressed gene-transcripts, and microarrays from the collection of Genevestigator microarray data base (Hruz et al., 2008) revealed considerable similarity to a number of perturbations. These included exposure of *Arabidopsis* roots to zoospores of the parasitic Oomycet *Phytophthora parasitica* for 2.5 h (cf. Attard et al., 2010) as well as exposure to KCl, heat, osmotica (in this case mannitol), hypoxia, and potassium starvation (**Figure 3**). A more detailed analysis between the neighbor-perception microarray and the named microarray by Attard et al. (2010) revealed that the overall transcriptomic response correlates highly significantly ($r = 0.32$; $p < 0.001$) (**Figure 4A**). It is also pinpointed that among the named 20 most strongly induced gene-transcripts from the neighbor-perception microarray 13 are also induced in the

microarray by Attard et al. (2010) while only five minor mismatches are found (**Figure 4C**). In repressed gene-transcripts twelve matches were found as opposed to six mismatches (**Figure 4B**). To test for any contamination of the plant material used in this study by the plant pathogen *Phytophthora* sp. a PCR-based *Phytophthora* screen was performed (**Figure 5**). This showed that no infection by any *Phytophthora* species was detectable. It is thereby indicated that the observed similarity in gene expression to *Phytophthora* exposition studies is not due to an actual infection by *Phytophthora* pathogens, but to similar plant reactions to root neighbors (and / or associated microorganisms).

To further analyze the similarity between root-root contact and root-pathogen contact a MapMan analysis (Usadel et al., 2005) concerning functional (sub-)categories with involvement in pathogen and pest attack was carried out (**Figure 6**). Three categories with significant regulation were identified. Two were significantly repressed ("Brassinosteroids" in the category of "Hormone Signaling" and "Heat Shock Proteins"). Highly significant induction was found for "PR-proteins", where 14 significantly induced and no repressed gene products were found. Further significant categories were not detected; however, when taken together the four sub-categories subsumed under "Transcription Factors" comprise a considerable number of induced genes products. Two of these belong to the WRKY domain transcription factor family (*At2g30250* and *At1g29280*), eight to the MYB domain transcription factor family (*At5g49620*, *At4g33450*, *At4g09460*, *At3g04030*, *At3g55730*, *At3g27220*, *At1g17950*, *At3g09370*), and four to the MYB-related transcription factor family (*At5g47390*, *At5g01200*, *At1g74840*, *At3g49850*) (**Figure 6**). A further three induced gene products of transcription factors belong to the ethylene-responsive element binding protein family (*At2g23340*, *At3g16770*, *At4g25480*), and one to the C2C2(Zn) DOF zinc finger family (*At3g50410*) (**Figure 6**). A possible involvement of these transcription factors in the activation of PR-proteins is thereby indicated, and

Table 2 | Significantly regulated functional categories.

BIN code	BIN designation	Direction of regulation	Elements	Number of sig. induced genes	Number of sig. repressed genes
1.1	PS.lightreaction	Induced***	136	19	0
1.1.1	PS.lightreaction. Photosystem II	Induced**	55	8	0
1.1.1.2	PS.lightreaction. Photosystem II.PSII polypeptide subunits	Induced***	44	8	0
1.1.40	PS.lightreaction. Cyclic electron flow-chlororespiration	Induced**	8	3	0
1.1.6	PS.lightreaction.NADH DH	Induced**	10	3	0
1.2.4	PS.photorespiration.glycine cleavage	Repressed*	6	0	2
1.3	PS.calvin cycle	Repressed	31	0	6
7	OPP	Repressed**	31	0	5
8	TCA / org. transformation	Repressed***	76	1	11
8.1	TCA / org. transformation.TCA	Repressed ***	41	0	8
8.1.1	TCA / org. transformation.TCA.pyruvate DH	Repressed*	13	0	3
8.1.1.1	TCA / org. transformation.TCA.pyruvate DH.E1	Repressed**	5	0	2
11.1.1	Lipid metabolism.FA synthesis and FA elongation.Acetyl CoA Carboxylation	Repressed*	7	1	3
13	Amino acid metabolism	Repressed***	225	1	20
13.1	Amino acid metabolism.synthesis	Repressed***	153	0	14
13.1.2	Amino acid metabolism.synthesis.glutamate family	Repressed**	8	0	3
13.1.2.3	Amino acid metabolism.synthesis.glutamate family.arginine	Repressed***	7	0	4
13.1.3	amino acid metabolism.synthesis.aspartate family	Repressed*	39	0	6
13.1.3.4	Amino acid metabolism.synthesis.aspartate family.methionine	Repressed*	20	0	4
16.1.2	Secondary metabolism.isoprenoids.mevalonate pathway	Repressed***	16	0	5
16.5.1	Secondary metabolism.sulfur-containing.glucosinolates	Repressed*	54	1	7
16.5.1.1	Secondary metabolism.sulfur-containing.glucosinolates.synthesis	repressed**	31	1	7
16.5.1.1.4	Secondary metabolism.sulfur-containing.glucosinolates.synthesis.shared	Repressed***	3	0	2
17.2.1	Hormone metabolism.auxin.synthesis-degradation	Repressed**	10	0	3
17.3	Hormone metabolism.brassinosteroid	Repressed**	49	0	6
17.3.1	Hormone metabolism.brassinosteroid.synthesis-degradation	Repressed**	31	0	5
17.3.1.2	Hormone metabolism.brassinosteroid.synthesis-degradation. sterols	Repressed***	19	0	5
17.3.1.2.2	Hormone metabolism.brassinosteroid.synthesis-degradation. sterols.SMT2	Repressed***	3	0	2
20.1.7	Stress.biotic.PR-proteins	Induced**	203	13	0
20.2.1	Stress.abiotic.heat	Repressed*	151	1	9
21.99	Redox.misc	Repressed*	6	0	2
23	Nucleotide metabolism	Repressed*	157	3	12
23.1.2	Nucleotide metabolism.synthesis.purine	Repressed*	15	0	3
23.4.1	Nucleotide metabolism.phosphotransfer and pyrophosphatases.adenylate kinase	Repressed*	6	0	2

(Continued)

Table 2 | Continued

BIN code	BIN designation	Direction of regulation	Elements	Number of sig. induced genes	Number of sig. repressed genes
25	C1-metabolism	Repressed***	33	0	7
25.1	C1-metabolism.glycine hydroxymethyltransferase	Repressed*	6	0	2
29	Protein	Repressed**	3123	109	133
29.1.20	Protein.aa activation.phenylalanine-tRNA ligase	Repressed***	3	0	2
29.2	Protein.synthesis	Repressed***	515	19	53
29.2.1	Protein.synthesis.ribosomal protein	Repressed***	371	14	36
29.2.1.1.1	Protein.synthesis.ribosomal protein.prokaryotic.chloroplast.30S subunit	Induced*	25	5	1
29.2.1.1.3.1	Protein.synthesis.ribosomal protein.prokaryotic.unknown organellar.30S subunit	Repressed*	14	1	4
29.2.1.2	Protein.synthesis.ribosomal protein.eukaryotic	Repressed***	236	1	25
29.2.1.2.1	Protein.synthesis.ribosomal protein.eukaryotic.40S subunit	Repressed*	88	0	7
29.2.1.2.2	Protein.synthesis.ribosomal protein.eukaryotic.60S subunit	Repressed***	148	1	18
29.2.1.2.2.19	Protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L19	Repressed**	4	0	2
29.2.1.2.2.57	Protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L7A	Repressed**	5	0	2
29.2.2.50	Protein.synthesis.misc ribosomal protein.BRIX	Repressed*	6	0	2
29.2.3	Protein.synthesis.initiation	Repressed**	84	3	12
29.5.11.20	Protein.degradation.ubiquitin.proteasom	Repressed***	53	0	13
29.5.11.3	Protein.degradation.ubiquitin.E2	Induced*	37	6	1
29.5.2	Protein.degradation.autophagy	Induced*	20	4	0
29.5.3	Protein.degradation.cysteine protease	Induced*	87	9	1
30.9	Signaling.lipids	Induced***	5	4	0
30.99	Signaling.unspecified	Repressed*	7	0	2
35	Not assigned	Induced***	7639	286	139
35.1.1	Not assigned.no ontology.ABC1 family protein	Induced**	11	3	0
35.2	Not assigned.unknown	Induced***	5386	208	89
35.3	Not assigned.disagreeing hits	Induced*	63	7	1

Asterisks denote statistically significant differences in Wilcoxon Rank Sum tests (Benjamini Hochberg corrected) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. BIN code and BIN designation are numerical codes and short descriptions of hierarchically organized functional categories according to the MapMan tool (Usadel et al., 2005).

in fact, at least one of these induced transcription factors [*At3g16770*, *ERF72*, \log_2 (signal ratio) = 1.56] has previously been shown to induce certain pathogen responsive genes (Ogawa et al., 2005). Likewise, the involvement of transcription factors of the WRKY family (see above) in pathogen response has repeatedly been described [reviewed by Rushton et al. (2010)].

General transcriptional responses were evaluated on the basis of functional categories and sub-categories (Table 2). The significant regulations of functional categories including all sub-categories were analyzed on the basis of the induction and repression (positive and negative \log_2 -ratios) of all elements assigned to the respective functional (sub-)category according to the MapMan system [(sub-)BIN, Table 2]. Most BINs and subBINs were not significantly regulated, a considerable number was repressed and a smaller number was induced. The

repressed BINs included OPP, TCA, and lipid metabolism. The functional category of photosynthesis (PS) is divided into the induced subBINs connected to light reaction and two repressed subBINs in photorespiration and Calvin cycle. Plastids in roots conduct no light reaction, and 13 out of 19 induced genes assigned to PS.lightreaction were plastid-encoded. Without light such plastid encoded genes can still be transcribed to considerable amounts, but the translation of the resulting mRNA is highly dependent on light and the biogenesis of plastids to chloroplasts. In roots, the according mRNA is therefore not translated into proteins (Mayfield et al., 1995; Spermulli, 2000). Some functional categories connected to cell energy status were repressed, e.g., OPP, TCA, and “Acetyl CoA carboxylation” in “lipid metabolism.” The few induced BINs include “Pathogenesis Related Proteins” in “Biotic Stress” as well as “Lipids” in the BIN “Signaling.”

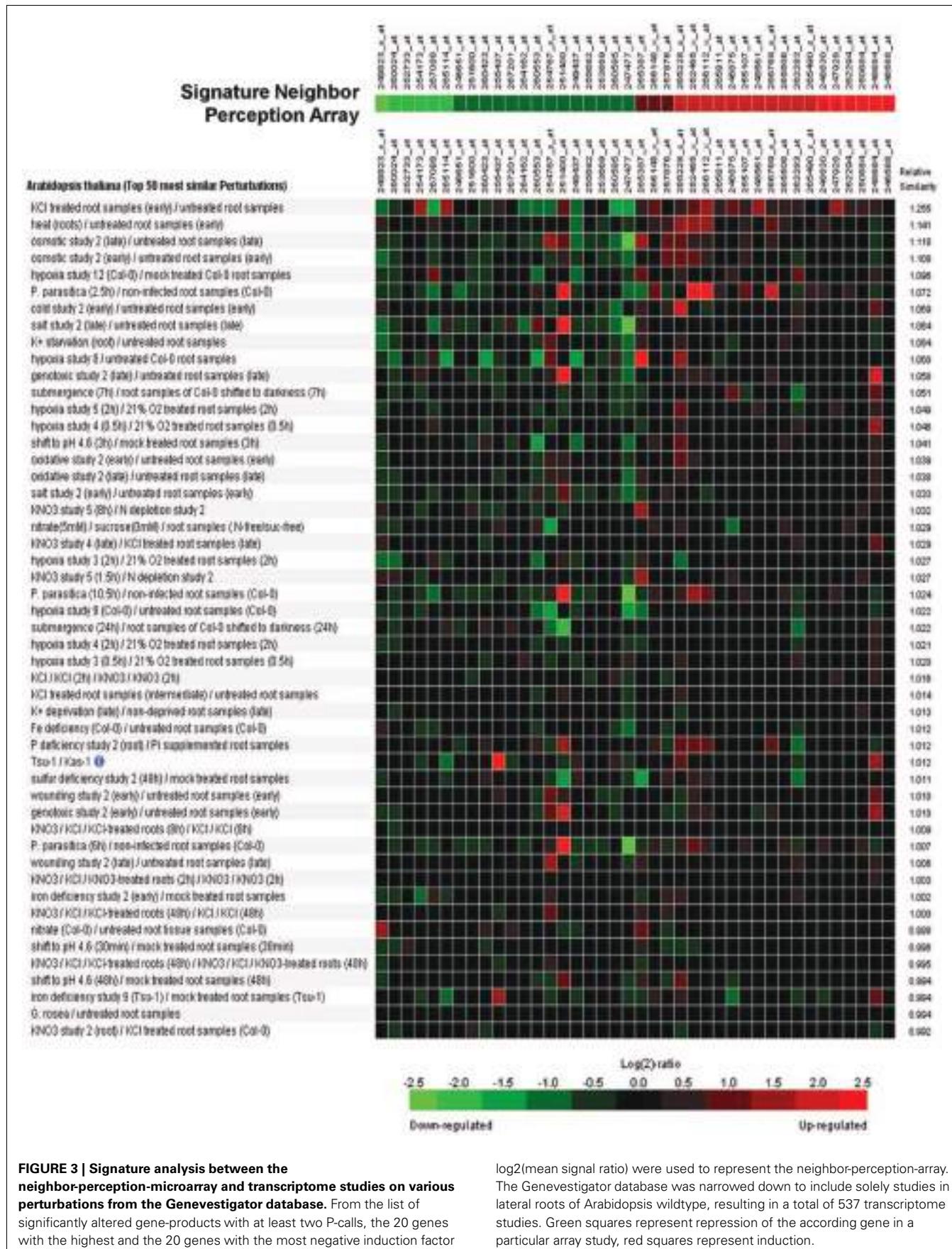
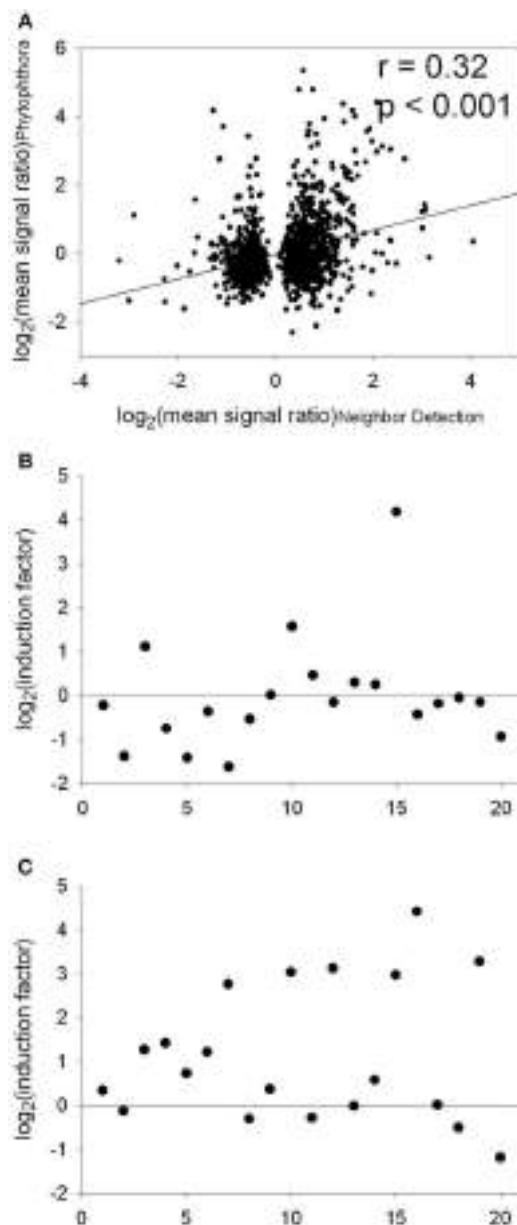


FIGURE 3 | Signature analysis between the neighbor-perception-microarray and transcriptome studies on various perturbations from the Genevestigator database. From the list of significantly altered gene-products with at least two P-calls, the 20 genes with the highest and the 20 genes with the most negative induction factor

log₂(mean signal ratio) were used to represent the neighbor-perception-array. The Genevestigator database was narrowed down to include solely studies in lateral roots of *Arabidopsis* wildtype, resulting in a total of 537 transcriptome studies. Green squares represent repression of the according gene in a particular array study, red squares represent induction.



COMPARISON TO INTRASPECIFIC COMPETITION

Interestingly, a recent study by Masclaux et al. (2012) examined the transcriptional outcome of intraspecific competition in roots. Our comparison included all genes that were mutually

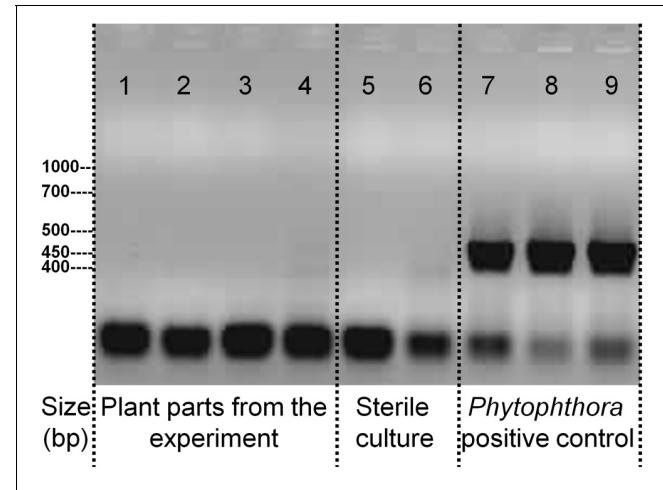
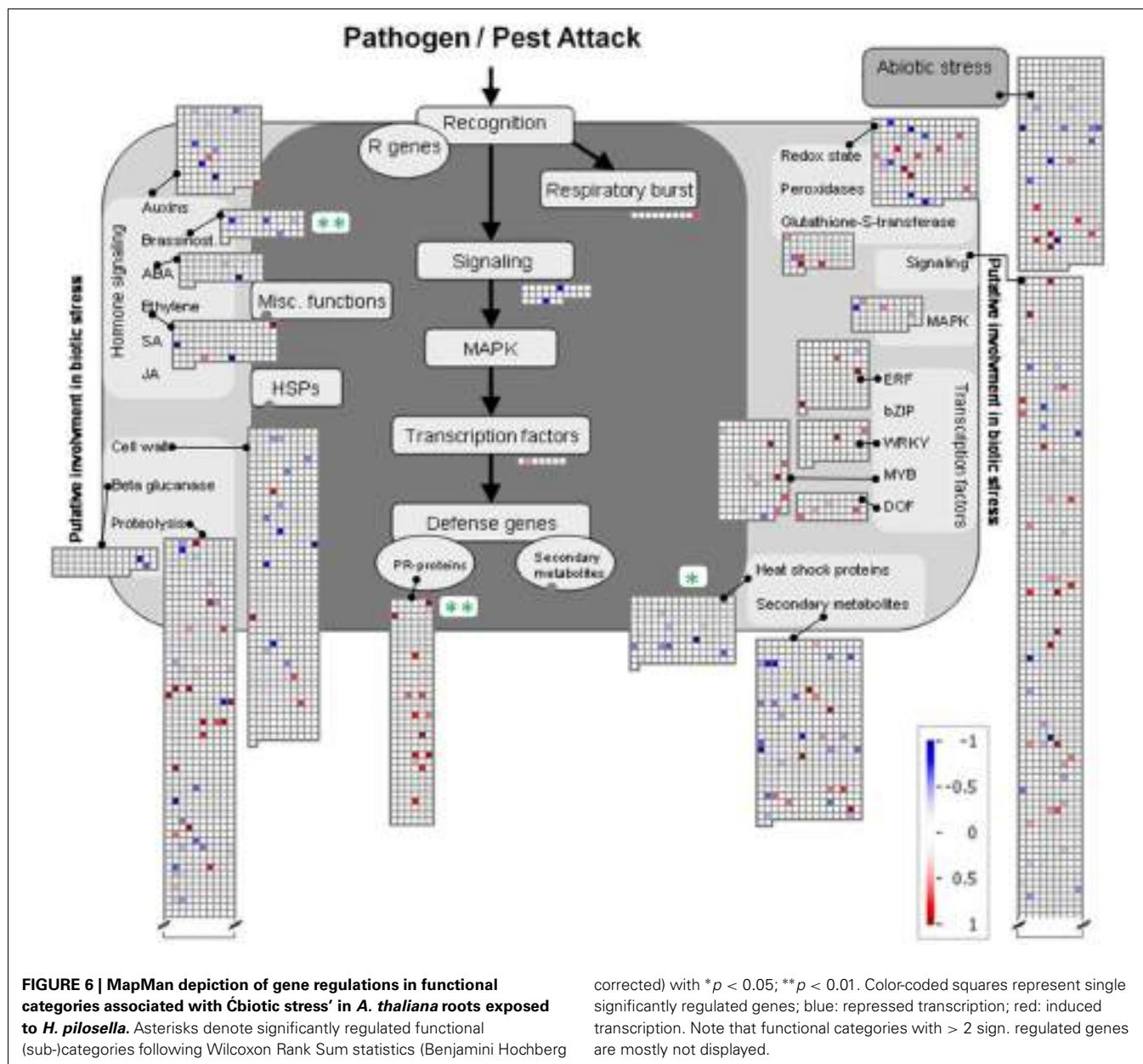


FIGURE 5 | PCR-test for the presence of DNA specific to the parasitic Oomycet genus *Phytophthora* in the experiment. Applied PCR-primers are specific to the entire genus. Displayed samples are from the experiment itself with 1. *A. thaliana* roots, grown in presence of *H. pilosella*, 2. *A. thaliana* leaf, grown in presence of *H. pilosella*, 3. *H. pilosella* roots, 4. *A. thaliana* roots, grown in absence of *H. pilosella*; or are samples raised under sterile conditions with 5. *A. thaliana* roots, 6. *H. pilosella* roots; or are inoculated samples from separate cultures with 7. *Phytophthora* (pure culture), 8. *A. thaliana* roots, 9. *H. pilosella* roots.

evaluated in both arrays ($N = 13, 150$), where numbers were reduced especially by cases with too few presence calls in our array. The number of genes exclusively induced in our array was $N = 600$, in the array by Masclaux et al. (2012) it was $N = 117$, and the overlap was $N = 18$ (Figure 7). This number of overlapping induced genes was significantly higher than would be expected from pure proportionality, i.e., in the absence of any concordant reaction (value expected from pure proportionality would have been 6.34; $p = 0.028$). In the case of repressed genes our array contained $N = 580$ exclusive cases, the array by Masclaux et al. (2012) contained $N = 62$ exclusive cases and the number of overlapping genes was $N = 6$ [not significantly different from number expected from proportionality (3.03)].

The list of genes that are concordantly induced or repressed, respectively, in both of these arrays possibly reflects those genes in *Arabidopsis* roots that are in general responsive to interaction with other plants. Reactions to nutrient depletion might be included as a secondary effect of neighboring roots. The induced genes (Table 3) comprise two genes from the functional category “biotic stress-PR-proteins” (BIN 20.1.7) as well as two genes from the functional category “signaling.receptor kinases” (BIN 30.2). Both among the induced and in repressed genes (Tables 3, 4) we find entries that are involved in the regulation of transcription (BIN 27.3; RNA.regulation of transcription). Therefore, both in intraspecific competition (Masclaux et al., 2012) and in interspecific interaction with *H. pilosella* genes with functions in pathogen response are induced, while only a smaller common core of these is a true commonality between the two setups.



DISCUSSION

ALTERATION OF ROOT DISTRIBUTION AS AVOIDANCE OF POTENTIAL COMPETITION

We found that in the presence of a neighbor, *A. thaliana* places more roots toward the margin than toward the center of the pot (Table 1, Figure 2), i.e., the neighbor root is avoided by root segregation (Schenk et al., 1999). This reveals unambiguously that the neighbor plant was detected and had a high impact on root distribution with an effect size of $r > 0.5$ (Table 1), which allows us to further analyze the mechanisms of neighbor perception (see below). It also reveals that in this experiment *Arabidopsis* avoids intense root overlap and potential competition. A spectrum from avoidance to confrontation of neighbor root systems has been found for different species in different experiments (Schenk et al., 1999 for segregation, Bartelheimer et al., 2006;

Semenchenko et al., 2007 for aggregation). To discuss the question what was the cause of the observed root segregation when a neighbor was present, at least two non-exclusive answers are possible [also reviewed by Hodge (2012)]. The first possibility is the perception of the neighbor by mechanisms beyond resource depletion. The second involves resource depletion due to consumption by neighbor roots. As for the perception of neighbor roots, they may be attributed to physical contact (Mahall and Callaway, 1991) or to root exudates (Bais et al., 2006) or to associated microbial organisms and substances of microbial origin (Steenhoudt and Vanderleyen, 2000) (a detailed discussion on possible functional mechanisms is found in the paragraphs below). As for perception of local resource depletion as the second possible explanation (Schenk et al., 1999; Nord et al., 2011), it is known that roots often proliferate less, where resources

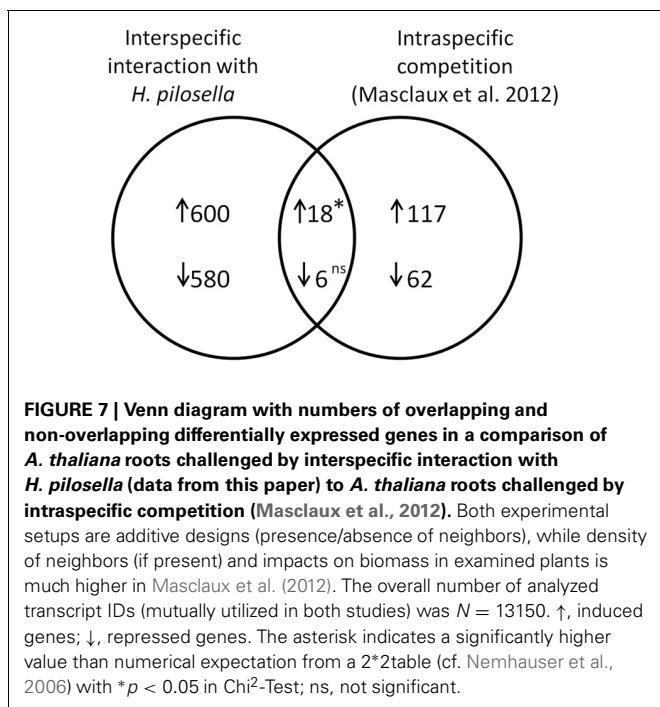


FIGURE 7 | Venn diagram with numbers of overlapping and non-overlapping differentially expressed genes in a comparison of *A. thaliana* roots challenged by interspecific interaction with *H. pilosella* (data from this paper) to *A. thaliana* roots challenged by intraspecific competition (Masclaux et al., 2012). Both experimental setups are additive designs (presence/absence of neighbors), while density of neighbors (if present) and impacts on biomass in examined plants is much higher in Masclaux et al. (2012). The overall number of analyzed transcript IDs (mutually utilized in both studies) was $N = 13150$. ↑, induced genes; ↓, repressed genes. The asterisk indicates a significantly higher value than numerical expectation from a 2^*2 table (cf. Nemhauser et al., 2006) with $p < 0.05$ in Chi²-Test; ns, not significant.

are scarce and allocate more growth to where resource availability is higher (Gersani et al., 1998; Hodge, 2004). Indeed, some signs for moderate resource depletion were found, since the signature analysis (**Figure 3**) detected similarities between the neighbor-perception microarray and experiments on potassium starvation as well as (to a lower extend) on other nutrient deficiencies. Consequently, local resource depletion may well have played a role as cue for the presence of a neighbor in the study at hand. However, signs of resource competition like reduced plant biomass or affected root/shoot ratios were weak, as indicated by small effect sizes of $r = 0.20$ and 0.21 , respectively, and non-significant (**Figure 2, Table 1**). It is thus unlikely that resource depletion alone caused the described reactions. In fact, a recent study by Cahill et al. (2010) on *Abutilon theophrasti* found resource availability and neighbor perception to act in concert with segregation occurring solely when both, neighbor presence and uniform distribution of resources, were given. In the case of our study, resource availability was not varied between treatments and depletion likely was low. Considering the results by Cahill et al. (2010) it is therefore well conceivable that *A. thaliana* in our study reacted in a similar information-integrating manner as *Abutilon theophrasti*: segregative root placement, when a neighbor is present and resources are relatively homogenously distributed.

Under natural conditions, root segregation as was found in our experiment is likely an essential strategy for annual species to maximize resource access. Detecting the neighbor early on will optimize this process, because this allows reactions even before the, often negative, interaction takes place. With neighbor perception as a prerequisite to optimize a plant's growth strategy, it is clear that we need to find out more about its mechanisms.

BELOWGROUND NEIGHBOR PERCEPTION AND TRANSCRIPTOME ANALYSIS

We found that the presence of a neighbor led to a high number of differentially transcribed genes Table S1. This allows the application of bioinformatic approaches to the topic of interspecific neighbor perception. These analyses included the detection of significantly regulated functional categories as well as a signature analysis for similarity with a broad variety of microarrays that cover the impact both of biotic and abiotic environmental factors. In addition, our data allows for comparisons of differentially expressed genes to those found in previous studies on similar topics (Broz et al., 2008; Biedrzycki et al., 2011; Masclaux et al., 2012).

FUNCTIONAL CATEGORIES: PATHOGEN RESPONSE GENES INDUCED DURING NEIGHBOR PERCEPTION

The most striking finding from the transcriptome analysis was the induction of genes coding for pathogenesis-related proteins (**Table 2, Figure 6**). These genes are known to respond to a number of different biotic stresses (Stintzi et al., 1993; Sels et al., 2008), but so far little is known about their role in plant neighbor perception. These PR proteins are a clear indication that not resource depletion but biotic signals mediated the detection of *H. pilosella* roots. We suggest that such biotic cues could be either the neighbor root itself, including its exudates, or it could be microorganisms associated to the neighbor root.

The presence of *Phytophthora* sp. itself was ruled out (**Figure 5**), which was pivotal after the detection of a high transcriptional similarity to a setup that challenged roots with *Phytophthora* oospores. Still, non-pathogenic microorganisms will have been present due to the non-sterile growth conditions of our setup. *H. pilosella* is a species strongly colonized by Vesicular Arbuscular Mycorrhiza (VAM) when raised on sand (personal observation), while *A. thaliana* is non-mycorrhizal. VAM as an agent of neighbor perception is a possibility, though support from the literature for this notion is weak: VAM are known to repress rather than induce genes coding for PR-proteins (Ginzberg et al., 1998; Shaul et al., 1999), which would be the opposite of what was observed in our transcriptome analysis. In addition the signature analysis (**Figure 3**) which compares a variety of arrays with the one at hand found only weak resemblance to one array that tested for the effect of the VAM species *Gigaspora rosea* on *Arabidopsis* roots (**Figure 3**). Similar to mycorrhiza, bacteria colonizing *H. pilosella* roots would be encountered by *A. thaliana* roots and could have an impact on gene expression. In fact, different plant species are known to have specific root microflora (Hartmann et al., 2009). Well known examples of bacteria impacting on plant gene activity are *Pseudomonas fluorescens* inducing systemic resistance to pathogens (Pieterse et al., 1996, 1998; Léon-Kloosterziel et al., 2005) and *Bacillus subtilis* (Rudrappa et al., 2008) and *Paenibacillus alvei* (Tjamos et al., 2005) being involved in the induction of PR-proteins in *Arabidopsis*.

The second explanation is non-exclusive with the one above and is the perception of the neighbor root itself and/or its exudates. The possibility that exudates cause the pronounced transcriptomic effect and therefore play an essential role during

Table 3 | List of induced transcripts regulated in concordance with transcriptional responses to intraspecific competition as examined by Masclaux et al. (2012).

Transcript ID	NAME and/or Description	Gene information		Ratio (log2)	
		BIN code	BIN name	Neighbor perception (this study)	Intraspecific competition (Masclaux et al., 2012)
At1g71697	CHOLINE KINASE 1	11.3.2	Lipid metabolism.Phospholipid synthesis.choline kinase	0.60	0.80
At5g40170	RECEPTOR LIKE PROTEIN 54	20.1.7	Stress.biotic.PR-proteins	0.98	1.16
At1g72920	Toll-Interleukin-Resistance domain family protein	20.1.7	Stress.biotic.PR-proteins	0.77	3.66
At3g19010	2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein	21.2	Redox.ascorbate and glutathione	0.56	1.17
At2g29720	CTF2B / monooxygenase activity, oxidoreductase activity	26.7	Misc.oxidases-copper. flavone etc.	0.73	1.64
At5g47390	MYB-like transcription factor family protein	27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	0.39	0.75
At3g59700	LECTIN-RECEPTOR KINASE 1 / Receptor kinase-like protein family	30.2.19	Signaling.receptor kinases.legume-lectin	0.71	1.54
At1g53440	Leucine-rich repeat transmembrane protein kinase	30.2.8.2	Signaling.receptor kinases.leucine rich repeat VIII-2	0.77	0.96
At5g61210	SOLUBLE N-ETHYLMALEIMIDE-SENSITIVE FACTOR ADAPTOR PROTEIN 33	31.4	Cell.vesicle transport	0.637	1.076
At1g59910	Actin-binding formin homology 2 family protein	35.1.20	Not assigned.no ontology.formin homology 2 domain-containing protein	0.358	1.919
At5g28630	Unknown protein with unknown function	35.1.40	Not assigned.no ontology.glycine rich proteins	0.482	2.681
At3g55840	Hs1pro-1 protein / ortholog of sugar beet HS1 PRO-1 2	35.2	Not assigned.unknown	1.166	3.441
At1g05340	Unknown protein with unknown function	35.2	Not assigned.unknown	1.060	0.933
At3g45730	Unknown protein with unknown function	35.2	Not assigned.unknown	0.972	1.38
At3g51890	Clathrin light chain protein	35.2	Not assigned.unknown	0.671	0.972
At2g28570	Unknown protein with unknown function	35.2	Not assigned.unknown	0.568	0.883
At2g42950	Magnesium transporter CorA-like family protein	35.2	Not assigned.unknown	0.426	0.783
At2g32210	Unknown protein with unknown function	35.2	Not assigned.unknown	0.291	2.342

neighbor perception is supported by results from the related field of kin recognition research. A study by Biedrzycki et al. (2010) demonstrated exudates from strangers and siblings to cause different root growth in *A. thaliana*. A subsequent transcriptome study found considerable impact of kin vs. stranger exudates on gene activity (Biedrzycki et al., 2011) and, even more interestingly, also found hints for the involvement of PR-genes in

this process. Biedrzycki et al. (2011) found three genes with roles in pathogen defence among their 20 genes most induced by foreign exudates (*PDF1.3*, *PDF1.2b*, *CA1*). While these three genes were not significantly induced in our data set, some other 13 PR-coding genes were, and so was the entire functional category “PR proteins” (Figure 4). PR-proteins have different antimicrobial functions (antifungal, anti-Oomycete, chitinases,

Table 4 | List of repressed transcripts regulated in concordance with transcriptional responses to intraspecific competition as examined by Masclaux et al. (2012).

Transcript ID	Description (name)	Gene information		Ratio (log2)	
		BIN code	BIN name	Neighbor perception (this study)	Intraspecific competition (Masclaux et al., 2012)
At5g10130	Pollen Ole e 1 allergen and extensin family protein	20.2.99	Stress.abiotic.unspecified	-0.951	-1.031
At5g62340	Plant invertase/pectin methylesterase inhibitor superfamily protein	26.18	Misc.invertase/pectin methylesterase inhibitor family protein	-1.218	-0.805
At3g49940	LOB (Lateral organ boundaries) DOMAIN-CONTAINING PROTEIN 38	27.3.37	RNA.regulation of transcription.AS2, lateral organ boundaries gene family	-0.939	-0.776
At3g04070	NAC domain containing protein 47	27.3.27	RNA.regulation of transcription.NAC domain transcription factor family	-0.334	-1.269
At5g60680	Unknown protein with unknown function	35.2	Not assigned.unknown	-0.359	-1.325
At3g19680	Unknown protein with unknown function	35.2	Not assigned.unknown	-0.431	-0.965

(1 → 3)-β-D-glucanases and others) but no clarified functions in plant-plant interaction. Their induction is the outcome of diverse and partly interconnected signal transduction pathways that involve different receptor proteins, plant hormones and transcription factors (Hammond-Kosack and Jones, 2000; Sels et al., 2008). Apparently, the induction of PR-proteins can thus be seen as a common outcome of pathogen perception and plant neighbor perception. This similarity, and especially the similarity between this data set and *Phytophthora*-affected transcriptome (Figures 3, 4), points to common features during the perception of microbes and neighboring plants.

Irrespective of whether the neighbor is perceived directly (root itself) or indirectly (associated microorganisms), the outcome for the *Arabidopsis* root is a reaction to the plant neighbor involving PR-proteins.

What cue elicits this reaction cannot be answered here. One might speculate about a possible role of oligogalacturonide (OGA) fragments from decomposition of neighbor roots' mucigels (Reymond et al., 1995; Ridley et al., 2001), because OGAs are known to bind to receptors located in the plasma membrane and to elicit plant defense responses including the induction of PR-proteins (Ridley et al., 2001; Aziz et al., 2007). Also, depending on concentration and species, they can both decrease or increase root growth (Bellincampi et al., 1993; Hernández Mata et al., 2006; Camejo et al., 2011). Considering the speculative nature of this, the exact molecular mechanisms of neighbor perception remain to be clarified.

GENES RESPONDING TO ROOT INTERACTION

Belowground interactions involve a lot of complex processes, most of which are hard to study. It would greatly facilitate research in root interaction to have good knowledge of genes that respond to interaction with neighbor roots. Data bases today hold no functional categories like plant-plant interaction, even though

plant-plant encounters are ecologically highly relevant for plant fitness. Data presented by this paper show that root interaction affects a multitude of genes (6.4% in this case). The comparison of our data to the microarray data of Masclaux et al. (2012) (Figure 7; Tables 3, 4) basically represents a comparison of reactions in roots challenged by intraspecific competition on the one hand vs. interspecific interaction with *H. pilosella* on the other hand. This comparison also involves differences in the intensity of competition [strong in the setup by Masclaux et al. (2012), weak in our setup] and likely also in nutrient depletion [presumably strong in the setup by Masclaux et al. (2012), presumably weak in our setup]. In spite of these discrepancies, it was found that in induced genes there were more commonalities than would be expected just from proportionality. This makes sense biologically, because it appears that to some extent the perception of neighbors involves the same set of genes irrespective of whether the neighbor is of the same or of a different species. The transcripts concordantly regulated between the two setups might be considered a core group regulated in response to a broad range of neighbors. This core group comprises two signaling receptor kinases, genes involved in the regulation of transcription (incl. an induced myb-like transcription factor), and two pathogenesis-related proteins (namely the RECEPTOR LIKE PROTEIN 54 and a Toll-Interleukin-Resistance domain family protein) (Tables 3, 4). Though this data can merely base on two studies, it is noteworthy that genes from these groups have a principal potential to perceive and transduce stimuli (by the signaling receptor kinases) and to regulate reactions (by the transcription factor) that include the induction of the named pathogenesis-related proteins.

At the same time, there are also large differences between the two data sets. While to a large extent these are likely due to the mentioned discrepancies in competition intensity and resource depletion between setups, it is also evident that some

of the differences do potentially result from neighbor identities and reactions thereupon. A picture emerging from this is that in general the perception of neighbor roots involves a multitude of genes including numerous genes coding for pathogenesis-related proteins. A relatively small common core of genes is regulated during the very general perception of a neighbor, irrespective of its identity [e.g., the *RECEPTOR LIKE PROTEIN 54* (At5g40170)], while for the larger part of genes regulation depends on the specific identity of the neighbor.

To date, transcriptome data sets on interspecific root interaction are very rare, and comparisons are hampered by methodological differences. In a study on *Centaurea maculata* growing with either a strong or a weak competitor Broz et al. (2008) found 43 genes with significantly regulated transcripts. Among these, 26 genes were induced in the presence of a weak competitor, which is a situation remotely resembling our set-up. Just one of these genes was also significantly regulated in our data: the adenine nucleotide translocator, COR13 (At3g08580) was significantly repressed in both studies. Clearly, more studies with different species combinations are needed to identify genes that are typically involved in neighbor perception. A future challenge will be to establish sound knowledge on genes that respond to plant-plant interactions and—equally important—which genes respond differentially depending on the type of interaction, e.g., from competitive to facilitative, with distant to close relatives, under stressful to benign conditions. Further studies will also need to prove the robustness of these findings under different environmental conditions, e.g., different soil types. On the one hand it is clear that the perception of neighbors *per se* is a very common phenomenon, as reactions in root distribution caused by neighbor plants have been found by different studies on soil substrates as diverse as sand (Bartelheimer et al., 2006; Mommer et al., 2010), sand/topsoil mixture (Cahill et al., 2010), sand/loam/potting soil mixture (Mommer et al., 2010), agricultural soils like anthrosol (Li et al., 2006), and even artificial substrates like gel growth medium (Fang et al., 2013). However, the details and generality of our main finding (the similarity between transcriptional responses to plant neighbors

and responses to pathogens) remain to be corroborated by further studies. The comparison of sterile to non-sterile conditions provides yet another approach to further elucidate the role of microorganisms in this context.

CONCLUSIONS

For the inventory of its biotic environment a plant needs detection mechanisms to allow optimized morphological and physiological responses. The belowground presence of a neighbor largely impacts on root distribution and gene transcription. Transcriptome analyses in roots reveal pronounced similarities between responses to plant neighbors and responses to pathogens. Transcriptome comparisons between setups with intra- and interspecific interaction corroborate this finding and bring forward a core of consistently involved genes. This hints to conserved mechanisms and conserved responses to a broad range of biotic taxa encountered in the rhizosphere. In an ecological context it is revealed that a root may detect a neighbor directly or indirectly (by associated microfloras) without or before detecting resource depletion, which can save valuable time for the plant to prepare for potential interactions. Future studies need to explore transcriptional differences brought about by different neighbors and in different species.

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SUPPLEMENTARY MATERIAL

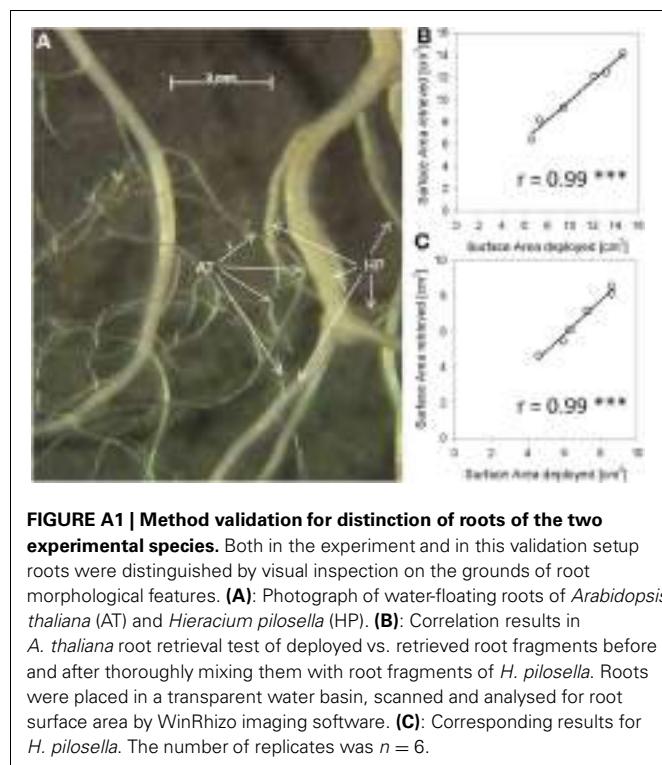
The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Functional_Plant_Ecology/10.3389/fpls.2013.00296/abstract

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APPENDIX



Space sequestration below ground in old-growth spruce-beech forests—signs for facilitation?

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Scientists are currently debating the effects of mixing tree species for the complementary resource acquisition in forest ecosystems. In four unmanaged old-growth spruce-beech forests in strict nature reserves in southern Sweden and northern Germany we assessed forest structure and fine rooting profiles and traits (≤ 2 mm) by fine root sampling and the analysis of fine root morphology and biomass. These studies were conducted in selected tree groups with four different interspecific competition perspectives: (1) spruce as a central tree, (2) spruce as competitor, (3) beech as a central tree, and (4) beech as competitor. Mean values of life fine root attributes like biomass (FRB), length (FRL), and root area index (RAI) were significantly lower for spruce than for beech in mixed stands. Vertical profiles of fine root attributes adjusted to one unit of basal area (BA) exhibited partial root system stratification when central beech is growing with spruce competitors. In this constellation, beech was able to raise its specific root length (SRL) and therefore soil exploration efficiency in the subsoil, while increasing root biomass partitioning into deeper soil layers. According to relative values of fine root attributes (rFRA), asymmetric below-ground competition was observed favoring beech over spruce, in particular when central beech trees are admixed with spruce competitors. We conclude that beech fine rooting is facilitated in the presence of spruce by lowering competitive pressure compared to intraspecific competition whereas the competitive pressure for spruce is increased by beech admixture. Our findings underline the need of spatially differentiated approaches to assess interspecific competition below ground. Single-tree approaches and simulations of below-ground competition are required to focus rather on microsites populated by tree specimens as the basic spatial study area.

Keywords: *Fagus sylvatica*, *Picea abies*, root system stratification, fine root biomass (FRB), fine root length (FRL), fine root surface area index (RAI), specific root length (SRL), specific root surface area (SRA)

INTRODUCTION

There is an on-going scientific debate about the effects of mixing tree species on forest ecosystem functioning in terms of productivity and resource acquisition. Most of the recent publications indicate a superiority of mixed-species stands compared to pure stands (Chen et al., 2003; Kelty, 2006; Erickson et al., 2009; Lei et al., 2009). For mixed forests with European beech (*Fagus sylvatica* L.) and Norway spruce [*Picea abies* (L.) Karst], Pretzsch and Schütze (2009) have presented a thorough analysis based on material from Southern Bavaria (Germany) presenting evidence for overyielding of the mixed stands above ground and growth acceleration of Norway spruce due to niche separation. These findings are supported by the previous review of Knoke et al. (2007) reporting a productivity increase compared to monospecific spruce and beech stands, but also higher stability against disturbances like storms, which was previously found by Schütze et al. (2006). All these considerations are mainly focused on species interaction and productivity aspects above ground, while root-system interactions play a minor role.

However, studies on fine root distribution in mixed forest with European beech (*Fagus sylvatica* L.) show evidence of species

interaction and its effects on tree performance and stability. Several studies in managed mature beech-spruce mixtures in Germany and Austria found indications for vertical root system stratification (Rothe, 1997; Schmid, 2002; Bolte and Villanueva, 2006) supporting the idea of complementary resource acquisition of mixed spruce and beech stands both above- and below-ground. Lei et al. (2012a) also found a shift of fine root allocation in Norway spruce in the presence of other tree species studying fine root competition in a multispecies experiment with younger trees (BIOTREE, Germany). In contrast, publications from multispecies broadleaved forests with beech at Hainich National Park (Germany) found no evidence for root system stratification and overyielding below ground (Meinen et al., 2009a,b; Jacob et al., 2013). The different habit of rooting distribution found for beech in different tree species mixtures with either an indication of root system stratification (e.g., MacQueen, 1968; Büttner and Leuschner, 1994; Hendriks and Bianchi, 1995; Rust and Savill, 2000) or none (e.g., Curt and Prévosto, 2003; Meinen et al., 2009b) is explained by the variation in growth and space occupation dynamic of beech and its competitors which may be related to their different successional status (Bolte and Villanueva, 2006;

Meinen et al., 2009a). Beech has been identified as being favored in interspecific competition below ground with less competitive tree species like Norway spruce [*Picea abies* (L.) Karst.] or oak (*Quercus* spp., Büttner and Leuschner, 1994; Bolte and Villanueva, 2006; Rewald and Leuschner, 2009).

Due to climate change, mixtures of European beech with Norway spruce may attain new attraction in forestry in both the hemi-boreal zone and the montane-temperate zone, since beech is supposed to: (1) expand its distribution range northwards (Bradshaw and Lindbladh, 2005), (2) be more resistant than spruce to an increase in abiotic and biotic stress due to warming, drought, and insect attacks, and (3) gain on productivity compared to spruce (Bolte et al., 2010, in press; Grundmann et al., 2011). However, we lack knowledge on the root system structure and distribution in natural mixed spruce-beech forests to evaluate the coherence or difference of fine-rooting comparing temperate and hemiboreal spruce-beech forests. Moreover, more information is needed about spatial effects of interspecific competition and constellations of tree mixture on fine root distribution and structure, since almost all previous studies provided results only on a stand scale.

The present study was aimed at (1) finding evidence for vertical root system stratification in the unmanaged spruce-beech forests, (2) quantifying effects of spruce-beech competition in different mixture constellations on fine root structural traits, and (3) evaluating the competitive status of both species below ground considering different levels of interspecific competitive pressure.

Our present study includes a temperate near-natural forest within the high montane zone of the Harz Nationalpark (Northern Germany) with climate and site conditions comparable to the hemi-boreal forests of our other two study sites in Southern Sweden (see Table 1). We applied a sophisticated sampling design that allows us to differentiate spatial effects of interspecific competition and tree mixture constellations. The

results will be discussed comparing our findings with recent knowledge from studies on spruce-beech interactions.

MATERIALS AND METHODS

SITE AND STAND DESCRIPTION

The two Swedish old-growth forests with Norway spruce [*Picea abies* (L.) Karst.] and European beech (*Fagus sylvatica* L.) are located in the boreo-nemoral zone (Sjörs, 1999) within the counties of Halland and Småland (Table 1). The Halandish site at “Rågetåsen” (Halmstad district) lies at the western fringe of the southern Swedish highlands with cool and humid climate, high precipitation rates and countless peat bogs and lakes. The site “Siggaboda” is situated within the south-eastern part of the forest and lake district of Småland near to the county borders to Blekinge and Skåne. Precipitation is distinctively lower than at the luv side, however, bogs and lakes are frequent due to the generally cool climate and a climatic water surplus. Comparable climatic conditions can be found at the German montane spruce-beech forest “Rehberg” within the “Harz Nationalpark” (Lower Saxony).

At the Swedish site, the bedrock is formed mainly by metamorphic rocks (granitic gneiss) covered with moraine sediments whereas at the Harz site both metamorphic “Hornfels” and sedimentary “Grauwacke” sandstones prevail, partly covered by loess (eolic silt sediment). All sites have comparably low amounts of fine-textured soil and are riddled with boulders. Soil traits of all three sites are comparable with a moderate to thick accumulation of organic material 5–12 cm; the humus type is moder to raw humus (Rågetåsen, Rehberg) or raw humus (Siggaboda) with a high C/N ratio. Fine soil material is dominated by silt (40–65%) and sand (18–52%); the clay proportion is relatively low (8–17%) and the texture can be classified as sandy silt or silty sand, respectively. The soil type is a Haplic Podzol (BGR, 2007) with a high moisture status. Beside several small fens at the

Table 1 | Location and site parameter of the three study sites.

	Rågetåsen	Siggaboda	Rehberg
Location	Southern Sweden, Halland	Southern Sweden, Småland	Northern Germany, Lower Saxony (Harz)
Geographic coordinates	56°51' N 13°06' E	56°27' N 14°33' E	51°43' N 10°33' E
Elevation (m a. s.l.)	140–160	140–165	651–700
Exposure	SE	varying	SE
Mean annual temperture (°C)	6.4	6.0	6.1
Precipitation (mm a ⁻¹)	1200	700	1200
Humus type	Moder to raw humus	Raw humus	Moder to raw humus
Bedrock	Gneiss	Gneissic granite	“Grauwacke” sandstones and “Hornfels” (partly with Loess overlay)
Soil texture	Sandy silt	Silty sand	Sandy silt
Soil type	Podzolic Cambisol	Podzolic Cambisol	Podzolic Cambisol
Moisture-status	Good (partly boggy)	Good (partly boggy)	Good
Nutrition status	Poor to moderate	Poor to moderate	Moderate
Stand age (years)	Spruce and beech >130 (+ natural regeneration)	Beech up to 230, spruce up to 210 (+ natural regeneration)	Spruce and beech around 150 (+ natural regeneration)
Proportion of spruce/beech (%)	50/50	60/40	65/35

Swedish sites, the soils are drained and quite acidic, occurring in the aluminum and iron buffer range at Siggaboda and Rehberg and the ion exchange buffer range at Rågetaåsen (Ulrich, 1983, **Table 2**). The amount of exchangeable base cations is limited; the higher base cation supply in the humus layer and “Ahe” horizon at Rehberg is likely due to repeated soil liming to compensate for anthropogenic acidic deposition (**Table 2**, Dammann and Guericke, 2002). Site moisture status is sufficient (partly boggy at the Swedish sites) and nutrient status poor to moderate at all sites.

All three mixed stands are dominated by spruce with higher mean values of stem density, diameter at breast height (*dbh*), tree heights, and basal area (BA) (**Table 3**). The Swedish stands are denser with higher BAs, in particular of spruce, whereas the spruce and beech trees at the German site Rehberg feature larger dimensions (both *dbh* and height).

Field sampling

Forest stand structures were recorded in a 1 ha square core plot (100 × 100 m) in the center of the semi-natural forest representing a typical section of the old-growth stand. We subdivided the plot using a 20 m-grid (cf. procedure for German forest nature

reserves, Meyer et al., 2001). In total 36 grid points of the core plot were leveled with an ultrasonic hypsometer (Vertex III, Haglöf Inc. Sweden) and a compass (PM-5/400PC, Suunto Inc., Finland) and subsequently semi-permanently marked with wooden stakes.

Within the entire 1 ha core plot, we assessed each tree with a *dbh* (tree diameter at 1.3 m above the ground) of 7 cm and larger in winter 2004 at Siggaboda and in spring 2005 at the other sites. For each tree, species, cardinal location coordinates of the stem using above mentioned equipment and *dbh* with a girth tape were recorded. Subsequently, a tagging system (Signumat, Latschenbacher, Austria) was used for temporarily (between 2004 and 2007) marking and numbering each measured tree.

We studied fine root structure of mature trees focusing on tree groups with one central tree with its mostly four–five competitors (mean value 4.84). Therefore, beech-spruce tree groups representing four different types of interspecific competitive situations were selected: (A) spruce as the central tree, (B) spruce as competitors, (C) beech as the central tree, and (D) beech as competitors. This design was completed by monospecies groups with comparable intraspecific competitive status (**Figure 1**). All directly neighboring trees with crown interaction were defined as competitors. Fine root sampling was conducted in a 8 × 8-point grid of variable width from 1.12 to 5.36 m (**Figure 2**). Total sample area was determined by the distance between the central tree and the farthest competitor (i.e., half size of the quadrat's edge, see **Figure 2**), and ranged from 61 to 1408 m². With this approach it was possible to assess the rooting system of the central tree with different extension but a comparable root sample number. Root sample number was reduced by excluding grid points outside the central tree—competitors range defined as a polygon area within competitors stem position (**Figure 2**). The resulting sample area was then 21 m² to 402 m². This interspecific sampling

Table 2 | Selected chemical soil properties of the three stands.

	Rågetaåsen	Siggaboda	Rehberg
HUMUS LAYER (Of/Oh)			
Thickness (cm)	5	>10	9
pH (KCl)	3.3–3.9	3.0–3.6	4.7–5.2
C/N ratio	23.8–23.9	28.9–31.0	22.5–27.6
MINERAL SOIL (Ahe HORIZON)			
Depth (cm)	0–5	0–10	0–8
pH (KCl)	4.4	3.4	4.4
CEC (μmol _c g ⁻¹)	94.9	75.1	51.1
Al + Fe (% CEC)	58.9	64.8	38.2
K + Ca + Mg (% CEC)	29.9	30.0	58.8
MINERAL SOIL (Bhs HORIZON)			
Depth (cm)	5–10	10–16	8–24
pH (KCl)	4.5	3.6	3.7
CEC (μmol _c g ⁻¹)	55.3	66.6	127.5
Al + Fe (% CEC)	75.0	81.9	76.7
K + Ca + Mg (% CEC)	16.3	13.2	22.8
MINERAL SOIL (Bv–Cv HORIZON)			
Depth (cm)	10–50+	16–45+	24–46+
pH (KCl)	4.8	4.7	4.0
CEC (μmol _c g ⁻¹)	18.5	54.2	84.5
Al + Fe (% CEC)	77.5	83.1	85.9
K + Ca + Mg (% CEC)	15.9	12.8	13.0

The ranges between minimum and maximum values of *n* combined samples consisting of at least 4 single samples taken at every subplot are displayed. Of, incompletely decomposed organic layer; Oh, humified organic layer containing amorphous organic material; Ahe, humified and eluviated mineral top soil horizon; Bhs, humified and podsolic mineral soil horizon; Bv–Cv, cambic mineral soil horizon; CEC, effective cation exchange capacity; Al + Fe, exchangeable aluminum and iron ions; K + Ca + Mg, sum of exchangeable base cations.

Table 3 | Traits of the stand structure for spruce and beech of all mixed stand plots (*n* = 6 plots per stand).

Stand	Rågetaåsen	Siggaboda	Rehberg
STEM DENSITY (TREE NUMBER per ha)			
Spruce	370 ± 343	310 ± 169	55 ± 41
Beech	148 ± 61	179 ± 170	43 ± 31
Total	518 ± 264	489 ± 175	98 ± 35
MEAN dbh (cm)			
Spruce	30.1 ± 14.3	32.3 ± 13.3	59.8 ± 6.8
Beech	28.4 ± 9.3	24.6 ± 14.4	46.7 ± 10.4
Total	29.3 ± 11.5	28.1 ± 13.8	52.7 ± 11.0
MEAN TREE HEIGHT (m)			
Spruce	21.7 ± 7.1	23.7 ± 5.6	33.1 ± 2.1
Beech	16.2 ± 6.8	16.8 ± 4.1	26.6 ± 3.5
Total	18.9 ± 7.2	19.9 ± 5.8	29.6 ± 4.4
MEAN BASAL AREA (m² ha⁻¹)			
Spruce	17.7 ± 12.4	34.6 ± 28.1	14.8 ± 10.5
Beech	10.1 ± 6.4	7.3 ± 5.9	8.3 ± 6.4
Total	27.8 ± 10.3	41.9 ± 23.1	23.1 ± 8.9

Mean values ± standard deviations are displayed.

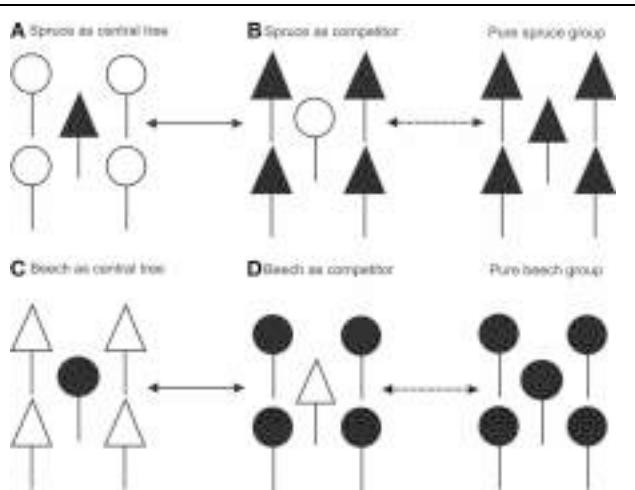


FIGURE 1 | Sampling design of tree groups with varying competitive status of mixed Norway spruce and European beech, and comparison with monospecies groups (only for analyses of mixed species representation).

until fine soil was reached. In a few cases when large boulders covered several grid points the central sample points were skipped. All variations from the regular grid design were recorded with the exact position of the moved sample points.

Processing of root samples

All soil core samples including soil or humus material and roots were soaked in water overnight. We separated root substance from soil using the floating method described by Böhm (1979). The watered samples were filled into trays and roots floating on top of the water were sieved off using a 1-mm mesh. We repeated this procedure until only stones were left in the soil sample. Root material was extracted from the fine soil organic matter of the humus samples by gentle washing. Roots and the remaining soil organic matter floating dispersed in the water-filled trays were separated manually. Washed root samples were kept in de-ionized water at 4°C until sorting.

We classified all root parts less than or equal to 2 mm in diameter as fine roots and separated them from coarse roots which were not investigated further. Fine root sorting addressed species (spruce, beech, other tree and shrub/herb species) and vitality (live, dead). We applied morphological criteria for the identification of dead root material: dead root parts exhibits a dark discoloration of the central cylinder and a decreased flexibility of root segments (cf. Bauhus and Messier, 1999a). Living spruce and beech roots were identified visually according to root elasticity and root cortex properties: spruce roots are elastic with a relatively thick and irregularly structured brownish cortex, whereas beech roots are less elastic and the red-brown cortex is thin with lines along the longitudinal axis (Schmid, 2002). We studied fine root attributes of spruce and beech with the digital image analysis system “Win-RHIZO V3.10” (Régents Instruments Inc., Canada). The sorted live fine root parts of spruce or beech were placed in a transparent water filled tray (10 × 15 cm) to facilitate root spreading. The system scanned all fine root fragments and the image analyses calculated the architectural traits, root length, and root surface for all fine roots. This method is proved to be reliable; only negligible errors for fine root structure measurements through root overlapping and abutment were found in a test-study by Bauhus and Messier (1999b).

All recorded root fragments were then dried for 48 h at 40°C and weighed in order to measure living fine root biomass ($FRB, g m^{-2}$). The reported measurements allowed the calculation of specific root length ($SRL, m g^{-1}$) and specific root surface area ($SRA, cm^2 g^{-1}$) from the ratio of fine root length (FRL) and root biomass, and fine root surface area and rooting biomass, respectively.

Data analyses

The data was included in a relational database running on the open source database system PostgreSQL and analyzed with the software package Statistica 9 (StatSoft, Inc. 2009) and SAS JMP 9 (SAS Institute Inc. 2010). Differences between mean root traits were assessed either by the Kolmogorov-Smirnov-two-sample test, or by the Kruskal-Wallis H test when more than two samples or groups were compared. We used the respective tree BA per hectare of either spruce or beech as a reference unit for the

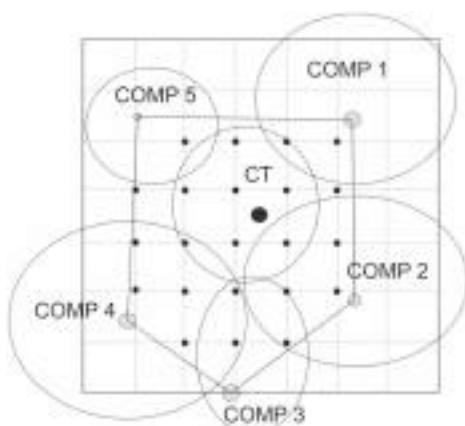


FIGURE 2 | Grid design for root sampling; CT, central tree; COMP, competitor. Black dots showing the positions of the soil core sampling.

design was repeated three times at each of the three sites whereas we sampled only two intraspecific group constellations per plot (beech, spruce) that was only used as a reference for analyses of mixed species representation (see following chapter on data analyses).

At each grid point soil cores with a surface area of 20 cm² were taken using a soil auger (d ~ 5 cm) until the solid bedrock layer was reached. The maximum mineral soil depth sampled varied from 20 to about 40 cm. Various studies revealed that the maximum of fine roots is found in the top soil layers (estimated >80%, cf. Büttner and Leuschner, 1994; Rothe, 1997; Hertel, 1999). Where the exact grid point position coincided with a solid boulder we moved the sample point within the grid orientation

comparison of fine root attributes (*FRA*: biomass, length and surface, cf. Schmid, 2002; Bolte and Villanueva, 2006) of trees or tree groups with different competitive situations (*CT*, Central tree; *COMP*, competitors), and thus variable species abundance above-ground (Equations 1, 2):

$$FRA_{ad, CT} = \frac{FRA_{CT}}{BA_{CT}} \quad (1)$$

$$FRA_{ad, COMP} = \frac{FRA_{COMP}}{\sum_1^n BA_{COMP}} \quad (2)$$

where *FRA* are the life fine root attributes in terms of *FRB* (g m^{-2}), *FRL* (m m^{-2}), and *RAI* ($\text{cm}^2 \text{m}^{-2}$) of a central tree (*CT*) or *n* competitors (*COMP*), respectively. *BA* ($\text{m}^2 \text{ha}^{-1}$) is the BA of either spruce or beech, and *FRA_{ad}* are fine root attributes adjusted to the same *BA* ($1 \text{ m}^2 \text{ha}^{-1}$) of either one central tree or of *n* competitors (*FRB_{ad}*, $\text{kg m}^{-2} \text{BA}$; *FRL_{ad}*, $\text{km m}^{-2} \text{BA}$; *RAI_{ad}*, $\text{m}^2 \text{m}^{-2} \text{BA}$).

The fine root representation of either central tree (*CT*) or competitor (*COMP*) constellations of spruce and beech were further assessed by calculating relative fine root attributes (*rFRA*, Schmid, 2002). The *rFRA* indicator relates the adjusted fine root attributes (*FRA_{ad}*) in mixed groups with either *CT* or *COMP* constellations to those of pure stand groups (Equation 3). This enables the assessment of under- (<1) or overrepresentation (>1) of beech and spruce below ground growing in different mixed stand constellations under interspecific competition.

$$rFRA = \frac{FRA_{ad, mix}}{FRA_{ad, pure}} = \frac{FRA_{ad, CT/COMP}}{FRA_{ad, pure}} \quad (3)$$

RESULTS

OVERALL PLOT MEANS OF FINE ROOT ATTRIBUTES

Overall core sample means for living fine root attributes show differences between spruce and beech as well as between the two Swedish sites and the German site. At all three sites beech had a 1.3–1.5 fold higher living *FRB* than spruce; an even higher beech-spruce ratio of 2.2–3.3 was found for living *FRL* and a ratio of 1.7–2.4 for living fine root area index (*RAI*, Table 4). Total fine root attributes of beech and spruce are quite comparable for both Swedish sites whereas the German Rehberg site has considerably lower amount of fine roots with less than half of *FRB*, *FRL*, and *RAI*. The lower fine root abundance of trees at the German site is in line with an also lower stand density (see Table 3). Since tree abundance above and below ground is correlated on microsite level (Bolte and Villanueva, 2006; Rewald and Leuschner, 2009; Lang et al., 2010), following detailed comparisons of the different intraspecific group constellations based on all three sites are only valid when using adjusted fine root attributes (cf. Equations 1, 2).

VERTICAL FINE ROOT DISTRIBUTION

For both spruce and beech, central trees (*CT*) attained higher adjusted fine root abundance (*FRB*, *FRL*, *RAI*) along the vertical rooting profile than the competitors (*COMP*, Figure 3). This result, however, should be regarded with caution, since it may

be biased by comparing the complete rooting area of the central tree with only partial inclusion of the competitors' rooting area. When comparing non-biased allocation shape of fine root abundance among different soil depths (Figure 3), central beech trees (*CT*) exhibited significantly higher fine root abundance in both the humus layer and deeper soil layers compared to beech competitors (*COMP*). This was not the case for spruce with a quite equal allocation of fine root proportion along the profile for both *CT* and *COMP* constellations. A spatial separation of the fine roots of spruce and beech was visible for the deeper soil horizons (10–40 cm) when growing with beech as the central tree (3 C, solid line, right) and spruce as competitors (3 B, dashed line, left) resulting in high beech and low spruce fine root quantities. This partially vertical stratification was mainly due to high plasticity of beech fine rooting as a central tree. No stratification was visible in the organic layer and top soil where both species reached their maximum abundances.

Total means of specific *SRL* and specific fine root surface area (*SRA*) attained values for mixed beech (*CT* and *COMP*) of *SRL* $12.7 \pm 5.5 \text{ m g}^{-1}$ and of *SRA* $367.3 \pm 125.9 \text{ cm}^2 \text{ g}^{-1}$ compared to mixed spruce of *SRL* $6.6 \pm 3.8 \text{ m g}^{-1}$ and of *SRA* $253.6 \pm 108.1 \text{ cm}^2 \text{ g}^{-1}$ (Kolmogorov-Smirnov-two-sample test, $p < 0.05$). However, total *SRL* and *SRA* differences between *CT* and *COMP* constellations within both species were not significant.

The vertical profiles of *SRL* and *SRA* support the idea of higher plasticity of beech fine rooting (Figure 4). Whereas beech competitors attained significant higher *SRL* and *SRA* in the humus layer, these values tended to be higher for central beech trees in deeper soil horizons (significant for *SRL* in 20–30 cm depth). For spruce in contrast, we did not find such changes between central spruce trees (*CT*) and its competitors (*COMP*) rooting behavior. However, significant higher *SRL* and *SRA* values of *CT* constellations in the humus layer (*SRL*, *SRA*) and the top soil (*SRA*, 0–5 cm depth) points to higher competitive investments for rooting space sequestration in the upper soil

Table 4 | Live fine root ($d \leq 2 \text{ mm}$) attributes for spruce and beech of all mixed stand plots ($n = 6$ plots per stand).

Stand	Rågetaåsen	Siggaboda	Rehberg
LIVE FINE ROOT BIOMASS <i>FRB</i>, DRY WEIGHT ($\text{g} \cdot \text{m}^{-2}$)			
Spruce	171 ± 212	192 ± 184	82 ± 78
Beech	253 ± 205	250 ± 211	106 ± 116
Total	424 ± 267	442 ± 211	188 ± 128
LIVE FINE ROOT LENGTH <i>FRL</i> ($\text{km} \cdot \text{m}^{-2}$)			
Spruce	0.9 ± 1.0	0.9 ± 0.8	0.5 ± 0.4
Beech	3.0 ± 2.5	2.2 ± 1.7	1.1 ± 1.1
Total	3.9 ± 2.4	3.1 ± 1.6	1.6 ± 1.1
LIVE FINE ROOT AREA INDEX <i>RAI</i> ($\text{m}^2 \cdot \text{m}^{-2}$)			
Spruce	3.6 ± 4.1	3.7 ± 3.4	2.0 ± 1.6
Beech	8.8 ± 7.2	7.2 ± 5.7	3.3 ± 3.3
Total	12.4 ± 7.1	10.9 ± 5.2	5.3 ± 3.3

Mean values \pm standard deviations of total cores to bedrock depth are displayed; $n = 154$ samples for Rågetaåsen, $n = 148$ samples for Rågetaåsen, $n = 123$ samples for Rehberg.

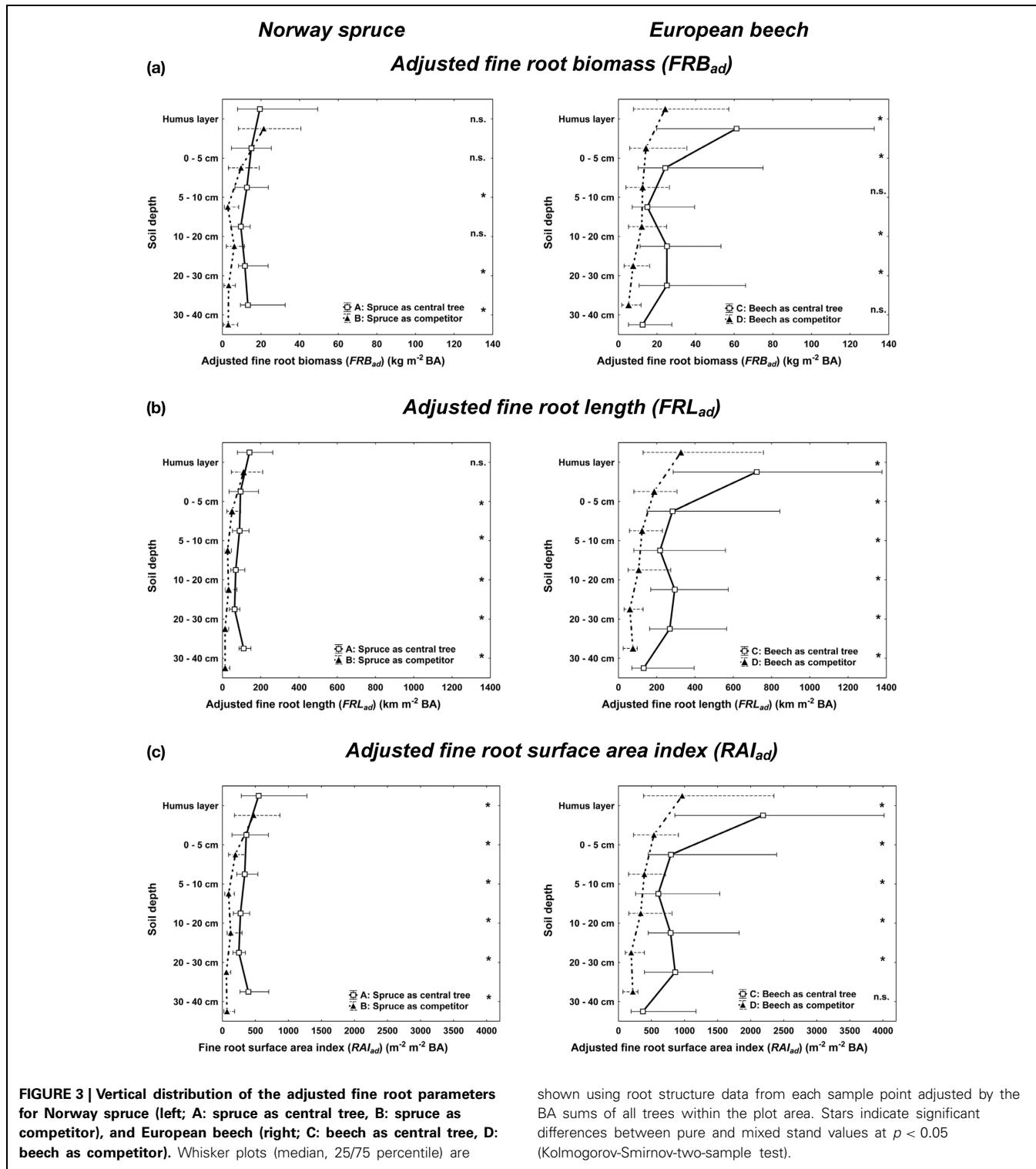


FIGURE 3 | Vertical distribution of the adjusted fine root parameters for Norway spruce (left; A: spruce as central tree, B: spruce as competitor), and European beech (right; C: beech as central tree, D: beech as competitor). Whisker plots (median, 25/75 percentile) are

shown using root structure data from each sample point adjusted by the BA sums of all trees within the plot area. Stars indicate significant differences between pure and mixed stand values at $p < 0.05$ (Kolmogorov-Smirnov-two-sample test).

horizons of single central spruce trees (CT), whereas single beech trees (CT) invested more in deeper soil horizons with less competitive abundance of spruce roots. Comparing beech and spruce as interspecific competitors (COMP), one can observe quite similar vertical rooting behavior (both fine root attributes, *FRA* and structural traits, *SRL* and *SRA*), beside the above mentioned

fact that the overall level of beech is much higher than that of spruce.

FINE ROOT REPRESENTATION

For the analyses of fine root representation (*rFRA*, cf. Equation 3) in mixed CT and COMP constellations (Table 5) we used root

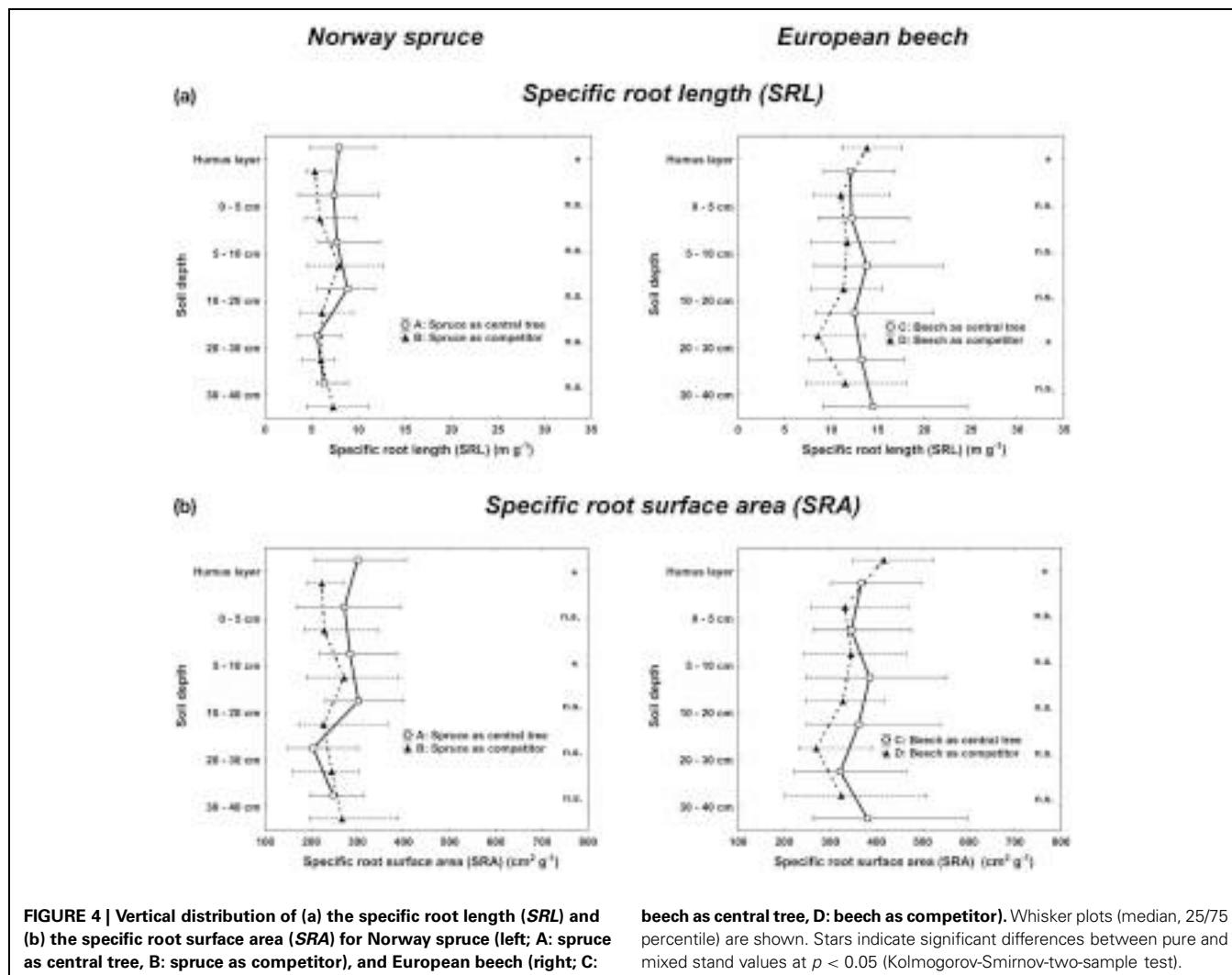


FIGURE 4 | Vertical distribution of (a) the specific root length (SRL) and (b) the specific root surface area (SRA) for Norway spruce (left; A: spruce as central tree, B: spruce as competitor), and European beech (right; C:

beech as central tree, D: beech as competitor). Whisker plots (median, 25/75 percentile) are shown. Stars indicate significant differences between pure and mixed stand values at $p < 0.05$ (Kolmogorov-Smirnov-two-sample test).

samples from pure groups of spruce or beech as reference. This enabled the assessment of the effects of *CT* (A: spruce, D: beech) and *COMP* (B: spruce, D: beech) constellations on interspecific tree species representation compared to the intraspecific one (**Table 5**, *rFRA*). Two major findings were derived: (1) spruce is underrepresented in fine root abundance in mixed stands (mean *rFRA* < 1) whereas beech is overrepresented (mean *rFRA* > 1); (2) both beech overrepresentation and spruce underrepresentation address *CT* constellations with beech as the central tree (B). This indicates that beech rooting was favoured by an asymmetric interspecific competition of both species when several spruce competitors grew together with a single beech tree. But this was not the case for *CT* constellations with central spruce and beech competitors where we found a quite symmetric interspecific competition. Combining results from the existing mixtures (means of A + D) of central spruce (*CT*) and competing beech (*COMP*) or vice versa (means of B + C) one can assess fine root representation effects of the mixtures compared to either beech or spruce pure groups. It turned out that due to the low fine root representation of spruce no existing mixture reaches the high

fine root representation of pure beech. However, both mixture constellations (means A + D, B + C) lead to higher fine root representation compared to the pure spruce plots.

DISCUSSION

OVERALL PLOT MEANS OF FINE ROOT ATTRIBUTES

The presented means of the *FRB* ($d \leq 2$ mm) of the mixed stand plots (and not stand scale) in Sweden ($424-442 \text{ g m}^{-2}$, **Table 4**) fit quite well to overall means of an extensive literature study for boreal and temperate forests with 399 ± 239 and $362 \pm 182 \text{ g m}^{-2}$, respectively (Finér et al., 2011, **Table 5**, original sampling depth). The lower *FRB* mean for the German Rehberg mixed plots (188 g m^{-2}) is related to the remarkably low tree abundance above ground (BA, basal area) compared to the Swedish plots. The positive relationship between tree dimension (BA) and soil exploration (*FRB*) on microsite and tree level has been demonstrated by Bolte and Villanueva (2006) in mature spruce-beech mixed stands indicating generally stem-centered *FRB* distribution of either spruce or beech with higher values near to large trees of the same species (cf. Rewald and Leuschner, 2009 and also Lang

Table 5 | Total means of all stands (\pm standard deviation) and relative mixed stand representation compared to pure stand (rFRA, Equation 3) for different adjusted fine root attributes related to central tree (CT), competitor (COMP) status.

Spruce	A: CT (n = 209)	B: COMP (n = 169)	Pure stand (n = 55)	rFRA A: CT/B: COMP/mean
Fine root biomass (FRB _{ad}) (kg·m ⁻² BA)	61.8 ± 144.6	44.9 ± 45.8	67.9 ± 72.3	0.91/0.66/0.79
Fine root length (FRL _{ad}) (km·m ⁻² BA)	336.8 ± 725.2	224.7 ± 194.1	344.0 ± 356.4	0.98/0.65/0.82
Fine root area index (RAI _{ad}) (m ⁻² ·m ⁻² BA)	1356.1 ± 3052.8	922.3 ± 814.9	1382.0 ± 1418.2	0.98/0.67/0.82
Beech	C: CT (n = 209)	D: COMP (n = 169)	Pure stand (n = 60)	rFRA C: CT/D: COMP/mean
Fine root biomass (FRB _{ad}) (kg·m ⁻² BA)	164.9 ± 234.7	131.4 ± 225.4	135.9 ± 104.9	1.21/0.97/1.09
Fine root length (FRL _{ad}) (km·m ⁻² BA)	1752.3 ± 2263.1	1334.3 ± 1887.1	1495.2 ± 1071.6	1.17/0.89/1.03
Fine root area index (RAI _{ad}) (m ⁻² ·m ⁻² BA)	5271.3 ± 7131.0	4166.6 ± 6215.6	4425.8 ± 3156.2	1.19/0.94/1.07

The samples do not originate from the same distribution (Kruskal-Wallis H-test, $p < 0.05$).

et al., 2010 conspecific beech plots). Values of live FRL and RAI are found to reflect the resource exploitation ability of plants better by focusing on the sensitive parameter *root length* for soil exploration and *root surface* for resource uptake (cf. Fitter, 2002). Our mixed plot means (Table 4) are in the range of previously reported overall means for boreal and temperate forests of FRL 2.6–6.1 and RAI 4.6–11.0 (Jackson et al., 1997) except of the lower FRL value in Rehberg due to above mentioned reasons.

VERTICAL FINE ROOT DISTRIBUTION

There is an on-going scientific debate about vertical root system stratification or segregation in mixed stands which is considered to be a major reason of overyielding below ground, i.e., the increase of FRB in mixed vs. pure stands (Schmid, 2002; Meinen et al., 2009a; Lei et al., 2012a; Brassard et al., 2013; Jacob et al., 2013; Smith et al., 2013). There seems to be a general effect of different admixed species and number of mixed species. Root system stratification on stand level was found mainly in (1) two-species systems, mixtures of (2) conifers with broadleaved species as well as of (3) early- or mid-successional tree species with late-successional species. Regarding late-successional, broadleaved beech, root system stratification was observed when admixed in (mainly) two-species stands to Scots pine (*Pinus sylvestris*, MacQueen, 1968; Curt and Prévosto, 2003), Douglas fir (*Pseudotsuga menziesii*, Hendriks and Bianchi, 1995), Norway spruce (Rothe, 1997; Schmid, 2002; Bolte and Villanueva, 2006), Sessile oak (*Quercus petraea*, Büttner and Leuschner, 1994; Leuschner et al., 2001) and common ash (*Fraxinus excelsior*, Rust and Savill, 2000). No evidence for root system stratification was found in multispecies mixtures with beech and other broadleaved tree species (Smith et al., 2013), and including additional late-successional species like winter lime (*Tilia cordata*, Meinen et al., 2009b; Jacob et al., 2013). Our result of only partial root system stratification (FRB_{ad}, FRL_{ad}, and SRA_{ad}) of central beech and competing spruce in deeper soil

horizons (Figure 3) draws the attention to the question *where* and under *which conditions* root system stratification occurs. Stratification may only occur in stand areas where the root systems of the competing tree species considerably overlap. In particular beech fine rooting are found not to be “territorial” (Lang et al., 2010) and may exploring soils near to an inter-specific competitor (e.g., Büttner and Leuschner, 1994; Rewald and Leuschner, 2009). However, we found in our design several sample points with no considerable fine root abundance of either spruce or beech, and consequently no root system stratification. In a previous study (Bolte and Villanueva, 2006) we focussed our root sampling on the overlapping rooting zones of spruce and beech between conspecific groups of both species. There, we found strong root system stratification. This indicates that sampling design and specific location of root sampling affect results on vertical rooting and stratification, and limits the generalization options for entire stands. Thus, rooting information taken from specific structural sub-strata or from location of even unknown structural stratum should be treated with care when using them for general statements on a stand level. Another condition for root system stratification is the availability of (non- or less-occupied) rooting space in deeper soil horizons. Apart from chemical restrictions like oxygen deficiency (e.g., Pezeshki and Santos, 1998) as well as soil acidification and related mobilization of root-toxic aluminium ions (Cronan and Grigal, 1995), physical restrictions and varied conditions for the exploration of deeper soil horizons like a massive bedrock layer or boulder occurrence may limit fine root system stratification. An important condition for active root system stratification by shifting fine root abundance to lower soil layers is a successful change of rooting behavior, and thus acclimation, in terms of “optimality” (“Optimality theory,” Bloom et al., 1985; Parker and Maynard Smith, 1990; Eissenstat and Yanai, 1997). There has to be a positive effect in the “cost/gain ratio” to invest resources to change its rooting, and the selected plasticity (Grime et al., 1986; De

Kron and Mommer, 2006). Where a large resource availability gradient from high availability in the top soil to a lower one in the deeper soil exists there is little “gain” to change rooting behavior. The same applies if the favourable soil space is already occupied by tree species with similar rooting plasticity and foraging strategy that could be explained by similar plant strategy type (Grime, 1979) and successional status (Bolte and Villanueva, 2006; Meinen et al., 2009a; Jacob et al., 2013). In our study, the different strategy of mid-successional conifer spruce with more conservative rooting and late-successional broadleaved beech with plastic rooting points to root system stratification in the overlapping zone. However, physical and chemical rooting restrictions in the boulder-rich soils with nutrition status of the mineral soil seemed to counteract a complete stratification (with beech as central tree) and to stimulate beech to compete intensively with spruce in the humus layer and uppermost soil horizons.

SRL of living fine roots can explain economic aspects of fine root system morphology (Ostonen et al., 2007), as does specific *SRA* (*SRA*, Bolte and Villanueva, 2006). Our *SRL* means for mixed spruce (6.6 m g^{-1}) and mixed beech (12.7 m g^{-1}) lie within the lower part of the range for spruce and beech ($d < 2 \text{ mm}$) of $4.5\text{--}26$ and $5.7\text{--}31.5 \text{ m g}^{-1}$, respectively, reported by the extensive meta-analyses of Ostonen et al. (2007). The *SRL* and *SRA* means (spruce $253.6 \text{ cm}^2 \text{ g}^{-1}$, beech $367.3 \text{ cm}^2 \text{ g}^{-1}$) are similar to those reported for mixed spruce by Bolte and Villanueva (2006) but lower than those for mixed beech. Along the vertical profile (Figure 4), *SRL* for mixed beech and spruce are lower than those found for young mixed stands by Lei et al. (2012b). The higher *SRL* and *SRA* values for beech compared to spruce correspond to ideas of basic differences of deciduous angiosperms and conifers, latter having thicker roots and thus lower *SRL* and *SRA* (Bauhus and Messier, 1999a; Lei et al., 2012b).

In line with above mentioned “optimality theory” and economic “cost/gain evaluations,” *SRL* is used as an indicator parameter for space sequestration efficiency (SSE, Grams et al., 2002): fine root systems with a higher *SRL* are supposed to sequester rooting space with lower “carbon” costs (Ostonen et al., 2007). In this respect, beech is more efficient to explore root space which is one explanation for higher rooting plasticity of beech compared to spruce. However, higher soil exploration efficiency (SSE) of beech does not mean that resource exploitation is more efficient compared to spruce (Lei et al., 2012b). In contrast to other studies which report a decrease of *SRL* with increasing soil depth (Bauhus and Messier, 1999a; Lei et al., 2012b), mixed central beech (CT) increased *SRL* in deeper soil layers. This indicates additional soil exploration activities at low carbon costs in soil depths from 20 cm downwards fitting well to the findings of changed root system partitioning favouring deeper soil horizons for increasing complementary resource exploitation and “underground niche separation” (Parrish and Bazzaz, 1976).

FINE ROOT REPRESENTATION

The relative fine root representation is a measure to compare mixed species abundance (*FRB*, *FRL*, *RAI*) adjusted to the same unit of above ground performance ($1 \text{ m}^2 \text{ BA per hectare}$) with adjusted values for monospecies plots (cf. Schmid, 2002; Bolte

and Villanueva, 2006). The underrepresentation of mixed spruce (Table 5, mean *rFRA*) is in line with other studies reporting an over-proportional reduction of fine root abundance in beech mixtures with spruce (Schmid and Kazda, 2002; Bolte and Villanueva, 2006), oak (Büttner and Leuschner, 1994; Leuschner et al., 2001; Rewald and Leuschner, 2009), common ash (Rust and Savill, 2000), or Douglas fir (Hendriks and Bianchi, 1995). This finding is contrasted by a slight overrepresentation of beech fine root abundance indicating an asymmetric interspecific competition of spruce and beech that was also found by Schmid (2002) but not by Bolte and Villanueva (2006). Previously reported ideas of a low belowground competitive ability of spruce compared to beech in mixed mature stands (Schmid, 2002; Bolte and Villanueva, 2006) are supported by this study. In particular, spruce competitors (COMP) surrounding a single beech tree (CT) have a low fine root representation possibly reflecting intensive above ground competition between central beech and spruce competitors leading to increased biomass partitioning toward aboveground tree compartments. Beech fine root representation on the other hand is favored by interspecific competition with spruce. The found morphological belowground plasticity (variation of rooting depth in CT constellation and of *SRL/SRA*) reflects the high crown plasticity (Dieler and Pretzsch, 2013) and the overall “foraging” ability of beech in relation to different growth resources. According to the coherence (beech) or dissimilarity (spruce) of competitive response above and below ground, the linkage of competition assessments above and below ground are of increasing interest.

CONCLUDING REMARKS

The results of the presented study exhibited an only partially vertical root system stratification in the subsoil in the three spruce-beech old-growth stands and depended on specific mixture constellation: beech central tree with spruce competitors. In this mixture constellation, beech was able to raise *SRL* and with this soil exploration efficiency in the subsoils while increasing root biomass partitioning toward deeper soil layers. Moreover, asymmetric below-ground competition was observed favoring beech toward spruce in a mixed constellation with central beech. We conclude that beech fine rooting is facilitated in the presence of spruce by lowering competitive pressure compared to intraspecific competition whereas the competitive pressure for spruce is increased by beech admixture. This is most obvious when central beech trees are admixed with spruce competitors. Our findings underline the need of spatially differentiated approaches to assess interspecific competition below ground. Since tree competition is a process that affects tree at an individual scale, stand scale analyses should be complemented by single tree approaches, above and below ground. In line with the recent development of crown competition assessments and stand simulation methods (e.g., Pretzsch et al., 2002; Nagel et al., 2006), single-tree approaches and simulations of below-ground competition are required to focus rather on microsites populated by individual specimens as the basic spatial study area.

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Fine-root carbon and nitrogen concentration of European beech (*Fagus sylvatica* L.) in Italy Prealps: possible implications of coppice conversion to high forest

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Fine-root systems represent a very sensitive plant compartment to environmental changes. Gaining further knowledge about their dynamics would improve soil carbon input understanding. This paper investigates C and N concentrations in fine roots in relation to different stand characteristics resulting from conversion of coppiced forests to high forests. In order to evaluate possible interferences due to different vegetative stages of vegetation, fine-root sampling was repeated six times in each stand during the same 2008 growing season. Fine-root sampling was conducted within three different soil depths (0–10; 10–20; and 20–30 cm). Fine-root traits were measured by means of WinRHIZO software which enable us to separate them into three different diameter classes (0–0.5, 0.5–1.0 and 1.0–2.0 mm). The data collected indicate that N concentration was higher in converted stands than in the coppiced stand whereas C concentration was higher in the coppiced stand than in converted stands. Consequently the fine-root C:N ratio was significantly higher in coppiced than in converted stands and showed an inverse relationship with fine-root turnover rate, confirming a significant change of fine-root status after the conversion of a coppice to high forest.

Keywords: fine-root carbon, fine-root nitrogen, stand characteristics, coppice conversion, *Fagus sylvatica* L. fine roots, fine roots soil depth, beech fine roots

INTRODUCTION

The Italian National Forest Inventory (SIAN, 2013) indicates that more than 60 percent of Italian forests are maintained under a coppice regime. This situation stems from when there was a high demand for small timber, firewood and charcoal. Now, based on social and economic factors, there is a trend to convert traditional coppice management to high-standard management (Nocentini, 2009). This conversion entails the transition from a condition where a number of stems grow contemporaneously on a single stool to a condition where only one stem is left to continue its growth so that it assumes a larger dimension. At the same time, tree density per hectare is usually decreased.

Forest management practices such as coppice conversion to high forest routinely involve thinning operations. These practices modify stand characteristics (i.e., tree density, canopy cover, stand basal area) and related environmental variables (i.e., soil moisture and temperature, irradiance), leading to changes in the ecophysiological behavior of trees (Aussenac, 2000). In particular, conversion results in considerable alteration of almost all micro-environmental factors that characterize a coppice stand. Various studies show that increase in canopy gap size causes an increase of both seasonal average soil temperatures and soil temperature extremes (Liechty et al., 1992; Hashimoto and Suzuki, 2004). Moreover, seasonal and diurnal differences in maximum-minimum air temperatures increase 15 cm above soil surface as well (Carlson and Groot, 1997).

In the attempt to shed some light on the effects of forest conversion, we studied the effect of conversion of a coppice stand to a high-standard management based on the fine-root component (roots <2 mm in diameter). Our rationale was that fine roots represent the component of a root system that is most sensitive to climate and microclimate variations (Aussenac, 2000; Fotelli et al., 2002), and to stressful conditions such as drought, competition, and herbivory (Lopez et al., 1998; Chiatante et al., 1999, 2005; Glen and Robert, 2006; Withington et al., 2006; Di Iorio et al., 2011; Montagnoli et al., 2012a). Few previous studies showed that stand conversion induces a decrease in the fine-root standing biomass (Lopez et al., 2003; Tufekcioglu et al., 2005). In addition, Fotelli et al. (2002, 2004) reported both an increase and a decrease of fine root biomass in thinned forests, depending on site exposure, whereas Lopez et al. (2003) confirmed that fine-root production is positively affected by management operations. We recently found that the conversion of a beech stand from coppice to high forest may be indirectly responsible, in the fine root component, for a decrease of total biomass and an increase of turnover rate, i.e., a decrease of life-span (Montagnoli et al., 2012b; Table A1). We also found that fine-root biomass production may be transiently stimulated by conversion. Therefore, our earlier findings suggest that the fate of fine roots after conversion is a factor that could be considered in the measurement of a forest carbon stock that will be used as an indicator of sustainable forest management (www.fao.org/forestry/ci/en/, 2013).

The work reported here builds on previous studies on beech stand conversion. We focus on carbon and nitrogen concentrations in fine roots because these two parameters can be used as indicators of the construction and maintenance costs, respectively, for fine-root biomass (Pregitzer et al., 1997, 2002). In fact, the C concentration of fine roots is associated with construction costs (Gordon and Jackson, 2000; Guo et al., 2004) whereas N concentration is associated with their metabolic activity, respiration and root longevity (Ryan, 1991; Pregitzer et al., 1998; Withington et al., 2006). As a consequence, the C:N ratio can provide an indication of the life-span of fine roots (Withington et al., 2006), i.e., the higher the fine-root C:N ratio, the longer their life-span and the lower the fine-root turnover rate (Pregitzer et al., 2002; McCormack et al., 2012). In particular, we hypothesized that fine-root population in the coppice stand, characterized by a lower fine-root turnover rate than converted stands, might have higher fine-root C:N ratio. This would result in an inverse relationship between fine-root C:N ratio and fine-root turnover rates previously measured by soil coring method.

MATERIALS AND METHODS

SITE DESCRIPTION

The study area was located in the catchments of the Telo stream in the Lombardy Alps (Intelvi Valley, NW Italy, 45° 59' N, 9° 07' E) approximately from 1160 to 1200 m above sea level between Lakes Como and Lugano. This area is characterized by a sub-continental climate, with a mean annual precipitation of 1600 mm, occurring in two main periods (April–May and October–November) and a mean annual temperature of 10–11°C. Generally, the area is snow-covered from late October to late March. The 2008 temperatures and precipitations are in accordance with the general trend and magnitude of the past 80 years (weather data from Consorzio dell'Adda, Lombardy, 1920–2000). According to the World Reference Base for Soil Resources (IUSS Working Group WRB, 2006), soil type is Leptosol 40–50 cm deep. Sampling plots were placed in three beech forest stands with different characteristics due to forest management. The three stands were adjacent to each other and located on the same slope (average between 28 and 30°) facing south-west. No significant differences in soil characteristics were recorded between the three stands. Specifically, three beech stands were considered: a residual coppice stand (CpS), the only one left in the area, cut once 40 years ago and then allowed to re-grow from stumps and never recut; two converted stands from coppice to high forest cut in 1994 (CvS 1994) and 2004 (CvS 2004), respectively. Cutting consisted in reducing the number of stems per stool to one per stool, and eliminating exceeding stools thereby reducing stand tree density, and transforming the coppice to high forest. Information from forest inventory indicates similar coppice management practices and stand characteristics for the whole study area. Since beginning of 90s, conversion thinning to high forest fractionated the area in several stands differing in cutting time and tree density. The three selected stands were considered as three different stages in a beech forest successional development with CvS 2004 and CpS representing the younger and older stage, respectively. Therefore, CpS with canopy cover of 94% was considered as the “time zero” before the management change occurred.

STAND CHARACTERISTICS

Soil temperature was measured during the growing season. Measurements were taken next to the soil cores. On each sampling date, six measurements were made at three soil depths: 5 cm, 15 cm, and 25 cm. Soil temperature was measured using a high accuracy thermometer with a stainless steel probe (mod. CheckTemp 1). The probe utilizes a high-tech NTC thermistor sensor that makes it possible to obtain an extremely high accuracy ($\pm 0.3^\circ\text{C}$) in a very short time. During the growing season, the mean soil temperature (0–30 cm depth) was lower in the CpS than in both CvSs (Montagnoli et al., 2012b; **Table A1**).

The tree number and diameter at breast heights (dbh) were surveyed on seven selected 20-m diameter circular-shaped sampling plots per stand (a total of 2199 m² per stand). In order to estimate above-ground biomass, a site specific allometric relationship, which estimates branch and stem biomass from tree dbh, was developed (Montagnoli et al., 2012b). The CpS had higher tree density and above-ground biomass than both CvSs (**Table A1**). Canopy cover, measured with the hemispherical photo method (Rich, 1990), showed the same trend observed for tree density. In fact, in the CpS, tree canopy almost completely covered the soil surface whereas in the CvS 2004 the cover decreased to half that of the CpS (**Table A1**). In all three stands the vegetation was dominated by European Beech, while the understory profile was characterized by differences in species and relative abundance between stands. The CpS was characterized by few *Fagus* seedlings, herbaceous species covered 5% of the stand and mosses covered 35%. In the CvS 1994, seedlings of *Fagus* covered up to 15% of the soil surface. Herbaceous species covered from 20 to 50% of the soil and mosses covered only 5% of the soil surface. In the CvS 2004, seedlings of *Fagus* covered up to 15% and seedlings of *Betula pendula* covered 2%. Herbaceous species covered up to 85% of the soil surface and mosses only 1%. More detailed information about stand characteristics were provided in Montagnoli et al. (2012b).

FINE ROOT MEASUREMENTS

Fine roots were collected at different soil depths using a motor-driven portable root soil core sampler [adapted from Ponder and Alley (1997)] during the 2008 growing season (between May and October). In each stand, four permanent 10-m² plots were set. Two soil cores (4 cm diameter × 30 cm deep) were randomly collected in each plot. Samples were taken when the soil was free of snow cover. Fine roots were sampled on six dates approximately every 30 days for a total of 144 cores (8 cores × 3 stands × 6 collecting dates). The soil cores were separated into three soil layers: 0–10 cm including the humus layer (0–2/3 cm), 10–20 cm and 20–30 cm from the soil surface. Samples were stored in plastic bags at 4°C until processed. Each sample was washed automatically in a filtering nylon bag (300 µm mesh) using a washing machine [adapted from Benjamin and Nielsen (2004)].

Soil-free roots were sorted into color, texture, and shape under a 10× stereomicroscope (Vogt and Persson, 1991). Live fine roots were scanned at resolution of 400 dpi and divided in three subsamples based on three diameter size classes (0–0.5; 0.5–1.0; 1.0–2.0 mm) by using WinRhizo Pro V. 2007d (Regent Instruments Inc., Quebec). Each subsample class was scanned and

analyzed in order to obtain the mean class diameter. Subsamples were then oven-dried, weighed and stored in sealed vials to further chemical analysis.

FINE ROOT CARBON AND NITROGEN CONCENTRATIONS

Fine-root subsamples were ground in liquid N₂ with mortar and pestle and analyzed for C and N concentrations with a CHN-analyzer (NA-2000 N-Protein; Fisons Instruments S.p.A., Rodano [MI], Italy). The analyzer was calibrated with an atropine standard, and every 10th sample with an atropine sample. The mean total N and C recovery rate for nutrient analysis of atropine was 100.48% (1 SE = 0.6%) and 101.02% (1 SE = 0.22%), respectively.

STATISTICAL ANALYSIS

Statistical analyses were carried out using the SPSS software package version 12.0 (SPSS Inc, Chicago IL, USA). Fine-root C concentration and fine-root C:N ratio data did not meet the normal distribution and homoscedasticity. A square root transformation produced normal distributions and equal variances. It was not necessary to transform fine-root N concentration data. General linear model (two-way ANCOVA) was performed with forest stand and time as fixed factor and fine-root diameter and soil-depth as covariates. A *post-hoc* multicomparison test (Bonferroni test with a 5% rejection level) was performed on estimated marginal means to detect significant differences between forest stands and sampling times.

RESULTS AND DISCUSSION

We measured C and N concentrations in fine roots in three beech forest stands: one maintained as coppice; the other two had been converted to high forest (in 1994 and 2004, respectively) but had a different tree density. All the three stands presented the same soil structural characteristics. Conversion resulted in a decrease in tree density in the CvS 2004, whereas tree density in the CvS 1994 was intermediate between that of CpS and the CvS 2004. The decrease in tree density increased light and soil temperature within the stand (**Table A1**), but we cannot exclude that the reduced tree density also affected other environmental factors. Given related effects in the fine-root turnover rate due to stand characteristic variations introduced during conversion (Montagnoli et al., 2012b), it is conceivable these changes could correlate to N and C concentrations in fine roots.

NITROGEN CONCENTRATION

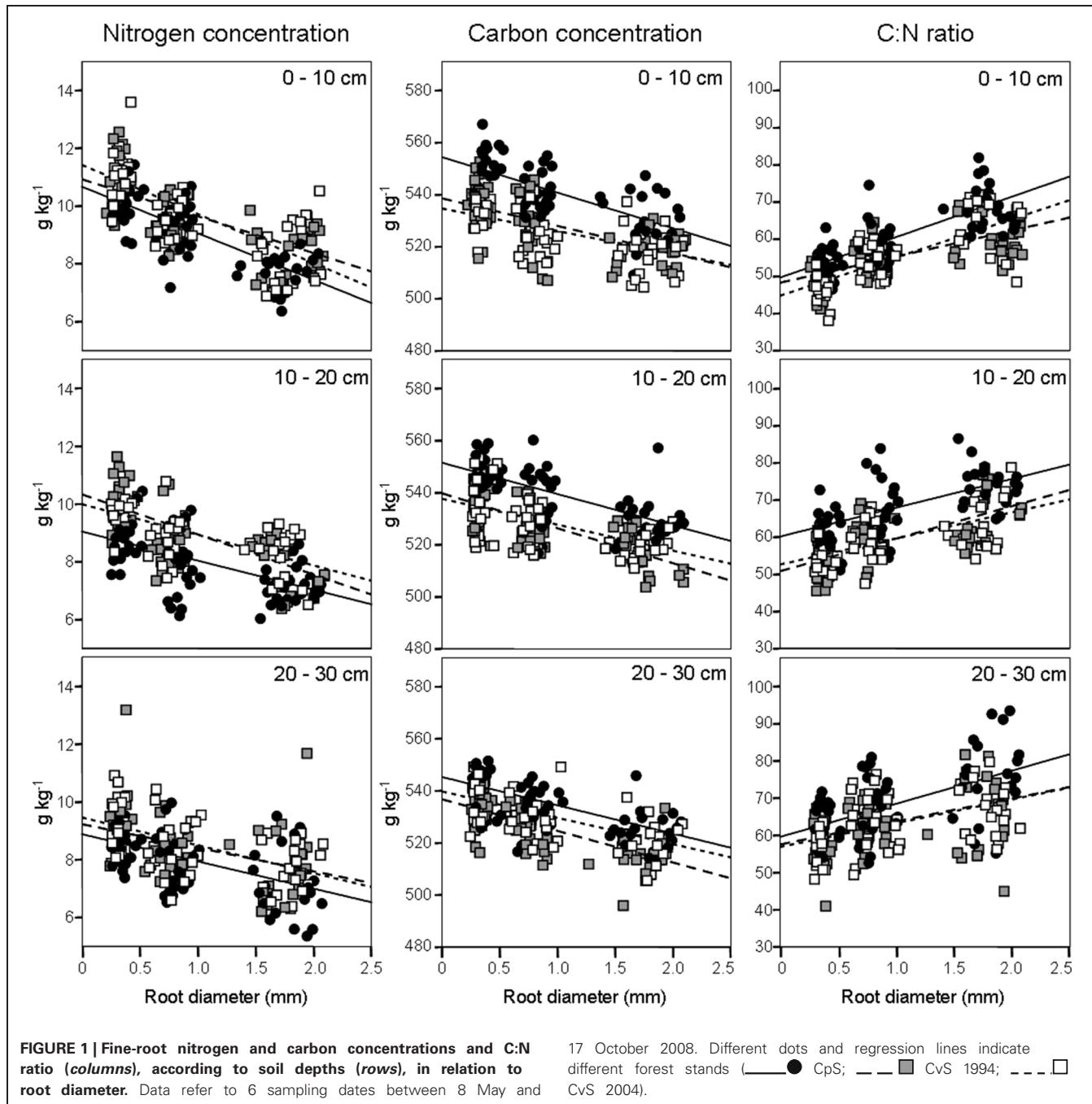
We evaluated N concentrations of fine roots belonging to the three different diameter classes excavated from the three different forest stands at three different soil depths (**Figure 1**). The N concentration was higher in fine roots with a diameter smaller than 0.5 mm, which live in the most superficial soil layer (0–10 cm). The N concentration in fine roots decreased as root diameter increased in all soil layers considered (**Figure 1; Table 1**). These findings are in accordance with findings of other similar studies which suggested that N concentration is related to root diameter with the highest concentrations in the thinnest root branches (Gordon and Jackson, 2000; Li et al., 2010) located

in the uppermost soil layer (Pregitzer et al., 1998; Persson and Ahlström, 2002; Ayres et al., 2004; Li et al., 2010; Montagnoli et al., 2010). Fine-root diameter showed a significant interaction with soil-depth ($p = 0.002$, **Table 1**). Indeed, the decrease of N-concentration in relation to soil depth differed depending on fine-root diameter class considered. Very fine roots (<0.5 mm) at 20–30 cm depth showed 16% less N than at 0–10 cm depth, while fine roots with diameter 0.5–1.0-mm and 1.0–2.0-mm showed, respectively, 14 and 7% less N in deeper roots. This result agree with that reported by Pregitzer et al. (1998). The stronger depth-related variation of very fine roots (<0.5 mm) will remain to deepen and could be related to their uptake function, which means that very fine roots are more sensitive to changes in soil features than larger roots. We have limited our investigation to fine roots with a maximum diameter of 2 mm and have divided them into diameter classes. Although we do not know whether functional differences exist between these three diameter classes, we cannot exclude that, also in our case, fine roots with a diameter smaller than 0.5 mm could play a role in N-uptake function as suggested by Guo et al. (2008) and Hishi (2007). If this is the case, we could speculate that fine roots belonging to the two larger diameter classes might play a role in transport and storage function (Hishi, 2007; Guo et al., 2008).

Regarding forest stands, we found significant lower N values in CpS than both CvSs (Bonferroni test, $p < 0.001$), mainly at a depth of 10–20 cm (11% less N, **Figure 1**). Higher N concentrations in both CvSs could be related to the higher soil temperatures, as found in other studies (Geßler et al., 1998; Fotelli et al., 2002, 2004; Nahm et al., 2006).

Previous studies showed that the N concentration in fine roots is directly related to their metabolic activity and respiration, and inversely to their longevity (Ryan, 1991; Pregitzer et al., 1998; Withington et al., 2006; McCormack et al., 2012). Therefore, our finding that N concentration was significantly higher in the two CvSs than in the CpS suggests that variations introduced by conversion may be responsible for the increased metabolic activities of fine roots, which in turn, would lead to an increase of their growth rate (Valverde-Barrantes et al., 2007) and a shorting of their life-span. This hypothesis is consistent with our previous findings (Montagnoli et al., 2012b) that the turnover rate of fine roots increases, and consequently the life-span decreases, as a result of change in stand characteristics due to conversion operations.

In our experiment, variations in the fine-root N concentration were similar in the three beech forest stands during the vegetative season (from May to October, **Table 2**). In all three stands, the N concentration significantly decreased during spring, and returned to similar values at the end of the growing season. This pattern of N concentration variation is in line with the report that temperate forests are characterized by seasonal variations of N concentration (Cerasoli et al., 2004; Nahm et al., 2006). Therefore, in analogy with Millard (1989) and Fotelli et al. (2002), we suggest that also in our beech forest stands the decrease in N could be associated to utilization of the N reserve in order to support newly developing tissues and the increase with restoration of the N-depleted reserves.



CARBON CONCENTRATION

Previous studies identified considerable differences in C concentration in the fine roots of different species, and showed that C concentration is related positively to root diameter (Gordon and Jackson, 2000; Pregitzer et al., 2002). In contrast, another study reported that C concentration was highest in roots with the thinnest diameter (Goldfarb et al., 1990). We found that C concentration did not differ among soil depths ($p = 0.144$) and significantly decreased with increasing root diameter (Table 1; Figure 1). The same result was found by Gaul et al. (2009) in a Norway spruce forest. Moreover, fine-root C concentration was

significantly higher in the CpS than in both CvSs (Bonferroni test, $p < 0.001$) highlighting a higher investment of carbon in CpS than CvS fine roots, although differences are small (1.5–3% of variation, Figure 1). Our findings could be related to a higher content of secondary metabolites (i.e., lignin and tannins, Harborne, 1980). In fact, secondary metabolites have a C content higher than compounds like cellulose and other sugars (Chua and Wayman, 1979; Krässig, 1993), therefore an increase in secondary metabolites would result in an increase in total C concentrations. Alternatively, we cannot exclude that a higher C concentration could derive from a lower cellulose and/or

total-non-structural carbohydrate (TNC) content (Nguyen et al., 1990; Guo et al., 2004), e.g., less secondary xylem and/or lower starch concentration in CpS fine roots, although this still need further investigation.

In regard to the seasonal variation, Goldfarb et al. (1990) suggested that C concentration in fine roots is higher in early summer than in spring or autumn. We confirm the significant variation of C concentration in fine roots during the year with a peak in July or August depending upon the forest stand (**Table 2**). The peak of C concentration found by us during summer could be related to the maximum vegetative activity which requires a reduction of investment in TNC. This possibility is in accordance with data of Cerasoli et al. (2004) who reported, during the growing season, the highest C concentration in roots while TNC levels were the lowest. The rapid decrease of C concentration following

the peak could be related to the end of the growing season and therefore to the need to restore the sugar reserve (Nguyen et al., 1990).

FINE-ROOT C:N RATIO

Fine-root C:N ratio data in the present study ranged from 39.6 to 93.4 (**Figure 1**) and were of the same magnitude as other published values for the same tree species (Persson and Ahlström, 2002; Ayres et al., 2004; Zang et al., 2011). Moreover, we found an increase of fine-root C:N ratio with depth that was related to the depth-dependent pattern of N concentration (**Figure 1**). For the same reason, the fine-root C:N ratio significantly increased during spring, and returned to similar values at the end of the growing season (**Table 2**).

It is known (as mentioned above) that fine-root C:N ratio has a direct relation with fine-root life-span (Pregitzer et al., 2002; Tjoelker et al., 2005; Withington et al., 2006; Gaul et al., 2009; McCormack et al., 2012). It can also cast light on the relationship between costs for fine-root biomass construction (in term of C concentration) and costs for biomass maintenance (in terms of N concentration) (Pregitzer et al., 1997, 2002). In accordance with our hypothesis, the fine-root C:N ratio differed significantly between forest stands ($p < 0.001$, **Table 1**) and was significantly higher in the CpS than in both CvSs (Bonferroni test, $p < 0.001$), independently from soil depth (**Figure 1**; **Table A2**). Moreover, fine-root C:N ratio was inversely related to fine-root turnover rate [data from Montagnoli et al. (2012b)] with a significant inverse power regression ($R^2 = 0.863$; $p = 0.007$; **Figure 2**). In particular, these results highlight that in terms of cost-benefit ratio, the higher C investment in the construction of CpS fine roots is balanced by lower maintenance cost (lower N concentration) and by higher lifespan.

In conclusion, our study shows that changing in stand characteristics due to conversion operations (from coppice to high forest) affects C and N concentrations of fine roots. Converted stands showed lower fine-root C concentration and higher fine-root N concentration than coppice. Consequently the fine-root C:N ratio was significantly higher in coppiced than in converted stands. These results support our previous finding (Montagnoli et al., 2012b) that a coppiced forest left to grow for 40 years

Table 1 | General linear model analysis (two-way ANCOVA) for the effects of forest stand and time on fine-root N and C concentrations and C:N ratio.

Parameter	F	p-value
NITROGEN CONCENTRATION		
Fine-root diameter (c)	69.138	<0.001
soil-depth (c)	60.160	<0.001
forest stand	19.794	<0.001
time	7.850	<0.001
Fine-root diameter (c) × soil-depth (c)	9.457	0.002
CARBON CONCENTRATION		
Fine-root diameter (c)	235.575	<0.001
soil-depth (c)	2.509	0.114
forest stand	67.113	<0.001
time	11.143	<0.001
C:N RATIO		
Fine-root diameter (c)	152.441	<0.001
soil-depth (c)	74.921	<0.001
forest stand	17.693	<0.001
time	10.095	<0.001

Fine-root diameter and soil-depth were treated as covariates (c). Non-significant interactive effects were excluded from the model.

Table 2 | Estimated marginal mean values (means adjusted for soil-depth and diameter class covariates, $N = 36$) of fine-root nitrogen and carbon concentration and C:N ratio.

Sampling date	Nitrogen concentration (g kg^{-1})			Carbon concentration (g kg^{-1})			C:N ratio		
	CpS	CvS 1994	CvS 2004	CpS	CvS 1994	CvS 2004	CpS	CvS 1994	CvS 2004
8 May	8.49ab x	9.36a y	9.29a y	536.5a x	521.8a y	528.9ab y	62.8ab x	58.7ab x	57.9a x
20 June	7.69b x	8.95ab y	8.60b y	535.6a x	526.4ab y	523.9a y	71.0c x	62.7b y	59.9a y
12 July	8.15ab x	8.37a x	8.75ab x	536.6a x	533.5c xy	529.1ab y	67.4bc x	65.2b x	65.8b x
26 August	8.32ab x	8.79a x	8.77ab x	544.9b x	529.9bc y	534.3b y	66.8abc x	62.7b x	58.2a x
24 September	8.63a x	8.94ab x	9.18ab x	541.7ab x	528.3abc y	529.8ab y	63.5ab x	59.9ab x	60.4ab x
17 October	8.82a x	9.62a y	9.72a y	534.0a x	521.3a y	523.6a y	60.0a x	55.8a x	59.3a x

a, b, and c indicate significant differences between sampling dates. For each sampling date x and y indicate significant differences between stands (Bonferroni test, $p < 0.05$).

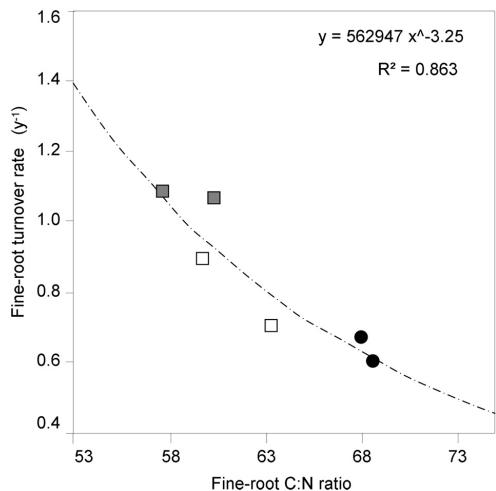


FIGURE 2 | The relationship between fine-root C:N ratio and fine-root turnover rate. C:N ratio data are estimated marginal mean values (means adjusted for diameter class covariates, $N = 72$). Significant turnover rate data ($p < 0.05$) are from Table 3 in Montagnoli et al. (2012b) and refer to two different soil depths. Different symbols indicate different forest stands (● CpS; ■ CvS 1994; □ CvS 2004).

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is characterized by fine roots with a longer life-span than those living in stands recently converted to high forest. Therefore, in our coppiced forest, fine-root carbon stock lasted longer than converted. Moreover, the thinnest root component (<0.5 mm) appears to be more sensitive to changes in stand characteristics than other root diameter classes.

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APPENDIX

Table A1 | Beech above-ground characteristics, live fine-root biomass and soil temperature of the three investigated stands.

Forest stand	Density (trees ha ⁻¹)	Canopy cover (%) ^a	Above-ground biomass (Mg ha ⁻¹) ^b	Live fine-root biomass (g m ⁻²) ^c	Soil temperature (°C) ^d
CpS	724 ± 35	94.2 ± 0.6	248.5 ± 15.6	230.0 ± 17.2	10.24 ± 0.30
CvS 1994	279 ± 24	74.2 ± 5.5	123.7 ± 7.3	144.8 ± 14.7	11.26 ± 0.32
CvS 2004	167 ± 20	54.3 ± 3.2	91.8 ± 20.2	119.4 ± 13.7	12.23 ± 0.36

Data shown are means ± S.E. dbh, diameter at breast height.

^aCanopy cover values are the mean of 10 replicates.

^bAbove-ground biomass values are the mean of seven replicates.

^cFine-root biomass values are the mean of 32 samples (eight sampling time X four plots).

^dSoil temperature (0–30 cm) is referred to the mean of three soil depths (5, 15, and 25 cm) and each value is the mean of four replicates for eight sampling dates (May 2008–April 2009). Data are from Montagnoli et al. (2012b).

Table A2 | Fine-root nitrogen and carbon concentrations and C:N ratio of three diameter classes.

Soil depth(cm)	Diameter class 0–0.5 mm			Diameter class 0.5–1 mm			Diameter class 1–2 mm		
	CpS	CvS 1994	CvS 2004	CpS	CvS 1994	CvS 2004	CpS	CvS 1994	CvS 2004
NITROGEN									
0–10	10.3 ± 0.3	10.6 ± 0.3	11.2 ± 0.3	9.7 ± 0.4	9.0 ± 0.3	9.4 ± 0.3	8.2 ± 0.4	8.4 ± 0.3	8.1 ± 0.3
10–20	8.9 ± 0.2	10.2 ± 0.3	9.8 ± 0.1	8.0 ± 0.3	8.8 ± 0.2	8.9 ± 0.3	7.2 ± 0.2	7.9 ± 0.3	8.2 ± 0.2
20–30	8.6 ± 0.2	9.3 ± 0.4	9.5 ± 0.2	8.1 ± 0.3	7.9 ± 0.2	8.3 ± 0.3	7.3 ± 0.4	7.7 ± 0.5	7.8 ± 0.2
CARBON									
0–10	551.4 ± 2.3	536.8 ± 2.6	533.3 ± 1.9	540.4 ± 2.0	528.3 ± 3.1	525.2 ± 2.3	527.3 ± 3.3	519.4 ± 1.8	519.7 ± 2.6
10–20	548.8 ± 1.4	536.6 ± 2.6	536.8 ± 3.7	538.8 ± 3.5	526.6 ± 2.0	528.2 ± 2.1	530.1 ± 3.0	518.0 ± 2.6	521.9 ± 1.8
20–30	541.7 ± 2.5	534.5 ± 2.0	538.8 ± 3.3	536.2 ± 2.2	525.7 ± 2.0	530.9 ± 2.4	525.9 ± 2.3	515.0 ± 2.7	521.4 ± 2.4
C:N RATIO									
0–10	53.8 ± 1.4	51.0 ± 1.2	47.8 ± 1.1	57.1 ± 2.4	59.4 ± 2.3	56.3 ± 1.7	66.0 ± 2.6	62.5 ± 2.0	65.5 ± 2.3
10–20	62.3 ± 1.5	53.1 ± 1.3	54.8 ± 0.9	68.5 ± 2.4	60.2 ± 1.5	59.8 ± 1.6	73.9 ± 1.7	66.9 ± 2.3	64.1 ± 2.1
20–30	63.6 ± 1.4	58.1 ± 2.0	57.3 ± 1.5	67.6 ± 2.5	67.0 ± 1.5	64.7 ± 2.2	74.6 ± 3.6	69.6 ± 3.4	67.7 ± 2.0

Values refer to three soil depths each covering 10 cm and three different forest management stands. Each value represents a mean of 24 samples ± S.E.



Influence of transplant size on the above- and below-ground performance of four contrasting field-grown lettuce cultivars

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Background and aims: Modern lettuce cultivars underperform under conditions of variable temporal and spatial resource availability, common in organic or low-input production systems. Information is scarce on the impact of below-ground traits on such resource acquisition and performance of field-grown lettuce; exploring genetic variation in such traits might contribute to strategies to select for robust cultivars, i.e., cultivars that perform well in the field, even under stress.

Methods: To investigate the impact of below-ground (root development and resource capture) on above-ground (shoot weight, leaf area) traits, different combinations of shoot and root growth were created using transplants of different sizes in three field experiments. Genetic variation in morphological and physiological below- and above-ground responses to different types of transplant shocks was assessed using four cultivars.

Results: Transplanting over-developed seedlings did not affect final yield of any of the four cultivars. Small transplant size persistently impacted growth and delayed maturity. The cultivars with overall larger root weights and rooting depth, "Matilda" and "Pronto," displayed a slightly higher growth rate in the linear phase leading to better yields than "Mariska" which had a smaller root system and a slower linear growth despite a higher maximal exponential growth rate. "Nadine," which had the highest physiological nitrogen-use efficiency (g dry matter produced per g N accumulated in the head) among the four cultivars used in these trials, gave most stable yields over seasons and trial locations.

Conclusions: Robustness was conferred by a large root system exploring deep soil layers. Additional root proliferation generally correlates with improved nitrate capture in a soil layer and cultivars with a larger root system may therefore perform better in harsh environmental conditions; increased nitrogen use efficiency can also confer robustness at low cost for the plant, and secure stable yields under a wide range of growing conditions.

Keywords: lettuce, transplanting, root activity, nutrient use efficiency

INTRODUCTION

In organic or low-input production systems, nutrient availability is more dependent on the soil's biological, chemical, and physical processes that influence mineralization of organic fertilizers than in conventional, high-external input production systems. Indeed, in conventional systems fertilization is provided in a mineral form and nutrients are therefore readily available for uptake by the plants once they are sown or transplanted. In lettuce, the impact of variable temporal or spatial shortage of water and nutrients common in organic production systems may significantly reduce final yields, as shown by Kerbiriou et al. (2013). In lettuce, like in other crop plants, breeding has mainly focused on above-ground characteristics, and modern cultivars have been bred for high-input production systems; these cultivars are characterized by large heads and small root systems (Johnson et al., 2000).

The small root systems perform sufficiently in such intensive systems.

Current cultivars also have a shallow root system, concentrated in the top 0.20 m of the soil profile (Johnson et al., 2000) which limits the access to deeper soil zones rich in water and nutrients that have leached through the profile. This root morphotype can affect shoot performance under organic conditions, which entail high temporal and spatial variability of resources availability. Exploring the impact of morphological (e.g., spatial configuration) and physiological (e.g., resource capture efficiency) root traits on shoot growth of lettuce may thus be interesting when evaluating the field performance of cultivars under organic conditions. Such investigation might be valuable in breeding programmes, as a mean to select genotypes with desirable root traits increasing tolerance to abiotic stresses and consequently

improved yield stability (Bengough et al., 2006). One way to study the impact of below-ground processes—i.e., root growth and resource capture—on shoot growth of lettuce in field conditions is to impact the equilibrium existing between root and shoot growth, by, for instance, altering the root:shoot ratio during the growth. An easy way to manipulate the root:shoot ratio of lettuce during growth is to use different root:shoot ratios at transplanting.

Transplanting is a common horticultural practice, which aims at increasing productivity in horticultural systems. In Western Europe, field-grown lettuce crops are established from transplants raised in compact peat blocks in greenhouses; because seeds germinate faster and more uniformly in peat blocks than in the field, transplanted crops are more competitive toward early weed infestation (Maltais et al., 2008) and provide a more uniform stand, thus facilitating crop scheduling (Cattivello and Danielis, 2008), reducing cropping time and allowing more plantings per year in the same field. However, transplanting induces a major stress in lettuce cultivation: lettuce seedlings in the optimal stage for transplanting (5–7 leaf stage) often suffer from mechanical root pruning (decapitation of the root tip; Biddington and Dearman, 1984) when seedlings are pulled out of the tray. The loss of root tips and root hairs due to root pruning at transplanting disturbs the root:shoot ratio and induces a “recovery phase” during which shoot growth is suppressed until the previous root:shoot ratio is restored (Bar-Tal et al., 1994a).

During this “recovery phase” capture of water (Grossnickle, 2005) and of nutrients (Bar-Tal et al., 1994b) is impaired to levels below requirements. Moreover, there is an imbalance in root and shoot hormones (Overvoorde et al., 2010) and additional assimilates are allocated to the roots to heal root injuries and restore root growth (Bastow Wilson, 1988). Nevertheless, moderate root pruning at transplanting, despite the need for a “recovery phase,” seems to hardly affect final yields: for instance, Bar-Tal et al. (1994a) found that fruit number or total fresh fruit yield were not significantly reduced in tomato plants whose roots were mildly pruned at transplanting, compared with plants whose roots stayed intact at transplanting. In a recent study, Ros et al. (2003) found that 40% root pruning of rice seedlings at transplanting had only a small effect on shoot growth, reducing grain yield and straw dry matter at maturity by a mere 10%. These findings were established for crops like rice, that require a long field growth; it is unclear what the consequences of root pruning could be on a short-cycle crop like lettuce, which is usually harvested within 100 days of field growth (Mou, 2011).

The small or short-lasting effect of root pruning on shoot growth implies that plants are plastic and able to overcome physical damage and adjust to their environment. Plants developed strategies to overcome the loss of root tips and root hairs at transplanting and to compensate for the subsequent impaired resource capture. For instance, Bar-Tal et al. (1994b) found that root pruning in tomato temporarily increased relative growth rate of the pruned roots compared to the intact roots and that nitrogen uptake per unit root volume was larger for plants with pruned root systems than for intact ones. Cattivello and Danielis (2008) showed that chemical root pruning in a selection of vegetables (asparagus, celery, Treviso chicory, fennel, lettuce, and parsley)

resulted in a more fibrous and branched root system and had no long-term impact on yield.

In lettuce, the contribution of root traits to field performance has not yet been investigated. It is not clear yet how plastic the plants are in displaying an adaptive response to stresses in the field, and what the contribution is of root morphological (changes in root spatial exploration) or root physiological (resource uptake for instance) traits to shoot development. We used different types of shocks caused by transplanting as a proxy for stress induction. By creating three levels of stress using three growth stages (i.e., differences in root:shoot ratios and in size) at transplanting, we expect to observe different responses in shoot growth that may be explained by below-ground cues, such as root growth and nitrate uptake.

Moreover, breeders assume that there might be considerable genetic variation in the capacity of lettuce plants to recover from transplanting, based on field observations (Velema and Koper, pers. commun.). This suggests that cultivars may develop various strategies below- and above-ground to overcome the disturbance in root:shoot ratio created by transplanting. This study also aims at identifying genetic variation in the physiological below- and above-ground responses to different types of transplant shocks.

MATERIALS AND METHODS

CULTIVAR CHOICE AND GROWING TRANSPLANTS

Four commercial butter head cultivars, “Mariska,” “Matilda,” “Nadine,” and “Pronto,” were chosen. These were known for their robust performance in the field, but also for differences in growth pattern. In a previous pilot study they also showed contrasting rooting patterns (deep vs. superficial) (Den Otter and Lammerts van Bueren, 2007). These cultivars are commonly sold to conventional and organic growers for cropping in spring, summer, and autumn seasons and have been performing consistently over many years (Enza Zaden, pers. commun.).

Seeds used in each of these experiments originated from seed lots produced under the same environmental conditions. Seeds were sown in 4 × 4 × 4 cm organic peat blocks (Jongerius, Houten, Netherlands) after breaking seed dormancy by exposure to 4°C for 24 h. Transplants were raised in a greenhouse with day temperature of 20°C and night temperature of 15°C.

EXPERIMENTAL DESIGN

Three trials were implemented at two different locations: Wageningen (51.97° N, 5.67° E, Netherlands) in spring 2009 and 2010 and Voorst (52.23° N, 6.08° E, Netherlands) in summer 2009. Each trial included three repetitions. The experimental set up was a complete randomized block design, each block consisting of 12 plots featuring all combinations of four cultivars and three transplant sizes.

FIELD CONDITIONS

For each trial, weather data (air temperature, radiation, rainfall) were recorded daily (Voorst) or hourly (Wageningen) at the nearest weather station (for the Wageningen trials, data were collected from <http://www.met.wau.nl/> and for the Voorst trials, data were collected from the on-farm weather station). Soil temperatures were measured at 4–5 depths (0–0.1, 0.1–0.2, 0.2–0.3,

0.3–0.4, and 0.4–0.5 m) using a data logger. Air and soil temperatures recorded during the growing season at Wageningen in spring 2009 were fairly conducive to crop growth, average daily air temperatures ranging from 9.5 to 20°C and average daily soil temperatures at –0.25 m ranging between 10 and 16°C. Rainfall was rather limited during the experiment (**Table 1**) but there was no drought stress. In contrast, rainfall during the early spring trial at Wageningen in 2010 was abundant, but air temperatures were rather low: during 36 days (i.e., half of the growing period) the daily mean temperature did not exceed 9.5°C. Average daily soil temperatures recorded at –0.25 m ranged between 6 and 15°C during growth, and did not exceed 10°C during the first month of growth. Experiment Voorst 2009 was conducted during late spring under warm weather. The average daily air and soil temperatures at –0.25 m were 16.5 and 17°C, respectively, with air temperatures above 13°C during 85% of the growing period. Soil temperatures at –0.25 m ranged between 15.5 and 20°C. Cumulated degree-days (based on air temperatures), as well as cumulated rainfall and irrigation (in the case of Voorst 2009) at each sampling date for each trial, are shown in **Table 1**.

TREATMENTS

Transplanting shocks were used as a proxy for stress induction: seedlings at different growth stages at the moment of transplanting presented different qualities of transplants; three contrasting transplant sizes were obtained by staggered sowings with intervals of 2 weeks. These differences in growth duration before transplanting resulted in intertwined variations in shoot characteristics (number of leaves, and consecutive leaf area) and in root characteristics (root length and mass, not measured at transplanting because of the organic matter in the peat blocks), and associated with the latter also in different levels of damage of the root system at transplanting:

- “Over-Developed” (OD) transplant size: 7–9 leaf-stage, developed root system largely emerging out of the peat block, many roots tips mechanically removed at transplanting, both changing the root:shoot ratio and causing mechanical damage, in addition to the physiological shock of rather large seedlings;
- “Normally Developed” (ND) transplant size: 5-leaf stage, only few roots emerging out of the peat block, some root tips

mechanically removed at transplanting, hardly any mechanical damage or root:shoot ratio change;

- “Under-Developed” (UD) transplant size: 3-leaf stage, no visible roots emerging from the peat block except the tap root which was damaged at transplanting; the shock here was mainly the early transplanting of rather small seedlings.

Crop plants raised from these treatments are called “OD plants,” “ND plants,” and “UD plants,” respectively.

In Voorst 2009 damage caused by a hail storm hastened final harvest by approximately 2 weeks, and therefore harvested plants were not fully mature; as UD plants formed heads very late they were not harvested. The final harvest date in the Wageningen trials was determined according to the marketable stage of head maturation for the ND plants. All treatments were harvested at the same date, no matter head maturation stages (which was visually not affected by the treatments at final harvest).

FIELD MANAGEMENT

All trial fields had been organically managed and were selected for uniform management in the past and for adequate soil structure. They were fertilized prior to transplanting with 100 kg/ha nitrogen, from seaweed pellets (9% N, 3% P, 3% K + 3% MgO, EcoFertiel, EcoStyle, Appelscha, Netherlands). Weeding was done manually every week. Irrigation was only provided at Voorst in 2009: 10 mm water was given 20 days after transplanting.

MEASUREMENTS

Calculation of thermal time

Cumulated degree days at each sampling date were calculated as the sum, between the date of transplanting and the sampling date, of the degrees above 4°C (base temperature for lettuce), based on an average daily temperature:

$$\text{CDD}_{\text{sampling } x} = \sum_{\text{day } 0}^{\text{sampling date } x} \left[\frac{(T_{\max} + T_{\min})}{2} - T_{\text{base}} \right]$$

where T_{\max} and T_{\min} correspond to the maximum and to the minimum temperatures recorded on a certain day, respectively.

Table 1 | Planting and harvesting dates and cumulated thermal time (CDD) and rainfall at the three sampling moments for each of the three field trials.

Planting date	Wageningen 2009		Voorst 2009		Wageningen 2010	
	1 April 2009		25 May 2009		23 March 2010	
	CDD ^a	Rainfall (mm)	CDD	Rainfall (mm)	CDD	Rainfall (mm)
Root sampling 1	111	7.3	152	20.0	152	35.5
Root sampling 2	224	21.5	253	77.4	252	60.4
Root sampling 3	325	32.4	420	83.4	347	91.3
Final harvest date	31 May 2009		30 June 2009		31 May 2010	

^a Cumulated Degree-Days (°Cd) after planting at sampling date based on air temperature, using a base temperature of 4°C.

Shoot measurements

Fresh weight, dry weight, total leaf area, and total number of leaves of three plants per plot were assessed weekly. Final harvest took place 6–10 weeks after transplanting depending on trial. For samples taken at final harvest total nitrogen in the head was measured using the Kjeldahl method. Physiological Nitrogen Use Efficiency (NUE, g DM g⁻¹ N in head) was calculated based on the head [N] (g N kg⁻¹ DM) extracted by the Kjeldahl method: NUE = 1/head [N].

Root measurements

Roots outside the peat block of three plants per plot were sampled at three moments during growth, and at two positions (“central” and “peripheral”) for each plant using the method described by Van Noordwijk et al. (1985) (**Figure 1**). Using a cylindrical auger of 0.07 m diameter and 0.1 m height, samples were taken every 0.1 m over a depth of 0.5 m. For each sample, roots were rinsed from soil and most organic matter using a rinsing machine and remaining organic matter was then manually removed using tweezers. Root samples were subsequently scanned and root length was measured using WinRhizo Pro 2007 (v2005b, Regent Instruments, Québec, Canada). Root dry matter was measured after drying the root samples at 105 °C for 24 h. Root Mass Density per layer (mg root dry weight g⁻¹ soil) was calculated as root dry weight measured in the sample taken with the auger, divided by the product of the volume of soil in the sample taken and the bulk density of that soil (based on dry weight).

Soil measurements

Soil samples were taken simultaneously on the opposite side of the same plants (**Figure 1**). For three plants per plot, soil samples were pooled to account for plant-to-plant variation. Soil moisture content was recorded after drying at 40°C for 48 h and soil nitrate content (soil [NO₃]) was measured using an Ion Selective Electrode (ThermoFisher, Waltham, MA, USA) using the method described previously by Sibley et al. (2009) and also used in Kerbiriou et al. (2013). As a measure for the difference between treatments in estimated NO₃ capture, the difference between

the average soil [NO₃], based on pooled data for all cultivar × transplant size combinations within a layer, and the soil [NO₃] measured on an individual plot was expressed as percentage difference in estimated NO₃ capture. This was calculated as:

$$\% \text{ difference for sample } i = 100 \times (([\text{NO}_3]_i / [\text{NO}_3]_{\text{avg}}) - 1)$$

Where

$[\text{NO}_3]_i$ = observed [NO₃] in sample *i* on sampling date *d* and for soil layer *l*

$[\text{NO}_3]_{\text{avg}}$ = the average observed [NO₃] in all samples on sampling date *d* and for soil layer *l*.

STATISTICAL ANALYSES

Dry weight and total leaf area data of all harvests for each trial were pooled per plot and a regression analysis was performed using the expolinear model of Goudriaan and Monteith (1990) to obtain estimates of the curve fit parameters for each combination of transplant size × cultivar × replicate. Then a two-way ANOVA was performed on those parameters to determine main effects of stage at transplanting (UD, ND, and OD), cultivar and their interactions, followed by a Tukey test *p-value* ≤ 0.05 to determine the statistical significance of the differences.

Moreover, for each sampling date for each trial a two-way ANOVA was performed followed by the Tukey test at *p-value* ≤ 0.05 to determine the statistical significance of the differences.

Curve fitting and statistical analyses were performed with Genstat 15th Edition (Hempstead, UK).

RESULTS

EFFECT OF TRANSPLANT SIZE ON SHOOT GROWTH AND DEVELOPMENT

The overall effects of transplant size on dry matter accumulation and total number of leaves decreased in time after transplanting (cf. **Figure A1**). Differences between the Over-Developed- (“OD”) or the Under-Developed (“UD”) plants and the Normally-Developed (“ND”) plants when expressed in percentages were larger for dry matter accumulation than for total number of leaves, and these differences disappeared faster for the OD plants than for the UD plants (cf. **Figures A1A, A1B**). After 200°Cd there was less than 20% difference in dry matter between the OD and the ND plants, whereas this level was reached by 500°Cd for the UD plants. No cultivar differences were observed. The same trends were observed in all experiments.

Dry matter accumulation

Differences in growing conditions affected the dry matter accumulation of the four cultivars, independently of stage at which they were transplanted, although all followed a typical expolinear growth pattern (Goudriaan and Monteith, 1990; **Figure 2**). Overall warmer growing conditions recorded during Voorst 2009 led to a higher maximal relative growth rate during the initial exponential growth phase, a lower maximal growth rate during the linear growth phase, and a reduced “lag phase” (time at which the asymptote of the expolinear growth curve meets the time abscissa, cf. **Figure 2**), compared to the trials conducted in Wageningen in 2009 and 2010 (**Table 2**).

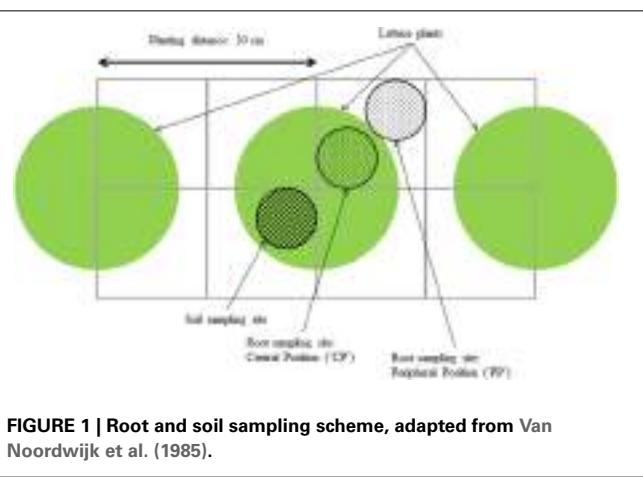
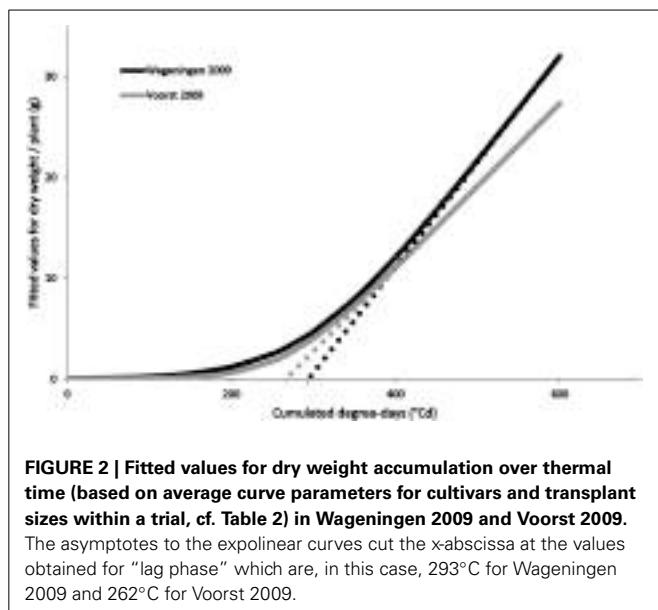


FIGURE 1 | Root and soil sampling scheme, adapted from Van Noordwijk et al. (1985).



Maximal relative growth rate during exponential phase. During the exponential growth phase, OD plants had a significantly smaller maximal relative growth rate than ND and UD plants, while no differences were observed between ND and UD in Wageningen 2009 and 2010. In Wageningen 2009 "Mariska" had the highest maximal relative growth rate for all transplant sizes. The two-way interaction was not significant in Wageningen 2009 and 2010, while it was in Voorst 2009. Here the same trend was observed as in the Wageningen trials but only the maximal relative growth rate of "Mariska" ND plants was different from all other treatments.

Maximal growth rate during the linear phase. No significant effect of transplant size was recorded on the maximal growth rate during the linear phase in any of the three trials. "Mariska" had a significantly lower growth rate than the other cultivars in the linear phase for all transplant sizes in Wageningen 2009 and Voorst 2009. The same trend was observed in Wageningen 2010, albeit not significant (p -value = 0.058). No two-way interactions were significant.

"Lag phase". UD plants had a longer lag phase in both Wageningen trials than OD and ND plants. In Voorst 2009, OD plants had a shorter lag phase than ND plants (Table 2). In Wageningen 2009 and Voorst 2009, "Mariska" had a significantly shorter lag phase than other cultivars across transplant sizes (Table 2). No two-way interactions were significant.

Dry weight at final harvest. While there was no significant effect of transplant size on dry weight at final harvest in Wageningen 2009, cultivar differences were visible, with "Mariska" having the lowest dry weight at final harvest and "Nadine" performing the best (Table 3). In Wageningen 2010, significant interactions between transplant size and cultivar effects were recorded. No significant difference at p = 0.05 was found between cultivars within the UD and the ND transplant size. OD plants of "Matilda"

and "Nadine" had higher final dry weights than OD plants of "Mariska." Whereas UD plants of "Matilda" and "Pronto" had significantly smaller dry weights at final harvest compared to ND and OD plants of these cultivars, for "Mariska" and "Nadine" there was no significant effect of transplant size on dry weight at final harvest.

In Voorst 2009, OD plant had significantly higher dry weight at final harvest than ND plants (Table 3). "Matilda" had a significantly higher final dry weight per plant than other cultivars across transplant sizes, whereas "Mariska" had the lowest dry weight at final harvest across transplant sizes.

(Shoot dry weights measured at intermediate root samplings are presented in the supplementary materials, Tables S1, S2).

Leaf area expansion

Interestingly no significant cultivar effect was found on the curve fit parameters of an expolinear model on leaf area expansion (Table 4). On the other hand, size at transplanting significantly affected the leaf area expansion rates of the plants both during the exponential and the linear growth phases.

Maximal relative leaf area expansion rate during the exponential phase. In Wageningen 2009 and Wageningen 2010, UD plants of all cultivars had a significantly higher maximal relative leaf expansion rate during the exponential phase than ND and OD plants (Table 4). In Wageningen 2009, OD plants had a significantly lower maximal relative leaf expansion rate during the exponential phase than ND plants (Table 4).

Maximal leaf area expansion rate during the linear phase. In Wageningen 2009, the leaf expansion rate of the UD and ND plants of all cultivars was reduced during the linear phase compared to OD plants (Table 4).

"Lag phase". A significantly longer lag phase was found for the OD plants of all cultivars compared to ND and UD plants (Table 4) only in Wageningen 2009.

EFFECT OF TRANSPLANT SIZE ON ROOT GROWTH AND RESOURCE CAPTURE

Root dry weights

In Voorst 2009, overall measured root dry weights were much lower than in Wageningen 2009 and Wageningen 2010 due to the precocious termination of the trial (Table 5).

In Wageningen 2009 and Voorst 2009, no significant transplant size effect was found on root weight at final harvest. On the other hand, significantly lower root weights were observed for all cultivars of UD plants compared to OD- and ND plants in Wageningen 2010 (Table 5). In this trial, no significant cultivar effect was measured, whereas these were recorded in Wageningen 2009 and Voorst 2009. In both trials, "Mariska" had—on average for all transplant sizes—a lower total root weight per plant than "Pronto" (Table 5).

(Root dry weights measured at intermediate root samplings are presented in the supplementary materials, Tables S3, S4).

Table 2 | Values for curve fit parameters when applying an expolinear model (Goudriaan and Monteith, 1990) for dry matter accumulation against thermal time, for combinations of transplant sizes and cultivars in each of three experiments.

Cultivar:	Maximal relative growth rate in the exponential phase ($\text{mg g}^{-1} (\text{°Cd})^{-1}$)				Maximal growth rate in the linear phase ($\text{mg DM m}^{-2} (\text{°Cd})^{-1}$)				“Lag phase” (°Cd)						
	Mariska	Matilda	Nadine	Pronto	Mariska	Matilda	Nadine	Pronto	Mariska	Matilda	Nadine	Pronto			
<i>TS^f</i>	Wageningen 2009				<i>Tr.^d</i>	Wageningen 2009				Wageningen 2009					
OD ^a	15.9	14.4	15.4	14.7	15.1a	91	109	110	112	106a	267	306	299	304	294ab
ND ^b	17.7	16.5	16.8	16.8	16.9b	93	102	113	104	103a	265	292	292	283	283a
UD ^c	18.8	16.5	16.3	17.9	17.4b	93	104	116	106	105a	279	318	323	296	304b
Cv. ^e	17.5b ^g	15.8a	16.2ab	16.5ab		92a	105b	113b	107b		271a	305b	305b	294ab	
	Wageningen 2010				<i>Tr.</i>	Wageningen 2010				<i>Tr.</i>	Wageningen 2010				<i>Tr.</i>
OD	16.0	14.9	14.7	14.9	15.1a	103	180	145	140	142a	255	311	291	286	286a
ND	16.5	18.2	17.3	16.8	17.2b	132	140	125	130	132a	283	273	274	278	277a
UD	16.7	19.3	19.4	18.6	18.5b	126	129	123	113	123a	316	319	309	309	313b
Cv.	16.4a	17.5a	17.1a	16.7a		121a	149a	131a	128a		285a	301a	291a	291a	
	Voorst 2009				<i>Tr.</i>	Voorst 2009				<i>Tr.</i>	Voorst 2009				<i>Tr.</i>
OD	21.1a	18.5a	20.7a	16.5a	19.2	68	100	78	101	87a	208	267	222	277	244a
ND	41.3b	24.8a	25.2a	22.4a	28.4	48	105	75	80	77a	218	330	303	303	288b
UD	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cv.	31.2	21.7	23.0	19.5		58a	103b	76ab	90b		213a	299b	262b	290b	

^a “Over-developed” transplant size.^b “Normally developed” transplant size.^c “Under-developed” transplant size.^d Mean for transplant size across cultivars.^e Mean for cultivar across transplant sizes.^f Transplant size.^g Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is indicated for each single parameter within an experiment and at the level of main factors cultivar or transplant size when the two-way interaction was not significant, and at the level of transplant size \times cultivar when the interaction was significant.

Root mass densities over the soil profile

Figure 3 shows the root mass densities for the four cultivars under the three transplant sizes over the soil profile at the third root sampling date, both at the central- and at the peripheral sampling position (cf. Figure 1).

Apparently, the most important element of variation in root spatial (horizontal and vertical) exploration (as measured by root mass densities over the soil profile at the different sampling positions) was conferred by the growing season: whereas under the rather optimal conditions in Wageningen 2009 (Figures 3A–D), the root mass density measured in the top 0.1 m at the central sampling position was rather identical to the root mass density measured at the peripheral position for all cultivars, with the exception of “Nadine” (Figure 3C), under the much cooler conditions in Wageningen 2010 a larger root mass density was measured at the central position compared with the peripheral sampling position (Figures 3E–H). The same pattern was observed, although to a lesser extent, under the rather warm conditions in Voorst 2009 (Figures 3I–L). The transplant sizes did not influence the root mass density distribution over the soil profile in any of the three trials.

Relationship between NO_3^- capture from the Soil and RLD (Root Length Density)

The NO_3^- capture and corresponding root proliferation data are provided in Figure 4. In this figure the percentage difference in Root Length Density (RLD) or in NO_3^- capture between a particular combination of cultivar \times transplant size in a given layer, and the average value obtained for the pooled data per layer has been plotted (cf. Materials and Methods). It is surmised that additional RLD is correlated to additional NO_3^- capture in a layer.

Effect of OD transplant size on NO_3^- capture and root proliferation. In Wageningen 2009, no clear pattern emerged showing a higher RLD being proportionally correlated with a higher NO_3^- capture in a layer. Mainly only the OD plants of “Pronto” showed a higher efficiency in NO_3^- capture from the soil in all layers (Figure 4A), but this was not accompanied by a higher RLD than average in these layers (Figure 4B). Conversely, the OD plants of “Matilda” had a higher than average RLD in the 0.3–0.5 m layers but this was not combined with a higher relative NO_3^- capture. “Nadine’s” OD plants showed an overall reduced RLD throughout the soil profile. In Wageningen 2009 the correlations

Table 3 | Average shoot dry weights (g per plant) of the four cultivars at final harvest, after establishment from three different transplant sizes in each of three trials.

Harvest Date	CDD ^f (°Cd)	TS ^h	Mariska	Matilda	Nadine	Pronto	
Wageningen 2009							<i>Tr.^e</i>
May 25th, 2009	474	OD ^a	30.3 ± 2.3 ^g	32.1 ± 3.1	33.9 ± 4.9	33.1 ± 2.0	32.3a
		ND ^b	31.0 ± 2.1	32.3 ± 2.1	35.3 ± 2.4	33.0 ± 3.2	32.7a
		UD ^c	30.2 ± 2.5	30.0 ± 2.0	33.3 ± 2.7	33.0 ± 2.1	31.6a
		Cv. ^d	30.5a ⁱ	31.5ab	34.1c	32.8bc	
Wageningen 2010							<i>Tr.</i>
May 30th, 2010	400	OD	25.4 ± 2.2abcde	34.0 ± 4.7g	31.3 ± 2.6fg	29.7 ± 3.5efg	30.1
		ND	29.1 ± 2.1cdefgh	33.0 ± 3.8fg	29.4 ± 2.9defg	28.5 ± 3.5bcd	30.0
		UD	23.5 ± 1.8ab	24.0 ± 3.1abc	24.3 ± 3.5abcd	22.4 ± 4.7a	23.6
		Cv.	26.0	30.4	28.3	26.9	
Voorst 2009							<i>Tr.</i>
June 29th, 2009	420	OD	18.5 ± 1.9	22.6 ± 2.7	20.5 ± 2.5	21.5 ± 2.7	20.8b
		ND	13.1 ± 2.0	17.5 ± 2.1	14.2 ± 2.2	14.8 ± 3.3	14.9a
		UD	—	—	—	—	
		Cv.	15.8a	20.1c	17.4ab	18.2b	

^a “Over-developed” transplant size.^b “Normally developed” transplant size.^c “Under-developed” transplant size.^d Mean for cultivar across transplant sizes.^e Mean for transplant size across cultivars.^f Cumulated Degree-Days.^g Standard error of the mean.^h Transplant Size.

ⁱ Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is within an experiment and at the level of main factors cultivar or transplant size when the two-way interaction was not significant and at the level of transplant size \times cultivar when the interaction was significant.

Table 4 | Values for curve fit parameters when applying an expolinear model (Goudriaan and Monteith, 1990) for leaf area expansion against thermal time.

Cultivar:	Maximal relative growth rate in the exponential phase ($\text{mm}^2 \text{cm}^{-2} (\text{°Cd})^{-1}$)				Maximal leaf expansion rate in the linear phase ($\text{cm}^2 \text{m}^{-2} (\text{°Cd})^{-1}$)				Time lost during canopy development before all radiation is intercepted (°Cd)						
	Matilda	Mariska	Nadine	Pronto	Matilda	Mariska	Nadine	Pronto	Matilda	Mariska	Nadine	Pronto			
	TS ^f	Wageningen 2009			Tr. ^d	Wageningen 2009			Tr.	Wageningen 2009			Tr.		
OD ^a	1.46	1.36	1.51	1.36	1.42a	40.6	43.1	41.9	45.2	42.7b	309	350	330	353	335b
ND ^b	1.64	1.65	1.56	1.57	1.60b	34.9	27.3	38.5	36.3	34.2ab	291	285	321	307	301a
UD ^c	1.77	1.67	1.73	1.83	1.75c	37.6	24.5	27.9	35.1	31.3a	305	301	309	300	304a
Cv. ^e	1.62a ^g	1.56a	1.60a	1.59a		37.7a	31.6a	36.1a	38.8a		301a	312a	320a	320a	
Wageningen 2010					Tr.	Wageningen 2010			Tr.	Wageningen 2010			Tr.		
OD	1.53	1.58	1.38	1.57	1.52a	31.5	40.0	45.5	35.0	38.0a	290	304	355	302	313a
ND	1.54	1.63	1.56	1.65	1.60a	41.8	36.5	36.0	38.2	38.1a	321	308	325	308	316a
UD	1.83	1.65	1.84	1.70	1.75b	30.9	40.0	29.1	35.5	33.9a	308	366	329	352	339a
Cv.	1.63a	1.62a	1.60a	1.64a		34.7a	38.9a	36.9a	36.2a		307a	326a	336a	321a	

^a “Over-developed” transplant size.^b “Normally developed” transplant size.^c “Under-developed” transplant size.^d Mean for transplant size across cultivars.^e Mean for cultivar across transplant size.^f Transplant size.

^g Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is indicated for each single parameter within an experiment and only at the level of main factors cultivar and transplant size as the two-way interactions were not significant.

Table 5 | Average estimated root dry weights (g per plant) of the four cultivars at third root sampling, after establishment from three different transplant sizes.

Harvest Date	CDD ^f (°Cd)	TS ^h	Mariska	Matilda	Nadine	Pronto	
Wageningen 2009							Tr. ^e
May 11th, 2009	325	OD ^a	0.39 ± 0.14 ^g	0.47 ± 0.27	0.44 ± 0.12	0.48 ± 0.21	0.44a
		ND ^b	0.35 ± 0.14	0.55 ± 0.26	0.47 ± 0.18	0.53 ± 0.22	0.48a
		UD ^c	0.36 ± 0.10	0.42 ± 0.15	0.46 ± 0.17	0.47 ± 0.17	0.43a
		Cv. ^d	0.37a ⁱ	0.48ab	0.46ab	0.49b	
Wageningen 2010							Tr.
May 25th, 2010	347	OD	0.61 ± 0.22	0.68 ± 0.12	0.52 ± 0.17	0.67 ± 0.20	0.62b
		ND	0.58 ± 0.17	0.63 ± 0.22	0.66 ± 0.32	0.74 ± 0.22	0.65b
		UD	0.40 ± 0.07	0.48 ± 0.19	0.57 ± 0.22	0.55 ± 0.17	0.50a
		Cv.	0.53a	0.60a	0.59a	0.65a	
Voorst 2009							Tr.
June 29th, 2009	420	OD	0.18 ± 0.03	0.28 ± 0.06	0.24 ± 0.12	0.29 ± 0.09	0.25a
		ND	0.12 ± 0.10	0.24 ± 0.11	0.15 ± 0.05	0.29 ± 0.10	0.20a
		UD	—	—	—	—	—
		Cv.	0.15a	0.26bc	0.20ab	0.29c	

^a "Over-developed" transplant size.^b "Normally developed" transplant size.^c "Under-developed" transplant size.^d Mean for cultivar across transplant sizes.^e Mean for transplant size across cultivars.^f Cumulated Degree-Days.^g Standard error of the mean.^h Transplant size.ⁱ Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is within an experiment and at the level of main factors cultivar or transplant size as the two-way interaction was not significant.

were clearer, with an overall higher NO₃ capture being positively correlated with a slightly higher RLD in all layers for all cultivars (**Figures 4C,D**). In Voorst 2009, the capture of NO₃ for the OD plants in all layers did not differ from the average, although the RLD was increased compared with the average for all cultivars through the soil profile, except for "Mariska" (**Figures 4E,F**).

Effect of UD transplant size on NO₃ capture and root proliferation. In Wageningen 2009, overall NO₃ capture was not extremely impaired by a somewhat smaller RLD (**Figures 4G,H**). "Matilda" showed the most pronounced impaired NO₃ capture in the 0–0.4 m layers, although this was not associated with a lower RLD in these layers. In Wageningen 2010, NO₃ capture of UD plants was reduced compared with the average in all layers, and this was well correlated with a reduced RLD throughout the soil profile (**Figures 4I,J**).

Root:shoot ratios over time

Table 6 provides details on the average root:shoot ratios of the four cultivars at the three root sampling dates. Over time the root:shoot ratios declined in all experiments and during the entire period of measurement, except in Wageningen 2009 between the first and second sampling, associated with the low temperatures

during the initial growth period in that experiment. Plants in the Voorst 2009 experiment had considerably lower root:shoot ratios than plants in the Wageningen 2009 and Wageningen 2010 experiments at all samplings, in line with the very low root mass observed in the Voorst 2009 experiment. Differences in root:shoot ratios between transplant sizes were only observed in the Voorst 2009 experiment at the second sampling: the normally developed transplants had a higher root:shoot ratio than the over-developed transplants in all cultivars. The same trend was also visible at the first sampling but could not be proven statistically. This general lack of treatment effect even at early stages shows how short-lived the effect of root damage associated with the transplanting actually was and how plastic dry matter partitioning over roots and shoots can be. Significant differences in root:shoot ratio amongst cultivars were found at later sampling dates, but were not always consistent across experiments and were not repeatable over samplings. However, "Pronto" showed consistently high values and "Mariska" consistently low values when cultivar differences proved significant (**Table 6**).

Physiological nitrogen use efficiency (NUE) and nutritional status of the plant

Physiological nitrogen use efficiency. Significant interactions were found between transplant sizes and cultivar effects on

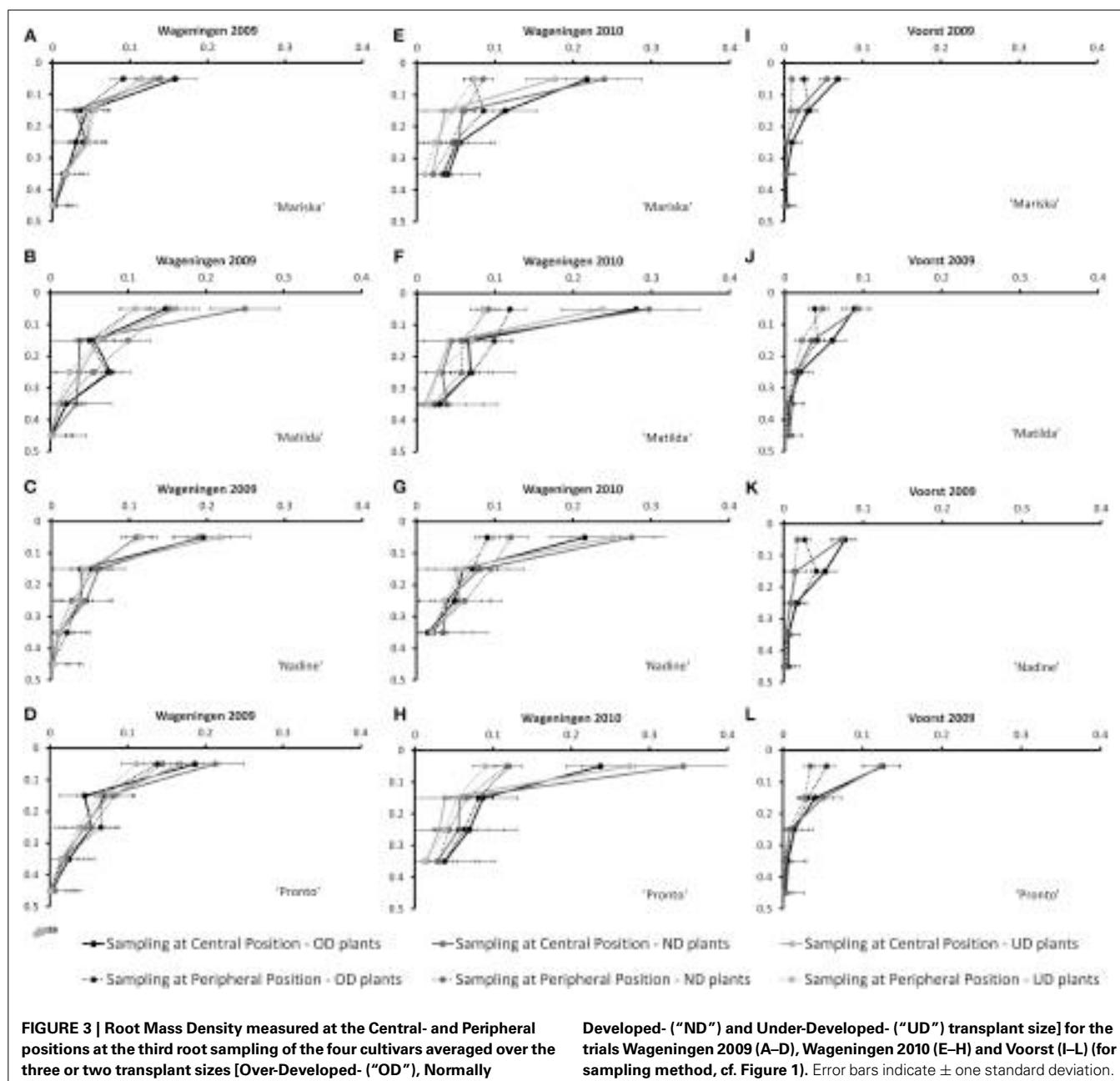


FIGURE 3 | Root Mass Density measured at the Central- and Peripheral positions at the third root sampling of the four cultivars averaged over the three or two transplant sizes [Over-Developed- ("OD"), Normally

Developed- ("ND") and Under-Developed- ("UD") transplant size] for the trials Wageningen 2009 (A–D), Wageningen 2010 (E–H) and Voorst (I–L) (for sampling method, cf. Figure 1). Error bars indicate \pm one standard deviation.

physiological NUE (defined as g dry weight per g nitrogen found in the plant) in Wageningen 2010 and Voorst 2009 (Table 7). In Wageningen 2009, OD and UD plants had a significantly reduced physiological NUE compared to ND plants. Overall, "Nadine" showed to have a higher physiological NUE whatever transplant size was applied, compared to "Mariska." In Wageningen 2009, this cultivar had the lowest physiological NUE. In Wageningen 2010, OD and ND plants of "Matilda" had a significantly higher physiological NUE than OD plants of "Mariska."

In Voorst 2009, physiological NUE values were lower than values obtained for the Wageningen trials (Table 7). No significant

difference in physiological NUE values was found between transplant sizes or between cultivars. Only within the ND plants, "Nadine" had a significantly higher physiological NUE than the other cultivars.

Nutritional status of the plant. Figure 5 shows the relationship between the nutritional status of the plant (shoot [N]) and its estimated root dry weight for the three trials at the respective final harvests. The alignment of the data obtained for the three trials highlights that the final harvests took place at different nutritional statuses of the plants which were proportionally related to root dry weight.

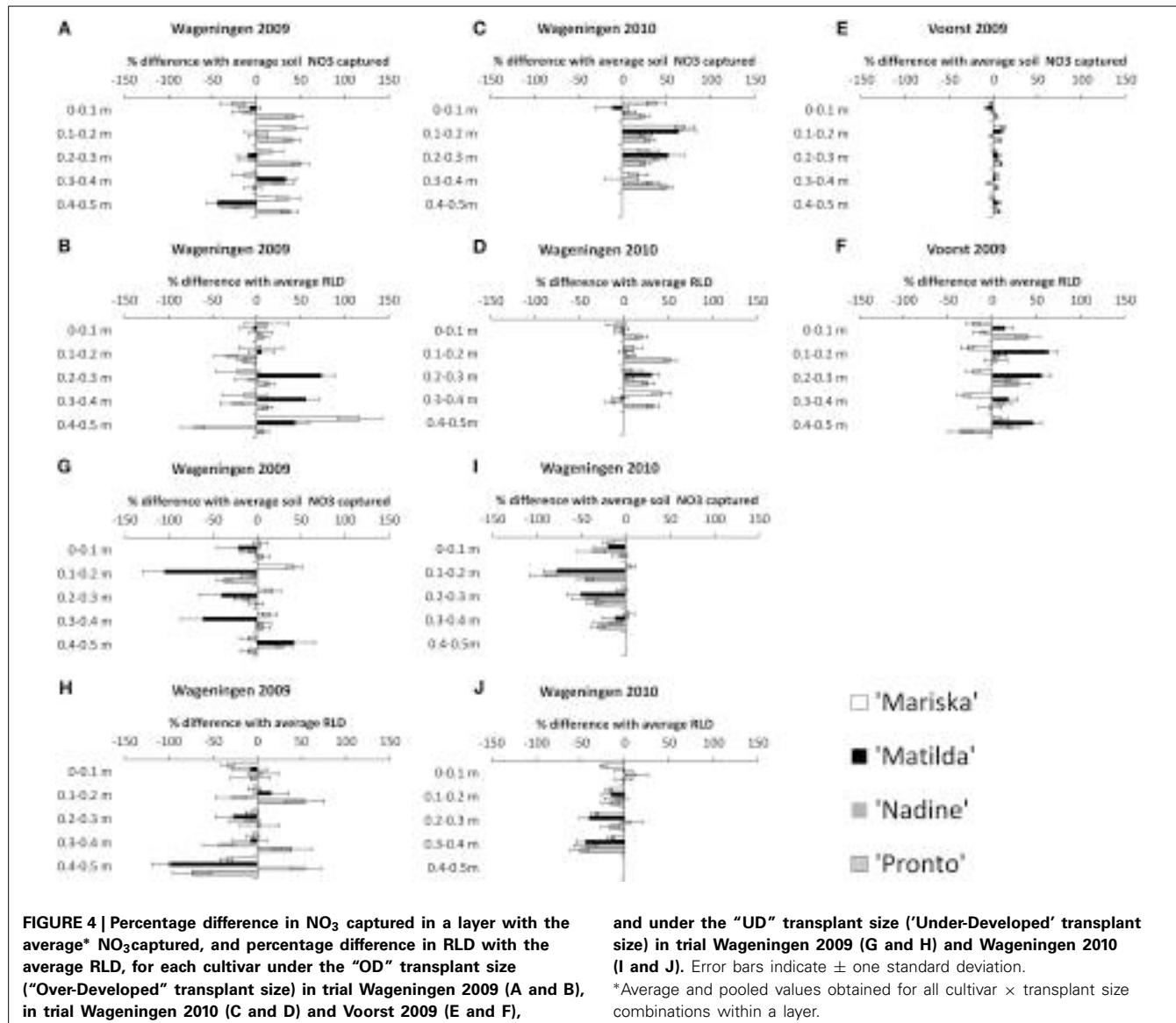


FIGURE 4 | Percentage difference in NO₃ captured in a layer with the average* NO₃ captured, and percentage difference in RLD with the average RLD, for each cultivar under the “OD” transplant size (“Over-Developed” transplant size) in trial Wageningen 2009 (A and B), in trial Wageningen 2010 (C and D) and Voorst 2009 (E and F).

and under the “UD” transplant size (“Under-Developed” transplant size) in trial Wageningen 2009 (G and H) and Wageningen 2010 (I and J). Error bars indicate ± one standard deviation.

*Average and pooled values obtained for all cultivar × transplant size combinations within a layer.

DISCUSSION

Transplanting four cultivars at three different transplant stages gave a significant insight into the impact of below-ground physiological processes developed by lettuce to overcome the short-lived stresses created by altering the initial root:shoot ratio and to maintain shoot growth. Strong Treatment × Environment interactions were visible in these trials.

SEASONS AND SOIL CONDITIONS IMPACTED TRANSPLANT SIZE EFFECT ON SHOOT AND ROOT GROWTH: TREATMENT X ENVIRONMENT INTERACTIONS

The early spring growing seasons in the Wageningen 2009 and 2010 trials were to a certain extent similar in terms of photoperiod and soil conditions (texture, CEC, etc.) although the Wageningen 2010 trial experienced slightly more rainfall (Table 1) and a colder start (cf. Materials and Methods) than the Wageningen 2009 trial; in contrast, the Voorst 2009 trial was conducted later in the

season, under higher soil and air temperatures and likely higher levels of radiation (not recorded), which led to much higher relative growth rates during the initial growth phase (Table 2). On the other hand, whereas maximal growth rates during the linear phase reached average values between 100 (Wageningen 2009) and 130 (Wageningen 2010) mg DM m⁻² (°Cd)⁻¹, these rates remained below 100 mg DM m⁻² (°Cd)⁻¹ for Voorst 2009 (Table 2). This influenced the effects of transplant sizes to a large extent, as the differences between the OD and the ND plants were significant in the Voorst 2009 trial but not in the early spring trials in Wageningen (Table 3). In Voorst 2009, the warm growing conditions even led to failure of UD plants, of which head formation and maturation did not occur within the time frame of the experiment, despite the higher cumulated thermal time.

Figure 5 shows that the root dry weight of the plants under the various transplant sizes was not driven by the transplant

Table 6 | Average root:shoot ratios of the four cultivars at first, second and third root sampling, after establishment from three different transplant sizes in each of three trials.

Harvest date	CDD ^f (°Cd)	TS ^h	Mariska	Matilda	Nadine	Pronto	
FIRST ROOT SAMPLING							
April 15th, 2009	111	OD ^a	0.107 ± 0.082 ^g	0.113 ± 0.129	0.091 ± 0.056	0.133 ± 0.035	0.111a
		ND ^b	0.115 ± 0.051	0.133 ± 0.091	0.072 ± 0.023	0.114 ± 0.038	0.108a
		UD ^c	0.095 ± 0.041	0.083 ± 0.059	0.061 ± 0.023	0.073 ± 0.040	0.078a
		Cv. ^d	0.105a ⁱ	0.110a	0.075a	0.107a	
April 26th, 2010	152	OD	0.111 ± 0.106	0.089 ± 0.025	0.114 ± 0.041	0.086 ± 0.022	0.100a
		ND	0.094 ± 0.057	0.121 ± 0.046	0.108 ± 0.088	0.071 ± 0.036	0.099a
		UD	0.087 ± 0.047	0.087 ± 0.048	0.095 ± 0.055	0.091 ± 0.037	0.090a
		Cv.	0.097a	0.099a	0.106a	0.083a	
June 8th, 2009	152	OD	0.070 ± 0.029	0.078 ± 0.041	0.072 ± 0.041	0.069 ± 0.041	0.072a
		ND	0.070 ± 0.047	0.127 ± 0.162	0.072 ± 0.035	0.071 ± 0.050	0.085a
		UD	–	–	–	–	–
		Cv.	0.070a	0.102a	0.072a	0.070a	
SECOND ROOT SAMPLING							
April 28th, 2009	224	OD	0.082 ± 0.049	0.102 ± 0.060	0.099 ± 0.040	0.130 ± 0.043	0.103a
		ND	0.110 ± 0.022	0.109 ± 0.041	0.084 ± 0.033	0.082 ± 0.040	0.096a
		UD	0.086 ± 0.019	0.132 ± 0.049	0.103 ± 0.037	0.128 ± 0.058	0.112a
		Cv.	0.093a	0.115a	0.095a	0.113a	
May 10th, 2010	252	OD	0.039 ± 0.012	0.035 ± 0.007	0.048 ± 0.017	0.052 ± 0.026	0.043a
		ND	0.043 ± 0.018	0.029 ± 0.010	0.043 ± 0.013	0.063 ± 0.034	0.045a
		UD	0.049 ± 0.018	0.043 ± 0.009	0.062 ± 0.017	0.057 ± 0.018	0.053a
		Cv.	0.044ab	0.036a	0.051bc	0.058c	
June 17th, 2009	253	OD	0.010 ± 0.007	0.012 ± 0.007	0.012 ± 0.006	0.022 ± 0.011	0.014a
		ND	0.017 ± 0.012	0.028 ± 0.033	0.021 ± 0.019	0.029 ± 0.019	0.024b
		UD	–	–	–	–	–
		Cv.	0.014a	0.020a	0.016a	0.025a	
THIRD ROOT SAMPLING							
May 11th, 2009	325	OD	0.028 ± 0.011	0.038 ± 0.027	0.033 ± 0.008	0.040 ± 0.019	0.035a
		ND	0.026 ± 0.011	0.044 ± 0.021	0.034 ± 0.014	0.045 ± 0.024	0.037a
		UD	0.028 ± 0.007	0.042 ± 0.017	0.042 ± 0.017	0.037 ± 0.015	0.037a
		Cv.	0.028a	0.041b	0.036ab	0.040ab	
May 24th, 2010	347	OD	0.029 ± 0.011	0.024 ± 0.006	0.021 ± 0.008	0.026 ± 0.009	0.025a
		ND	0.023 ± 0.006	0.026 ± 0.011	0.028 ± 0.014	0.029 ± 0.009	0.027a
		UD	0.021 ± 0.005	0.025 ± 0.010	0.030 ± 0.012	0.032 ± 0.014	0.027a
		Cv.	0.024a	0.025a	0.026a	0.029a	

(Continued)

Table 6 | Continued

Harvest date	CDD ^f (°Cd)	TS ^h	Mariska	Matilda	Nadine	Pronto	Tr.
Voorst 2009							
June 29th, 2009	420	OD	0.009 ± 0.002	0.012 ± 0.003	0.012 ± 0.007	0.013 ± 0.004	0.012a
		ND	0.009 ± 0.007	0.014 ± 0.007	0.010 ± 0.003	0.019 ± 0.007	0.013a
		UD	—	—	—	—	—
		Cv.	0.009a	0.013b	0.011ab	0.016c	

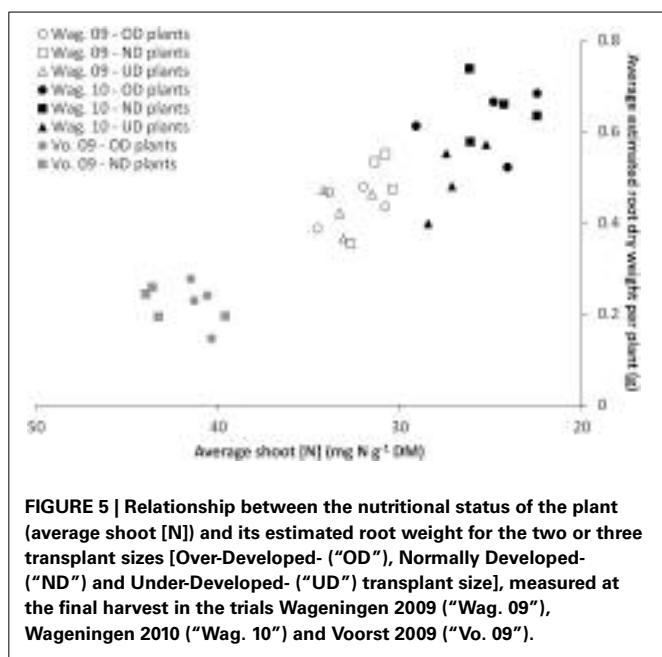
^a "Over-developed" transplant size.^b "Normally developed" transplant size.^c "Under-developed" transplant size.^d Mean for cultivar across transplant sizes.^e Mean for transplant size across cultivars.^f Cumulated Degree-Days.^g Standard error of the mean.^h Transplant Size.ⁱ Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is within an experiment and at the level of main factors cultivar or transplant size when the two-way interaction was not significant and at the level of transplant size × cultivar when the interaction was significant.**Table 7 | Average physiological NUE (g DM g⁻¹ N in head) of the four cultivars at final harvest, after establishment from three different transplant sizes.**

Harvest date	CDD ^f (°Cd)	TS ^h	Mariska	Matilda	Nadine	Pronto	Tr.
Wageningen 2009							
May 25th, 2009	474	OD ^a	29.1 ± 1.8 ^g	29.6 ± 1.9	32.7 ± 3.0	31.3 ± 3.6	30.7a
		ND ^b	30.6 ± 1.5	33.0 ± 4.5	33.1 ± 2.5	32.0 ± 2.3	32.2b
		UD ^c	30.3 ± 1.3	30.2 ± 2.7	31.8 ± 1.8	29.3 ± 1.5	30.4a
		Cv. ^d	30.0a ⁱ	31.0ab	32.5b	30.9ab	
Wageningen 2010							
May 30th, 2010	400	OD	34.8 ± 4.2a	45.1 ± 4.9d	41.7 ± 3.0cd	40.5 ± 3.0abcd	40.5
		ND	39.2 ± 6.2abcd	44.8 ± 3.8d	41.5 ± 3.8bcd	38.4 ± 2.4abc	41.0
		UD	35.5 ± 3.4ab	37.1 ± 2.8abc	39.7 ± 2.2abcd	36.6 ± 2.2abc	37.2
		Cv.	36.5	42.3	41.0	38.5	
Voorst 2009							
June 29th, 2009	420	OD	24.9 ± 1.8ab	24.1 ± 0.6ab	24.6 ± 0.6ab	24.2 ± 0.6ab	24.5
		ND	23.1 ± 0.8a	22.8 ± 1.0a	25.6 ± 3.0b	23.0 ± 0.7a	23.6
		UD	—	—	—	—	—
		Cv.	24.0	23.4	25.1	23.6	

^a "Over-developed" transplant size.^b "Normally developed" transplant size.^c "Under-developed" transplant size.^d Mean for cultivar across transplant sizes.^e Mean for transplant size across cultivars.^f Cumulated Degree-Days.^g Standard error of the mean.^h Transplant Size.ⁱ Means with different letters indicate a significant difference at $p \leq 0.05$ —means separation with lettering is within an experiment and at the level of main factors cultivar or transplant size when the two-way interaction was not significant and at the level of transplant size × cultivar when the interaction was significant.

size and/or the cultivars, but rather a function of the nutritional status i.e., the growth stage. The higher shoot N concentration for some treatments is an indication of physiologically younger plants. Here shoot N is diluted over less biomass as shown by the

smaller dry weights. Comparison of these data with the root:shoot ratio and shoot dry weight data in **Tables 7** and **3**, respectively shows that the harvested plants at the lower shoot N concentration also had a higher root:shoot ratio. This may have been



related to the functional equilibrium change under reduced plant nitrogen status (Poorter and Nagel, 2000).

UNBALANCED ROOT:SHOOT RATIO CREATED BY ROOT PRUNING AT TRANSPLANTING HAS SHORT-LASTING EFFECTS ON SHOOT GROWTH

Root pruning at transplanting using overdeveloped seedlings did not impact the yield at final harvest in the Wageningen trials (**Table 3**). The mechanical damage inflicted to the roots of the OD plants at transplanting did not impact root growth either, as no significant difference was found between the OD and the ND plants in total root weight at any sampling date or in RLD at any soil depth for any sampling date (data not shown). Any impact of the treatment on the root:shoot ratios had already disappeared at first sampling in the Wageningen experiments and only showed itself temporarily in Voorst 2009 (**Table 6**). For the three trials, OD plants showed an overall lower maximal relative growth rate (**Table 2**) and an overall lower maximal leaf expansion rate (**Table 4**) during the exponential phase compared with the ND plants, which was caused by their bigger size at transplanting compared to ND plants (therefore a lower amount of tissue produced per amount of existing tissue in the exponential phase). However, this did not influence the start of the linear growth phase, as no significant difference in lag phase was found for dry weight accumulation (**Table 2**) or leaf expansion (**Table 4**), except in Wageningen 2009. These results suggest that for the lettuce cultivars used in this study, a mild root pruning at transplanting is not a large stress for shoot growth and does not affect final yield in the early spring season. The moderate soil and air temperatures, light intensity and radiation (not recorded) in the Wageningen trials led to a slower shoot growth, especially in the exponential phase (**Table 2**), and consequently required less from the roots to sustain the growth. This may explain why the stress created by root pruning was not crucial for shoot growth for these trials. In contrast, the higher air and soil temperatures recorded in

the Voorst trial (late spring/early summer) increased the shoot growth rates in the exponential phase (**Table 2**) and emphasized the important role of a larger root system in this trial to sustain the growth of larger shoots such as the OD plants. This was very visible in the results, as the cultivars with the largest root weight ("Matilda" and "Pronto," **Table 5**) under both the OD and the ND transplant size, performed better in terms of shoot weight (**Table 3**) than "Mariska" which had the smallest root weight (**Table 5**).

TRANSPLANTING UNDERDEVELOPED PLANTS IMPACTS ROOT AND SHOOT GROWTH TO A LARGE EXTENT

Transplanting UD seedlings in open field conditions imposes considerable physiological stress on growth and development of the plant. UD plants were not able to recover from transplant shock and to catch up with ND plants during the experiments in terms of dry weight accumulation, especially for Wageningen 2010 (Supplementary materials, Tables S1, S2 and **Table 3**). Vos et al. (1996) showed that leaf initiation and potential leaf size are largely determined before leaves actually appear, i.e., the number of leaves and the size of the leaves are determined already in the apex. They hypothesized that stress at an early growth stage may disturb the physiological mechanisms controlling leaf initiation in the apex, and may therefore affect later field performance over a longer time, as observed in our experiments. The smaller size at transplanting impacted shoot growth: the UD plants' smaller leaf area at transplanting increased the maximal relative growth rate/leaf expansion rate during the exponential phase (**Table 2**) which increased the lag phase, as the UD plants required more time to finalize the exponential growth period. As a result UD plants had slightly smaller heads and delayed maturity (data not shown). In practice, transplanting smaller plants, delaying maturity, translates into a longer period in the field and consequently some financial loss for the grower.

The transplanting shock did not only affect shoot growth and development. We surmise that the shock imposed on the plants by transplanting underdeveloped seedlings also disturbs root initiation and leads to a smaller root system for the UD plants compared to the ND plants, as observed in Wageningen 2010 (**Table 5**), the trial with lowest soil temperatures. The smaller root system was not compensated by an improved NO_3^- capture capacity, as shown clearly for Wageningen 2010 in **Figure 4**.

GENETIC VARIATION IN ROOT:SHOOT GROWTH STRATEGIES

The four cultivars were chosen according to their different growth patterns in the field as well as their specific root mass distributions over the soil profile as observed previously by Den Otter and Lammerts van Bueren (2007). The diverse strategies exhibited by the cultivars to overcome the transplant shock seemed rather consistent across years.

"Mariska" was a cultivar which had the smaller root system overall (**Figures 5A,E,I**). For this cultivar, root pruning tended to increase total root mass consistently in Wageningen 2009 and 2010 (**Table 5**) which underlines a powerful root regeneration capacity. In practice, the cultivar Mariska is often preferred for the early spring growing season, when weather conditions force growers to delay the planned planting date. They are then faced

with overdeveloped transplants, a situation from which the cultivar is known to recover easily (K. de Jong, pers. commun.). This research shows that for “Mariska” this high root regeneration capacity is however a trade-off for shoot growth, as the larger assimilate allocation to the roots was at the expense of the shoot, which tended to be lighter than that of the other cultivars at final harvest (**Table 3**).

In contrast to “Mariska,” “Matilda,” and “Pronto” were the two cultivars which had the largest root system (**Table 5**), whereas Pronto often had the highest root:shoot ratio (**Table 6**). Such a large root system may have contributed to their steady good field performance across transplant size, locations, and years (**Table 3**); indeed developing more roots, especially in deeper soil layers (as it was measured for these cultivars in layers 0.1–0.2 and 0.3–0.4 m, **Figures 3B,E,J** for “Matilda” and **Figures 3D,F,L** for “Pronto”) increased resource capture quantitatively and consequently conferred a proportional advantage for shoot performance. Besides, the results of this study suggest that these cultivars are relatively robust, as their response to transplant shock (either root pruning or underdeveloped transplant size) was consistent over locations and seasons. In practice, these cultivars are often preferred by “hobby” gardeners as robust cultivars when growing conditions are less controlled and less optimal, which confirms our findings. However, it must be underlined that the field conditions under which the trials were carried out in this study were rather optimal, as no strong drought or nitrate leaching occurred. It might be that a larger proportion of assimilates allocated to root proliferation as displayed by “Matilda” and “Pronto” could be a trade-off for final yield in case of less optimal field conditions, e.g., temporary drought or spatial limitation in nitrate availability. Other physiological mechanisms involved in nitrate capture e.g., improved nitrate inflow per unit root length (Vuuren et al., 1996) may then confer robustness.

Finally, “Nadine” is a cultivar that had a relatively smaller root system but had a higher physiological NUE than the other cultivars (**Table 7**). This cultivar performed consistently in all three experiments under all transplant sizes, underlining the fact that not only the capacity to take up resources from the soil is important, but also the internal ability to use these resources in order to ensure adequate shoot growth despite environmental stresses.

CONCLUDING REMARKS

This study investigated the effect of different types of transplant shocks, created by root pruning or underdeveloped transplant size, on field performance of lettuce, and the role of below-ground traits in overcoming such disturbances. The results of three field experiments showed that the mechanical damage inflicted at transplanting to the roots of overdeveloped transplants has short-lasting effects on shoot growth and does not impact final yield.

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This suggests that the plants respond quickly to such a shock by adaptive responses at the root level, and are able to restore the initial root:shoot ratio fast enough not to impact final yield. Strategies to overcome the mechanical damage at the root level include high root regeneration capacity, which however, can be trade-off for shoot yield as shown for “Mariska.”

On the other hand, a large transplant shock, created by transplanting underdeveloped seedlings, cannot be overcome by lettuce; the results showed that transplanting undeveloped seedlings has lasting effects on overall root and shoot growth: slower growth results in smaller plants that mature later.

Overall, more roots in deeper layers, as observed for “Matilda” and “Pronto,” was linked to stable field performance despite transplant shock across trials, locations, and seasons, and may therefore constitute a trait of robustness for lettuce, as we hypothesized. If a more developed root system enables the plants to sustain growth during temporary periods of drought or nitrate shortage by capturing resources from deeper soil layers, the ability to efficiently transform the captured resources into shoot mass is also an important trait for robustness, as found for “Nadine” in these trials.

Monitoring spatial and temporal changes in below-ground cues and measuring their effects on above-ground parameters were only feasible in this study by using a limited set of cultivars, selected on the basis of specific criteria. In no way do we suggest that our results are fully representative for the genetic variation present among the numerous lettuce varieties. Instead, this study, together with a previous paper reporting on the spatial and temporal dynamics of root development and resource capture in lettuce (Kerbiriou et al., 2013), will provide the basis for a conceptual framework to design a strategy to breed lettuce for robustness, which will be used to interpret results obtained from a large set of lettuce varieties trialed in diverse environmental conditions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material (Tables S1, S2, S3, S4) for this article can be found online at: http://www.frontiersin.org/Functional_Plant_Ecology/10.3389/fpls.2013.00379/abstract

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APPENDIX

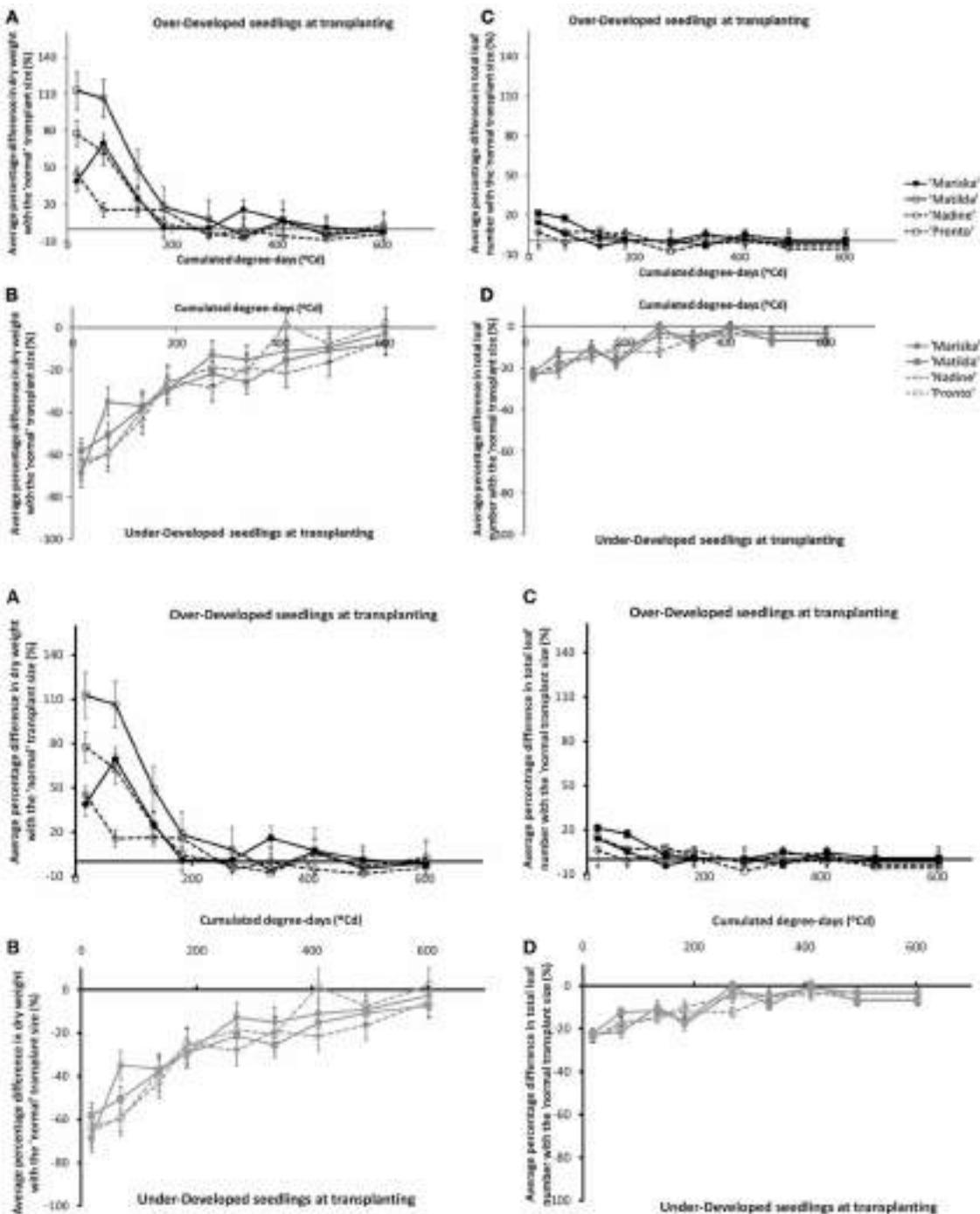


FIGURE A1 | Average percentage difference in dry weight of plants originating from Over-Developed (A) and Under-Developed (B) seedlings, in comparison with the dry weights of plants originating from Normally Developed seedlings, and average percentage difference in total number of leaves of plants

originating from Over-Developed (C) and Under-Developed (D) seedlings in comparison with the number of leaves counted on plants originating from Normally Developed seedlings for the four cultivars (trial Wageningen 2009). Error bars indicate \pm one standard deviation.



A statistical approach to root system classification

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Plant root systems have a key role in ecology and agronomy. In spite of fast increase in root studies, still there is no classification that allows distinguishing among distinctive characteristics within the diversity of rooting strategies. Our hypothesis is that a multivariate approach for “plant functional type” identification in ecology can be applied to the classification of root systems. The classification method presented is based on a data-defined statistical procedure without a priori decision on the classifiers. The study demonstrates that principal component based rooting types provide efficient and meaningful multi-trait classifiers. The classification method is exemplified with simulated root architectures and morphological field data. Simulated root architectures showed that morphological attributes with spatial distribution parameters capture most distinctive features within root system diversity. While developmental type (tap vs. shoot-borne systems) is a strong, but coarse classifier, topological traits provide the most detailed differentiation among distinctive groups. Adequacy of commonly available morphologic traits for classification is supported by field data. Rooting types emerging from measured data, mainly distinguished by diameter/weight and density dominated types. Similarity of root systems within distinctive groups was the joint result of phylogenetic relation and environmental as well as human selection pressure. We concluded that the data-define classification is appropriate for integration of knowledge obtained with different root measurement methods and at various scales. Currently root morphology is the most promising basis for classification due to widely used common measurement protocols. To capture details of root diversity efforts in architectural measurement techniques are essential.

Keywords: root system diversity, classification, plant functional types, cluster analysis, root architecture model, taxonomy

INTRODUCTION

The evolution of root systems¹ is closely related to plant colonization of terrestrial ecosystems (Kenrick, 2002; Sperry, 2003) where plants require roots for anchorage, water, and nutrient acquisition. Although roots are a key organ for plant adaptation to variable environments and therefore for biodiversity (Cornwell and Grubb, 2003), they have long been neglected in plant biology and agronomy. Recently, however, root systems receive increasing attention as a key for a “second green revolution” (Lynch, 2007; Gewin, 2010) leading to more resource efficient plants.

A proper characterization of root system diversity is essential for various purposes, such as crop improvement, prediction of

changes in species distribution under global change, or exploration of root functions in the carbon cycle. To capture diversity, a classification scheme for root systems is needed. It is a major shortcoming that to-date we lack such a scheme and that only few recent contributions try to fill this gap (e.g., Zobel and Waisel, 2010). Most botanical textbooks do not go beyond a very general developmental distinction between tap and fibrous root systems, concentrating more on specialized morphological adaptations (e.g., haustorial roots, storage roots) that occur in certain species (Bresinsky et al., 2008).

An explicit proposal of root system classification was presented by Fitter (1987) based on root topology. Fitter (1987) derived a topological index as a measure of functional diversity in resource acquisition by different species.

Another classification approach based on the developmental origin of roots (Cannon, 1949) was recently re-proposed by Zobel and Waisel (2010). This scheme is based on the distinction between a primary, secondary, and tertiary root system. The primary system consists of the primary (pole) root originating from the embryo radicle, basal roots originating from the

¹According to Troll (1943) the term “radication” would be more appropriate than the term “root system.” He defined a root system to consist of branches from a single root (e.g., from the pole-root or a single shoot-borne root). Following this definition, all shoot-borne roots of monocots form distinctive systems, while the term radication comprises all of these root systems of a plant. However, as most root studies use the term “root system” instead of radication, we also make use of this term.

hypocotyle/mesocotyle and lateral roots that emerge from these axes. The secondary system is built from shoot-borne roots and their laterals, while the tertiary system refers to fine roots below 0.6 mm diameter. Zobel and Waisel (2010) and Hochholdinger et al. (2004) demonstrated the distinct genetic control of these root types. Still when considering the entire root system, a developmental classification only results in a very general distinction between tap root dominated and shoot-borne root systems. This allows a differentiation between homorhizy in monocotyledonous and allorrhizy in dicotyledonous species, while having limited capacity to capture more detailed distinctions within these groups.

A classification of root systems via their geometrical shape was suggested by Kutschera and Lichtenegger (1997). Based on extensive *in situ* observation of excavated root systems from over 1100 species, they distinguished between 11 fundamental rooting types from cord like (dominant vertical growth with few lateral extension) to discoidal (dominant surface near lateral extension).

A classification for woody species, combining morphological and functional attributes, was presented by Wahid (2000) who defined seven classes of different root foraging types.

These root system classification schemes presented so far differ in the traits they use for systematization and in the degree to which they capture taxonomic plant diversity. Traditional plant systematics used phylogenetic relationship to define species as a basic taxonomic unit with distinctive morphological attributes (e.g., Simpson, 2005). Fitter (1987) however questions the possibility to develop a species based root classification system and advocates a more functional approach to capture distinctive root system types.

Functional classifications have been used in plant ecology to define groups of species with shared biological characteristics that relate directly to function rather than phylogeny (Lavorel et al., 1997). Westoby and Leishman (1997) therefore speak of an ecological rather than a taxonomic classification. While functional/physiological traits can be used directly for classification (e.g., Wahid, 2000), the main idea is that the classification scheme has a functional rather than a phylogenetic meaning.

The idea of functional types rather than species as a classification unit seems particularly useful to be applied to root systems. Roots are morphologically less differentiated compared to shoots. Therefore, it is likely that species differences are often rather continuous than discrete regarding their root systems. Distinct, discontinuous groups of rooting types still might be found as a result of functional plant adaptation to different environments (e.g., Schenk and Jackson, 2002).

Functional classification schemes can be obtained using subjective, deductive and data-defined methods (Gitay and Noble, 1997). The subjective method derives functional groups based on field experience. It takes for granted that functional groups exist in an ecosystem and that they can be defined inductively by using plant attributes considered as essential grouping variables due to botanical expert knowledge and experience. The deductive approach uses an a priori established idea of important processes or properties in an ecosystem and deduces functional categories and related sets of traits from these premises. The data-defined approach uses multivariate statistics to seek for clusters

that emerge from a set of attributes. The matrix of classification attributes for plant functional groups often depends on the objectives and context of a study. To obtain a generalized classification, some authors therefore suggested defining a core data set to extend the comparability between single studies (Weiher et al., 1999). Gitay and Noble (1997) recommended testing the uniqueness of any classification result for repeatability, using the same traits measured at different times or sites, for congruency, using different classification attributes, and for convergence, using data sets collected for different purposes.

The objective of this discussion paper is to present a functional classification of root systems. We show the shortcoming of single trait based comparative root research and suggest a new data-defined approach using multivariate statistics to derive functional classification of root systems. We hypothesize that such an approach can provide efficient classifiers to capture root system diversity. The main purpose of the paper is to encourage an exchange on root system classification and to suggest the use of multivariate methods as a way to obtain additional explanatory benefits from available root data sets in the frame of functional classification.

MATERIALS AND METHODS

The classification scheme we suggest in this paper is first introduced by giving details on the applied statistical procedure. Then we present the data sets we use to exemplify the approach. Finally we suggest a core data set for classification and explain how our approach contributes to its elaboration.

STATISTICAL CLASSIFICATION APPROACH

The main purpose of the paper is to suggest a data-defined statistical classification method. We followed similar approaches used in ecology to obtain plant functional types, particularly the work of Gitay and Noble (1997). Our procedure is based on principal component analysis (PCA) and biplot inspection of root classification attributes, followed by cluster analysis using principal component based rooting types as composite classification variables.

PCA is a procedure to convert a multivariate dataset of various trait variables into few uncorrelated variables (principal components) that account for most of the variance existing in the original dataset. PCA is useful when some redundancy in measured variables can be assumed, i.e., mutual correlation among traits which are measuring the same construct. The extracted principal components optimally describe the common meaning of the single trait variables they contain. We used the SAS procedure PROC FACTOR to perform PCA. The number of principal components is determined based on the Kaiser criterion (Kaiser, 1960), retaining any component with an eigenvalue greater than 1.00. Also the increase in the explained proportion of variance by each further principal component is an indicator of how many components to keep. To improve the interpretability of principal components, different data transformations can be applied. A common way is to use rotation, i.e., a linear transformation of the factor solution. We used the orthogonal varimax rotation that results in uncorrelated principal components. Also other variable transformations can be useful to obtain principal

components that better satisfy the interpretability criterion. The SAS procedure PROC PRINQUAL was used for this purpose.

An important result of PCA is the graphical representation of the solution via biplots. Biplots contain the single variables (i.e., root traits) as vectors and the single objects (i.e., species, cultivars) as points. The length of a vector is equal to the variance of the corresponding variable. A narrow angle between vectors of two variables indicates that they are strongly associated to each other. The origin of the biplot represents the average value for each variable. The more distant from the origin the projection of an object on a variable vector, the more this object deviates from the average in the variable. Inspection of biplots allows deciding which variables are essential to represent the overall variance of a studied object, i.e., which traits are necessary to obtain a comprehensive picture of a root system. This provides a way to ensure a high degree of congruency (i.e., same classification result is obtained with different sets of attributes) in the subsequent clustering. Also the repeatability of the classification result can be ensured when previously testing relations between the single variables over different years or sites.

The principal components as distinctive composite variables of different rooting types are then used in cluster analysis. Cluster analysis is a distance based method to identify common groups of species. This was done with the SAS procedure PROC CLUSTER. We notice here that for any statistical approach a possible influence of different mathematical clustering procedures on the uniqueness of the classification solution has to be taken into account (e.g., Milligan and Cooper, 1985; Lo Siou et al., 2011). Botanical expert knowledge is required when comparing the results of different clustering procedures that might not yield the same result as well as in the decision on the number of meaningful clusters. PROC CLUSTER however provides some statistical decision support on the number of clusters to retain (cubic clustering criterion, pseudo- F , pseudo- t^2).

SIMULATION OF ROOT SYSTEM ARCHITECTURES

To exemplify the classification approach with a comprehensive root data set, we used a total number of 288 simulated root systems. They covered a wide range of diversity ranging from shallow laterally extending dichotomous root types to deep primary root dominated herringbone types. Some examples are shown in Figure 1.

For simulating the different root architectures we used the model of Leitner et al. (2010a). This Matlab based model uses L-systems (Prusinkiewicz and Lindenmayer, 1990) for the construction of branched root geometries. Basic production rules for root axes include root growth, root branching, and different types of tropism. Root axis elongation follows a negative exponential function as used by Pagès et al. (2004). Every root axis of a certain order produces lateral branches. Each axis is divided into three zones: the basal and apical zones near the base and the tip of the root, respectively, where no branches are produced, and the branching zone where new roots of successive order are created. Within the branching zone, a predetermined number of branches emerge. The spacing between the branches is determined by the section growth rule. The rule allows branches to occur at any point along the root axis and not only at segment

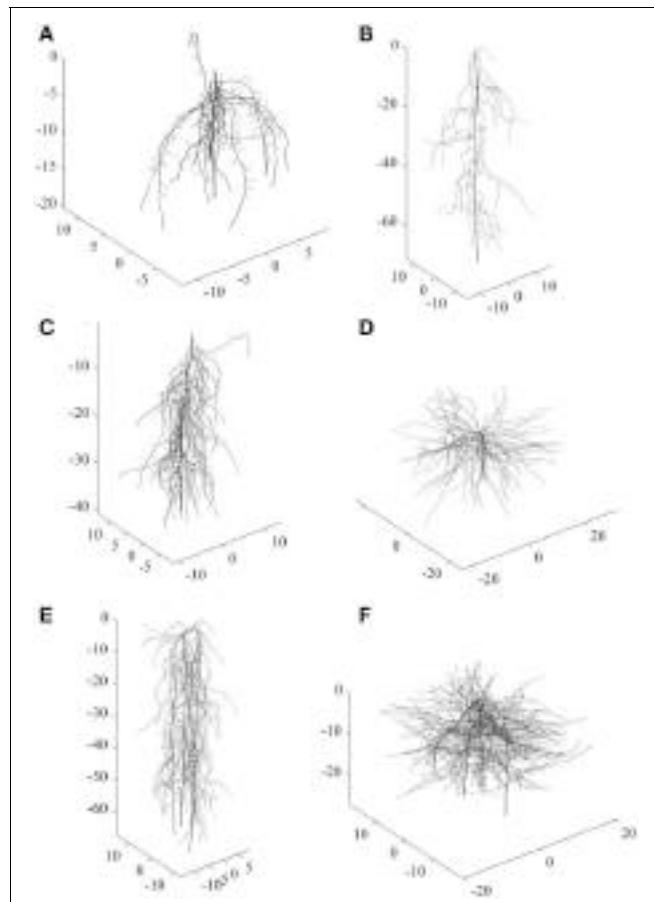


FIGURE 1 | Examples of simulated root systems corresponding to six main clusters. (A–C) are tap root systems (one zero-order axes) differing in vertical root distribution and branching intensity, **(D–F)** are shoot-borne root systems (four zero-order axes) with different vertical distribution and branching intensity. **(A)** and **(B)** are more herringbone systems while **(D)** and **(F)** are typical dichotomous systems.

edges with fixed segment length. The model allows simulation of different tropisms which cause root tip deflection. At the root system level both primary root dominated tap root systems as well as shoot-borne dominated fibrous root systems can be created via an adequate initial L-System string. In the case of a tap root system, the initial string consists of a single root tip of a zero order root. In the case of fibrous root systems, a number N_0 of initial axes as well as the angle of their initial growth within a cone radius r_0 is defined. Importantly the model includes a defined standard deviation for each parameter giving certain randomness in each simulation run even for the traits that were set by the user. For example the effect of soil particles on the direction of root growth is considered indirectly by some random variation of the growth direction.

Representation of real root architectures by the simulation model was originally validated by Leitner et al. (2010b) using digitalized images of excavated root systems of several plant species from the root atlas series by Kutschera (1960). Their study confirmed the proper biological basis of the parameter

and production rules used by the model to simulate plant root systems.

The range of model parameters to simulate the diverse root architectures are given in **Table 1** based on different literature sources.

In our study the main objective of model application was to determine the key traits that shape root system diversity and to explore the most efficient classification variables among the various traits that make up the complex overall architecture of root systems. The results should also support the elaboration of a core data list and the development of targeted measurement strategies for root system classification.

For this model application some new evaluation tools were included into the code to obtain shape and topological root attributes for subsequent classification. Parameters obtained from the simulated root architectures were (i) macroscopic shape of lateral and vertical distribution according to Vrugt et al. (2001), (ii) topological branching parameters according to Fitter (1987) i.e., magnitude, altitude and external path length, and (iii) mean axes morphological traits (total root surface, average diameter, length per root order). These root attributes were then submitted to the statistical classification approach described in Statistical Classification Approach.

MEASURED ROOT SYSTEM MORPHOLOGY

Application of the classification scheme to measured data was done using two samples of morphological traits from field experiments. Root traits were analyzed from soil cores (7 cm diameter) where roots were washed free from soil and then quantified by image analysis. Image analysis with WinRhizo followed the procedure described in Himmelbauer et al. (2004).

The first data set comprised morphological traits of different species used as cover crops for agro-environmental purposes. In this specific case root samples in the surface layer were taken to investigate root effects on soil structural properties in surface soil (Bodner et al., 2012). The sample comprised four fabaceae (*Vicia sativa* L., *Lathyrus sativus* L., *Trifolium alexandrinum* L.,

Melilotus officinalis L.) two brassicaceae (*Sinapis alba* L., *Raphanus sativus* var. *oleiformis* L.), one biginacea (*Phacelia tanacetifolium* Benth.), one linacea (*Linum usitatissimum* L.), one polygonacea (*Fagopyrum esculentum* MOENCH.), and one poacea (*Secale cereale* L.). Additionally two species mixtures were included (Mixture 1; *Secale cereale* L., *Trifolium incarnatum* L., *Vicia villosa* ROTH.; Mixture 2: *Phacelia tanacetifolium* Benth., *Sinapis alba* L., *Vicia sativa* L.).

Samples were taken from surface soil (2–7 cm soil depth) with two subsamples per plot (one sample on the plant row and one sample between rows) in three replicates. Although root parameters have been measured for a specific purpose in this study (influence on soil structure), the convergence criterion (Gitay and Noble, 1997) claims that the same classification should be obtained when the same species would have been sampled for a different purpose including other root traits. Beside basic morphological traits obtained by WinRhizo, we calculated root diameter ratio as an attribute describing lateral root extension. This was done by comparing root diameter in and between plant rows. The ratio between the two sampling positions indicated the change in diameter between branches near the primary axes (row) and more distant laterals (interrow). Furthermore, homogeneity between different root orders was approximated by the root diameter range, obtained by the coefficient of variation of root length in different diameter classes. A low diameter range means an even distribution of root length over all diameter classes.

The second data set originated from root samples of 12 cereal genotypes of different species and cultivars within a species. The data were obtained to determine differences in root systems relevant for drought resistance (Nakhforoosh et al., 2012). They comprised seven *T. turgidum* subsp. *durum* cultivars (Floradur and SZD3146 from Austria, Clovis from France, Matt from Arizona, 7060, 7063, and 7094 from CIMMYT, Mexico), two *T. monococcum* subsp. *monococcum* from Turkey (PI428154, PI428165), two *T. turgidum* subsp. *turanicum* (Kamut from Middle East, TRI5254 from Europe), and one *T. timopheevii* from Georgia (W9).

Four replicate soil cores were taken on the plant row. Samples from three soil depth (10–20 cm, 30–40 cm, 50–60 cm) were analyzed by WinRhizo. In this case depth distribution was calculated by the slope of a linear regression of root length densities vs. depths. Beside the morphological data also root capacitance (Chloupek, 1977) was measured and included in the analysis.

Measured data were first analyzed for each trait by univariate analysis of variance using SAS PROC MIXED. In case of repeated measurements over depths the mixed model was used with an AR(1) correlation structure for the repeated factor (Piepho et al., 2004). Subsequently, for those traits with significant differences at $p < 0.05$ mean comparison was done with a two-sided *t*-test. Thereafter multivariate analysis with PCA and clustering for root classification based on the morphological attributes was used as described above.

ROOT TRAIT CORE DATA SET

The proposal for a preliminary root trait core dataset was derived from a literature survey. The objective was to estimate current availability of root data that could be used for classification. We did not pretend a comprehensive literature review, but a coarse

Table 1 | Parameters and values used for simulating different root architectures.

Parameter	Values	References
Number of zero-order axes	1, 4	Kutschera et al., 2009
Number of laterals per branching zone	20, 40, 60	Werner et al., 2001; Pasternak et al., 2005; Hund et al., 2009
Highest (4th order) lateral branching	Yes/no	Kutschera et al., 2009
Branching angle (zero-to-first order axes)	65°, 90°	Osmont et al., 2007; Nagel et al., 2009
Inter-branch distance at zero-order root	0.2, 0.5, 2.0 cm	Fitter, 1987; Arredondo and Johnson, 2011
Inter-branch distance at first-order root	0.1, 0.2 cm	–
Tropism type	Gravitropism, Exotropism	Rosen et al., 1999; Pagès et al., 2004

overview of the frequency certain traits are measured. Therefore, we limited the survey to a keyword search in the Scopus database over the last 10 years. The number of hits in Scopus was used as indicator for the potential availability of datasets for the respective traits. A preliminary hierarchy among root trait was established based on the scale of observation. Different root attributes at each hierarchical level were listed and usual measurement methods indicated. In the frame of our classification approach biplot trait vector direction reveals which traits are essential to capture the overall diversity in a sample and thereby supports the elaboration of a core data set. Based on our simulation sample, covering several classes of root attributes, we show which class of traits is essential for a core data set according to our results.

RESULTS AND DISCUSSION

The present discussion paper is motivated by the increasing need to understand root system diversity and functioning in several fundamental and applied research disciplines. For this purpose a common classification scheme is required to identify the distinctive root properties among different plant species. Here we suggest a data-defined procedure for functional root system classification and exemplify the approach with selected data sets.

UNIVARIATE ANALYSIS RESTRICTS COMPARATIVE ROOT RESEARCH

Comparative root studies have been done in ecology (e.g., Schenck and Jackson, 2005) and agronomy (e.g., Kashiwagi et al., 2005). Whenever large samples are involved, decision on the root parameter to use for comparison is strongly influenced by measurement constraints (e.g., Manschadi et al., 2007; Chloupek et al., 2010). In most cases it is uncertain if the selected trait is efficient to identify differences in the sample and captures the functional pattern of interest. The predominant method to decide on the differences within a sample is the application of univariate analysis of variance for each single trait.

Figure 2 gives an example for this procedure.

The cover crop sample (**Figure 2A**) contained species from different plant families described by morphological root traits. Legume species were similar in root attributes related to diameter, while they differed in root surface area. They had a higher average diameter with less difference between row and inter-row sampling position (diameter ratio), and they had a more even root length distribution over different diameter classes (diameter range). On the contrary *Brassica* species were similar in total root surface area, but they differed in diameter related parameters. Similarity between *L. usitatissimum* and *P. tanacetifolia* could be expected from surface area, average diameter, and diameter range, while the two species clearly differed in the row-to-inter-row diameter ratio.

The cereal sample (**Figure 2B**) compares more closely related species of one family using root:shoot ratio, root length density, average diameter, and root depth distribution. The exotic genotypes (*T. monococcum* subsp. *monococcum*, *T. timopheevii*) represented extremes in most traits, in some cases (length density, depth distribution) with marked differences to the other genotypes, which however were not significant in all cases. The European *T. turgidum* subsp. *durum* genotypes were very similar in length and diameter, while showing different root:shoot

ratio and depth distribution. Again their differences to neighboring genotypes (e.g., Mexican CIMMYT durum cultivars) were not clearly significant. Again the decision on distinctive groups of root systems within the sample and any interpretation of possible causes is hardly possible.

The following problems can be identified from these examples when relying on univariate analysis of variance for root system comparison:

- i. Ranking of species depends on the trait used. When using different traits, the conclusive picture remains unclear.
- ii. Univariate analysis implicitly claims that traits are independent from each other, while they can be functionally linked. Separate evaluation of each trait therefore cannot provide a comprehensive comparison on the root system level.
- iii. It is unlikely that a single root trait is sufficient to identify structural adaptation or functional behavior on its own. In most situations simultaneous consideration of more than one trait is required to understand adaptation and functioning (e.g., root response to soil compaction; Bengough et al., 2006).
- iv. The outcome of single trait comparison is probably more study specific (different sites, different purposes) compared to multivariate approaches that capture the whole root system. This will easily lead to contradicting results and difficult inter-comparison between studies.

Comparative root research thus requires an approach that is more adapted to a multi-trait system which is hardly captured, neither structurally or functionally, by separate analysis of single attributes.

PRINCIPAL COMPONENT BASED ROOTING TYPES

To distinguish between different plant functional types, ecological research at the whole plant level combined morphological, physiological, phenological, and reproductive attributes (Duckworth et al., 2000). Fitter (1987) followed the general idea of functional classification and used a deductive approach based on the assumption that topological traits are most adequate to capture functional differences between root systems. Still it was not demonstrated that the topological classifiers most efficiently capture root system diversity compared to other root traits. In order to avoid any a priori decision on key classifiers we opted for a more open, data-defined approach via multivariate statistics (e.g., Kindscher and Wells, 1995; Naeem and Wright, 2003).

Following the plant functional type concept from ecology, we define rooting types, built from a distinctive combination of single traits, to be used as classifiers. Methodologically they are obtained by PCA, merging the single traits into independent composite variables (principal components). Hatcher (1994) underlines the importance of interpretability, i.e., the new variables should be biologically meaningful constructs. In a drought resistance study, Araus et al. (2007) demonstrated this by using principal components of several aboveground morphological attributes as classifiers for different regional origin of wheat species.

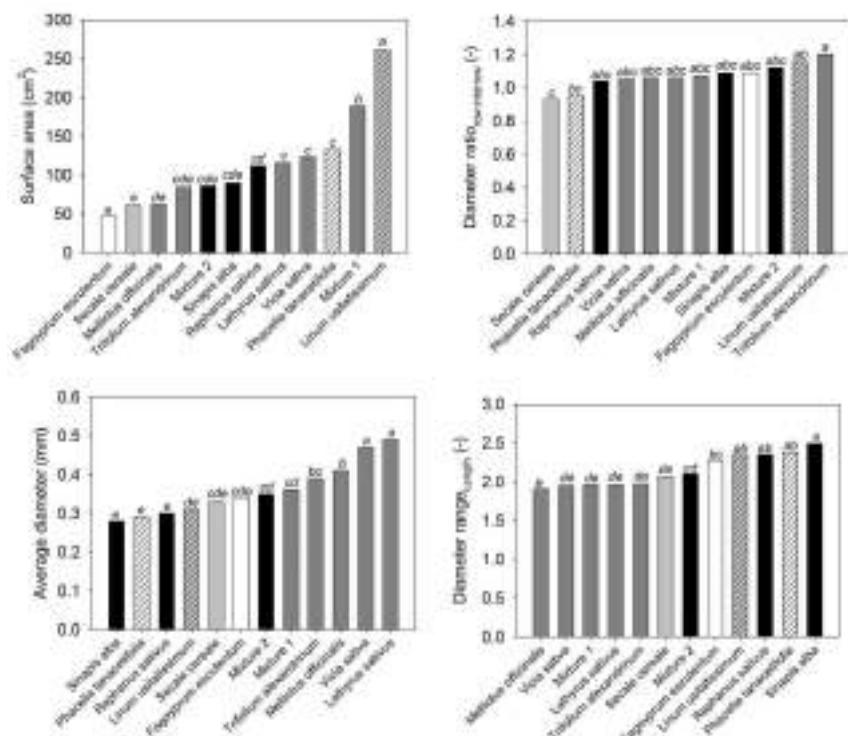
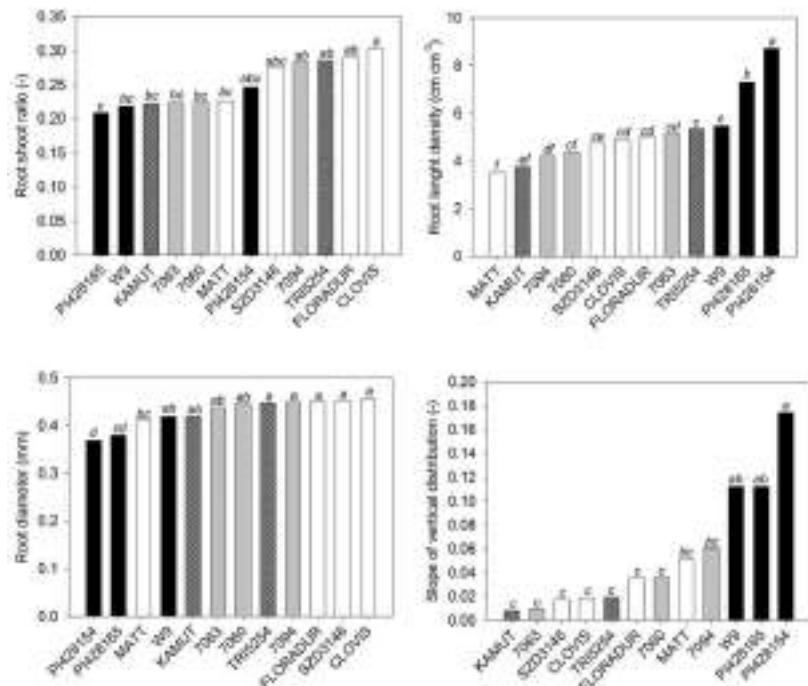
A Cover crop species sample**B Cereal genotype sample**

FIGURE 2 | Analysis of variance of selected root morphological traits from (A) the cover crop and (B) the cereal genotype sample. Change in neighboring bars of species order shows that single trait analysis leads to

different conclusions for each trait. (Statistical comparison of means is indicated by lowercase letters. Species sharing a common letter are not significantly different from each other at $p < 0.05$).

Figure 3 shows the biplots obtained from our three example data sets. Each biplot contains the root traits vectors and the location of the single species (objects) according to their principal component scores. For better visibility, trait vectors and objects for the simulation sample are shown on separate biplots.

For the simulated root systems the first principal component (PC1) captures density related attributes and Fitter's topological parameters, while the second principal component (PC2) is related to diameter and rooting depth mainly. According to the principal component scores, six distinct rooting types can be identified. Three groups are located along PC1, differing mainly in surface area, fine root length, and external path length. All these parameters capture the overall rooting density independent of shape or branching. Two further groups with a positive PC1 differed by their location along PC2, one containing roots with positive the other with negative PC2 scores. Thus, here vertical root distribution (tap root length, depth distribution) was the main distinction. The last group was located in the lower left quadrant with negative PC1 and PC2 scores. These systems are small sized herringbone roots with low branching and restricted depth penetration.

In the field sample with cover crop species of different plant families, trait vectors show a distinction between density dominated (high root:shoot ratio, high surface, length and fine length) rooting types vs. coarse (low specific root length) diameter dominated types. Root systems related to the density dominated

rooting type are in the direction of a positive PC1, while those of the coarse diameter dominated rooting type are in the direction of a positive PC2. In the quadrants at the right side of the biplot (positive PC1) *L. usitatissimum*, *P. tanacetifolia* but also *S. alba* and the mustard-phacelia dominated mixture 1 are found. *Fabaceae* species are all found in the upper left quadrant, in the direction of the diameter vector and in the opposed direction of (fine) root length. High diameter/low density root systems shared by the *Fabaceae* species are probably related to root-microbial interactions which are characteristic for this plant family. The high root diameter of legumes is supposed to have evolved for properly hosting their root symbionts (Eissenstat, 1992).

In the cereal genotype sample the two fundamental rooting types are also related to high (positive PC1) and high diameter/mass (positive PC2). Here also depth distribution is contained in PC1, with surface near root systems at the right side of the biplot (positive PC1) and deep rooted species at the left side (negative PC1). This indicates a trade-off between high resource exploitation potential by dense root systems and strong exploration capacity of deep rooting types (Fitter et al., 1991; Fitter, 2002). Wild genotypes (*T. monococcum* subsp. *monococcum*, *T. timopheevii*) are located on the right hand of the biplot (positive PC1), particularly in the lower quadrant. Thus, they are characterized by root systems of high density with a surface near concentration of root axes. *T. turgidum* subsp. *durum* cultivar Matt and the *T. turgidum* subsp. *turanicum* variety Kamut

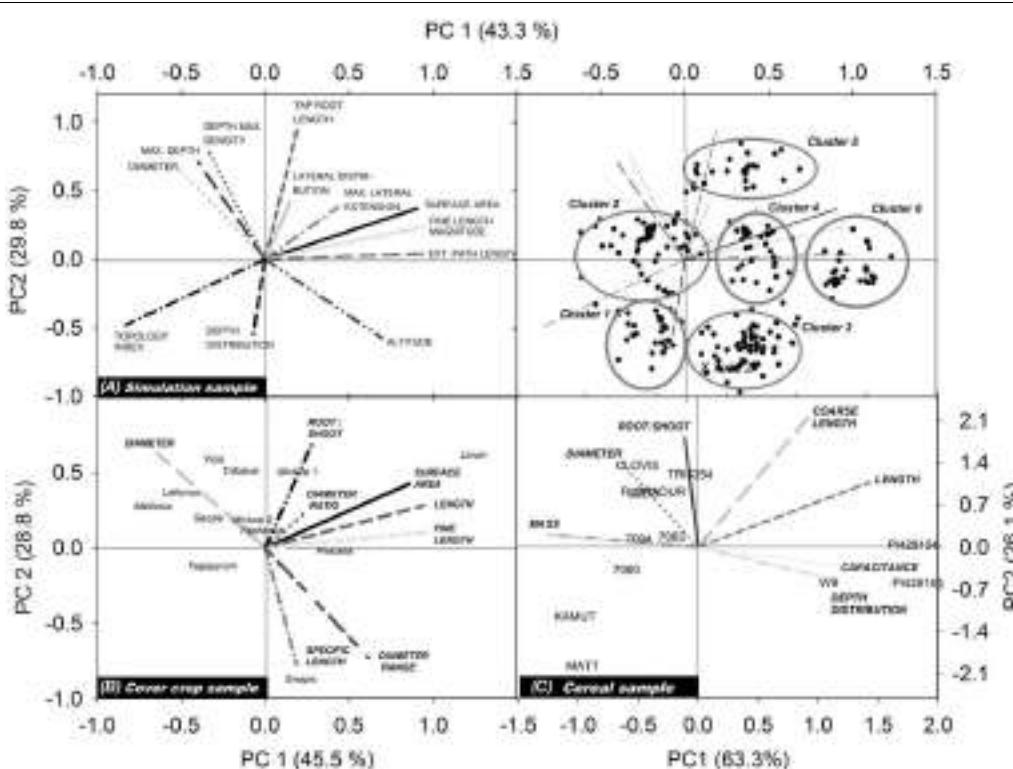


FIGURE 3 | Biplots showing trait vectors and location of the single objects from (A) the simulation sample, (B) the cover crop species sample, and (C) the cereal genotype sample. For

better visualization of the simulation results, trait vectors and objects are shown in separate biplots (for explanation of root traits, cf. section Measured Root System Morphology).

are both located in the lower left quadrant, sharing a high negative value of PC2. They show a rooting type with below average density, and high root allocation to deep soil layers. The central European genotypes are all located in the upper left quadrant with high PC2 and negative PC1, i.e., showing a deep rooting, diameter dominated rooting type. CIMMYT genotypes are found around the biplot origin suggesting an intermediate rooting type.

In spite of the different number and type of root traits in each data set, in all cases there was a high influence of rooting density traits (e.g., length, fine length, and surface area) on the first principal component, while the second principal component was always positively related to high average diameter and dominance of coarse root axes. The common meaning underlying the principal components is demonstrated quantitatively by a significant relation of principal component scores of common root traits between the different samples (simulation vs. cover crop sample: $r^2 = 0.65$, $p = 0.02^*$; simulation vs. cereal sample: $r^2 = 0.82$, $p = 0.002^{**}$; cover crop vs. cereal sample: $r^2 = 0.65$, $p=0.05^*$). This proves that the principal components indeed provided meaningful constructs expressing distinct rooting types.

In our examples principal component based rooting types captured between 74% (cover crop sample) and 89% (cereal sample) of the overall variability. A third principal component would have increased the explained variance between 6.6% (simulated sample) and 14.5% (cover crop sample) and might be considered for some cases.

FUNCTIONAL CLASSIFICATION BASED ON ROOTING TYPES

Our data-defined approach intends to build functional classification from principal component based rooting types integrating all available root trait information. Each species is characterized quantitatively by principal component scores that identify its association with the distinct rooting types.

The classification step is then done using cluster analysis. This statistical method determines the distance between the species contained in a sample. The resulting dendograms illustrate the number of functional groups that emerge from the data, containing all species with similar rooting types. The emerging groups are an adequate functional classification unit to be used for further interpretation of underlying causes (e.g., genetic relationship, environmental adaptation). **Figure 4** shows the dendograms calculated from our three example data sets.

For the simulated root sample, statistical criteria (cubic clustering criterion, pseudo- F and pseudo- t^2) suggests six groups to be retained in the dendrogram. There are two clearly distinctive clusters with highest average distance. These are related to the number of zero order axes (one vs. four zero-order roots). However, the total number of clusters with distinctive groups to be considered in the data-set is six. These groups differ by (i) the number of zero order axes, (ii) the number of lateral branches, and (iii) the inter-branch distance between laterals along the zero-order axis. The three first clusters from top to bottom along the dashed line of the dendrogram all represent tap-root systems with increasing number of lateral branches. Inter-branch distance on the tap root is similar in cluster one and three, while cluster two had around twice the length between branches. Clusters four to six contain systems with four zero-order roots (approximation

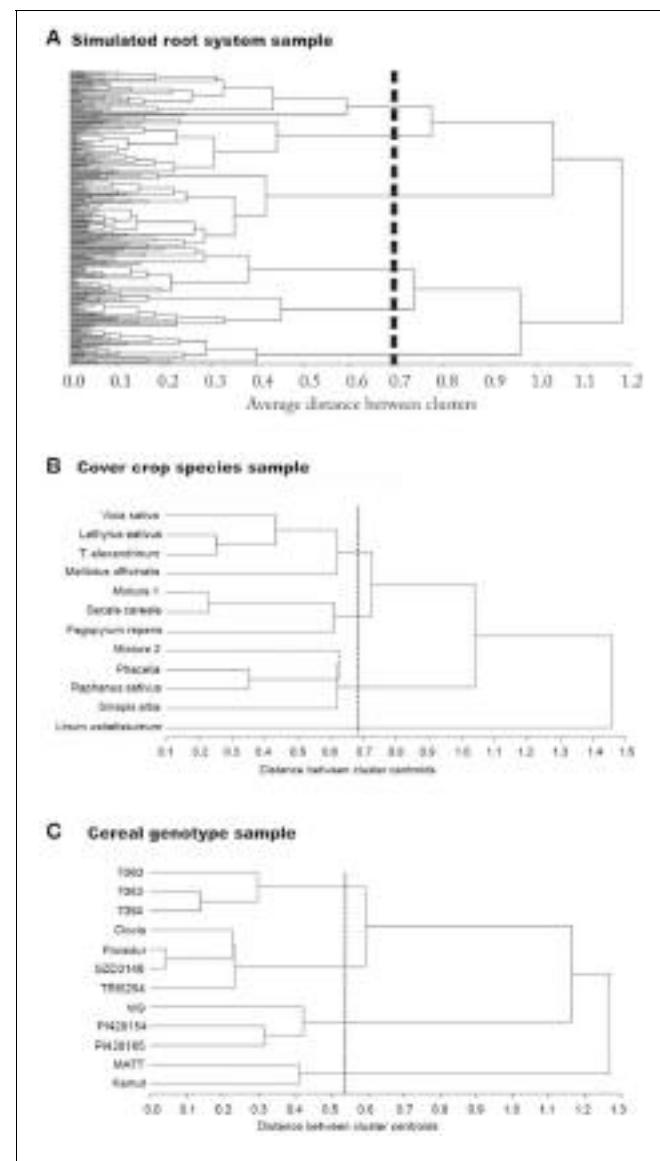


FIGURE 4 | Dendograms showing the classification result from principal component based rooting types used as classifiers. Results are from (A) the simulation sample, (B) the cover crop species sample, and (C) the cereal genotype sample.

of shoot-borne root systems) again with increasing number of lateral branches from four to six. Inter-branch distance on the zero-order roots is highest in cluster five and lowest in cluster six.

Taking into account the root traits contained in the principal component based rooting types, clusters one, two, and six mainly differ due to their rooting density (PC1 score), while for clusters three to five the main distinctive criterion is their depth distribution (PC2 score). Considering root topology as classifier, clusters one and two fall in the category of low branched herringbone systems, while cluster six represents a strongly branched dichotomous system.

Using shape and morphological indicators only, four distinctive clusters would have been retained. The same

number of cluster would have been obtained when only considering topological indicators. When reducing classification variables to single axes morphological attributes (diameter, surface area, fine lengths) only, three distinctive groups would have been identified (dendrograms not shown).

The classification results from the simulated root data show that developmental type (branching from a single zero-order axis originating from the embryonic radicle vs. branching from several zero-order axes originating from shoot nodes) is indeed a fundamental distinctive criterion at a high hierarchical level. This is in agreement with the recent developmental classification of Zobel and Waisel (2010) showing the decisive role of root origin to understand different root system structures. Several morphological properties related to rooting density strongly depend from developmental type. Still the results also highlight our criticism on developmental classifications. They are restricted to a very coarse distinction (tap vs. shoot-borne systems) and insufficient to reveal all existing groups with distinctive rooting types.

To extract all six distinctive groups identified by statistical indicators, density related, shape related (distribution), and topological classifiers are required. Topological classification parameters from Fitter (1987) and morphological attributes with macroscopic shape parameters are equally efficient classifiers when used separately (four clusters). Still a disadvantage of the topological approach is the difficult measurement of topological branching structure. This is reflected by the low number of studies reporting this type of data (cf. **Table 2**). With quickly increasing *in situ* imaging possibilities however we might expect better architectural data for topological classification in future (Zhu et al., 2011; Ingram et al., 2012).

On the contrary adequate spatial sampling schemes for morphological traits (Bengough et al., 2000) to derive shape parameters as used here (Vrugt et al., 2001) provide more easily available classifiers that also capture most of the diversity resulting in a meaningful classification. Axes morphology and macroscopic distribution seem to be a reasonable compromise between coarse distinction due to development type and the fine differentiation that requires topological branching information.

Table 2 | Traits at different observation scales for a comprehensive, hierarchically ordered core data set to classify root systems, common measurement methods, and Scopus database hits from keyword search.

Hierarchical level	Parameter	Method	Hits
Whole plant traits	Root biomass	Dry weight	2166
	Root: shoot ratio		1165
Root system shape	Maximum rooting depth	Excavation	27
	Maximum lateral extension	Curve fitting to morphological data	1
	Depth distribution		303
	Lateral distribution		41
Root developmental traits	Number of seminalroots	Root observation on young plants (e.g., gel chambers, blotting paper)	53
	Emergence of shoot-borne roots		18
	Initiation of lateral branching		13
	Maximum number of lateral branching orders		8
Root branching traits	Average branching angle	2D and 3D <i>in situ</i> observation and image analysis	10
	Distance between lateral branches		2
	Link length (internal, external)		4
	Topology (magnitude, external path length, altitude)		23
Axes morphology	Root length (surface) density	Destructive sampling (soil cores) and image analysis	473
	Average root diameter		550
	Root length/surface per diameter class		90
	Decrease of root diameter per root order		13
Root anatomical and physiological traits	Specific root length		249
	Xylem vessel number and diameter	Root anatomical cuts and microscopic measurement	153
	Cortex thickness		51
	Suberization/lignification	CO ₂ -flux	36
Root respiration	Root respiration		422
	Aquaporin abundance	PCR	308

For the cover crop species data set, four groups are suggested by the cubic clustering criterion. The dendrogram clearly separates legumes at one end, while *L. usitatissimum* forms a separate group at the opposite side. The density dominated rooting types (*Brassicaceae*, *P. tanacetifolia*, mixture 1) form a common group, while *S. cereale*, *F. esculentum*, and the legume-rye mixture 2 are an intermediate group between the diameter and density dominated rooting types. The distinction between the large diameter dominated legume systems and the density dominated *Brassica* systems can be interpreted functionally as being associated either with strong exploitative potential (fine roots, high root-soil contact surface) or high explorative capacity (Fitter, 2002), in the case of legumes also influenced by rhizobia colonization.

At the taxonomic order of plant family the cover crop species data revealed that only for certain families a distinctive rooting pattern could be identified. *Fabaceae* clearly clustered together sharing a high diameter/low density rooting type (high PC2). Also *Brassica* species were in a joint cluster (density type with positive PC1). However, they shared their cluster with species from other families. This suggests that either morphological descriptors are insufficient to capture differences in the root systems of these plant families, or that indeed these species, used as autumn grown cover crops, have similar rooting types due to similar environmental adaptation in spite of belonging to different families.

The cereal genotypes fall into four distinct functional groups. Mexican CIMMYT durum genotypes are grouped at one end of the tree, while Matt and Kamut are located at the other end. The latter form a low density deep rooting type (low PC1 and low PC2), while the CIMMYT genotypes are an intermediate type (PC1 and PC2 near origin). A separate group is formed by *T. monococcum* subsp. *monococcum* and *T. timopheevii*, characterized by a density dominated shallow rooting type (high PC1). A fourth cluster contains Central European durum cultivars as well as the European *T. turgidum* subsp. *turanicum* genotype with high diameter rooting types of intermediate density and depth (high PC2 and PC1 near origin).

The cereal data set shows the relation between regional origin and rooting type. Genotypes from summer dry climates (Arizona *T. turgidum* subsp. *durum* var. Matt and Middle Eastern *T. turgidum* subsp. *turanicum* var. Kamut) share a common cluster, different from all others, in spite of belonging to different subspecies. Also *T. turgidum* subsp. *turanicum* var. TRI5254 with unspecified European origin falls in a common cluster with the Central European durum species, which seems to be related to regional origin. In case of the exotic genotypes (*T. timopheevii* and *T. monococcum* subsp. *monococcum*), sharing a common cluster of different sub-species too, probably low breeding intensity is the main reason for their similar root systems (Reynolds et al., 2007). Their overall habitus rather resemble natural grass species than modern cereal crops. The cluster formed by CIMMYT durum cultivars could be expected as they have a common genetic background. Although regional origin is important in many cases, still similarity in the genome seems to be a basic factor underlying the joint clusters: *T. turgidum* subsp. *turanicum* and subsp. *durum* both have genome BA^u, while *T. monococcum* (A^m) and

T. timopheevii (GA^m) both contain the A^m genome (originating from *T. uratu*).

The classification results from the two field samples confirms that meaningful groups can be extracted based on principal component based rooting types. In most cases possible causes for common groups of species could be identified. Distinctive groups showed a relation to the genetic background, suggesting that also at the level of root systems, similarity due to phylogenetic relation can be expected. However, also common environmental adaptation and breeding background seem to be relevant causes for root system formation. In some cases a more accurate distinction might be obtained when topological root attributes would be included. However, the various potential causes for common clusters also underlines the idea of a functional classification where environmental driving forces might have a dominant impact on the rooting type beyond phylogenetic relationship.

A CORE DATA SET FOR CLASSIFICATION

We have shown that PCA provides meaningful and efficient classifiers that integrate all available root information without a priori deciding on their importance for comparative classification. Some authors working on functional classifications suggested core data sets to improve the comparability among classification results (Weiher et al., 1999). Optimally the traits in a root core data set should fulfill three main criteria: (i) they should provide a biologically meaningful and comprehensive root system description; (ii) they should contain traits that efficiently distinguish between root systems and capture as much detail of species differences as possible; (iii) it should comprise traits that are readily measureable with agreed protocols.

Table 2 suggests a list of root core traits covering different structural and functional scales of a root system. The hierarchical order of traits assumes that discrete classes of root systems can be found on higher scale (overall root system size and shape, developmental type), while at lower scale (single axes morphology, physiological functioning) differences are more continuous. However, important exceptions from this rule may exist (e.g., strongly distinctive anatomical attributes). We also give related measurement methods and trait availability via the number of hits from a Scopus database search. Database review clearly shows that root information is mainly available at the level of total root biomass (27% of studies). Also single axes morphology (8%) and some root physiological traits (e.g., respiration, aquaporines; 6%) were reported more frequently. On the contrary architectural traits are rarely measured, particularly in mature root systems, and some relevant physiological parameters (e.g., root resistances) were absent at all in our database screening.

Reich (1993) suggests that the number of traits required for a minimum data set can be reduced by statistical methods exploring the covariance between traits. Within our data-defined classification approach, this can be done previously to clustering during biplot inspection. When trait vectors have a similar direction, they show joint variability. In this case some descriptors can be substituted by others (e.g., more easily measureable traits), without losing information on the whole system diversity and ensuring the congruency of a subsequent classification result (cf. Appendix). Also trait stability and thereby expected repeatability

of the classification result can be assessed via biplot inspection in case of multi-location or multi-year data sets. While for example root diameter is a rather stable trait, root distribution and root-to-shoot ratio strongly respond to different environmental conditions (cf. Appendix). For traits strongly responding to different environmental conditions, a plasticity index (e.g., Bell and Sultan, 1999; Valladares et al., 2006) could be included to capture root-soil interaction effects on root system structure.

Also root architecture models could be an important tool to support elaboration of a core trait list for classification. To our knowledge after the publication of Fitter (1987) and his successive studies, we are the first in applying a root architecture model in the context of classification. The main intention was to determine strong classifiers to distinguish different root systems within the architectural diversity to be expected in nature. Our results suggest that information on axes morphology, root system shape and branching (topology) is required to capture all distinctive groups within a large sample. The lack of architectural measurement data can only partially be covered by other root attributes such as macroscopic shape descriptors.

Classification results presented in this study were limited to differences in root structural attributes rather than functionality (e.g., water or nutrient uptake). When including functionality, other traits (e.g., branching angles, tropisms) could become more important for capturing distinctive rooting types. The relation among a classification based on structural vs. strictly functional/physiological attributes could be studied theoretically when calculating functional differences resulting from the different root architectures simulated here.

Although there is still a lack of certain type of root data (e.g., mature root system architecture), the exponential increase in root studies since the 1960s strongly suggests that a general classification of root systems it is not mainly a problem of lacking data, but rather of an agreed effort and method to join existing knowledge. An open data-defined classification scheme presented here could contribute to close this gap in root research.

CONCLUSIONS

This paper presents a data-defined approach for classification of root systems. The objective is to provide a frame to identify the main distinctive root system properties among different species, analyze potential driving forces in the evolution of root system diversity, and study the functional implication of different rooting strategies. We follow the concept of

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“plant functional types” used in ecology and ecophysiology to search for common groups on a functional rather than a phylogenetic basis. We propose a data-defined approach where distinctive groups are obtained via statistical data exploration methods without a prior decision on a selected classifier.

Our main conclusions are that root morphological description with adequate spatial data provides reliable attributes to classify different types of root systems. This was shown by both simulated and field measured root data that allowed identification of main common rooting types based on average axes morphological attributes. Still a more accurate classification could be expected when integrating topological information. Root system types are the joint result of phylogenetic relation and environmental as well as human selection pressure. A functionally based classification is therefore most appropriate to capture diversity among root systems. The data-defined approach can integrate the increasing number of knowledge on root structure and functioning. PCA and biplot based data inspection provide methods to determine key traits for a core data set and ensures a high degree of stability in cluster based grouping.

The data-defined classification method encourages integration of results from different measurement scales and research foci to capture the overall root system diversity for a broad classification scheme. Currently numerous information on average axes morphology and spatial distribution is available as a result of efficient image analysis tools. Our study suggests that such morphological data sets (length, diameter, depth distribution) would constitute a reliable initial step for classification beyond the established coarse distinction based on developmental type. For a detailed classification of root functional types however a core data set requires quantitative knowledge on root branching. Future integration of root functionality will further extend the basis of our approach and highlight the role of functional vs. structural attributes for classification of root functional types.

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APPENDIX

In **Table 1** we suggested a core set of root attributes that provide a comprehensive description of the root system. **Figure A1** gives an example of root classifiers at different hierarchical levels. Qualitative descriptors can be derived for each attribute, resulting in a composite descriptive characterization of a given rooting type.

An initial distinction is made from whole plant allometric relations such as root:shoot ratio which capture assimilate allocation between aboveground and belowground organs. Following Kutschera and Lichtenegger (1997) a major distinction among root system types is expected from the overall root system shape which can be defined by a geometrical form. The geometrical form or spatial distribution is influenced by developmental type (dominance of the primary root, number of basal roots, extensiveness of shoot-borne roots) as well as the topological connection between axes. These classifiers are thus placed on the next hierarchical orders. The morphological description of single root axes via average descriptors or derived attributes (e.g., diameter or frequency distributions) logically follows on the subsequent level. Finally root anatomical and physiological characteristics are set at the basis of the hierarchical scheme. An example of root classification attributes and distinctive categories at each hierarchical level is shown in the Appendix.

For a stable classification result, it is essential to test the root attributes used. Within the data-defined approach we presented, this can be done at the step of biplot inspection. Particularly biplot analysis serves to investigate the relation among traits used as classifiers. Knowledge of collinear relations between data is important to ensure the congruency of classification in case of using different classifiers. This is exemplified in **Figure A2A**. Vectors of similar direction express joint variability of traits, while a perpendicular direction shows traits not related to each other (**Figure A2A**). When using principal components for clustering, dendograms remain stable as long as all main vector directions in the biplot are covered by traits. Thereby the description of the overall variability of the system is conserved (**Figure A2B**). Thus, traits showing similar vector direction might be substituted by one another.

Also stability over years or sites (repeatability of classification) can be assessed in this way (**Figure A3**). It can be seen that two of the traits (root:shoot, slope of depth distribution) have a roughly perpendicular angle between their vectors. Also the length of the vector is different. Thus, these traits are very sensitive to environmental conditions (years, sites). Root diameter and root length on the contrary show similar vector directions and length revealing their stability over the 2 years. The latter traits therefore can be expected to result in higher repeatability when used for cluster based classification.

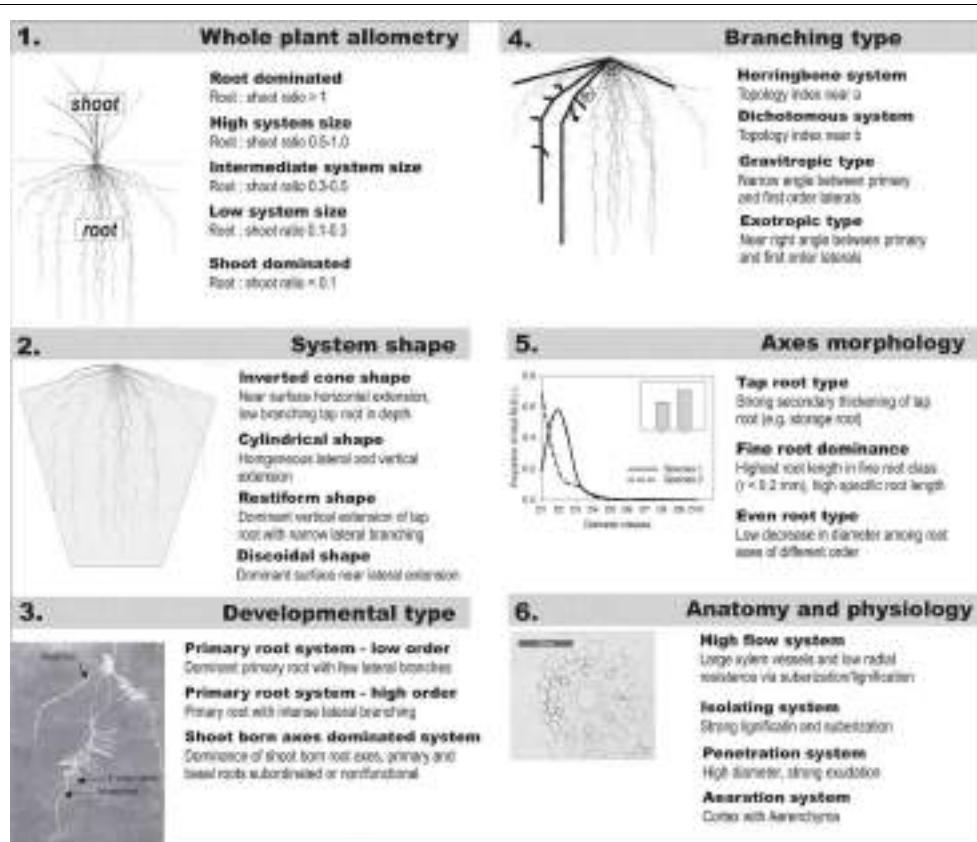


FIGURE A1 | Examples of possible descriptive categories for hierarchically ordered root traits within a comprehensive core data set to be used for classification of root system types.

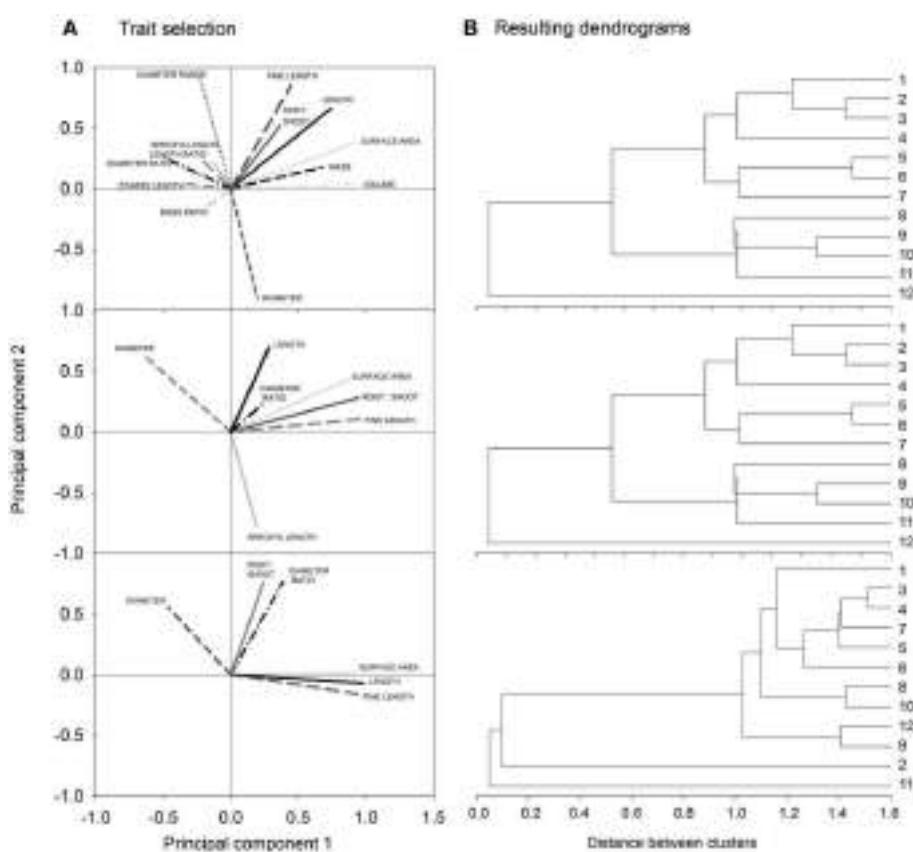


FIGURE A2 | Example for biplot inspection (A) to ensure congruency of subsequent grouping via cluster analysis (B). Biplots are used to visualize the mutual relation between different traits and to determine which traits are essential to conserve the overall variance in a multi-trait root system

characterization (Data from species experiment, cf. Measured Root System Morphology; Root:shoot is root-to-shoot dry matter ratio; mass, length and diameter ratio are indicators of lateral distribution calculated by dividing the respective parameter measured in and between plant rows).

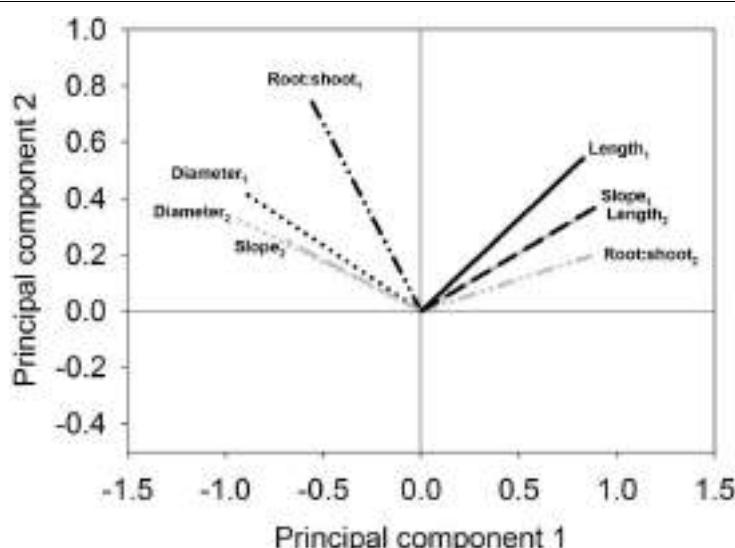


FIGURE A3 | Example of biplot to check expectable repeatability of groups in subsequent clustering. Measured morphological traits of the same genotypes at the same site over 2 years. (Subscripts

indicate first and second year sampling; data from *Triticum* experiment; slope is calculated from the root length density decrease over soil depth).



Descendant root volume varies as a function of root type: estimation of root biomass lost during uprooting in *Pinus pinaster*

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Root systems of woody plants generally display a strong relationship between the cross-sectional area or cross-sectional diameter (CSD) of a root and the dry weight of biomass (DW_d) or root volume (V_d) that has grown (i.e., is descendant) from a point. Specification of this relationship allows one to quantify root architectural patterns and estimate the amount of material lost when root systems are extracted from the soil. However, specifications of this relationship generally do not account for the fact that root systems are comprised of multiple types of roots. We assessed whether the relationship between CSD and V_d varies as a function of root type. Additionally, we sought to identify a more accurate and time-efficient method for estimating missing root volume than is currently available. We used a database that described the 3D root architecture of *Pinus pinaster* root systems (5, 12, or 19 years) from a stand in southwest France. We determined the relationship between CSD and V_d for 10,000 root segments from intact root branches. Models were specified that did and did not account for root type. The relationships were then applied to the diameters of 11,000 broken root ends to estimate the volume of missing roots. CSD was nearly linearly related to the square root of V_d , but the slope of the curve varied greatly as a function of root type. Sinkers and deep roots tapered rapidly, as they were limited by available soil depth. Distal shallow roots tapered gradually, as they were less limited spatially. We estimated that younger trees lost an average of 17% of root volume when excavated, while older trees lost 4%. Missing volumes were smallest in the central parts of root systems and largest in distal shallow roots. The slopes of the curves for each root type are synthetic parameters that account for differentiation due to genetics, soil properties, or mechanical stimuli. Accounting for this differentiation is critical to estimating root loss accurately.

Keywords: root system architecture, forest trees, 3D digitizing, *Pinus pinaster*, uprooting, structural root biomass, fractal branching analysis, biomechanics

INTRODUCTION

Root system architecture is one of the primary aspects of plant structure insofar as it influences plant anchorage in the soil and the way plants absorb water and nutrients (Lynch, 2005). However, far less is known about root system architecture than about aboveground architecture because roots are almost entirely hidden in the soil (Böhm, 1979). Due to their spatio-temporal distribution, coarse and fine roots are not studied in the same way, with the categories generally divided at 2 mm diameter (Böhm, 1979). Coarse root distribution varies greatly as a function of position in the root system, whereas fine roots have a more homogeneous distribution at the stand level. Improved methods for quantifying coarse root system architecture (CRSA) of entire trees would therefore be valuable for a number of applications, ranging from studies of tree biomechanics to those of root carbon sequestration (Brunner and Godbold, 2007).

Although there are techniques of growing plants that allow for relatively easy access to roots, these methods often alter root structure and function (Poorter et al., 2012). For example, CRSA is largely altered by growth in a container as soon as the roots reach the wall of the pot. Moreover, soils are complex media and it is nearly impossible to accurately reproduce soil structure in pots. While measurements made on containerized roots may be useful in some contexts, the role of roots in other contexts, such as studies of ecosystem functioning, can only be studied in natural environments (Danjon et al., 2013). Similarly, transparent interfaces placed in the soil give only partial information on CRSA and can modify root growth. Although larger pots decrease the problem of roots intersecting container walls and allow roots to be scanned with computed tomography (CT; Mooney et al., 2012), root system size is still quite limited and only partial information can be inferred with respect to the root system architecture of mature trees. In the field,

ground-penetrating radar (GPR) shows promise as a non-invasive measurement technique, but, to date, it has only been used for stand level biomass estimations (Butnor et al., 2003) and in methodological studies (Danjon and Reubens, 2008). Therefore, the root architecture of larger plants should ideally be studied in the field using excavated or uprooted root systems (Danjon and Reubens, 2008).

However, coarse root field studies are time-consuming and data are not available for complete root systems because roots break while being removed from the soil. In non-cultivated soils, roots have to be disentangled from the roots of other plants, stones, or woody debris. Excavating soil from roots causes fewer roots to break than pulling roots from the soil, e.g., with heavy machinery. Retrieving broken roots after mechanical uprooting is time-consuming and not always successful. However, excavation is often prohibitively time-consuming when manual tools are used (Puhe, 1994). Excavation is more rapid when done with pressurized water (e.g., Richardson and Dohna, 2003; Tarroux et al., 2010), but requires special equipment and conditions, such as a water source, sloping ground, and shallow rooting. Roots growing under large neighboring trees cannot be recovered. Lost roots result in an under-evaluation of biomass at the stand level, which might be substantial. Perhaps the largest impact on analysis is when individual root system architecture is studied. For example, in a study of susceptibility to windthrow (Danjon et al., 2005), the relative volume of sinkers leeward of the tree was dramatically underestimated if a large sucker was lost in that sector. Resources for removal are always limited, resulting in a trade-off between the proportion of the root system that can be recovered and the number of root systems that can be accessed.

For a given species, a strong relationship between cross-sectional area (CSA) or cross-sectional diameter (CSD) and descendant root biomass (DW_d) or volume (V_d) is assumed in numerous papers (Nielsen and Hansen, 2006). Based on this, techniques of fractal branching analysis (FBA) have been developed to assess CRSA features. Generally, architectural parameters are measured on a small sample of excavated branches, and related to the proximal CSA of all second-order roots (van Noordwijk et al., 1994). There are related techniques for estimating root biomass lost during excavation (e.g., Heth and Donald, 1978).

Fractal branching analysis is based on the assumption that a root system is comprised of self-similar substructures that have consistent tapering and branching properties (van Noordwijk et al., 1994). FBA has been largely used to model the structure of root branches from their proximal diameters. FBA is based on a low number of parameters, including root taper, the CSA shared between the main root and its branches, and inter-lateral branching length. The relationships established in FBA literature demonstrate that there is a basis for estimating missing root volume from CSDs.

Present methods for estimating root biomass lost during excavation are based on the relationship between the CSD and the dry weight (DW_d) of all descending roots for a fairly intact branch (Whittaker and Woodwell, 1968; Heth and Donald, 1978; Le Goff and Ottorini, 2001). Once known, this relationship can be applied to diameters at broken ends of roots to estimate the missing biomass.

Root systems are generally composed of a set of distinct root forms, which researchers have quantitatively differentiated into root types (woody plants are reviewed by Danjon et al., 2013). To study phosphorus uptake in a crop plant (*Phaseolus*), Rubio and Lynch (2007) defined five root categories: the taproot, lateral roots branching from the taproot, basal roots, laterals originating from basal roots, and roots originating from the hypocotyl. Plants can also grow roots from shoots (Zobel and Waisel, 2010). At least four types of roots were used by Köstler et al. (1968) to describe root systems in temperate forest trees: the taproot, shallow roots, secondary sinkers, and oblique roots. Collet et al. (2006) defined five root types in *Quercus petraea* seedlings. Jourdan and Rey (1997) classified roots of *Elaeis guineensis* using seven categories. These were based on branching orders, but included subclasses for orientation (horizontal or vertical) and depth (shallow or deep). Finally, Danjon et al. (2005) used nine root categories to assess relationships between root architecture and wind-firmness in mature *P. pinaster* root systems (see below). A subset of six of these root categories was used to perform an in-depth phenotyping of RSA in *Robinia pseudoacacia* seedlings (Khuder, 2007).

Fractal branching analysis models have tended to treat all roots as a single type, which may be one reason why they have had poor predictive ability (Van Noordwijk and Purnomasidhi, 1995; Danjon and Reubens, 2008). One exception in the FBA literature is Richardson and Dohna (2003); although they did not assess root type explicitly, they showed that FBA parameters do vary as a function of root diameter. Kalliokoski et al. (2010) showed that root tapering could be larger in shallow roots (especially in the zone of rapid taper, ZRT) than in oblique roots, and could decrease with branching order. Nielsen and Hansen (2006) also fitted separate equations between proximal CSA and DW_d for horizontal and vertical roots.

Most studies have pooled all roots to estimate missing biomass or volume. However, cases in which DW_d or V_d were estimated when stratified by root category found stronger relationships. These include cases in which CRSA has been incorporated in an approximate way, such as an analysis using three diameter classes (Le Goff and Ottorini, 2001). Given that branching and tapering parameters may vary as a function of root type (Danjon and Reubens, 2008), estimates of missing volume or biomass may be more accurate if they take such information into account. It should be noted that the relationship between CSA and DW_d is strong partially because root tissue density tends to vary little among root types (Danjon et al., 2006). However, the relationship can have a large inter-stand and inter-species variability (Nielsen and Hansen, 2006; Kalliokoski et al., 2010).

The objectives of this study were (1) to test the hypothesis that the root volume originating from a section varies as a function of root type and (2) to present and apply a new method of assessing the root volume or biomass lost during uprooting. This method avoids the process of collecting and weighing roots, other than to determine root wood density, if required. We used a database of 3D root system architecture that was compiled from a stand of *P. pinaster* trees over time (trees were 5, 12, or 19 years when uprooted). The database included 49 trees, 11,000 roots, and 60,000 root segments. Relationships between CSD and descendant volume were derived for all intact roots in each of 10 architectural

types. These relationships were then applied to the broken tips of all root axes to estimate missing volumes.

MATERIALS AND METHODS

ROOT SYSTEM DATASETS

The root systems used in this study came from the control plot in a fertilization \times irrigation experiment; the design is described thoroughly in Trichet et al. (2008). The experiment took place on a 5.6 ha stand of *P. pinaster* in Pierrotton, France, 20 km south-west of Bordeaux. In that region, mean annual rainfall is 850 mm and mean annual temperature is 13°C. The water table generally fluctuates close to the soil surface during rainy winters, but sinks to 1.5 m depth in late summer. The experimental plot was 60 m asl and was underlain by a moderately humid, sandy spodosol, with a discontinuous deep hard pan at approximately 70 cm depth. The plot was surrounded by 0.5–1.2 m deep ditches.

In spring 1993, a field was prepared by first removing stumps that remained from a clear-cut, and then plowing the soil to 0.3 m depth. One-year-old *P. pinaster* seedlings were subsequently planted at 2 m \times 4 m spacing. Seedlings were of local provenance and were in 200 cm³ turf plugs before planting. Major storms damaged the stand in December 1999 and February 2009. The first storm toppled 20% of trees in the control plot; these were later straightened and secured with cables for 2 years. Trees were harvested for root architectural analysis when trees were 5, 12, and 19 years old (**Table 1**). The respective datasets will be referred to as L5, L12, and L19.

In the L5 dataset, trees were selected from across the diameter at breast height (DBH) range represented in the stand.

Trees were uprooted by pulling the stem upward with a logging crane after loosening the soil with hand tools. Further details of measurement and uprooting are given in Danjon et al. (1999a,b). In the L12 dataset, the sample consisted of seven of the largest DBH trees, as well as five trees that spanned the range of DBH values in the stand (Danjon et al., 2007). In L19, only the largest, most dominant trees were sampled (Augusto et al., 2013). In L12 and L19, trees were uprooted after removing soil from shallow roots with an air-lance, loosening the soil between the shallow roots with the bucket of a mechanical shovel, and then pulling the stem vertically. Details of uprooting and measurement for the L12 and L19 datasets are given in Danjon and Reubens (2008).

Digitizing in 3D was performed using a Fastrack positional measurement system with a Long Ranger magnetic source (Polhemus, Colchester, VT, USA). Each root was divided into approximately 20 cm long segments so as to record changes in direction or diameter, as well as branching points. Spatial coordinates (X, Y, and Z values) were measured at the base of each root axis and at the end of each segment with the digitizer's stylus. The largest and smallest root diameters were also entered for each of these cross sections. In recording L5 and L12, diameters were measured with an analog caliper (0.5 mm resolution). In L19, diameters were measured with a Mitutoyo 700-126 or 700-128 plastic digital caliper (0.1 mm resolution). All excavated roots with basal diameters larger than a given threshold were digitized (**Table 1**). The taproot was considered the first-order root.

Several additional features were recorded during measurement. These included the positions of intra-tree root grafts (except

Table 1 | Characteristics of the three root architecture datasets used for regressions and estimation of missing root volume.

Variable	Unit		
Dataset name		L5	L12
Tree age	Year	5	12
Total trees		30	12
DBH	cm	5.8	17.4
Standard deviation DBH	cm	1.2	3
Basal diameter threshold	cm	0.2	0.2
Depth limit definition	Shallow roots Deep roots	cm or % cm or %	–15 –40
Mean root system	Length Volume	cm cm ³	3988 2731
Total	No. roots included No. segments included	2877 22740	3851 18176
Selected for analysis	Segments (QR ₀) Axes (QR ₀) Segments (LR ₁) Axes (LR ₁)	% % % %	15.7 17.4 14.6 17.3
		16 19.2 16.3 19	26 37 18.5 23.3

Depth limits were used to classify each segment by root type; fixed depths were used for L5, whereas depth limits corresponding to a percentage of maximal rooting depth by tree were used for L12 and L19. Ages were based on the dates that plants germinated.

for L5), ball-shaped growths of unknown origin that occasionally appeared on roots, and forks, which we defined as multiple, higher-order roots extending from a single, lower-order root. We distinguished normal forks from traumatic forks, as multiple root axes often form where tips are killed or roots cut (note that these are called traumatic reiterations elsewhere; Collet et al., 2006; Danjon et al., 2013).

ANALYSIS OF ROOT ARCHITECTURE

We performed a quantitative architectural analysis (Barthélémy and Caraglio, 2007) to determine the functional role of individual roots with respect to maintaining the stability of the tree. This entailed matching segments to one of nine structural classes that occur in tree root systems; the classes are based largely on position and orientation, and therefore correspond to the biomechanical properties that they convey (Danjon et al., 2005; Danjon and Reubens, 2008). An additional class for the higher-order shallow roots was added. Classes were defined as follows (**Figures 1 and 2**):

- (1) Root stump; the portion of the taproot that has a large diameter, and where most shallow lateral roots originate (Nicoll et al., 1995).
- (2) Taproot; the largest root originating at the distal part of the root stump and growing in a vertical direction.

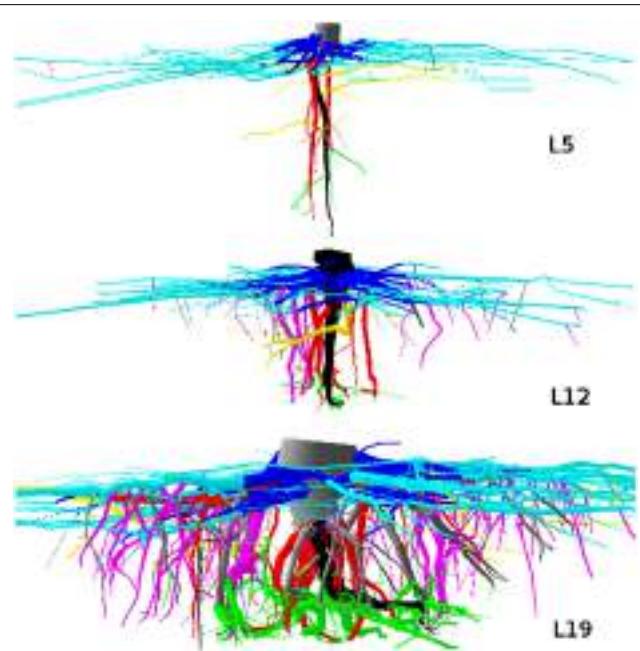


FIGURE 1 | 3D reconstruction of one tree from each dataset, side view. L5: 2.3 m image width, tree 16, 10.7% root volume lost. L12: 3 m image width, tree 725, 6.9% root volume lost. L19: 3 m image width, tree 4601, 3.51% volume root loss. View is from the West. Segments are coloured as a function of their root type: dark gray, root stump; black, taproot; dark blue, shallow roots in the zone of rapid taper (ZRT); light blue, shallow roots beyond ZRT; red, sinkers from ZRT; magenta, sinkers beyond ZRT; yellow, intermediate depth horizontal roots; green, deep roots; gray, oblique roots above the deep limit. Shallow roots are clipped.

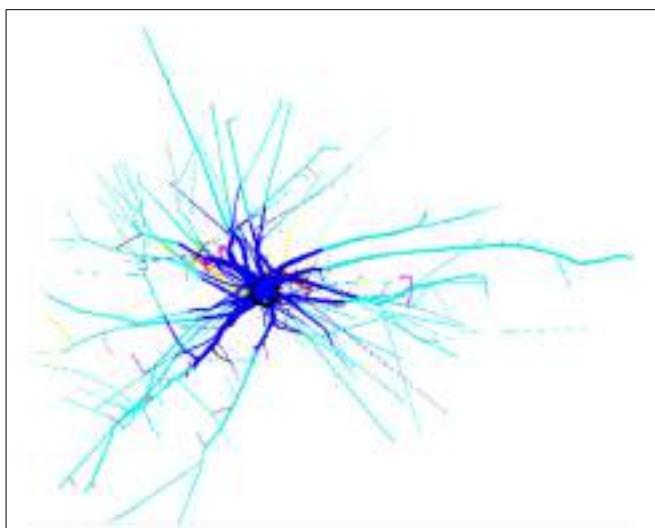


FIGURE 2 | 3D reconstruction of one tree from dataset L12, top view. 4 m image width, tree 725. Coloration follows **Figure 1**. Shallow roots are not clipped.

- (3) Shallow roots in the ZRT; the proximal part of second-order shallow roots (also third-order roots if they originate from a fork).
- (4) Shallow roots beyond the ZRT; the distal part of second-order shallow roots (also third-order roots if they originate from a fork).
- (5) Sinker roots extending from the first-order root or from the ZRT of shallow roots.
- (6) Sinker roots extending from shallow roots beyond the ZRT.
- (7) Intermediate-depth horizontal roots.
- (8) Deep roots; those that originate below a threshold value.
- (9) Oblique roots that originate above the deep-root limit.
- (10) Shallow roots with branching order >2 (or >3 in the case of forks).

The limit between horizontal, oblique and vertical roots was set to 30° and 60°, respectively. The depth limits between shallow, intermediate-depth and deep roots are indicated in **Table 1**. We used fixed limits for L5 and a percentage of maximal depth for L12 and L19, according to Danjon et al. (2005). In L5, the limit for the ZRT was fixed to a radial distance of 2.5 times the DBH. In L12 and L19, the ZRT extended from the root base to the last segment for which the taper from root origin was larger than 2% per cm for L12 and 1.25% per cm for L19 (Danjon et al., 2005).

Using AMAPmod (Pradal et al., 2008), we computed several characteristics for each of the 60,000 distal cross sections in the database or the roots originating from them:

- (1) Cross-sectional diameter measured over bark; where cross sections were elliptical and two diameters were measured, CSD was computed as the quadratic mean.
- (2) Total root volume originating from the section, V_{d0} .
- (3) Root volume originating from the section, but including only segments with diameters larger than 1 cm, V_{d1} .

- (4) Sum of the distal cross-sectional areas (cross sectional areas; break points) of all root segments descendant from the section, $\Sigma \text{CSA}_{\text{end}}$.
- (5) Sum of descendant root graft surfaces (Danjon et al., 2005), ΣS_{graft} .
- (6) Sum of the distal CSAs of roots ending in a traumatic fork, $\Sigma \text{CSA}_{\text{fork}}$.

We used these computations to determine the relationship between the CSD or the CSA and the total root volume originating from that cross-section, excluding segments with non-characteristic taper:

- (1) Bases of large roots are often thickened for reinforcement; we excluded the most proximal segment of each root in L12 and L19.
- (2) *Pinus pinaster* roots often form intra-root-system grafts, where a large root can taper abruptly after crossing a small root, and the small root increases after the graft (Danjon et al., 2005). Therefore, segments for which $\Sigma S_{\text{graft}} > \text{CSA}/20$ were excluded.
- (3) When a root is cut or dies at a given point and a traumatic fork is formed, some root volume is lost and the architecture is disturbed. Therefore segments for which $\Sigma S_{\text{fork}} > \text{CSA}/20$ were excluded.
- (4) Segments with oversized diameters that corresponded to growths were excluded.

The L5 selection of segments for model training did not include sinkers beyond the ZRT, and included very few deep root segments, at this age, these roots were fine and were not measured.

STATISTICAL ANALYSIS

We assessed the relationship between CSD and the two measures of V_d . First, we assessed structural roots defined as sections larger than 1 cm, segments in the root stump, and segments bearing branches without break points >1 cm in diameter:

$$\sqrt{V_{d1ij}} = \lambda_i + \gamma_i \text{CSD}_{ij} + \varepsilon_{ij} \quad (\text{LR}_1)$$

where V_{d1ij} is the volume of section j in root type i , λ_i is the effect of root type i , γ_i is a root type-dependent slope, and ε_{ij} is a residual term. A simplified LR₁ model was also generated, in which four root types were specified rather than ten. In some studies, log–log transformations have been used to obtain a linear relationship between CSD or CSA and DW_d (Le Goff and Ottorini, 2001; Nielsen and Hansen, 2006). For our database, this transformation did not yield Gaussian-distributed residuals, whereas the square root transformation of V_{d1ij} did. The log–log transformation also strongly reduced the spread of the points in the original plot (Poorter and Sack, 2012).

Second, we included all reasonably intact root branches in the model, except root stumps. Specifically, roots branches were considered intact when the sum of distal CSAs was small compared to the proximal CSA ($\Sigma \text{CSA}_{\text{end}} < \text{CSA}/8$). To ensure that long, gradually tapering roots were included, we did not remove roots for which $V_d > \text{CSA} \times 60$ cm. Below 1 cm CSD, the relationship

between CSD and $\sqrt{V_{d0}}$ was slightly curvilinear, because below about 2 mm CSD, roots keep approximately the same diameter over several meters (Danjon et al., 2009a). To account for the properties of thin segments, a quadratic term was added to the model:

$$\sqrt{V_{d0ij}} = \lambda_i + \gamma_i \text{CSD}_{ij} + \beta_i \text{CSD}_{ij}^2 + \varepsilon_{ij} \quad (\text{QR}_0)$$

where β_i denotes the root type-dependent quadratic effect of the CSD.

Although the residuals of preliminary models were Gaussian-distributed, the variances were still heterogeneous. Consequently, we used a variance power function to account for heteroscedasticity in the models, i.e., $\text{Var}(\varepsilon_{ij}) = \sigma^2 \text{CSD}_{ij}^{2\theta_i}$ (Pinheiro and Bates, 2000, p. 211). Note that the parameter θ_i in the variance function is root type dependent.

Both models were fitted using a generalized least squares (GLS) estimator (function *gls* in the R package *nlme*; R Core Team, 2012). The two models were compared to simpler nested models using Akaike's information criterion (Pinheiro and Bates, 2000, p. 84) to ensure that they were not overparameterized (Table 2). A potential random effect of tree was also tested using a mixed model approach, but the effect was not significant. The empirical correlations calculated from the within-subject residuals (cf. Fortin et al., 2008) were small in all three datasets, demonstrating that there was no need for a random effect of tree in the models.

The amount of lost roots was then estimated for all broken root ends using the QR₀ model, because it could be used also for the small CSDs. Because the back transformation of the predictions to the original scale is subject to a bias (Gregoire et al., 2008), we used a naive correction that consisted of adding the prediction error variance to the squared estimate (cf. Fortin et al., 2008).

RESULTS

A good fit was obtained for all models, except with the QR₀ model in the L5 dataset (Figures 3–6; Tables 2–5). Two curves were parabolic (Figure 7): that for shallow roots beyond the ZRT and that for higher-order shallow roots. In these cases, the few roots with the largest CSD had a low descendant root volume. Thus, QR₀ may not deliver accurate estimation outside the range of CSD values used for estimation. However, this did not cause inaccuracies in estimates of lost volume, because the CSDs of the largest broken root ends were much smaller than the largest CSDs used to develop the models (1.5 cm in shallow roots beyond the ZRT and 1 cm in higher-order shallow roots). As a result of the selection procedure, large root sections within the ZRT itself were not included in LR₁ (Figures 3–5).

Root type strongly influenced the intercepts, first-order, and second-order model coefficients for both models in all three of the datasets ($P < 0.001$; Figures 3–5; Tables 3 and 5). γ_i varied from 3.5 to 9 between deep roots and shallow roots. As a consequence, the predicted V_d for a 2 cm CSD root in L19 reached 43 cm³ in deep roots, 102 cm³ in intermediate depth roots, and 290 cm³ in shallow, second-order roots beyond the ZRT. Rankings of root categories were fairly consistent across the three datasets. Roots could be roughly divided into four categories according to

Table 2 | Summary of GLS regression models assessed for descendant root volume.

Dataset	λ_i	γ_i	β_i	Degrees of freedom	AIC	Log likelihood ratio	Grouping	Model retained
Structural roots only								
L5	Global	Global	/	4	13275	13300	a	
L5	Root type	Root type	/	28	12215	12386	b	LR ₁
L5	Root type	Root type	Root type	37	11978	12204	c	
L12	Global	Global	/	4	11525	11548	a	
L12	Root type	Root type	/	31	10390	10571	b	LR ₁
L12	Root type	Root type	Root type	41	10327	10566	c	
L19	Global	Global	/	4	21190	21215	a	
L19	Root type	Root type	/	31	19526	19723	b	LR ₁
L19	Root type	Root type	Root type	41	19493	19753	c	
All roots								
L5	Global	Global	/	4	14125	14150	a	
L5	Root type	Root type	/	25	11614	11768	b	
L5	Root type	Root type	Root type	33	11346	11550	c	QR ₀
L12	Global	Global	/	4	12354	12378	a	
L12	Root type	Root type	/	28	8785	8952	b	
L12	Root type	Root type	Root type	37	8663	8884	c	QR ₀
L19	Global	Global	/	4	28561	28588	a	
L19	Root type	Root type	/	28	23973	24159	b	
L19	Root type	Root type	Root type	37	23665	23911	c	QR ₀

Data were either restricted to root segments from structural roots or included all root segments. 'Global' denotes that the parameter was fixed for all root types, 'Root type' denotes that it varied by root type, and '/' denotes that it was not included in the model.

AIC is Akaike's information criterion; significant differences between models at a 5% level are indicated as letters.

γ_i in LR₁ (**Tables 3 and 5**) and the shape of the curve in QR₀ (**Figures 3–5**):

- (1) Shallow roots beyond ZRT and higher-order shallow roots, for which γ_i seems to increase with age from 8 to 9.5.
- (2) Shallow roots in ZRT, intermediate depth roots, and root stump, for which γ_i increased approximately with age from 6 to 7.5, except the stump in L5.
- (3) Sinkers and oblique roots, for which γ_i was near 5.5.
- (4) Deep roots and the taproot, for which γ_i was near 4, except in L5.

When root types were grouped into four classes (**Table 4**), the above mentioned values for γ_i were indeed found, and confidence intervals were smaller.

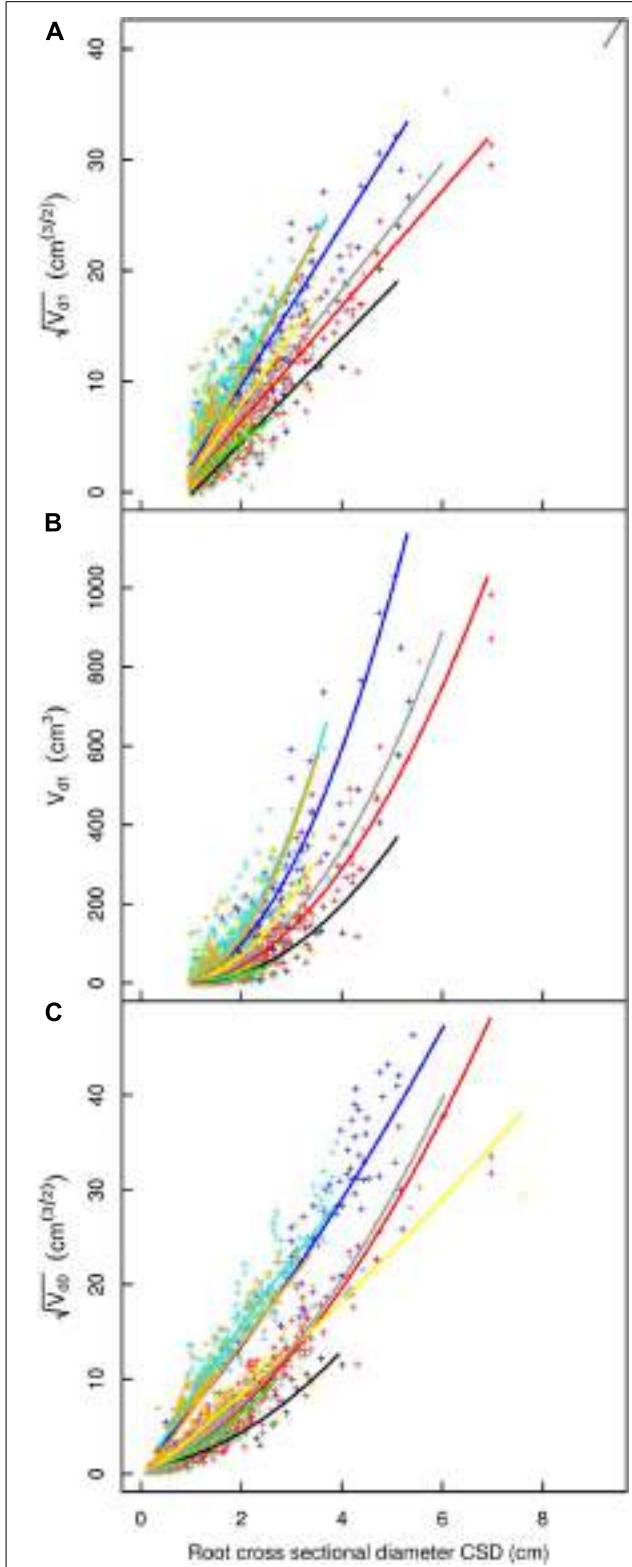
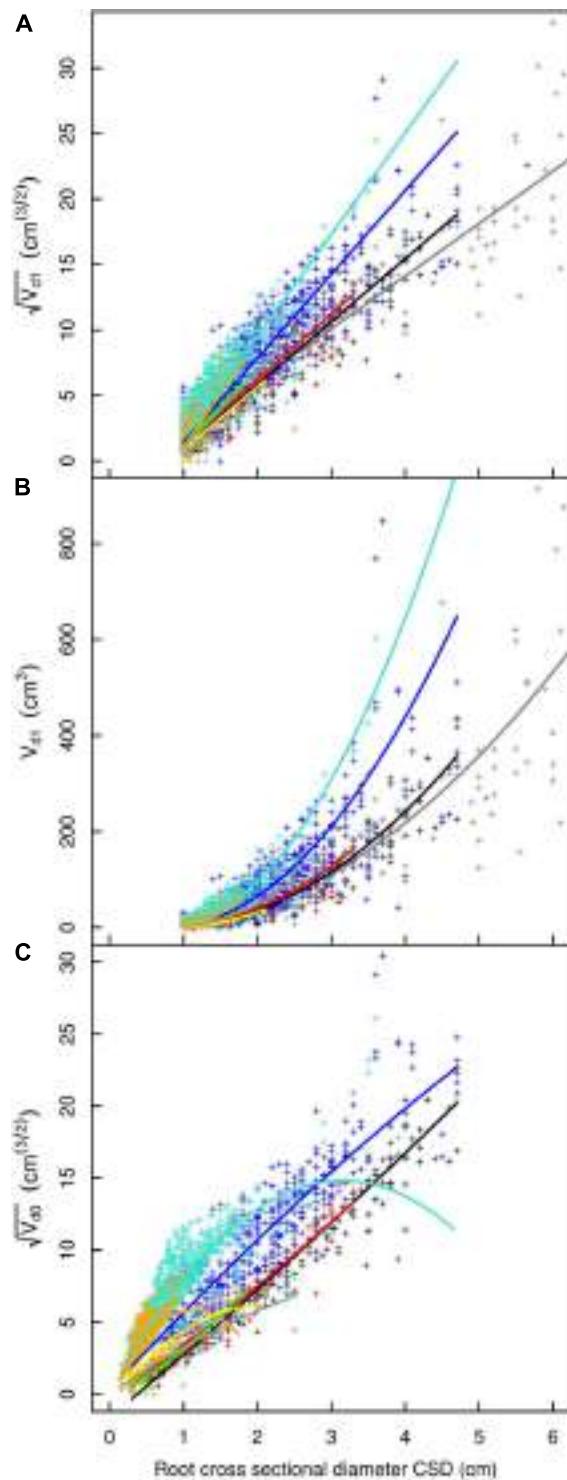
At the root system level, missing root biomass varied as a function of tree age and of root type (**Table 6**). In younger trees, 12–23% of root biomass was lost per tree, almost exclusively in higher-order roots and in shallow roots beyond the ZRT. The contribution of other root types was marginal. In L12, 2–7% of the root volume was missing at tree level. For L5, most of the missing root volume originated from the shallow roots beyond the ZRT (1.3–3.5%) and in higher-order shallow roots (0–2.3%). In L19, the amount of missing roots varied from 2 to 4% and shallow roots contributed only 70% of the missing volume. In L19, the

largest contribution to missing biomass (1066 cm³, or 17% of lost volume, in tree no. 5329) came from a single, 3 cm CSD shallow root that was beyond the ZRT.

The percentage of missing root volume in each root type varied widely (**Table 6**). It was generally close to zero for the taproot, shallow roots within the ZRT, and sinkers below ZRT; these three root types form the central part of the root system. Missing volume had high mean values for shallow second-order roots beyond the ZRT, decreasing with age from 27 to 15 to 10%, though it was still larger in higher-order shallow roots. The amount of roots lost in the other categories varied between the values for the two aforementioned categories, namely around 8% in L12 and L19, with a high inter-tree variability. L5 trees possessed only a few roots in the intermediate depth, deep, and oblique classes, but these were largely broken. When root types were not used for estimation, the percentage of missing root volume was largely underestimated in L12 and L19, but largely overestimated in L5 (**Table 6**).

DISCUSSION

This study has shown that the relationship between root CSD and descendant volume varies largely as a function of root type. In quantifying these relationships, we have demonstrated that it is possible to accurately estimate the amount of missing root material from datasets describing 3D root system architecture. Estimation



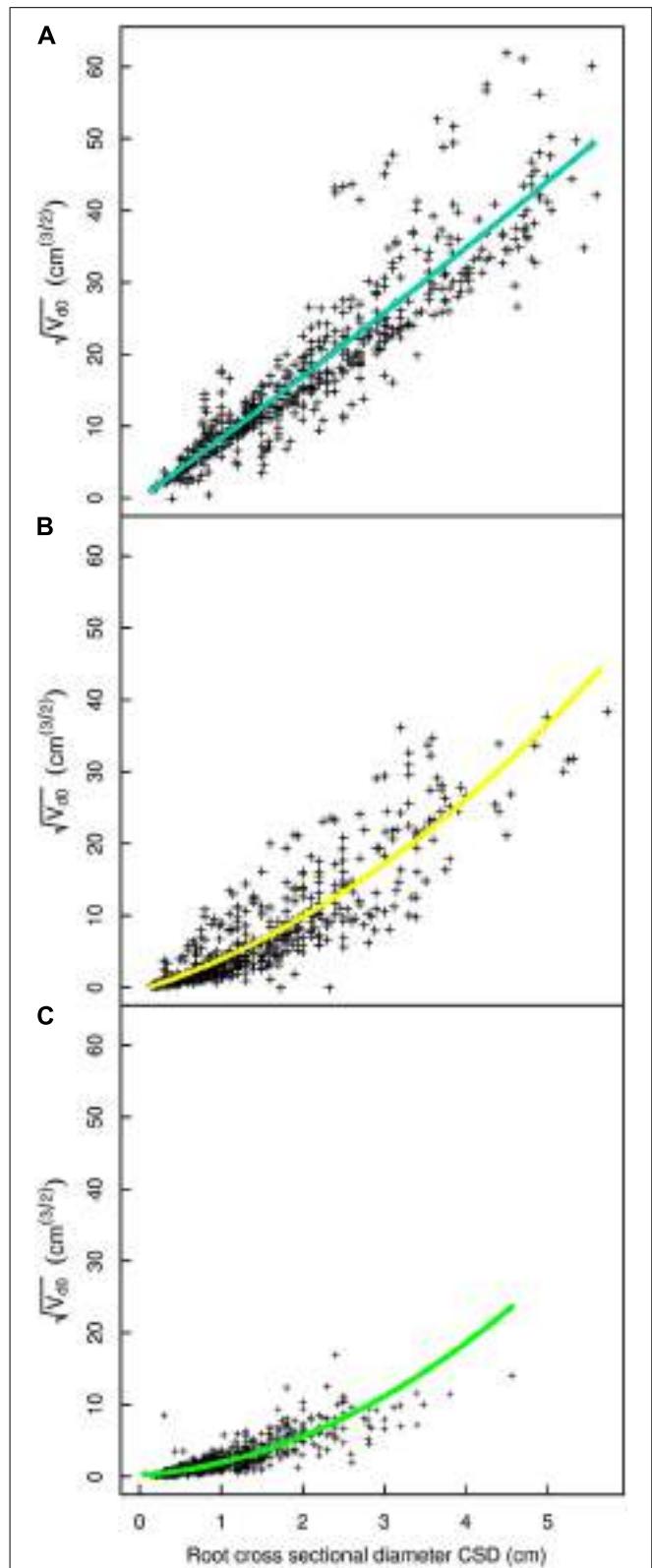
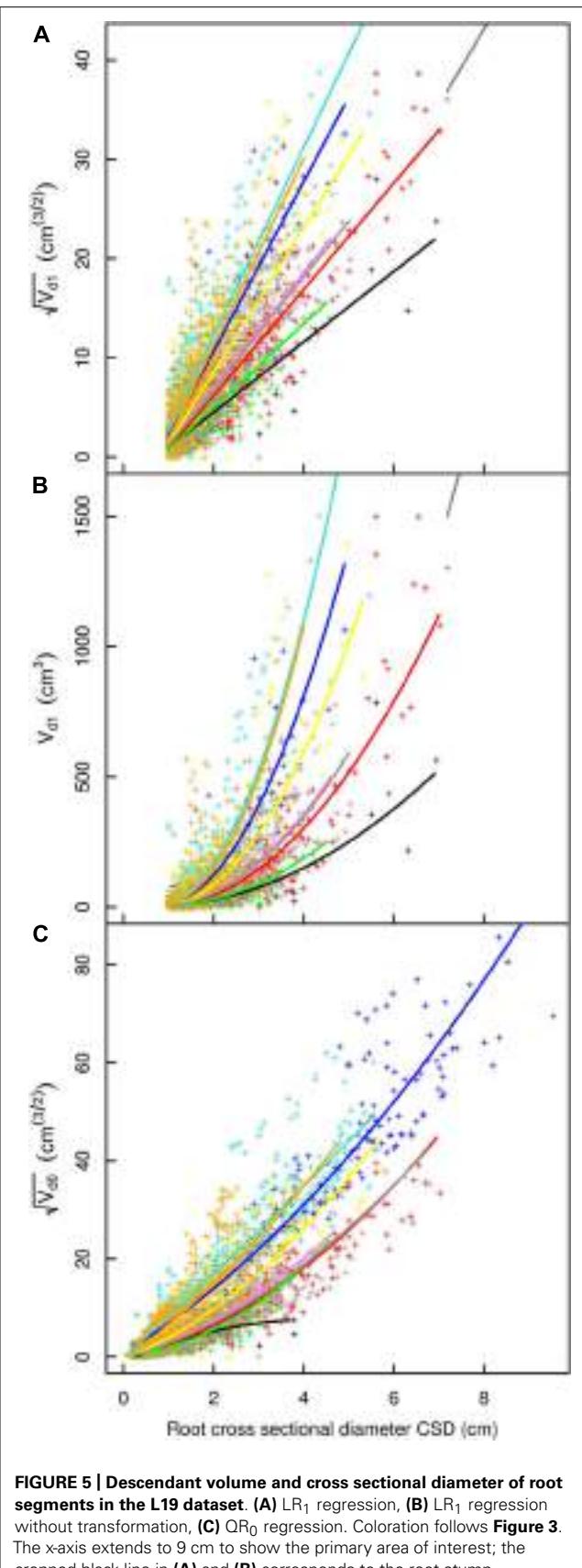


Table 3 | Results of the LR₁ regression, generalized least squares linear regression per root type between the cross sectional diameter and the square root of descendant root volume for each of the three separate datasets, structural roots only, including the root type-dependent variance parameter. Parameter values and 95% confidence intervals (CI).

Parameter	Root type	L5		L12		L19	
		Value	CI	Value	CI	Value	CI
$\hat{\lambda}_i$ (root type-dependent intercept)	Root stump	-1.92	± 1.84	-25.84	± 11.58	-18.06	± 27.38
	Taproot	-3.74	± 0.38	-4.88	± 1.61	-2.60	± 1.86
	Shallow in ZRT	-4.96	± 0.29	-4.69	± 0.78	-6.45	± 1.50
	Shallow beyond ZRT	-6.03	± 0.32	-5.43	± 0.67	-6.70	± 0.86
	Higher-order shallow	-7.08	± 1.48	-7.03	± 1.03	-7.60	± 0.82
	Sinkers from ZRT	-3.77	± 0.49	-4.02	± 0.40	-4.49	± 0.49
	Sinkers from beyond ZRT	NA	NA	-4.55	± 0.80	-4.38	± 0.52
	Intermediate depth	-3.90	± 0.91	-4.67	± 0.79	-6.03	± 0.69
	Deep	-4.72	± 2.94	-2.23	± 1.11	-3.40	± 0.54
	Oblique	-4.91	± 1.15	-4.51	± 0.47	-4.47	± 0.40
	Root stump	4.01	± 0.39	7.15	± 0.62	7.65	± 1.28
	Taproot	4.80	± 0.18	4.68	± 0.70	3.55	± 0.84
$\hat{\gamma}_i$ (root type-dependent slope)	Shallow in ZRT	6.40	± 0.19	7.18	± 0.45	8.56	± 0.91
	Shallow beyond ZRT	7.78	± 0.26	8.21	± 0.49	9.48	± 0.65
	Higher-order shallow	8.15	± 1.32	8.63	± 0.81	9.42	± 0.64
	Sinkers from ZRT	4.99	± 0.32	5.20	± 0.24	5.35	± 0.26
	Sinkers from beyond ZRT	NA	NA	5.80	± 0.55	5.74	± 0.34
	Intermediate depth	4.78	± 0.63	6.29	± 0.59	7.29	± 0.49
	Deep	5.90	± 2.31	3.43	± 0.75	4.20	± 0.37
	Oblique	5.87	± 0.92	5.69	± 0.32	5.65	± 0.25
	$\hat{\sigma}^2$	1.09	± 0.04	1.34	± 0.06	1.60	± 0.06
	Root stump	0.99	± 0.07	0.79	± 0.07	1.02	± 0.09
	Taproot	0.35	± 0.10	0.45	± 0.29	0.65	± 0.24
$\hat{\theta}_i$ (root type-dependent variance parameter)	Shallow in ZRT	0.85	± 0.08	0.73	± 0.15	0.98	± 0.23
	Shallow beyond ZRT	0.93	± 0.13	1.17	± 0.17	1.40	± 0.14
	Higher-order shallow	1.06	± 0.58	1.15	± 0.27	1.52	± 0.15
	Sinkers from ZRT	0.31	± 0.18	0.48	± 0.11	0.66	± 0.08
	Sinkers from beyond ZRT	NA	NA	-0.26	± 0.29	0.59	± 0.11
	Intermediate depth	-1.66	± 1.29	0.92	± 0.21	1.12	± 0.11
	Deep	0.27	± 2.40	-0.13	± 0.35	0.41	± 0.13
	Oblique	0.78	± 0.35	0.47	± 0.13	0.70	± 0.08

is possible provided that the root dataset contains sufficient information on reasonably intact root axes and segments from which to derive a relationship. Basing the estimate on volume makes for a significant time savings over methods that require biomass to be measured for roots of varying sizes. Root loss estimations are much more accurate when roots are classified by type, and the numerical relationships used to estimate missing volume are stratified according to these classes. Root volume data can be subsequently converted to root biomass if necessary, provided that root wood density data are available (Danjon et al., 2008). Given that root tissue density varies little by root type, if at all (it did not vary significantly for mature *P. pinaster* (Danjon et al., 2006), the

effort needed to collect appropriate root density measurements will generally be minimal.

Parameter estimates for the model with structural roots only (LR₁) were highly reliable because only complete branches were used, excluding the root stump. In contrast, for QR₀ estimates, all of the branches used were broken. Given that quadratic models are flexible, they should not be used outside the range of the data used to estimate their parameters. A quadratic term was needed to cope with the curvature of the relationship for small values of CSD. Defining the optimum criteria to distinguish weakly vs. strongly broken branches is not without challenges. Roots within the ZRT that had large cross sections were not included in the LR₁

Table 4 | Same as Table 3, but with only four root categories: (1) tap root and deep roots, (2) sinkers and oblique roots, (3) shallow roots in ZRT, intermediate depth horizontal roots and root stump, and (4) shallow roots beyond ZRT and higher-order shallow roots.

Parameter	Root type	L5		L12		L19	
		Value	SE	Value	SE	Value	SE
$\hat{\lambda}_i$ (root type-dependent intercept)	Taproot and deep roots	-3.70	0.36	-3.39	0.76	-3.16	0.48
	Sinker and oblique roots	-4.05	0.45	-4.13	0.28	-4.29	0.26
	ZRT, intermediate depth and root stump	-4.37	0.26	-4.47	0.31	-6.09	0.51
	Shallow beyond and higher order	-6.29	0.32	-6.36	0.65	-7.65	0.67
$\hat{\gamma}_i$ (root type-dependent slope)	Taproot and deep roots	4.78	0.17	4.19	0.46	4.01	0.32
	Sinker and oblique roots	5.18	0.31	5.36	0.18	5.49	0.16
	ZRT, intermediate depth, and stump	5.85	0.18	6.47	0.20	7.54	0.35
	Shallow beyond and higher order	7.96	0.26	8.73	0.50	9.91	0.52
$\hat{\delta}^2$		1.07	0.04	1.34	0.06	1.61	0.05
$\hat{\theta}_i$ (root type-dependent variance parameter)	Taproot and deep roots	0.37	0.10	0.36	0.20	0.46	0.11
	Sinker and oblique roots	0.48	0.16	0.45	0.09	0.67	0.06
	ZRT, intermediate depth, and stump	1.12	0.06	0.86	0.07	1.05	0.07
	Shallow beyond ZRT and higher order	1.00	0.13	1.29	0.16	1.52	0.12

model because at least one root with a broken tip >1 cm generally descended from them.

VARIATION IN TAPER

The largest difference in γ_i was between deep roots and shallow roots beyond the ZRT. For a given diameter in the L19 dataset, LR₁ estimates that deep roots had about six times less (and sinkers had about two times less) root volume descending from them than shallow second-order roots beyond the ZRT. In FBA studies, the taper at branches, when mentioned (Soethe et al., 2007), is close to 1 (i.e., the sum of CSAs proximal and distal to the branch are equal). This is in accordance with the pipe model (Shinozaki et al., 1964). Thus, the slope γ_i mainly accounts for tapering between branches of all descendant root segments in an integrative way and also to branching in fine roots. This means that deep roots, the taproot, and to a lesser extent sinkers and oblique roots, have a high overall tapering rate. This probably occurs for two reasons: (1) shallow roots develop more rapidly because they have a significantly higher potential to contribute to plant productivity than deep roots (Korndoerfer et al., 2008), and (2) root growth is restricted by unfavorable soil conditions in deeper soil horizons (e.g., low nutrient content, hard pans, or water tables). It is therefore surprising that CSA has been considered a good predictor of descendant root biomass in the literature. One reason is that this relationship was mainly assessed in a single root type, with the proximal diameter used for estimation located in the ZRT. The other reason is that a high correlation can simply be caused by a wide range of CSA size values (Poorter and Sack, 2012). For example, in Drexhage and Colin (2001), where the root CSA ranges from 2 to 120 cm², the r^2 is around 0.9 but there is still large variation that is orthogonal to root CSA. Kalliokoski et al. (2008) and Heth and Donald (1978) also found a very large variation orthogonal to root CSA (close to 1:10). It should also be noted that, if γ_i

varies as a function of root type, FBA parameters will also vary as a function of root type (Kalliokoski et al., 2010). In *R. pseudoacacia* seedlings, tapering between branches scored around 7% for the root stump, 20% for the taproot, and only 5% in laterals (Khuder, 2007).

Variability in taper among root types may be larger than what we computed. For example, large horizontal shallow roots with gradual taper can branch into secondary sinkers with moderate taper, which themselves can branch into deep roots with steep taper. Moreover, even if the branches kept for QR₀ computations were less broken than the others, they were still broken. Therefore, γ_i for unbroken shallow roots without sinkers is probably distinctly larger than 9. In the same way, the descendant volume of ZRT segments also included a large amount of shallow roots beyond the ZRT. That is why the differences in γ_i between the two root types were small even though taper in the ZRT was steep. Because deep roots did not bear other types of roots, their γ_i was close to 3.5 in limiting soils. Deep roots that tapered steeply were observed in the field, especially in the vicinity of the hard pan.

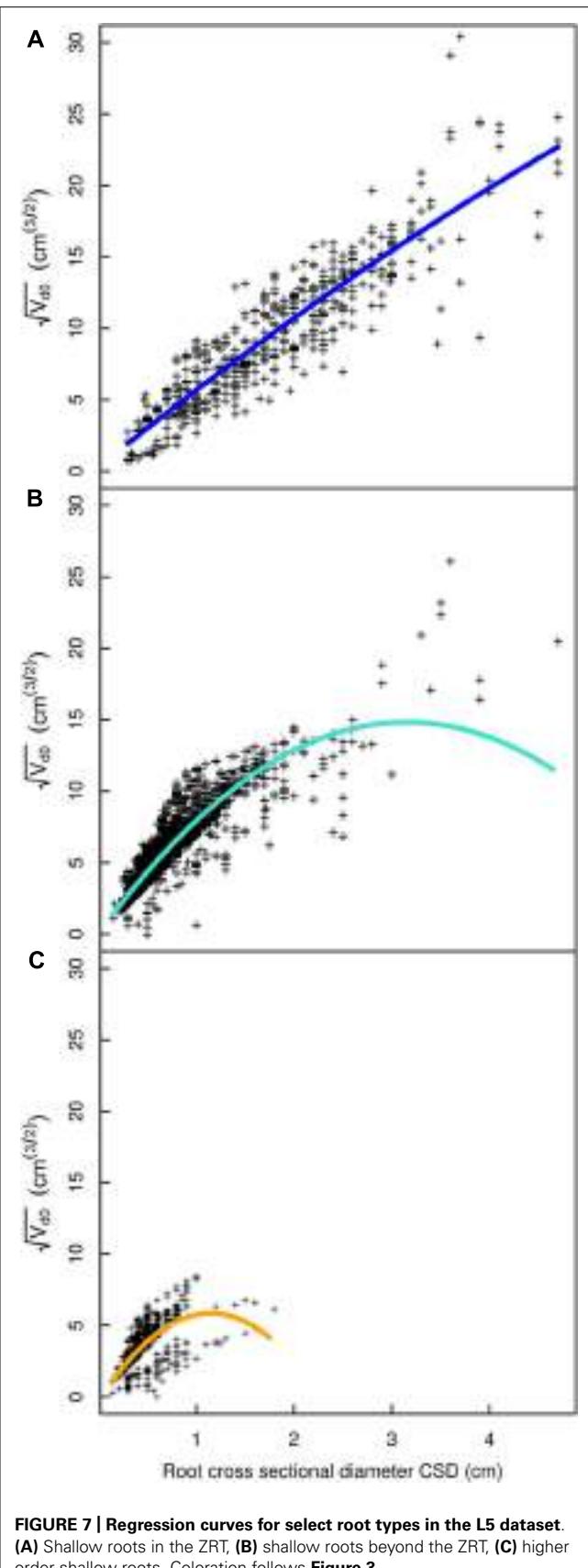
The large variability of V_d for first-order roots can be explained by the structure of the root. For mechanical reasons, the top of the first-order root (the root collar) has a diameter that is generally larger than the DBH of the tree (Coutts, 1987). Below-ground, the diameter increases in the zone where large shallow roots originate (the root stump), with a large diameter maintained through the region where shallow roots originate. The first-order root tapers strongly below this point to form the taproot. Prediction of V_d in the first-order root would therefore be unreliable using a single set of parameters for both the root stump and the taproot. In our database the root stump was never broken, so there was no practical reason to estimate its descendant volume.

Table 5 | Parameters of the QR₀ generalized least squares polynomial regression for each root type, between the cross sectional diameter and the square root of descendant volume for each of the three datasets, for all roots. Parameter values and 95% confidence intervals (CI).

Parameter	Root type	L5		L12		L19	
		Value	CI	Value	CI	Value	CI
$\hat{\lambda}_i$ (root type-dependent intercept)	Taproot	-1.60	± 0.92	0.56	± 1.98	-1.04	± 1.56
	Shallow in ZRT	0.27	± 0.39	0.46	± 0.69	0.76	± 0.74
	Shallow beyond ZRT	0.06	± 0.16	-0.22	± 0.57	-0.13	± 0.29
	Higher-order shallow	-0.32	± 0.42	-0.78	± 0.24	-0.82	± 0.17
	Sinkers from ZRT	-0.43	± 0.34	-0.15	± 0.15	-0.19	± 0.18
	Sinkers from beyond ZRT	NA	NA	-0.66	± 0.66	-0.26	± 0.31
	Intermediate depth	-0.38	± 0.99	-0.55	± 0.20	-0.40	± 0.18
	Deep	-0.07	± 0.95	-0.10	± 0.09	0.19	± 0.12
	Oblique	0.01	± 0.82	-0.15	± 0.08	-0.29	± 0.12
$\hat{\gamma}_i$ (root type-dependent slope)	Taproot	4.26	± 0.85	0.71	± 2.47	4.08	± 2.67
	Shallow in ZRT	5.55	± 0.57	5.88	± 1.11	5.63	± 0.87
	Shallow beyond ZRT	9.37	± 0.38	8.58	± 0.85	8.32	± 0.55
	Higher-order shallow	10.78	± 1.39	7.41	± 0.59	6.92	± 0.46
	Sinkers from ZRT	3.62	± 1.10	2.20	± 0.36	2.03	± 0.40
	Sinkers from beyond ZRT	NA	NA	3.27	± 1.60	2.70	± 0.63
	Intermediate depth	6.28	± 2.69	4.24	± 0.52	3.55	± 0.50
	Deep	2.27	± 4.25	1.82	± 0.43	0.82	± 0.40
	Oblique	3.58	± 2.35	2.23	± 0.30	2.57	± 0.35
$\hat{\beta}_i$ (root type-dependent quadratic effect of the cross sectional diameter)	Taproot	0.08	± 0.17	0.60	± 0.61	-0.48	± 0.82
	Shallow in ZRT	-0.17	± 0.16	0.31	± 0.25	0.49	± 0.15
	Shallow beyond ZRT	-1.49	± 0.20	-0.33	± 0.26	0.10	± 0.17
	Higher-order shallow	-4.70	± 1.07	-0.10	± 0.26	0.50	± 0.21
	Sinkers from ZRT	0.17	± 0.63	0.68	± 0.15	0.64	± 0.13
	Sinkers from beyond ZRT	NA	NA	0.37	± 0.81	0.60	± 0.23
	Intermediate depth	-1.50	± 1.59	0.11	± 0.22	0.77	± 0.20
	Deep	0.92	± 4.15	0.71	± 0.46	0.95	± 0.25
	Oblique	-0.35	± 1.31	0.73	± 0.22	0.55	± 0.16
$\hat{\sigma}^2$		1.39	± 0.04	1.21	± 0.03	1.76	± 0.03
$\hat{\theta}_i$ (root type-dependent variance parameter)	Taproot	0.13	± 0.11	0.26	± 0.40	0.59	± 0.50
	Shallow in ZRT	0.57	± 0.08	0.93	± 0.10	0.94	± 0.08
	Shallow beyond ZRT	0.73	± 0.06	0.91	± 0.12	1.08	± 0.06
	Higher-order shallow	0.31	± 0.08	0.52	± 0.08	0.99	± 0.05
	Sinkers from ZRT	1.46	± 0.26	1.05	± 0.11	0.91	± 0.10
	Sinkers from beyond ZRT	NA	NA	1.13	± 0.53	0.72	± 0.10
	Intermediate depth	0.23	± 0.26	0.89	± 0.14	1.07	± 0.07
	Deep	1.87	± 0.34	1.84	± 0.12	1.07	± 0.06
	Oblique	0.50	± 0.36	1.51	± 0.09	1.12	± 0.09

All trees in this study were from the same species, the same genetic provenance, and grown in the same stand. As no tree effect was detected, these trees probably exhibit low intra-population genetic variability, low plasticity to the micro-environment, and a minimal effect of tree size for the studied relationship. A small but

significant increase in γ_i was observed with age for certain root types. However, we could not assess whether there is a global age effect, because, in L5, the number of segments was only sufficient to provide reliable estimates in the first-order root and in shallow roots.



A further improvement of our method would be to split the estimates of lost root volume into root types or root diameter categories (Le Goff and Ottorini, 2001). This technique could also be used to estimate structural root length and root number. However, fine roots make up a large proportion of root length and number. It could also be used to estimate the number of fine roots branching from the structural roots, possibly on a sub-sample of intact root branches carefully excavated and digitized, including counts of fine roots. A recurrent procedure could be used to compute parameters of QR_0 from all the segments of the root systems whether they carry large broken root ends or not. This would entail attributing a lost root volume (from QR_0) to all broken root ends of the database, and recomputing a corrected descendant root volume for each segment.

ROOT LOSS IN LARGER TREES

The low fraction of missing roots in our database can be partly attributed to the fact that we worked in shallow, sandy soils, excavated during the wet season, and dealt with root systems that were mainly composed of long, shallow roots and sinkers. The amount of lost roots is expected to be larger without preliminary removal of understory plants and soil preparation. Root loss would also be large in stony, hard, or deeper soils, or for heart root systems (i.e., those with a large proportion of oblique roots originating from the root stump; Danjon et al., 2013). Even in deep soils with deep-rooting species, only few structural roots can be found below a 4 m depth (Christina et al., 2011). While low, the root biomass losses reported here may be slight overestimates. If our assumption of constant root tissue density was incorrect, and density decreased with distance from the root collar (Danjon et al., 2006), biomass in distal roots would have been lower than reported.

The reason that most of the structural root biomass could be easily extracted is that a rigid structure is formed in *P. pinaster* by the root stump, taproot, shallow roots within the ZRT, and sinkers originating from the ZRT; these classes also made up the largest portion of the root biomass (Danjon et al., 2005). When root systems were extracted vertically, the taproot usually remained intact and the large sinker roots originating within the ZRT were also recovered. However, much larger losses occurred in the shallow roots beyond the ZRT. After we removed understory vegetation, litter, and the upper soil surface, most of the thicker shallow roots were removed within a radius of 3 m by manual pulling. However, as these roots taper very gradually, about 15% of their volume was still lost in larger trees. As a point of comparison, in a prior study that used four *Fagus sylvatica* trees (Le Goff and Ottorini, 2001), the estimate of lost root biomass varied from 5 to 35%. Heth and Donald (1978) experienced an average loss of 1.6% in 38 cm DBH *Pinus radiata* trees and an average loss of 10.6% in 47 cm DBH trees. Niijima et al. (2010) reported a 23% mean loss in a sample of 121 tropical trees ranging from 0.4 to 116 cm DBH, and suggested that the proportion of root loss increases with tree size. We observed the opposite relationship with tree size. Our uprooting technique was more efficient for larger trees, and structural roots of the L5 trees broke more easily than those of L12 and L19.

Table 6 | Estimation of the percentage of root volume lost downstream of the breaking point, as part of the total volume (above) and within each root type (below).

Stand:	L19							L19		L12		L5		
	Tree number	725	4601	4824	4832	4864	5306	5329	Mean	SD	Mean	SD	Mean	SD
Contribution of each root type to root loss														
Order 1		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.0	0.0	0.0	0.0	0.0	0.1
Shallow in ZRT		0.06	0.00	0.09	0.01	0.06	0.01	0.00	0.0	0.0	0.1	0.1	0.1	0.2
Shallow beyond ZRT		2.58	2.22	0.95	2.01	1.26	0.47	1.05	1.5	0.8	2.3	0.8	9.2	3.2
High order shallow		0.62	0.45	0.54	1.44	1.42	1.28	0.77	0.9	0.4	1.2	0.7	6.2	2.1
Sinkers from ZRT		0.01	0.03	0.04	0.17	0.02	0.00	0.00	0.0	0.1	0.1	0.1	0.1	0.1
Sinkers from beyond ZRT		0.07	0.19	0.16	0.36	0.30	0.10	0.14	0.2	0.1	0.2	0.2	NA	NA
Intermediate depth		0.09	0.14	0.17	0.22	0.57	0.44	0.31	0.3	0.2	0.3	0.2	0.0	0.1
Deep		0.03	0.19	0.08	0.11	0.08	0.10	0.04	0.1	0.1	0.1	0.1	0.8	0.8
Oblique		0.16	0.29	0.11	0.19	0.17	0.15	0.26	0.2	0.1	0.3	0.2	1.0	1.9
Total tree		3.61	3.51	2.15	4.53	3.86	2.56	2.60	3.3	0.8	4.4	1.5	17.5	5.1
LR0WC % difference		-24%	-14%	+1%	-17%	-11	+6%	-13%	-10%	11	-46%	7.8	+86%	23
QR0WC % difference		-44%	-28%	-13%	-29%	-27	-12%	-14%	-23%	11	-47%	8.7	+64%	19
% loss in each class														
Order 1		0.01	0.00	0.00	0.01	0.00	0.00	0.41	0.1	0.2	0.0	0.0	0.4	0.7
Shallow in ZRT		0.26	0.00	0.75	0.07	0.28	0.04	0.01	0.2	0.3	0.2	0.2	1.7	7.0
Shallow beyond ZRT		14.50	11.70	6.45	14.30	10.90	5.21	9.75	10.4	3.6	15.1	4.2	27.2	9.8
High order shallow		13.50	11.30	13.60	9.76	18.30	13.40	5.52	12.2	4.0	22.0	11.4	56.1	9.4
Sinkers from ZRT		0.10	0.54	0.38	1.30	0.17	0.04	0.04	0.4	0.5	0.8	0.6	11.5	13.6
Sinkers from beyond ZRT		5.55	3.64	16.20	9.56	8.98	4.18	2.95	7.3	4.7	11.5	7.6	NA	NA
Intermediate depth		2.87	10.40	3.71	4.57	13.20	8.64	3.57	6.7	4.0	7.7	5.0	34.2	22.3
Deep		3.41	3.69	13.60	4.03	2.86	4.90	6.96	5.6	3.8	9.3	9.5	51.0	22.5
Oblique		4.68	4.05	4.67	2.91	4.47	1.86	3.42	3.7	1.1	8.6	7.5	40.7	18.5

LR0WC and QR0WC: % difference in estimation of total root loss without taking into account root types compared to estimations obtained using QR₀. Left: estimation for each tree in L19. Right: mean and standard deviation in L5, L12, and L19.

Bold values are summary values. Italicized values correspond to analyses which do not take root types into account.

APPLICATION

The approach described here to estimate root losses during uprooting can be used to improve estimates of forest carbon storage. Regional carbon stocks in forests are quantified using stem volume measurements that are taken during forest inventories. Carbon stored in root systems are incorporated through a biomass expansion factor (FEB), or the ratio between total biomass and stem biomass. An estimate of FEB in a mature *P. pinaster* stand that did not account for missing roots yielded a value of 1.585 (Bert and Danjon, 2006). However, using the 4% coarse root loss found here, the corrected FEB would increase to 1.598. Similarly, the ratio between total woody biomass and aboveground woody biomass, referred to as the root expansion factor (REF) originally scored 1.26, but would rise to 1.27 after being corrected. The original root mass fraction (RMF) of 20.6% would change to 21.2% after correction. Correction for missing roots is also useful for assessing the percentage of root biomass or mineral mass exported from a forest by root system harvesting (Augusto et al., 2013).

Root loss diminishes strongly with increasing diameter. Therefore, during uprooting of larger trees, one must avoid losing

roots with CSD larger than about 1.5 cm, especially in shallow roots with large γ_i . A better estimation of γ_i and lost root biomass in shallow roots may have been achieved by carefully excavating a few shallow roots per tree before uprooting.

In some cases, model coefficients may need to be derived from a different set of plants than those for which missing volume is estimated. This is the case in plants that are excavated quickly and in which a large amount of root material is lost. For example, rapid uprooting is necessary in studies using high throughput phenotyping (Danjon et al., 2009b; Trachsel et al., 2011). An alternative to using separate plants for missing volume estimation would be to carefully uproot and digitize a small, stratified sample of roots specifically for deriving regression parameters for the root types of interest.

ROOT SYSTEM DIFFERENTIATION

The fact that roots experience diverse growth conditions, even within a single plant, may limit the applicability of conventional fractal branching models (e.g., Soethe et al., 2007) in problems like estimating the root volume lost in excavation. For instance,

root tapering and related fractal properties may be altered by growth responses to mechanical stimuli (Danjon et al., 2005), soil geometry (Nicoll et al., 2006), soil layer properties, and resource availability (Pierce et al., 2013). Models that account for variation in root morphology and architecture, especially those accounting for root types (Jourdan and Rey, 1997; Collet et al., 2006) are probably better suited to reconstructing root biomass from partial measurements of root systems. This study also demonstrates that the prediction accuracy of root properties from proximal diameter measurements is substantially higher when multiple trees and whole root systems are used for analysis. FBA has generally been performed with modest numbers of branching points (250 branching points in Richardson and Dohna, 2003, 200 per species and stand in Kalliokoski et al., 2010). Descendent volume has also been assessed with modest numbers of root samples (27 in Niiyama et al., 2010; 400 in Heth and Donald, 1978; 100 in Le Goff and Ottorini, 2001; three per tree in 417 trees from four species in 20 stands in Nielsen and Hansen, 2006).

High variability in taper between root types is likely to be observed in most woody species, given that roots can generally be classified as shallow, sinker, and deep roots. There was high variability in the database used herein, despite the fact that it described a single, monospecific stand with a single provenance. Exceptions may be found: for example, roots specialized for starch storage, roots with a specialized mechanical function (the tap-root in *Q. petraea*; Reubens et al., 2009), or adventitious roots originating in stems, as in the banyan, mangrove, and certain orchids. The datasets of Kalliokoski et al. (2010) and Nielsen and Hansen (2006) suggest that there can also be a large inter-species and inter-soil-type variability for γ_i .

Insofar as it describes a rate of tapering, the magnitude of γ_i may vary with root type consistently across species. For example, the magnitude may correspond to the strategy used for anchorage, water uptake, or nutrient absorption. These parameters may therefore have similar utility to topological indices, which describe the connectivity of branches along the spectrum from herringbone to

dichotomous, and the associated soil exploration and exploitation potentials (Fitter, 1987). The variability of γ_i , e.g., among species within a genus or among ages within a species, may be useful indicators of the degree to which roots are differentiated into multiple functional classes. One approach would be to assess the degree of differentiation for each root system of interest using the ratio of γ_i for a reference type (e.g., distal shallow roots) and γ_i of a given root type. By this measure, differentiation for deep roots scored 2.3 in the studied stand. Limitations with respect to inferring and comparing below-ground strategies using this approach include the fact that root taper is difficult to measure and the inconsistency of root types among species. While it appears that the γ_i of proximal structural roots is mainly associated with anchorage, and the γ_i of shallow distal roots is mainly associated with absorption, the basis of γ_i must be better understood before it can be used to infer functional significance and the degree of differentiation among root types. Also, the variability of γ_i for each root type can be better characterized. To better understand the variation in the relationship between CSD and V_d , the variability of fractal branching parameters (mainly taper between branches) in the root system, and its variation as a function of root type should be examined. Further research will be needed, spanning multiple species and wide array of substrate conditions.

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Disentangling who is who during rhizosphere acidification in root interactions: combining fluorescence with optode techniques

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Plant–soil interactions can strongly influence root growth in plants. There is now increasing evidence that root–root interactions can also influence root growth, affecting architecture and root traits such as lateral root formation. Both when species grow alone or in interaction with others, root systems are in turn affected by as well as affect rhizosphere pH. Changes in soil pH have knock-on effects on nutrient availability. A limitation until recently has been the inability to assign species identity to different roots in soil. Combining the planar optode technique with fluorescent plants enables us to distinguish between plant species grown in natural soil and in parallel study pH dynamics in a non-invasive way at the same region of interest (ROI). We measured pH in the rhizosphere of maize and bean in rhizotrons in a climate chamber, with ROIs on roots in proximity to the roots of the other species as well as not-close to the other species. We found clear dynamic changes of pH over time and differences between the two species in rhizosphere acidification. Interestingly, when roots of the two species were interacting, the degree of acidification or alkalinization compared to bulk soil was less strong than when roots were not growing in the vicinity of the other species. This cutting-edge approach can help provide a better understanding of plant–plant and plant–soil interactions.

Keywords: plant roots, interaction, green fluorescent protein, pH planar optodes, rhizotrons, rhizosphere, maize, bean

INTRODUCTION

The main root functions are to ensure both uptake of water and nutrient resources as well as provide an anchorage function for the whole plant. Moreover, Darwin (1880) considered roots to act as the plant brain integrating information from multiple sources. Despite these key functions of roots for whole plant performance, root ecophysiology and ecology have until relatively recently been a field of research weighed down by seemingly unsolvable difficulties in following root growth *in situ* in natural substrates. The soil–root–rhizosphere system has until recently been considered a black box that is hard to reach and to study (Faget et al., 2013).

Roots are continuously interacting with their environment, not only with their direct abiotic environment (as in the rhizosphere), but also interacting with biotic neighbors such as roots of neighboring plants, microbes, and soil fauna (Bonkowski et al., 2009). Alone when considering root interactions with the abiotic environment in the soil, processes occur at very variable spatial and temporal scales. Recent years have shown important breakthroughs in understanding the complex interplay of how roots both react to and affect their environment (de Kroon and Mommer, 2006; Schreiber et al., 2011; de Kroon et al., 2012; Postma and Lynch, 2012). It is well documented that plant roots are able to actively alter the biogeochemistry of their vicinity, the rhizosphere (Hiltner, 1904; Hinsinger et al., 2003, 2005, 2009). This interaction of plant roots with the soil causes a highly complex spatial and temporal pattern of micro niches that are potentially characterized

by large differences in, e.g., soil water content, soil pH, nutrient availability, microbial community structure and activity. There are several drivers for this interaction, but root foraging for the resources water and nutrients are of most importance. Foraging and uptake of nutrients can cause strong variations in soil pH. For example, during the uptake of nitrate or ammonium, plant roots release OH⁻ (hydroxyl ions) or H⁺ (protons) in order to maintain electro-neutrality across the root membrane (Marschner and Romheld, 1983; Colmer and Bloom, 1998; Hinsinger et al., 2003). On the other hand, plant roots are able to release large amounts of organic acids such as citric acid, in order to mobilize nutrients (e.g., phosphorous) when they are bound to soil particles and therefore inaccessible for direct uptake (Jones et al., 2003; Lambers et al., 2006). Both processes can create pH gradients of more than one pH unit from the root surface to the bulk soil. Additionally, when considering the dynamic growth of plant roots, it quickly becomes clear that the further elucidation of plant soil interactions is not a trivial task but very important for understanding plant performance, especially under stressful conditions. This point becomes even more pertinent, when the target plants are crops such as maize or bean and when the aim of the research is to sustain or even to improve the yield of crops in low-input agro-ecosystems.

When different species are sharing the same soil volume, they have to forage for the same essential resources that are often limiting and to explore and adapt to their environment to be able to uptake sufficient resources for maintaining their growth. Major

advances in root research both in ecology and ecophysiology have shown that roots respond both to nutrient availability (Hodge, 2004; Cahill et al., 2010) but also to the presence of different microfauna groups in the soil (Bonkowski et al., 2009) as well as the presence of a neighboring plant (Callaway et al., 2002; de Kroon and Mommer, 2006; de Kroon, 2007). Some studies suggest that both kin recognition (Dudley and File, 2007; Dudley et al., 2013) and recognition of the genetic identity of neighbors can influence the proliferation of roots and root allocation (Gagliano et al., 2012; Fang et al., 2013). Gagliano et al. (2012) found that the identity of the neighbor affected the allocation to roots and shoots, as well as affecting germination of seeds. Such studies finding communication between plants beyond direct resource-based competition have received a number of critical responses (Klemens, 2008), but the number of studies finding evidence for such communication is on the rise (de Kroon, 2007; Gagliano et al., 2012). This is clearly a research field with ample need for further studies to back-up and test theories and outcomes, and novel methods being established will no doubt provide important new insights to the issue of the question of plant interactions and whether non-resource-based competition is important compared to resource-based competition. Our posit, is that novel combinations of non-invasive methods for studying roots (Rewald et al., 2012; Faget et al., 2013) can now provide important tools to explore rhizosphere interactions with more ease and will allow important new insights. For further validation and elucidation of these topics an approach is missing which enables us to investigate and understand *in situ* rhizosphere processes of plants in more detail, either growing alone or intercropped with plants of different species.

Although studying the dynamics of root growth is still a challenge, new methods are allowing us to follow roots *in situ* (Faget et al., 2013) and even to separate the roots of different species (Faget et al., 2009; Rewald et al., 2012). One of these methods uses fluorescent roots of genetically transformed plant species using fluorescent protein (FP; green fluorescent protein, GFP; Faget et al., 2009, 2010, 2012, or red fluorescent protein, RFP; Faget et al., 2013). At the same time, other methods have been developed to study rhizosphere-scale processes, such as pH, CO₂, and O₂ concentrations with the technique of planar optodes (Blossfeld and Gansert, 2012; Blossfeld, 2013). The FP method relies on the ability of genetically transformed roots to express fluorescing proteins and thus be visible at certain excitation and emission wavelengths, whereas the optode method uses indicator dyes on the planar optodes that get excited by specific light and emit characteristic fluorescence patterns in proportion to the concentration of the measured substance, e.g., H⁺. Planar optodes provide new opportunities to study rhizosphere processes *in situ* and dynamically over time (Blossfeld et al., 2011, 2013). There are several approaches for fluorescence detection and we refer the reader to the scientific literature for detailed comparison and evaluation of the advantages and disadvantages of the different approaches (Holst and Grunwald, 2001; Stahl et al., 2006; Gansert and Blossfeld, 2008). In studies where roots of different individuals (either of the same species or of another species) are interacting, however, it is often desirable to be able to identify which root within the region of interest

(ROI) of the optode belongs to which species or genotype. For this reason, we hereby combined the GFP and planar optode methods in order to achieve the combined goal of following rhizosphere processes and being able to identify which species is which underground.

Within this context we asked:

- (1) Whether we can combine the planar optode and the FP methods to visualize rhizosphere pH changes during root–root interactions between species, using the FP method to assign species identity to roots and the optode method to measure the rhizosphere pH changes.
- (2) As a consequence we asked, whether we can localize specific rhizosphere processes and link them to specific plant species and their interactions?

We approached these questions by setting up an experiment with two plant species, maize and bean (*Zea mays* and *Phaseolus vulgaris*) with roots growing in rhizotrons either with or without close contact with roots of the other species. We measured selected ROIs within the rhizosphere of the rhizotrons using the planar optode method, and GFP maize to be able to identify which species is contributing to what extent to the specific pH measured in the intercropped rhizosphere.

MATERIALS AND METHODS

PLANT MATERIAL

The maize line ETH-M72_{GFP} expressing the GFP was grown alone or together with common bean (*P. vulgaris* "Fadenlose"). The maize genotype ETH-M72 was genetically transformed to include the gene for GFP (ETH-M72_{GFP}). The transformation construct contains the gfp gene flanked by the ubiquitin promoter (ubi::gfp) and the nopaline synthase (NOS) terminator. It was cloned into the PUC19 vector, which contains the gene for ampicillin resistance (ampR) at the restriction sites *SpeI* and *XbaI*. The gfp gene was cloned into the cassette at the NcoI and SalI sites. The expressed GFP is reported to have a fluorescence peak between 500 and 520 nm when excited by light at 450–470 nm (Faget et al., 2009, 2010, 2012).

EXPERIMENTAL CONDITIONS

Seeds of the two species were germinated on blotting paper before seedlings of comparable size were transplanted into rhizotrons. The rhizotrons had one side covered in plexiglass that is removable so that planar optodes can be installed on ROIs and roots growing on the surface are visible to the naked eye. The rhizotrons with dimensions of (400 mm × 200 mm × 20 mm) were filled with 2/3 soil (sieved with 4 mm mesh) and 1/3 sand (washed two times with deionized water). The soil and sand were mixed and each rhizotron received 1.3 l of mixed substrate. All rhizotrons were kept in a climate chamber (12 h light, 240 µmol m⁻² s⁻¹ PAR, 65% humidity, 24.5°C day, and 18.5°C night). All rhizotrons were placed at an angle of 30° from the vertical with black cover on the transparent side to prevent the roots from incident light and each rhizotron received 100 ml of 1/3 the full Hoagland's nutrient solution at the start of the experiment. The rhizotrons additionally received 30 ml of 1/3 the full Hoagland nutrient solution per day. The full Hoagland nutrient solution used contained the

following minerals: 5 mM KNO₃, 5 mM Ca(NO₃)₂, 2 mM MgSO₄, 1 mM KH₂PO₄, 0.09 mM Fe EDTA, 0.01 mM MnCl₂, 0.001 mM CuSO₄, 0.001 mM ZnSO₄, 0.05 mM H₃BO₃, and 0.0005 mM Na₂MoO₄.

The aim of this study was to for the first time combine pH measurements using planar optodes with GFP methods in roots to discriminate between roots of different species growing adjacent to one another and hence be able to follow pH dynamics of roots whose species identity we knew.

Our setup had an intercropping factor with three levels: (i) one maize individual growing together with one bean seedling, (ii) one maize individual growing together with two bean seedlings, and (iii) a control level of one maize individual growing alone (in order to visualize rhizosphere pH dynamics without close contact between roots of the two species). The limited number of bean seedlings allowed no cultivation of single grown bean plants, therefore we used ROIs of bean roots growing without neighbors within the intercropping rhizotrons. So, for example, in **Figure 3**, the pH data depict values for maize from maize growing in its own rhizotron, whereas the pH data for bean depict values from a bean root growing without close neighbors but in an intercropped rhizotron. In intercropped rhizotrons we therefore had two ROIs with planar optodes attached, corresponding to maize root next to bean, bean root growing without a neighbor.

It is important to note that the spatial scale of a planar optode ROI is very much smaller than that of the whole rhizotron, such that we considered the ROIs as replicates in most cases [e.g., see **Figure 5**; see arguments in Hurlbert (1984) on the issue of spatial scale and pseudo-replication in experiments]. Seedlings were transplanted into four rhizotrons per factor level (i.e., $n = 4$) on Day 1.

Four days after transplanting (DAT), all roots had reached half way to the bottom of the rhizotrons. Planar optodes were placed into the rhizotrons on 5 DAT. The number of optodes was limited and therefore not all rhizotron replicates could be investigated at each time point: for the evaluation of pH dynamics particular ROIs within every single optode were determined according to the following scheme: central on surface of maize/bean root, bulk soil close to maize/bean root (i.e., 6–10 mm off the root surface) and bulk soil between the roots of both species.

Additionally, it turned out that after the placement of the optodes two intercropping rhizotrons could not be included in the further analysis because the roots of maize and bean grew together too close in order to separate individual pH signals.

During the course of the experiment, some ROIs showed an unexpectedly strong pH drop which was out of the range of the calibration curve (see chapter below). This caused a reduction of number of replicates during data analysis. In particular the number of replicates changed as follows: maize $n = 4$ DAT 6–8 and 14, $n = 3$ DAT 12; bean $n = 4$ DAT 6–8, $n = 3$ DAT 12–14; bulk soil close to bean/maize $n = 4$ DAT 6–14; bulk soil between roots $n = 4$ DAT 6–8, $n = 2$ DAT 12–14. Conventional and fluorescent pictures (for FP) as well as pH measurements using the planar optodes were taken on the following days of the experiment: 6 (morning and afternoon), 7 (afternoon), 8 (afternoon), 12 (afternoon), and 14 (morning).

GFP TECHNIQUE

To identify the plant species crossing the optode region in a first step, the plant roots of maize and bean grown along the transparent plate of the rhizotrons were imaged with a conventional camera system and with an adapted lighting system-filtered camera to excite the FPs as described in Faget et al. (2009). In this paper, to adapt the previously developed method for minirhizotron to rhizotron with the transparent plate, we used a digital camera Canon, G10 mounted on a tripod. The conventional camera systems use ambient light and photograph the roots at the interface of the soil with the transparent plexiglass window of the rhizotrons. For the adaptation of this system to GFP, we mounted a filter (LONG 515 nm, Edmund Optics, Barrington, USA) in front of the camera to allow only roots expressing the GFP to be visible under excitation light (at wavelengths of 440–460 nm); further details including the components and standardized protocol are given in Faget et al. (2010).

At harvest, a closer identification was necessary to assess the root identity under the optode by removing the sensor and re-screening this area with conventional and fluorescent imaging techniques.

OPTODE TECHNIQUE

Depending on the optical setup, different spatial and temporal resolutions can be achieved. In our experimental design we used the setup as recently described in Blossfeld et al. (2013). In particular, we used a fluorescent detection system with a field of view of 15 mm × 12 mm and a pixel resolution of 12 μm . In detail, this detection system is based on a modified USB-microscope device that consists of a light-emitting diode (LED) ring (470 nm) functioning as the excitation light source, filters, lens, and the complementary metal-oxide-semiconductor (CMOS) chip. The detection system is connected via USB to a PC and powered by this connection. Thus, this system is highly flexible and even portable, when using a notebook. The RGB images [24-bit, 1280 × 1024 (1.3 megapixel)] created by this detection system contain the raw, i.e., untreated sensor response. Hence, these red, green, blue (RGB) images needed to be analyzed with an image processing software (VisiSens; PreSens GmbH, Regensburg, Germany). This software calculates the ratio of red to green in the emitted fluorescence response (so-called *R*-value) provided by the color channels of the CMOS chip. This is possible because the optodes were made of two different dyes that are either analyte-sensitive or analyte-insensitive. The intensity of the green fluorescence of the analyte-sensitive dye is driven by the analyte concentration, whereas the intensity of the red fluorescence of the analyte-insensitive dye is not. The CMOS chip captured the red and green fluorescence in one single image and therefore the created *R*-value then provided a two-dimensional quantitative map of the measured parameter, i.e., the pH.

Several optode sensor foils (size 10 mm × 20 mm, product code SF-HP5-OIW; PreSens GmbH) were fixed at the transparent front plate of the rhizotrons with plants growing in them. The positioning was done 5 DAT when the roots had reached almost the lower third of the rhizotrons. By adding the planar optodes at this time point, we ensured that the placement of the planar optode was at a ROI (size 2 cm × 1 cm). We chose our ROIs in the

following manner: we placed the optode on a zone where the tip of a growing root was just inside the area covered by the optode; this allowed for measurement of pH changes in most of the optode region without direct root contact at time point zero, as well as the dynamic measurement of pH changes as the root(s) grew through the ROI, i.e., behind the optode.

The rhizotrons were closed again after the placement of the planar optodes and first daily measurements were performed after one day of equilibration. The soil moisture and temperature was monitored in four rhizotrons via frequency domain reflectometry (FDR)-probes (Model: 5TE, Decagon Devices Inc., 2365 NE Hokins Court, Pullman WA 99163) parallel to the daily measurements and ranged between 26.6 and 36.3% (volumetric water content, VWC) as well as 24.3 and 25.1°C in the afternoon.

CALIBRATION OF PLANAR OPTODES

Prior to the start of the experiment, the optical setup together with the planar optodes was calibrated. This was achieved by using a small transparent vessel containing defined pH buffer solutions (mixture of K_2HPO_4 and KH_2PO_4 , controlled with standard pH glass electrodes) and a small replicate of the planar optode batch installed on the inside of this vessel.

The average R -value (R_m) of this replicate for each given pH was recorded and used as input parameter for a fitting function. The relationship between R_m and the given pH can be described by a sigmoidal Boltzmann equation (Eq. 1). This equation was adapted from (Blossfeld and Gansert, 2007) by exchanging the parameter Φ with the parameter R . This equation can be transformed in order to calculate the pH from the measured R -value during the experiment (Eq. 2). This equation was also adapted from Blossfeld and Gansert (2007) by exchanging the parameter Φ with the parameter R .

$$R_m = \frac{R_{\min} - R_{\max}}{\{1 + \text{Exp}[(pH_m - pH_0) dpH]\}} + R_{\max}, \quad (1)$$

$$pH_m = pH_0 + dpH \times \ln \left[\frac{(R_{\min} - R_{\max})}{(R_m - R_{\max}) - 1} \right], \quad (2)$$

where R_m is the calculated/measured R -value, R_{\min} and R_{\max} represent the upper and lower range of the fitting; pH_0 is the inflection point and dpH the slope of the fitted curve. The Boltzmann fit clearly demonstrates that the sensitivity of the sensor was highest between pH 6 and pH 7 and lowest below pH 5 and above pH 8 (Figure 1).

RESULTS AND DISCUSSION

Figure 2 shows photographs through the transparent window of the rhizotrons of maize roots on the left side (**Figures 2A,B**) and bean roots on the right side (**Figures 2C,D**) growing alone with no neighbors in the proximal rhizosphere. The upper row (**Figures 2A,C**) was taken before harvest showing the position of the planar optodes on the root systems through the window interface. Just before harvest, the planar optodes were taken away to identify and measure the exact location of the roots behind the optode sensors (**Figures 2B,D**).

The pH monitoring via the optodes revealed that the investigated species modified their rhizosphere pH creating very distinctive patterns; **Figure 3** shows the evolution of pH measured by

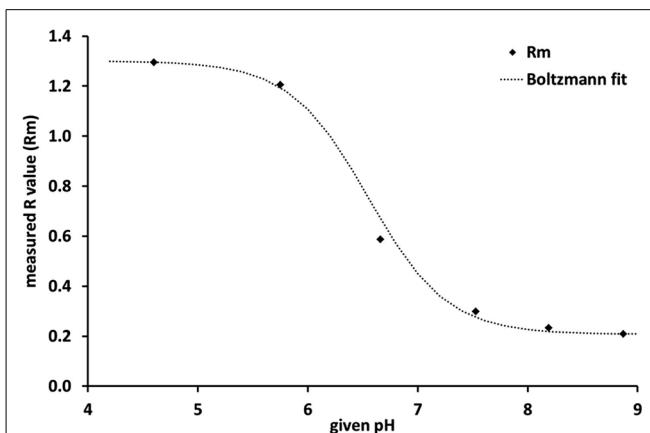


FIGURE 1 | Calibration curve of the planar optodes, where R_m is the measured R -value, i.e., the ratio of red to green in the emitted fluorescence response. The steep slope of the Boltzmann fit between pH 6 and pH 7 indicates that the sensor is most sensitive within this pH range.

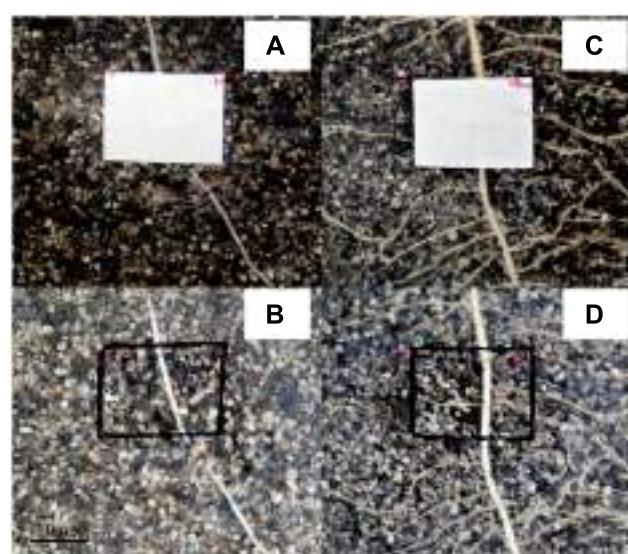


FIGURE 2 | Photographs of the experimental setup as seen through the transparent window of the rhizotrons with and without optodes installed. The pictures show roots growing with no neighbor of another species nearby. Panels (A,B) are photographs of maize roots while (C,D) are of bean roots. Panels (A,C) were shot at the time of destructive harvesting which corresponded to DAT 14 (DAT, days after transplanting) and we can clearly see the roots crossing the planar optodes. The optodes where removed as seen in (B,D) to precisely locate the root trajectories under the sensors. The scale is given by the optodes which measure 10 mm × 20 mm regions of interest (ROIs).

the planar optodes over time. We found clear dynamic changes of pH over time and differences between the two species in rhizosphere acidification both when roots grew alone and in interaction between the species.

Initially, the roots of maize growing alone acidified the rhizosphere on average by 0.75 pH units compared to the bulk soil pH (**Figure 3A**). This rhizosphere acidification was not constant over time, but changed instead to a net alkalization of up to 0.62 pH

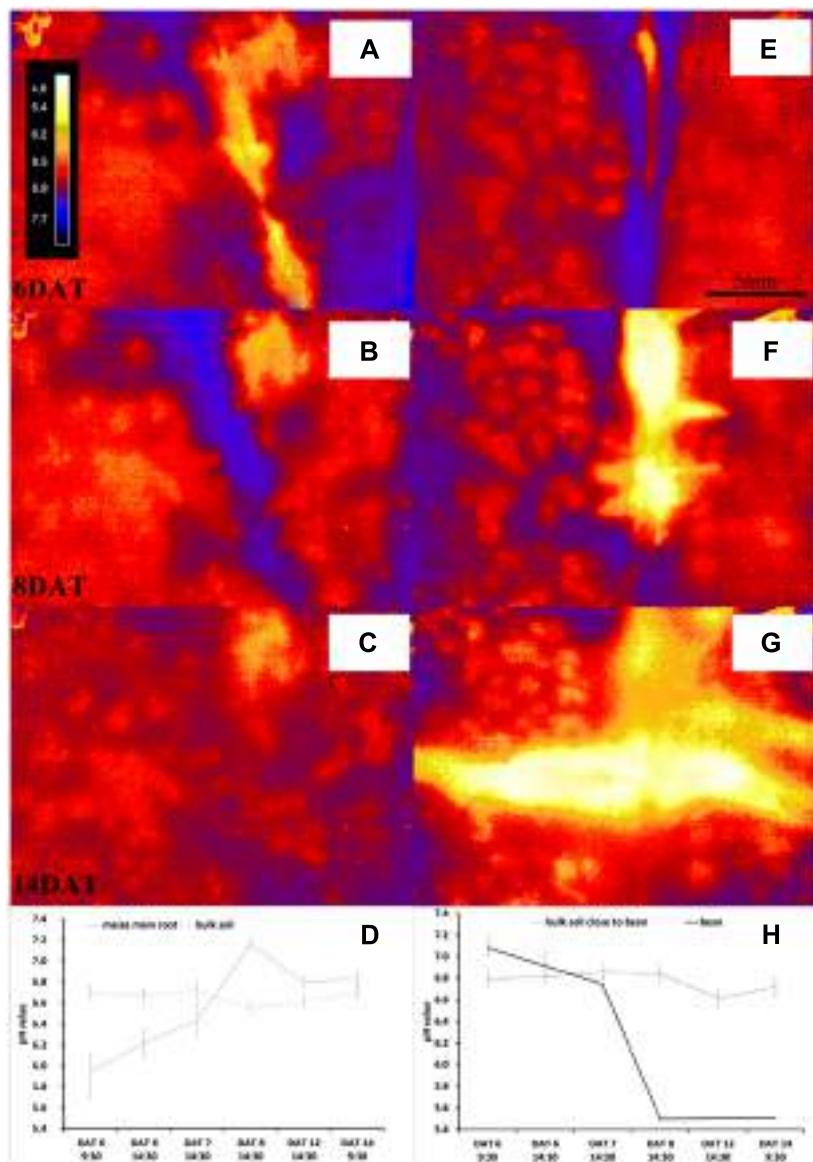


FIGURE 3 | Dynamics over time of pH measured with the optodes for the rhizosphere and bulk soil of roots of maize (A–D) or bean (E–H) growing alone. Panels (A–C,E–G) show the pH maps of the respective ROIs at a scale ranging from 4.6 to 7.7 pH units at DAT 6 (A,E), DAT 8

(B,F), and DAT 14 (C,G), respectively; the scale here is 20 mm × 10 mm. Panels (D,H) show the evolution of the mean pH value (\pm SD) of all pixel within the ROI at the root surface of maize (D) and bean (H) over time growing in separate rhizotrons.

units on 8 DAT (Figure 3B). In the later phase of the experiment the rhizosphere pH came closer to the bulk soil pH, which varied between pH 6.55 and pH 6.72 (Figure 3C).

Interestingly, the single grown bean roots showed the opposite behavior (Figures 3D,H). The rhizosphere of this young bean roots was 0.29 pH units higher than the initial bulk soil pH of 6.79 (Figure 3E). However, from 8 DAT onward, the bean roots acidified the rhizosphere in such a strong manner that the sensor signal was below pH 5.5 (Figure 3F). The young lateral roots of bean acidified the rhizosphere right from their emergence onward and it cannot be excluded that some of the acidic molecules diffused along the lateral roots to the main roots (Figure 3G). It should also

be noted that the bean roots formed no nodules during the course of the experiment. Since both species were grown in the same substrate and all rhizotrons received the same watering regime with the same nutrient solution, this contrasting pattern is very interesting. Since the only source of nitrogen in the rhizosphere of all plants was derived from the nitrate of the nutrient solution, we expected that an uptake of this nitrate would cause an alkalization of the rhizosphere (Marschner and Romheld, 1983; Colmer and Bloom, 1998; Cousins and Bloom, 2003). This was what we found around the maize roots, but not the bean roots, although we cannot confirm with our study that this is the mechanism behind the pH patterns we found. The bean rhizosphere pH response is

difficult to interpret given that there were no nodules on the roots and hence no sign of N₂-fixation occurring, which would have potentially explained the acidification over time as protons are released during fixation (Bolan et al., 1991). Another explanation could be the species-specific ability to mobilize phosphorous (P) in the rhizosphere. It has been reported that under P-limitation but high nitrate content non-nodulated roots of faba bean heavily acidified the rhizosphere, whereas maize roots alkalinized their rhizosphere when growing under the same conditions (Li et al., 2007). However, we have not measured the P-content of the plants and the soil after the experiment in order to verify this explanation. Thus, the patterns found now need further testing with more replication, further soil, and plant analysis and with a variety of species in addition to maize and bean.

Figure 4 shows the evolution of pH over time when roots of both species grew within the proximity of the other. **Figures 4A–C** show the variations in acidification and alkalinization of the rhizosphere at 6, 8, and 14 DAT, respectively. **Figures 4D,E** allow us to identify which roots belonged to which species (using the GFP method and conventional photography): in interaction, we see an acidification, then alkalinization followed by an acidification of the maize root, with a clear acidification of the bean root over time (**Figures 4A–C**). Overall (see **Figure 5** for more detailed views of the pH changes) we found that the pattern of rhizosphere acidification over time was similar to that found when the roots of one

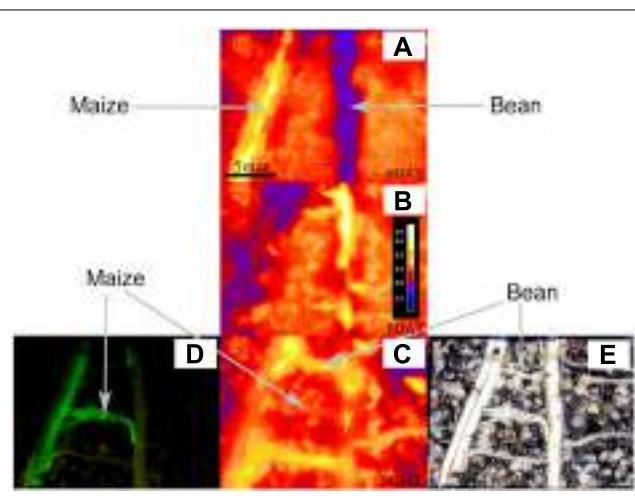


FIGURE 4 | This figure shows the potential of combining fluorescence (GFP) with optode pH methods, illustrating what each method can contribute to understanding who is who during rhizosphere pH changes. The figure shows the ROIs for the rhizosphere and the bulk soil of roots of maize and bean growing in close proximity to each other. Panels (A–C) show the pH maps of pH measured with the optodes for the rhizosphere and bulk soil of roots of maize and bean growing in close proximity: at a scale ranging from 4.6 to 7.7 pH units at DAT 6 (A), DAT 8 (B), and DAT 14 at the end of the experiment (C). Panels (D,E) show photographs of these same ROIs taken on DAT 14 at harvesting after having removed the optode for locating and identifying roots. Panel (D) was photographed under blue light to excite the maize expressing the GFP, allowing the identification and exact location of the maize roots (the roots tips and meristematic areas are even brighter than the remaining tissue). Panel (E) shows a conventional photograph that is complementary to (D) shot in conventional light, where all the roots form maize and bean are visible.

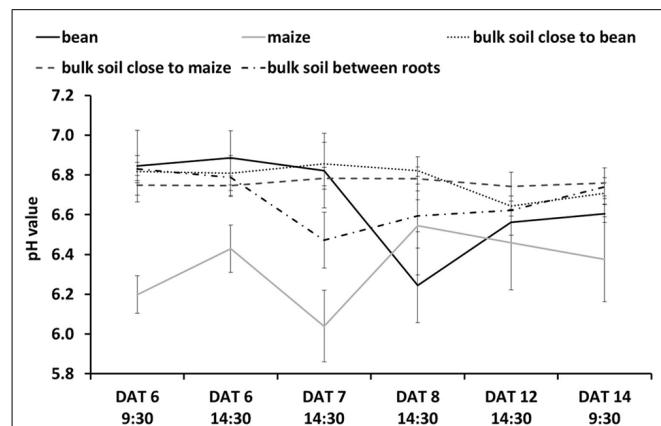


FIGURE 5 | pH Dynamics over time as related to different ROIs either in the rhizosphere of maize or bean (i.e., positioned centrally on the individual root), in the bulk soil close to maize or bean roots (i.e., positioned 6–10 mm away from the individual root), or in the bulk soil between maize and bean roots (i.e., positioned centrally between roots with >6 mm distance between them). Values are means and standard errors of the mean of all pixels (approx. 2500–3500 pixels for the rhizosphere and 10000–15000 pixels for bulk soil) in the individual ROI. Note that these mean values are derived solely from intercropping rhizotrons, such that they are composed of values from rhizotrons with maize intercropped with one or with two bean individuals. For maize $n = 4$ DAT 6–8 and 14, $n = 3$ DAT 12; bean $n = 4$ DAT 6–8, $n = 3$ DAT 12–14; bulk soil close to bean/maize $n = 4$ DAT 6–14; bulk soil between roots $n = 4$ DAT 6–8, $n = 2$ DAT 12–14.

species were not in the proximity to the other, but the intensity of the pH changes was about 0.6 pH units lower.

We cannot yet explain why we found a less strong change in pH compared to bulk soil (**Figure 5**) when roots of the two species were directly interacting. Further studies should help identify whether this was due to the species interactions and some kind of plant–plant communication or other more resource-based competitive outcomes (see Faget et al., 2013 for discussion of this topic).

Without the GFP method it would have been impossible to distinguish by eye, which root belonged to which species and thus which pH activity could be assigned to the maize or to the bean root zones (as is the case in **Figures 2** and **3**). At harvest time (14 DAT) the optode was removed and the roots were imaged (**Figure 4E**). This conventional photograph is helpful to visualize the location of different roots behind the optodes but alone does not allow one to identify to which species they belong. By using the GFP method, as in this case maize roots expressing the GFP, it was possible to separate maize from bean roots and to then compare pH dynamics in specific ROIs.

Figure 4D clearly shows the maize roots in fluorescent green, differing from the bean roots in pale color or even not visible on the GFP-image but only on the conventional image. Here we can see that some of the lateral roots belonged to maize and some to bean, which would not have been visible to the naked eye. This then explains why not all visible lateral roots acidified their rhizosphere and why the acidification of the upper and lower lateral roots is not as prominent as in the single root observations (**Figure 5**).

In **Figure 4D** one can clearly see that only the acidifying roots belong to the bean plant and the central lateral roots belong to the

maize plant. Hence, by combining the GFP method with the planar optode methods, it is now possible to follow the pH variation of the rhizosphere during plant–plant interactions and precisely indicate which species had what kind of influence on the rhizosphere properties, even if the mechanism behind the patterns requires further complementary studies.

Combining these methods should also allow one to compare the integrated effect of roots growing alone or with neighbors on rhizosphere pH (or O₂ or CO₂) with outcomes when roots are interacting directly. For example in our study, we found that the modification of the rhizosphere pH when roots of two species were directly interacting was similar to the roots growing alone (**Figure 5**). The maize still tended to alkalinize the rhizosphere and the bean still acidified it, but the intensity of the modification by the roots of both species was reduced.

We also found that the pH of the bulk soil in proximity of either the maize roots or bean roots did not show strong variation while the pH of the bulk soil in between the two roots systems suggests it may be an averaging of the pH values for roots growing alone.

Our approach has the potential to prove very useful in so-called *guided sampling*. High-throughput phenotyping of plant traits is currently a burgeoning field in plant sciences (Rascher et al., 2011; Nagel et al., 2012; Fiorani and Schurr, 2013), and allows for large screening of many genotypes and species. At times, high-throughput phenotyping can benefit greatly from more detailed lower-throughput methods such as the described planar optode method for studying processes in the rhizosphere at particular points in space or time deemed particularly interesting. The planar optode method can report differences in rhizosphere (metabolic) activity of different roots, including hotspots of root activity in the main or lateral roots at different times. Information derived from the optodes and the GFP-images could then be used directly for guided sampling of specific root/rhizosphere sections for analysis of compounds, enzymes, microbial communities, etc. One of the main limitations would come from the need to use genetic modified plant material. This is a pre-condition in order to be able to distinguish roots from different species. GFP-transformed

Arabidopsis thaliana is readily available, whereas it is not available yet for many other plant species since transformation involves a considerable amount of work.

Another area of research where we deem that the application of these two methods may be very promising is in plant–plant interaction studies in ecology and ecophysiology. In these research fields, a range of different theories to explain patterns found in nature are being tested based on both resource-based and non-resource-based competition, novel communication pathways between plants (Zavala and Baldwin, 2006; Gagliano et al., 2012), as well as considering the role of positive interactions between plants as well as competition (Temperton et al., 2007; Brooker et al., 2008).

Not only GFP is available but different colors have now been made available in a number of mainly agriculturally interesting species which will make possible for us to be able to distinguish and study root–root interactions within populations as well as communities in the longer run. For example, maize expressing the GFP was combined with wheat expressing the RFP and rape-seed as wild type in Faget et al. (2013). At the same time, planar optodes can measure not only pH but also CO₂, O₂, and ammonium (Stromberg, 2008) and the size of the optodes available for research is increasing such that whole rhizotrons can soon follow plant–soil dynamics over time. This combination of novel methods for studying root biology and ecology should pave the way to an improved understanding of both root–soil and root–root interactions.

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Better to light a candle than curse the darkness: illuminating spatial localization and temporal dynamics of rapid microbial growth in the rhizosphere

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The rhizosphere is a hotbed of microbial activity in ecosystems, fueled by carbon compounds from plant roots. Basic questions about the location and dynamics of plant-spurred microbial growth in the rhizosphere are difficult to answer with standard, destructive soil assays mixing a multitude of microbe-scale microenvironments in a single, often sieved, sample. Soil microbial biosensors designed with the *luxCDABE* reporter genes fused to a promoter of interest enable continuous imaging of the microbial perception of (and response to) environmental conditions in soil. We used the common soil bacterium *Pseudomonas putida* KT2440 as host to plasmid pZKH2 containing a fusion between the strong constitutive promoter *nptII* and *luxCDABE* (coding for light-emitting proteins) from *Vibrio fischeri*. Experiments in liquid media demonstrated that high light production by KT2440/pZKH2 was associated with rapid microbial growth supported by high carbon availability. We applied the biosensors in microcosms filled with non-sterile soil in which corn (*Zea mays* L.), black poplar (*Populus nigra* L.), or tomato (*Solanum lycopersicum* L.) was growing. We detected minimal light production from microbiosensors in the bulk soil, but biosensors reported continuously from around roots for as long as six days. For corn, peaks of luminescence were detected 1–4 and 20–35 mm along the root axis behind growing root tips, with the location of maximum light production moving farther back from the tip as root growth rate increased. For poplar, luminescence around mature roots increased and decreased on a coordinated diel rhythm, but was not bright near root tips. For tomato, luminescence was dynamic, but did not exhibit a diel rhythm, appearing in acropetal waves along roots. KT2440/pZKH2 revealed that root tips are not always the only, or even the dominant, hotspots for rhizosphere microbial growth, and carbon availability is highly variable in space and time around roots.

Keywords: rhizosphere, microbiosensor, *lux*, roots, *Pseudomonas*, *Zea mays*, *Solanum lycopersicum*, *Populus nigra*

INTRODUCTION

Most terrestrial plants grow in environments where restricted quantities of water or mineral nutrients (e.g., nitrogen, phosphorous) limit plant growth. Plants invest a significant amount of fixed carbon into root tissue and rhizodeposition to acquire these limiting resources. As roots grow, they release carbon, in the process stimulating the growth and activities of surrounding microbial community (Wardle, 1992; Cheng et al., 1996). van Veen et al. (1991) estimate, for example, that for every 10 grams of carbon assimilated by a plant, an estimated 4 grams are contributed to the soil as rhizodeposition. These rhizodeposits provide energy supporting growth and activity of microbes in the rhizosphere (Lynch and Whipps, 1990). Microbial growth and activity, in turn, affect nutrient availability to plants, via immobilization of nutrients into microbial biomass, release of mineral nitrogen during decomposition of organic matter, or via

a soil “microbial loop” in which protozoa grazing on rhizosphere microbes release waste ammonium (Helal and Sauerbeck, 1983; Clarholm, 1985; Bottner et al., 1988; Dormaar, 1990; Wheatley et al., 1990; Darrah, 1991; Haider et al., 1991; De Nobili et al., 2001; Kuzyakov, 2002; Cardon and Gage, 2006; Jones et al., 2009; Kuzyakov and Xu, 2013).

Basic questions about plant-spurred microbial growth and activity in the rhizosphere are difficult to answer with standard, destructive soil assays that mix a multitude of microbe-scale microenvironments in a single, often sieved, sample. Is the energy contribution from roots to microbes a one-time occurrence as the root tip passes by in the soil, or do plants continue to release carbon at the same location again and again? Do known shoot-root carbon allocation patterns in various plant species translate to similar temporal (or spatial) patterns of carbon availability to free-living rhizosphere microbes?

Living soil microbial biosensors, engineered to “report” conditions in their local microenvironment (and/or their response to those conditions), offer the possibility of gathering the continuous *in situ* information necessary to begin answering such questions.

Microbial biosensors consist of a host strain (usually bacterial) that contains inserted DNA (on the chromosome or a plasmid), coding for an environmentally controlled promoter, driving expression of an easily assayed reporter gene (e.g., *inaZ*, *gfp*, *lux*) (Hansen and Sørensen, 2001; Gage et al., 2008). The expression of the reporter molecule is thus tied to the activity of the promoter within the host organism. The ability to choose a promoter that scales with a metabolic process or is activated by a specific compound in the environment contributes to the great flexibility of biosensors. Investigators have made use of this flexibility to investigate pollutants such as naphthalene (King et al., 1990) and PCB's (Boldt et al., 2004), water potential around plant roots (Herron et al., 2010), as well as more common soil and root-derived compounds such as sucrose and tryptophan (Jaeger et al., 1999), nitrate (DeAngelis et al., 2005), and galactoside sugars (Bringhurst et al., 2001). Common reporter systems include a number that require destructive harvest for measure (*lacZ*, *phoA*, and *inaZ*) as well as a number that yield a visible readout (e.g., *gfp*, *lux*). The value of microbial biosensors as measurement devices is tied to the great numbers that can be applied to a system, design flexibility, the sensitivity of microbes to low activities of inducing signal (Hansen and Sørensen, 2001) and the specificity of the biosensor to “bioavailable” forms of that signal.

We used the common soil bacterium *Pseudomonas putida* KT2440 to host plasmid pZKH2, which contains a fusion between the neomycin phosphotransferase II (*nptII*) promoter, cloned from Tn5 (Rothstein et al., 1980; Axtell and Beattie, 2002; Herron et al., 2010), and *lux* reporter genes cloned from the marine bacterium *Vibrio fisheri*. Bioluminescence, while quite common in marine bacterial species, is very rare in terrestrial organisms (Stewart and Williams, 1992). The application of marine *lux* genes under control of known promoters and incorporated into terrestrial bacteria offers the opportunity to track luminescence from these specific bacteria in a dark soil system. The *nptII* promoter in plasmid pZKH2 is constitutive, in the transposon Tn5 it drives the expression of genes that confer antibiotic resistance, and *nptII* has been shown to function in a large number of bacterial species (Labes et al., 1990; Joyner and Lindow, 2000; Wright and Beattie, 2004) isolated from multiple environments. In *Pseudomonas* species, *PnptII* has been reported to drive transcription at moderate levels under a variety of conditions (Axtell and Beattie, 2002; Wright and Beattie, 2004; Goymer et al., 2006; Herron et al., 2010; Park et al., 2010).

Initial results showed *P. putida* KT2440 carrying the pZKH2 plasmid (KT2440/pZKH2) produces light only when growing rapidly. Luminescence does not indicate the presence/absence of particular compounds; instead, luminescence signals the integrated microbial perception of the local environment, indicating sufficient energy and substrates are available to support microbial growth and light production as well.

We characterized the behavior of the light emission response from the biosensors using traditional growth curve studies, pulsed carbon availability experiments, and substrate amendments into soil. The biosensor was then applied into plant-soil microcosms planted with species known from the literature to have distinct internal vascular architectures and/or root and carbon allocation patterns. Corn (*Zea mays* L.) is well-known to elongate rapidly in soil and produce abundant mucilage and other rhizodeposits at the growing root tip (see McCully, 1999). We explored spatial and temporal patterns in KT2240/pZKH2 luminescence near corn root tips growing at different rates. Members of the genus *Populus* are known to store newly-fixed carbon in starch during the day, then break it down end of day for shipment to roots, producing a strong diel oscillation in belowground carbon allocation (Dickson, 1991). We explored whether *Populus nigra* L. stimulated rhythmic luminescence from KT2240/pZKH2 over several diel cycles. Finally, tomato (*Solanum lycopersicum* L.) is known to have highly modular internal vascular architecture (e.g., Zanne et al., 2006), with root-shoot physiological units operating relatively independently. We explored whether biosensor luminescence exhibited coordinated temporal or spatial patterns among roots, or through time. Rather than using a biosensor to detect the presence of a pre-determined substrate (e.g., galactosides, Bringhurst et al., 2001) or condition (e.g., water potential, Herron et al., 2010), the KT2240/pZKH2 biosensor design has the advantage of reporting periods of rapid growth on any substrate the microbe can use (exudates, secretions, rhizodeposited cap cells), without requiring identification or quantification of particular substrate components.

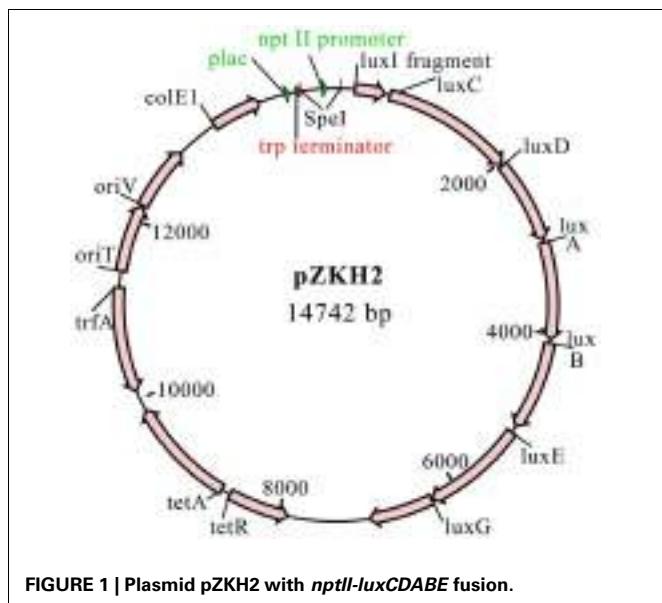
METHODS

PLASMID CONSTRUCTION

E. coli strains XL1Blue MRF' (Stratagene) and XL1Blue MRF' Km were used as hosts for all plasmids. The strains were grown in LB broth or plates with antibiotics as needed (tetracycline 10 µg ml⁻¹; ampicillin 100 µg ml⁻¹; kanamycin 25 µg ml⁻¹). All electroporations were at 1.8 kV. Plasmids were isolated from LB cultures using a Qiagen Miniprep kit (Qiagen).

Joerg Graf (University of Connecticut) generously provided the plasmid pLM2819 (Stewart and McCarter, 2003) containing the full *luxCDABE* cassette cloned from *Vibrio fisheri*. Initially, the *lux* operon was cut from pLM2819 with KpnI and cloned into a KpnI-cut pBluescriptSK(–), resulting in pDG115. A *trp* terminator was cloned into the XbaI site of pDG115, upstream of the *lux* operon, to make pDG117.

A promoterless-*lux* construct, pCAP40, was created by excising the *trp* terminator-*lux* fragment from pDG117 as a KpnI-XbaI fragment and ligating it into plasmid pCM62 (Marx and Lidstrom, 2001). The *nptII* promoter for the *nptII-lux* construct was amplified from Tn5 by PCR using the primers 5' GGACTAGTGTCAAGGCTGTTACAGCTC 3' and 5' CTACTAGTTCATGCGAACGATCCTC 3'. These primers include SpeI restriction sites (underlined). The *nptII* fragment was cloned into pGEM T-Easy (Promega). This plasmid was digested with SpeI and the *nptII* fragment was ligated to SpeI-cut, dephosphorylated pCAP40. The resulting plasmid was called pZKH2 (Figure 1).



BACTERIAL STRAINS, GROWTH MEDIA AND CHEMICALS

Both plasmids pZKH2 and pCAP40 were moved by triparental mating into *P. putida* KT2440 using plasmid pRK600 as the helper plasmid. In preparation for broth and soil-based experiments, *P. putida* KT2440/pZKH2 and *P. putida* KT2440/pCAP40 were streaked on LB Tetracycline ($10 \mu\text{g ml}^{-1}$) plates and grown at 30°C .

For broth experiments, bacteria were grown in M9 minimal medium (Sambrook et al., 1989) amended with citrate, succinate, or glucose (0.15–0.4% w/v). All cultures were amended with tetracycline at $10 \mu\text{g ml}^{-1}$ unless noted otherwise. Bacterial strains were inoculated into 2.5 ml of medium in 18×150 -mm tubes and grown at 30°C with constant shaking (120 rpm). In cases when bacteria were needed for testing in peristaltic experiments and soil chambers, a single colony was inoculated into 20 ml of M9 medium with tetracycline and glucose (0.15–0.4% w/v) and grown in 250 ml Erlenmyer flasks at 30°C with constant shaking (120 rpm). Optical density values are reported for cultures measured in 96-well or 48-well plates. For comparison with optical densities measured in a standard cuvette with a 1 cm pathlength, optical densities from these well plates should be scaled with a multiplier dependent on path length. For example, the path length of the medium is approximately 1/3 of a cm for 100 μl inoculations in 96-well plates. Optical densities should be multiplied by 3 in order to compare 96-well plate data to cultures measured in a standard cuvette with a 1 cm pathlength.

PLATE READER EXPERIMENTS

Influence of growth phase on light production

KT2440/pZKH2 and KT2440/pCAP40 were grown as described above until mid-exponential phase in M9 medium, then centrifuged, washed in M9 medium three times, and re-suspended to an optical density of 0.1 OD₅₉₅. Bacteria were inoculated in triplicate, to 0.005 OD₅₉₅, into the wells of a Falcon 48-well plate

(BD Biosciences, Franklin Lakes, NJ) filled with 200 μL of M9 amended with tetracycline. For KT2440/pZKH2, triplicate wells were amended with citrate (0.1, 0.2, 0.4, 0.6% w/v) or with succinate (0.4%, 0.6%). For KT2440/pCAP40, only citrate (0.1, 0.2, 0.4, 0.6% w/v) was tested. Bacteria were grown at 30°C and shaken every 5 min in a plate reader (Synergy HT Multi-Detection Microplate Reader, Bioteck). Optical density was measured every 5 min at 595 nm and luminescence was read every 30 min from the bottom of the plate with sensitivity set at 125, until stationary phase was reached. The software packages used to alternate optical density and luminescence measurements were Automate 4 (Network Automation, Los Angeles) in conjunction with KC4 (Bioteck, Winooski, VT). Specific luminescence was calculated as the light emission measured by the Synergy plate reader in each well divided by the optical density of the suspension of bacteria in that well.

Influence of carbon, M9, and oxygen availability on light production

In 48-well plate experiments, a sharp decline in light production was consistently observed in late exponential growth. Normalized light production dropped to zero in stationary phase. We tested whether oxygen, mineral nutrient, or carbon (energy) limitations were the cause of the decline.

Six independent cultures of KT2440/pZKH2 were grown as inoculant, three in M9 amended with citrate (0.15% w/v), and three in M9 amended with glucose (0.15% w/v), all with tetracycline ($10 \mu\text{g ml}^{-1}$). At mid-exponential growth, cells were spun down, washed in M9 medium three times, then each independent culture was re-suspended to an optical density of 0.1 in M9 minimal medium (no carbon). Ten microliters (10 μl) of bacterial suspensions were inoculated into 190 μl of M9 medium amended with glucose (0.1% w/v) or citrate (0.1% w/v), in a 48 well plate.

Three hundred and eighty minutes after dilution into fresh medium, M9+glucose cultures were in late exponential growth (and specific luminescence had just begun to decline); M9+citrate cultures were in stationary phase and luminescence had declined to zero. The 48-well plate was removed from the plate reader. Triplicate wells were assigned to oxygen, mineral nutrient, carbon and control treatments. A pipette was used to bubble air in the wells that received an oxygen amendment. Carbon amendment wells received a boost of 0.3% carbon source of the same type they had been growing in (3 μl addition of 20% w/v citrate or glucose). Mineral nutrient wells received a 50% increase in M9 nutrients (10 μl addition of $10 \times$ M9 salts). Control wells received 3 μl of distilled and deionized water. The plate was returned to the plate reader and OD₅₉₅ and luminescence tracked for another 600 min.

EXPERIMENTS ON FILTER DISCS

Dynamic response to pulsed carbon availability

KT2440/pZKH2 was grown in 20 ml of M9 amended with tetracycline ($10 \mu\text{g ml}^{-1}$) and glucose (0.15% w/v). This low concentration of glucose was used to minimize the amount of energy stored within the bacteria as polyhydroxyalkanoates (PHAs) prior to the experiment (Huijberts et al., 1992). After reaching stationary phase (~ 0.08 OD) the bacteria were spun down, washed three times and resuspended in M9 to a concentration of 0.005 OD in

a flask with 20 ml of M9 minimal medium without tetracycline or carbon. The culture was incubated for another 24 h to help exhaust energy supplies. Bacteria were re-suspended to an optical density of 0.0001 for the experiment.

2.5 ml of the bacterial suspension was filtered onto each of five disposable 26-mm 0.45-micron syringe filters (Corning Cat. 431220, Fisher Scientific). One control filter was not inoculated with bacteria to serve as a blank. All filters were flushed with 30 ml of M9 (no carbon) to remove any contaminants that could serve as a carbon source. All filters were arranged on a board with individual pieces of Tygon tubing fitted to the front and rear of the filters. The board with filters was placed inside a light tight box at a distance of 15 cm in front of a Princeton Instruments Versarray CCD camera. This camera contained a 1024×1024 back-thinned chip cooled to -70°C (Acton PI 1 kb Versarray Cooled Camera, Princeton Instruments, Trenton, NJ) fitted with a 25 mm (0.95 na) lens (Universe Kogaku, Oyster Bay, NY). The tubes leading into each of the filters connected to lines on a multi-channel peristaltic pump (Zellweger Analytics, Suffolk, GB) that delivered a steady flow of M9 solution at a rate of 0.5 ml min^{-1} (no carbon added, no tetracycline added). Filters + bacteria equilibrated for 12 h.

The filters that had received bacteria were broken into two treatments: Treatment 1 (2 replicates), bacteria received a constant flow of M9 for the duration of the experiment; Treatment 2 (3 replicates), bacteria received a flow of M9, followed by a pulse of M9 amended with glucose (0.1%) for 180 min, followed by a return to M9.

Images were captured with the Versarray CCD camera every 5 min for the duration of the experiment and pixels were binned 10×10 . A background image was obtained prior to the experiment and subtracted from each image. The camera was controlled using WinView/32 software (Princeton Instruments). Total light emitted for each filter disc was analyzed for each image using a macro written for ImageJ v 1.38 (Rasband, 1997–2007).

EXPERIMENTS IN SOIL – NO PLANTS

Soil used in both soil experiments without plants was a 1:1 mix of sand and Connecticut loam obtained from the Ecology and Evolutionary Biology Greenhouses at the University of Connecticut, Storrs and sieved to 2 mm.

Detection of biosensor luminescence in soil supported by distinct carbon substrates

We tested whether we could detect luminescence from the biosensor inoculated into soil with three different labile, low molecular weight carbon substrates. KT2440/pZKH2 was grown over 48 h in M9 and tetracycline ($10 \mu\text{g ml}^{-1}$) amended with 0.4% citrate, 0.4% glucose, or 0.4% acetate (w/v) at 30°C . The cultures were then spun down, washed three times with M9, and re-suspended to three optical densities (0.02, 0.04, 0.08) in each of the three media treatments. The citrate-amended M9 culture was also used as the source of bacteria for a fourth treatment—M9 minimal medium with no added carbon. Twenty-four Eppendorf tubes were each filled with $200 \pm 10 \text{ mg}$ of soil. For each of two replicates of each M9+carbon combination and optical density, 40 μl of bacterial suspension was added to the soil in the Eppendorf

tube. The soil was then mixed with a dissecting needle and vortexed to achieve an even mixture of cell suspension and soil. Soils were then deposited into the wells of a 96-well plate. A glass cover slip, treated with Sea-Drops Anti Fog solution (McNutt Corp., Bellingham, WA) was placed over the wells. The plate was placed in a dark box 15 cm in front of a CCD camera (Retiga EX CCD camera, 12 bit 1360×1360 pixels, QImaging, Burnaby, BC) with a Navitar TV Zoom 7000 lens. Sixteen minute exposures were captured at 3×3 binning every 40 min, for 18 h. The camera was controlled by Openlab software (Improvision) on a Macintosh G4. Images were analyzed using NIH Image v 1.37.

Response of biosensor luminescence in soil to repeated pulses of carbon and mineral nutrients

Building on the plate reader experiment (detailed above) testing the influence of carbon and M9 on light production, biosensors were inoculated into soil and luminescence monitored during repeated pulses of added carbon and various mineral nutrients.

Bacteria were grown in M9 minimal medium amended with glucose (0.4% w/v) and tetracycline ($10 \mu\text{g ml}^{-1}$) into stationary phase. Bacteria were spun down and washed two times before being re-suspended in distilled water to an optical density of 0.08. The 1:1 sand and loam mix was packed into a 20 cm (wide) \times 27.5 cm (tall) \times 2.5 cm (thick) container. The surface of the soil was sprayed with 2 ml of KT2440/pZKH2 suspended in distilled water using a 25 ml reagent sprayer (Kontes, Vineland, NJ USA), then covered with a 20×27.5 cm sheet of borosilicate glass for 24 h. Following the 24 h, twenty-two 13-mm nylon filter discs (Millipore, Boston, MA) were attached to the soil surface using stainless steel pins. The discs served as the vehicle for delivering nutrients to soil bacteria underneath them.

Seven treatments (six replicated three times and one treatment, #7, below, replicated four times) were applied in a random pattern among the 22 filter discs. All pre-treatments were pipetted onto the filter discs in 20 μl volumes and left for 48 h; mineral nutrients were added at the same concentrations as found in M9 minimal medium. The seven pre-treatments were:

- (1) Carbon (as glucose $\text{C}_6\text{H}_{12}\text{O}_6$, 0.4% w/v)
- (2) Nitrogen (as NH_4Cl)
- (3) Sulfate (as MgSO_4)
- (4) Phosphorous (as a mix of Na_2HPO_4 and NaH_2PO_4 to yield pH of 6.2)
- (5) Nitrogen, sulfate, phosphorous (as above)
- (6) Phosphorous, sulfate, carbon (as above)
- (7) M9 + Carbon (M9 with glucose 0.4% w/v)

Following the 48 h initial exposure to these pre-treatments, each filter disc received a daily aliquot of nitrogen, phosphorous, carbon or water. We used the PI Versarray camera to capture images of the soil with discs at 115 min exposures with 2×2 binning, over 100 h.

EXPERIMENTS IN SOIL – SOIL MICROCOSMS WITH PLANTS

Soil was prepared by using a 1:1:1 mix of loam, sand, and peat obtained from the Ecology and Evolutionary Biology Greenhouses at the University of Connecticut, Storrs. Soil was

not sterilized, and was packed into tall, thin microcosms measuring 1 cm (thick, from glass to back) \times 22 cm (wide) \times 27 cm (tall). Soil in the chamber was brought to approximately 20% soil moisture and lightly fertilized with Peters Professional 20-20-20 fertilizer.

Seeds of four species were used in experiments: *Zea mays* L. (sweet corn) (Kandy Korn "EH," The Page Seed Company, Greene, NY), *Solanum lycopersicum* L. (tomato) (Celebrity F1, Johnny's Selected Seeds, Winslow, ME), *Capsicum annuum* L. (green pepper) (Yolo, B + T World Seeds, Aigues-Vives, France), and *Artemesia tridentata* var. *vaseyana* (mountain sagebrush, grown from seed gathered in northern Utah, USA). Cuttings of poplar (*Populus nigra* L.) were generously provided by Dr. Rachel Spicer, Harvard University.

Seeds were surface sterilized by soaking in 50% ethanol (1 min) followed by 10% bleach (5 min), then rinsing twice in sterile DI. Seeds germinated on an agar plate until the radical had emerged, then were planted at 1 cm depth in soil. A glass sheet was placed over the soil surface and clamped into place using large binder clips. Tinfoil and cardboard were placed over the surface of the glass sheets prevent light from reaching roots. Microcosms were placed in the greenhouse leaning at a 30° angle from vertical with the glass side face down to promote growth of long roots through soil at the surface of the glass.

Following 10–14 days of growth, microcosms were removed from the greenhouse and the volume of soil was doubled to allow new roots that emerged near the shoot-root interface to grow toward depth in fresh soil. The glass sheet was removed and a short, clear acetate sheet was placed on the surface of the existing soil, extending from the base of the microcosm up to within 5 cm of the top of the soil. One cm thick strips of neoprene foam gasket were positioned to double the thickness of the soil chamber, and an additional 1 cm of soil was added and moistened with a general household sprayer with tap water. The glass and clamps were returned to the microcosm and the microcosm was returned to the greenhouse. Plants were inspected every 2–3 days and when new roots were growing in the chamber, the microcosm was chosen for application of bacteria. The limited number of roots growing in the fresh soil simplified the analysis of patterns of luminescence from bacteria located near individual roots, particularly for work with corn. Tomato and poplar root systems were more complex.

Bacteria were grown in 20 ml of M9 medium amended with glucose (0.4% w/v) and tetracycline ($10 \mu\text{g ml}^{-1}$) in 250 ml flasks. Bacteria were spun down and re-suspended in 15 ml of M9 medium to an optical density of 0.08 OD without tetracycline and without carbon. For application of KT2440/pZKH2, the glass was removed from the side of the chamber and a 25 ml reagent sprayer (Kontes, Vineland, NJ USA) was used to apply an even distribution of bacteria onto the exposed surface of the roots and soil. Following the application of the bacteria, a clear perforated bread bag (25 holes per square cm, Whole Foods, Austin, TX USA) was attached in front of the soil to support the soil and allow air flow.

The microcosm was placed in a 60 \times 40 \times 40 cm Rubbermaid container (Newell Rubbermaid, Atlanta GA) that had been made light tight using a combination of black duct tape and dark-room cloth material. The soil surface faced the Versarray CCD

camera lens at approximately 15 cm distance. Heat produced by the camera required that the camera body itself be placed outside of the box. A 15 \times 15 cm piece of sheet metal was machined with a hole that allowed the threading of the C-mount lens to extend through and thread into the body of the camera. The sheet metal was connected to the sides of the light-tight box with two layers of blackroom cloth and black duct tape. This connection allowed the camera and lens position to be moved relative to the position of roots growing down the surface of the soil, but did not allow light into the box. The stem and leaves of the plant extended up out of the box through a hole so that the aboveground portion of the plant was exposed to light from a halogen lamp on a 12 h light/dark cycle. The light was filtered through 5 cm of water to remove infrared heat. A light-tight seal was made with Play-Doh (Hasbro, Pawtucket, RI) kept from drying using parafilm. Reflective insulation (Reflectix, Markleville, IN) on top of the box also kept halogen light from heating up the box. Temperature inside the box was monitored using a temperature logger (Onset Corporation, Bourne, MA USA) and maintained within 1°C between dark and light cycles.

Images of luminescence were captured as 55 min exposures with 1 \times 1 or 2 \times 2 binning. Following every 55 min exposure, a timer turned on a very weak indiglo nightlight inside the box (AmerTac Model E-22A, generating $<1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ as measured by a LiCor 250A meter equipped with LiCor 190A PAR sensor). While the indiglo light was on, the camera captured a 3-second bright-field image of the microcosm's soil-root surface. This alternating image acquisition was programmed using a macro in the WinView/32 imaging software, for 3–7 days of plant growth. Images were analyzed using ImageJ. For corn, light was quantified in 1 mm or 2 mm increments along the length of individual roots in areas of 25 pixels, up to 90 mm back from root tips, over time. For tomato, a complex root system developed prior to application of bacteria and the whole system was imaged over time. For poplar, individual regions of interest approximately 1 cm in length were selected from mature, non-woody roots at most several days old, and luminescence was quantified through time.

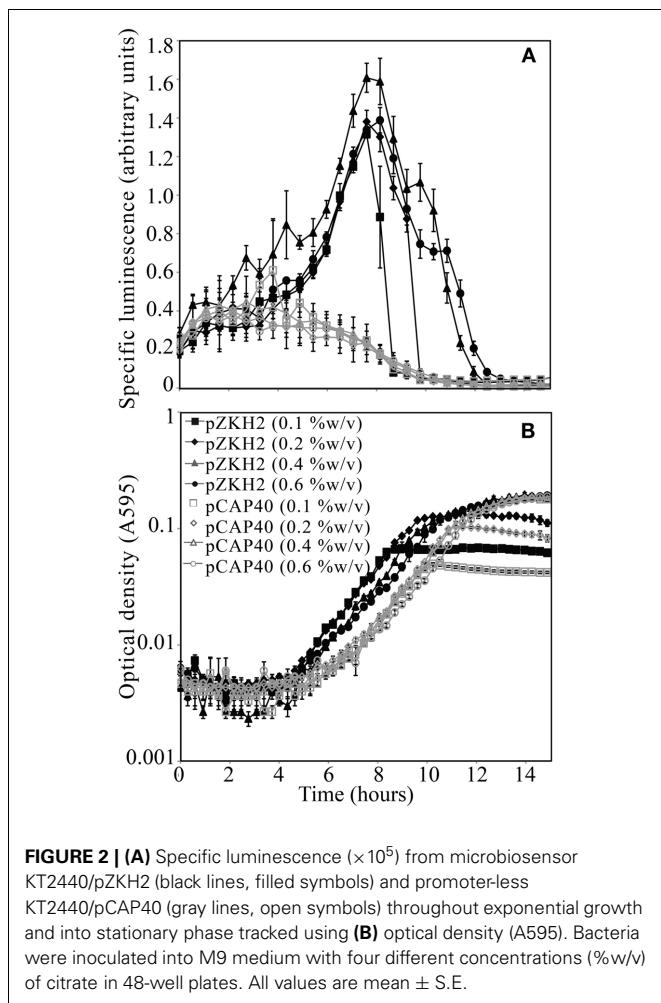
RESULTS

PLATE READER EXPERIMENTS

Influence of growth phase on light production

For the biosensor *P. putida* KT2440/pZKH2 (black lines), calculated specific luminescence (Figure 2A) peaked during mid-exponential growth (Figure 2B); specific luminescence decayed rapidly to zero in late log to stationary phase. Specific luminescence from the promoter-less construct *P. putida* KT2440/pCAP40 (gray lines) exhibited no mid-exponential growth peak (Figures 2A,B), but equaled specific luminescence from the biosensor both early in the growth cycle when cell numbers were very low, and in late log to stationary phase (when luminescence dropped to zero).

The mid-exponential peak in specific luminescence was observed for the biosensor *P. putida* KT2440/pZKH2 in both citrate and succinate, at all concentrations (Figures 3A,B). Peak luminescence persisted longer at higher concentrations.



Influence of carbon, M9, and oxygen availability on light production
 Three hundred and eighty minutes into a typical growth experiment, KT2440/pZKH2 growing in M9+citrate had reached stationary phase, and specific luminescence had dropped to zero (**Figure 4A**). Specific luminescence increased again only when more citrate was added, which also spurred more growth (after a lag). Adding oxygen, mineral nutrients (M9) and water (a control) had no effect. In contrast, KT2440/pZKH2 growing in M9+glucose was still in late exponential phase at 380 min., and specific luminescence had only just begun to decline. Addition of more glucose did not increase luminescence and spurred only minimally more growth, hours later. Addition of O₂, mineral nutrients, or water also had no effect (**Figure 4B**).

EXPERIMENTS ON FILTER DISCS

Dynamic response to pulsed carbon availability

KT2440/pZKH2 biosensors deposited on filter discs and flushed continuously with M9 salts containing no glucose exhibited low light production through time (**Figure 5**, open diamonds). KT2440/pZKH2 biosensors exposed to 180 min of 0.1% glucose in M9 responded with strongly increasing specific luminescence, starting approximately 1 h after exposure to high glucose.

Luminescence increased and had just reached a plateau when glucose flow stopped, then luminescence declined to baseline over approximately 2.5 h.

EXPERIMENTS IN SOIL – NO PLANTS

Detection of biosensor luminescence in soil supported by distinct carbon substrates

Luminescence detected from soil is a function of specific luminescence and bacterial population size, both of which are likely affected by carbon substrates available to support growth. To test whether the camera systems we had available were sensitive enough to detect luminescence in soil, KT2440/pZKH2 was inoculated into soil in various combinations of cell densities, carbon types and concentrations, with each treatment loaded into a 96-well plate and imaged by a Retiga EX CCD camera.

KT2440/pZKH2 that was inoculated with M9+glucose medium into soil yielded strong luminescence that was easily detected by the camera; inoculation with citrate yielded detectable but much lower luminescence (**Figure 6**). Inoculation with M9+acetate resulted in no increase in luminescence, either because populations of luminescing bacteria were too small, or specific luminescence was low on acetate, or both. In the case of bacteria supplemented with media containing glucose or citrate, the timing of peak light production was related to the inoculation density. Inoculations at an OD of 0.08 yielded peak luminescence earliest, followed by the inoculations of 0.04 and 0.02 OD, as would be expected from a combined population-size effect tempered by decreasing light production as bacteria approach stationary phase.

Response of biosensor luminescence in soil to repeated pulses of carbon and mineral nutrients

We tested the ability of the Princeton Instruments cooled Versarray CCD camera to detect luminescence from biosensors in soil, and the repeatability of the KT2440/pZKH2 biosensor response in soil to pulses of carbon, nutrient, and water availability provided via filter discs, over multiple days. Only data from filter pre-treatment groups 5, 6, and 7 are shown in **Figure 7**; pre-treatment groups 1, 2, 3, and 4 yielded similar results. No matter the pre-treatment, and no matter the day, addition of glucose to any filter resulted in orders of magnitude increase in luminescence (**Figure 7**, all peaks labeled “C”). Small increases in luminescence were induced by water (e.g., see 80 and 100 h, panels **7B,C,D**) and by mineral nutrients delivered in water (e.g., N at 10 h, panel **7B**; P and S panel **7C** at 10 h; M9 panel **7D** at 10 h). Additions of these solutions may have mobilized small pockets of soil carbon that were previously unavailable to the biosensors.

One treatment, on one day, yielded an anomalous result—the addition of N on the first day after the pre-treatment of M9 + C (**Figure 7D**). It is possible that the bacteria were nitrogen limited before the addition of the N, but the discs that received M9 with an equivalent level of nitrogen did not ramp up light production significantly. The anomalous result is likely either a case of carbon being mobilized by the liquid addition of N from surrounding pockets not in contact with the bacteria, or the experimenter adding carbon in that spot by accident.

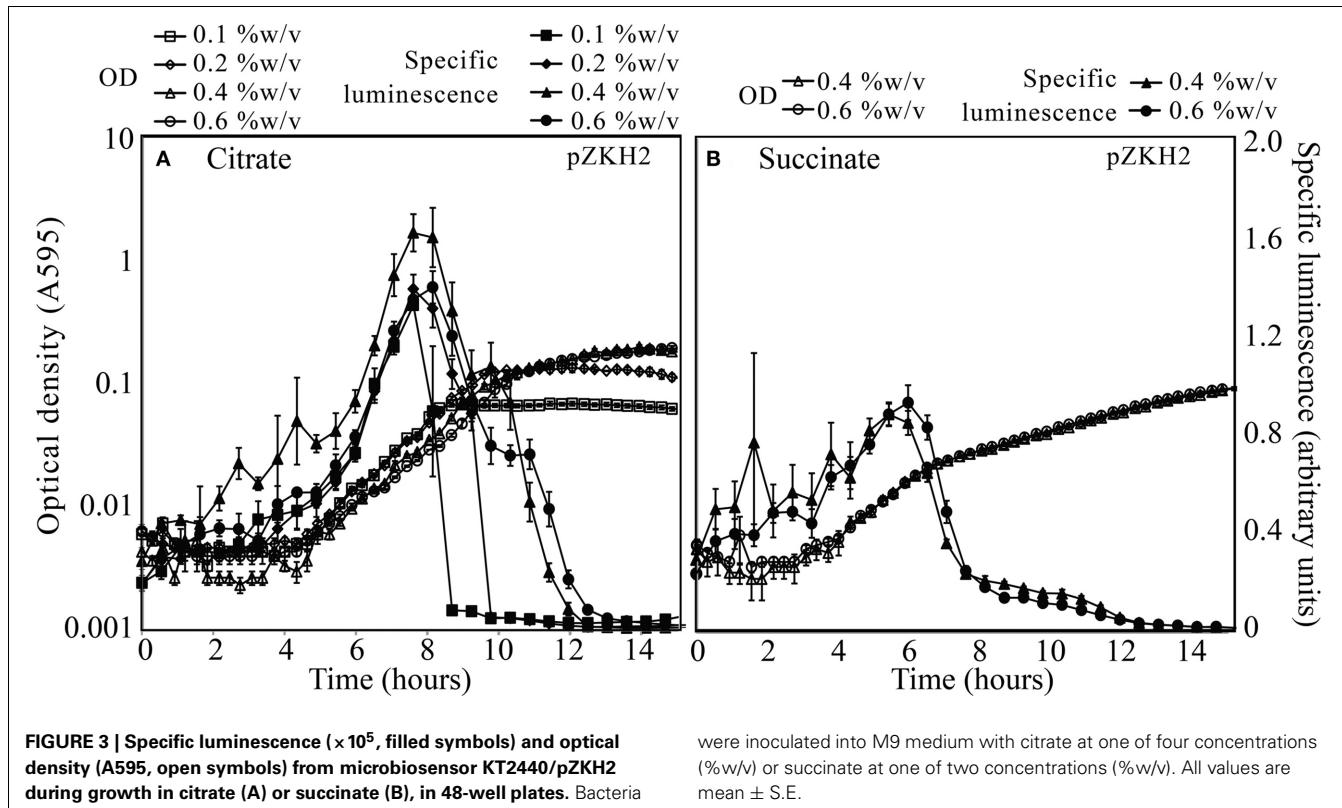


FIGURE 3 | Specific luminescence ($\times 10^5$, filled symbols) and optical density (A595, open symbols) from microbiosensor KT2440/pZKH2 during growth in citrate (A) or succinate (B), in 48-well plates. Bacteria

were inoculated into M9 medium with citrate at one of four concentrations (%w/v) or succinate at one of two concentrations (%w/v). All values are mean \pm S.E.

EXPERIMENTS IN SOIL – SOIL MICROCOSMS WITH PLANTS

KT2440/pZKH2 produced light on all type of plants that were inoculated including corn (*Z. mays* L.), tomato (*S. lycopersicum* L.), pepper (*C. annuum* L., data not shown), sagebrush (*Artemisia tridentata* var. *vaseyana*, data not shown) and black poplar (*P. nigra* L.). The biosensor produced similarly high intensities of light when inoculated on corn, tomato and pepper (pepper data not shown). When inoculated on sagebrush (data not shown), whose overall growth rate is not as fast as the crop species used, KT2440/pZKH2 produced lesser but easily measurable amounts of light. KT2440/pZKH2 produced the smallest output of light on *P. nigra*. Selected results from corn, tomato, and poplar are described in detail below.

Corn

KT2440/pZKH2 consistently emitted a high amount of light on growing corn roots. Results from monitoring two corn plants are shown in Figures 8A–F. Both image series demonstrate a clear pattern of light emission associated with the tips of corn roots as they grow through the soil. For plant 1 (Figures 8A–C), the major peak of light emission occurs 22–32 mm behind the growing root tip along the root axis, though another peak in luminescence also occurs \sim 1–4 mm behind the root tip. A bright-field image of the root is shown at left; the regular grid of dots is the grid of air holes in the bread bag covering. Luminescent regions are visible from the false color superimposed on the outline of the corn root in all four black panels in Figure 8A. Intensity of luminescence is indicated by color, with lower values

blue, increasing through yellow and then red for highest luminescence. Figure 8B shows the distribution of luminescence along the root at 1 mm intervals, from 0 to 90 mm behind the tip, with hourly measurements binned into 12-h increments. These binned data are separated along the Y axis for clarity, with earlier times lower, progressing to later times in the experiment higher in the graph. As time progressed, soil drying slowed root growth and the peak of light emission shifted closer to the tip (Figure 8B). The distance between the peak of light emission and the root tip is linearly related to the growth rate of the root (Figure 8C). Similar results from a second corn plant are shown in Figures 8D–F.

Black poplar

Figure 9A shows a bright-field image of one microcosm planted with *P. nigra*, and the regions of interest (ROIs) defined in ImageJ from which luminescence was quantified over time, and graphed in Figure 9B. The ROIs illustrated are in root locations that are mature; tissue is no longer extending or expanding but the root is still young (not woody) and cortex is still intact. Though overall luminescence from biosensors around these mature regions decreased over the 6 days of the experiment, the biosensor luminescence associated with all five roots also exhibited a strong diel rhythm throughout the experiment.

Tomato

Figure 9C shows a bright-field image of an established, mature tomato root system (at left), and a subset of a series of luminescence images taken over 4 days. Luminescence images progress in

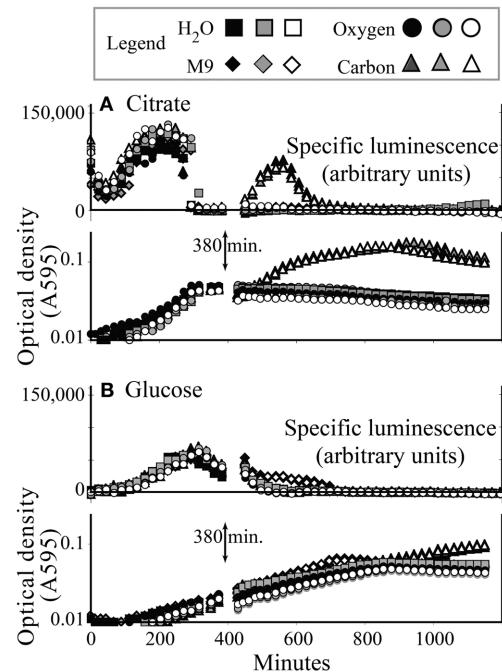


FIGURE 4 | Induction of specific luminescence from microbiosensor KT2440/pZKH2 during growth in citrate (A) or glucose (B), in 48-well plates. At 380 min., triplicate wells were spiked with carbon, oxygen, M9 medium, or water. Growth and luminescence data are separated for clarity. Each replicate is shown separately.

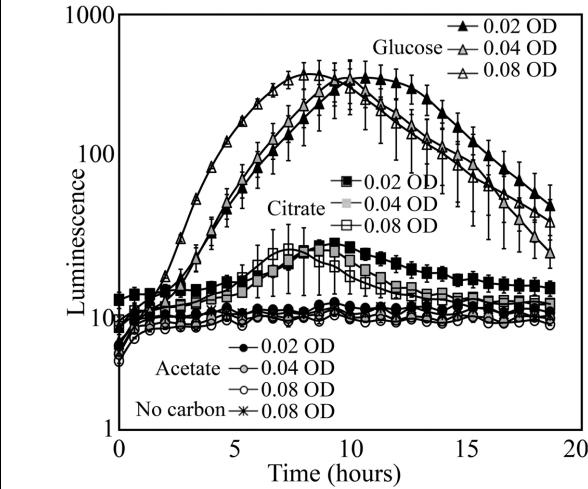


FIGURE 6 | Luminescence detected using a Retiga EX CCD camera from populations of the microbiosensor KT2440/pZKH2 established in soil in 96-well plates. Bacteria were grown in M9 with 0.4% (w/v) citrate, glucose, or acetate as carbon source, spun down and re-suspended to three optical densities for each source, then inoculated into soil. All values are mean \pm S.E.

this tomato root system is included in online supplementary materials.

DISCUSSION

Interactions among roots and soil microorganisms occur within a complex, heterogeneous matrix of soil grains and organic matter. Organic compounds are released and root cap cells distributed into the soil dynamically as root systems become established. These compounds influence soil bacteria and fungi at spatial scales difficult to measure, with dynamics that cannot be captured with destructive soil sampling techniques. Experiments simplifying the root-microbial system by isolating plants and roots into hydroponic or other culture systems have been valuable for detecting diel patterns of root exudation, types of compounds released from roots into solution (and the effects of the presence of microbes on that release), and signaling compounds exchanged by roots and microbes. Microbial biosensors used in non-sterile soil complement these approaches by providing information on local microenvironmental resources and conditions experienced by the microbes themselves.

Microbiosensor design is flexible and can be tailored to particular questions via choice of host organism, promoter, and reporter genes (Gage et al., 2008). Here we used a host bacterium that is native to the rhizosphere environment, and a reporter system with a constitutive promoter (*nptII*) fused to the *luxCDABE* operon. The great strength of the system is that the light output that emerges reflects the growth and metabolic activity of a soil bacterium that is native to the rhizosphere. It might be expected that because the *nptII* promoter is constitutive, luminescence on a per-cell basis would be relatively constant during microbial growth. However, the results of liquid culture experiments (Figures 2–4) revealed a strong peak

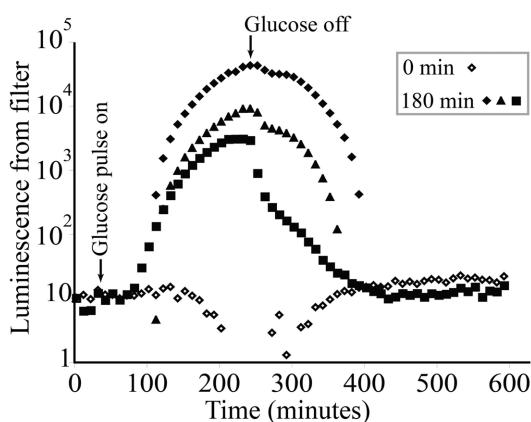


FIGURE 5 | Luminescence from populations of microbiosensor KT2440/pZKH2 established on 0.45 μ m syringe filters and receiving M9 medium at a constant flow rate of 0.5 ml min⁻¹. Starting at approximately 40 min, 3 replicates were exposed to a 180-minute pulse of 0.1% (w/v) glucose in M9, then returned to M9 alone. Each glucose-treated replicate is shown separately to illustrate dynamics more clearly (0 min indicates no glucose added).

time left to right, top row then bottom row, within Figure 9C, spaced at 6 h intervals. Biosensor luminescence was highly dynamic, progressing in acropetal waves along root axes during the course of the experiment. A video of luminescence around

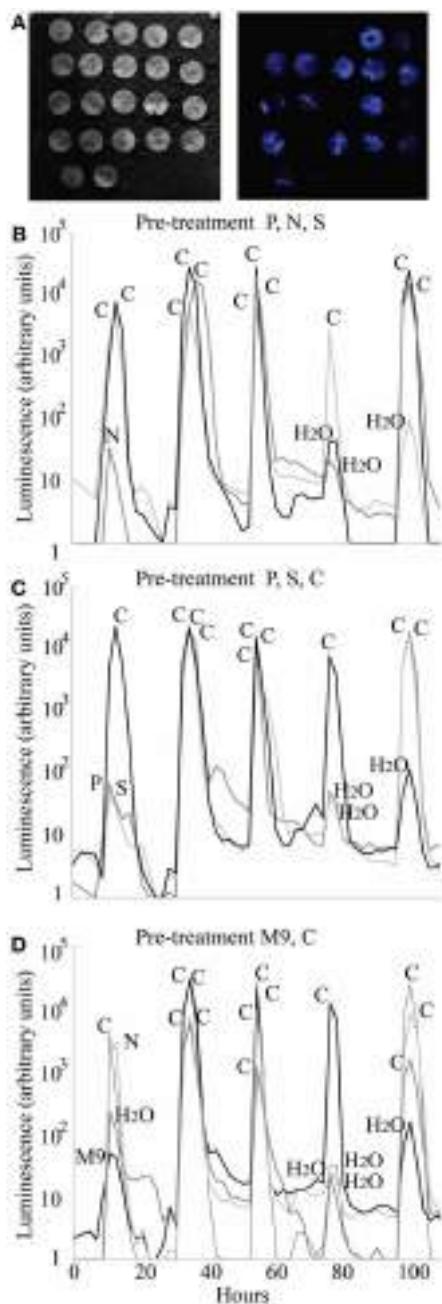


FIGURE 7 | Luminescence detected using a Versarray CCD camera from populations of microbiosensor KT2440/pZKH2 inoculated onto soil and provided with pulses of carbon, various mineral nutrients, and water, all delivered via filter discs pinned to the soil surface. Panel (A) shows brightfield (left) and false-color luminescence (right) images of the filters. Panels (B,C,D) show luminescence from replicate filter discs within three of seven pre-treatments (see text), over five days, as pulses of carbon, nutrients, and water were applied.

of light production at mid-exponential growth followed by the rapid decrease in light production as cells moved into stationary phase. Light production by these microbiosensors is therefore a signal that all conditions and resources in the biosensors'

local environment are conducive to (and supporting) rapid growth.

The decrease in light production initiated as cells move from late exponential to stationary phase is likely connected with the high energy demand of bioluminescence. *P. putida* KT2440/pZKH2 contains the full *lux* operon *luxCDABE*. Light production requires the dual oxidation of a reduced flavin mononucleotide (FMNH₂) and an aldehyde molecule (RCHO). *luxA* and *luxB* code for the alpha and beta-subunits of the luciferase enzyme responsible for carrying out that oxidation. *luxCDE* encode a multi-enzyme reductase complex responsible for the regeneration of aldehyde (RCHO) (Meighen, 1988), requiring one NADPH and ATP (Stryer, 1988). Overall, close to 20 ATP molecules are estimated to be required to produce one quantum of light (van der Meer et al., 2004), imposing a significant metabolic burden on bacteria.

Results from multiple experiments are consistent with the idea that the *lux* system is competing with other cellular activities for energy. Our working hypothesis for the growth stage dependent behavior of the biosensor is that as cells transition from late exponential growth toward stationary phase, competition within the cells for dwindling pools of energy intensifies and light production rapidly decreases (e.g., Figure 2). Figure 4A shows that when KT2440/pZKH2 growing in M9+citrate had reached stationary phase, specific luminescence increased again very rapidly when more citrate was added (suggesting *lux*-related machinery was still present and ready to act when energy became available), whereas growth resumed after a short lag. The contrasting behavior of biosensors spiked with glucose in liquid culture, Figure 4B, where no increased luminescence or growth were observed when glucose was added, resulted because the cells were still growing exponentially; they had not reached stationary phase. Biosensors already had sufficient resources to grow exponentially, they were already highly luminescent, and neither growth rate nor luminescence increased quickly with added glucose. However, repeated application of glucose to filter disks pinned to soil seeded with KT2440/pZKH2 resulted in consistent pulses of light production, over a number of days (Figure 7), consistent with the idea that carbon limits microbial growth in bulk soil (Cardon and Gage, 2006).

Studies on *E. coli* carrying a plasmid with a full complement of *lux* genes (*luxCDABE*) (Rattray et al., 1990) found a similar effect of growth stage influencing overall light production. However, when Rattray et al. (1990) exogenously supplied dodecyl aldehyde to *E. coli* strains carrying only *luxABE* on the plasmid, the strain was able to express light consistently across growth stage. The supply of aldehyde subverted the dependence of the luciferase reaction on fatty acids diverted away from the cells' normal lipid production.

The amount of luminescence from microbiosensors in soil recorded by the camera system is influenced by both the luminescence per cell (specific luminescence, influenced by promoter activity, *lux* machinery turnover, and energy pool sizes) and by the population size of the biosensors per unit area. We imaged at relatively low magnification to capture behavior of the luminescent signal emanating from populations of bacteria, through time, across entire root systems or for millimeters behind growing

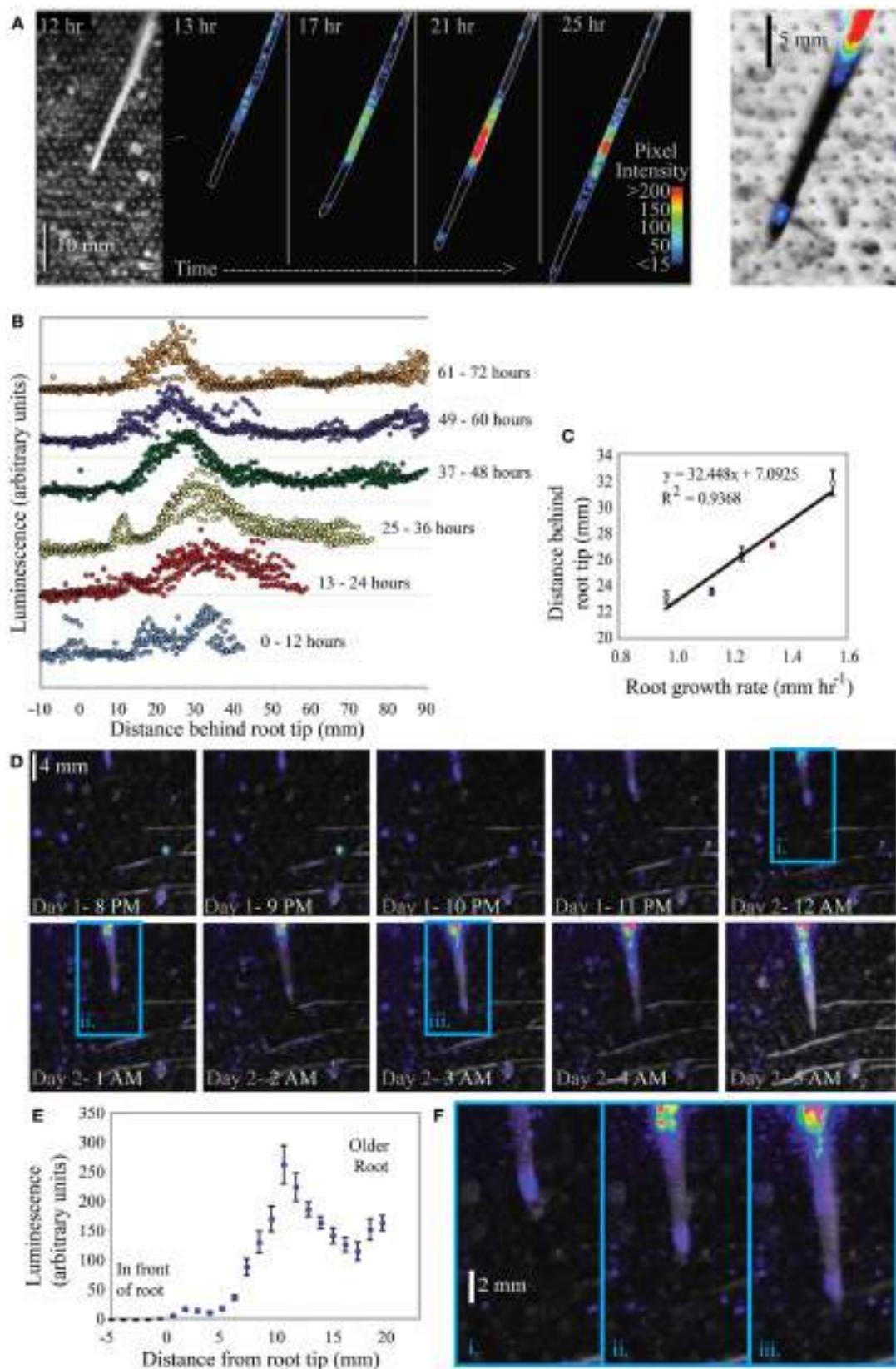


FIGURE 8 | Continued

FIGURE 8 | Luminescence from microbiosensor KT2440/pZKH2 inoculated into microcosms containing 3-week old *Zea mays* (corn). Panel (A) shows a bright field (left) and a small subset of images from a time series of false-colored luminescence around a single growing corn root, along with a closeup (right) of false-color luminescence superimposed on an inverted brightfield image of the corn root tip. Panel (B) charts luminescence quantified hourly along the root, binned into six

time frames. Panel (C) graphs the distance of maximum luminescence from the root tip against root growth rate. Panel (D) shows a portion of a similar image series from another corn plant, and panel (E) shows the distribution of luminescence along the root axis averaged over the 25 h experiment. Panel (F) includes zoomed portions of images from the panel (D) series (indicated by i, ii, and iii), showing false-color luminescence close behind the growing root tip.

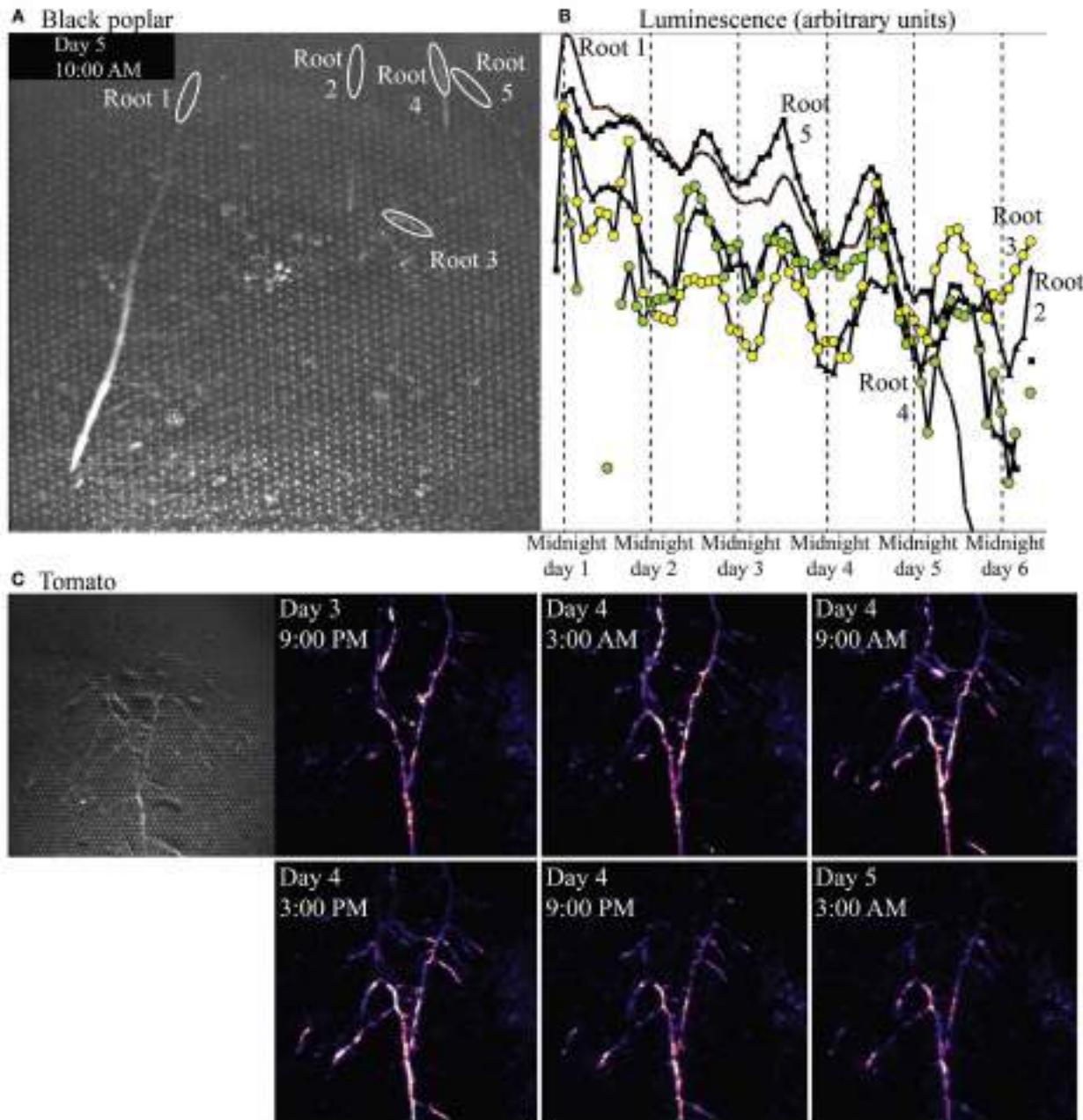


FIGURE 9 | (A) Bright-field image showing roots of *Populus nigra* (black poplar) established in a soil microcosm, and the rhizosphere regions of interest associated with mature zones of five roots. Panel (B) shows luminescence quantified from those

regions over time. Panel (C) shows a brightfield (left) and time series of false-color luminescence images (left to right, top row then lower row) from a microcosm planted with *Solanum lycopersicum* (tomato).

root tips. Light production was informative; darkness was not. Where the camera detected light, conditions and resources must have been conducive for growth and energy production, and populations of microbiosensors must have built to sufficient size to produce detectable signal. Darkness, in contrast, could result from e.g., insufficient resources for light production, conditions not conducive to growth of sufficient numbers of microbiosensors, and death of sensors (grazing protozoa were present in these non-sterile soils).

Within these constraints for interpretation, our results from soil microcosm experiments with plants are consistent with biosensors reporting conditions in which rapid growth can occur. Not surprisingly, luminescence was most often seen clearly associated with live roots for all plants tested—the rhizosphere is a hotspot for microbial activity in ecosystems, where carbon fixed by plants and released or lost by roots spurs microbial growth and activity. Plant species-specific patterns, however, were also observed.

Rhizodeposition by corn roots is known from the literature to be very high (McCully, 1999), and in our experiments was substantial enough to support bright luminescence even within millimeters of the root tip. Again, to detect this luminescence using the camera, not only did biosensor cells need to luminesce, but biosensor population sizes had to be large enough that the camera could detect light from them. The clear linear dependence of the location of maximum luminescence (along the root axis) on root growth rate is consistent with it taking time for the population of biosensors to build up sufficiently to produce maximum detectable luminescence. The slower the root grows, the closer to the tip are locations that have been exposed to high rhizodeposition for long enough for a microbial population to grow larger. All plant roots grow by adding new cells at the tip, building root tissue at the tip through soil (Raven and Edwards, 2001). A root growth rate of 1 mm h^{-1} (as in corn **Figure 8C**) is a measure of the position of the root tip in soil over time, but does not represent a pushing of the entire long root axis through soil at that rate. As cells are built on the tip of the root, the cells behind the tip generally remain in place in soil (though they do expand before maturing). Using the growth rate and maximum luminescence data in **Figure 8C**, we can estimate that the position at which maximum luminescence occurs along the root is approximately 22–23 h old (average $22.5 \pm 0.6 \text{ h SE}$, for the five points shown in **Figure 8C**). Interestingly, using the high magnification images in **Figures 8A,F**, it is clear there is another, but less bright, “hotspot” of luminescence just 1–4 mm behind the root tip. Again using root growth rate data, we can calculate that this location is just a few hours old, yet resources there are high enough that luminescence is detectable. This time frame is consistent with the data from labile carbon pulse experiments in **Figure 5**, where an increase in glucose available to filter-immobilized bacteria resulted in increased luminescence detectable by a camera after approximately 1 h.

Results from black poplar, however, provide an informative contrast. Luminescence was never detected by the camera at or near the growing tips of poplar roots, and luminescence overall was very low around all roots. We suspect this may be

because populations of biosensors did not build up to sufficient sizes, rapidly enough, for detection at the tips; we have noted in other experiments that *Sinorhizobium*-based microbiosensors do not proliferate easily around roots of poplars, for as yet unknown reasons. However, low luminescence was detected around mature regions of the roots, and quantification of that luminescence over time revealed a very strong diel cycle over several days (**Figure 9B**), with peak luminescence near noon, and lowest luminescence near midnight. Strong diel patterns in allocation of carbon from shoots to roots are known in the forestry literature for species within the genus *Populus* (e.g., Dickson, 1991). Starch builds up in leaves during the day, then is mobilized and carbon shipped belowground as sucrose at night. We do not yet know whether such a strong pattern of daytime starch buildup and nighttime starch mobilization exists in black poplar, but the diel pattern in luminescence (suggesting rhythmic diel carbon availability in the rhizosphere) that we observed would be nearly half a day out of phase with such a diel carbon allocation pattern within the plant.

In contrast to this coordinated, rhythmic but very low biosensor luminescence along multiple poplar roots, luminescence from biosensors associated with tomato roots was bright and did not follow a diel rhythm. The luminescence pattern observed day 5 at 3 AM, for example, is different from the pattern observed day 4 at 3AM (**Figure 9C**). Biosensor luminescence progressed in waves along root axes acropetally (toward root tips) during the course of the experiment. The supplementary video shows an initial surge of light from all areas of the microcosm as microbiosensors use available resources, eventually settling into a pattern of dynamic luminescence restricted largely to the rhizosphere.

CONCLUSION

It has long been known that different plant roots release different kinds of carbon compounds, that shoot-root allocation patterns vary widely, and that plant roots grow at different rates depending on environmental conditions. Biosensor *Pseudomonas putida* KT2440/pZKH2 adds an integrated microbial report on whether rhizosphere conditions and resources in the Pseudomonads' local soil microenvironment are supporting rapid growth. From the perspective of the rhizosphere being a key commodities exchange in ecosystems, this integrated view is essential—many resources may support microbial growth, and a range of conditions exist around plant roots. KT2440/pZKH2 luminescence indicates that energy is available for bacterial growth around roots beyond the moment that the root tip grows past a particular site. KT2440/pZKH2 luminescence also reveals that not all root tips are equal. Though a poplar root tip may be similar in size to a root tip of corn or tomato, it drives very different microbial response; root biomass is not necessarily a strong predictor of the capacity of a root system to spur microbial growth and activities. KT2440/pZKH2 also reports that availability of carbon is variable in space and time around plant roots, sometimes in a coordinated or predictable spatial or temporal pattern based on plant allocation or vasculature. But no matter the ecosystem function of

interest, sufficient microbial biomass hosting the genetic capacity for that function must build up before the microbes' effects can be exerted.

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