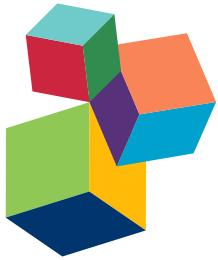


HARVESTING PLANT AND MICROBIAL BIODIVERSITY FOR SUSTAINABLY ENHANCED FOOD SECURITY

EDITED BY: Laurent Laplaze, Francesca Sparvoli, Khaled Masmoudi and Charles Thomas Hash

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HARVESTING PLANT AND MICROBIAL BIODIVERSITY FOR SUSTAINABLY ENHANCED FOOD SECURITY

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Pearl millet field after harvest near Nioro (Senegal).
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The World population will reach 9 billion by 2050, with the majority of this growth occurring in developing countries. On the other hand, one in nine of the World's population suffers from chronic hunger, the vast majority of which live in developing countries. We therefore need to find new and sustainable solutions to feed this increasing population and alleviate the predicted negative impact of global changes on crop production.

This e-Book deals with new strategies to improve food security and livelihoods in rural communities, reduce vulnerability, increase resilience and mitigate the impact of climate change and land degradation on agriculture. This collection of 18 articles addresses the major abiotic factors limiting crop production worldwide, how to characterize and exploit the available plant biodiversity to increase production and sustainability in agrosystems, and the use of beneficial microbes to improve production and reduce the use of fertilizers and pesticides.

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Editorial: Harvesting Plant and Microbial Biodiversity for Sustainably Enhanced Food Security

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Keywords: food security, breeding, biodiversity, inoculation, microbiome, drought tolerance, Salinization, climate change

The Editorial on the Research Topic

Harvesting Plant and Microbial Biodiversity for Sustainably Enhanced Food Security

According to the United Nations, the World population will reach 9 billion by 2050, with the majority of this growth occurring in developing countries. More than half of global population growth is expected to occur in Africa. On the other hand, one in nine of the World's population suffers from chronic hunger, the vast majority of which live in developing countries (FAO et al., 2015). We therefore need to find new and sustainable solutions to feed this increasing population and alleviate the predicted negative impact of global changes on crop production. This e-Book summarize current research to improve food security and livelihoods in rural communities, reduce vulnerability, increase resilience, and mitigate land degradation in developing countries.

Several reviews and articles addressed the current status and strategies to deal with the major abiotic factors limiting crop production. Sultan and Gaetani review the current predictions of future climate in West Africa, one of the most dynamic area for demographic growth, as well as its expected impact on crop production and scenario for adaptation. Drought episodes are expected to occur more frequently and Ndour et al. review how structural-functional plant models can be used to understand water acquisition and to breed varieties with increase tolerance to water deficit. Beside, about 6% of the arable land is affected by salinization worldwide. The problem is mostly concentrated in arid and semiarid regions, where it seriously threatens agricultural sustainability and food security. Salinity induces a rapid osmotic stress that reduces shoot growth, and a slower ionic stress that accelerates senescence of older leaves (Munns and Tester, 2008). Adaptation to salinity is a quantitative character, which is controlled by different genetic pathways (DeRose-Wilson and Gaut, 2011). Hanin et al. discuss how our current knowledge on sodium accumulation and transport and how beneficial plant-microbe interactions can be used to create varieties and agricultural practices to improve yield in salt-affected areas. Phosphorus availability is another major limiting factor for crop production and the limited amount of available high quality rock phosphate deposits to make fertilizer reinforce the need to find alternative strategies to improve P acquisition and use efficiency. Gemenet et al. discuss the various strategies that could be used to achieve these goals for sorghum and pearl millet in the context of West Africa. Vandamme et al. show that genetic diversity exists in rice for P accumulation in seeds and could be used to limit the removal of P from agricultural soil. Indeed, keeping P in the biomass that can be recycled back to the soil rather than the grain could limit P depletion.

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Clearly, there is a need to characterize and exploit available plant biodiversity to increase production and sustainability in agrosystems. In their review, Mondal et al. discuss new breeding strategies that could be used to exploit the genetic diversity in wheat. Pearl millet is an important crop for food security in arid and semi-arid areas. The characterization of the genetic diversity of cultivated pearl millet in Senegal by Diack et al. reveals a genetic differentiation between early- and late-flowering varieties and a very large and untapped potential for breeding.

Root traits (including root-beneficial microbe interactions) are potential new targets to breed new varieties with improved resource use efficiency. The review by Schmidt et al. discusses how domestication and modern breeding indirectly affected root development and rhizosphere ecology in maize and how these could be new targets to create varieties with more efficient resource acquisition. Similarly, Passot et al. analyzed early root development and root anatomy in pearl millet. They demonstrate that there is diversity for root traits such as root growth or branching that could be used for breeding.

Beneficial microbes are another potential lever to improve production and reduce the use of fertilizers and pesticides. While many studies have revealed the potential beneficial impact of microbes, little are used in agriculture. The review article by Parnell et al. presents the factors that need to be taken into account to develop inoculants for agriculture and give some examples of products available and their use. Two original research papers report the impact of microorganisms that could be used as bio-fertilizer. Zhang et al. report the impact of *Trichoderma longibrachiatum* T6 on wheat tolerance to salt stress, and provide evidence that the beneficial effect involves the antioxidative defense system of the plant. Similarly, Akram et al. provide evidences that *Staphylococcus sciuri* strain SAT-17 alleviates salt-induced cellular damages in maize plants and enhances growth. Pitzschke et al. also report that seeds of the

hardy crop quinoa contain bacteria of the genus *Bacillus* that could change the host's redox status and induce a primed state that might enhance plant tolerance to abiotic stresses.

Ecological intensification of agroecosystems could also be achieved using plant association. For instance, Wahbi et al. show that intercropping wheat and faba bean gave better crop productivity than rotation practice, impacting soil microbial functionalities. Tropical trees of the *Casuarinaceae* family can enter nitrogen-fixing symbiosis with the soil actinomycete *Frankia* sp. and are widely used to rehabilitate including salinized soils. Ngom et al. show that salt stress reduces symbiosis formation in *Casuarina glauca* and *C. equisetifolia* and that *Frankia* nitrogen-fixation efficiency rather than *in vitro* salt tolerance is important to improve salt tolerance of inoculated plants.

Finally, two original research articles deal with crop quality characters for food and industrial uses. Neglected and underutilized crops might represent an alternative to current staple crops, especially in marginal lands such as those of the arid and semi-arid regions of sub-Saharan Africa (Naylor et al., 2004). Ghebrehiwot et al evaluated the use of flours from *Eragrostis curvula*, a wild relative of tef (*Eragrostis tef*), for preparing injera, a typical sour bread consumed as staple food in Ethiopia and Eritrea. Cassava is an important crop for food security and a major source of starch for industry (Li et al., 2017). In their article, Karlström et al. report that cassava varieties with amylose-free starch have no limited impact on yield and could therefore be planted to increase the revenues of farmers.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Agriculture in West Africa in the Twenty-First Century: Climate Change and Impacts Scenarios, and Potential for Adaptation

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West Africa is known to be particularly vulnerable to climate change due to high climate variability, high reliance on rain-fed agriculture, and limited economic and institutional capacity to respond to climate variability and change. In this context, better knowledge of how climate will change in West Africa and how such changes will impact crop productivity is crucial to inform policies that may counteract the adverse effects. This review paper provides a comprehensive overview of climate change impacts on agriculture in West Africa based on the recent scientific literature. West Africa is nowadays experiencing a rapid climate change, characterized by a widespread warming, a recovery of the monsoonal precipitation, and an increase in the occurrence of climate extremes. The observed climate tendencies are also projected to continue in the twenty-first century under moderate and high emission scenarios, although large uncertainties still affect simulations of the future West African climate, especially regarding the summer precipitation. However, despite diverging future projections of the monsoonal rainfall, which is essential for rain-fed agriculture, a robust evidence of yield loss in West Africa emerges. This yield loss is mainly driven by increased mean temperature while potential wetter or drier conditions as well as elevated CO₂ concentrations can modulate this effect. Potential for adaptation is illustrated for major crops in West Africa through a selection of studies based on process-based crop models to adjust cropping systems (change in varieties, sowing dates and density, irrigation, fertilizer management) to future climate. Results of the cited studies are crop and region specific and no clear conclusions can be made regarding the most effective adaptation options. Further efforts are needed to improve modeling of the monsoon system and to better quantify the uncertainty in its changes under a warmer climate, in the response of the crops to such changes and in the potential for adaptation.

Keywords: West African monsoon, climate change, impacts, adaptation, agriculture

INTRODUCTION

Climate has a strong influence on agriculture, considered as the most weather-dependent of all human activities (Hansen, 2002) with impacts on food security (Schmidhuber and Tubiello, 2007). Both variability and change in climate affect food production availability, stability of food supplies, food utilization, access to food and food prices everywhere in the world (Schmidhuber and Tubiello, 2007). It is especially true in Sub-Saharan Africa which is known to be particularly vulnerable to climate change due to a combination of naturally high levels of climate variability, high reliance on rain-fed agriculture and limited economic and institutional capacity to cope with and adapt to climate variability and change (Challinor et al., 2007; Müller et al., 2010; Roudier et al., 2011). Indeed, under its current climate Sub-Saharan Africa is already facing recurrent food crises and water scarcity triggered or exacerbated by climate variability and extreme events such as droughts, excessive rains and floods which affect agricultural productivity and hence rural household food security (Dilley et al., 2005; Haile, 2005). This chronic food insecurity may even increase in the future since the food demand is expected to be multiplied by more than five in Africa by 2050 (Collomb, 1999).

Climate change and its impact on food security are additional strains on the agriculture sector in Africa. The last Intergovernmental Panel on Climate Change (IPCC, 2014) highlighted that: “warming of the climate system is unequivocal, and since the 1950s, many of the observed changes are unprecedented over decades to millennia. The atmosphere and ocean have warmed, the amounts of snow and ice have diminished, and sea level has risen. Changes in many extreme weather and climate events have been observed since about 1950. Recent climate changes have had widespread impacts on human and natural systems.” Moreover, “continued emission of greenhouse gases will cause further warming and long-lasting changes in all components of the climate system, increasing the likelihood of severe, pervasive and irreversible impacts for people and ecosystems.” In this context, crop productivity, which is directly tied to climate variability, appears particularly exposed to current and future climate change impacts. Indeed, “many studies covering a wide range of regions and crops show that negative impacts of climate change on crop yields have been more common than positive impacts.” Moreover, “rural areas are expected to experience major impacts,” and “all aspects of food security are potentially affected by climate change, including food production, access, use, and price stability.” At the turn of the twenty-first century, West Africa has been identified among the primary observed climate change hot-spots, and among the most persistent and early emerging prominent hot-spots foreseen for the twenty-first century, because of the observed and projected widespread increase in mean temperature and extreme hot-season occurrence (Turco et al., 2015). Given the particularly strong deep connection between crop production and climate variability in West Africa since agriculture is mostly rain-fed and crop management (use of fertilizers and pesticides combined with modern cultivars) remains low (Dingkuhn et al., 2006), the detected sensitivity to recent and future climate change makes the region a hotspot even in terms of food production and security.

In the context described above, better knowledge of how climate will change in West Africa and how such changes will impact crop productivity is crucial to inform policies that may counteract the adverse effects. Furthermore, the ability to identify the most suitable crop varieties and practices with the most robust characteristics for withstanding climate change, is crucial for formulating adaptation strategies in this region where farmers are already able to select adapted varieties (e.g., late or early millet) or to adapt their practices (e.g., delayed or early sowing) to a changed environment (Dingkuhn et al., 2006). However, although there is a growing literature on the impact of climate change on crop productivity in Africa, there are large uncertainties in climate change projections, in the response of crops to such changes and in the adaptation of agricultural systems to future climate conditions (Challinor et al., 2007; Roudier et al., 2011). Thus, this paper provides a comprehensive overview of climate change impacts on agriculture in West Africa based on the recent scientific literature.

This review is based on a wide review of the literature on climate variability and change in West Africa and associated impacts on crop productivity. Given the sensitivity of the topic, the available literature is vast (more than 200 papers are cited in the references), the review presented here does not claim to be exhaustive and certainly misses many studies. However, an effort has been done to present a selection of the most important results, with a special attention to the recent studies. Moreover, the extensive and coordinated discussion of the crop productivity problem and the related climate dynamics aspects represents the noticeable novelty of this review. Section Climate Change Scenarios of this review paper provides observed evidences of climate change in West Africa and gives some robust features about expected changes in the next decades. Section The Impact on Crop Yield and Potential for Adaptation investigates how such climate changes affect crop production as well as potential for adaptation for the major crops in West Africa. Each section attempts to stress the most robust results in the screened literature but, more importantly, includes a discussion about limitations and uncertainties. The reader is invited to read the cited papers for more details on any specific aspects discussed in this review.

CLIMATE CHANGE SCENARIOS

West African Climate and Monsoon Dynamics

The West African climate is deeply tied to the West African monsoon (WAM) system, which develops in May over the Guinean coast ($\sim 5\text{--}10^\circ\text{N}$), reaches the maturity in August in the Sahel ($\sim 10\text{--}15^\circ\text{N}$), and finally retreats to the coast in October (Sultan and Janicot, 2003; Cook, 2015), concentrating in this period more than 70% of the annual precipitation in the region (CLIVAR, 2015). The monsoonal rainfall is a key element of the regional climate, especially in the semiarid Sahel, where vegetation is highly sensitive to precipitation variability, at time scales from intraseasonal to interannual (Philippon et al., 2007; Martiny et al., 2010; Taylor, 2011). Moreover, the atmospheric

circulation characterizing the monsoonal system is associated with mineral dust emission (Bou Karam et al., 2007; Wang et al., 2015) and thermal anomalies (Guichard et al., 2009; Fontaine et al., 2013) in the region.

The WAM is the response to the land-sea thermal contrast triggered by the seasonal cycle of incoming insolation at the surface, which favors the inland penetration of the deep convection associated with the intertropical convergence zone (ITCZ; Thorncroft et al., 2011). In the lower troposphere, the atmospheric circulation is characterized by a southwesterly moist flow from the Gulf of Guinea, contrasting a dry northeasterly flow crossing the Sahara desert. This intertropical front can be regarded as the northern boundary of the WAM, and at the peak of the monsoonal season it is displaced around 20°N (Issa Lélé and Lamb, 2010). In the mid troposphere, the circulation is dominated around 12°N by the African easterly jet, originated by the meridional thermal gradient between the vegetated Guinean coast and the Sahara desert (Thorncroft and Blackburn, 1999). The African easterly jet is the wave guide for synoptic disturbances propagating westward along the Guinean coast and the Sahelian belt, known as African easterly waves (Poan et al., 2015). These disturbances are particularly important in triggering the monsoonal precipitation through the initiation and organization of mesoscale convective systems and squall lines during the monsoonal season (Cretat et al., 2015). The annual evolution of the WAM thermodynamic features (moisture fluxes and convergence), and of the associated rainfall distribution, is strongly impacted by the emergence of the Atlantic cold tongue, and the installation of the Saharan heat low. The Atlantic cold tongue is a cold pool which characterizes the equatorial eastern Atlantic Ocean from boreal spring to early summer, and its variability influences the timing of the monsoon onset over the Guinean coast and the intensity of the inland precipitation (Druyan and Fulakeza, 2015). The Saharan heat low is a lower tropospheric thermal depression over the Sahara desert west of 10°E, developing in response to the surface heating over West Africa in boreal summer (Lavaysse et al., 2009). The Saharan heat low onset is closely linked to the WAM onset in late June, and its variability modulates the longitudinal distribution of the monsoonal precipitation in the Sahel, being strong Saharan heat low phases associated with wet/dry anomalies in eastern/western Sahel (Lavaysse et al., 2010).

Multi-Time Scales Variability

In the twentieth century, the West African climate has been characterized by the variability of the WAM, showing a succession of long lasting wet and dry periods. This climate variability has been particularly relevant in the Sahel, where a large scale drought during the 70s–80s has been followed by a partial recovery of precipitation at the turn of the twenty-first century (Trenberth et al., 2007). The main driver of the WAM variability at time scales from intraseasonal to multidecadal is the global ocean sea surface temperature (SST; Pomposi et al., 2015; Rodríguez-Fonseca et al., 2015).

The observed 40-day variability of the WAM is mainly related to SST anomalies in the Indian Ocean associated with the Madden-Julian oscillation, which trigger convection

disturbances traveling along the Equator and modulating the WAM precipitation (Pohl et al., 2009; Mohino et al., 2012).

The SST variability in the Tropical Atlantic is the main driver of the monsoonal circulation at the interannual time scales, through the land-sea thermal gradient which influences the meridional displacement of the precipitation belt, with the strongest impact on the Guinean coast (Polo et al., 2008; Losada et al., 2010). The Mediterranean Sea plays a role in modulating the interannual variability of the monsoonal precipitation over the Sahel, by feeding the convergence over the Sahel with moisture transported across Sahara (Fontaine et al., 2010; Gaetani et al., 2010). The WAM interannual variability is also remotely influenced by the SST variability in the Tropical Indian/Pacific Oceans, which may induce stationary waves propagating along the Equator and interacting over the Sahel (Rowell, 2001; Mohino et al., 2011b). These regional and remote connections are not stationary and are modulated at decadal and multidecadal time scales (Fontaine et al., 2011a).

The multidecadal variability of the WAM dynamics results from the combination of diverse low frequency global ocean signals (Mohino et al., 2011a). On the one hand, the warming of the Tropical Ocean, associated with global warming and positive phases of the interdecadal Pacific oscillation, favors dry conditions in the Sahel, through the inhibition of the tropical convection (Bader and Latif, 2003; Villamayor and Mohino, 2015). On the other hand, positive phases of the Atlantic multidecadal variability, by displacing northward the ITCZ, favor precipitation in the Sahel (Zhang and Delworth, 2006; Ting et al., 2009). The severe drought that affected the Sahel during the 70s–80s has been attributed to a negative Atlantic multidecadal variability phase, concomitant with a positive interdecadal Pacific oscillation phase, in a global warming context (Mohino et al., 2011a).

Other than to the SST forcing, the West African climate is highly sensitive to land surface conditions and processes. Vegetation-associated land surface processes have in West Africa the largest climate impact worldwide, especially in summer (Ma et al., 2013), and the Sahel shows the strongest soil moisture/climate coupling (Koster et al., 2006). In this context, it has been shown that the vegetation degradation has a role in the drought events in the Sahel, through the increase in albedo and the reduction of evaporation, leading to reduced net radiation and inhibited convection, respectively, which in turn weaken the monsoonal circulation (Xue, 2004).

Modeling the West African Climate

In the last 15 years, a big effort has been made to understand climate variability and change in West Africa. The African Monsoon Multidisciplinary Analysis program (AMMA; <http://amma-international.org/>), launched in 2002 and involving a number of research institutions in the international scientific community, was the first large scale coordinated program aiming to improve the understanding of the WAM system and its influence on the physical, chemical and biological environment, regionally, and globally. The AMMA community is still active to provide the underpinning science to assess the impacts of WAM variability on health, water resources, food security and

demography in the West African countries, and to define and implement monitoring and prediction strategies (Redelsperger et al., 2006). Specifically addressed to climate modeling issues, the WAM Modeling and Evaluation project (WAMME; Druyan, 2011) is an initiative designed to evaluate the performance of global and regional climate models (GCMs and RCMs, respectively) in simulating the WAM dynamics and associated precipitation.

In the context of the Coupled Model Intercomparison Project Phase 3 and 5 (CMIP3 (Meehl et al., 2007) and CMIP5 (Taylor et al., 2012), respectively, a World Climate Research Programme (WCRP, <http://www.wcrp-climate.org/>) standard experimental protocol for studying the output of coupled atmosphere-ocean GCMs, climate variability in West Africa is extensively studied, with promising but still unsatisfying results. Specifically, state-of-the-art climate models in both CMIP3 and CMIP5 exercises show low skill in simulating the observed WAM variability (amplitude, phases, and trends), and sizable uncertainties affect projections in the twenty-first century, ranging from dry to wet conditions in the Sahel (Biasutti, 2013). Although coupled models generally well reproduce the relationship between the regional atmospheric circulation and the monsoonal precipitation, during both the twentieth and the twenty-first century, the same models show discrepancies in future projections (Biasutti et al., 2009). Therefore, model shortcomings can be firstly related to the ability in reproducing the large scale mechanisms which influence the regional atmospheric circulation, and especially the teleconnections with the global SST teleconnections (Biasutti et al., 2009; Rowell, 2013). An important source of uncertainty in the modeling of climate change in West Africa is also the model responses to the direct and indirect CO₂ radiative forcing in the atmosphere: the former rapidly warms the continental surface, inducing a positive response in the WAM precipitation; the latter slowly warms the ocean surface, inducing dry conditions (Giannini, 2010). It has been shown that wet and dry model biases over West Africa may be related to an unbalanced model response to the direct and indirect CO₂ forcing (Gaetani et al., 2016). At a regional scale, limitations in the model representation of SST in the Tropical Atlantic (Roehrig et al., 2013), surface heat fluxes (Xue et al., 2010), vegetation feedback (Kucharski et al., 2013), land use (Bamba Sylla et al., 2016), and mineral dust atmospheric concentration (Tompkins et al., 2005) are sources of incorrect simulations of the temporal and spatial variability of the WAM precipitation. Finally, the coarse resolution typical of GCMs limits the model ability to simulate the intense and organized convection characterizing the WAM (Vellinga et al., 2016). The assessment of model performances is critical to understand the sources of errors and limit uncertainties, but an overall and objective evaluation is a particularly difficult task, because results may differ depending on the specific variable analyzed and the metrics used. In the CMIP5 archive, a discrimination in the model performances for the historical climate may be achieved, but uncertainty in the projections is not reduced when skillful models are selected (Rowell et al., 2016). This suggests that the underlying assumption relating the model shortcomings in simulating past, present and future climate in West Africa is incorrect, being the assumption that

the same modeled processes lead to errors in the simulation of the historical climate and uncertainty in projected change (Rowell et al., 2016). Therefore, further research, based on the understanding of the mechanisms that drive the errors and uncertainty in projected changes, is needed to discriminate model performances.

In the CMIP5 exercise, a specific effort had been devoted to climate prediction at decadal time scales (10–30 years), which is recognized as a key planning horizon in a socioeconomic perspective (Doblas-Reyes et al., 2013). Results demonstrate that the WAM variability at decadal time scales is influenced by both the global SST natural variability and the green-house gases (GHG) external forcing, and the prediction skill is highly model dependent (Gaetani and Mohino, 2013; Martin and Thorncroft, 2014; Otero et al., 2015). Specifically, highest skill models are characterized by the ability in reproducing the WAM connection with, primarily, the Atlantic multidecadal variability (Gaetani and Mohino, 2013) and, secondly, with the relative SST difference between the subtropical North Atlantic and the tropics and Mediterranean SST (Martin and Thorncroft, 2014).

In the framework of the Coordinated Regional Climate Downscaling Experiment (CORDEX, <http://www.cordex.org/>), a WCRP initiative for the assessment and comparison of RCM skills in diverse regions, CORDEX-Africa provides a set of state-of-the-art simulations and predictions for the West African climate at high resolution (Nikulin et al., 2012). The availability of reliable climate simulations at high spatial-temporal resolution is crucial for a robust assessment of climate impacts at regional scale, and the CORDEX-Africa exercise shows encouraging results for West Africa. The dynamical downscaling of GCMs, operated at higher resolution by the RCMs, leads to improvements in the simulation of the atmospheric circulation, temperature and precipitation climatology, as well as the occurrence of wet and dry spells, the frequency of heavy rain events, and the drought geographical distribution (Laprise et al., 2013; Buccignani et al., 2016; Buontempo et al., 2015; Diasso and Abiodun, 2015; Dosio et al., 2015), although the biases in the lateral boundary conditions provided by the driving GCMs may significantly affect the RCMs outputs (Laprise et al., 2013; Dosio et al., 2015). Being the GCM biases more pronounced over the Tropical Atlantic, the RCM performances are in general better over the Sahel than in the Guinean coast, which is more influenced by the local SST variability (Paxian et al., 2016). Uncertainties in the simulation of daily precipitation are also observed, mainly related to the diverse convection schemes utilized in the CORDEX-Africa models (Klutse et al., 2016). However, the spread in the individual model performances is substantially improved when the ensemble mean is computed (Klutse et al., 2016).

Recent Climate Change

After the devastating drought of the 70s–80s, West Africa is nowadays experiencing a partial recovery of precipitation, with a coherent increase in the annual rainfall in the Sahel (29–43 mm/year per decade in the period 1983–2010; Maidment et al., 2015). This recovery is characterized by a modification of the seasonal cycle, showing a delay of the monsoon retreat in the

Sahel (two days per decade in the period 1983–2010; Sanogo et al., 2015), and by a change in the rainfall regime, showing a decrease in the number of rainy days and an increase in the proportion of annual rainfall associated with extreme events (17% in the period 1970–1990 and 21% in the period 2001–2010; Panthou et al., 2014). This precipitation recovery is accompanied by a stable rainfall/vegetation trend (Hoscilo et al., 2015). The recent climate change is also characterized by modifications in terms of atmospheric circulation and surface temperature. The meridional overturning cell associated with the monsoonal circulation is shifted $\sim 1^\circ$ northward, with changes in the convection belt in West Africa and the subsidence over the Mediterranean region (Fontaine et al., 2011b). Moreover, an amplified warming of the Sahara desert is detected (Cook and Vizy, 2015), and the Saharan heat low shows an intensification (Lavaysse et al., 2015) with reduced desert dust emission in summer (Wang et al., 2015). The origin of this climate change signal in the Sahara region has been related to the direct radiative forcing of the increased CO₂ concentration (Gaetani et al., 2016) and to an augmented moisture availability in the lower troposphere over the desert, triggering a water vapor-temperature feedback (Evan et al., 2015). The changes in the regional atmospheric dynamics accompanies positive temperature anomalies and extremes in spring and summer in the Sahel (Fontaine et al., 2013; Russo et al., 2016). Using a network of 90 *in situ* observations in West Africa, Moron et al. (2016) found that the linear trends of annual mean maximum and minimum temperature equal respectively $+0.021^\circ\text{C}/\text{year}$ and $+0.028^\circ\text{C}/\text{year}$.

The debate on the origin of the recent precipitation recovery in West Africa and the associated modifications in the regional atmospheric dynamics is open and heated, and the positions may be conveyed into two main arguments. On the one hand, the recovery is ascribed to the northward migration of the ITCZ in response to the SST warming at end of the twentieth century, which was stronger in the Northern Hemisphere than in Global Tropical Ocean (Park et al., 2014). A role of the warming of the subtropical North Atlantic in providing the moisture to feed the monsoonal system has been identified (Giannini et al., 2013). On the other hand, a dominant role of the direct GHG radiative forcing is hypothesized, acting by warming the surface and increasing evaporation over the continental surface (Dong and Sutton, 2015).

Future Projections

In the CMIP5 exercise, a positive trend in the WAM precipitation results from the multi-model mean in the twenty-first century, though the individual model projections are characterized by a large spread (Biasutti, 2013). Indeed, about 50% of the model runs in the CMIP5 archive shows a robust positive trend, about 25% shows a robust decreasing trend, while the trend is negligible in the remaining 25% (Biasutti, 2013). In the models predicting wet conditions, these are related to the direct radiative effect of the increase in GHG concentration, leading to local increased evaporation and vertical instability (Hoerling et al., 2006; Giannini, 2010). On the contrary, models projecting dry conditions simulate reduced moisture transport and deep convection over land as a response to the global ocean warming,

which heats the troposphere and imposes stability (Held et al., 2005; Caminade and Terray, 2010). Therefore, the competition between the response of the land-atmosphere system to the local GHG radiative forcing, and the response mediated through the warming of the global SST, emerges as a key component of the West African climate change (Bony et al., 2013; Gaetani et al., 2016), and understanding the relative impact of these two diverse forcings represents a task of primary importance for the climate modeling community.

The future projection in precipitation simulated by climate models in the twenty-first century is not spatially homogeneous over the Sahel. Indeed, future wet conditions in central-eastern Sahel (east of $\sim 0^\circ\text{E}$) contrast with dry anomalies over western Sahel (west of $\sim 0^\circ\text{E}$), and these sub-regional trends are more robust than the trend simulated in the extended Sahelian belt (Monerie et al., 2012, 2013; Biasutti, 2013). The rainfall excess expected in central-eastern Sahel is mainly linked to a strengthening and northward shift of the meridional overturning circulation over West Africa, reinforcing the monsoonal flow, with a feedback in the lower levels from the increased temperature and evaporation associated with the GHG radiative forcing (Monerie et al., 2012). The projected dry spot over western Sahel is associated with a reinforcement of the African easterly jet and modifications in the overturning zonal circulation connecting the Indian and Atlantic Oceans, which result in anomalous subsidence on its descending branch over subtropical North Atlantic (Monerie et al., 2012). Moreover, this east-west anomaly dipole in precipitation is consistent with the recently observed long term intensification of the Saharan heat low (Lavaysse et al., 2015). The projected rainfall trends result to be gradually enhanced and extended in future scenarios with a global warming of $2\text{--}4^\circ\text{C}$ and beyond, showing an approximately linear amplification with no tipping points being reached (James and Washington, 2013; James et al., 2014). The twenty-first century evolution of the WAM precipitation simulated by a subset of the CMIP5 models is illustrated in **Figure 1**.

The WAM seasonal cycle is also affected by climate change in the twenty-first century. The projected precipitation increase in the central-eastern Sahel is characterized by a robust increase of the rainfall amounts in September–October (70% of the CMIP5 model runs; Biasutti, 2013). This results in a delay of the monsoon withdrawal, with a lengthening of the monsoon season (Monerie et al., 2016). The moisture transport dominates the water budget change in September, while the local recycling role is prominent in October (Monerie et al., 2016). Conversely, the drying of the western Sahel appears to be concentrated in June–July in 80% of the CMIP5 model runs (Biasutti, 2013). The future modifications in the WAM seasonal cycle are accompanied by coherent changes in the African easterly wave activity, showing a reduction in late spring and early summer and a large increase between July and October, although large differences exists in African easterly wave projections between high- and low-resolution models (Skinner and Diffenbaugh, 2014; Martin and Thorncroft, 2015).

In contrast to the uncertainties affecting the future projection of the West African rainfall, a broad consensus characterizes the

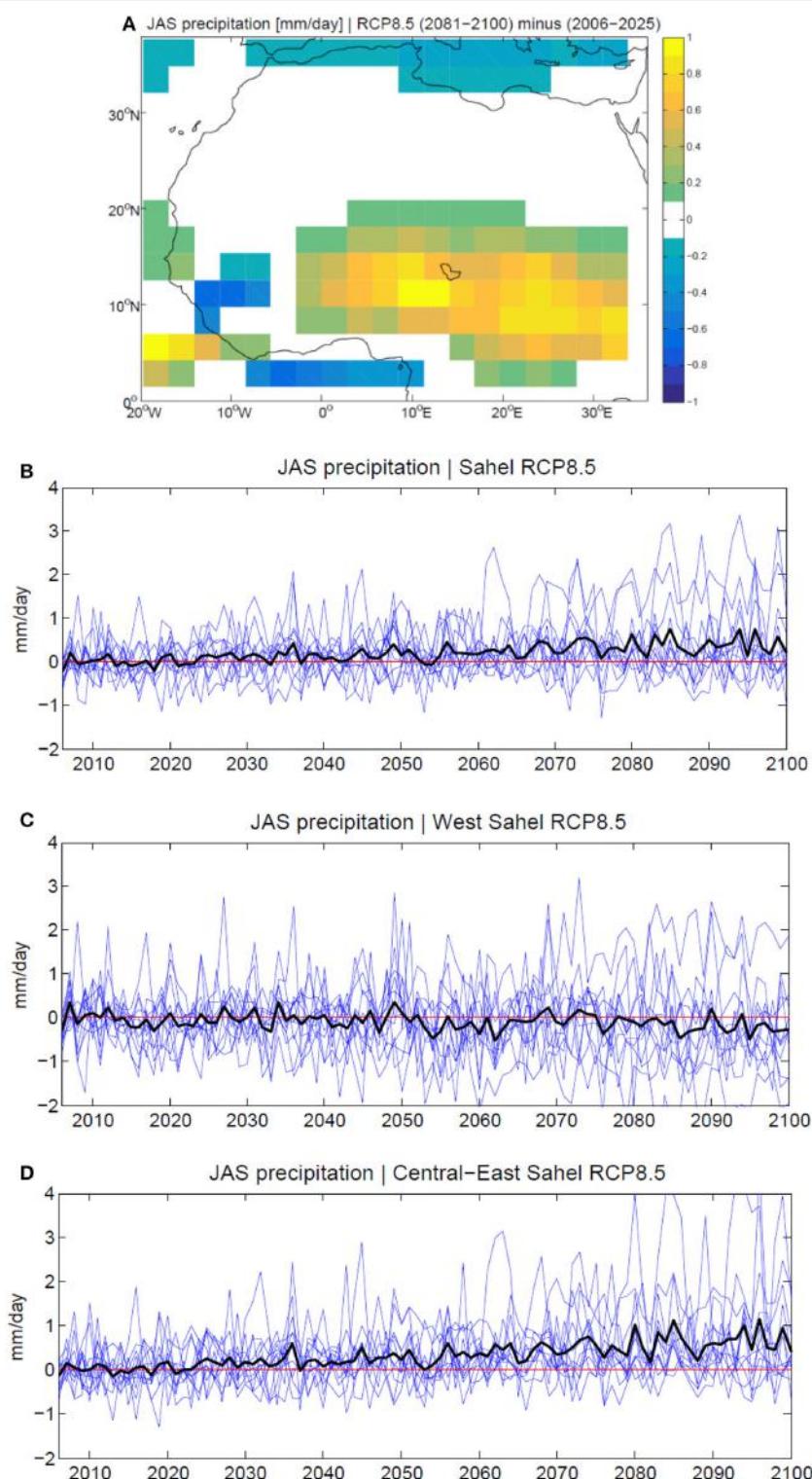


FIGURE 1 | WAM precipitation evolution in the twenty-first century, simulated by 12 CMIP5 models in the RCP8.5 scenario (van Vuuren, 2011). (A)

Projected change in multi-model mean of the July-to-September (JAS) precipitation [mm/day] at the end of the twenty-first century (2081–2100), represented by computing the difference with the period 2006–2025. Significance is estimated through a Student's *t*-test at 90% level of confidence. Time series of the WAM precipitation averaged in (B) Sahel [15°W – 30°E , 7 – 20°N], (C) western Sahel (west of 5°W) and (D) central-eastern Sahel (east of 5°E). The twenty-first century anomalies are computed regarding the period 2006–2015. The models analyzed are: BCC-CSM1-1, CanESM2, CCSM4, CNRM-CM5, FGOALS-g2, HadGEM2-CC, IPSL-CM5A-LR, IPSL-CM5B-LR, MIROC5, MPI-ESM-LR, MPI-ESM-MR, MRI-CGCM3. For data availability and accessibility, the reader may refer to the CMIP5 web portal at <http://cmip-pcmdi.llnl.gov/cmip5/availability.html>.

model simulations of the surface temperature for the twenty-first century. The future change in the monsoonal regime will be accompanied by a general warming of the African continent, with a maximum over the Sahara desert, ranging between 3 and 7°C, depending on the model and the emission scenario (Monerie et al., 2012; Dike et al., 2015). Boreal winter in West Africa will be also affected by a 2–3°C warming, with the strongest anomalies over the Guinea coast (Dike et al., 2015).

High resolution RCMs provide a detailed description of the future climate change in West Africa, generally agreeing with GCMs on the temperature projection in the region. A robust warming is predicted throughout the twenty-first century, although even large differences (more than 1°C) with the driving GCMs exist locally (Laprise et al., 2013; Dosio and Panitz, 2016). This will be accompanied, in the mid-twenty-first-century, by an increase in the number of heat wave days, by 20–120 days per year over the Sahel, by 20–60 days over Western Sahara, and by 5–40 days over eastern Sahara (Vizy and Cook, 2012). Moreover, half of the CORDEX-Africa projections suggests that heat waves that are unusual under present climate conditions in West Africa, will occur on a regular basis by 2040 under high emission scenarios (Russo et al., 2016). Finally, in the mid-twenty-first-century, daily maximum and minimum temperatures are projected to increase, and the daily diurnal temperature range to decrease, by 0.3–1.2°C during boreal spring and fall over West Africa, and by 0.5–1.5°C during boreal summer over the Sahel (Vizy and Cook, 2012).

The number of dry days is predicted to decrease by 3–7% over central Africa in spring and over eastern Sahel in summer. Conversely, the occurrence of extreme wet days will increase over West Africa by 40–60% (1–4 days) and the southern Sahel by 50–90% (1–4 days), uniformly during boreal summer. The associated changes in extreme wet rainfall intensity show a regional response, including a 30–70% decrease over northern Niger and northeastern Mali, and a 10–25% increase over Senegal, southern Mali, Burkina Faso, northern Nigeria, and southern Chad (Vizy and Cook, 2012). However, future RCM rainfall projections are affected by large uncertainties. On the one hand, RCMs tend to inherit the biases of the driving GCMs, so that a RCM downscaling several GCMs reproduces the inter-GCM spread, though with a reduced amplitude (Buontempo et al., 2015; Dosio and Panitz, 2016). On the other hand, a RCM may project its own trend regardless the inter-model spread of the driving GCMs, due to the differences in the specific physical formulation of RCMs and GCMs (Laprise et al., 2013; Buontempo et al., 2015; Saini et al., 2015).

Finally, it has been recently pointed out that the projected modification in the atmospheric dynamics over North Africa may impact the Saharan dust emission and atmospheric concentration, leading to a significant negative trend in the twenty-first century (Evan et al., 2016). Other than on human health in the region, expected to be benefitted, the reduction in dust concentration may have a positive feedback on the monsoonal precipitation, through a reduction in the associated surface cooling and lower troposphere heating, favoring atmospheric instability (Yoshioka et al., 2007; Ji et al., 2016).

THE IMPACT ON CROP YIELD AND POTENTIAL FOR ADAPTATION

Predicting Crop Yield from GCM Simulations

Crop Models

Predicting the potential impacts of climate change on crop yields requires a model of how crops respond to future conditions induced by anthropogenic climate change, such as: warmer temperatures, more frequent extreme temperatures, possible changes in rainfall mean, seasonality spatial and temporal distribution. In addition, there is a direct impact of atmospheric composition on crops with elevated levels of carbon dioxide acting to increase crop yields through the stimulation of photosynthesis and reduction of drought stress (Tubiello et al., 2007; Leakey, 2009) while elevated levels of atmospheric ozone which are expected in developing countries like Africa (Royal Society, 2008) can lead to yield losses (Van Dingenen et al., 2008). Crop models typically simulate the response of the crop to variability and change in weather and climate related to temperature, precipitation and radiation, and atmospheric CO₂ concentration (Ewert et al., 2015). There are numerous crop models with different levels of sophistication (Di Paola et al., 2016) and several reviews can be found in the literature, describing the concepts and limitations (see for instance Boote et al., 1996; White et al., 2011; Affholder et al., 2012; Ewert et al., 2015; Di Paola et al., 2016). Crop models can be roughly divided into two categories: statistical models trained on historical yields and some simplified measurements of weather, such as growing season average temperature and precipitation (Lobell and Burke, 2009) and process-based crop models which simulate explicitly the main processes of crop growth and development (see for instance Ewert et al., 2015). **Table 1** shows a selection of models that have been used to assess the impact of climate change on yields of various crops in West Africa. If the use of process-based models for climate change impact and risk assessment studies has become increasingly important (Tubiello and Ewert, 2002; Challinor et al., 2009; White et al., 2011; Rötter et al., 2012; Angulo et al., 2013; Ewert et al., 2015) since they are able to simulate impacts of climate, CO₂ concentrations on bio-physical processes (e.g., phenology, photosynthesis, respiration, transpiration, and soil evaporation) and other production constraints such as N limitations, these models require extensive input data on cultivar, management, and soil conditions as well as calibration and validation data that are often unavailable in Africa (Lobell and Burke, 2010). Even in the presence of such data these models can be very difficult to calibrate because of a large numbers of uncertain parameters (Iizumi et al., 2009; Tao et al., 2009). Furthermore, research effort in crop modeling has focused on the world's major food crops such as wheat, maize, rice, and sorghum and the simulation of crops common in African farming systems (sorghum, millets, yam) is less well developed as well as simulations of crops grown as intercrops across Africa (Challinor et al., 2007; White et al., 2011). Ensemble modeling including a variety of crop models is thus highly recommended to enable a quantification of the uncertainty (Challinor et al., 2009). In this context, extensive model intercomparisons such

TABLE 1 | A selection of crop models (including combination between crop models) that have been used to assess the impact of climate change on yields of various crops in West Africa in the recent scientific literature.

Crop model	Area	Crop	References
EPIC	Nigeria	Cassava, maize, millet, rice, sorghum	Adejuwon, 2006
Empirical	Niger	Millet	Ben Mohamed et al., 2002
EPIC + PHYGROW + NUTBAL	Mali	Cotton, cowpea, groundnut, maize, millet, sorghum	Butt et al., 2005
AEZ + BLS	Sub-Saharan Africa	Global	Fischer et al., 2005
IMPACT + DSSAT	Sub-Saharan Africa	Global, maize, millet, rice, sorghum, wheat, soybean, groundnut	Nelson et al., 2009
CERES – maize	West Africa	Maize	Jones and Thornton, 2003
CERES – maize + Empirical	Niger, Nigeria, Mali, Guinea, Ivory Coast, Cameroun	Maize	Lobell and Burke, 2010
GEPIC	Sub-Saharan Africa, West Africa	Global, cassava, maize, millet, rice, sorghum, wheat	Liu et al., 2008
Empirical	West Africa	Cassava, groundnut, maize, millet, rice, sorghum, wheat, yams	Lobell et al., 2008
LPJmL	West Africa	Global	Müller et al., 2010
MOS (empirical)	Benin	Beans, cassava, cotton, groundnut, maize, rice, sorghum, yams	Paeth et al., 2008
Empirical + BLS	West Africa	Global	Parry et al., 2004
DSSAT	Niger, Burkina Faso	Millet (two cultivars), sorghum	Salack, 2006
Empirical	West Africa	Cassava, groundnut, maize, millet, sorghum	Schlenker and Lobell, 2010
DSSAT	Gambia	Groundnut, maize, millet late, millet early	Smith et al., 1996
Cropsyst	Cameroon	Bambara nut, groundnut, maize, sorghum, soybean	Tingem and Rivington, 2009
Empirical	Niger	Cowpea, groundnut	Vanduivenbooden et al., 2002
SARRA-H + APSIM	West Africa	Sorghum (two cultivars)	Sultan et al., 2014
SARRA-H	West Africa	Millet (three cultivars), Sorghum (three cultivars)	Sultan et al., 2013
CROPGRO	Cameroon	Cotton	Gerardeaux et al., 2013
EPIC + GEPIC + LPJ-GUESS + pDSSAT + PEGASUS	Burkina Faso, Senegal	Maize, Wheat, Soybean, Rice, Millet, Sorghum, Sugarcane, Beans, Cassava, Cotton, Sunflower, Groundnut	Deryng, 2015
SARRA-H + EPIC	Niger, Benin	Maize, Millet	Ramarohetra et al., 2015
DSSAT	Niger	Millet	Rezaei et al., 2014
EPIC	Benin	Yam (early and late cultivars)	Srivastava et al., 2015
EPIC	Benin	Maize	Gaiser et al., 2010
ORCHIDEE	West Africa	C4 crop	Berg et al., 2013
GLAM	West Africa	Groundnut	Parkes et al., 2015
GEPIC	Sub-Saharan Africa	Maize	Folberth et al., 2014
DSSAT + APSIM	Senegal, Ghana	Maize, Millet, Peanut	Adiku et al., 2015
EcoCrop	Africa	Maize, millets, sorghum, banana, and beans	Jarvis et al., 2012

as the ones conducted throughout the Agricultural Model Intercomparison and Improvement Project (AgMIP; <http://www.agmip.org/>; Rosenzweig et al., 2014), which includes Sub-Saharan Africa as one of the target region (Adiku et al., 2015), are likely to improve substantially the characterization of the threat of crop yield losses and food insecurity due to climate change.

Link with Climate

The use of climate projections from GCMs to force crop models is challenging and raises several important issues. First, combining GCMs and process-based crop models raises a scale mismatch since climate models typically operate on spatial scales much larger than the processes governing the yields at the plot scale and most factors affecting crops such as soil properties and farming

practices (Baron et al., 2005; Challinor et al., 2009). To overcome this issue, climate data can be downscaled to the scale of a crop model with two types of downscaling approaches that can be sometimes combined (see for instance Zorita and von Storch, 1999). Statistical downscaling relies on the use of empirical relationships between mesoscale and local climate observed variables to relate GCM output to local climate (Zorita and von Storch, 1999). An alternative approach is the use of dynamical downscaling which offers a self-consistent approach that captures fine-scale topographic features and coastal boundaries by using regional climate models (RCMs) with a fine resolution (\sim 10–50 km) nested in the GCM (Paeth et al., 2011; Glotter et al., 2014). The use of dynamical downscaling in long-range climate projections has recently increased with the growth of computing

resources and large simulations databases of downscaled climate outputs are available for intercomparison and impacts assessment (Glotter et al., 2014). For instance the international Coordinated Regional Climate Downscaling Experiment Africa (CORDEX Africa) simulations are now publicly available and used in the literature, including a downscaled subset of GCMs simulations with different RCMs (Diallo et al., 2016). However, although it can improve weather and climate variability (Feser et al., 2011; Gutmann et al., 2012) as well as crop yield projections (e.g., Mearns et al., 1999, 2001; Adams et al., 2003; Tsvetsinskaya et al., 2003), it is important to keep in mind that downscaling is an additional source of errors and uncertainties to crop yield projections. For example, when different RCMs were used to downscale atmospheric re-analyses to force the SARRA-H crop model in Senegal, Oettli et al. (2011), large differences were found in the simulated sorghum yields depending on the RCM used. More recently, Ramarohetra et al. (2015) conducted a sensitivity analysis of the WRF model and found that a change in the physical parameterizations of a single RCM as well as internal variability of the RCM can lead to major changes in the simulation of crop yields of millet and maize in West Africa. As alternative to downscaling, the use of large-area crop modeling has grown in recent years (Challinor et al., 2004, 2009; Tao et al., 2009). This approach offers the possibility of using the outputs from climate models directly in a process-based way, suppressing the needs for downscaling, has grown in the literature (Challinor et al., 2004, 2009). Several models have been used in West Africa like the GLAM model used to simulate groundnut (Parkes et al., 2015) or LPJ-ml (Müller et al., 2010) and ORCHIDEE (Berg et al., 2011, 2013) which are part of Earth System vegetation models in which they account for tropical croplands.

The second issue raised by the use of GCM for assessing climate impacts is that climate models show significant biases in simulating current climate with sometimes insufficient skill for GCM outputs to be used directly as inputs for impact models without prior bias correction (Semenov and Barrow, 1997). If bias-correction is often included into statistical downscaling, the skill of representing the present-day climate can be very low using regional downscaling (Oettli et al., 2011). Since impact models ultimately rely on the accuracy of climate input data (Berg et al., 2010), the errors inevitably propagated into the combined climate/crop modeling (Oettli et al., 2011; Glotter et al., 2014; Ramarohetra et al., 2015). For instance, using two RCMs and the DSSAT-CERES-maize crop model over the United States, Glotter et al. (2014) showed that although the RCMs correct some GCM biases related to fine-scale geographic features, the use of a RCM cannot compensate for broad-scale systematic errors that dominate the errors for simulated maize yields. Moreover, Ramirez-Villegas et al. (2013) suggested that the use of raw GCM outputs can even affect the estimation of the climate change impact on crop yields by significantly under- or overestimate cropping system sensitivity by 2.5–7.5% for precipitation-driven areas and 1.3–23% for temperature-driven areas. Thus, careful evaluation of climate models using regional key drivers of crop yields (Berg et al., 2010; Ramirez-Villegas et al., 2013; Guan et al., 2015) is needed to make the best use of climate change simulations for impact research. Large errors have been found

in the simulation of the WAM rainfall by climate models which usually suffer from too much drizzle and a large bias in rainfall frequency, large errors in simulating seasonal rainfall as well as an underestimation of the interannual variability which can subsequently bias simulated crop yield (Baron et al., 2005; Berg et al., 2010; Ramirez-Villegas et al., 2013; Guan et al., 2015). Significant biases have also been found CMIP5 simulations for mean temperature and diurnal temperature ranges in West Africa (Ramirez-Villegas et al., 2013). To overcome this issue, climate impact studies generally require some level of climate data bias correction. The simplest correction method is the delta method used by Müller et al. (2010) or Sultan et al. (2013) which consists to add a computed mean annual anomaly between future and current simulated climates of a given GCM to a current observation-based dataset. Promising results are obtained by Oettli et al. (2011) when applying a more complex bias correction technique (Michelangeli et al., 2009) to climate model outputs. In particular the authors showed that means and standard deviations of simulated yields of sorghum in Senegal are much more realistic with bias corrected climate variables than those using raw climate models outputs.

Another important issue which has already been discussed in Section Climate Change Scenarios is the large plausible range of future climate changes at the regional scale of West Africa. Although there are some robust features in climate change scenarios in the region (see Section Climate Change Scenarios), there is a wide spread in current climate model projections of regional rainfall changes over West Africa, especially with respect to summertime rainfall totals (Druyan, 2011) which are crucial for yields of staple food crops in West Africa (Berg et al., 2010; Guan et al., 2015). Up to now, using the largest number of GCMs from the CMIP5 ensemble of around 36 GCMs remains the best way to represent the range of climate futures in impact assessment. Knox et al. (2012) showed that increasing the number of climate models used to force crop models reduces the median range and outliers about the mean change in future yields. Important biases or underestimation of uncertainties can be expected from climate impact assessments based on subsets of CMIP datasets, and similarly from downscaled or bias-corrected datasets (like CORDEX) which are based on a restricted subset of GCMs. This point is illustrated by McSweeney and Jones (2016) who investigated how well the widely used Inter-Sectoral Impact Model Inter-comparison Project (ISI-MIP) subset of five CMIP5 models (see for instance Adiku et al., 2015) represent the plausible range of future climate changes. They found that the fraction of the full range of future projections captured by the ISI-MIP subset is sometimes very low depending on the variable, the season and the region especially for summer rainfall and temperatures in the Western part of West Africa (McSweeney and Jones, 2016).

Assessing Climate Impacts

The Overall Signal

Although there is a growing literature on the impact of climate change on crop productivity in tropical regions, it is difficult to provide a consistent assessment of future yield changes because of large uncertainties in regional climate change projections, in the

response of crops to environmental change (rainfall, temperature, CO₂ concentration), in the coupling between climate models and crop productivity functions, and in the adaptation of agricultural systems to progressive climate change (Challinor et al., 2007; Roudier et al., 2011). These uncertainties result in a large spread of crop yield projections indicating a low confidence in future yield projections. As an example of the diversity of yield scenarios that have been produced, Roudier et al. (2011) found that the response of crop yield to climate in change in West Africa can vary from -50% to +90% in a selection of 16 publications. This range is even larger in the review made by Müller et al. (2010) which showed that projected impacts relative to current African production levels range from -100% to +168%. This range reflects the variety of regions, crops, climate scenarios and models and crop models chosen in the studies.

To identify the main sources of uncertainty and establish robust estimates of the aggregate effects of climate change on crop yields, meta-analyses were conducted at the global scale by Challinor et al. (2014) to contribute to the food security and food production systems chapter of the Fifth Assessment Report (AR5) of the IPCC and at the regional scale, including West Africa (Roudier et al., 2011; Knox et al., 2012). Meta-analyses that combine and compare results from numerous studies are widely used in epidemiology and medicine and can be a useful way of summarizing the range of projected outcomes in the literature and assessing consensus. The meta-analysis conducted by Challinor et al. (2014) used a data set of more than 1700 published simulations to evaluate yield impacts of climate change and adaptation which is the largest pool of data from diverse modeling studies ever used for a global synthesis of this kind (Rötter, 2014). The meta-analyses published by Knox et al. (2012) and Roudier et al. (2011) are based on a smaller data set (1144 and 347 published simulations respectively) but concern specific regions: Asia and Africa in database compiled by Knox et al. (2012) and only West Africa in the database compiled by Roudier et al. (2011). These latter two meta-analyses also include the response of relevant crops in Africa (maize, sorghum, millet, rice, cotton, cassava, groundnut, yam) while the meta-analysis conducted by Challinor et al. (2014) includes only major crops such as maize, rice and wheat; maize and rice being the only crops of the study grown in West Africa. Interestingly, while there are all based on different approaches and different samples, the three studies came out with similar conclusions on how climate change will affect crop yield in West Africa and how this response varies across the different assumptions and methodological choices. While the magnitude of the response of crop yield to climate warming scenarios varies considerably in the simulations reported by Challinor et al. (2014), Knox et al. (2012) and Roudier et al. (2011), the sign of the change is mostly negative with a mean yield reduction of -8% was identified in all Africa (Knox et al., 2012) and -11% in West Africa (Roudier et al., 2014). Maize was found to be the most affected crop in West Africa and in the Sahel by Knox et al. (2012). Without adaptation, the mean response of major crops (mostly maize and rice) to climate change depicted by Challinor et al. (2014) in tropical regions is a yield reduction.

This robust yield loss is already significant at moderate levels of local warming (+2°C) but is more consensual and stronger in the second half of the century when the additional radiative forcing is amplified. If this negative impact on crop yield was already depicted in the previous IPCC report, it suggested such yield loss would only occur when exceeding 3–4°C local warming which might be due to an overestimation in previous studies of the yield benefits of enhanced atmospheric CO₂ (Rötter, 2014).

Such robust evidence of future yield loss in West Africa also confirmed in previous review of the literature (Challinor et al., 2007; Kotir, 2010; Müller et al., 2010) can be surprising in regards to the diverging projections in a warmer climate of summer monsoon rainfall. This is because of the adverse role of higher temperatures in shortening the crop cycle duration and increasing evapotranspiration demand and thus reducing crop yields, irrespective of rainfall changes (Schlenker and Lobell, 2010; Roudier et al., 2011; Berg et al., 2013; Sultan et al., 2013). Potential wetter conditions or elevated CO₂ concentrations hardly counteract the adverse effect of higher temperatures (Sultan et al., 2014) while dryer conditions can strongly amplify the yield losses (Schlenker and Lobell, 2010; Roudier et al., 2011; Sultan et al., 2013, 2014).

Crop Model Differences

The response of the crop to climate change is subject to uncertainty that can arise from several sources (Challinor et al., 2009). In particular, significant differences were found in yield response from process-based vs. statistical models. Knox et al. (2012) and Roudier et al. (2011) both found that the dispersion around the mean is greater using process-based crop models. Furthermore, Challinor et al. (2014) found that statistical models predict a greater negative impact of climate on crop yields. The review of Müller et al. (2010) based on recent climate change impact assessments (14 quantitative, six qualitative) in Africa also stressed this larger dispersion with projected impacts relative to current production levels range from -84% to +62% in process-based and from -57% to +30% in statistical assessments. The larger dispersion of process-based crop models can be induced by the fact that they incorporate more complex factors in the yield response to climate change (CO₂ effect, rainfall distribution, extreme temperatures) but also that the lack of sufficient data for accurate calibration and validation (Lobell and Burke, 2010; Lobell et al., 2011) and site specific parametrization of the crop management options and cultivars (Müller et al., 2010) in developing countries such as in Africa increase uncertainty in the crop response. More recently, systematic intercomparison studies of climate change impacts in West Africa were conducted using five process-based crop models (EPIC, GEPIC, LPJ-GUESS, pDSSAT, and PEGASUS; see Deryng, 2015) and two process-based crop models (DSSAT and APSIM in Adiku et al., 2015; SARRA-H and APSIM in Sultan et al., 2014) using the same forcing climate datasets. They all found a general agreement in the sign of the crop yield response to climate change scenarios while the amplitude of the impact varied strongly across models and simulated crops.

Regional Differences

Important regional differences have been found in the response of crop yield to climate change. Roudier et al. (2011) found that cropped areas in the Soudano-Sahelian zone are likely to be more affected by climate change than those located in the Guinean zone. This difference can be explained by the projections of future climate in Africa which show a greater warming over continental Africa (particularly in the Sahel and Sahara) while the temperatures of the Guinean zone, which are influenced by the Atlantic Ocean, are expected to increase more slowly.

Using simulations of nine bias-corrected CMIP5 climate models and two crop models (SARRA-H and APSIM), Sultan et al. (2014) found a West-East dipole in the impacts of crop yield to climate change in West Africa. Indeed, in broad agreement with the full CMIP5 ensemble, their subset of bias-corrected climate models depicted a robust change in rainfall in West Africa with less rain in the Western part of the Sahel (Senegal, South-West Mali) and more rain in Central Sahel (Burkina Faso, South-West Niger) in the decades of 2031–2060 compared to a baseline of 1961–1990. In response to such climate change, but without accounting for direct crop responses to CO₂, mean crop yield of sorghum decreases by about 16–20% and year-to-year variability increases in the Western part of the Sahel, while the eastern domain sees much milder impacts. This West-East dipole is confirmed by the study of Deryng (2015) which uses a set of five global climate models and six different global gridded crop models to assess climate change impacts on crop productivity in semi-arid croplands by the 2030s under the RCP 8.5 scenario. Without including the effect of elevated CO₂ on crop photosynthesis and water demand, the author shows in Senegal, where three over five GCMs simulate drier conditions a median decrease of rainfed crop ($-8.5 \pm 9.9\%$) while in the Eastern part of West Africa in Burkina Faso, where four of the five GCMs simulate wetter conditions, the results show a slight decrease ($-3.9 \pm 4.3\%$). This dipole was also found in the study of Adiku et al. (2015) which used DSSAT and APSIM to simulate climate change impacts on crop yields in two locations in Nioro (Senegal) and Navrongo (Ghana). The effect of climate change was higher in the Senegalese site than in the one in Ghana using both crop simulation models.

The Effect of Elevated CO₂

If rising atmospheric CO₂ concentrations directly contributes to climate change, it has the potential to increase crop water productivity by enhancing photosynthesis and reducing leaf-level transpiration of plants (Tubiello et al., 2007; Leakey, 2009; Deryng et al., 2016). Significant increases of crop yield due to elevated levels of CO₂ have been reported in experiments for different crops (Kimball, 1983; Kimball et al., 2002) and most of the recent modeling studies simulate the effect of elevated CO₂ (Deryng et al., 2016). However, there is an ongoing debate about the extent of impacts of CO₂ fertilization on crop yields in observations and models (Long et al., 2006; Ainsworth et al., 2008), especially in Africa where few field observations are unavailable to validate and further improve the models. In particular there is no free air carbon dioxide enrichment (FACE) experiments in Africa. Yet, the impact of higher atmospheric

CO₂ concentration is a major source of uncertainty in crop yield projections (Soussana et al., 2010; Roudier et al., 2011). For instance, by conducting a systematic comparison between yield response to climate change with, or without, CO₂ fertilization effect, Müller et al. (2010) found a yield increase of 8% in Africa (percent change in 2046–2055 relative to 1996–2005) with full CO₂ fertilization, and a yield loss of -8% without the CO₂ effect. More recently, Deryng (2015) found that simulated median yield of rain-fed crops in six countries of semi-arid areas (including Senegal and Burkina Faso in West Africa) increases by $4.7 \pm 9.6\%$ when including the effects of both climate change and elevated CO₂ concentrations while median yield decreases by $4.5 \pm 7.3\%$ when excluding the effects of elevated CO₂ concentrations. Sultan et al. (2014) also found that CO₂ fertilization would significantly reduce the negative climate impacts, increasing sorghum yields on average by 10%, and drier regions would have the largest benefits. However, other studies show lower differences between full and no CO₂ fertilization scenarios (Berg et al., 2013). Overall most studies conclude that benefits of elevated CO₂ will be greater for C₃ crops (e.g., soybean, groundnut) which are likely to accumulate more biomass and for C₄ crops in arid regions through increased water use efficiency (Berg et al., 2013; Sultan et al., 2014; Deryng et al., 2016). However, while showing benefits of higher CO₂ concentrations on water crop productivity, Deryng (2015) and Sultan et al. (2014) both show that it partially offsets the impacts from climate changes especially in the Western part of Africa where yield losses are expected even after accounting for CO₂ fertilization effect. Deryng (2015) found a decrease of crop yield of groundnut, millet, sorghum, and maize in Senegal by the 2030s even when including the effects of CO₂. The author also found a slight increase of crop yield of millet and sorghum in Burkina Faso when including CO₂ but yield of groundnut and maize decreases. Moreover, even if we can expect benefits from increasing CO₂ on crop productivity, nutritional value may nevertheless be compromised (Müller et al., 2014). Indeed, a meta-analysis conducted by Myers et al. (2014) demonstrated that CO₂ fertilization is likely to have adverse effects on the nutritional value of many key food crops by reducing the concentrations of essential minerals and protein with potential serious consequences in food security (Müller et al., 2014).

Adaptation Studies

Despite large uncertainty, there is a robust conclusion from the above section: agriculture in West Africa is at risk to be negatively affected by climate change. These potential adverse negative climatic changes effects are superimposed on top of high natural variability in seasonal rainfall, which historically has produced large inter-annual variations in rainfall and prolonged droughts (Giannini et al., 2008) and the recent increase in rainfall intensity and extreme heavy-rainfall events (Pantheou et al., 2014). Both climate variability and trend pose a challenge for the primarily rain-fed agriculture systems in West Africa. Since the 1970's, the largest food crises in Africa that required large-scale external food aid (1974, 1984/1985, 1992, and 2002) have been attributed fully or partially to extreme weather events (Dilley et al., 2005). Thus, any successful adaptations should be able to cope with

the short-term climate variability as well as reduce the negative impacts of climate change in the long term (Saba et al., 2013; Lobell, 2014). Hertel and Lobell (2014) distinguished between three categories of adaptation: (i) adaptation options based on current technology which can also identified as autonomous adaptation, (ii) adaptation involving a new technologies, and (iii) adaptations involving the institutional environment within which the producer is operating such as markets and policy and resulting from planned adaptation. Adjustments in planting and harvesting dates, varieties of crops to be grown (including combination between crops and cultivars as intercrop or the use of existing varieties more resistant to climate-induced stress), increase planting density and/or fertilizers use, use of crop residue as mulch are examples of options already available to farmers in West Africa to adapt to climate variability and change. Breeding more resilient crop varieties (Rötter et al., 2015), advanced breeding methods including more effective root system size, dehydrin genes, phenotyping (Araus et al., 2012; Setter, 2012; Vadez et al., 2012; Amelework et al., 2015); innovating water harvesting techniques (Lebel et al., 2015; Rockström and Falkenmark, 2015) belong to the second category of adaptation options. In the third category defined by Hertel and Lobell (2014), fertilizer subsidies, crop insurances (Berg et al., 2009), credits, climate services (access and use of weather and seasonal forecasts; Sultan et al., 2010; Roudier et al., 2012, 2014, 2016) are such important changes in the institutional and market environment of West Africa that would affect producer decisions. Assessing various possible adaptation options and their uncertainties is crucial for optimal prioritization of adaptation investments for supporting adaptation strategies in West Africa that may counteract the adverse effects of climate change. However, pointing out the most promising adaptation options remains challenging since there is a large scatter of possible results across locations and situations, indicating the need for a more contextual approach on regional and local scales (Challinor et al., 2014). We will thus give some examples of some recent studies who quantified the potential of adaptation for major crops in West Africa showing sometimes apparent contradictory and crop-specific results.

Millet and Sorghum

These two crops are among the main staple crops of sub-Saharan West Africa (64% of the total cereal production in 2000; FAOSTAT, 2012 data). On-farm surveys have shown the dominance of traditional cultivars of sorghum and millet characterized by a strong sensitivity to photoperiod (Traore et al., 2011). Photoperiod sensitivity would likely present some advantages in the event of future change in the timing of the rainy season. Indeed, it allows for flowering at the end of the rainy season for a wide range of planting dates and avoids incomplete grain filling, a problem for late maturing varieties faced with water shortage at the end of the rainy season (Dingkuhn et al., 2006). Furthermore, Sultan et al. (2013) found that traditional photoperiod-sensitive cultivars are less affected by temperature increase since the photoperiod limits the reduction of the crop duration. On the opposite, adverse impacts of climate change have been found to be the lowest on mean yield and yield

variability for photoperiod-insensitive cultivars, as their short and nearly fixed growth cycle appears to be more resilient to the seasonality shift of the monsoon, thus suggesting shorter season varieties could be considered a potential adaptation to ongoing climate changes (Sultan et al., 2014). This result is consistent with the study from Kouressy et al. (2008), which demonstrated that potentially high-yielding and photoperiod-insensitive cultivars display an advantage where the rainy season is short. Modeling studies (Turner and Rao, 2013; Sultan et al., 2014) suggest that while increasing fertilizer inputs and restoring nutrients imbalance in low-input, smallholder, sorghum farmers of Africa would increase overall food production and have fundamental benefits increasing food security (Vitousek et al., 2009), the trade-off is that it would increase the sensitivity of those systems to climate variability and increase adverse impacts of climate change.

Several studies also investigated new technologies for mitigating the adverse impacts of climate change on millet and sorghum production. Adiku et al. (2015) used two crop models DSSAT and APSIM to simulate millet cultivars adapted to future climate conditions. They found positive effects on crop yield whereas the benefits depend on the location, the crop and the climate model used for the simulation. Sultan et al. (2013) also found advantages of breeding varieties with higher thermal requirements which can partly counteract the shortening of crop-cycle duration in a warmer climate. Guan et al. (in press) used two crop models APSIM and SARRA-H to assess five possible and realistic adaptation options for the production of sorghum (late sowing, increase planting density and fertilizer use, increasing cultivars' thermal time requirement, water harvesting, and increase resilience to heat stress during the flowering period). They found that most proposed adaptation options are not more beneficial in the future than in the historical climate so that they do not really reduce the climate change impacts. Increased temperature resilience during grain number formation period is the main adaptation that emerges from this study.

Maize

Maize is the most important staple food and accounts for nearly 20% of total calorie intake in sub-Saharan Africa (SSA) (FAOSTAT, 2012, data). In their meta-analysis, Challinor et al. (2014) compared the effect of climate change on maize yields in the Tropics with and without adaptation; adaptation options including changes in planting dates, fertilizer use, irrigation, cultivar or other agronomic options. They concluded that in contrast to what has been published for wheat and rice in the temperate latitudes, there is no effect of adaptation in the Tropics and little evidence for the potential to avoid yield loss in maize yield since the varieties of crop grown are already adapted to high temperatures. Similar results were also found by Deryng et al. (2011) who reported substantial yield losses in developing countries located in the Tropics for maize even after allowing for adjustment of planting dates and varieties grown. Using simulations from the GEPIC model in Sub-Saharan Africa, Folberth et al. (2014) investigated different intensification options for growing maize under climate change. They found that intensive cultivation is predicted to result in lower yields

under future climate conditions and increased soil erosion while eco-intensification shows better yields. However, yield losses are simulated in all management scenarios toward the end of the century suggesting a limited effect of eco-intensification as a sole means of adapting agriculture to climate change. Finally, promising results of rainfall harvesting have been found by Lebel et al. (2015) which found that applying this technique to maize cultivation across Africa could mitigate 31% of yield losses attributable to water stress and increase maize yields by 14–50% on average under the projected climatic conditions of the 2050s.

Groundnut and Yam

Groundnut is an important crop for Nigeria, southern Mali, Ivory Coast, Burkina Faso, Ghana, and Senegal. Parkes et al. (2015) investigated the benefits of breeding cultivars of groundnuts with heat and water stress resistance as well as the potential of marine cloud brightening to reduce the rate of crop failures in West Africa using the GLAM model. The authors found that climate change will increase mean yields of groundnut and reduce the risk of crop failure in West Africa. This projected increase in yields is due to the carbon dioxide fertilization effect also to increased seasonal rainfall in the unique GCM simulation used in this study. Parkes et al. (2015) investigated the benefits of breeding cultivars of groundnuts with heat and water stress resistance as well as the potential of marine cloud brightening to reduce the rate of crop failures in West Africa. They found that water stress, rather than heat stress, is the main cause of crop failure in current and future climate and also demonstrated a positive impact of marine cloud brightening.

Yam is the second most important crop in Africa in terms of production after cassava. Srivastava et al. (2015) simulated the advantages of specific adaptation strategies using the EPIC model. They found that changing solely sowing date may less effective in reducing adverse climatic effects than adopting late maturing cultivars. Yet, combining different options such as coupling irrigation and fertilizer application with late maturing cultivars, highest increase in the yields could be realized.

Cassava

Using the EcoCrop model to investigate the response of important staple food crops for Africa including maize, millets, sorghum, banana, and beans to climate projections by 2030, Jarvis et al. (2012) found that cassava reacted very well to the predicted future climate conditions compared to other crops. Whilst most simulated crops in Africa were predicted to experience decreases in overall suitability in Africa, cassava always outperformed or (in the worst case) equaled the average and appeared as a highly resilient staple crop. Crop improvements toward greater drought tolerance and heat tolerance in localized pockets of West Africa and the Sahel could bring some additional benefits.

SUMMARY AND CONCLUSIONS

In this paper, an extensive review of the recent literature on the West African climate and impacts is used to draw a general picture of the main features of the regional climate,

the associated observed variability, the future change as well as expected impacts and potential for adaptation in the agriculture sector.

The dominant role of the WAM in determining the regional climate is highlighted, and the importance of the global SST in driving the multi-time scales variability is described (Rodríguez-Fonseca et al., 2015). In particular, the relationship of the WAM precipitation variability with the tropical ocean SST at the interannual time scales (Rowell, 2001; Polo et al., 2008; Losada et al., 2010; Mohino et al., 2011b), and with the extratropical ocean SST at multidecadal time scales (Zhang and Delworth, 2006; Ting et al., 2009; Mohino et al., 2011a; Villamayor and Mohino, 2015), is illustrated. The long lasting wet phase characterizing the Sahelian precipitation in the twentieth century up to the 70s, and the following severe drought affecting the Sahel culminating in the 80s, have been related principally to the SST variability associated with the Atlantic multidecadal variability (Mohino et al., 2011a). At the turn of the twenty-first century, the Sahel experienced a slight recovery of precipitation (Panthou et al., 2014; Maidment et al., 2015; Sanogo et al., 2015), but the attribution of this recovery is still debated. On the one hand, it is attributed to the differential warming between extratropical and tropical SST in the Northern Hemisphere, favoring the northward displacement of the ITCZ (Park et al., 2014). On the other hand, the recovery is attributed to the regional radiative warming produced by the CO₂ direct forcing, inducing a thermodynamic feedback on the monsoon system (Dong and Sutton, 2015). The rainfall recovery has been characterized by a modification of the precipitation regime, with higher intensity rainfall events concentrated in less rainy days (Panthou et al., 2014). Moreover, a widespread warming of the North African subcontinent, and an increase in the occurrence of climate extremes, such as heat waves ad hot summers, has been observed (Fontaine et al., 2013; Moron et al., 2016).

The same tendencies in temperature, precipitation and climate extremes are projected in the twenty-first century, in all the moderate-to-high emission scenarios, with the amplitude of the climate change signal growing proportionally with the projected global warming. The intensification of the hydrological cycle in the recent decades and in future projections has also been detected in the world's dry and wet regions, leading to an increased risk of flooding in dry regions as the climate warms (Donat et al., 2016). However, the future projections of the West African climate are affected by large uncertainties, especially regarding the monsoonal precipitation. Indeed, although around 50% of the CMIP5 GCMs agrees on the future positive trend, around 25% of the models project the opposite situation, weakening the prevision (Biasutti, 2013). The origin of this uncertainties is two-fold. On the one hand, the biases characterizing the SST simulated by the atmosphere-ocean climate models, which affect the mechanisms driving the multidecadal variability of the WAM system (Roehrig et al., 2013; Rowell, 2013). On the other hand, the diverse sensitivity of climate models to the effect of the projected increase in CO₂ concentration, which induces wet anomalies through the direct radiative warming of the surface at the regional scale, but at the same time inhibits the precipitation when the radiative forcing is

mediated by the global SST warming (Bony et al., 2013; Gaetani et al., 2016). Climate modeling of West Africa at the regional scale shows promising improvements of the GCM performances, although large uncertainties still persist. Firstly, RCMs are inevitably affected by the biases of the driving GCMs (Dosio et al., 2015). Secondly, RCMs experiments show high sensitivity to the physical parametrization, especially regarding convection (Klutse et al., 2016), which is crucial for the simulation of the monsoonal rainfall. Therefore, the climate modeling community is pushed for a further effort to improve the modeling of West African climate, in the direction of both understanding the physical mechanisms and reducing the climate model shortcomings.

There are many complex processes that drive the response of crop yield to such climate changes. These processes can act in a competing way as we can expect from the role of increased atmospheric CO₂ concentration which increase crop yield while warmer mean temperatures are likely to lead to crop yield losses. Such processes can interact together and their importance might depend on the region, the scale and the crop. The complexity of the risk posed by climate change and possible adaptation strategies have called for a number of climate change assessment studies especially in Africa where this risk can severely affect food security and impede development. Despite a large uncertainty in the published results and diverging future projections of summer monsoon rainfall which is key for rain-fed agriculture, a robust evidence of yield loss in West Africa emerges from these studies. This yield loss is mainly driven by increased mean temperature while potential wetter conditions as predicted in Central Sahel or elevated CO₂ concentrations for C3 crops and C4 crops in the arid zones of the Sahel can partly or totally counteract this effect. On the opposite, yield losses will be the highest for C4 crops in the Soudano-Sahelian zones and in areas where rainfall is expected to decrease like in the Western part of the Sahel. Identifying the most promising adaptation options is even more uncertain since uncertainty about climate impacts is then cumulated with uncertainty about the effectiveness of adaptations. Most adaptation options illustrated in this review are implemented in process-based crop models to adjust cropping systems (change in varieties, sowing dates and density, irrigation, fertilizer management) to future climate. Results of the cited studies are crop and region specific and no clear conclusions can be made regarding the most effective adaptation options.

Although substantial progress has been made in the assessment of the effect of climate change on crop yield and potential for adaptation in West Africa, large gaps still exist. Important processes like the effect of heat stress or ozone are missing in crop models (Ewert et al., 2015), most effort on model development and intercomparison are biased toward major crops in temperate regions and the African region generally suffers from a lack of sufficient data for accurate calibration and validation of crop models (Lobell and Burke, 2010). Furthermore, specific crop management options and cultivars of low intensive systems as mainly found in West Africa (mulching, species mixtures, intercropping and reduced tillage technologies) are not well represented in crop models (Hertel and Lobell, 2014; Ewert et al., 2015). If recent progress has been made to quantify the

potential for adaptation in integrated assessment and modeling approaches linking biophysical and economic models (Patt et al., 2010; Ewert et al., 2015), these approaches are built on assumptions which are more appropriate for the high income and developed countries with high adaptive capacity. Hertel and Lobell (2014) concludes that they present a risk to underestimate the impacts of climate change in the Tropics and a risk of overstating the efficiency of adaptations in regions like Sub-Saharan Africa.

As suggested by Challinor et al. (2009), an objective quantification of impacts uncertainty is a necessary step to go beyond syntheses or meta-analyses of published studies with large heterogeneity resulting from inherently uncoordinated studies. Large ensemble of climate simulations, downscaling techniques and crop simulation ensembles including different modeling approaches and sensitivity analyses are necessary for improved understanding of how climate uncertainties and errors propagate into impact estimates, a better quantification of crop model uncertainty as well as a better quantification of downscaling and bias-correction uncertainty (Ramirez-Villegas et al., 2013). In this respect, coordinated efforts such as the AgMIP initiative which aims to improve agricultural models including biophysical and socio-economic approaches at various scales and develop common protocols to systematize modeling for the assessment of climate change impacts on crop production represents a promising way toward more robust results (Rötter, 2014). While they are crucially lacking in Sub-Saharan Africa, observations are also a key to go forward in the quantification of uncertainty and possible reduction of its range. Most modeling work on climate impacts assessment needs quality data to validate and bias-correct climate simulations, calibrate, validate and force crop models or evaluate cropping systems adaptation. Improvement of quality, accessibility of data (including weather, soil, on-farm and experimental crop data, socio-economic data) as well as support for maintaining data over time and collecting long-term time series is of high importance in Sub-Saharan Africa. Finally, if there is evidence that farmers and farming systems are highly resilient to environmental changes, adaptation to climate change needs to be supported and facilitated by governmental, institutional and macro-economic conditions (Challinor et al., 2007). Adaptation to climate change cannot be achieved without a considerable institutional and political commitments for technical support or access to credit for instance (Thornton et al., 2011) and many of institutional, economic, informational, and social constraints are still ignored in modeling approaches of adaptation (Hertel and Lobell, 2014) which need to better account for both the biophysical and socio-economic determinants and specificities of agricultural systems in Africa.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Virtual Plants Need Water Too: Functional-Structural Root System Models in the Context of Drought Tolerance Breeding

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Developing a sustainable agricultural model is one of the great challenges of the coming years. The agricultural practices inherited from the Green Revolution of the 1960s show their limits today, and new paradigms need to be explored to counter rising issues such as the multiplication of climate-change related drought episodes. Two such new paradigms are the use of functional-structural plant models to complement and rationalize breeding approaches and a renewed focus on root systems as untapped sources of plant amelioration. Since the late 1980s, numerous functional and structural models of root systems were developed and used to investigate the properties of root systems in soil or lab-conditions. In this review, we focus on the conception and use of such root models in the broader context of research on root-driven drought tolerance, on the basis of root system architecture (RSA) phenotyping. Such models result from the integration of architectural, physiological and environmental data. Here, we consider the different phenotyping techniques allowing for root architectural and physiological study and their limits. We discuss how QTL and breeding studies support the manipulation of RSA as a way to improve drought resistance. We then go over the integration of the generated data within architectural models, how those architectural models can be coupled with functional hydraulic models, and how functional parameters can be measured to feed those models. We then consider the assessment and validation of those hydraulic models through confrontation of simulations to experimentations. Finally, we discuss the up and coming challenges facing root systems functional-structural modeling approaches in the context of breeding.

Keywords: functional structural plant model, drought, phenotyping, root system architecture, plant development and physiology, breeding

INTRODUCTION

At the advent of the Green Revolution in the 1960s, the world population was numbered at 3 billion people. Roughly 50 years later, it reached 7 billion. According to the 2015 revised world population prospects of the United Nations, median estimates place world population at almost 10 billion by the year 2050 (esa.un.org; Lee, 2011). Feeding the current and coming world is a key challenge,

and is strongly conditioned by the possibility of extending the practices of the Green Revolution in developing world regions such as Sub-Saharan Africa. Agricultural production worldwide is facing rising multifactorial problems among which are increasing pressure on arable lands, decreasing soil qualities, rising cost of fertilizers and energy, and climatic change. Regarding this last point alone, it is expected that changes in meteorological pattern (precipitation and temperature) will result in decreasing the mean yields of all crops (Knox et al., 2012) especially due to drought, one of the mains constraints for crop productivity (Lynch et al., 2014). This in turn will adversely impact food security in regions where the bulk of the population is coping with chronic hunger and malnutrition (Schmidhuber and Tubiello, 2007; Lobell et al., 2008; Lobell and Gourdji, 2012).

One way for breeders to deal with emerging drought episodes is to create cultivars with improved drought tolerance. This is particularly critical for the subsistence crops used in developing countries where people are almost completely reliant on the crop effective adaptive capacity for their sustenance (Sultan et al., 2013). One particular target of interest in this context is the plant root system. Roots are the organs responsible for the uptake of nutrients and water in the soil. Their efficiency depends on several factors, the main one being their spatial organization within the soil, or root system architecture (RSA) (Den Herder et al., 2010; Draye et al., 2010). The RSA is the result of the interaction between the genetic programming of developing roots and their response to a specific growth environment (Orman-Ligeza et al., 2014). Consequently, RSA developmental plasticity is of major importance as it determines the plant adaptability to environmental constraints such as those of drought-subjected environments (Tuberosa et al., 2002a,b; Lynch, 2007; Draye et al., 2010; Tardieu, 2012; Leitner et al., 2014b). Another important aspect of roots that has been largely overlooked is their functionality and in particular hydraulic processes that facilitate water transport and may explain a number of critical phenotypes, measured at the shoot level, for drought adaptation (see Vadez, 2014).

Historical breeding programs have been mainly focused on visible and easily quantifiable traits such as grain production, shoot biomass, or resistance to diseases and pests, all accessible traits from aboveground parts of the plant (Paez-Garcia et al., 2015). Breeding programs emerging from the Green Revolution have not directly focused on the root system. As root architecture was shown to be positively correlated with plant productivity (Lynch, 1995; Kell, 2011; Hufnagel et al., 2014), there is an increasing interest in developing plant breeding programs directed at “improving” RSA and developing new cultivars with higher soil resources exploitation efficiency or better tolerance to environmental stress such as drought (Wasson et al., 2012). For example in sorghum, QTLs for nodal root angle have been identified and shown to co-locate with QTL for traits related to drought adaptation (Singh et al., 2010, 2012; Mace et al., 2012). However, carrying out root traits-based breeding calls for identifying root-specific phenes related to the optimization of soil exploration and water and nutrient uptake in various environments (Lynch et al., 2014). A prerequisite for this is to be able to phenotype and to select desirable root parameters

(Paez-Garcia et al., 2015). However, root systems have long been neglected in breeding programs specifically because they are hard to phenotype. The rising interest in root breeding has thus sparked the development of a wide spectrum of root phenotyping techniques covering a large panel of growth conditions (Paez-Garcia et al., 2015). Those techniques can be used to characterize and quantify root growth and development parameters necessary for breeding programs. An example of such technique in the scope of drought research are lysimetric systems, where plants are grown in large tubes offering space and soil volume similar to field conditions. There, roots are not extracted destructively but plant water extracted by the roots—i.e., their functionality with regards to water—can be dynamically monitored throughout the crop cycle (Vadez et al., 2014).

In addition to the emergence of numerous varied phenotyping systems for roots, traditional breeding approaches can now also benefit from the mechanistic understanding coming from the field of functional-structural plant modeling (Xu et al., 2011). Mechanistic modeling approaches offer the possibility to integrate knowledge of plant development and physiology and to assess it against varied environment, leading to more reliable breeding (Lynch, 2013, 2015; Lynch et al., 2014). Functional-structural plant model (FSPM) approaches focus on the modeling of development, growth and function of all parts of the plant (cells, tissues, organs...) at different level of details in their spatio-temporal context. FSPMs are models that rely on an explicit and accurate description of the considered plant structure, and their efficiency is consequently tightly linked to the progress of phenotyping techniques. FSPM link the structure of the plant to its physiological processes, which are themselves driven by environmental factors (Godin and Sinoquet, 2005; Vos et al., 2007, 2010). The development of FSPMs is interdisciplinary by nature and uses various concepts, tools and software originating from a wide range of disciplines (DeJong et al., 2011). It can involve scientists with backgrounds ranging from plant physiology, plant development, soil science, mathematics, computer science, cellular biology, physics, to ecology and agronomy. For instance, to encode multiscale plant architecture, Godin and Caraglio (1998) used nested graphs which originated from mathematics and have been extensively used in others fields such as economy, networks and telecommunications, genetics and physics. Beyond encoding the plant structure, this multiscale formalism can also be used to simulate the development of plant architecture (Boudon et al., 2012; Ong et al., 2014).

Using FSPM to model the behavior of a crop root system can help understanding the extent of the impact of RSA on a given physiological process (DeJong et al., 2011). *In silico* approaches offer the advantage of fully mastering the studied system and allow to accurately assess the influence of each parameter on its functioning through sensitivity analysis (Han et al., 2012). FSPM have notably been used to simulate and study the development of plants in the context of water acquisition and transportation (Doussan, 1998; Roose and Fowler, 2004a; Doussan et al., 2006; Javaux et al., 2008; Couvreur et al., 2012; Lynch et al., 2014). In a breeding context, FSPMs can be very useful as they use a reverse-engineering approach to identify plant mechanisms likely to be beneficial under specific stress environment scenarios.

In this review, some examples will be covered showing how crop simulation models can predict the effect of certain rooting traits on crop performances across time and geographical scale (e.g., Vadez et al., 2013; Kholová et al., 2014). FSPMs can also serve as a basis for the development of ideotypes by highlighting the parameters most likely to influence the adaptability to environmental constraints (Lynch, 2013; Lynch et al., 2014).

We focus here on the design of FSPMs that can be used in the broader context of research on root-related drought tolerance. First we will present the different phenotyping techniques existing for root architectural and physiological study and their limits, and will go over the root traits of interest for breeders. We will then present the integration of the generated data within architectural models, and how those data-driven architectural models can be coupled with functional hydraulic models useful for breeding studies. Finally we will discuss the assessment and validation of FSPMs hydraulic models through confrontation of simulations to experimentations.

ROOT SYSTEM PHENOTYPING METHODS

Designing a functional-structural plant model (FSPM) presupposes to gather data related to plant structure and physiological processes that will serve as inputs to feed the model (DeJong et al., 2011). Plant phenotyping is the process of identifying and recording qualitative and quantitative traits that are depicting plant development and its functional aspects at different levels of organization (cell, tissue, organ, whole-plant scale) (Granier and Vile, 2014). Phenotyping strategies include skills and techniques that allow monitoring plant development and its response to different growth conditions in order to describe a full architectural and/or physiological outline in time and space. Many phenotyping techniques ranging from laboratory and greenhouse to field-based methods have been developed over the recent years (Paez-Garcia et al., 2015) and while most of them were applied to plant shoots (Berger et al., 2012; Araus and Cairns, 2014), a number of those allow for the characterization of root architecture.

The choice of a root phenotyping system depends on several factors, among others, plant species (annual vs. perennial), targeted traits of interest, studied developmental phase of the plant (early vs. terminal), necessity to gather 2D or 3D data, possibility to sacrifice the plant (destructive vs. non-destructive measurements), time scale of the growth kinetics (days vs. months) and costs (Paez-Garcia et al., 2015). The diversity of root phenotyping systems that have been developed over the years now allows researchers to choose the setup most adapted to their questions of interest (Kuijken et al., 2015) (**Table 1**).

One simple way that can help to categorize and choose among root phenotyping systems is to consider them from a throughput point of view, throughput being estimated both by the scaling of the experimental setup (how many experimental units can be deployed in parallel), and the time it takes to collect data per experimental unit. Lab and greenhouse-based phenotyping systems tend to allow for high-throughput phenotyping experiments (several hundred to several thousands

of plants in parallel and/or quick data acquisition), allowing to test large number of seedlings in highly controlled and repeatable conditions (**Table 1**). These high-throughput methods are critical for QTL or GWAS studies aiming to link the plasticity of the RSA to genetic markers or specific genes or alleles that may be breeding target of interest. Medium throughput systems can typically deal with tens of plants at the same time and usually focus more on the spatial and temporal resolution of the data harvesting. Whether, they are lab or field based, these systems are often used to generate the architectural and physiological parameters used both for FSPM calibration and validation. On the lower throughput scale are methods requiring either costly technological tools (e.g., x-ray tomography) or significant data acquisition time (e.g., fine scale shovelingomics). In addition to the low throughput, root x-ray tomography is still not perfectly mastered, being subjected to potential loss of information and added noise due to the low resolution of the generated images (Mooney et al., 2011) and the fact that automated 3D reconstruction of root system is carried out based on statistical modeling approaches (Mooney et al., 2011; Kuijken et al., 2015).

An important parameter to take into account when choosing a root phenotyping system is the balance between the need for controlled conditions and observation of the “real” development of the root. Lab and greenhouse based methods such as rhizotrons often constrain the root system growth into a 2D structure of limited size, which can rapidly impede root system growth. On the contrary, systems allowing for permanent accessibility of the root for observation and sampling (e.g., hydroponics and aeroponics) imply a lack of mechanical medium to support the RSA and to impact on its development, meaning that the pertinence of observed architectural phenotypes in those setups is debatable. While theoretically less structurally limiting, field-based methods need specific setup such as rainout shelters and irrigation systems to offer controlled conditions and to precisely take into account environments effects on root development, as well as strongly limit the extend of possible root system observation and measurement (Paez-Garcia et al., 2015). Intermediate strategies such as rhizolysimeters can offer rather unlimited growth under controlled (or at least monitored) conditions, but they require substantial structural investment to be practical.

Plant structure phenotyping procedures can typically be separated in three phases: firstly the acquisition of the architectural (and/or physiological) data within the phenotyping system of choice through imaging, secondly the analysis of the generated image to extract quantitative data regarding the characters of interest, and thirdly the subsequent analysis of this quantitative data to extract meaningful information such as mathematical laws describing a growth process. Regarding root architecture characterization, the first step is mainly limited by the difficulty of accessing to root systems either visually or physically, an issue for which several solutions have been devised (**Table 1**). The second step however is generally highly dependent on image analysis capacities and constitutes the main bottleneck of root phenotyping studies (Furbank and Tester, 2011).

From low to high throughput phenotyping systems, morphological and structural information is mainly generated

TABLE 1 | Overview of existing root phenotyping systems.

Plant cultivation system	Growth media (localization)	Throughput	Destructive and dimensionality	Description	References
1. X-Ray computed tomography	Soil (lab and greenhouse)	Very low (single plant at a time)	No/3D	This technique use X-ray to image root structure within a soil column. It generates stacks of projections which need to be combined and analyzed to reconstruct the 3D structure of the root system.	Mooney et al., 2011; Mairhofer et al., 2012; Mairhofer and Zappala, 2013; Koebernick et al., 2014
2. Shovelomics	Soil (field-based)	Low (Single to tens of plants in parallel, depending on available workforce)	Yes/3D	As the name imply, this method involves the manual and/or mechanical excavation of plants root systems from the soil. Roots can be measured <i>in situ</i> while being excavated, or phenotyped after washing and preparation.	Trachsel et al., 2011; Bucksch et al., 2014
3. Rhizotrons	Substrate (lab, field)	Low to medium (up to tens of plants in parallel)	No/2D	Rhizotrons are composed in principle of a succession of plates enclosing a thin layer of substrate. One at least of the external plates is transparent, and the rhizotron is built so that the root system grows in part or in total against this transparent plate, allowing for its imaging. In field conditions, the rhizotron can actually be a full trench along which the root system growth is observed.	Colin-Belgrand et al., 1989; Neufeld et al., 1989; Singh et al., 2010, 2012
4. Rhizolysimeters	Soil (field-based)	Low to medium (Tens to hundreds of plants in parallel)	No/3D	Rhizolysimeters are concrete, steel or PVC columns which are filled with soil and used to grow plants. The column can either be equipped with sensors or "windows" allowing for the observation and measurement of the plant as it grows by.	Eberbach and Hoffmann, 2013
5. Minirhizotron	Soil (field-based)	Low to medium (Tens to hundreds of plant in parallel)	No/3D	This particular system is based on transparent observation tubes which are permanently inserted in the soil. These tubes allow for the passage of a camera to image roots growing along the minirhizotron wall.	Iversen et al., 2012; Maeght et al., 2013
6. Growth and luminescence observatory (GLO-Roots)	Soil (lab)	Medium (tens of plants in parallel)	No/2D	Derived from the rhizotron principle, this system makes use of bioluminescent transgenic plants to image the growth of the root in soil.	Rellán-Álvarez et al., 2015
7. Rhizoscope	Liquid medium + solid support (glass beads) (lab)	High (hundreds of plants in parallel)	No/2D	This system is akin to a rhizotron. The main difference is that the growth substrate is replaced by transparent glass beads between which liquid medium is circulated. The glass beads can be removed to expose the root system for easy imaging and/or sampling.	Audebert et al., 2010
8. Clear pot method	Soil (greenhouse)	High (hundreds of plants in parallel)	No/3D	Again a variation on the rhizotron principle. Here plants are grown in transparent pots filled with soil or other potting medium. Seeds are planted close to the pot wall to enable high- throughput imaging of roots along the clear pot wall.	Richard and Hickey, 2015
9. Rhizoslides	Paper-based (lab, greenhouse)	High (hundreds of plants in parallel)	No/2D	This setup consists in growing the plants on germination paper supported by plexiglass plates and partially immersed in nutritive liquid medium, allowing for direct imaging of seedling growing on the paper.	Le Marié, 2014
10. Rhizoponics	Liquid medium (lab)	Very high (thousands of plants in parallel)	No/2D	Similar to rhizoscope systems in that it combines hydroponics and rhizotrons. The system is made of a nylon fabric supported by an aluminum frame. The set-up is immersed in a tank filled with liquid media.	Mathieu and Lobet, 2015
11. Root aerponics	Air (lab)	Very high (thousands of plants in parallel)	No/3D	In this system plant are grown out of any kind of substrate and root are subjected to regular misting to provide water and nutrient. The root system is fully accessible at all time, albeit slumped due to growing without mechanical support.	de Dorlodot et al., 2005

Adapted from Paez-Garcia et al. (2015).

as 2D images that need to be processed into quantitative data representing a 3D structure (Kuijken et al., 2015). And because the root system is a 3D structure (even in 2D rhizotron), any sufficiently old root system will exhibit overlapping of roots in 2D projection pictures. This greatly complicates the extraction of root structure from images and has lead to the development of a wide range of image analysis algorithms and software applications to help automatically extract root structure from noisy images. These image analysis software usually offer functions to quantify root features such as root number, length or angles which can be used to calibrate or validate root FSPM (Godin and Sinoquet, 2005; Vos et al., 2007, 2010; Lynch et al., 2014). Kuijken et al. (2015) recently reviewed all currently available image processing software applicable to root phenotyping. Their increasing number results in a large variety of software solutions for root systems analysis (Lobet et al., 2013). However, this diversity also led to the proliferation of independent computational methods and framework to represent and store root architectures. It is a hindrance that limits the possibility to exchange data between labs or to use different software on the same dataset. To resolve this issue, a common root architectural description was recently developed. Emerging from an international joined effort by several groups working in root phenotyping and modeling, the Root System Markup language (RSML) has been specified to ensure root architecture data transferability between software, thus promoting research exchanges within the scientific community and given rise to a standard format upon which to build central root model warehouses (Lobet et al., 2015).

ROOT ARCHITECTURE PHENOTYPING IN A BREEDING CONTEXT

The phenotyping methods described above are still not widely used in the context of breeding programs, in part because the link between measurable traits and their usefulness in the context of breeding is not always evident, and in part because

of somewhat limited throughput of analysis compared to the genomics methods of analysis that can be used to support breeding programs such as GWAS for example (based on thousands to tens of thousands of plants) (Spindel et al., 2015; Biscarini et al., 2016; Gao et al., 2016; Iwata et al., 2016). Yet, breeding effort targeting several aspects of the RSA have been successfully undertaken in different crops (**Table 2**). For example Tuberosa et al. (2002a,b) identified QTLs for seminal root traits in a maize recombinant inbred line population and found a certain degree of co-location between QTLs for seminal root traits and QTLs for yield performance across different water regimes in the field. In chickpea a major QTL for root traits (depth, density) was identified (Varshney et al., 2014), from phenotypic data generated in a PVC tube system where plants were grown and root extracted and scanned at 35 days after sowing (see Kashiwagi et al., 2005 for a method). In sorghum, genotypic variation for nodal root angle was identified (Singh et al., 2010, 2012) and these traits are seen as a potential target for breeding program for either deep rooting (narrow angle), or rooting in the scope of skipped-row planting that requires shallow root angle. Subsequently, a phenotyping platform was developed at a scale that allowed phenotyping of a mapping population and QTLs for nodal root angle have been identified and shown to co-locate with QTL for traits related to drought adaptation. These three examples, taken from a wider variety of uses of root traits in breeding (**Table 2**) illustrate how simplified techniques (i.e., a hydroponic system, or root angle measurements between two thin plates) can be sufficient to pinpoint genotypic variation in traits that are strongly related to field-based performance.

ROOT SYSTEM ARCHITECTURAL MODELING

The ability of roots to ensure the hydro-mineral nutrition of the plant is dependent on RSA (Lynch, 1995, 2007; Comas et al., 2013; Lynch et al., 2014), but also on the root hydraulic characteristics. Root systems appear highly plastic, and their

TABLE 2 | Structural and functional root traits identified as potentially relevant for drought-resistance breeding.

Traits	Species	QTLs	Sources
Root length	Rice, Wheat, Maize	Yes	Price et al., 2002; Tuberosa et al., 2002a,b; MacMillan et al., 2006; Courtois et al., 2009
Root biomass	Rice	Yes	Courtois et al., 2003
Root thickness	Rice, Maize	Yes	Zheng et al., 2000; Tuberosa et al., 2002a,b
Total root biomass	Wheat, Maize	Yes	Tuberosa et al., 2002a,b; Sharma et al., 2011
Root length density (RLD)	Chickpea	No	Kashiwagi et al., 2005
Seminal root angle	Wheat	Yes	Christopher et al., 2013
Number of seminal roots	Wheat	Yes	Christopher et al., 2013
Crown root angle	Maize, Sorghum	Yes	Giuliani et al., 2005; Singh et al., 2010, 2012
Rooting depth	Wheat, Chickpea	No	Sayar et al., 2007; Varshney et al., 2014
Crown root diameter	Maize	Yes	Giuliani et al., 2005
Xylem vessel size and number	Rice, Wheat	Yes	Richards and Passioura, 1989; Uga et al., 2008
Root cortical aerenchyma	Maize	Yes	Mano and Omori, 2009

structure is the result of complex interactions between genetic and environmental regulations. Those interactions generate dynamic feedback loops in which the heterogeneousness of the soil environment modifies the plant growth, which in turn modify the soil by harvesting water and nutrient from it, and so forth. One way to investigate and solve such complex feedback system is to use models.

Being hidden underground, root systems are particularly challenging to model. Nevertheless, a lot of architectural root models have been developed over the last 40 years. In all cases, the very first step of the modeling process consists in choosing an adequate representation (i.e., formal encoding) for the root structure.

Due to the inherent difficulty to assess the precise root spatial distribution in soil, the first root system architectural model where actually continuous models based on estimates of root density distribution within the soil through time and depth (Dupuy et al., 2010). An early example of such models used diffusion equation to model the dispersion of root within the soil (Page and Gerwitz, 1974). However, density-distribution models relied on synthetic parameters such as a single root density descriptor and were based on the hypothesis that roots distribute regularly throughout the soil. Such an assumption is not verified in field conditions where a discontinuous distribution of roots is observed, presumably due to heterogeneous distribution of environmental effects. As such, simple continuous models cannot easily take into account effects such as root clustering which is instrumental for resource uptake (Dupuy et al., 2010). As a consequence, rather than focusing on the precise developmental regulation of RSA, these types of continuous architectural models are better suited to give synthetic descriptions of RSA in global environments. As they can be used to infer missing or imprecise architectural information, continuous models are best used to investigate RSA when root systems are partially or totally inaccessible, for instance when studying mature trees or field grown plants. In order to be able to investigate RSA in heterogeneous soil conditions in the field, continuous model can be further coupled with statistical approaches allowing for the description of root density statistical maps through the soil (Chopart and Siband, 1999). The main limitation of those coupled models is their reliance on calibration data that need to be generated from tedious *in situ* excavation and manual measurement of different parts of the root system in soil (Chopart and Siband, 1999).

RSA emerge from the interaction between root developmental processes and their environment. As they do not consider individual roots, continuous models cannot easily account for the feedback existing between root and soil. Therefore, new approaches were needed to understand how soil is explored by the plant at the individual root axis level (Pierret et al., 2007). This consideration gave rise to the development of more complex root models. Those models are based on the explicit description of root development, growth and branching processes resulting in 1D, 2D, or 3D models (Dunbabin et al., 2013). Such discrete and explicit models consider root architecture through its complete discrete topology and geometry and can be based on several distinct mathematical formalisms (Godin, 2000; Balduzzi et al.,

2017). Two of the most popular formalisms used to represent discrete plant architecture in general are multi-scale tree graphs (MTGs) (Godin and Caraglio, 1998; Godin et al., 1999, 2005) and L-systems (Prusinkiewicz and Lindenmayer, 1990).

MTGs were developed based on the concept of plant modularity and aim to describe individual parts of the plants as tree graphs, themselves included in an arborescent structure (Godin et al., 1997). MTGs allow topological and geometrical encoding of any kind of plant and were used as a standard to describe plant development and architecture of a broad range of species (Godin and Caraglio, 1998; Danjon et al., 1999; Godin, 2000; Guédon et al., 2001; Danjon and Reubens, 2008; Fournier et al., 2010; Garin et al., 2014; Griffon and de Coligny, 2014). The MTG formalism has notably been used as the principal data structure for the OpenAlea platform (<http://openalea.gforge.inria.fr>), a software environment dedicated for plant modeling which integrates algorithms designed for creating, parsing, modifying and extending MTGs (Pradal et al., 2008, 2015), as well as algorithm to convert MTGs to the recently defined RSML formalism and conversely (Lobet et al., 2015).

While MTGs can be extended to provide dynamical consideration of plant architecture, they are inherently static structural descriptions. Another way to encode plant architecture is to see it as the result of iterative developmental processes and try to express it using a procedural formalism. This is the view chosen in the L-systems formalism (Lindenmayer, 1968). This formalism uses a symbolic language to provide a description of the plant as a bracketed string of characters. Each character stands for a given plant developmental module (meristems, organs, metamers, segments, axes, etc.). Developmental rules are specified as rewriting rules for each possible type of characters, indicating whether it stays the same or is replaced by another character or group of characters at each iteration. The repetitive and recurrent nature of plant structure thus allow to capture and to recreate plant developing architecture through time by discretizing the plant as a set of characters and specifying a reduced set of rewriting rules (Prusinkiewicz, 2004).

Since their first formalization, L-systems have been implemented and extended through different modeling languages and systems, notably cpfg (Prusinkiewicz and Lindenmayer, 1990; Prusinkiewicz and Karwowski, 1999), lpfg (Karwowski and Prusinkiewicz, 2003), XL (Kniemeyer and Kurth, 2008) and more recently L-Py (Boudon et al., 2012). This latest installment of L-system formalism implementation was designed to allow mutual conversion between L-strings and MTGs. This offers the possibility to use the large set of available built-in components, tools and algorithms already designed for MTGs in conjunction with L-systems (Boudon et al., 2012). While both structural and procedural formalisms were initially designed with plant aerial part structure in mind, both have been used with success to generate discrete explicit root system models. For instance the mature RSA of *Pinus pinaster* was reconstructed from 3D-digitizing data using a MTG approach (Danjon et al., 2005). In the same species, MTG-type simulated root systems were used to investigate plant anchorage and its response to architectural modification (root wounding, absence of tap root, pruned root systems, etc.) (Khuder et al.,

2007; Danjon and Reubens, 2008). Another example is Root Box (Leitner et al., 2010b) that represents root growth and architecture using L-systems. It is encoded in Matlab and was applied to the study of maize root system. This model uses a modular approach to integrate the interplay between root and soil and can be used to compute complex root system properties such as root length density distribution for different soil models (Leitner et al., 2010a). The model code is publicly available (<http://www.boku.ac.at/marhizo/simulations.html>) and has already been coupled with different soil models to simulate the influence of chemotropism on root growth (Schnepp et al., 2011), the effect of root exudation on phosphate acquisition (Schnepp et al., 2012), or the impact of root architecture on water acquisition under different hydrological conditions (Tron et al., 2015).

Beyond MTGs and L-systems, another formalism is the fractal approach, which uses mathematical concepts initially developed for the study of geometric patterns in nature and, in particular, to characterize self-similar patterns. This specific formalism has notably been used to develop a static 3D architectural model of *Gloricidia sepium* root system (Ozier-Lafontaine et al., 1999). This model was able to efficiently predict root branching patterns and some root traits at plant level such as root dry matter, total root length and root system diameter (van Noordwijk and Mulia, 2002; Doussan et al., 2003).

From a breeding point of view, all those encoding formalisms give convenient access to root structure descriptors and allow for easy quantification of plant root system shape. However, by themselves they are not enough to understand how the root structure emerges and thus need to be coupled with mechanistic developmental rules. In addition, once proper formalisms are integrated in root models, and their functions validated, the conditions in which the root phenotypes that emerge from these models have a demonstrated effect on crop productivity will have to be validated. For instance in maize, it was shown that reduced lateral root branching was beneficial for crop adaptation to water stress because of a reduced carbon cost of that root type (Zhan et al., 2015). In such case, a clear link could be conceived between some well-defined encoding formalism and its expression in the form of a phenotype of importance for a breeding perspective.

Many 3D mechanistic dynamic root architectural simulation models have been developed since the 80s to investigate root growth and function (Dunbabin et al., 2013). They usually rely on the description of different mechanistic developmental rules depending on root branching order and/or root type. Those rules need to be calibrated against data obtained through large-scale phenotyping or specific experimental measurements. Different root types can be characterized through developmental descriptors or criteria such as growth rate, branching variability, branching density, tropism efficiency, radial growth, etc. Each criterion is considered a distinct parameter for the generation of the 2D or 3D structural models (Figure 1, Pagès, 2002). Development of the whole root system is simulated in discrete time points on the basis of the specified morphogenetic rules (Doussan et al., 2003; Prusinkiewicz, 2004). These rules govern initiation (branching), emergence and growth of new axes but can also integrate rules for root senescence and / or rule

describing the influence of various tropisms on root development (thigmotropism, hygrotropism, chemotropism, gravitropism, ...). One of the first 3D mechanistic root model was developed by Deans and Ford (1983). This model was able to simulate a 16-year-old excavated root system of *Sitka pruce* and allowed investigation of wind impact on the tree development and stability (Deans and Ford, 1983). It inspired the development of subsequent 3D mechanistic root models of others species using the same method to describe the elementary growth and branching processes of root systems. An example of such later model is ROOTMAP (Diggle, 1988a,b) that simulated root growth and architecture of fibrous root systems. It considered mechanistic parameters for growth (e.g., root-elongation rate) and branching (branching angle, branching density, time of branching delay, branching order, etc.). This model was used to simulate a broad array of lupin genotypes with a high accuracy using data acquired from semi-hydroponic phenotyping system (Chen et al., 2011). It was since then extended to integrate a 3D soil model, thus representing root system plasticity in a mixed soil environment and allowing to model nutrient uptake dynamics from that environment (Dunbabin et al., 2002). Pagès et al. (1989) used the same approach as Diggle (1988a,b) to produce a 3D RSA model of maize using empirical observations to define morphogenetic rules and different growth processes depending on root branching order and inter-node root origin (Pagès et al., 1989). Based on the concepts developed by previous models (Diggle, 1988a,b; Pagès et al., 1989), the SimRoot model was designed with better focus on visualization, taking into account the spatial heterogeneity of root growth processes through a kinematic description of variation of growth features along root axes (Lynch et al., 1997). It has been calibrated using empirical datasets acquired from different growth environments and was used to predict precisely the growth of maize and bean root systems (Ge et al., 2000; Postma and Lynch, 2010). SimRoot has also been extended to integrate interactions between root systems, phosphorus uptake efficiency (Ma et al., 2001), carbon allocation (Nielsen and Lynch, 1994; Walk et al., 2006; Postma and Lynch, 2011) and shoot/root exchanges by coupling with the LINTUL model (Postma and Lynch, 2011; Dunbabin et al., 2013). Another generic mechanistic root model is RootTyp (Pagès et al., 2004). Contrary to previous models that differentiated root behavior depending on their branching order, RootTyp relies on the explicit determination of different root types (with different growth properties such as branching density or elongation rate) independently of their branching order (Pagès et al., 1989). RootTyp has been used to represent a large variety of plant root systems and was parameterized using various architectural datasets (Collet et al., 2006; Garré et al., 2012). It integrates stochasticity through inclusion of randomness in some geometrical or topological parameters (e.g., root trajectories). As of particular interest in the context of study of root-driven drought tolerance, it is to be noted that RootTyp was also extended to provide dynamic description of water supply within the soil environment (Doussan et al., 2006; Dray and Pagès, 2006).

One common denominator for all those mechanistic models to be useful as predictive tools is their dependence on the

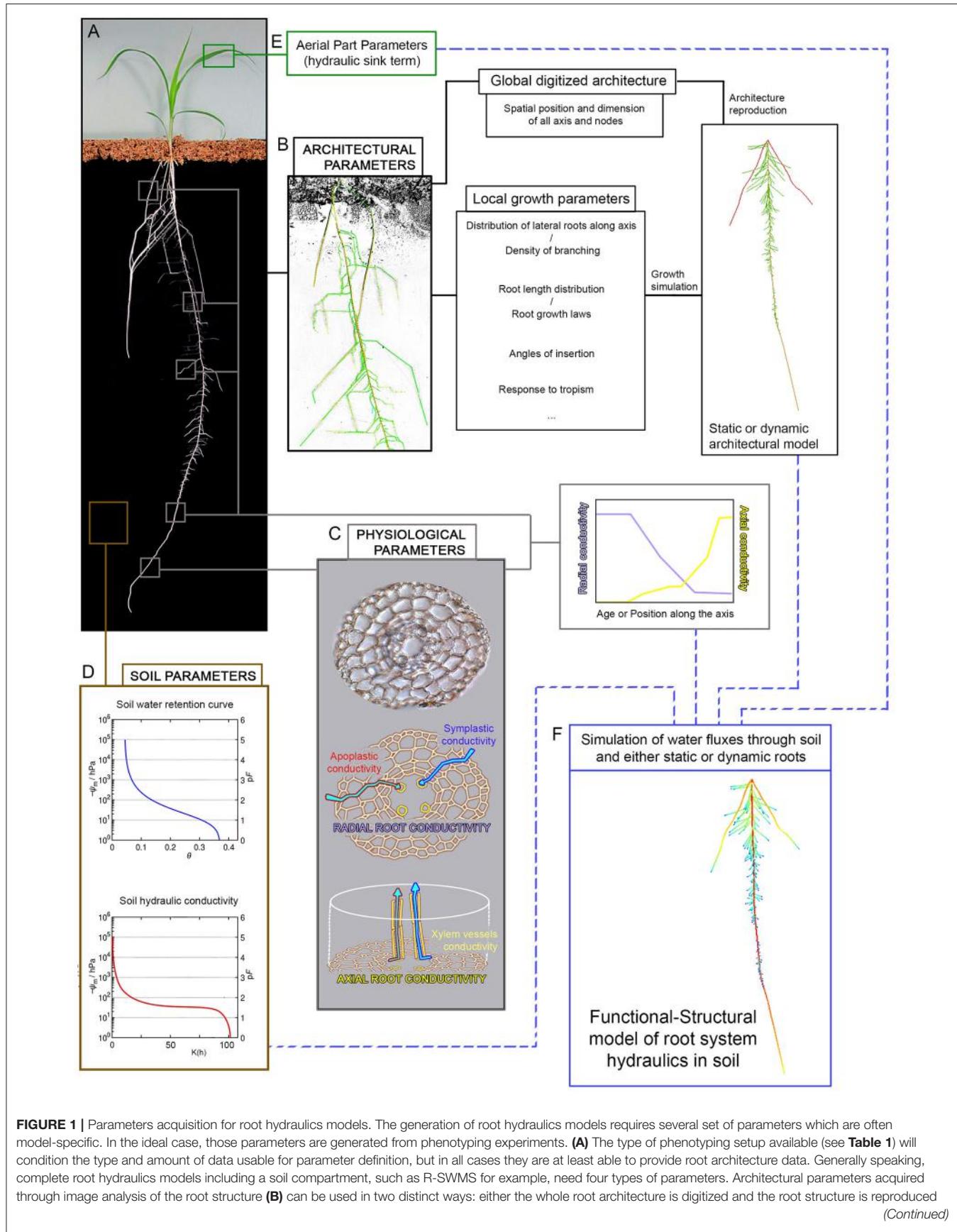


FIGURE 1 | Parameters acquisition for root hydraulics models. The generation of root hydraulics models requires several set of parameters which are often model-specific. In the ideal case, those parameters are generated from phenotyping experiments. **(A)** The type of phenotyping setup available (see **Table 1**) will condition the type and amount of data usable for parameter definition, but in all cases they are at least able to provide root architecture data. Generally speaking, complete root hydraulics models including a soil compartment, such as R-SWMS for example, need four types of parameters. Architectural parameters acquired through image analysis of the root structure **(B)** can be used in two distinct ways: either the whole root architecture is digitized and the root structure is reproduced

(Continued)

FIGURE 1 | Continued

computationally, or the root architecture is used to determine local growth parameters which can in turn be used to create representative root architecture through growth simulation. Physiological parameters (**C**) relating to water transport are principally acquired through additional histological and physiological measurement. Radial root conductivity is a function of apoplastic and symplastic water transport and is hard to evaluate, often needing to be estimated through proxy such as pressure probe measurement in outer cells layers. Axial root conductivity is dependent on xylem vessel size and shape and can be partially extrapolated from cell measurement and application of Hagen-Poiseuille's law. Those two parameters need to be evaluated along the root axis and/or for different root ages to generate profiles of conductivity. Soil parameters needed (**D**) are soil water retention and soil hydraulic conductivity profiles, as well as an eventual description of the soil structure. Finally, depending on the model, aerial part parameters (**E**) can be more or less explicit and are used to express a hydraulic sink term driving water absorption by the root. Taken altogether, these four sets of parameters can be used to simulate the dynamic of water fluxes through the soil and roots and to study the patterns of water distribution under a given environment (**F**).

previously described phenotyping methods (**Table 1**) to provide the architectural data necessary both for model rule calibration and for validation of model predictions (**Figure 1**). Architectural data can be generated using any of the described phenotyping platforms, as each of those allow for the possibility to capture the structure of the root system in some way. Depending on the considered model and on the nature of the image analysis procedure following root system imaging, parameter calibration can be done from global descriptors (for example statistical distribution of root densities in the soil, or on the contrary the entire and precise description of the root structure) or from quantitative measurement of local specific architectural traits or growth laws (root densities, root length distribution or root growth speed, root angle of insertion, etc.). In ideal cases, at least two independent datasets will be used for parameter definition, one to calibrate the model and one to validate its predictions.

Following parameter definition, the models are expected to provide digital root architectures based on the input parameters, either reproduced from structural data or digitally grown from local growth laws. These simulated architecture need then to be validated against the additional datasets. Depending on the model and on the nature of these datasets, this validation can be done either by direct structural comparison of architecture, or through the use of indirect descriptors (for example, amount of root biomass by soil horizons, distribution of root length between the different root orders, etc.). In some specific circumstances, only a single dataset may be available both for calibration and validation. In this case, it is still possible to proceed by calibrating the model using only part of the available data, validating it against the rest of the data, and repeating this procedure for all possible combination of sub-dataset.

CONCEPTION OF FUNCTIONAL-STRUCTURAL MODELS OF ROOT HYDRAULICS

We have gone over the different solutions available to gather data on root structure through phenotyping, how this data can be encoded using various mathematical formalisms and then used to provide calibration and rules for various mechanistic models of root development. Those mechanistic developmental models can then be further coupled with functional processes in order to provide an integrated view that could ultimately support model-assisted breeding programs (**Figure 1**).

One such functional process is water uptake and transport by plant roots. Drought is one of the main limiting factors for plant productivity (Lobell and Gourdji, 2012), therefore using models to understand where, when and how water is absorbed and transported from the soil by the plant roots could help improving water use both by optimizing plant RSA through ideotype-assisted breeding and through model-directed changes in agricultural management practices (Lynch, 2007; Blum, 2009; Palta et al., 2011; Lynch et al., 2014). It would be also important to couple these modeling approaches with experimental setup that allow a very precise evaluation of water extraction. For instance, the use of a lysimeter system for close monitoring of plant water use (Vadez et al., 2014) has shown genotypic variation in sorghum germplasm for the capacity to extract water from the soil profile (Vadez et al., 2011). It would be interesting to investigate which rooting trait or traits can explain the contrasting water extraction characteristics and whether these can be exploited in further breeding programs. Such traits may be macroscopic and related to the repartition of different root types in the soil, or microscopic and linked to cellular processes or structure (e.g., root hair or xylem cells size, changes in tissue conductivity through differential aquaporin expression, etc...).

The dynamics of water uptake from the soil is a very complex issue which depends both on the properties of a dynamic biological system (the root) and of a physical heterogeneous system (the soil). Studies of water dynamics in soil were initiated in the 1960s with the innovative work of Gardner (1960) which was more focused on soil than plant properties but nevertheless served as the basis for subsequent works to better understand root-soil exchange processes. These later works introduced roots in the form of sink terms in soil water distribution models to represent soil water uptake by roots (Feddes, 1974; Molz, 1981; Homaei et al., 2002; Dardanelli et al., 2004). Those models mainly use a continuous root system representation, describing it through root length density descriptors. As the feedback between root system growth and its environment has been proven to play a major role in the dynamics of soil resources uptake (Doussan et al., 2003; Lynch et al., 2014), FSPMs had to be able to deal with developmental feedback.

Specific root-soil FSPMs have been developed to investigate the interaction between soil water hydrodynamics and root system development. Clausinger and Hopmans (1994) proposed a detailed FSPM-type root hydraulic model that coupled a 3D root architectural model with a 3D transient water flux model. They modeled the interaction between root growth and soil water distribution to simulate water uptake in crops. In their

model, processes governing root development are expressed as a function of local soil conditions, and the water sink root uptake term is expressed as a function of transpiration and root length. The soil itself is discretized and a finite-element grid is used as the basis for soil properties computation. Benjamin et al. (1996) combined a 2D root model of corn root system with a 2D water model to simulate the effects of root patterns on water uptake. Somma et al. (1998) extended the model of Clausnitzer and Hopmans to express water uptake activity of roots as a function of root age and to additionally simulate solute transport and nutrient uptake.

Beyond those models based essentially on soil water fluxes, the dynamic of water fluxes in the plant tissues was also the focus of early modeling studies where plants were considered using an electrical analogy (van den Honert, 1948). In this first modeling effort and subsequent works, plant tissues are represented using a network of hydraulic resistances behaving as electrical resistances, and water transport is governed by pure physical consideration (van den Honert, 1948; Zimmermann, 1978). The parameterization of those models however required to be able to determine the value of the hydraulic resistance of plant tissues. This lead to the extensive development of measurement methods allowing for the estimation of physiological parameters of water transports in tissues, attested by the extensive work of groups such as those of Steudle (Hüsken et al., 1978; Steudle, 1993, 2000) and Sperry (Sperry et al., 2002; Sperry, 2011; Sperry and Love, 2015). Interestingly, these measurement methods essentially allow for the determination of water conductance rather than resistance. As those two physical values are inversely linked, models of water transport in plant tissues based on electrical analogy can use either value for their parameterization, provided that the equations are accordingly tweaked. Those models essentially need two types of conductances (or resistances) for their parameterization: namely axial and radial conductances (resistances) (**Figure 1**). Axial conductance is directly linked to the structure of the vascular tissue. Under the assumption that the vascular strands are lengthy regular cylinders, it can be directly computed from application of generic Hagen-Poiseuille law. This predicted axial conductivity values may need to be adjusted depending on the proportion of embolized vessels existing in the considered species. Computation of radial resistance on the contrary will depends the level of details of the considered model, as it is the results of the combination of conductances of the cells comprised in the concentric tissue layers of the plant axis.

In 1998, Doussan et al. proposed a novel approach integrating in a single framework knowledge about water flux in the soil, plant water uptake, plant vascular structure, global root tissue conductivities and RSA. In this approach, the architectural model of Pagès et al. (1989) was combined with the biophysical description of water fluxes in root tissues considered as a network of radial and axial water conductances. Calibrated using both measured and estimated conductances from tree root phenotyping, this model can simulate root water fluxes through computation of water potentials along the conductance network. Lately, Couvreur et al. (2012) extended this approach by computing analytical solutions to water flow equations for complex hydraulic architecture in simulations of water fluxes

distribution under drought. Chopard (2004) simulated water transfer in soil and root systems using a 3D root architectural model based on MTG formalism and integrating water transport processes within differentiated root types. Integrated models can also result from the conjunction of several pre-existing independent models. For instance, Javaux et al. (2008) developed the R-SWMS model from the conjunction of the models of Doussan et al. (1998, 2006) and Somma et al. (1998), coupling a mechanistic root development model with deep integrated knowledge of soil hydrology processes. R-SWMS can be used to simulate various water distribution and uptake rules under a wide variety of environmental conditions (Draye et al., 2010; Couvreur et al., 2012). Beyond being used to estimate water absorption by the roots, those models can also be extended to study nutrient uptake, such as was done by Roose and Fowler (2004a,b) in the case of phosphate uptake. Latest modeling development also focuses on the crucial problematic of model scaling and extrapolation (Meunier et al., 2017). The majority of plant root and soil hydraulics models are designed and parameterized from experimental data generated in lab or greenhouse, while they should ideally be intended to give prediction regarding the behavior of field-grown plants. Meunier et al. (2017) recently proposed a numerical solution to extrapolate global root behavior from sets of local variables that can be easily measured in lab or greenhouse. However, this solution can only be applied to root systems presenting a highly regular structure, and the problem of model field-projection for irregular, life-like root systems still need to be addressed.

VALIDATION OF ROOT HYDRAULICS FUNCTIONAL-STRUCTURAL MODELS

Root hydraulic FSPMs can be used to predict the dynamics of water distribution in the plant-soil continuum (Roose and Fowler, 2004a,b; Doussan et al., 2006; Javaux et al., 2008; Moradi et al., 2011; Couvreur et al., 2012). The quality of those predictions is dependent on the correct calibration of architectural and functional characteristics of roots and hydraulic properties of soil from phenotyping and physical measurement techniques. It is also known that the genetic of the plant can have profound role on the hydraulics of the root system. For example, Ehlert et al. (2009) showed different hydraulic conductivities in maize genotypes treated with a range of aquaporin inhibitors. Moreover, for the predictions to be of use in model-assisted breeding, they must themselves be validated against observable environmental and physiological parameters.

Validation methods of water fluxes prediction use various non-destructive and non-invasive imaging techniques to allow for the real-time observations of water content in root-soil systems (Doussan et al., 2006; Garrigues et al., 2006; Perret et al., 2007; Pohlmeier et al., 2008; Carminati et al., 2010; Moradi et al., 2011). The predictions of the Doussan et al. (2006) root hydraulic model were confronted to an experimental system aimed at monitoring dynamic water depletion around roots in soil (Garrigues et al., 2006). The principle of this system is to measure changes in light transmission value between different

water saturation level of the soil matrix and use those to compute the uptake of water by the root system throughout the soil. This experimental setup is a derivative of rhizotron systems (**Table 1**) and can theoretically be applied to any kind of root/soil system, scaling up to fully grown crops such as mature maize root system. This setup showed that the prediction of the root hydraulic model were qualitatively and quantitatively representative of the water dynamics observed within the root-soil system, with greater water depletion occurring close to root base. Regarding phenotyping systems where the root architecture is not directly apparent but rather embedded in the soil, alternative techniques need to be used to quantify water movement through soil and roots, such as magnetic resonance imaging (MRI). Pohlmeier et al. (2008) used MRI to monitor changes in water uptake dynamics in soil. Both soil water content and root architecture can be imaged using this technique, and imaging results revealed that water uptake is greater in zones were the root densities are the highest. This technique is however still limited to imaging in-lab experimental setup of small dimension and is not yet directly applicable in field or for rhizolysimeters. The creation of portable MRI apparatus is one of the challenges that need to be addressed in order to be able to validate hydraulic model prediction in large scale and *in situ* phenotyping platforms. Alternatively to MRI, Carminati et al. (2010) used neutron radiography to image 2D water content distributions in soil under drought conditions and following rewetting in order to investigate the role of rhizosphere in water uptake and drought tolerance. While chemical and physical characteristics of the rhizosphere have been proven to be different from that of the bulk soil (Strayer et al., 2003; Gregory, 2006; Hinsinger et al., 2009), the effects of these specific properties on water uptake are usually neglected in root hydraulics models. 2D water distribution patterns observed using neutron radiography showed that the water content of the rhizosphere is higher than that of the bulk soil during drying and reversely during rewetting. These observations were used to determine the respective water retention curves of rhizosphere and bulk soil. Given these parameters, a simulation of water flux for a single root according to the model of Gardner (1960) suggested that the rhizosphere actually acts as a buffer to soften the impact of drought and provide smooth water availability in time of water stress. Moradi et al. (2011) further advanced this line of inquiry, using neutron tomography to quantify and visualize water content dynamics in 3D with high spatial resolution in the rhizosphere of three different plant species. They observed increased water content in soil next to roots (rhizosphere) and the observations were consistent in the three species (chickpea, white lupin and maize), confirming the conclusions of Carminati et al. (2010). The measured experimental water retention profiles were used in a simplified 3D analytical model which again confirmed the conclusion of the previous 2D model, highlighting the importance of the rhizosphere in water uptake processes and its potential interest as a target for drought-tolerance breeding programs. In another set of studies, Zarebanadkouki et al. (2012, 2013, 2014) used neutron radiography coupled with injection of deuterated water D_2O to actually trace water fluxes within

roots. D_2O was injected into roots and its transport dynamics were tracked closely using time-series neutron radiography. To quantify the local transport of D_2O through convective fluxes, a diffusive-convective model was developed, taking into account the different water pathway available in plant tissues (apoplastic, cell-to-cell). Model predictions on D_2O fluxes were in harmony with experimental measures of axial flow of D_2O inside the roots of 24-days old lupin plants.

In all those instances, the prediction of the different root hydraulics FSPMs could be validated against experimental data in the lab, further confirming the interest of such models to investigate the behavior of plant regarding water acquisition. However, those various validation methods all suffer from similar limitation in that they cannot easily be transposed to field-based measurement and thus are yet almost exclusively limited to the validation of lab-based and greenhouse-based predictions. This point, among others, constitutes one of the challenges that need to be addressed in order to promote the use of FSPM model in future breeding programs. In particular, a link needs to be made between the root phenotype that these models are able to predict, and root or plant phenotypes that would have a demonstrated effect on crop performance in the field conditions. Another limit of the current validation methods is the lack of way to generate anisotropic hydraulic environments in the existing phenotyping platforms. In order to precisely predict the impact of water availability in the environment on the root architecture, the models need to integrate rules expressing retroaction existing between root growth and water acquisition. These rules in turns need to be parameterized and validated against experimental data. Current phenotyping platform do not allow for easy control of the local hydraulic potential of the root environment: field based assays are limited to controlling global irrigation; rhizotrons grown plants are usually irrigated either from the top down or from the bottom up, resulting on a gravity induced water gradient in the soil; liquid media-based setup are by definition saturated in water and aeroponics systems are isotropic in term of water availability. They are currently several ways to improve on this situation and we will only list a few possibilities for some of those phenotyping systems, whether or not these are currently being pursued by different groups: rhizotrons can be improved by predefined subdivision of the soil into compartments with different hydraulics properties; another possibility would be to devise a setup to provide water at different points of the rhizotron, perhaps through distribution of capillary dripping through the plates of the setup—this last possibility could also be adapted to rhizolysimeters setups through differential water feed along the column; liquid based setup can be improved using water-retentive beads instead of glass beads as a mechanical support, modifying the proportion and/or distribution of those beads to affect the pattern of hydraulic potential around the root system. These new systems would allow the modeler to have access to data regarding the response of the root to changes in its environment and could also be used to facilitate testing for hypothetical drought scenario by selectively depriving parts of the root system from water. This would in turn help the models predict and validate the optimal root architecture for a given water-constrained environment.

LINKING MODELS PREDICTION TO BREEDING

While high-tech phenotyping methods such as X-Ray tomography and models of functional root architecture can appear to be too theoretical to be of use in breeding studies at a first glance, there is actually a growing body of literature regarding models based on root phenotyping successfully predicting performance improvement associated with the selection of certain root traits. For example, a study in sorghum showed that the advantage of lines introgressed with staygreen QTL came from the capacity to restrict transpiration under high evaporative demand. The virtual crop model predicted a clear advantage from this trait in terms of grain yield. It was then found that this trait, measured at the shoot level, could be related to differences in the root hydraulic conductance and indeed contrasting lines were identified, having different root hydraulic characteristics (Kholová et al., 2014). Another example in maize used modeling to predict the maize yield changes in corn over the last century, and showed that changes in root architecture were the most likely reason for the increases in yield (Hammer et al., 2009). A modeling study in chickpea showed that increasing the speed of root growth, which related to rooting depth and rooting density, was likely to lead to a faster depletion of the soil water, bringing about a yield penalty (Vadez et al., 2012). On the contrary, increasing the depth of water extraction was the mean by which yield could be increased. While the former traits were related to root expansion and branching, the latter deals very likely with a different root architecture with more profuse rooting / branching at depth. Similar work was done in wheat, showing again the value of more profuse density at depth (Manschadi et al., 2006).

UPCOMING CHALLENGES OF FSPM APPROACHES

In the wake of global climatic changes and of increasing concerns regarding the limits of the agricultural methods inherited from the Green Revolution, a rising opinion is that a Second Green Revolution will actually come both from the consideration of plant roots and from the use of models and systems biology to promote more mechanistically-driven breeding and more rational agricultural practices (Lynch, 2007; Lynch et al., 2014).

We have seen here how FSPMs built upon the advances of phenotyping and modeling techniques can provide insight on the mechanisms of root development and water acquisition. Those predictive models have three-fold interests. First, they allow for quantification of the respective contribution of each parameter of the root system to water acquisition through sensitivity analysis approaches, and thus help focusing breeding efforts on the most important phenes. Second, they can be used to search through the plant structure-function-space for integrated root ideotypes best adapted to various environmental scenarios. Those ideotypes can then be used as target and guideline for subsequent breeding projects (Lynch, 2013).

Third, they offer the opportunity to rapidly and cheaply assess the effect of alternative agricultural strategies *in silico* before deploying them to field assays. For instance, root hydraulics FSPMs can be used to test various timing and magnitude of irrigation strategies and to optimize these in regards to the dynamics of the plant water acquisition capacities. Yet, despite all those advantages, root hydraulics FSPMs are still faced with a certain number of challenges that need to be addressed.

We have already mentioned some of those challenges. For instance, automated image analysis is the current bottleneck of most root system phenotyping approaches (Furbank and Tester, 2011; Roose et al., 2016). This issue could be solved in two ways, either by diminishing the amount of noise in the generated data or by improving the analysis of noisy data. Diminishing the noise-to-signal ratio can be done by improving upon phenotyping systems to allow better visualization and capture of the root structure, using for instance technological advances such as plant MRI (Stingaci et al., 2013; Metzner et al., 2015), neutron radiography (Leitner et al., 2014a) or simultaneous imaging of the root system at different angles to overcome the root overlapping issue. By contrast, the improvement of noisy data analysis will rely mainly on computer vision advances and on the development of novel signal-detection algorithms. Moreover, even with fully automated approaches, the plant community will face new challenges such as the reproducibility of computational experiments (Cohen-Boulakia et al., 2017) and the management of very large amount of data (Pradal et al., 2017). The development of new computational methods in Phenotyping (e.g., scientific workflows) and the availability of very large distributed infrastructure (i.e., cloud, grid) will be needed to tackle the new challenges that appear with the need to process very large amount of data in an automated and reproducible way (Bucksch et al., 2017).

Another challenge is the difficulty to acquire data regarding root architecture and physiology in the field, where the conjunction of FSPMs and breeding approaches should ideally takes place. One way to address this challenge will be through technological progress, such as the development of underground radar techniques or transportable MRI apparatus which will allow for non-destructive *in situ* imaging of root structures and water fluxes in soil. Local physico-chemical properties of the soil could also be explored using underground sensor such as optodes which can currently be used to fine-map rhizotron or rhizoboxes but would need to be improved for field use. The acquisition of data regarding the physiological status of the different part of the root system within the soil is a more problematic issue that will require ingenious inventions in the domain of markers of physiological status. Another way to tackle this issue would be to develop integrated models coupling underground to aboveground functional processes. This would allow indirectly assessing the behavior of the underground parts of a plant through measurement of aboveground traits such as sap flow, stomata conductance or leaf-temperature, thus facilitating field-validation of model prediction.

We also mentioned the fact that while the rhizosphere was shown to play a critical role in the buffering of water stress during

drought or submergence episodes (Carminati et al., 2010; Moradi et al., 2011), no current root model includes it (Dunbabin et al., 2013). As such, one of the challenges for root hydraulic FSPMs is to integrate the rhizosphere layer as a dynamic interface between roots and soil. This presupposes additional investigation of the precise dynamics of the rhizosphere deposition and evolution throughout root development, and of its interaction with the soil processes. Depending on the complexity of the rhizosphere dynamics, its consideration might require either the extension of existing models or the development of completely new root FSPMs model paradigms.

More generally speaking, some of the main challenges for root FSPMs in the future are centered upon ecosystem integration. For example, it is known that soil microorganisms increase plant growth and tolerance to water stress and result in changes in root system morphology (Azcón-Aguilar et al., 1996), but no current root model take that into account. It would thus be interesting to consider the integration of biotic interactions in future root FSPM development. Of particular interest would be the investigation of the influence of mycorrhizae on water acquisition by plant roots. Of course, including this partnership in root FSPMs would necessitate being able to observe and quantify the dynamics of mycorrhizae development in soil, to estimate its impact on root developmental and physiological processes, and to measure water fluxes going from the soil to the plant through the fungi.

Regarding the topic of processes integration, current root hydraulic FSPMs can be used either to simulate water fluxes given static root architectures, or to simulate root growth under water acquisition-related developmental feedbacks. However, root development regulation does not only depend on soil water content and as such, root FSPMs need to evolve toward integration of the full range of regulatory processes impacting root growth and development, such as mechanistic description of nutrient perception and tropisms at the microscopic scale.

On the topic of scale integration, if root FSPMs are to be used as breeding tools, they will increasingly need to be able to integrate quantitative and qualitative knowledge from both extremities of the scale range. At the microscopic scale, root FSPMs will have to integrate rules for the genetic and hormonal regulation of root growth and physiology at the cellular level. For instance in the context of drought, it has been demonstrated that abscisic acid controls water stress tolerance mechanisms in later steps of root growth (Kholová et al., 2010). Other phytohormones such as auxin are also implicated in the complex feedback systems of root developmental regulation and environmental perception (Lavenus et al., 2013). Several models of mechanistic regulation by auxin of the different steps of root branching (initiation and emergence) have already been proposed (Lucas et al., 2008; Péret et al., 2012, 2013). The processes described in those models rely essentially on the dynamical reorientation of auxin fluxes at the cellular level by changes in auxin transporters expression and localization. In the particular case of root emergence, there is a clear link between cellular hydraulics and the auxin regulatory processes (Péret et al., 2012). This hint at further coupling mechanisms

that will need to be explored to explicitly link the hydraulic state of the whole root system to its cellular development and resulting architecture. While one may argue that considering microscopic scale processes would unnecessarily complicate the FSPMs, the identification of explicit morphogenetic mechanisms at the cellular level would be of tremendous help to link FSPMs prediction with genetic studies such as QTL or GWAS analysis. The culmination of this would be the possibility to model the impact of changes at the level of a single component of the genetic network on the development and physiology at the root system scale.

On the other side of the scale range, at the macroscopic level, two more specific challenges remain. First, root FSPMs will need at some point in time to be connected to shoots FSPMs. While it is easier to consider roots and shoots independently, there is a necessary developmental coordination between aerial and underground plant organs. Some simple models connecting shoot and root already exists (Sperry et al., 2016) and will need to be expended upon so that both roots and shoots FSPMs can benefit from the creation of unified virtual plants models. Second, root FSPMs are mainly used to consider a single plant in interaction with its environment. In the context of breeding and agricultural production, a plant being virtually alone in its environment is an exceptionally rare case. Root FSPMs will thus need to be adapted to investigate crop-like situation including inter-individual competition and/or cooperation. It will also be interesting to use multiple parallel distinct root FSPM to study inter-specific interactions and their potential impact on agricultural practices such as inter-cropping.

In the end, the future of functional-structural plant models represents both an incredible opportunity and an incredible source of technical and intellectual challenges, which will require scientific cooperation through fields far out-reaching plant biology. While it remains to be seen whether the Second Green Revolution will actually precede or follow the advent of true virtual plants successfully integrating all biological scales, one thing is for certain: virtual plants have taken root.

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AN, VV, CP, and ML all contributed to the redaction of this review.

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New Insights on Plant Salt Tolerance Mechanisms and Their Potential Use for Breeding

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Soil salinization is a major threat to agriculture in arid and semi-arid regions, where water scarcity and inadequate drainage of irrigated lands severely reduce crop yield. Salt accumulation inhibits plant growth and reduces the ability to uptake water and nutrients, leading to osmotic or water-deficit stress. Salt is also causing injury of the young photosynthetic leaves and acceleration of their senescence, as the Na⁺ cation is toxic when accumulating in cell cytosol resulting in ionic imbalance and toxicity of transpiring leaves. To cope with salt stress, plants have evolved mainly two types of tolerance mechanisms based on either limiting the entry of salt by the roots, or controlling its concentration and distribution. Understanding the overall control of Na⁺ accumulation and functional studies of genes involved in transport processes, will provide a new opportunity to improve the salinity tolerance of plants relevant to food security in arid regions. A better understanding of these tolerance mechanisms can be used to breed crops with improved yield performance under salinity stress. Moreover, associations of cultures with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi could serve as an alternative and sustainable strategy to increase crop yields in salt-affected fields.

Keywords: salinity, tolerance mechanisms, transport of sodium, detoxification pathways, beneficial soil microorganisms, engineering of plant salinity tolerance

INTRODUCTION

It is expected that world population will continue to grow and exceed nine billion by 2050 (Department of Economic and Social affairs of the United Nations, 2015¹). Therefore, the global food production must increase substantially to ensure food security for the growing population. However, food production is seriously threatened by various environmental factors and soil salinity is one of the major stresses adversely affecting plant growth and crop productivity, especially in arid and semi-arid regions. Worryingly, these regions continue to expand and they represent today 40% of the world's land surface where two billion people are living, mostly in developing countries (UNEP, 1992; Flowers and Yeo, 1995). As a result, more irrigation with brackish water is unavoidable and salinization becomes a serious agricultural concern worldwide. Therefore,

¹<https://www.un.org/development/desa/en/news/population/2015-report.html>

engineering crops with enhanced salt stress tolerance traits is one of the most important challenges for modern agriculture.

This review focuses on the state of the art regarding the effect of soil salinity on plant growth and gives an overview of mechanisms controlling salt stress tolerance from sensing and signaling to gene expression and adaptive plant responses. Such knowledge is primordial for setting molecular approaches to enhance plant salinity tolerance. We will also discuss the potential of beneficial soil microorganisms in providing sustainable alternatives for improving crop production in saline soils.

SALINIZATION IN ARID AND SEMI-ARID REGION AND THE PROBLEM OF LAND DEGRADATION

According to standard definition, saline soils are those which have an electrical conductivity (EC) of the saturation soil-paste extract of more than 4 dS/m at 25°C, which corresponds to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (Munns and Tester, 2008; USDA-ARS, 2008). When grown on soils with an EC value above 4, crops significantly reduce their yield. Salts may include chlorides, sulfates, carbonates and bicarbonates of sodium, potassium, magnesium, and calcium, the diverse ionic composition of salt-affected soils results in a wide range of physiochemical properties. In the case of saline-sodic soils growth is hindered by a combination of high alkalinity, high Na^+ , and high salt concentration (Eynard et al., 2005). In this regard, it is important to distinguish between soil salinization and soil sodicity.

Soil salinization is referred as the accumulation of soluble salts in the soils (Bockheim and Gennadiyev, 2000). This is particularly favored by arid and semi-arid climates with evapotranspiration volumes being greater than precipitation volumes along the year.

Soil sodicity is a term given to the amount of Na^+ detained in the soil. High sodicity (more than 5% of Na^+ of the overall cation content) causes clay to swell excessively when wet, hence limiting severely air and water movements and resulting in poor drainage.

Salts may arise naturally in subsoil (primary salinization) or maybe be introduced (secondary salinization) by soil amendments, inorganic fertilizers, and most importantly irrigation with brackish water (Carillo et al., 2011). As a result, the total area of salt-affected lands in the world is estimated at more than 800 million hectares (ha), which account for more than 6% of the world's total land area. Of the current 230 million ha of irrigated land, 45 million ha (19.5%) have been already damaged by salt (FAO, 2016).

As NaCl is the most soluble and widespread salt, plants have evolved mechanisms to tolerate/exclude it while allowing acquisition of other nutrients available at low concentrations, such as phosphate, potassium, and nitrate.

IMPACT OF SOIL SALINIZATION ON PLANT GROWTH AND SURVIVAL

High soil salinity impacts the growth of numerous plant species especially glycophytes (salt-sensitive compared to salt-tolerant halophytes species), wherein fall major crops. Salt stress tolerance level varies from one species to another and, for cereal crops, bread wheat is a moderately salt-tolerant crop (Maas and Hoffman, 1977). In field conditions, the wheat crop yield will be reduced in the presence of 100 mM NaCl (10 dS/m), whereas rice cannot survive up to maturity under such conditions. Barley (*Hordeum vulgare*), the most tolerant cereal, can tolerate up to 250 mM NaCl (equivalent to 50% seawater), beyond which the survival rates drop drastically. Other cereals, such as durum wheat (*Triticum turgidum* ssp.), maize (*Zea mays*), and sorghum (*Sorghum bicolor*) are less tolerant to salinity (Maas and Hoffman, 1977).

The reduction in plant growth following salt exposure is due to two phases, osmotic stress and ionic toxicity (Munns and Tester, 2008). Upon a salt stress, the first phase is a rapid response to an increase in the osmotic pressure of the soil solution, whereas the second one is a slower response and takes place after the accumulation of Na^+ in photosynthetic tissues. Although they can be clearly identified in most plants and under various salt stress conditions, these two phases are not obvious under high salinity or in the case of Na-hypersensitive plant species such as rice (Munns and Tester, 2008).

It is noteworthy that the overall leaf/shoot development is more sensitive to salinity than root growth and it is assumed that less expanded leaves would decrease the water use by the plant, hence allowing it to conserve soil moisture and prevent a further rise in the salt concentration in the soil. Knowledge about the mechanism by which leaf growth and shoot development are down-regulated under salt stress is relatively scarce, but within days following a salt stress, there is evidence for the involvement, in this inhibitory mechanism, of long distance signals consisting of mainly hormones, as it was previously reported in barley (Munns et al., 2000).

Moreover, salt may affect plant growth indirectly by decreasing the rate of photosynthesis and stomatal conductance (Brugnoli and Lauteri, 1991). Decrease in stomatal aperture is considered as the most dramatic response that occurs soon after plant exposure to salinity owing to the osmotic effect of salt outside the roots (Munns and Tester, 2008).

Stomata are the main structures responsible for gas exchange control, and salt stress affects not only stomatal opening but also their size and density, resulting in a decrease in stomatal conductance. Consequently, rates of transpiration (i.e., water loss) and photosynthesis (CO_2 uptake) are also reduced. Indeed, in cotton plants submitted to salt stress treatments, a substantial reduction in photosynthesis has been associated with a decrease in total chlorophyll content and distortion in chlorophyll ultrastructures (Zhang L. et al., 2014). It has been also evoked that sink to source feedback inhibition would moderate the rate of photosynthesis to match the reduced demand arising from growth inhibition (Paul and Foyer, 2001).

On the other hand, the reduced rate of photosynthesis increases the formation of reactive oxygen species (ROS), and enzymatic antioxidant activities such as superoxide dismutase (SOD), catalase (CAT), and various peroxidases (Apel and Hirt, 2004; Foyer and Noctor, 2005; Logan, 2005). These functionally inter-related enzymes act in a coordinated manner to ensure a balance between the rate of formation and removal of ROS. However, previous studies in *Arabidopsis* have suggested that the mode of coordination between different components of the ROS removal network is complex, since ROS are also used by plants as signaling molecules controlling different processes such as growth, development, and stress responses (Mittler et al., 2004).

SALT TOLERANCE MECHANISMS IN PLANTS

Sensing and Signaling to Regulate Plant Salt Stress Response

To tolerate salt in soil solution, plants deploy a variety of traits to control cell function and development that relies on signal perception, signal integration, and processing. The development of high-throughput sequencing technologies during the last few years has generated huge quantity of data and led to the discovery of several signaling molecules. Among them, are those involved in the activation of signaling pathways that can enhance plant ability to tolerate salt stress. ROS, despite their potential toxicity, have the advantage of being versatile signaling molecules with regard to their properties and mobility within cells. The mitogen-activated protein kinase (MAPK) can trigger plant response to biotic and abiotic stresses by activating the antioxidant enzymes. Many different MAPKs cascades are activated upon ROS accumulation. These include the ROS-responsive MAPKKK, MEKK1, MPK4, and MPK6 (Xing et al., 2008; Jammes et al., 2009). ROS signaling is tightly linked to cellular homeostasis and highly integrated with hormonal signaling networks, allowing plants to regulate development processes, as well as adaptive responses to environmental constraints (Miller et al., 2010). Increased generation and accumulation of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) cause oxidative damages in the apoplastic compartment and lipid peroxidation of cellular membranes, and have an extensive impact on ion homeostasis by interfering with ion fluxes (Baier et al., 2005). Excess of ROS levels are particularly scavenged by antioxidant metabolites such as ascorbate, glutathione, tocopherols, and by ROS detoxifying enzymes such as SOD, ascorbate peroxidase (APX), and CAT. Moreover, increased ROS levels can cause salicylic acid accumulation contributing to plant defense, cell death, and induced stomatal closure (Khokon et al., 2011). Recent advance in considering the important role of ROS in plant salt responses was the discovery of a coupled function of plastid heme oxygenases and ROS production in salt acclimation (Xie et al., 2011). These findings strongly suggest involvement of the chloroplast to nucleus signaling pathway in plant salt adaptation. In depth study on cross-species expression of a

SUMO conjugating enzyme has provided considerable insight into the links between ROS, ABA (abscisic acid) dependent signaling and the sumoylation pathways in plant salt and drought tolerance (Karan and Subudhi, 2012).

To cope with salt stress, plants have developed the ability to sense both the hyperosmotic and the ionic Na^+ components of the stress. However, the molecular identities of the plant Na^+ and hyperosmotic sensors remain unknown until now. Nevertheless, plant hyperosmotic sensors are likely to be coupled with Ca^{2+} channels due to the rapid rise in cytosolic Ca^{2+} levels within seconds of exposure to NaCl or mannitol (Knight et al., 1997; **Figure 1**). The Ca^{2+} signal occurs in roots and in several cell types (Martí et al., 2013). This finding indicates that hyperosmotic stress may be sensed by a mechanically gated Ca^{2+} channel (Kurusu et al., 2013). In a recent study, Choi et al. (2014) suggested that Ca^{2+} -dependent signaling plays a role in the process of systemic signaling in response to salt stress. Thus, local salt stress of the root tip leads to the spreading of a Ca^{2+} wave that propagates preferentially through cortical and endodermal cells to distal shoot tissues at speeds of up to 400 $\mu m/s$. Salt stress-induced long-distance Ca^{2+} wave is dependent on the activity of the ion channel protein Two Pore Channel 1 (TPC1), which appears to contribute to whole-plant stress tolerance. In *Arabidopsis*, the vacuolar cation channel TPC1 is involved in propagation of calcium waves and mediates the passage of K^+ and Na^+ . TPC1 plays an important role for cation homeostasis and vacuolar storage function (Larisch et al., 2016). These results suggest that plants do possess a sensory network that uses ion fluxes moving through defined cell types to rapidly transmit information between distant sites within the organism. Downstream of Ca^{2+} signaling, calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) with CBL-interacting protein kinases (CIPKs) may become active and transduce the hyperosmotic signal to downstream protein activity and gene transcription (Weinl and Kudla, 2008; Boudsocq and Sheen, 2013).

On another hand, potassium as an essential macroelement, is needed at large amounts to be taken up from the soil and transported throughout the plant and enable efficient growth and development (Ahmad and Maathuis, 2014). Under salinity the increase in cytoplasmic Na^+ and reduction of K^+ result in changes of membrane potential, osmotic pressure, turgor pressure, calcium signaling, ROS signaling, etc. Recent results on ion fluxes in glycophyte *Arabidopsis thaliana* and the halophytic relative *Thellungiella halophila* showed lower Na^+ fluxes and higher K^+/Na^+ selectivity of ion currents in the roots and root protoplasts of the halophyte under salt treatment (Volkov and Amtmann, 2006; Amtmann, 2009). Maintenance of K^+ homeostasis is essential for enzyme activities, ionic and pH homeostasis, and cytosolic K^+ is considered to be an attribute of plant adaptive responses to a broad range of environmental constraints (Shabala and Pottosin, 2014). In addition, a strong correlation between the root's K^+ retention ability and plant salinity stress tolerance was reported for several species including wheat (Cuin et al., 2012), barley (Wu et al., 2015), and *Brassica* species (Chakraborty et al., 2016). In fact, this strong correlation between K^+ retention and net Na^+

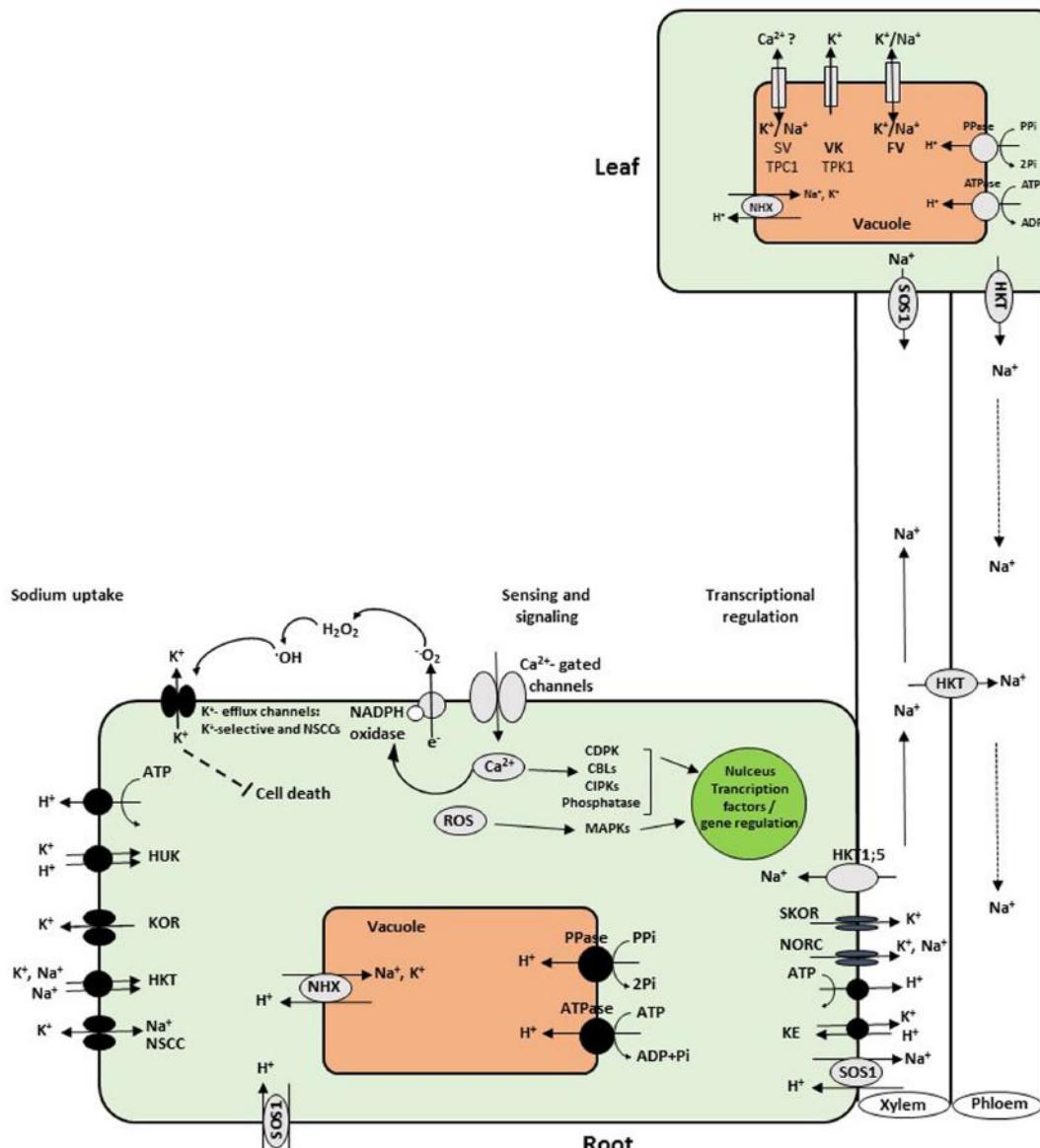


FIGURE 1 | Schematic overview of sodium uptake into roots and transport mechanisms into leaves. Na⁺ enters root cells and cross the plasma membrane via NSCCs, CNGCs, members of the HKT gene subfamily 1, and apoplastic pathways. To cope with salt stress, Na⁺ is sensed by the hyperosmotic and ionic sensors leading to activated Ca²⁺, ROS, and hormone signaling pathways. CDPKs, CBLs, CIPKs, MAPK become active and transduce signal downstream gene transcription in the nucleus. This signaling pathways result in activation of detoxification mechanisms, including the plasma membrane Na⁺/H⁺ antiporter (SOS1), HKT, and the tonoplast Na⁺, K⁺/H⁺ exchanger (NHX). SOS1 extrudes Na⁺ from the cortex cells at the root-soil interface, while at the xylem parenchyma cells; it loads Na⁺ into xylem sap. The HKT1 protein mediates the reverse flux and unloads Na⁺ from the xylem vessels to prevent overaccumulation in photosynthetic tissues. Other candidates for loading Na⁺ to the xylem are the outward-rectifying K⁺ channels KORC and NORC. At the tonoplast membrane, NSCCs include the slow vacuolar (SV) and fast vacuolar (FV) conductances, whereas the vacuolar K⁺ (VK) channel is selective for K⁺, while TPC1 is perfectly leaky for Na⁺ (Maathuis and Amtmann, 1999; Shabala and Cuin, 2007; Kronzucker and Britto, 2011). To maintain low concentration of Na⁺ in leaves, it is either retranslocated with HKT gene through the phloem to lower leaves and down to the roots, or detoxified by sequestration into the vacuole with NHX proteins. NSCCs, nonselective cation channels; HUK, HKT, high potassium affinity transporter; ROS, reactive oxygen species; CDPKs, calcium-dependent protein kinases; CBLs, calcineurin B-like proteins; CIPKs, CBL-interacting protein kinases; MAPK, mitogen-activated protein kinase; KOR, outward-rectifying K⁺ channels; NORC, nonselective outward-rectifying channels.

uptake observed in *Brassica* species argue toward involvement of GORK (outward-rectifying potassium selective) channels as a major pathway for the salt stress-induced K⁺ efflux from *Brassica* roots. GORK is central for Na⁺-induced K⁺ loss from

root epidermis. GORK channels are activated by membrane depolarization (Véry et al., 2014), and their gating is strongly dependent upon the extracellular K⁺ concentration (Anschütz et al., 2014).

On another hand, it is well established that electrolyte leakage which is considered as a hallmark of plant cell response to abiotic (including salinity) and biotic stresses, is based mainly on K⁺ efflux (Palta et al., 1977). This stress induced K⁺ leakage is often accompanied by ROS generation and leads to cell death (Demidchik et al., 2014). In addition, there are K⁺ outwardly rectifying channels that are induced by ROS and especially hydroxyl radicals (Demidchik et al., 2003, 2010). Therefore, under stress K⁺ leakage, ROS and plant cell-death (PCD) seem to be intimately connected. Such hypothesis was elegantly strengthen by Demidchik et al. (2010) who showed through pharmacological and genetic approaches that blocking K⁺-channel and the lack of functional GORK both inhibit the stress-induced activation proteases and endonucleases that lead to PCD.

Transport of Sodium and Detoxification Pathways

Na⁺-influx pathways into roots occur via different channels and transporters. Na⁺ may cross the plasma membrane through nutrient channels and calcium-permeable nonselective cation channels (NSCCs), including the cyclic nucleotide-gated channel (CNGC) and the glutamate-like receptor (GLR), which represent a likely entry point of Na⁺ into the cell (Tyerman and Skerrett, 1999; Guo et al., 2008; Deinlein et al., 2014). The role of NSCC is not restricted to mediate Na⁺ influx, which triggers K⁺ efflux via KORG, but they can also contribute to K⁺ efflux directly. Under salt stress and for efficient storage of Na⁺ to the vacuole, NSCC may play important role by preventing Na⁺ leak to the cytosol and without perturbing K⁺ release (Pottosin and Dobrovinskaya, 2014). This leads to the activation of K⁺-efflux channels (GORK) and the loss of K⁺ from the cell, stimulating cell-death enzymes (Demidchik et al., 2010). These functions might be regulated by ROS-activated ion channels in plant roots. Salt-induced K⁺ efflux in relation to Na⁺ influx, which depolarizes the membrane, increasing the driving force for K⁺ efflux and causing the activation of outward-rectifying K⁺ channels (Shabala et al., 2006; Cuin et al., 2008).

The several HKT-type transporters were shown to be involved in Na⁺/K⁺ symport in different plant species including *Arabidopsis*, rice and wheat. Based on sequence and transport analyses, HKT transporters can be classified into two distinct subgroups class I and II, with the first being more Na⁺-selective transport and the second as Na⁺-K⁺ co-transporter (Munns and Tester, 2008; Deinlein et al., 2014). The rice Na⁺ transporter OsHKT2;1 (previously named OsHKT1) has been shown to mediate Na⁺ influx into roots under K⁺ starvation (Horie et al., 2007). Other members of the HKT gene subfamily 1, are thought to mediate Na⁺ influx in root cells and to regulate the Na⁺ distribution between roots and shoots (Sunarpi et al., 2005; Horie et al., 2009).

The Na⁺/H⁺ antiporter SOS1 extrudes Na⁺ from the cortex cells at the root-soil interface, thereby reducing the net uptake of Na⁺. In contrast, at the xylem parenchyma cells, SOS1 loads Na⁺ into xylem sap, whereas HKT1-like proteins mediate the reverse flux and unload Na⁺ from xylem vessels to prevent

Na⁺ overaccumulation in photosynthetic tissues (Figure 1). Na⁺ enters the xylem by efflux out of stellar cells and is subsequently transported to aerial plant tissues. HKT1-like proteins may facilitate the translocation of Na⁺ to the upper shoot or back to the roots by unloading ions from the xylem and transported to the phloem via symplastic diffusion (Berthomieu et al., 2003; Sunarpi et al., 2005). Studies on xylem parenchyma-localized class I HKT transporters have led to the identification of an essential mechanism for plants to protect photosynthetic organs from Na⁺ overaccumulation and balancing to high K⁺/Na⁺ ratio in plants (Sunarpi et al., 2005). In *Arabidopsis*, the phloem recirculation model proposed by Berthomieu et al. (2003) suggests that Na⁺ is loaded into shoot phloem cells by AtHKT1;1 and then transferred to roots via the downstream phloem flow. In fact, it is generally accepted that AtHKT1;1 mediates the retrieval of Na⁺ from the xylem sap, thereby restricting the amount of Na⁺ reaching the young photosynthetic tissues (Berthomieu et al., 2003; Sunarpi et al., 2005).

Potential candidates for the control of xylem loading of Na⁺ are the outward-rectifying K⁺ channels KORC and NORC (Wegner and de Boer, 1997).

To balance the toxic effect of Na⁺ accumulation and to control ion homeostasis during salinity stress, plants require the maintenance of stable K⁺ acquisition and distribution (Schroeder et al., 1994). The tonoplast-localized Na⁺/H⁺ exchangers (NHX1 and NHX2) and the plasma membrane-localized Na⁺/H⁺ antiporter (SOS1), are thought to play important roles in osmoregulation and to maintain low cytoplasmic Na⁺ concentration in plant cells (Shi et al., 2002; Apse et al., 2003; Brini et al., 2007a; Olias et al., 2009). Most NHX proteins are essential for Na⁺ detoxification through sequestration into the vacuole, whereas SOS signaling pathways were responsible to export Na⁺ outside the cell. The concept that vacuolar NHX proteins were capable of exchanging Na⁺ and H⁺ across the tonoplast as claimed by Apse et al. (1999) and Blumwald (2000), was challenged by the biochemistry of NHX proteins. Indeed, NHX proteins were shown not to discriminate between Na⁺ and K⁺ nor having preference for K⁺ transport (Jiang et al., 2010). Moreover, it has been shown that NHX1 and NHX2 proteins of *Arabidopsis* play a comparatively greater role in K⁺ homeostasis, rather than in Na⁺ sequestration (Leidi et al., 2010; Bassil et al., 2011; Barragan et al., 2012). In addition, NHX1 overexpression in tomato conferred tolerance to NaCl, which was related to the preferential accumulation of K⁺ in vacuoles and improved K⁺ retention after stress imposition, but did not enhance the ability to compartmentalize toxic Na⁺ ions into the vacuole (Leidi et al., 2010).

Adaptive Mechanisms of Salt Tolerance at the Cell and Organ Level

Research carried out on salinity tolerance mechanisms was mainly performed on model plants (i.e., *A. thaliana*) and only few cases were reported on crop plants. In glycophytes, salinity tolerance is mainly achieved through more than one strategy operating either simultaneously or separately, depending on the duration and intensity of the stress. According to Chakraborty

et al. (2016), the overall superior salinity tolerance observed in *Brassica napus* was achieved by the high osmotolerance matched by the moderate tissue tolerance and superior K⁺ retention ability in the leaf mesophyll. Moreover, Chakraborty et al. (2016) provide strong evidence that higher salt tolerance in *B. napus* is conferred by at least three complementary physiological mechanisms: (i) higher Na⁺ extrusion ability from roots correlated with increased expression and activity of plasma membrane SOS1-like Na⁺/H⁺ exchangers; (ii) better root K⁺ retention ability resulting from stress-inducible activation of H⁺-ATPase and the ability to maintain a more negative membrane potential under saline conditions; and (iii) reduced sensitivity of *B. napus* root K⁺-permeable channels to ROS. Shabala et al. (2015) has argued that changes in SOS1 activity strongly correlated with changes in net K⁺ and H⁺ fluxes, making the involvement of the H⁺-ATPase/GORK tandem system as a potential sensor a plausible hypothesis. Therefore, it was suggested that Na⁺ exclusion from uptake plays an important but not a crucial role as a determinant of genetic variability in salinity stress tolerance in *Brassica*. At the root level, exclusion of ~95% of salt entering the roots back to the soil solution constitutes the major adaptive trait that plants will undertake to avoid the toxic effect of high salinity in the shoots. Species exhibiting significant genotypic variation in Na⁺ accumulation in leaves have shown a correlation between salt tolerance and Na⁺ exclusion. This is the case in sensitive species like rice and durum wheat (Munns, 2005), but also in more salt-tolerant species like barley (Chen et al., 2005). Indeed, a strong correlation between salt exclusion and salt tolerance does exist in many species (Munns and James, 2003). Further removal of sodium from xylem to translocate back into roots is another way to prevent Na⁺ overaccumulation in photosynthetic tissues. QTL analyses for salinity resistance have suggested that similar xylem Na⁺-unloading mechanisms are essential for salt tolerance in rice and wheat (James et al., 2006). In both cases, major salt tolerance QTL map to regions that include *HKT1;5* orthologs, encoding a more Na⁺-selective class I HKT transporter (Byrt et al., 2007). When reaching the stem, sodium will either be stored or controlled in its long distance transport. Partitioning of sodium into leaf sheath/petiole during its export through xylem to the leaves, could conceivably help to maintain low salt concentration in the transpiration stream (James et al., 2006; Byrt et al., 2007). However, its retranslocation through the phloem in the lower leaves and down to the roots is considered as relatively limited. To avoid raising cytosolic Na⁺ concentration and balance the toxic effect of Na⁺ accumulation, Garcia de la Garma et al. (2015) reported extensive vesicle trafficking of Na⁺ between the plasma membrane and Na⁺-rich vacuolar compartment in salt-acclimated tobacco BY2 cells. However, and as mentioned above, the enhancement of salt tolerance observed on transgenic tomato plants overexpressing AtNHX1 was related to larger K⁺ vacuolar pools and improved K⁺ retention rather than a compartmentalization of toxic Na⁺ ions into the vacuole (Leidi et al., 2010). The lack of correlation between greater salt tolerance and the enhancement of Na⁺ accumulation in different species overexpressing NHX proteins has been specified by Rodríguez-Rosales et al. (2009) and Jiang et al. (2010). The maintenance of

K⁺ acquisition with the exclusion of Na⁺ from photosynthetic leaves has been indeed found to be highly correlated with plant salt tolerance (Hauser and Horie, 2010).

STRATEGIES TO IMPROVE SALT TOLERANCE IN CROPS

Interaction with Beneficial Soil Microorganisms to Improve Salinity Tolerance

Interactions with beneficial soil microorganisms including symbiotic nitrogen-fixing bacteria (*Frankia* and rhizobia) and mycorrhizal fungi can have a large impact on plant tolerance to salt stress. The impact of salt stress on these symbioses has been reviewed elsewhere and will be not covered in this review (Swaraj and Bishnoi, 1999; Zahran, 1999; Serraj and Adu-Gyamfi, 2004; Evelin et al., 2009; Porcel et al., 2012; Ngom et al., 2016b).

Symbiotic associations with arbuscular mycorrhizal fungi (AMF) are found in roughly 80% of terrestrial plant species (Smith and Smith, 2011). These microsymbionts play a critical role in plant nutrition and profit from plant carbon in return (Smith and Read, 2010). In addition, they enhance plant performance and resistance to abiotic stresses (Sadhana, 2014). AMF can alleviate salt stress in host plants by enhancing water absorption capacity, nutrient uptake and accumulation of osmoregulators to increase osmotic potential of cells. Studies have reported that mycorrhizal colonization can reduce the uptake of Cl⁻ ions while preventing Na⁺ translocation to shoot tissues under salinity (Evelin et al., 2009). AMF have been known to occur naturally in saline environments (Evelin et al., 2009). For example, AMF (*Glomus intraradices*, *Glomus versiform*, and *Glomus etunicatum* predominantly) were observed in the severely saline soils of the Tabriz plains of Iran, where soil salinity levels range from 7.3 to 92.0 dS/m (Aliasgharzadeh et al., 2001). The effects of mycorrhizal symbiosis on plant salinity tolerance have been studied in many species including *Medicago sativa* (Azcon and El-Atrash, 1997), *Sesbania aegyptiaca*, and *Sesbania grandiflora* (Giri and Mukerji, 2004), *Z. mays* (Feng et al., 2002; Krishnamoorthy et al., 2016), *Capsicum annuum* (Kaya et al., 2009), *Olea europaea* (Porras-Soriano et al., 2009), *Citrus tangerine* (Wu et al., 2010), *Gossypium arboreum* (Tian et al., 2004), and *Lycopersicon esculentum* (Al-Karaki, 2000, 2006; Al-Karaki and Hammad, 2001; Latef and Chaoxing, 2011). In all these species, AMF improved plant salinity tolerance, leading to enhanced plant growth and yield (Azcon and El-Atrash, 1997; Giri and Mukerji, 2004; Diouf et al., 2005; Kaya et al., 2009; Porras-Soriano et al., 2009; Wu et al., 2010), nutrient acquisition (Feng et al., 2002; Giri and Mukerji, 2004; Diouf et al., 2005; Kaya et al., 2009; Porras-Soriano et al., 2009; Wu et al., 2010; Krishnamoorthy et al., 2016), chlorophyll content (Feng et al., 2002; Giri and Mukerji, 2004; Kaya et al., 2009), proline concentration (Diouf et al., 2005; Kaya et al., 2009), and promoting higher accumulation of soluble sugars in roots (Feng et al., 2002; see Table 1). In tomato, mycorrhizal colonization significantly improved fruit

TABLE 1 | Example of beneficial soil microorganisms enhancing plant salinity tolerance.

Beneficial microorganisms inoculum	Plant species	Reference
Mycorrhizal fungi		
<i>Glomus claroideum</i>	<i>Olea europaea</i>	Porras-Soriano et al., 2009
<i>Glomus clarum</i>	<i>Capsicum annum</i>	Kaya et al., 2009
<i>Glomus intraradices</i>	<i>Acacia auriculiformis</i> <i>A. mangium</i> <i>Cucurbita pepo</i> <i>O. europaea</i>	Diouf et al., 2005 Diouf et al., 2005 Colla et al., 2008 Porras-Soriano et al., 2009
<i>Glomus macrocarpum</i>	<i>Sesbania aegyptiaca</i> <i>S. grandiflora</i>	Giri and Mukerji, 2004 Giri and Mukerji, 2004
<i>Glomus mosseae</i>	<i>Citrus tangerine</i> <i>Medicago sativa</i> <i>Zea mays</i> <i>O. europaea</i> <i>Gossypium arboreum</i> <i>Lycopersicon esculentum</i>	Wu et al., 2010 Azcon and El-Atrash, 1997 Feng et al., 2002 Sheng et al., 2008 Porras-Soriano et al., 2009 Tian et al., 2004 Al-Karaki, 2000, 2006 Al-Karaki and Hammad, 2001 Latef and Chaoxing, 2011
<i>Paraglomus occultum</i>	<i>C. tangerine</i>	Wu et al., 2010
<i>Rhizophagus intraradices</i>	<i>Zea mays</i>	Krishnamoorthy et al., 2016
Rhizobia		
<i>Rhizobium</i> spp. strain AC-2	<i>A. nilotica</i>	Bala et al., 1990
<i>Rhizobium</i> spp. strain AC-1	<i>A. nilotica</i>	
<i>Rhizobium</i> spp. strain L-10	<i>L. leucocephala</i>	
<i>Rhizobium</i> spp. strain P-4	<i>Prosopis juliflora</i>	
<i>Rhizobium</i> PMA63/1	<i>A. ampliceps</i>	Zou et al., 1995
<i>Rhizobium tropici</i> CIAT899	<i>Phaseolus vulgaris</i>	Dardanelli et al., 2008
<i>Rhizobium etli</i> ISP42		
<i>Rhizobium</i> strain USDA 208	<i>Glycine max</i>	Elsheikh and Wood, 1995
<i>Bradyrhizobium</i> strain RCR 3407		
Frankia		
Crushed nodule suspension	<i>Casuarina equisetifolia</i>	Ng, 1987
Crushed nodule suspension	<i>Alnus glutinosa</i>	Oliveira et al., 2005
Ccl3 strain	<i>C. equisetifolia</i>	Ngom et al., 2016a
CeD strain	<i>C. glauca</i>	
	<i>C. equisetifolia</i>	Ngom et al., 2016a
	<i>C. glauca</i>	
Dual inoculation		
Mixed spores from <i>Glomus</i> , <i>Gigaspora</i> , and <i>Acaulospora</i> genera + <i>Sinorhizobium terangae</i>	<i>A. saligna</i>	Soliman et al., 2014
<i>Glomus mosseae</i> + <i>Mesorhizobium mediterraneum</i>	<i>Lathyrus sativus</i>	Jin et al., 2010
<i>Glomus intraradices</i> + <i>Bradyrhizobium</i> strains Aust 11c and Aust 13c	<i>A. auriculiformis</i> <i>A. mangium</i>	Diouf et al., 2005
<i>Glomus clarum</i> + <i>Azospirillum brasiliense</i>	<i>Vigna sinensis</i> <i>Vicia faba</i>	Rabie et al., 2005 Rabie and Almadini, 2005
<i>Rhizophagus intraradices</i> + <i>Massilia</i> sp. RK4	<i>Zea mays</i>	Krishnamoorthy et al., 2016
Crushed nodule suspension + <i>Glomus intraradices</i>	<i>A. glutinosa</i>	Oliveira et al., 2005

fresh weight and fruit yield under salt stress (Latef and Chaoxing, 2011). The fruit yield (kg/plant) increased by 33.3 and 106% at 50 and 100 mM salinity levels, respectively. A similar positive effect was reported in the squash *Cucurbita pepo* leading to better fruit yield production in saline conditions (Colla et al., 2008). These benefits of mycorrhizal fungi under saline conditions depend on the symbiotic associations and could therefore be improved by selection of efficient fungal strains.

Rhizobia are a group of Gram-negative soil heterogeneous bacteria that are also able to form nitrogen-fixing nodules on the roots, or occasionally the shoots, of legumes (Young, 1996) and *Parasponia* species (Akkermans et al., 1978). Bacteria are accommodated intracellularly in nodule cells and fix dinitrogen for plant growth, while being supplied with carbon sources by the plant (Pawlowski and Demchenko, 2012). Many studies have demonstrated that inoculation with suitable *Rhizobium* sp. increase plant dry weight in legumes including *Acacia nilotica*,

Leucaena leucocephala, *Prosopis juliflora* (Bala et al., 1990), *Acacia ampliceps* (Zou et al., 1995), *Phaseolus vulgaris* (Dardanelli et al., 2008), and soybean (Elsheikh and Wood, 1995) under salt stress. These beneficial effects on plant growth result from an effective N₂-fixing symbiosis, as acetylene reduction activities were detected even at high salinity levels, depending on the *Rhizobium*-legume associations (Bala et al., 1990; Elsheikh and Wood, 1995; Zou et al., 1995). Indeed, under saline conditions, the salt-tolerant strains of *Rhizobium* sp. formed more effective N₂-fixing symbiosis with *A. nilotica*, *L. leucocephala*, *P. juliflora* (Bala et al., 1990), *A. ampliceps* (Zou et al., 1995), and soybean (Elsheikh and Wood, 1995) than did the salt-sensitive strains. These results indicate that biological N₂-fixation under saline conditions may be improved by inoculation with a salt-tolerant *Rhizobium* strain. However, the tolerance of the legume host is the most important factor determining the success of compatible *Rhizobium* strains in forming effective symbioses under conditions of high soil salinity (Craig et al., 1991). Thus, a screening of both symbiotic partners is necessary for obtaining an efficient N₂-fixing symbiosis under saline soils (Zahran, 1999). Salinity tolerance of legumes could be better improved by associated *Rhizobium* with mycorrhizal fungi (Diouf et al., 2005; Jin et al., 2010; Soliman et al., 2014) and/or plant growth promoting rhizobacteria (PGPR; Rabie and Almadini, 2005; Rabie et al., 2005; Bano and Fatima, 2009). Compared to control treatments, dual inoculation of *Rhizobium* bacteria and mycorrhizal fungi under salt stress, enhanced plant nutrition, growth parameters and proline concentration in *Acacia saligna* (Soliman et al., 2014), *Lathyrus sativus* (Jin et al., 2010), *Acacia auriculiformis*, and *Acacia mangium* (Diouf et al., 2005). Co-inoculation with *Rhizobium* and PGPR including *Azospirillum brasilense* and *Pseudomonas* species showed the same beneficial effects in *Z. mays* (Bano and Fatima, 2009), *Vigna sinensis* (Rabie et al., 2005), and *Vicia faba* (Rabie and Almadini, 2005), which were more pronounced in plant inoculated with AMF, in addition to *Rhizobium* and PGPR (Rabie and Almadini, 2005; Rabie et al., 2005). In Cowpea plants, dual inoculation with AMF and nitrogen-fixing bacteria such as *A. brasilense* increased plant nitrogen content by 230% against 151 and 94% in plants inoculated separately with nitrogen-fixing bacteria and AMF, respectively, at 7.2 dS/m salinity (Rabie et al., 2005).

Frankia is a genus of Gram-positive filamentous actinobacteria that can induce the formation of nitrogen-fixing nodulation the roots of 260 species, belonging to eight dicotyledonous families (Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Eleagnaceae, Rhamnaceae, Daticaceae, and Coriariaceae), collectively called actinorhizal plants (Benson and Silvester, 1993). Like rhizobia-legume symbioses, bacteria are hosted in root nodules and fix atmospheric nitrogen (Perrine-Walker et al., 2011). Some actinorhizal plants such as Casuarinaceae trees are largely used in land reclamation pros including salinized land (reviewed in Diagne et al., 2013). Several studies have therefore reported strategies to improve the tolerance of these plants to salt stress. Similarly to what occurs in legume-rhizobia symbioses, it has been reported that inoculation with the microsymbiont *Frankia* improves the host plant salinity tolerance (Reddell et al., 1986; Ng, 1987; Oliveira

et al., 2005; Ngom et al., 2016a). *Frankia* strains CcI3 and CeD significantly improved *Casuarina glauca* and *Casuarina equisetifolia* plant growth, shoot, root, and total dry weight, proline and chlorophyll contents according to the symbiotic association (Ngom et al., 2016a). According to Ng (1987), inoculated *C. equisetifolia* plants exhibited greater growth (shoot, root, and total dry weight) compared to uninoculated plants under saline conditions. This increase in dry weight was associated with increase in the total nitrogen content of the nodulated plants even at 500 mM NaCl. In saline anthropogenic sediment (conductivity of 5,980 µS/cm), *Alnus glutinosa* plants inoculated with *Frankia* spp. alone significantly increased the growth parameters (total leaf area, shoot height, root collar diameter, and total dry weight), the leaf N content by 197% and the chlorophyll a + b content by 478%, as compared to uninoculated controls (Oliveira et al., 2005). The increased levels of N indicates the effectiveness of the nitrogen fixation process under salinity, and depends on the plant-*Frankia* isolate associations (Reddell et al., 1986). Furthermore, these beneficial effects were significantly greater when *A. glutinosa* plants were inoculated with both *Frankia* spp. and a mycorrhizal fungi, *G. intraradices*. Dual inoculation increased the leaf N, P, and K contents and chlorophyll a + b by 277, 240, 129, and 531%, respectively, suggesting the ability of both microsymbionts in improving actinorhizal plants nutrition under salt-stressed conditions (Oliveira et al., 2005). *Frankia* isolates exhibited diversity in their response to salt stress. Salinity affected their *in vitro* growth and N₂ fixation depending on the isolate. Among strains studied, *Casuarina* isolates seem to be more tolerant to salinity than others (Ngom et al., 2016b). Under saline conditions, the effects of inoculation of actinorhizal plants by salt-tolerant vs salt-sensitive *Frankia* strains remain poorly studied. Nevertheless, Reddell et al. (1986) showed that under salt stress, *Frankia* ys, collected from *Casuarina obesa* at 1.3 mg Cl⁻/g soil, was able to increase the dry weight of shoot and nodule and the nitrogen content in *C. obesa* plants better than *Frankia* cb, collected from *Casuarina cunninghamiana* at 0.1 mg Cl⁻/g soil. Among three *Frankia* isolates used separately as inoculums of *C. glauca*, Thr which was more *in vitro* sensitive to salt stress, was the most effective strain *in planta*. A recent study showed that inoculation of *C. glauca* plants with the salt-sensitive CcI3 strain improved plants growth under saline conditions while in *C. equisetifolia* plants the salt-tolerant strain CeD was more effective (Ngom et al., 2016a). These results suggest there is no correlation between *in vitro* salt tolerance of *Frankia* strains and their effectiveness in association with plants under salt stressed conditions (Girgis et al., 1992; Ngom et al., 2016a). Thus, as what was observed in *Rhizobium*-legumes symbiosis, effectiveness of established actinorhizal symbioses to saline conditions is primary dependent on the salt tolerance of the host plant (Girgis et al., 1992).

Taken together, these studies indicate that AMF and nitrogen-fixing bacteria could be used to increase salt tolerance both in crops and in plants used for saline soils rehabilitation. Co-inoculation with different beneficial microbes has the potential to further increase tolerance. These beneficial microbes improve plant growth and nutrition and promote higher accumulation

of osmolytes such as proline and sugars in saline environments. This leads to higher crop yield but also to products with better nutritional properties (Latef and Chaoxing, 2011). To fully exploit the beneficial effect of these symbioses, they need to be taken into account in breeding programs for salt-resistant crop varieties. Breeder will need to identify QTLs that control the response of the plant to these beneficial microbes in normal and salt-stress conditions. These QTLs can then be included in breeding programs along more classical salt-resistance QTLs. Furthermore, the development of industrial scale microbial inoculants for salinized soils will be required.

Salt Tolerance in Crops through Marker-Assisted Selection and Genetic Engineering

Conventional plant breeding approaches through which beneficial traits can be introgressed into elite varieties have been adopted since a long time to generate stress tolerant varieties (for review, see Ashraf, 2010; Varshney et al., 2011). Hence, traditional breeding allowed the development of new salt-tolerant rice and wheat varieties (Munns et al., 2006; Singh et al., 2010). However, as salinity tolerance is a multigenic trait, such approaches have only limited success, which can explain the absence of commercially available salt-tolerant crops. Moreover, as a procedure, plant breeding is time consuming and labor intensive, relies on well characterized germplasms and can result in introducing undesirable traits along with the selected one. Therefore, biotechnological approaches including molecular breeding and genetic engineering seem to be more attractive alternatives.

Plant Salinity Tolerance through Genetic Engineering

In the last 30 years, tremendous progress has been made toward the isolation and molecular characterization of genes involved in plant salt stress responses. These candidate genes can be classified into two main groups: effectors and regulatory genes. The first group includes mainly genes encoding ion transporters, channels, enzymes involved in osmolyte biosynthesis, antioxidant systems and protective proteins such as heat shock proteins and late embryogenesis abundant (LEA) proteins. The second group is composed of genes involved in transcriptional and post-transcriptional regulation as well as in signaling pathways. Within this group we find essentially transcription factors, protein kinases, phosphatases, and proteases. Numerous reports described how the overexpression of these genes can improve plant tolerance to various abiotic stresses including salinity (for review, see Ashraf, 2009; Türkan and Demiral, 2009; Cominelli et al., 2013; Roy et al., 2014; Fita et al., 2015; see Table 2).

Ion transporters

Ion transporters are obvious candidates among effectors since they participate in salt detoxification and ion homeostasis. Overexpression of genes involved in Na^+ transport was therefore largely investigated. The *Arabidopsis* vacuolar Na^+/H^+ antiporter (AtNHX1) was among the first candidate genes which when

overexpressed lead to enhanced salinity tolerance (Apse et al., 1999). These transgenic plants were reported to exhibit higher ability for vacuolar sequestration of Na^+ to avoid its toxic accumulation into the cytoplasm. Later on, overexpression of AtNHX1 and related NHX proteins from various sources have been shown to increase salt tolerance in other plant species including tomato (Zhang and Blumwald, 2001), *B. napus*, wheat and cotton (Pardo et al., 2006; Munns and Tester, 2008). Interestingly, the tomato AtNHX1 overexpressing plants are more salt stress tolerant, with an accumulation of NaCl in leaves but not in fruits. However, and as was indicated above, its role in salt stress tolerance may also result from its capacity to facilitate K^+ uptake at the tonoplast (Leidi et al., 2010). Nevertheless, engineering of vacuolar cation/ H^+ antiporter coupled with its H^+ -translocating pyrophosphatase (H^+ -PPase) often leads to increased salt stress tolerance. Therefore, overexpression of the wheat TNHX1 and TVP1 improves salt stress tolerance in *Arabidopsis* (Brini et al., 2007a), tobacco (Gouiaa et al., 2012), and tomato (Gouiaa and Khoudi, 2015). Alone the *Arabidopsis* H^+ -PPase (AtAVP1) was also demonstrated to increase stress tolerance in a crop plant. Indeed, Schilling et al. (2013) reported that barley transgenic plants overexpressing AtAVP1 are more tolerant to salinity under greenhouse conditions but they also showed increased shoot biomass production and grain yield in a saline field.

On another hand, the HKT family and the SOS pathway play also a relevant role in controlling Na^+ transport within the plant and have been considered in transgenic approaches to increase salt stress tolerance in crops. However, knowing their role in the removal of Na^+ from the xylem sap into the surrounding xylem parenchyma cells, improving salinity tolerance using HKTs can be successful only when its expression is targeted to the stele or driven by salt-inducible promoter (Møller et al., 2009; Roy et al., 2014). Recently, the overexpression of *Ncl* gene (homologous to the Na^+/H^+ antiporter gene family) into a Japanese soybean salt-sensitive cultivar Kariyutaka, resulted in improved salt tolerance in transgenic soybean. A close association was observed between the high expression of the salt tolerance gene *Ncl* in the root, the lower accumulation of Na^+ , K^+ , and Cl^- in the shoot under salt stress and salt tolerance in the transgenic lines (Do et al., 2016).

Osmolyte accumulation

Under salt stress, along with Na^+ exclusion from the cytoplasm, plant cell accumulates a wide range of compatible solutes or osmolytes to balance the osmotic pressure of ions in vacuoles. Sucrose, proline, and glycine betaine are among the most studied osmolytes accumulating upon salt stress in some plant species including halophytes (Flowers et al., 1977). Therefore, plant engineering for higher accumulation of these compounds was considered as a possible way in improving crop tolerance to salinity. The bacterial *mt1D* gene encoding the mannitol-1-phosphate dehydrogenase, enzyme involved in mannitol biosynthesis was, early on, successful in enhancing salt tolerance after its ectopic expression in tobacco (Tarczynski et al., 1992, 1993). Similarly, transgenic rice overexpressing choline oxidase showed increased levels of glycine betaine and enhanced tolerance to salinity and cold (Sakamoto et al., 1998).

TABLE 2 | Example of genes leading to improvement of salt stress tolerance of crop plants through genetic engineering.

Transgene	Function	Donor	Transgenic plant	Description	Reference
Na ⁺ /H ⁺ antiporter (<i>AtNHX1</i>)	Vacuolar sequestration of Na ⁺ and K ⁺ ?	<i>Arabidopsis</i>	Tomato <i>Brassica napus</i> Wheat Cotton	Enhanced salt tolerance with higher accumulation in leaves but not in fruits Maintenance of seed yield and seed oil quality under high salinity Improved grain yield in saline soils Increased fiber yield under salt stress	Zhang and Blumwald, 2001 Zhang et al., 2001 Xue et al., 2004 He et al., 2005
H ⁺ -pyrophosphatase (<i>AVP1</i>) <i>AtNHX + AVP1</i>	Vacuolar membrane-bound proton pump	<i>Arabidopsis</i>	Cotton Barley Cotton Tomato	Increased fiber yield under salt stress in field conditions Higher biomass production and grain yield in saline field Further enhancement of salt tolerance compared to single-gene overexpressing plants Increased salt stress tolerance compared to single gene overexpression	Pasapula et al., 2011 Schilling et al., 2013 Shen et al., 2014 Gouiaa and Khoudi, 2015
<i>Ncl</i>	homologous to NHX gene family	Soybean	Soybean	Improved salt tolerance	Do et al., 2016
Mannitol-1-phosphate dehydrogenase <i>mt1D</i>	Mannitol biosynthesis	<i>E. coli</i>	Tobacco	Increased salt tolerance	Tarczynski et al., 1992
Choline synthase <i>codA</i>	Betaine biosynthesis	<i>E. coli</i>	Rice	Enhanced tolerance to salinity and cold	Sakamoto et al., 1998
delta 1-pyrroline-5-carboxylate synthase (<i>P5CS</i>)	Proline biosynthesis	<i>Arabidopsis</i>	Tobacco	Increased tolerance to drought and salt stress	Kishor et al., 1995
ascorbate peroxidase (<i>AtAPX</i>)	ROS-scavenging	<i>Arabidopsis</i>	Tobacco	Enhanced tolerance to salt and osmotic stress	Badawi et al., 2004
Late embryogenesis abundant protein (<i>HVA7</i>)	Osmoprotection	Barley	Rice	Enhanced tolerance to salt and osmotic stress	Xu et al., 1996
Transcription factor <i>DREB1B/CBF1</i>	Transcription regulation	<i>Arabidopsis</i>	Rice	Enhanced tolerance to salinity and drought	Oh et al., 2005
Transcription factor <i>DREB1A/CBF3</i>	Transcription regulation	<i>Arabidopsis</i>	Rice	Increase in salinity tolerance	Hu et al., 2006
<i>SNAC1</i>	Transcription regulation	Rice	Rice	Increase in salinity tolerance	Hu et al., 2008.
Transcription factor <i>SIAREB1</i>	Transcription regulation	<i>Tomato</i>	Tomato	Enhanced tolerance to salinity and water stress	Orellana et al., 2010
Transcription factor <i>ZFP179</i>	Transcription regulation	Rice	Rice	Increase in salinity tolerance	Sun et al., 2010
calcium-dependent protein kinase <i>OsCDPK21</i>	Calcium signaling	Rice	Rice	Increase in salinity tolerance	Asano et al., 2011
MAP kinase <i>GhMPK2</i>	MAPK signaling	Cotton	Tobacco	Enhanced tolerance to salinity and drought	Zhang et al., 2011

Also, plants such as tobacco transformed with delta 1-pyrroline-5-carboxylate synthase (*P5CS*) gene exhibited higher proline production, correlated with increased tolerance to drought and salt stress (Kishor et al., 1995). Similarly, transgenic rice plants expressing the mothbean *P5CS* gene under constitutive or stress inducible promoter showed significant tolerance to high levels of NaCl (Su and Wu, 2004).

Antioxidant systems and protective proteins

Despite their importance as signaling molecules regulating cellular responses to various stresses (for review, see Apel and Hirt, 2004), ROS can also damage plant tissues during salinity stress by perturbing enzyme, cell wall and membrane function. Plants detoxify then ROS generated by salt stress by up-regulating antioxidative enzymes such as SOD, CAT, APX, and glutathione peroxidase. Therefore, overexpressing ROS-scavenging enzymes

were shown to promote tolerance of plants to various stresses including salinity (Rodriguez-Rosales et al., 1997; Roxas et al., 1997; McKersie et al., 1999; Badawi et al., 2004; Miller et al., 2008). For example, the overexpression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit (Badawi et al., 2004).

Other proteins like osmotin and LEA proteins contribute in alleviating salt stress by protecting macromolecules from damages caused by ion toxicity and/or water deficit. HVA7, a LEA from barley, when transferred to rice, confers water and salt stress tolerance (Xu et al., 1996). It is worth to note that, among the different groups, the group 2 of LEA proteins known as dehydrins are particularly interesting and were shown to enhance plant tolerance to various stresses (Hanin et al., 2011). Indeed, Brini et al. (2007b) showed that the expression of the wheat dehydrin DHN-5 in *A. thaliana* led to an increase in salt and osmotic stress

tolerance, but also evidence is provided for the involvement of DHN-5 in other abiotic and biotic stress responses (Brini et al., 2011; Drira et al., 2015).

Transcription factors and signaling proteins

Plant response to salinity is complex and involves multiple genes involved in distinct or overlapping regulatory pathways. Therefore, the engineering of a single downstream effector gene as indicated above, albeit efficient in some circumstances might have limited success when one considers multiple stress combination as occurring in the field. In contrast, regulatory proteins such as transcription factors and signaling proteins will gain increasing interest as they are expected to modulate the expression of numerous downstream genes involved in stress responses. It is well documented that transcription factors belonging to the families of DREB, NAC, MYB, MYC, Cys2/His2 zinc finger, bZIP, AP2/ERF, and WRKY are relevant in salt stress tolerance (Golldack et al., 2011, 2014).

In this regard, several transcription factors such as DREBs, MYCs, AP2/ERFs, and NACs were tested in model plant species and few crops. In some cases, the overexpression of these transcription factors was successful to enhance salinity tolerance in crops (for review, see Lata and Prasad, 2011; Lata et al., 2011; Turan et al., 2012; Nuruzzaman et al., 2013).

The expression of DREB1B/CBF1 or DREB1A/CBF3 under the control of the cauliflower mosaic virus 35S promoter in *Arabidopsis* plants increases significantly tolerance to freezing, drought, and high salinity stresses (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). Similarly, transgenic rice plants constitutively expressing DREB1A/CBF3 were reported to be more tolerant to drought and salinity (Oh et al., 2005). Also, the overexpression of SNAC1 or SNAC2 (stress responsive NAC) resulted in an enhanced salinity tolerance (Hu et al., 2006, 2008).

SlAREB1 is a bZIP transcription factor from tomato (*Solanum lycopersicum*), member of the ABA-responsive element binding protein (AREB)/ABA-responsive element binding factor (ABF) subfamily. Its overexpression in tomato was reported to improve tolerance to water and salt stresses (Orellana et al., 2010). Likewise, Sun et al. (2010) have shown that the overexpression of ZFP179, a salt responsive gene encoding a Cys2/His2 zinc finger protein enhanced salt tolerance in rice.

Using strong and constitutive promoters to drive the expression of transcription factors is still, however, considered as a controversial strategy. The constitutive expression of transcription factors caused in some cases growth defects under standard conditions as was reported for the 35S:TaDREB1 rice transgenic plants overexpressing the bread wheat DREB1 gene that showed a dwarf phenotype (Shen et al., 2003). Therefore, one should reconsider the use of constitutive promoters and rather employ stress inducible/tissue-specific promoters to avoid secondary deleterious effects (Munns and Tester, 2008).

Moreover, effects of the overexpression of genes encoding signaling proteins such as kinases and phosphatases on salt tolerance were reported. As conserved signaling proteins at the crossroads of several signaling cascades, MAPKs play pivotal role in plant responses to various stresses. Many transgenic plants that have been engineered with MAPK cascade were reported to be

tolerant to salt stress. The ectopic expression of cotton *GhMPK2* improves salinity and drought tolerance in tobacco (Zhang et al., 2011). In addition, CDPKs, which are involved in salt stress response, were found efficient in transgenesis approaches, as was reported for the overexpression of *OsCDPK21* that resulted in increased salinity tolerance of transgenic rice (Asano et al., 2011). However, other MAPKs can have opposite effects and this was described for the rice OsMAPK33, the overexpression of which caused higher sensitivity to drought and salinity compared to wild-type plants (Lee et al., 2011). Regulators of MAPKs, the MAPK phosphatases (MKPs) can also be involved in the control of stress responses. Interestingly, while the *Arabidopsis* AtMKP1 acts as a negative regulator, the wheat counterpart acts as a positive regulator of salt stress responses (Ulm et al., 2002; Zaidi et al., 2016).

Noteworthy, it is still until now problematic to generate and commercialize crops more tolerant to salinity or to any other stresses. This can be due to several factors. First of all, the laboratory growth conditions are far different from field conditions. Field tests are often forbidden or restricted in several countries but mainly one needs to consider the reproducibility of the field tests and be able to measure and monitor any environmental changes that may affect crop yield (humidity, soil composition, light intensity,...). Second, often the stress tolerance of transgenic crop grown in a greenhouse or a growth chamber is assessed by measuring survival and/or recovery rates, while for farmers; the main trait for stress tolerance is crop yield. Moreover, in the first case the evaluation of stress tolerance level is limited to particular traits (plant height, leaf or root size) or development stages during the vegetative phase and only occasionally covers the reproductive stage. The need to conduct field trials under monitored conditions over several years in distinct environmental conditions is a necessary condition to ensure the development of sustainable genetically improved salt-tolerant crops.

Marker-Assisted Selection and Salt Stress Tolerance

One of the major limitations for the use of conventional breeding to improve salt tolerance in crops is its slowness which is closely linked to the complexity of this polygenic trait. Traditionally, the selection of crops raised from backcrosses of genetically diverse germplasms is limited to their phenotype analyses in the field. As an alternative to streamline this process, breeders use QTL analyses coupled with marker-assisted selection (MAS), referred as an approach linking a quantitative trait with a genetic marker that is polymorphic between parental lines (Ashraf and Foolad, 2013). Thus still, accumulating knowledge on plant salt stress response is a must to be able to develop confident and efficient markers. The fact that maize, rice, barley, sorghum, and soybean genomes are sequenced and the advances made in next generation sequencing (NGS) helps nowadays to develop high-resolution genetic maps to produce salt-tolerant crops. A quick search on ene database for QTL related to salt revealed 17 QTLs in *Oryza sativa* covering six chromosomes and delimited by several markers². “Saltol” was reported as a promising QTL for salinity

²<http://ene.org>

tolerance in rice that was identified after searching for more than 100 SSR markers in 140 recombinant inbred lines between Pokkali and IR29 (Thomson et al., 2010). "Saltol" is located on chromosome 1 and is important for the maintenance of the shoot ratio of Na^+/K^+ (Thomson et al., 2010). Other QTL analyses performed in wheat for Na^+ tolerance allowed the identification of Nax1 locus, which maps to the region of the TaHKT1;4 gene that contributes to Na^+ removal from xylem in the leaf sheath avoiding its over-accumulation in leaf blades (Huang et al., 2006). In soybean, genetic variation for salt tolerance has been described and was observed in wild and cultivated soybean species, suggesting that genetic improvement of salt tolerance is feasible (Lee et al., 2008; Qi et al., 2014). A major QTL for salt tolerance was constantly detected on soybean chromosome 3 (linkage group N) in different populations (Lee et al., 2004; Hamwieh et al., 2011). This QTL is likely to be the *Ncl* locus based on pedigree tracing (Lee et al., 2004). Recently, the introgression of the tolerance allele *Ncl* into soybean cultivar Jackson, using DNA MAS, produced an improved salt-tolerant line (Do et al., 2016).

Despite these successful examples, MAS based breeding pros are still limited because undesirable traits may be transferred with the QTL when wild relatives are used as donors and the results raised from QTL analyses must be confirmed in different conditions and genetic backgrounds. In fact, the cultivated soybean germplasm may be more efficiently used in breeding cultivars with improved salt tolerance compared with that of wild soybean, which generally possesses several undesirable agronomic traits.

Nevertheless, NGS technologies will significantly contribute into discovering molecular markers to obtain high density genetic maps, a prerequisite for a precise location and quicker cloning of new QTLs. Moreover, the advent of genomic selection should speed up the production of varieties combining several salt-resistance QTLs.

CONCLUSION

Over the last two decades, research made on *Arabidopsis* and a few crops shed light on several aspects of the molecular mechanisms controlling the salt stress tolerance. However, many challenges still lie ahead before successfully improving crop yield under saline conditions. Hopefully, available tools including molecular breeding and advanced biotechnology methods combined to the exploitation of the potential of soil microorganisms can speed up the release of salt-tolerant crop varieties. A combination of approaches will accelerate

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the identification and characterization of specific loci involved in tolerance to salinity that can be introgressed into elite sensitive varieties through molecular marker-assisted breeding. To achieve this goal, we need to have diversity in germplasm resources, high-throughput phenotyping platforms, genome sequencing of crops and their relatives. Furthermore, molecular genetic resources including mutation detection, gene discovery and expression profile, genome wide association studies, and powerful omics databases are also needed. Many traits related to salt stress tolerance have been identified and shown effective for engineering stress tolerance in model plants. The most impressive results were obtained when manipulating signaling factors, as they control a broad range of downstream events, which results in superior tolerance. Effective expression systems, including cell type-specific and stress-inducible promoters will be required to adapt the plant response to stress, lower the energy cost, according to the environmental constraints as using constitutive promoters can have severe drawbacks on plant growth or yield. Finally, the targeted genome editing using CRISPR-Cas9 technology has emerged as an alternative to classical plant breeding and transgenic (GMO) methods. CRISPR-Cas9 technology enables precision design of alleles that aid stress tolerance (Zhang H. et al., 2014), but in depth study of genome editing to engineer mechanisms of salt stress tolerance needs to be pursued in the coming years.

AUTHOR CONTRIBUTIONS

KM handled the entire process of preparing this paper and prepared the topic on salt tolerance mechanisms in plants. MH and CE prepared the topics related to salinization in arid and semi arid region and the problem of land degradation, the impact of soil salinization on plant growth and survival and salt tolerance in crops through marker-assisted selection and genetic engineering. Finally, LL and MN prepared the topic related to the interaction with beneficial soil microorganisms to improve salinity tolerance. All authors reviewed the manuscript.

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Overcoming Phosphorus Deficiency in West African Pearl Millet and Sorghum Production Systems: Promising Options for Crop Improvement

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West Africa (WA) is among the most food insecure regions. Rapid human population growth and stagnating crop yields greatly contribute to this fact. Poor soil fertility, especially low plant available phosphorus (P) is constraining food production in the region. P-fertilizer use in WA is among the lowest in the world due to inaccessibility and high prices, often unaffordable to resource-poor subsistence farmers. This article provides an overview of soil P-deficiency in WA and opportunities to overcome it by exploiting sorghum and pearl millet genetic diversity. The topic is examined from the perspectives of plant breeding, soil science, plant physiology, plant nutrition, and agronomy, thereby referring to recent results obtained in a joint interdisciplinary research project, and reported literature. Specific objectives are to summarize: (1) The global problem of P scarcity and how it will affect WA farmers; (2) Soil P dynamics in WA soils; (3) Plant responses to P deficiency; (4) Opportunities to breed for improved crop adaptation to P-limited conditions; (5) Challenges and trade-offs for improving sorghum and pearl millet adaptation to low-P conditions in WA; and (6) Systems approaches to address soil P-deficiency in WA. Sorghum and pearl millet in WA exhibit highly significant genetic variation for P-uptake efficiency, P-utilization efficiency, and grain yield under P-limited conditions indicating the possibility of breeding P-efficient varieties. Direct selection under P-limited conditions was more efficient than indirect selection under high-P conditions. Combining P-uptake and P-utilization efficiency is recommendable for WA to avoid further soil mining. Genomic regions responsible for P-uptake, P-utilization efficiency, and grain yield under low-P have been identified in WA sorghum and pearl millet, and marker-assisted selection could be possible once these genomic regions are validated. Developing P-efficient genotypes may not, however, be a sustainable solution in itself in the long-term without replenishing the P removed from the system in harvested produce. We

therefore propose the use of integrated soil fertility management and systems-oriented management such as enhanced crop-tree-livestock integration in combination with P-use-efficiency-improved varieties. Recycling P from animal bones, human excreta and urine are also possible approaches toward a partially closed and efficient P cycle in WA.

Keywords: phosphorus use efficiency, low-P tolerance, sorghum, pearl millet, sahel

INTRODUCTION

Much interest in food security has focused on depletion of non-renewable energy and land resources. Recently, the depletion of phosphorus (P) is receiving increased interest (Van Vuuren et al., 2010; Cordell and White, 2015) as a major limiting factor. Low soil P is considered to be one of the major constraints for food production in the whole of sub-Saharan Africa (SSA; Verde and Matusso, 2014) and is the main limiting macronutrient for the staple cereal crops of sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) in the Sahelian and Sudanian regions of West Africa (WA; Bationo and Mokwunye, 1991; Buerkert et al., 2001).

Phosphorus is essential for plant nutrition, serving as a component of DNA and cellular energy transport (Cooper et al., 2011; Obersteiner et al., 2013). The application of P-containing fertilizers is therefore important for food production in the context of P-deficiency. The P delivered by inorganic fertilizers is derived from rock phosphate, which is a non-renewable resource (Cordell et al., 2009; Cooper et al., 2011; Veneklaas et al., 2012). The high transportation and processing costs of inorganic P fertilizer make it generally too expensive and hard to access for many farmers in low-income and food-insecure countries (Obersteiner et al., 2013). A more intensive use of inorganic P fertilizers to increase the largely subsistence-oriented food production in WA is therefore not an option in the foreseeable future (Cordell et al., 2009; Obersteiner et al., 2013). Non-acidulated rock phosphate could be used as a substitute source of P (Bationo and Mokwunye, 1991) but farmers have not widely adopted this technology because it often does not produce visible results in the first year and most rock phosphates are not suitable for direct application. Given these conditions, breeding for crops which may produce higher yields under P-limited conditions appears to make an important contribution to an environmental friendly and economically feasible strategy in order to improve pearl millet and sorghum yields in WA under subsistence farmers' conditions.

The overall goal of the present article is to explore the prospects of using sorghum and pearl millet genetic diversity to contribute toward solving the P-deficiency issue in WA following a multidisciplinary approach. Specific objectives are to summarize:

- (1) The global problem of P scarcity and how it will affect WA small-scale farmers;
- (2) Soil P dynamics in WA soils;
- (3) Plant responses to P deficiency;
- (4) Opportunities to breed for improved crop adaptation to P-limited conditions, with the aspects of existing genetic

diversity, the question of direct versus indirect selection, availability of genomic tools to enhance phosphorus use efficiency in crops;

- (5) Challenges for improving sorghum and pearl millet adaptation to low-P conditions in WA, including lack of reliable screening procedures for accurate phenotyping and trade-offs between traits of interest; and
- (6) Systems approaches to address P deficiency in WA.

SOIL PHOSPHORUS DYNAMICS

The overall P dynamics in the soil-plant system is a function of the integrating effects of P transformation, availability, and utilization driven by soil, rhizosphere and plant processes (Shen et al., 2011). From a pedo-genetical perspective, soil P originates from primary minerals such as apatite, strengite, variscite, and vivianite (Shen et al., 2011). In magmatic parent rocks the phosphate concentration is generally low (100–300 ppm). Mainly sedimentary processes can lead to accumulation of phosphates that can then be used as mineable resources. However, these processes are rare and most sediments too, are characterized by low P concentrations. These low concentrations are a consequence of the parent material and its weathering (Table 1).

Through chemical weathering, mainly protolysis, the primary minerals are transformed to soluble orthophosphates. These have different fates: (i) Depending on the ions present in the soil solution (pH-dependent), they can react with calcium, iron (Fe), or aluminum (Al) to form secondary minerals. In addition, they can be absorbed to Fe and Al oxyhydrate surfaces and later on be occluded upon further growth of these minerals. Compared to the primary minerals, the secondary minerals have a very low solubility (Blume et al., 2010). (ii) The phosphate anion can be taken up by plants or soil-microorganisms from the soil solution and some of it is then converted to organic P forms, while other parts might be kept as phosphate, stored in the vacuole or active in the cytoplasm (Hinsinger, 2001; Shen et al., 2011; Verde

TABLE 1 | Descriptive statistics of total and plant available phosphorus in topsoil samples of South-West Niger based on Hammer (1994).

	Total P	Plant available P (Bray 1)
	mg kg ⁻¹	mg kg ⁻¹
Mean	272	7.6
Standard deviation	288	6.6
Min	90	0.5
Max	2110	36.6
n	65	53

and Matusso, 2014). (iii) Humic (organic matter) substances can also indirectly form complexes with the orthophosphates where the orthophosphates are bound by Al and Fe complexed by the humic substances to form humic-metal-P complexes (HMEP) and this form may account to between 50–80% of P in the soil solution (Gerke, 2010). Organic P mainly exists in form of inositol phosphates, phosphonates, active forms as orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates; as a consequence, P occurs in the soil either in inorganic or organic form (Verde and Matusso, 2014). The ratio of both fractions mainly depends on the concentration of organic matter in the soil, and the proportion of inorganic P increases with soil depth.

The inorganic primary and secondary P minerals and the organic P forms (once released from the organism) can become plant available upon chemical reaction (Turner et al., 2002; Condon et al., 2005; Shen et al., 2011). However, this plant available fraction usually represents only several percent of the total P stock. Soils in South-West Niger have on average have plant available P concentrations that are close to the absolute deficiency level ($4\text{--}5 \text{ mg kg}^{-1}$, Table 1).

The mobility of inorganic P in most soils is still poorly understood and hardly predictable because of the lack of appropriate methods for studying its speciation and biogeochemical behavior (Hinsinger, 2001). However, P availability in the soil depends on the types and amounts of clay and metal oxides, soil solution pH, ionic strength, concentrations of P and metals (Fe, Al and Ca) and the presence of competing anions, including organic acids (Hinsinger, 2001) as well as symbiotic interaction of the crop plants with micro-organisms (e.g., symbiosis of sorghum/pearl millet with mycorrhiza) that facilitate P uptake.

Phosphorus is taken up by plants either as dihydrogen phosphate ions (H_2PO_4^-) at low soil pH, or as hydrogen phosphate ions (HPO_4^{2-}) at high soil pH, and these occur in soil solutions only at very low concentrations (Raghothama, 1999; Hinsinger, 2001; Hammond et al., 2009). This is the reason why phosphate is leached from soils in only very low amounts under quasi-natural conditions.

Phosphorus acquisition happens by diffusion along the depletion gradient established by the plant and as such is a very slow process. It is therefore important to increase the amount of available P as close to the plant roots as possible in order to increase plant productivity (Hash et al., 2002). A series of responses are triggered in plants under P deficiency which either increase the ability of a plant to acquire P from the soil or the ability of the plant to use the P taken up more efficiently (Vance et al., 2003; Hammond et al., 2004; Jain et al., 2007; Hammond and White, 2008).

Phosphorus Deficiency in West Africa

Soils in Sahelian WA are either Entisols which are composed of quartz sand or Alfisols which have a clay accumulation horizon and are highly saturated with cations (Kang, 1985), using the soil classification system from United States Department of Agriculture/Natural Resources Conservation Service [USDA/NRCS], 1985). These soils have been shown to have

poor structural stability, low water retention, low nutrient holding capacity, low organic matter content, low effective cation exchange and are highly prone to drought (Kang, 1985; Bationo and Mokwunye, 1991). Soils in the sorghum and pearl millet growing areas of WA, the Sudano-Sahelian zone, have been shown to have low total and available P levels with an average total P of 109 mg kg^{-1} and available soil P of sometimes less than 2 mg kg^{-1} (Manu et al., 1991). These soils often have a low capacity to fix P, with sorption data for P in pearl millet growing areas ranging from 27 mg kg^{-1} to 252 mg kg^{-1} (Sanchez and Uehara, 1980). The low plant available soil P in WA can be attributed to several factors: (i) the Aeolian parent materials have low mineral reserves therefore lacking primary minerals for nutrient recycling; (ii) a proportion of P is in occluded form and therefore not available; (iii) low organic matter and the removal of organic residues from the fields (Charreau, 1974). Information on the agronomic practices including combining of organic and inorganic fertilizer, crop rotation, intercropping, among others, for sustainable management of sandy Sahelian soils with regard to P deficiency is provided by Bationo et al. (2007).

PLANT RESPONSES TO PHOSPHORUS DEFICIENCY

P-uptake or acquisition efficiency (PAE) and P internal utilization efficiency (PUTIL, sometimes also called PUE) are the two strategies of plant adaptation to P-limited conditions. Phosphorus-uptake efficiency can be defined as the total P in the above-ground plant organs at maturity per unit area. Phosphorus-utilization efficiency is the grain yield per unit of P taken up (Moll et al., 1982; Wang et al., 2010; Manschadi et al., 2014). Both strategies together result in the plants' P-use efficiency (grain yield per soil available P). Plant adaptations that may contribute toward P-uptake efficiency involve altered root morphology and architecture (Lynch and Brown, 2001; Lynch, 2007), symbioses with vascular arbuscular mycorrhiza (VAM; Smith and Read, 2008) to effectively explore a larger volume of soil, exudation of carboxylates (Lambers et al., 2006, 2011) and secretion of phosphatases (Vance et al., 2003; Richardson et al., 2009) to mobilize organic P forms. Release of citrate and other organic anions into the rhizosphere also helps prevent root damage by chelating aluminum ions (Al^{3+}) and lead to more plant available P by mobilizing previously bound P mainly by ligand exchange, dissolution and occupation of P sorption sites (Neumann and Römheld, 1999; Ma et al., 2001). Aluminum-stimulated malic acid secretion encoded by the *Alt1* locus originally identified in wheat by Delhaize et al. (1993) is another mechanism toward Al tolerance with the malic acid mainly secreted from root apices being able to protect seedlings from toxic Al levels. Phosphorus-uptake (acquisition) efficiency resulted in higher yield increase under P-deficient conditions in rice (Vandamme et al., 2016), WA sorghum (Leiser et al., 2014b) and pearl millet (Gemenet et al., 2015b) as compared to internal P-utilization efficiency. However, the higher P-uptake efficiency is expected to result in greater P removal from the cropping system with for example about $1\text{--}2 \text{ kg P ha}^{-1}$ removed

by higher P-uptake rice varieties (Vandamme et al., 2016). These two strategies (P-uptake efficiency and P-utilization efficiency) may be potentially independent and may offer additive benefits if they co-exist in the same genotype (Richardson et al., 2011) and if P-utilization efficiency can be properly separated from the confounding effects of P-uptake efficiency by proper phenotyping approaches (Vandamme et al., 2016; Rose et al., 2016). In WA where soils are characterized by low total P, with low P-fertilizer use, and where residues are removed from the farms (Bationo et al., 2007), combining both P-uptake and P-utilization efficiency would be of particular importance in order to reduce further mining of P from the soils and to enhance adaptation of WA crops to P-limited conditions.

OPPORTUNITIES TO BREED FOR IMPROVED CROP ADAPTATION TO PHOSPHORUS LIMITED CONDITIONS

Availability of Genetic Variation

The success of a breeding program depends on accessing useful levels of genetic variability for the target trait and an efficient selection method for increasing the frequency of desirable genes or gene combinations. WA sorghums were found to have large genetic variation for P-uptake and utilization efficiencies under P-limited conditions (Leiser et al., 2014b). Differences were observed, however, between specific sorghum germplasm pools. Guinea-race landrace and photoperiod sensitive sorghum varieties, for example showed higher P-uptake efficiency, whereas varieties bred from Caudatum-race introgressed materials, showed higher mean P-utilization efficiency corrected for harvest index (Leiser et al., 2014b). The potential benefits of combining these two pools is suggested by a genotype derived from an inter-pool population that combined superior levels of both P-uptake and utilization efficiency. It was also noted that no single P parameter (P-utilization or P-uptake) trait was appropriate as single selection criterion for enhancing yield under low-P conditions (Leiser et al., 2015).

Pearl millet was also found to possess large genetic variation for P-uptake efficiency, P-utilization efficiency and grain yield performance under P-limited conditions across large-scale regional evaluations in WA under P-deficiency (Gemenet et al., 2014, 2015b). The presence of important genetic variation for these traits in both pearl millet and sorghum indicates that classical breeding can be used to enhance performance under P-deficient conditions in WA.

Symbioses with VAM to enhance access to inorganic forms of P has also been examined in WA pearl millet and sorghum. Beggi (2014) found genetic variation for early VAM colonization and positive correlation of total root length infected with VAM and P-uptake efficiency among open-pollinated pearl millet varieties under low-P conditions. Beneficial effects of VAM infestation in pearl millet were also reported by Bielders et al. (2010) suggesting that this mechanism offers potential for improving P uptake efficiency in WA germplasm. Sorghum, however, although exhibiting significant variation for VAM colonization did not show any useful association between VAM and P-uptake

or grain yield under P-limited conditions (Leiser et al., 2016). Breeding for enhanced mycorrhiza colonization in sorghum was therefore concluded to be an ineffective way of enhancing sorghum adaptation to low-P conditions (Leiser et al., 2016).

Direct versus Indirect Selection for Genotypic Performance under Phosphorus-limited Conditions

A fundamental question for breeders targeting crop improvement for low-P environments is under what conditions selection should be conducted. Indirect selection under high-P conditions may provide higher heritability estimates but may also risk losing genotypes that are best under low-P conditions. Direct selection under low-P conditions on the other hand, may retain these genotypes, only if heritability estimates are not so low as to hinder effective differentiation (Atlin et al., 2001; Bänzinger and Cooper, 2001). Direct selection is expected to be more effective where the genetic correlation between the two contrasting fertility regimes is weak, with significant genotype × P-interactions of cross-over type, and when the broad-sense heritability estimates are similar under both low-P and high-P conditions (Falconer, 1952; Atlin and Frey, 1989). Direct selection under low-P conditions was found to be the strategy of choice for both sorghum (Leiser et al., 2012b) and pearl millet (Gemenet et al., 2014). However, higher error levels did occur under low-P as compared to high-P conditions indicating the importance of using effective experimental designs and statistical analysis methods to minimize error levels. Leiser et al. (2012a) showed that spatial adjustment approaches could reduce residual error and increase heritability under low-P trial conditions and thereby increase efficiency of direct selection in P-limited environments in WA.

Genomic Tools for Enhancing Adaptation to Low Soil Phosphorus in Crops

Numerous genomic tools offer promising options to applied breeding programs targeting low-P adaptation. The first validated quantitative trait locus (QTL) for P uptake efficiency named *PUP-1* was mapped by Wissuwa et al. (1998, 2002) in rice. Numerous QTLs for low-P adaptation traits such as P-uptake and utilization efficiencies, grain yield under low-P, among others, were subsequently reported in many crops (for a review, please see van de Wiel et al., 2016 and Wissuwa et al., 2016) including sorghum (Leiser et al., 2014a) and pearl millet (Gemenet et al., 2015c). Functional genomics approaches such as transcriptomics and metabolomics have also helped in the discovery of genes involved in adaption to low-P conditions. *PSTOL1* (*phosphorous-starvation tolerance 1*), the underlying gene of *PUP-1* (Gamuyao et al., 2012), for example, was discovered through transcriptomics. This gene encodes a protein kinase which enhances early root development in rice thus improving P-uptake efficiency. Homologs of this gene were also identified in sorghum and maize through DNA analysis (Hufnagel et al., 2014; Azevedo et al., 2015). In maize, several genes responsible for root development and morphology have been discovered and a relationship between some of these genes and adaptation to

low-P conditions established. The *rootless concerning crown and seminal roots* (RTC) genes encoding a *lateral organs boundaries* (LOB) domain which regulates embryonic seminal and post-embryonic shoot-borne root initiation (Taramino et al., 2007) was for example shown to be overexpressed in a P-efficient line as compared to a P-inefficient line in maize (de Sousa et al., 2012).

Advances in next generation genome sequencing techniques have greatly enhanced the power of gene/QTL discovery and selection through increased molecular marker density and improved resolution (Varshney et al., 2014). These genomic tools offer both increased genetic gain as well as the possibility to expedite the breeding process and reduce its costs. However, the large discrepancy between published results on genomic tools and their actual application in breeding programs has remained a major challenge because most published results are normally not validated for stability across environments and/or genetic backgrounds (Xu and Crouch, 2008). The link between allelic variation of the identified genes and field performance is thus not established in most cases (Wissuwa et al., 2016). With advances in both phenotyping and genotyping technics, however, application of these genomic tools are now becoming possible. The ability to sequence a large number of rice genotypes, for example, together with improved phenotyping for root traits under low-P have allowed precision in estimating trait-allele associations that can be reproduced under field conditions (Wissuwa et al., 2016). There is therefore need to validate the genomic regions associated with performance under P-limited conditions for both sorghum and pearl millet in order to use marker-assisted selection. Proper definition of breeding populations is also needed using the high density marker data from next generation sequencing techniques in order to enhance application of genomics-based selection in sorghum and millet.

CHALLENGES FOR IMPROVING SORGHUM AND PEARL MILLET ADAPTATION TO LOW SOIL PHOSPHORUS CONDITIONS IN WEST AFRICA

Lack of Reliable Screening Procedures for Accurate Phenotyping of Specific Adaptation Mechanisms

Most studies into plants' adaptation to P-limited conditions have mainly dwelled on P-uptake (acquisition) and little has been achieved for P-utilization efficiency. This is mainly because there are no reliable screening methods for P-utilization efficiency because in most cases genotypes used in such studies had different P-uptake capacities which masked the true effects of P-utilization efficiency. Genotypes that were P-uptake inefficient subsequently appeared to be more P-utilization efficient just because they produced relatively more biomass per unit of P taken up as compared to the P-uptake efficient genotypes (Rose et al., 2016). Improving P-utilization efficiency would therefore require evaluation of genotypes with equal P-uptake efficiency. This is still a challenge in a normal breeding program

where diverse genotypes are evaluated. P-uptake efficiency is also a function of the root system and its interaction with the rhizosphere. Screening for differences in the root system is a difficult undertaking because roots grow underground and often require destructive sampling. Recovery of the whole root system is therefore difficult and not amenable to evaluation of many genotypes as is normally the case in breeding programs. Controlled conditions using pots, lysimeters, gels and hydroponic systems offer alternative ways of studying root responses to P-deficiency. Screening procedures to estimate the size of plant root systems under controlled conditions (Otani and Ae, 1996; Subbarao et al., 1997a,b; Kaepller et al., 2000) and field conditions (van Beem et al., 1998; Fenglu and Mugo, 2002) based on non-destructive root capacitance measurements based mainly on models (Dalton, 1995; Dietrich et al., 2012) have been suggested. However, these methods have not been widely applied in practical breeding programs for several reasons. Controlled conditions, though easier to manage, do not wholly represent the conditions under which plants eventually grow under normal field conditions. Recent studies on tolerance to low soil P conditions in WA revealed minimal genetic correlation between controlled pot experiments and field conditions (Gemenet et al., 2015a). Even when carried out in the field, it is difficult to focus solely on root responses to P-deficiency because other stresses occur in combination with low soil P. The effects of soil P-deficiency on pearl millet growth in WA are confounded with drought, the soil physical and chemical environment and possibly unevaluated biological interactions (Gemenet et al., 2015a). Climate variability and change are further challenges to be considered (Haussmann et al., 2012). Differences between genotypic performances under contrasting P-levels were masked in the event of terminal drought stress, which agrees with the findings of Sinclair and Vadez (2002) that P-uptake is reduced to near zero during water stress. Beggi et al. (2015) also found contrasting effects of low soil P on the time to flowering across genotypes, and this would have different effects on yield responses to low soil P in combination with terminal water stress. Al and/or manganese (Mn) toxicities or calcium (Ca), and/or magnesium (Mg) deficiencies may also mask P effects during phenotyping under field conditions. Selecting specifically for low-P adaptation mechanisms alone may therefore prove ineffective for enhancing yield under field conditions. Selection gains for the target population of environments may only be achieved if the selection environments represent the complexity of factors appearing under on-farm field conditions.

Trade-offs between Adaptation Traits

There are trade-offs between water-uptake (deeper root systems) and P-uptake efficiency-related root traits (shallower foraging root systems; Ho et al., 2005; Lynch, 2011; Manschadi et al., 2014). This is of particular importance for pearl millet in Sahelian regions of WA where drought and low soil P typically overlap. Further research into the genetic variation of crop plants for root systems under field conditions is thus needed to identify genotypes with dimorphic rooting systems capable of both vigorous surface and deep soil horizon root growth (Lynch, 2007; Richardson et al., 2009, 2011).

The carbon costs of more extensive root growth, VAM colonization, or exudation of organic anions for increasing P-uptake, need to be considered (Lynch, 2007; Richardson et al., 2009, 2011; Ryan et al., 2012). The extent of carbon costs may differ by species. Pearl millet for example, showed a positive yield response to early colonization by VAM under low-P conditions (Bielders et al., 2010; Beggi, 2014) whereas sorghum showed a negative response of biomass and no relationship between VAM colonization and final grain yield performance (Leiser et al., 2016).

Increasing P-content of the grain may compromise zinc, iron and calcium bioavailability in human nutrition due to a higher content of phytate, the storage form of phosphorus in the grain, which may also become anti-nutrient (Buerkert et al., 1998; Manschadi et al., 2014). Breeding for lower P concentration in grains has been proposed to increase micronutrient bioavailability and to reduce loss of P from the farming system (Rose et al., 2010, 2016; Leiser et al., 2014b). Pearl millet and sorghum for example were found to partition on average 60 (Gemenet et al., 2015b) and 73% (Leiser et al., 2014b), respectively, of their total P to grain under low-P conditions in WA. Lower P-concentration in rice grain was capable of reducing P removal from the farming system by between 0.5–5 kg P ha⁻¹ in the medium to high yield production systems (Vandamme et al., 2016). Reduced P content in grains could be particularly important in WA where soil P deficiency and human micro-nutrient deficiency (“hidden hunger”) are extensive. However, reduction of seed P content can negatively impact germination, seedling establishment and final grain yield under low-P conditions (Raboy, 2009; Robinson et al., 2012; Rose et al., 2012; Vandamme et al., 2016). Lower P concentration in rice grains was, however, not found to affect seedling vigor regardless of soil P status (Pariasca-Tanaka et al., 2015). Also, a selection index that combined low grain-P content and high grain yield under low-P conditions was predicted to be among the most successful for WA sorghum (Leiser et al., 2014b). Targeted selection of reduced phytate P while maintaining the other P forms in the seed would be another option for maintaining Fe and Zn bioavailability. This option was found to reduce germination by 30% but not affect development of the germinated plants (Pilu et al., 2003). Such an approach could be studied further in sorghum and pearl millet. In addition, the effect of reduced P in grains on human nutrition needs to be studied further since P is also the most abundant element in the human body and a substantial amount of it is consumed through cereals (Welch et al., 2009; Rose et al., 2013). In this regard therefore, there seems to be no “one option fits it all” solution and options need to be optimized according to the needs and objectives of a given program.

SYSTEMS APPROACHES TO ADDRESSING THE PHOSPHORUS DEFICIENCY ISSUE IN WEST AFRICA

The use of P-efficient genotypes provides an opportunity to initially increase crop productivity which may subsequently

enable farmers to have surplus income to purchase fertilizer (Lynch, 2007). It will also help address the P-use inefficiencies that commonly occur where P fertilizers are used in large quantities by encouraging reduction in fertilizer use with no reduction in yield (Simpson et al., 2011; Weaver and Wong, 2011). However, genetic advancement of adaptation to low-P soils is not in itself a long-term solution to P deficiency problems in WA and will not offset the need for P input from fertilizer, application of locally available phosphate rock, P placement to seeds at sowing (Buerkert and Hiernaux, 1998) organic matter, manures, or other sources of P to replace P exports (Hash et al., 2002; Sánchez, 2010; Leiser, 2014; Gemenet, 2015). These approaches form part of the integrated soil fertility management (ISFM) practices that have been proposed for SSA and adopted by the Alliance for a Green Revolution in Africa (AGRA; AGRA, 2013). The ISFM approach is defined by Sanginga and Woomer (2009) as the application of soil fertility management practices, together with the knowledge to adapt these to local conditions, in order to maximize fertilizer and organic resource-use efficiency and crop productivity. The ISFM approach links back to farming-systems-oriented research which is an interdisciplinary, integrative, problem-oriented and farmer-centered approach (Darnhofer et al., 2012). Improved crop-livestock integration has been proposed as a strategy toward addressing the challenge of increasing productivity and making African smallholder farming systems more sustainable (Powell and Williams, 1995). In this case, animals produce manure for use in crop production, and crop residues are used as feed and fodder for livestock. Another widely used systems approach involves crop-tree-livestock integration. The extensive ‘agroforestry parklands’ of Sahelian WA involve various tree genera and species grown together with important annual crops in a system shown to provide soil cover that reduces erosion and buffers the impacts of climate change (Bayala et al., 2013). In addition, these trees and shrubs can also provide green fodder that complements crop residues for livestock feeds, firewood, and fruits and leaves for human consumption and for income generation (Zomer et al., 2009; Bayala et al., 2013). Integrated crop-tree-livestock systems adapted to specific local conditions could be maintained as another approach toward a sustainable P management with high internal efficiency. For instance, livestock, especially ruminants, in addition to feeding on crop residues and agro-forestry trees can also harvest P from pasture lands where no crops grow and the resulting manure is then transferred to croplands. Further, P could be recycled from livestock bones e.g., from slaughter houses if proper processing procedures are put in place (Keyzer, 2010). The fact that most of the P is removed from farming systems in the form of grain, coupled with the increasing rural-urban migration prevalent in the whole of SSA (Hove et al., 2013) imply that most P is lost as human waste in cities (Keyzer, 2010). However, it is possible to either directly recycle wastewater in urban agriculture or to extract P from urban wastewaters in the form of the mineral struvite ($MgNH_4PO_4 \cdot 6H_2O$) that then can be used as fertilizer in agriculture (Le Corre et al., 2009). It is therefore necessary to put in place policies that will enhance P recovery from human excreta from cities and return it to farming systems as an approach toward closing the human P cycle

(Childers et al., 2011). MacDonald et al. (2011) also proposed a global P fertilizer use strategy and reduction of P fertilizer use in areas with intense P surpluses and redistribution of such fertilizer to P-deficit croplands as another approach to reduce global P imbalances.

Farmers, researchers and other development partners operate within certain government legislative frameworks. In order to address the P deficiency issue in WA, governments within the region need to commit to sustainably reduce poverty and improve food security. The governments for example need to increase public investment in research and development activities; encourage private sector investments in agriculture through enabling policies; facilitate the generation and sharing of relevant new scientific knowledge (AGRA, 2013); facilitate breeding and improve seed systems of low-P adapted varieties (AGRA, 2014), among other commitments for example as made by SSA governments in the Maputo declaration of 2003.

Rock phosphate is a finite resource over which geopolitical tension may arise (Cordell et al., 2009), especially with the increasing global human population and food insecurity in WA (FAO et al., 2013). Tackling the issue of low-P in WA sorghum and pearl millet production systems will therefore contribute to prosperity and peace, but will require action by all players, from smallholder farmers to researchers, non-governmental organizations, international development partners and policy makers.

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AUTHOR CONTRIBUTIONS

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Genotypic Variation in Grain P Loading across Diverse Rice Growing Environments and Implications for Field P Balances

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More than 60% of phosphorus (P) taken up by rice (*Oryza* spp.) is accumulated in the grains at harvest and hence exported from fields, leading to a continuous removal of P. If P removed from fields is not replaced by P inputs then soil P stocks decline, with consequences for subsequent crops. Breeding rice genotypes with a low concentration of P in the grains could be a strategy to reduce maintenance fertilizer needs and slow soil P depletion in low input systems. This study aimed to assess variation in grain P concentrations among rice genotypes across diverse environments and evaluate the implications for field P balances at various grain yield levels. Multi-location screening experiments were conducted at different sites across Africa and Asia and yield components and grain P concentrations were determined at harvest. Genotypic variation in grain P concentration was evaluated while considering differences in P supply and grain yield using cluster analysis to group environments and boundary line analysis to determine minimum grain P concentrations at various yield levels. Average grain P concentrations across genotypes varied almost 3-fold among environments, from 1.4 to 3.9 mg g⁻¹. Minimum grain P concentrations associated with grain yields of 150, 300, and 500 g m⁻² varied between 1.2 and 1.7, 1.3 and 1.8, and 1.7 and 2.2 mg g⁻¹ among genotypes respectively. Two genotypes, Santhi Sufaid and DJ123, were identified as potential donors for breeding for low grain P concentration. Improvements in P balances that could be achieved by exploiting this genotypic variation are in the range of less than 0.10 g P m⁻² (1 kg P ha⁻¹) in low yielding systems, and 0.15–0.50 g P m⁻² (1.5–5.0 kg P ha⁻¹) in higher yielding systems. Improved crop management and alternative breeding approaches may be required to achieve larger reductions in grain P concentrations in rice.

Keywords: grain P concentration, P removal, P utilization efficiency, rice genotypes, P cycling

INTRODUCTION

Phosphorus (P) is a key nutrient limiting crop growth, and although it is needed by plants in lower total quantities than nitrogen (N) and potassium (K), its continued supply to crops is challenged by the finiteness of phosphate rock stocks worldwide. A large proportion of the P taken up by agricultural crops ends up in the food cycle without being recycled back to fields, leading to a continuous removal of P from fields (Smil, 2000; Senthilkumar et al., 2014; Wu et al., 2016). This results in high requirements for P inputs which come at a significant economic cost, and this cost is expected to increase in the future as high grade, readily accessible phosphate rock reserves are further depleted (Cordell et al., 2009; Senthilkumar et al., 2011, 2012). Where P removed from fields is not replaced by P-containing inputs, soil P stocks are gradually depleted, leading to soil degradation and a decline in productivity (Nziguheba et al., 2016). Highly negative P balances are commonly observed in agricultural fields in many developing countries (MacDonald et al., 2011; Fixen et al., 2015).

Improving the efficiency of P use in agriculture can be achieved by adapting agronomic management strategies to better exploit existing soil P stocks and new P inputs or by exploiting genotypic variation in P efficiency to breed more P-efficient crop cultivars (Simpson et al., 2011). Conventional P efficiency traits that have been targeted in crop improvement programs are P uptake efficiency (PAE, enhanced capacity of the plant to take up P from the soil) and P utilization efficiency (PUE, higher biomass production per unit of P taken up) (Wang et al., 2010; Rose and Wissuwa, 2012). In rice, 60–90% of P taken up by the crop is typically accumulated in the grains at maturity (Rose et al., 2010; Bi et al., 2013; Somaweedra et al., 2015) and hence removed from the fields at harvest. Enhanced P uptake efficiency leads to higher yields but also to increased P removal from fields (Henry et al., 2010). Improved P utilization efficiency can either lead to higher grain yields (at equal levels of P uptake and P removal) or to reduced P removal from fields (at equal grain yield) (Vandamme et al., 2015). As grains contain the majority of P in the rice plant at maturity, and grains—including husks—are removed from fields, Rose et al. (2010) proposed to directly breed for low grain P concentrations as a way to reduce P removal from fields. A similar effort to lower P concentrations in maize (*Zea mays*) grains was undertaken by Wardyn and Russell (1998) with the aim of reducing environmental pollution associated with cattle manure. Recent studies on various crops including rice have focused on reducing phytate levels in grains because of human and animal health concerns, but total seed phosphorus generally remained unchanged (Dorsch et al., 2003; Bryant et al., 2005; Raboy, 2009). Some concerns have been raised about potential negative effects of reduced grain P concentration on seedling vigor. A number of studies have shown that such a negative response of seedling vigor to low grain P concentration can occur but is genotype-specific (Rose et al., 2012; Pariasca-Tanaka et al., 2015). Furthermore, the negative impact of low grain P concentration was shown to be small compared to genotypic variation in seedling vigor and plant responses to externally applied P. Breeding for reduced grain P concentrations is only feasible, however, if genotypic

variation for this trait is sufficiently large to be exploited in breeding programs with a significant impact on removal of P from fields (Rose et al., 2013). In a field study in Japan, rice grain P concentration varied from 2.0 to 3.2 mg g⁻¹ among 38 diverse rice genotypes (Rose et al., 2010), suggesting that considerable genotypic variation exists for this trait in rice. In a field study at three locations in Laos by Inthapanya et al. (2000), grain P concentrations of 16 rice genotypes were lower (on average 1.6 mg g⁻¹) but significantly affected by a location × P rate × genotype interaction. The concentration of P in rice grains is determined by a complex interplay between P supply and other grain yield-determining factors, which can be environmental or genotypic in origin or affected by the interaction of both (Vandamme et al., 2015). In order to identify donor genotypes with a low grain P concentration and understand the implications of genotypic variation in grain P concentration for field P balances, it is essential to take into account this interplay by determining grain P concentration of rice genotypes grown in a wide range of environments with and without external P supply. This study therefore aimed to: (i) assess genotypic variation in grain P concentration of rice in a diverse range of rice growing environments, (ii) identify genotypes with a low grain P concentration irrespective of grain yield, and (iii) quantify the potential impact of the observed genotypic variation in grain P concentration on P removal from rice fields. For these purposes, a series of multi-location experiments was established at different sites across Africa and Asia within the framework of the Global Rice Science Partnership (GRiSP, www.grisp.net).

MATERIALS AND METHODS

Theoretical Framework

A typical nutrient response curve is characterized by an ascending area of the curve where yield increases with plant nutrient concentration, and a relatively level portion where yield is not limited by the specific nutrient (Bates, 1971). The portion of the curve where yield declines quickly with declining nutrient concentration is referred to as the “critical range.” Based on this, a theoretical relationship between grain yield and grain P concentration for a certain rice genotype grown across different environments or levels of P supply was drawn in **Figure 1**. In P-deficient environments, grain yield is limited by insufficient P uptake and this affects grain P concentrations which remain low (zone 1 on **Figure 1**). When P supply increases, increases in grain P concentration will depend on whether grain yield increases concomitantly. If other factors limiting grain yield exist, additional P available in the plant tissue will be distributed among a small amount of grain biomass and grain P concentrations may ultimately reach high levels (zone 2). In environments where P deficiency is the main yield-limiting factor, additional P uptake will lead to increases in grain P concentration and grain yield but—compared to zone 2—the additional P moving to grain may be partly diluted because of the grain yield increase (zone 3). Lastly, if P supply is in excess of that required by the crop for optimal growth, luxury P loading in grains at concentrations above the sufficiency range may occur (zone 4).

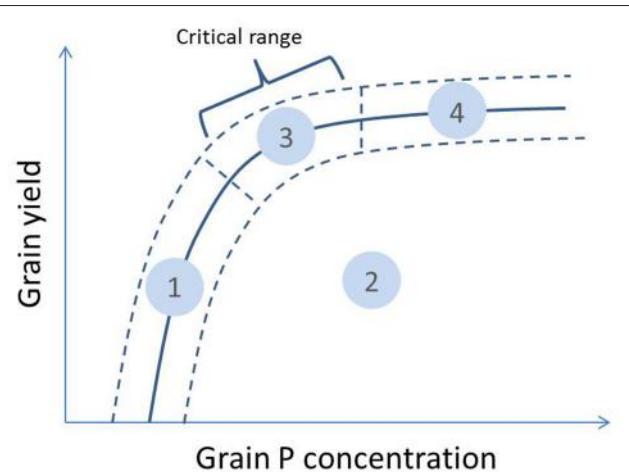


FIGURE 1 | Theoretical relationship between grain yield and grain P concentration of a genotype grown across different environments or levels of P supply (full line with uncertainty interval indicated with dashed lines). The different zones on the curve can be interpreted as: (1) low grain yield and low grain P concentration—grain yield restricted by P availability; (2) low grain yield and medium to high grain P concentration—grain yield restricted by other factors; (3) high grain yield and medium grain P concentration—no major restrictions to grain yield; (4) high grain yield and luxury grain P loading—no major restrictions to grain yield and very high P supply. The critical range is the portion of the curve where yield declines quickly with declining grain P concentration.

When comparing genotypes within the same environment, both a positive and negative relationship between grain P concentrations and grain yields can exist among genotypes. A positive relationship can occur when genotypes with a higher capacity to acquire P have a larger amount of P available for growth and consequently a higher P concentration in their biomass and also grains. A negative trend may occur when higher grain yield leads to lower grain P concentration due to a “dilution effect” (Batten, 1992; McDonald et al., 2008) or when higher grain yield is the result of superior P utilization efficiency. When applied to environmental variation, the positive and negative relationships between grain yield and grain P concentration translates into shifts from zone 1 to 3, and 2 to 3 in **Figure 1**, respectively.

Multi-Location Genotype Screening Experiments

Twenty three field trials were conducted between 2012 and 2014 using 10–75 genotypes per trial (**Table 1**). In total, 83 different genotypes were tested across locations. The selection of genotypes was based on initial data on grain P concentrations (Wissuwa et al., 2015), their previous performance under P deficiency (Mori et al., 2016) and on general adaptation to conditions at respective sites. In all the trials, with the exception of trials 3, 19, 20, 21, and 23 (see trial numbers in **Table 1**), genotypes were grown at two P rates (with and without P application) in different but neighboring field plots. In trials 3 and 19, genotypes were grown only with P application and in trials 20, 21, and 23, genotypes were grown only without P application.

Herein, each trial × P rate combination was considered as one environment and data were collected in 41 environments in total.

Detailed information on soil characteristics, fertilizer rates, experimental design, plot size, hill density and number of replicates is provided in **Supplementary Table 1**. A full list of all genotypes evaluated can be found in **Supplementary Datasheet 1**. Fourteen trials were conducted in West Africa, three in East Africa and six in Asia. Nine trials were conducted under irrigated lowland conditions (flooded) while 14 trials were conducted under upland (aerobic) conditions. Under upland conditions, rainfall was supplemented with irrigation if needed except for the trials in Burkina Faso and The Gambia which were strictly rainfed. The trials were established with three replicates in each environment, with the exception of trial 20 which was conducted with 2 replicates. The pH (1:5 H₂O) of the soils ranged from 4.7 to 6.9 and soil P availability (Bray-P) ranged between 1.3 and 18 mg P kg⁻¹. An alpha lattice design was used in 14 trials and a randomized complete block design in the other nine trials. Trials in upland environments were established by direct seeding (dibbling) and trials in lowland were established by transplanting seedlings that were raised in a nursery bed. Plot size ranged from 0.4 to 3 m² depending on the trial. Nitrogen and potassium (K) were applied in all the trials at rates ranging from 75 to 150 kg N ha⁻¹ and 30 to 50 kg K ha⁻¹, respectively. Where P was applied, its rates ranged from 22 to 30 kg P ha⁻¹.

At harvest, aboveground biomass was collected, separated into panicles and straw, and grains were manually threshed. Straw was oven-dried at 65°C until constant weight while filled grains were air-dried, weighed and their grain moisture content determined. Grain yields are presented at 14% moisture content. Grain and straw samples were ground and digested following different digestion protocols. In Africa, the samples were digested in sulfuric acid, salicylic acid, hydrogen peroxide, and selenium (Novozamsky et al., 1983) and plant P concentration was determined by colorimetry using a continuous-flow analysis system (Thomas et al., 1967). Samples from Asia were digested in a mixture of 3:1:1 nitric:perchloric:sulfuric acid and the P concentration in the extract was determined using the colorimetric vanadomolybdate assay (Murphy and Riley, 1962). Data on grain yield and grain P concentration for all 41 environments were compiled. For 34 and 35 out of these 41 environments, data on straw biomass and straw P concentration were also obtained, respectively.

Data Analysis

Firstly, single-environment ANOVAs were carried out to evaluate differences in grain yield, straw biomass, grain P concentration and straw P concentration among genotypes within each environment and to calculate least square means for these variables in each environment using SAS software (SAS Institute Inc., 2012). A mixed model (PROC MIXED) was used with genotype as a fixed factor and replicate and block nested into replicate (in the case of alpha lattice design) as random factors.

For the second part of the analysis, 30 genotypes were selected that had been grown in at least 15 out of the 41 environments. These genotypes, and information on their origin, species group

TABLE 1 | List of trial and environment numbers with information on year, country, site, rice growing environment, P treatment and number of genotypes, and probabilities of F-statistics for single-environment ANOVA for the effect of genotype on grain yield, grain P concentration, straw biomass and straw P concentration in each environment.

Env	Trial	Year	Country	Site	Rice growing environment	P treatment	No. of genotypes	Grain yield	Grain P conc	Straw biomass	Straw P conc
1	1	2012	Benin	Bohicon	Upland	+	39	**	ns	nd	nd
2	1	2012	Benin	Bohicon	Upland	-	39	**	ns	nd	nd
3	2	2012	Burkina Faso	Farako-ba	Upland	+	39	***	ns	nd	nd
4	3	2012	The Gambia	Yundum	Upland	+	39	*	ns	nd	nd
5	3	2012	The Gambia	Yundum	Upland	-	39	*	ns	nd	nd
6	4	2012	Benin	Cotonou	Upland	+	40	***	**	***	***
7	4	2012	Benin	Cotonou	Upland	-	40	***	***	***	***
8	5	2012	Benin	Cotonou	Lowland	+	75	***	*	***	***
9	5	2012	Benin	Cotonou	Lowland	-	75	***	***	***	***
10	6	2012	Philippines	Pangil	Lowland	+	19	**	***	***	***
11	6	2012	Philippines	Pangil	Lowland	-	19	ns	*	***	*
12	7	2013	Nigeria	Ibadan	Lowland	+	50	***	**	***	***
13	7	2013	Nigeria	Ibadan	Lowland	-	50	***	**	***	***
14	8	2013	Benin	Cotonou	Lowland	+	21	ns	***	*	nd
15	8	2013	Benin	Cotonou	Lowland	-	21	***	***	ns	nd
16	9	2013	Benin	Cotonou	Upland	+	12	ns	**	***	ns
17	9	2013	Benin	Cotonou	Upland	-	12	**	*	**	*
18	10	2013	Benin	Cotonou	Upland	+	12	*	*	***	**
19	10	2013	Benin	Cotonou	Upland	-	12	ns	**	ns	*
20	11	2013	Benin	Cotonou	Upland	+	12	**	*	**	ns
21	11	2013	Benin	Cotonou	Upland	-	12	*	*	**	*
22	12	2013	Benin	Bohicon	Upland	+	33	***	***	***	***
23	12	2013	Benin	Bohicon	Upland	-	33	***	***	***	***
24	13	2013	Burkina Faso	Farako-ba	Upland	+	33	ns	0.05	ns	ns
25	13	2013	Burkina Faso	Farako-ba	Upland	-	33	*	*	ns	*
26	14	2013	Nigeria	Ikenne	Upland	+	33	**	**	***	***
27	14	2013	Nigeria	Ikenne	Upland	-	33	***	*	***	**
28	15	2013	Benin	Cotonou	Upland	+	33	***	ns	**	**
29	15	2013	Benin	Cotonou	Upland	-	33	***	0.06	**	**
30	16	2013	Tanzania	Dakawa	Lowland	+	45	***	*	**	ns
31	16	2013	Tanzania	Dakawa	Lowland	-	45	***	**	ns	ns
32	17	2013	Tanzania	Ruvu	Lowland	+	43	***	**	***	***
33	17	2013	Tanzania	Ruvu	Lowland	-	43	***	0.07	***	ns
34	18	2013	Japan	Tsukuba	Upland	+	13	ns	***	ns	***
35	19	2013	Japan	Tsukuba	Upland	-	13	ns	***	ns	ns
36	20	2013	Japan	Tsukuba	Upland	-	13	*	ns	*	ns
37	21	2013	Philippines	Pangil	Lowland	+	18	*	***	***	***
38	21	2013	Philippines	Pangil	Lowland	-	18	***	**	***	**
39	22	2014	Sri Lanka	Bathalagoda	Lowland	-	20	***	***	nd	***
40	23	2014	Tanzania	Dakawa	Lowland	+	10	***	***	*	**
41	23	2014	Tanzania	Dakawa	Lowland	-	10	***	ns	**	ns

***P < 0.001, **P < 0.01, *P < 0.05, ns, not significant; nd, not determined.

and the number of environments in which they were grown, are presented in **Table 2**. The minimum and maximum number of genotypes taken into account per environment after this selection was 7 and 30, respectively, with an average of 20 (**Table 3**). A cluster analysis was then carried out with the aim to group environments based on average grain yield and grain

P concentration in each environment following the theoretical framework presented in **Figure 1**. The method of cluster analysis used was hierarchical complete-linkage clustering based on Euclidian distance using the R software version 3.3.0 (R Core Team, 2016). Subsequently, genotypic variation in grain yield, grain P concentration and straw P concentration was evaluated

TABLE 2 | Genotypes selected for cluster analysis with information on country of origin, genetic group and number of environments in which they were grown.

Genotype	Country of origin	Group	#Env
Apo	Philippines	IND	15
BJ1	India	IND (AUS)	30
Coarse	Pakistan	IND (AUS)	23
Dawebyan	Myanmar	IND	31
DJ123	Bangladesh	IND (AUS)	39
EMATA A16-34	Myanmar	IND	22
IR36	Philippines	IND	33
IR64	Philippines	IND	32
IR8	Philippines	IND	21
IR82635-B-B-143-1	Philippines	IND	25
IR82635-B-B-93-2	Philippines	IND	17
IR83399-B-B-52-1	Philippines	IND	19
ITA257	Nigeria	TRJ	31
Kalubala Vee	Sri Lanka	IND (AUS)	34
Kasalath	India	IND (AUS)	23
Mudgo	India	IND	38
NERICA1	Ivory Coast	Interspecific	25
NERICA10	Ivory Coast	Interspecific	23
NERICA3	Ivory Coast	Interspecific	21
NERICA4	Ivory Coast	Interspecific	30
PH218-5-3-8-3	Philippines	IND	19
Sadri Tor Misri	Iran	ADMIX	39
Santhi Sufaid	Pakistan	IND (AUS)	41
Seratous Heri	Indonesia	IND	15
Sigadis	Indonesia	IND	17
Surjamkuhi	India	IND (AUS)	38
Taichung Native1	Taiwan	IND	30
Tondok	Indonesia	TRJ	16
TOX1011-4-A2	Nigeria	TRJ	31
Yodanya	Myanmar	IND	28

ADMIX, admixture; IND, Indica; Interspecific, *O. sativa* × *O. glaberrima*; TRJ, tropical japonica; AUS, variety group from India/Bangladesh known for earliness and tolerance to stresses.

within each of the environment clusters. To avoid bias in genotype means across environments due to the unbalanced design of the multi-location trials (not all genotypes grown in all environments), standard scores of the outcome variables (grain yield, grain P concentration and straw P concentration) for each of the genotypes within each environment were calculated as follows:

$$Y_i(j), \text{std} = \frac{Y_i(j) - Y_j}{\sigma_j} \quad (1)$$

where $Y_i(j)$, std is the standard score for variable Y of genotype i within environment j , $Y_i(j)$ is the observed value for variable Y of genotype i within environment j , and Y_j and σ_j are the mean and standard deviation among genotypes for variable Y in environment j . Average standard scores per environment cluster were calculated for each genotype and compared among

genotypes within each environment cluster by a mixed model analysis in SAS with genotypes as fixed factor and environment as random factor, and standard errors of the differences were calculated.

The third part of the analysis was carried out using 14 genotypes that had been grown in at least 30 out of 41 environments. For each genotype, a response curve as illustrated in **Figure 1** was plotted using the observed values for the genotypes in each of the environments. To evaluate the maximum attainable yield at a range of grain P concentrations, boundary curves were then fitted using the method described by Shatar and McBratney (2004). First, outliers were selected and removed for the boundary curve analysis. Two types of outliers were distinguished: (1) data points with yields more than 100 g m^{-2} higher than other yield levels of data points within a range of $\pm 0.4 \text{ mg g}^{-1}$ in grain P concentration, and (2) data points with the lowest grain P concentration observed among environments for a particular genotype yet with a grain yield level higher than 300 g m^{-2} . The first type of outliers were removed to avoid bias in the upper part of the curve (maximum yield level) while the second type of outliers were removed to avoid bias in the lower and left part of the curves. Subsequently, quadratic spline boundary curves (Daouia et al., 2015) were fitted. For each of the genotypes, maximum grain yield and minimum grain P concentrations at various yield levels were then derived from the boundary curves, as well as minimum P removal at various grain yield levels. The minimum grain P concentrations were compared with average observed grain P concentrations in different grain yield level intervals for each genotype.

RESULTS

Environmental Variation and Genotypic Variation within Single Environments

Probabilities of F-statistics for the effect of genotype on grain yield, grain P concentration, straw biomass and straw P concentration within each environment are presented in **Table 1**. Significant differences ($P < 0.05$) in grain yield among genotypes were found in 85% of the environments, and for straw biomass in 80% of the environments. For grain and straw P concentration, significant differences were found in 73 and 74% of the environments, respectively. Differences among genotypes in these variables were equally detected in both environments with and without P applied. Least square means for grain yield, straw biomass, grain P concentration and straw P concentration in all the environments are presented in **Supplementary Datasheet 1**.

Average grain P concentrations across genotypes varied almost 3-fold among environments, from 1.4 to 3.9 mg g^{-1} (**Supplementary Datasheet 1**). On average across environments, grain P concentration tended to be higher under lowland conditions than under upland conditions (2.8 vs. 2.4 mg g^{-1}) and higher when P was applied (2.8 vs. 2.3 mg g^{-1}). Within environments, grain P concentration varied 1.3- to 2.7-fold among genotypes (**Supplementary Datasheet 1** and **Figure 2**). The difference between the minimum and maximum observed grain P concentration within one environment was on average

TABLE 3 | Mean grain yield, straw biomass and grain and straw P concentration in each environment (across selected genotypes) and the number of the environment cluster in which they were grouped by cluster analysis based on grain yield and grain P concentration.

Env	Year	Country	Site	Ecology	P treatment	No. of selected genotypes	Grain yield (g m ⁻²)	Straw biomass (g m ⁻²)	Grain P conc (mg g ⁻¹)	Straw P conc (mg g ⁻¹)	Env
											cluster
2	2012	Benin	Bohicon	Upland	—	24	287	nd	2.09	nd	1
3	2012	Burkina Faso	Farako-ba	Upland	+	24	237	nd	2.46	nd	1
5	2012	The Gambia	Yundum	Upland	—	22	349	nd	1.49	nd	1
11	2012	Philippines	Pangil	Lowland	—	16	82	112	1.77	0.50	1
19	2013	Benin	Cotonou	Upland	—	12	325	864	1.99	0.54	1
20	2013	Benin	Cotonou	Upland	+	12	241	658	1.83	0.32	1
21	2013	Benin	Cotonou	Upland	—	12	86	103	1.61	0.46	1
23	2013	Benin	Bohicon	Upland	—	25	133	168	2.19	0.50	1
25	2013	Burkina Faso	Farako-ba	Upland	—	26	75	80	1.58	0.48	1
27	2013	Nigeria	Ikenne	Upland	—	25	219	362	2.17	0.44	1
34	2013	Japan	Tsukuba	Upland	+	13	281	785	2.13	0.78	1
36	2013	Japan	Tsukuba	Upland	—	13	280	449	1.83	0.36	1
37	2013	Philippines	Pangil	Lowland	+	15	247	254	2.49	1.18	1
38	2013	Philippines	Pangil	Lowland	—	15	165	154	1.36	0.31	1
39	2014	Sri Lanka	Bathalagoda	Lowland	—	16	270	nd	1.57	0.22	1
41	2014	Tanzania	Dakawa	Lowland	—	7	322	289	2.12	0.81	1
6	2012	Benin	Cotonou	Upland	+	23	276	483	3.40	1.13	2
7	2012	Benin	Cotonou	Upland	—	23	268	420	3.23	1.42	2
8	2012	Benin	Cotonou	Lowland	+	30	323	421	2.85	1.37	2
10	2012	Philippines	Pangil	Lowland	+	16	115	156	3.31	1.74	2
12	2013	Nigeria	Ibadan	Lowland	+	23	249	338	3.21	0.81	2
13	2013	Nigeria	Ibadan	Lowland	—	22	299	358	3.20	1.01	2
14	2013	Benin	Cotonou	Lowland	+	19	254	332	3.53	1.53	2
22	2013	Benin	Bohicon	Upland	+	25	213	289	2.93	1.41	2
24	2013	Burkina Faso	Farako-ba	Upland	+	26	114	106	2.91	1.50	2
26	2013	Nigeria	Ikenne	Upland	+	24	319	474	3.06	1.03	2
29	2013	Benin	Cotonou	Upland	—	24	291	469	2.89	1.27	2
1	2012	Benin	Bohicon	Upland	+	24	363	nd	2.80	nd	3
4	2012	The Gambia	Yundum	Upland	+	22	402	nd	2.49	nd	3
9	2012	Benin	Cotonou	Lowland	—	30	444	474	3.11	1.23	3
16	2013	Benin	Cotonou	Upland	+	12	372	764	2.50	1.28	3
17	2013	Benin	Cotonou	Upland	—	12	414	675	2.13	1.12	3
18	2013	Benin	Cotonou	Upland	+	12	404	474	2.23	0.60	3
28	2013	Benin	Cotonou	Upland	+	26	357	534	2.83	1.25	3
30	2013	Tanzania	Dakawa	Lowland	+	24	555	900	2.70	0.92	3
31	2013	Tanzania	Dakawa	Lowland	—	25	469	782	2.86	0.87	3
35	2013	Japan	Tsukuba	Upland	—	13	367	682	2.45	0.67	3
40	2014	Tanzania	Dakawa	Lowland	+	8	538	549	2.26	1.11	3
15	2013	Benin	Cotonou	Lowland	—	19	452	428	3.90	1.43	4
32	2013	Tanzania	Ruvu	Lowland	+	25	493	715	3.61	1.62	4
33	2013	Tanzania	Ruvu	Lowland	—	25	368	660	3.66	1.54	4

1.2 mg g⁻¹ and ranged between 0.5 and 2.0 mg g⁻¹ (**Figure 2**). The lowest observed grain P concentration was 1.1 mg g⁻¹ for the genotype Dawebyan in environment 38 and the highest observed grain P concentration was 4.7 mg g⁻¹ for the genotype Kalubala Vee in environment 32 (**Supplementary Datasheet 1**).

Average straw P concentrations across genotypes varied widely among environments, between 0.2 and 1.7 mg g⁻¹ (**Supplementary Datasheet 1**). Within environments, straw P concentration varied between 1.8- and 7.5-fold among genotypes. The lowest observed straw P concentration was 0.1 mg g⁻¹ for the genotype Kalubala Vee in environment 39 and the highest

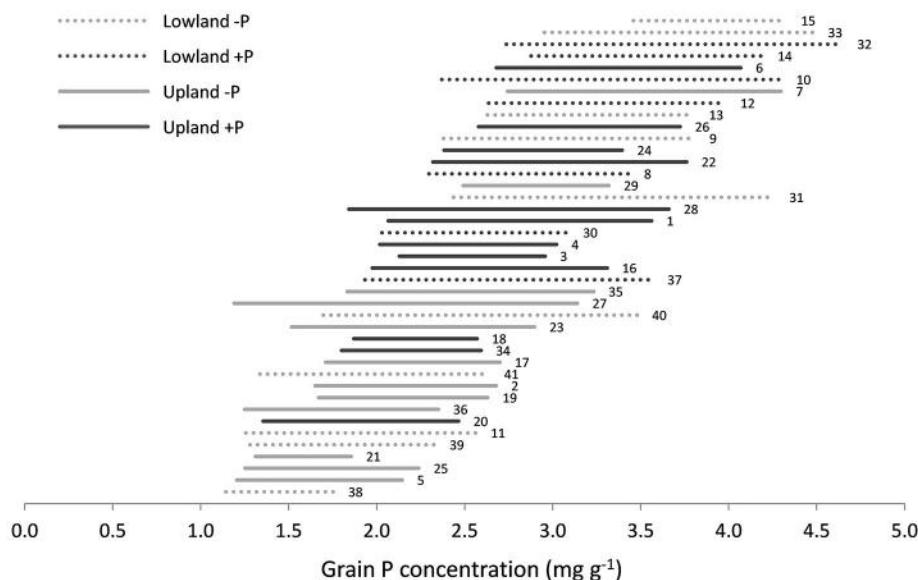


FIGURE 2 | Range in grain P concentration among genotypes in each environment with each line representing the minimum and maximum grain P concentration observed in a certain environment. Environments are sorted from lowest (down) to highest (up) mean grain P concentration. The labels next to the lines are environment numbers as presented in **Table 1**.

observed straw P concentration was 3.3 mg g^{-1} for the genotype PH228-2 in environment 22.

Genotypic Variation within Environment Clusters

A cluster analysis to group environments based on mean grain yield and grain P concentration was carried out using data of a selection of 30 genotypes (**Table 2**) and the environments were grouped in four environment clusters. Mean grain yield, straw biomass, grain and straw P concentration per environment and the cluster in which they were grouped are shown in **Table 3**. **Figure 3** visualizes the variation in grain yield and P concentration within and among the clusters, and mean grain yield, grain P concentration and straw P concentration per cluster are shown in **Table 4**. Environment clusters 1, 2, 3, and 4 comprised 16, 11, 11, and 3 environments, respectively. The first group (environment cluster 1) had relatively low grain yield and low grain P concentration, indicating that grain yield was restricted by P (P-limited environments). The second group (environment cluster 2) was characterized by relatively low grain yield but high grain P concentration. This indicated that a factor other than P was limiting grain yield. Environment cluster 3 was high-yielding with grain P concentrations around 2.5 mg g^{-1} , which can be considered typical for rice (Dobermann et al., 1998), and was identified as the environment group in which there were no major yield-limiting factors. The last environment group had high grain yields with very high grain P concentrations indicating that excessive P supply led to luxury P uptake in the plants. Twelve out of 21 environments where no P was applied were grouped in environment cluster 1. Environment cluster 4

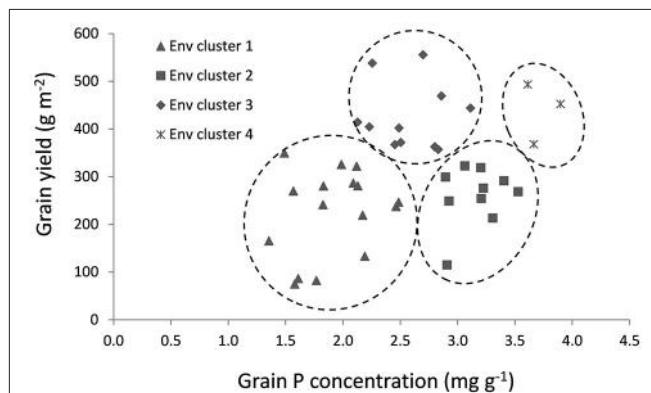


FIGURE 3 | Environments clustered based on mean grain yield and grain P concentration per environment.

(luxury P supply) only contained lowland environments, while other clusters contained both upland and lowland environments.

In environment cluster 1 (P-limited environments), the genotype Kasalath had the highest grain yield combined with the lowest grain and straw P concentration (**Table 4**). To the contrary, Sigadis had low grain P concentration combined with low grain yields. Santhi Sufaid had considerably lower than average grain P concentration and moderately higher than average grain yield. Kalubala Vee and Mudgo had high grain yields and high grain P concentrations indicating superior P uptake.

In environment cluster 2 (grain yield restricted by other factors), Santhi Sufaid was the genotype with the lowest grain P concentration while its yield was moderately higher than average.

TABLE 4 | Mean per cluster and standard scores per genotype for grain yield (GYId), grain P concentration (GrainP) and straw P concentration (StrawP) for 30 genotypes in different environment clusters.

	Env cluster 1 (<i>n</i> = 16)			Env cluster 2 (<i>n</i> = 11)			Env cluster 3 (<i>n</i> = 11)			Env cluster 4 (<i>n</i> = 3)		
	P-limited			Other yield limitation			No major limitation			Luxury P supply		
	GYId	GrainP	StrawP	GYId	GrainP	StrawP	GYId	GrainP	StrawP	GYId	GrainP	StrawP
Mean	g m ⁻²	mg g ⁻¹		g m ⁻²	mg g ⁻¹		g m ⁻²	mg g ⁻¹		g m ⁻²	mg g ⁻¹	
	225	1.92	0.53	247	3.14	1.29	426	2.58	1.00	438	3.72	1.53
Apo	0.20	0.71	-0.06	0.49	0.59	-0.06	0.06	0.06	-0.96	0.67	0.83	-0.65
BJ1	-0.57	-0.02	-0.72	-0.02	0.29	-0.09	-0.40	-0.15	-1.07	-0.72	-0.07	-0.01
Coarse	0.41	-0.27	-0.10	0.28	0.07	-0.74	-0.11	0.19	-0.61	-0.64	-0.63	-1.45
Dawebyan	-0.33	-0.35	-0.67	-0.20	-0.27	-0.47	0.04	-0.16	-0.07	0.32	-0.66	0.63
DJ123	0.14	-0.44	-0.33	0.61	-0.39	-0.84	0.80	-0.40	-0.14	0.33	-1.08	-0.90
EMATA A16-34	-0.64	-0.16	0.68	0.39	-0.39	-0.19	-0.97	-0.63	0.79	0.43	-0.53	0.29
IR36	-0.70	0.37	0.74	-0.61	-0.36	0.39	-0.35	-0.17	0.64	1.39	0.47	0.18
IR64	0.02	0.20	0.70	-0.19	-0.25	0.67	0.18	-0.27	0.96	0.77	-0.51	0.14
IR8	-0.36	0.84	0.28	0.12	-0.12	1.04	-0.66	-0.45	0.52	0.01	-0.38	-0.03
IR82635-B-B-143-1	0.30	0.05	0.32	0.23	0.21	-0.12	-0.16	-0.36	-0.22	0.19	0.03	0.61
IR82635-B-B-93-2	0.79	0.14	0.34	-0.01	0.39	0.58	0.43	-0.20	-0.51	-1.19	0.25	0.19
IR83399-B-B-52-1	0.49	-0.12	0.05	0.35	0.13	0.37	0.67	-0.10	-0.04	-0.14	1.64	-0.82
ITA257	-0.05	-0.10	0.10	-0.49	0.02	-0.20	-0.23	-0.31	-0.49	1.44	1.13	-0.64
Kalubala Vee	0.91	0.57	-1.08	0.35	1.18	-0.41	0.57	0.72	-0.95	-0.35	0.47	0.60
Kasalath	1.08	-0.89	-1.36	0.36	-0.28	-0.47	0.30	-0.11	-0.54	-1.39	0.07	-0.02
Mudgo	0.41	0.80	0.64	0.51	0.93	0.04	0.86	1.06	0.78	-0.57	0.34	-0.54
NERICA1	-0.37	0.46	0.73	0.08	0.07	-0.36	0.05	0.19	-0.50	-1.12	-0.70	0.02
NERICA10	-0.26	0.40	-0.36	0.03	0.36	-0.58	0.43	0.39	-0.12	-1.16	0.16	0.49
NERICA3	-0.03	-0.11	-0.27	-0.63	-0.06	-0.16	-0.12	0.15	0.48	0.89	-0.91	0.03
NERICA4	-0.29	0.11	-0.19	-0.08	-0.09	-0.70	-0.39	0.30	-0.21	-0.33	0.56	0.99
PH218-5-3-8-3	-0.92	0.23	0.56	-0.08	0.26	1.01	-0.49	0.75	0.95	-0.69	-0.90	0.06
Sadri Tor Misri	0.07	-0.10	0.41	0.16	-0.37	0.82	0.34	0.17	0.76	-0.53	-1.03	-0.94
Santhi Sufaid	0.37	-0.68	-0.10	0.21	-0.63	-0.29	-0.78	0.04	0.45	1.08	-0.77	-1.18
Seratus Hari	-0.62	0.03	-0.10	0.12	-0.11	0.18	-0.36	-0.23	-0.60	-0.17	0.79	1.23
Sigadis	-0.40	-0.79	0.10	0.52	-0.06	0.60	0.18	0.58	0.52	-0.90	-0.11	0.05
Surjamkuhi	-0.24	-0.34	-0.25	-0.20	-0.36	-0.41	-0.55	-0.19	0.17	-1.35	-1.52	1.62
Taichung Native1	-0.28	-0.19	0.89	-0.22	0.00	0.70	-1.12	-0.82	1.00	1.34	-0.04	0.28
Tondok	-1.21	0.48	1.01	-1.48	-0.52	0.63	-0.29	0.08	-0.74	-0.90	0.07	0.23
TOX1011-4-A2	0.06	-0.18	-0.54	-0.53	-0.10	-0.31	0.16	0.60	-0.09	0.74	0.31	0.51
Yodanya	0.53	0.15	-0.74	0.22	-0.15	-0.33	ns	ns	ns	ns	ns	ns
Prob of <i>F</i> -stat	***	**	***	**	**	***	**	*	***	***	ns	ns
SEDmin	0.33	0.34	0.33	0.40	0.40	0.37	0.39	0.40	0.39	0.53	0.73	0.75
SEDmax	0.65	0.71	0.63	0.60	0.59	0.55	0.79	0.78	0.76	0.80	1.27	1.13

Environments were clustered based on mean grain yield and grain P concentration. SED is standard error of the difference; ***P < 0.001, **P < 0.01, *P < 0.05, ns, not significant; for grain yield: dark color = high yield (desirable) to light color = low yield, for grain and straw P concentration: dark color = low P concentration (desirable) to light color = high P concentration.

Again, Kalubala Vee and Mudgo had high grain P concentrations associated with high grain yields.

In environment cluster 3 (no major yield-limiting factor), Santhi Sufaid again had low grain P concentrations but also lower than average grain yield. Tondok and EMATA A16-34 also had low grain P concentrations but this was associated with considerably lower than average grain yields. DJ123 had considerably lower grain P concentration while its grain yield was higher than average. As in environment cluster 1 and 2,

Mudgo and Kalubala Vee had high grain yields and high grain P concentrations. Data from environment cluster 4 (luxury P supply) have to be interpreted with care as only three environments were grouped in this cluster. Results tended to be similar as those for environment cluster 3 but variation in grain and straw P concentration among genotypes was not significant in this cluster.

Santhi Sufaid was the only genotype that had the lowest grain P concentration in two environment clusters (cluster 2 and 3)

and it was also among the three genotypes with lowest grain P concentration in environment cluster 1. DJ123 was the only genotype that had lower than average grain P concentration in all environment clusters combined with medium to high grain yield levels. Kalubala Vee and Mudgo had high grain P concentrations across all environment clusters, with Mudgo also having higher than average straw P concentrations while Kalubala Vee had considerably lower than average straw P concentrations indicating that its high grain P concentrations were associated with enhanced P translocation from the straw.

Boundary Curve Analysis

Santhi Sufaid and Surjamkuhi had a relatively low grain yield potential but had a steep slope of the boundary curve below 80% of their maximal grain yield, indicating that these genotypes were able to increase grain yields with only very limited increases in grain P concentrations (**Figure 4** and **Table 5**). Surjamkuhi scored particularly well in terms of minimum grain P concentrations at different grain yield levels, but had relatively high average grain P concentrations, meaning that in many cases it loaded more P than needed (**Table 5**). Santhi Sufaid did not score particularly well in terms of minimum grain P concentrations, but had low average grain P concentrations at low to medium grain yield levels (**Table 5**), and was the only genotype for which no grain P concentrations $>3\text{ mg g}^{-1}$ were observed at grain yield levels $<200\text{ g m}^{-2}$ (**Figure 4**). The genotypes TOX1011-4-A2 and ITA257 had grain yield potentials similar to those of Santhi Sufaid and Surjamkuhi, but the slope of the lower part of their boundary curves was notably less steep, meaning that these genotypes rapidly accumulated more P in their grains upon an increase in P supply. The genotype Mudgo had the highest grain yield plateau (maximum grain yield), followed by DJ123 and IR64, but these genotypes differed clearly in terms of grain P loading patterns (**Figure 4**). On the one hand, the slope of the boundary curve of DJ123 was much steeper than that of IR64, indicating that DJ123 efficiently utilized grain P while IR64 rapidly increased grain P loading upon an increase in P supply. On the other hand, Mudgo exhibited a slope of the boundary curve that was comparable to that of DJ123, however, compared to DJ123 and IR64 the lower part of its curve was shifted to the right meaning that in general Mudgo required a higher grain P concentration to reach certain grain yields especially at lower yield levels. Other genotypes exhibited intermediate responses.

Despite the clear differences in grain P loading patterns among genotypes that can be derived from the boundary curves in **Figure 4**, genotypic differences in minimum and average grain P concentrations were rather small in absolute terms (**Table 5**). Minimum grain P concentrations associated with grain yields of 150, 300, and 500 g m^{-2} varied between 1.2 and 1.7, 1.3 and 1.8, and 1.7 and 2.2 mg g^{-1} among genotypes respectively. **Table 6** shows that reductions in P removal potentially achieved by exploiting genotypic differences would be in the order of magnitude of <0.1 , 0.15, and 0.5 g P m^{-2} (equivalent to 1, 1.5 and 5 kg P ha^{-1}) at grain yield levels of 150, 300, and 500 g m^{-2} (equivalent to 1500, 3000, and 5000 kg ha^{-1}) respectively, and less when commonly grown genotypes such as IR64 or NERICA4 are considered as a reference. P removal from fields was, on

average across genotypes, 1.7, 1.8, and 1.4 times larger than P removal at minimum grain P concentrations for grain yield levels of 150, 300, and 500 g m^{-2} respectively (**Table 6**).

DISCUSSION

Variation in Grain P Concentration among Environments and Genotypes

Grain P concentration varied widely among environments and genotypes. Grain yields above 400 g m^{-2} were generally associated with minimum grain P concentrations of around 2 mg g^{-1} (**Figure 3**). However, at the same grain yield level of 400 g m^{-2} , average grain P concentrations up to 4 mg g^{-1} were observed in other environments because of luxury P supply or other constraints limiting a further yield increase. At lower yield levels of about 200 g m^{-2} , a similar 2-fold variation in grain P loading was observed. Our observations are comparable with those of Dobermann and Fairhurst (2000), who determined typical grain P concentrations in rice ranging from 1.7 to 2.3 mg g^{-1} under nutrient limitation, 2.4 to 2.8 mg g^{-1} under nutrient optimum, and 2.8 to 4.8 mg g^{-1} under nutrient surplus, and in agreement with the 2-fold range (2 – 4 kg t^{-1}) in P uptake per ton of rice grain yield reported by Dobermann et al. (1998). Especially at higher yield levels, where biomass export from fields is high, such large variation in grain P loading at equal grain yields is expected to have important implications for P removal rates, field P balances and subsequent fertilizer requirements. Indeed, at a yield level of 400 g m^{-2} , 1.6 g P m^{-2} (equivalent to 16 kg P ha^{-1}) is removed with grains from fields at grain P concentrations of 4 mg g^{-1} , compared to only 0.8 g P m^{-2} (equivalent to 8 kg P ha^{-1}) at grain P concentrations of 2 mg g^{-1} . In high-input, high-yielding systems, matching externally applied P with crop P demand, based on a targeted yield level, therefore appears a logical option to avoiding excess P uptake and excessive P removal from fields. This may involve fine-tuning fertilizer rates or innovative water management to manipulate P availability at different crop development stages. Since yields are known to respond to the most limiting nutrient, a crucial aspect for maximizing nutrient use efficiency and avoid excess accumulation of non-limiting nutrients without a concomitant yield increase is also the balanced use of fertilizers (Janssen, 1998; Dobermann and Fairhurst, 2000). This can be exemplified by the study of Bi et al. (2013) who found that grain P concentrations of rice decreased in response to increasing N rates and concluded this was at least partly due to a dilution effect. At lower grain yield levels, where high grain P loading but low grain yields are observed due to other yield limiting factors, agronomic management needs to focus on overcoming these other stresses to improve the amount of rice harvested per unit of P exported from fields with grains.

Fine-tuning nutrient availability is, however, not a straightforward approach in rice systems where small-scale farming dominates and soil analysis may not be readily available. Exploiting genotypic variation in grain P concentrations to breed P-efficient crop cultivars that minimize P removal from fields may therefore be a more sustainable or practical option

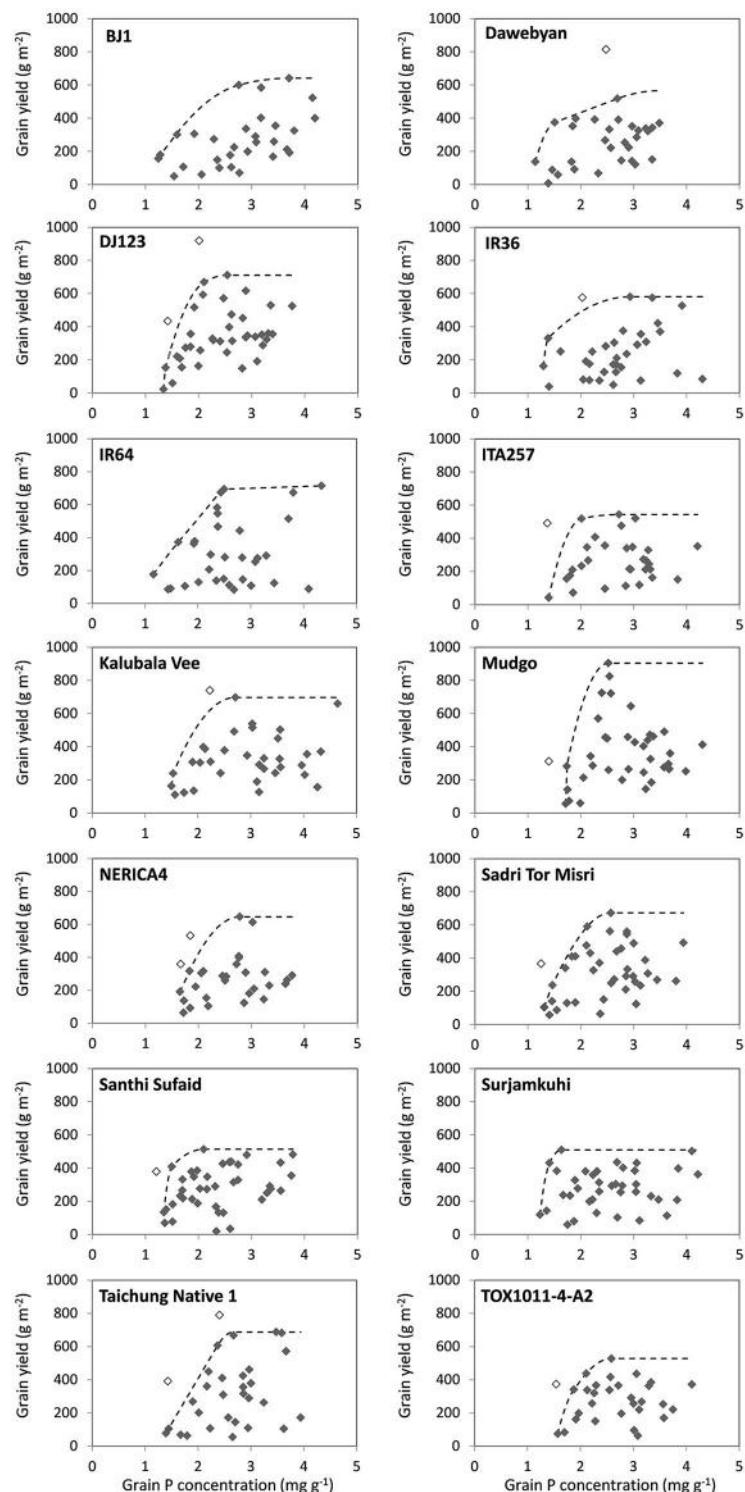


FIGURE 4 | Grain yield plotted against grain P concentration observed in different environments for 14 rice genotypes, and boundary curves estimating minimum grain P concentrations to reach certain grain yield levels. Empty dots are outliers not included in the boundary line analysis.

(Rose et al., 2010). A major aim of the present study was to investigate whether sufficient variation for grain P concentration exists among the rice genotypes tested to warrant the selection of

genotypes as donor varieties in a breeding program. To this end, genotypic variation in grain P concentration was investigated across diverse environments while concomitantly assessing

TABLE 5 | Minimum grain P concentration at various grain yield levels for 14 rice genotypes and average grain P concentration in different grain yield level intervals, and slope of boundary curves below 80% of maximum grain yield.

	Minimum grain P concentration (mg g^{-1})				Average grain P concentration (mg g^{-1})			Slope of boundary curve below 80% of max grain yield	
	At grain yield level				At grain yield level				
	150 g m^{-2}	300 g m^{-2}	500 g m^{-2}	80% of max grain yield	<200 g m^{-2}	200–400 g m^{-2}	>400 g m^{-2}		
BJ1	1.2	1.6	2.2	2.2	2.4	3.0	3.4	365	
Dawebyan	1.2	1.3	2.5	2.2	2.2	2.7	2.6	240	
D1J23	1.4	1.5	1.7	1.8	2.0	2.5	2.5	991	
IR36	1.3	1.4	2.1	1.9	2.5	2.6	3.1	378	
IR64	1.2	1.4	2.0	2.1	2.4	2.5	3.0	395	
ITA257	1.5	1.6	1.9	1.8	2.5	2.9	2.4	1052	
Kalubala Vee	1.5	1.6	1.9	2.1	2.5	3.0	3.2	651	
Mudgo	1.7	1.7	1.9	2.1	2.3	2.9	3.0	1311	
NERICA4	1.7	1.8	2.1	2.1	2.3	2.7	2.6	626	
Sadri Tor Misri	1.4	1.6	1.9	2.0	1.9	2.7	2.6	632	
Santhi Sufaid	1.4	1.4	1.9	1.5	1.9	2.4	2.7	1868	
Surjamkuhi	1.2	1.3	1.6	1.4	2.2	2.7	2.6	1907	
Taichung Native 1	1.5	1.8	2.2	2.3	2.6	2.5	2.9	543	
TOX1011-4-A2	1.6	1.8	2.3	2.1	2.4	2.8	2.6	681	

Minimum grain P concentration and slopes of boundary curves were determined based on the boundary curves presented in **Figure 4**.

TABLE 6 | Minimum P removal and estimated average P removal at various grain yield levels for 14 rice genotypes.

	Minimum P removal (g P m^{-2})			Estimated average P removal (g P m^{-2})				
	At grain yield level			At grain yield level				
	150 g m^{-2}	300 g m^{-2}	500 g m^{-2}	150 g m^{-2}	300 g m^{-2}	500 g m^{-2}		
BJ1	0.19	0.47	1.08	0.35	0.90	1.70		
Dawebyan	0.17	0.40	1.27	0.32	0.81	1.29		
D1J23	0.21	0.45	0.87	0.30	0.76	1.25		
IR36	0.19	0.41	1.03	0.38	0.79	1.57		
IR64	0.17	0.43	0.98	0.36	0.74	1.48		
ITA257	0.22	0.48	0.96	0.37	0.86	1.18		
Kalubala Vee	0.23	0.48	0.97	0.37	0.89	1.59		
Mudgo	0.26	0.52	0.94	0.35	0.87	1.48		
NERICA4	0.25	0.54	1.07	0.34	0.82	1.30		
Sadri Tor Misri	0.20	0.47	0.95	0.29	0.81	1.28		
Santhi Sufaid	0.20	0.42	0.94	0.29	0.72	1.35		
Surjamkuhi	0.19	0.39	0.78	0.34	0.80	1.31		
Taichung Native 1	0.23	0.54	1.09	0.38	0.75	1.43		
TOX1011-4-A2	0.24	0.54	1.16	0.36	0.84	1.29		

Average P removal was estimated based on average P concentrations observed in the grain yield intervals presented in **Table 5**.

grain yields. The 1.3–2.7-fold variation in grain P concentration within environments observed in this study is in agreement with earlier field studies that have evaluated genotypic variation in grain P concentrations in one environment for various cereals including rice (Rose et al., 2010), sorghum (*Sorghum bicolor*) (Leiser et al., 2014) and maize (*Zea mays*) (Wardyn and Russell, 1998). However, the roughly 2-fold variation in grain P concentration observed within environments does not imply that grain P concentration could be reduced by 50% through breeding. Within the set of genotypes tested, genotypes with

exceptionally high grain P concentrations were also included, and furthermore, a considerable part of the genotypic variation in grain P concentration within environments was related to differences in grain yield. The most promising genotype appeared to be Santhi Sufaid as it exhibited considerably low grain P concentrations in all types of environments irrespective of its grain yield level, while the genotype D1J23 was the only genotype that had lower than average grain P concentrations in all environment clusters combined with medium to high yield. The same genotypes were found in previous studies to exhibit

high PUE at the vegetative stage (Saito et al., 2015; Rose et al., 2016). The boundary line analysis showed that by using Santhi Sufaid as a donor in breeding for low grain P concentration, a reduction of 5 to maximally 20% in grain P concentration at equal yield levels can be achieved in popular rice genotypes such as IR64 and NERICA4.

Relationship between Grain P Concentration and Grain Yield and PUE

Different patterns with regard to the interaction between grain P concentration and grain yield could be distinguished. A first pattern involved genotypes with low grain P concentrations and medium to high grain yields, and can be exemplified by the performance of Kasalath in P-limited environments, which seemed to have a superior P utilization efficiency. A second pattern involving high grain P concentrations combined with medium to high grain yields was observed for the genotypes Kalubala Vee and Mudgo. For these genotypes, high grain P concentrations appeared to be the result of an outstanding P uptake capacity. The high P uptake capacity of Mudgo is in agreement with the results of Saito et al. (2015), where Mudgo had the highest P uptake among 7 genotypes, measured at the vegetative stage. At maturity, Mudgo was found to have 10–20% higher grain P concentrations and 20–50% higher straw P concentrations than Santhi Sufaid while yield levels were similar (Vandamme et al., 2016). For Kalubala Vee, the excessive P loading was at least partly the result of enhanced remobilization of P from the straw to grains during grain filling, as straw P concentrations for this genotype were exceptionally low across environments. A third pattern involved genotypes with low grain P concentrations combined with low grain yields, and this was observed for Tondok which nevertheless also had high straw P concentrations, indicating that for this genotype a major problem occurred with mobilizing resources to the panicles for grain production.

Reduced grain P concentration may either be the result of high PUE (biomass produced per unit of P uptake) in general (low P concentrations in all parts of the plant) or of a reduced translocation of P to the grains at equal grain yield which leads to a lower P harvest index (PHI) (Rose et al., 2013; Vandamme et al., 2015). In the first case, P concentration is expected to be lower than average in both grains and straw, and this was observed for a number of genotypes such as Kasalath and DJ123, and Santhi Sufaid at low to medium grain yield levels. This is not surprising for DJ123 and Santhi Sufaid, which have high PUE during vegetative growth (Saito et al., 2015; Rose et al., 2016). In the second case (lower PHI at equal grain yield), low grain P concentrations combined with higher than average straw P concentrations are expected. However, at maturity, straw P concentrations are affected by P mobilization to the grains which is in turn determined by a combination of all grain yield-determining factors contributing to the harvest index. To determine whether low grain P concentrations are the result of general PUE or lowered PHI at equal grain yield, data from grain P concentrations at maturity need to be compared with data from vegetative phase screening for PUE. In recent studies by

Wissuwa et al. (2015) and Rose et al. (2016), where PUE at the vegetative stage was compared among genotypes at equal total plant P content, Santhi Sufaid and DJ123 exhibited high PUE, indicating that the low grain P concentrations observed in these genotypes were indeed related to whole plant PUE.

Improvements of Field P Balances Given Current Levels of Genotypic Differences in Grain P Concentrations

The boundary line analysis showed that at low grain yield levels of around 150 g m^{-2} , potential improvements in field P balances that can be achieved by exploiting genotypic variation in minimum grain P concentration observed within the limits of this study are less than 0.1 g P m^{-2} (equivalent to 1 kg P ha^{-1}), which is a rather small amount of P especially in the short term. Based on this genotypic variation, breeding for low grain P concentration using a conventional approach does not seem to be a viable option for reducing P mining in low-yielding, low-input systems. At higher yield levels ($300\text{--}500 \text{ g m}^{-2}$), boundary curve analysis showed that breeding for low grain P concentration can improve field P balances by 0.15 to maximally 0.5 g P m^{-2} (1.5 to maximally 5.0 kg P ha^{-1}). This may seem a modest amount of P in absolute terms, particularly compared to generally recommended P application rates of $15\text{--}60 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$ ($= 6.5\text{--}26 \text{ kg P ha}^{-1}$) for rice (Fairhurst et al., 2007). However, in relative terms it constitutes a reduction of 20–40% in P removal with grains, and this would hence considerably reduce the maintenance fertilizer need or the amount of P mined in low input systems.

Reductions in P removal larger than estimated above are possible since boundary conditions (Shatar and McBratney, 2004) represent ideal cases when the utilization of P is optimized within the plant (any reduction in P uptake would reduce yield). These conditions rarely exist and data in Figure 4 show that grain P concentrations at yield levels of $400\text{--}500 \text{ g m}^{-2}$ can be 2-fold higher than boundary levels within each genotype. A highly P efficient genotype like DJ123 can achieve that yield level at a grain P concentration of $1.5\text{--}1.7 \text{ mg g}^{-1}$ under optimized conditions. One could use this P concentration in setting a target concentration in breeding efforts to be achieved not only at boundary conditions but across environments, thus effectively avoiding any excess loading of P into grains. This would reduce P offtake from fields and therefore reduce maintenance fertilizer requirements beyond levels discussed above. While excess loading of P is of no agronomic concern in high-input systems (because mining is not a concern), it should be limited to reduce the environmental impact associated with the consumption and poor utilization of P (Withers et al., 2001) and the negative effects on human and animal health related to phytate-rich grains (Raboy, 2009). Nevertheless, as desirable as it should be, capping P loading at some low P concentration around 1.5 mg g^{-1} does not appear feasible using conventional breeding as none of the genotypes studied had the ability to fully restrict luxury P loading into grains. Santhi Sufaid had a lower tendency to load excess P under conditions of additional stresses compared

to other genotypes, but on average still loaded around 50% P more than minimum levels needed to reach certain yield levels.

Toward Developing Rice Varieties with Reduced Grain P Loading

Consistent genotypic variation in grain P concentration of rice was observed across a wide range of rice growing environments, and Santhi Sufaid and DJ123 were identified as potential donors for breeding for low grain P concentrations. Improvements in P balances that could be achieved by exploiting this genotypic variation are in the range of less than 1 kg P ha⁻¹ in low yielding P deficient environments, and 1.5–5 kg P ha⁻¹ in higher yielding systems. The magnitude of that potential improvement is likely too small to justify breeding activities specifically targeting this trait, particularly since the current lack of selectable markers and the high degree of environmental variation constitute technical barriers for the successful implementation of a traditional breeding program for reduced grain P concentrations. A larger portion of the rice gene pool may have to be screened, ideally in a genome wide association approach, to identify donors and associated markers. In addition alternative options should be explored that may include screens of mutant populations (Raboy, 2009) and identification of candidate genes involved in the regulation of P loading into grains for potential genetic manipulation (Wang et al., 2015; Jeong et al., 2016).

The present study demonstrated that relatively high yield levels (>4 t ha⁻¹) were achieved at grain P concentrations around 1.5–1.7 mg g⁻¹, and Pariasca-Tanaka et al. (2015) showed that grain P concentrations as low as 1 mg g⁻¹ did not affect seedling vigor in some rice genotypes. Currently it is not known at what level a “safe” lower limit for grain P concentration that does not affect subsequent crop productivity would be, and additional research is needed to clarify this point. However, conceptually it is evident that maximum benefits in terms of reducing P mining, maintenance P requirements and environmental P fluxes would be achieved by efforts to cap grain P concentrations at such a lower limit by avoiding excess P loading to grains. Large (2-fold) across-environment variation in grain P concentrations at equal grain yield levels indicated that such excess P uptake is common and likely due to other yield-limiting factors leading to less than

optimal P utilization efficiency. Any effort to lower P removal by grains through genetic improvement should therefore be combined with improved crop management to overcome other yield-limiting factors and maximize utilization efficiency of P in grains and ultimately in cropping systems.

AUTHOR CONTRIBUTIONS

KS coordinated data collection in Africa and MW in Asia. KD, MF, KS, RV, DJ, ZS, LS, DS, YK, and TR conducted field trials and analyzed samples. EV, KS, and ID compiled and analyzed the data. EV wrote the manuscript with contributions from all co-authors. All co-authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01435>

Supplementary Table 1 | Information on the materials and methods used in each of the multi-location trials including location, rice growing environment, year, season, soil characteristics, fertilizer rates, experimental design, plot size, hill density, number of replicates and total number of genotypes for 41 environments.

Supplementary Datasheet 1 | Genotype × environment least square means for grain yield, straw biomass, grain P concentration and straw P concentration.

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Harnessing Diversity in Wheat to Enhance Grain Yield, Climate Resilience, Disease and Insect Pest Resistance and Nutrition Through Conventional and Modern Breeding Approaches

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Current trends in population growth and consumption patterns continue to increase the demand for wheat, a key cereal for global food security. Further, multiple abiotic challenges due to climate change and evolving pathogen and pests pose a major concern for increasing wheat production globally. Triticeae species comprising of primary, secondary, and tertiary gene pools represent a rich source of genetic diversity in wheat. The conventional breeding strategies of direct hybridization, backcrossing and selection have successfully introgressed a number of desirable traits associated with grain yield, adaptation to abiotic stresses, disease resistance, and bio-fortification of wheat varieties. However, it is time consuming to incorporate genes conferring tolerance/resistance to multiple stresses in a single wheat variety by conventional approaches due to limitations in screening methods and the lower probabilities of combining desirable alleles. Efforts on developing innovative breeding strategies, novel tools and utilizing genetic diversity for new genes/alleles are essential to improve productivity, reduce vulnerability to diseases and pests and enhance nutritional quality. New technologies of high-throughput phenotyping, genome sequencing and genomic selection are promising approaches to maximize progeny screening and selection to accelerate the genetic gains in breeding more productive varieties. Use of cisgenic techniques to transfer beneficial alleles and their combinations within related species also offer great promise especially to achieve durable rust resistance.

Keywords: wheat, genetic diversity, introgressions, disease resistance, pest resistance, cisgenesis, genomic selection, nutritional quality

INTRODUCTION

Wheat (*Triticum aestivum* L.), one of the key cereal crops, is grown on 222 million hectares worldwide and is a major source of calories and proteins globally (USDA, 2016). Wheat production has increased from 235 million tons in 1961 to an estimated 733 million tons in 2015 (FAOSTAT, 2014). The Green Revolution of 1960 and 1970s along with changes in policies, fertilizer use and

advances in agronomy has stimulated wheat productivity over past decades (Ziska et al., 2012). A highly cited example is the global success of two semi-dwarf wheat varieties “Sonali and Kalyan Sona” in the 1960s which helped wheat production advance from deficit to surplus in South Asia.

In recent years, changes in population trends, eating habits, and economic and socio-economic conditions, especially in Africa and Asia, have caused an increase in global wheat demand. Under the assumption of favorable growing conditions, the International Grain Council [IGC] (2014) estimated the wheat production and consumption demands till 2020. Based on their predictions, wheat productivity growth was estimated at 1.1% per year for next 5 years, which will make it possible to meet the consumption demands till 2020. However, in recent years, noticeable changes in temperature and rainfall at the global level have had an impact on wheat production. Various crop models have estimated yield reductions of 6–13% in wheat for each °C rise in temperature. Based on the current trends in wheat production, the predicted increase in wheat productivity by 2050 will be short of 1 t/ha which is required to meet the rising global demand (Figure 1). Increased climate variability, frequent extreme weather events, and new variants of pathogens and pests further jeopardize linear productivity growth into the future. Breeding wheat for climatic change tolerance and disease resistance combined with good agronomy can potentially improve wheat productivity to meet the future demands.

Wheat is an allopolyploid species that originated from a cross of the tetraploid species *Triticum turgidum* and the diploid species *Aegilopspustulosus* (Coss.) Schmalh. Wild tetraploid emmer wheat evolved from a hybridization of wild *Triticum urartu* tumanian ex Gandivan and an undiscovered species of the *Aegilops speltoides* Tausch lineage. During the process of

domestication genetic bottlenecks resulted in significant loss of diversity. There has been a keen interest in utilizing the genetic diversity of Triticeae species, which includes the primary, secondary, and tertiary gene pools (*Aegilops*, *Agropyron*, *Elymus*, *Hordeum*, *Leymus*, *Secale*, *Thinopyrum*, and *Triticum*). These gene pools are a rich source of genes that can be used to improve diverse traits such as disease resistance, micronutrient availability and abiotic stress adaptation. Novel alleles have been introgressed from nearly 52 species highlighting the genomic plasticity of wheat and the importance of exotic introgressions in wheat improvement (Wulff and Moscou, 2014).

In this review, we highlight the genetic diversity available in wheat for grain yield, adaptation to climate change, disease and insect pest resistance, and nutritional and end-use quality. We also discuss traditional approaches to introgression that are still successful and current technologies that are being used to characterize the genetic diversity and improve the efficiency of the introgression process. We also explore the role of new technologies such as genomic selection (GS) and cisgenesis to integrate diverse genes/alleles and accelerate the breeding process.

DIVERSITY IN WHEAT FOR:

Grain Yield Improvement and Climate Resilience

Grain yield *per se* is a polygenic trait, and yield improvements from alien introgressions are due to their positive impact on phenology, yield components (that is grain size, grain number, floret number, etc.), or through adaptive traits for abiotic stresses (such as heat, drought, and alkaline/acid soils) and

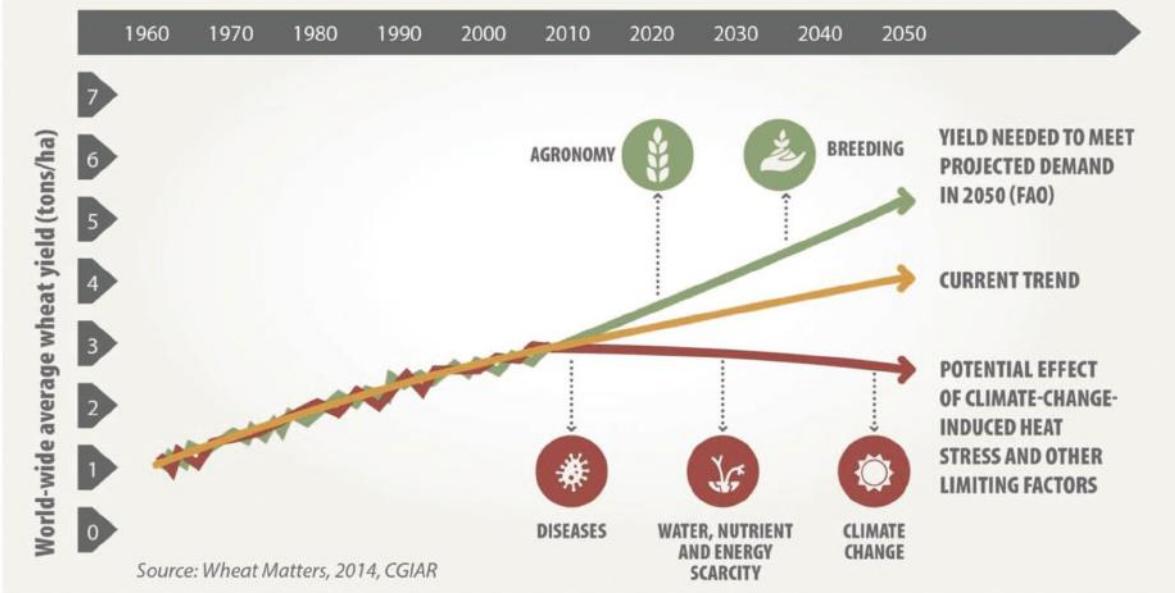


FIGURE 1 | Projected demand and yield trends in wheat under several scenarios. Source: CIMMYT (2014).

resistance to biotic stresses. Landraces, a crucial germplasm pool has been reported to contribute genes for grain yield improvement in irrigated environments or, in heat and drought stress environments (Reynolds et al., 2007a; Lopes et al., 2015). Direct varietal releases from simple crosses with landraces are rare, though a Turkish variety 'Gerek 79' is an exception (Smale and McBride, 1996). One of the best examples is the *Rht* dwarfing gene that was available through the Japanese variety 'Norin10' originating from a Japanese landrace Shiro Daruma (Reitz and Salmon, 1968; Dreisigacker et al., 2005). These dwarfing genes were utilized by Dr. Norman E. Borlaug to develop the high-yielding semi-dwarf wheat varieties that triggered the Green Revolution. Several other landraces also have had an impact on improving the germplasm pool: for example, 'Cheyenne,' a selection from landrace Crimea, founded the Nebraska wheat gene pool while 'Turkey Red' was used for winter wheat breeding in the USA Great Plains (Lopes et al., 2015). Studies on landraces from different regions of the world have identified potential sources for improvement of grain yield and climate resilience, for instance the drought tolerant variety 'Aragon 03' was developed from a selection of a landrace population 'Catalan de Monte' (Royo and Briceño-Félix, 2011). The potential of Mexican landraces to adapt to temperature and drought stress has been reported (Hede et al., 1999; Vikram et al., 2016). Further, allelic variation for specific plant traits such as improved 1000 kernel weight, biomass, and photosynthesis has also been identified in landraces (Lopes et al., 2015).

The development of synthetic hexaploid wheats has allowed the use of wild relatives such as tetraploid species (e.g., *Triticum dicoccum*) and the diploid species *A. tauschii* to transfer adaptive traits in to modern wheat. Genomic regions in *A. tauschii* can contribute to nearly 10% increase in grain weight (Röder et al., 2008) and improve grain yield (Börner et al., 2015). Synthetic wheats can be used to transfer such useful genetic variations. Studies have reported synthetic wheat lines that can extract more water from deeper soil, which under drought stress is an excellent adaptive trait (Reynolds et al., 2007b). Similarly, other synthetic derivatives with improved tolerance to water logging, high temperatures, and freezing have also been identified (Maes et al., 2001; Villareal et al., 2001; Yang et al., 2002).

Wild relatives of wheat also present a rich source of diversity. Species such as *Agropyron elongatum* (Host) Beauv. and *Agropyron cristatum* Gaertn. are reported to contribute to higher grain yields in wheat growing under optimal conditions. In certain wheat backgrounds, chromosome 7 Ag from *A. elongatum* increases grain yield up to 8% and carries leaf (Lr) and stem rust (Sr) resistance genes *Lr19* and *Sr25*, respectively (Singh et al., 1998). On further study this yield increase from *A. elongatum* was attributed to a better allocation of assimilates to the reproductive organs (Miralles et al., 2007). Another example is the 6P chromosome from the tetraploid species *A. cristatum*, which has been reported to increase number of florets, kernels and grain weight in wheat, in addition to improving resistance to the barley yellow-dwarf virus and powdery mildew resistance alleles (Wu et al., 2006; Wang et al., 2011).

One of the most widely used wheat relatives is rye (*Secale cereale* L.), which is well-documented as a rich source of biotic

and abiotic resistance/tolerance. Rye ($2n = 2x = 14$), is a diploid species, originating from the Near East (Hillman, 1978; Salamini et al., 2002), belongs to the tertiary gene pool of wheat, along with *Thinopyrum* and *Elymus* species (Harlan and de Wet, 1971). The first attempts to hybridize wheat and rye were conducted by Stephen Wilson (Wilson, 1873). The first stable amphiploid triticale (*Triticosecale Wittmack*) is attributed to Rimpau in 1888; thereafter, efforts were dedicated to producing wheat-rye hybrids (Ammar et al., 2004).

Several 100s of cultivars with the (1B)1R substitution or 1BL.1RS and 1AL.1RS translocations from Petkus rye were deployed between 1960 and 1990 (Rabinovich, 1998). During the 1990s, the 1BL.1RS translocation was present in 60% of wheat descending from lines developed at the International Maize and Wheat Improvement Center (CIMMYT) and nearly half of the commercial varieties (Rabinovich, 1998). In China, which is one of the major wheat growing countries, about 42% of the wheat cultivars released between 1960 and 2000 were (1B)1R genotypes, and the consistent yield gains over the years were partially attributed to the translocation (Zhou et al., 2007). Most of the desirable characteristics translocated from rye to wheat have been found in chromosome 1R that contributes to yield advantage (Villareal et al., 1998). Translocations from chromosomes 1RL and 1RS improve water use efficiency by promoting root and above ground biomass growth (Ehdaie et al., 2003; Hoffmann, 2008; Karki et al., 2014). Other rye chromosomes such as 3R, 4R, and 6R are also potential donors; introgressions from these regions could improve aluminum and acid soil tolerance in wheat.

Disease Resistance

Diseases, caused by both fungi and fungi-like pathogens pose a major threat to wheat production. Evolution of new virulence through migration, mutation, selection, and recombination of virulence genes occurs in all pathogens, but has been more frequent in those causing rust and powdery mildew. Yield losses due to diseases can be up to 70% in susceptible varieties (Singh et al., 2008). For example, in 1998, stem rust infections were reported in Uganda caused by a new race designated as Ug99. A series of reviews by Singh et al. (2006, 2008, 2011, 2015) has documented the significance, emergence, evolution and geographical spread of the Ug99 group as time progressed. Since its first discovery, 13 races within the Ug99 group have been identified across several countries in Africa and Middle East¹. Another example in recent years is of the stripe or yellow rust pathogen. Yellow rust (Yr) is found primarily in the Northern latitudes or cooler environments, however, Hovmöller et al. (2015) found 'Warrior' and 'Kranish,' two aggressive races of yellow rust originating from sexual recombination in the near-Himalayan region of Asia which can infect host under warmer temperatures.

One of the strategies to mitigate the threat from diseases is to identify and utilize diverse sources of durable resistance. Globally important fungal diseases of wheat caused by biotrophs (obligate parasites), include the three rusts; leaf or brown rust,

¹www.rusttracker.org

stripe or yellow rust and stem or black rust, caused by *Puccinia triticina*, *Puccinia striiformis* f. sp. *Triticici*, and *Puccinia graminis* f. sp. *Triticici*, respectively, powdery mildew caused by *Blumeria graminis* f. sp. *tritici*; whereas, those caused by hemibiotrophs (facultative parasites) include Fusarium head blight, *Septoria tritici* blotch, leaf blotch, spot blotch, and tan spot.

Resistance genes can be characterized as race specific and race non-specific, this classification dates back to 1962 when Van der Plank proposed the first theoretical concepts of disease resistance. Race specific genes confer resistance to one or a few races of a pathogen and are known to be based on 'gene for gene' interaction. Also known as 'major genes', they usually have large phenotypic effects, but may not confer complete resistance. Although incorporation of race-specific resistance genes may be promising, it increases the risk of faster breakdown. Some examples of major genes for rust resistance include *Lr19*, *Lr26*, and *Lr42* effective against leaf rust, *Yr5*, *Yr10*, and *Yr15* against yellow rust and *Sr22*, *Sr26*, and *Sr35* against stem rust. Race non-specific resistance, is usually effective in the post-seedling growth stage, thus commonly referred to as adult plant resistance (APR). Race-non specific resistance is generally quantitatively inherited and ranges from moderate resistance/moderate susceptibility to nearly complete resistance and interact additively with other non-specific resistance genes. Varieties with high levels of durable resistance to multiple pathogens can be developed by combining multiple race non-specific resistance loci, especially to those which are known to confer resistance to multiple diseases (Singh et al., 2008). Examples of these pleiotropic resistance genes are *Lr34*, *Lr46*, and *Lr67* which provide resistance to leaf, yellow and stem rust and powdery mildew. Because race non-specific resistance can provide broader and robust resistance to fight pathogen evolution it has been recommended for the high risk areas, for instance in East African highlands where wheat cultivation and pathogen evolution is continuous (Singh et al., 2008).

Though most rust resistance genes originated from hexaploid wheat, there are also many genes that originated from the wild relatives and other genera such as *Aegilops*, *Dasypyrum*, *Thinopyrum*, and *Secale* (Figure 2). As early as 1920 and 1930s, introgression of stem rust resistance from *T. turgidum* subsp. *durum* and *T. dicoccum* subsp. *Dicoccum Schrank ex Schubler* into bread wheat was reported (Hayes et al., 1920; McFadden, 1930). Both race-specific and non-specific genes have been identified from diverse genetic sources. For instance, *Lr9* from *Aegilops umbellulata* Zhuk, *Yr5* from *Triticum spelta* L., *Yr28* from *A. tauschii*, *Sr9e* from tetraploids and *Sr35* from *Triticum monococcum* L. are race-specific genes. Examples of race non-specific genes/APR include *Lr22a* from *A. tauschii*, *Yr36* from *Triticum dicoccoides* (Korn. Ex Asch. and Graebn) Schweinf, *Yr48* from synthetic hexaploid wheat PI610750 and *Yr52*, *56*, *57*, and *62* from landraces. Introgressions are also associated with multiple disease resistance as well, such as *Pm8/Sr31/Lr26/Yr9* from rye, *Sr36/Pm6* from *Triticum timopheevi* (Zhuk.) Zhuk., *Pch1* and *Sr38/Lr37/Lr17* from *Aegilops ventricosa* Tausch, and *Lr19/Sr25*, *Sr24/Lr24*, and *Sr26* from *A. elongatum* (Host) P. Beauvois (Sears, 1956; Friebe et al., 1996; Mago et al., 2005; Wulff and Moscou, 2014). Some genes introgressed from wild relatives

have been associated with negative linkage drag and therefore have not been widely deployed in breeding: examples include *Sr32* and *Sr37* identified in *A. speltoides* (McIntosh et al., 1995) and *T. timopheevi* (McIntosh and Gyarfas, 1971) respectively. Other temporarily designated genes that are common in high yielding wheat germplasm offer additional possibilities for combining resistance genes combinations.

Novel alleles from genetically diverse sources have also been identified for other important wheat diseases. For example, Fusarium head blight resistance genes are from genera *Roegneria*, *Hystrix*, *Elymus*, *Kengyilia*, and *Agropyron* (Wan et al., 1997) and other related species, e.g., *T. timopheevi*, *T. monococcum*, *Triticum karamyschevii* Neyski, and *T. militiniae* Zhuk and Migush (Cai et al., 2005). Genes conferring powdery mildew resistance have been reported from *T. dicoccoides* (Moseman et al., 1984), *Triticum carthlicum* Nevski, *T. monococcum* and *T. timopheevi* (Tomerlin et al., 1984). Some of the designated genes for resistance to powdery mildew, fusarium head blight and *Septoria tritici* blotch are given in Table 1. Wheat blast caused by *Magnaporthe oryzae* (anamorph. *Pyricularia oryzae*) is an emerging disease in the tropical parts of the Southern Cone of South Americas and was reported in Bangladesh as well. Though wheat blast is a recent disease, resistance has been identified in *A. tauschii* (Bockus et al., 2012) and in synthetic wheats (Cruz et al., 2010). The 2NS/2AS translocation from *A. ventricosa* was recently found to confer wheat blast resistance (Cruz et al., 2016), though unpublished reports from Paraguay have documented the emergence of new isolates virulent to this resistance. Both qualitative and quantitative resistance have been observed and the former has been validated at the seedling stage (Maciel et al., 2014). So far, eight resistance genes have been identified (i.e., *Rmg1* to *Rmg8*), of which only *Rmg2*, *Rmg3*, *Rmg7*, and *Rmg8* are host resistance genes against *Triticum* isolates of *Pyricularia oryzae*; the rest are non-host resistance genes (Anh et al., 2015). It is noteworthy that only *Rmg7* was identified in *T. dicoccum* (Tagle et al., 2015) whereas all are from bread wheat (Anh et al., 2015). Thus diverse resistant sources are available for both the existing and the emerging diseases in wheat.

Insect Pest Resistance

It is estimated that global yield losses due to insect pests in the pre-green revolution era were about 5.1%, however, the losses increased to 9.3% in the post-green revolution in 1990s (Dhaliwal et al., 2010). Insect pests are dynamic and highly adaptable. Changes in environmental temperature can modify their physiology, behavior, voltinism, and distribution. For instance, with warmer winters, the number of aphid generations per wheat growing cycle may increase (Hullé et al., 2010) and extend their distribution further (Macfadyen and Kriticos, 2012). It has also been proven that aphids can modify their behavior in response to either high or low temperature stress (Ma and Ma, 2012; Alford et al., 2014), enabling them to adapt in the presence of natural selection if genetic variation exists for such traits. While the work on disease resistance has tremendously contributed to protect wheat yields, control of arthropod pests has largely depended on the use of chemicals. A dramatic positive impact could be achieved through the introduction of new resistance

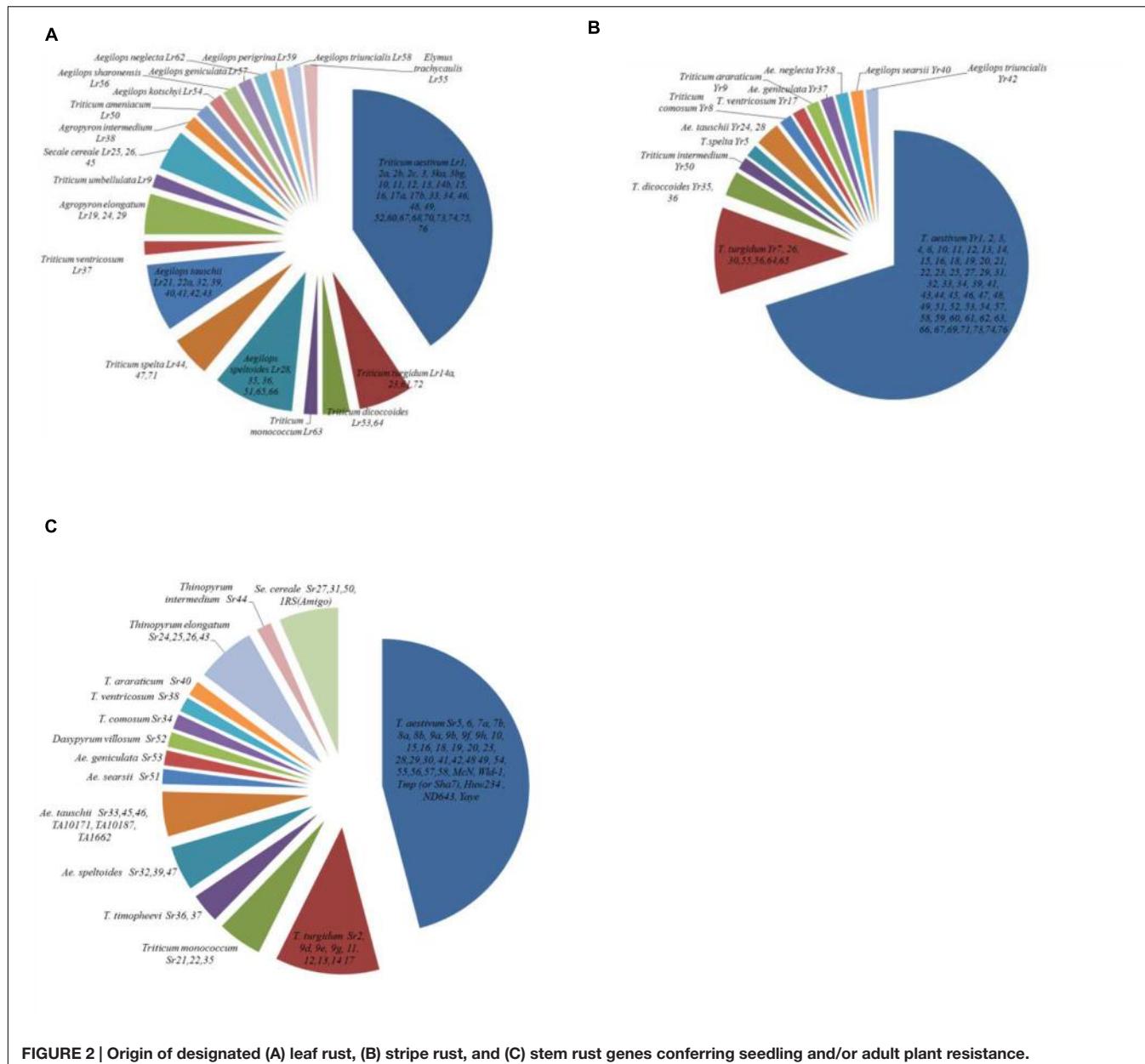


FIGURE 2 | Origin of designated (A) leaf rust, (B) stripe rust, and (C) stem rust genes conferring seedling and/or adult plant resistance.

genes (either singly or in combination with of multiple genes) to provide a broad spectrum of protection against multiple pathogens and insect biotypes.

There are several examples where genes from alien sources have been found to confer resistance to some of the most important wheat pests such as aphids *Schizaphis graminum* (Rondani), *Diuraphis noxia* (Mordvilko), *Rhopalosiphum padi* L. and *Sitobion avenae* (F.), the cecidomyiid *Mayetiola destructor* (Say), the nematode *Heterodera avenae* (Wollenweber) and the mite *Aceria tosichell* Keifer. Several wheat-related species have been found to be resistant to aphids; however, efforts to incorporate such resistance sources into wheat breeding pipelines are limited and there are only few a specific cases in which aphid resistant cultivars are purposely bred (i.e., *D. noxia* in

the USA and South Africa, and *S. graminum* in the USA). To determine the utility of such genetic resources for aphid resistance, Smith et al. (2004) evaluated 21 accessions from six species of *Aegilops* and one accession of *Triticum araraticum* Jakubz that were previously identified to be resistant to *R. padi* and found antibiotic effects on *S. avenae* and *D. noxia* in an *Aegilops neglecta* accession. Migui and Lamb (2003) evaluated resistance to *R. padi*, *S. avenae*, and *S. graminum* in 19 species related to wheat, and found that species such as *Triticum boeticum* Boiss., *A. tauschii* and *T. araraticum* had the higher levels of resistance to *R. padi*, whereas *A. tauschii* and *T. turgidum* had higher levels of overall resistance to *S. graminum*, and *T. araraticum* and *T. dicoccoides* had higher levels of overall resistance to *S. avenae*. However, for other destructive pests

TABLE 1 | Known genes for resistance to Powdery mildew, Fusarium head blight and *Septoria tritici* blotch from landraces, wild relatives and synthetic wheat.

Diseases	Source of Resistance	Genes
Powdery mildew (<i>Blumeria graminis</i> f. sp. <i>tritici</i>)	<i>Triticum monococcum</i> <i>Triticum urartu</i> <i>Triticum boeticum</i> <i>Triticum dicoccoides</i> <i>Triticum dicoccum</i> <i>Triticum carthlicum</i> <i>Triticum spelta</i> <i>Triticum sphaerococum</i> <i>Triticum timopheevii</i> <i>Aegilops tauschii</i> <i>Aegilops speltoides</i> <i>Aegilops longissimi</i> <i>Aegilops ovata</i> <i>Secale cereale</i> <i>Thinopyrum intermedium</i> <i>Haynaldia villosa</i> <i>Triticum</i> spp. <i>Leymus racemosus</i> <i>Elymus tsukushiensis</i> <i>Thinopyrum ponticum</i>	<i>Pm4d</i> , <i>Pm1b</i> , and <i>Pm1c</i> <i>PmU</i> <i>Pm25</i> , <i>PmTb7A.1</i> , and <i>PmTb7A.2</i> <i>Pm16</i> , <i>Pm26</i> , <i>Pm30</i> , <i>Pm31</i> , <i>Pm36</i> , <i>Pm41</i> , <i>Pm42</i> , and <i>MelW72</i> <i>Pm4a</i> , <i>Pm5a</i> , <i>Pm49</i> , and <i>Pm50</i> <i>Pm4b</i> , <i>Pm33</i> , and <i>Pm46</i> <i>Pm1d</i> , <i>Pm10</i> , and <i>Pm11</i> <i>Pm3b</i> and <i>Pm36</i> <i>Pm6</i> , <i>Pm27</i> , and <i>Pm37</i> <i>Pm2</i> , <i>Pm19</i> , <i>Pm34</i> , and <i>Pm35</i> <i>Pm12</i> and <i>Pm32</i> <i>Pm13</i> <i>Pm29</i> <i>Pm7</i> , <i>Pm8</i> , <i>Pm17</i> , and <i>Pm20</i> <i>Pm40</i> and <i>Pm43</i> <i>Pm21</i> <i>Fhb1</i> , <i>Fhb2</i> , <i>Fhb4</i> , and <i>Fhb5</i> <i>Fhb3</i> <i>Fhb6</i> <i>Fhb7</i>
Fusarium head blight (<i>Fusarium graminearum</i>)	Synthetic Wheat (Synthetic 6x, W7984, M3) <i>Triticum monococcum</i> (W7984)	<i>Stb5</i> , <i>Stb8</i> , <i>Stb16q</i> <i>TmStb1</i>
<i>Septoria tritici</i> blotch (<i>Mycosphaerella graminicola</i>)		

Friebe et al. (1996) and McIntosh et al. (2013).

such as, *Eurygaster integriceps* Puton, more work is required to find adequate resistance levels that can be incorporated in wheat cultivars (El Bouhssini et al., 2009). Friebe et al. (1996) made a comprehensive review of wheat-alien translocations that confer resistance to wheat biotic stresses. Here, some examples of resistance to diseases and pests translocated from rye are reviewed (see Table 2, where we summarize resistance sources by rye chromosome and diseases/pests).

End-Use Quality and Nutritional Quality

In addition to combating abiotic and biotic stresses while improving grain yield, wheat breeding must improve or at least maintain the nutritional and end-use quality. The wide variety of food products made from wheat flour has resulted in ongoing demand from the wheat processing industry for wheat with specific quality attributes. Additionally, dietary deficiencies of essential micronutrients such as zinc (Zn) and iron (Fe) are a major health concern in developing countries especially for pregnant women and children under age 5. An estimated 17.3% of the world's population is at risk for inadequate zinc intake, a factor highly correlated with stunted growth in children (Wessells and Brown, 2012). Genetic biofortification with natural genetic variation present in wild relatives, synthetics and landraces for micronutrient uptake from the soil and translocation in to wheat grain is a sustainable solution that can supplement micronutrient-deficient rural inhabitants with limited access to formal markets or health care systems (Velu et al., 2014).

In recent years, the focus has been on "biofortification" of wheat with micronutrients, specifically Zn and Fe.

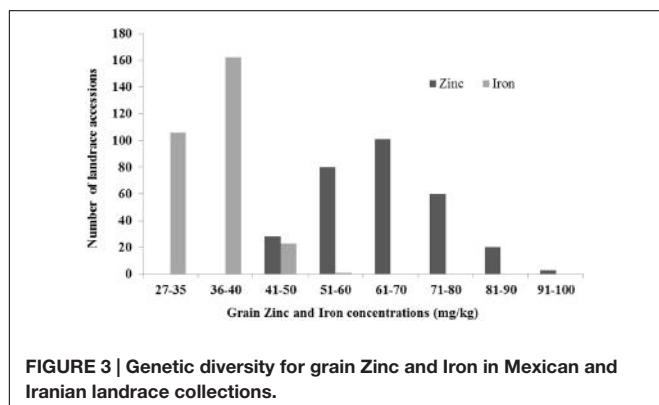
Evaluation of landraces and secondary gene pools (i.e., tetraploid and diploid progenitors of hexaploid wheat) for micronutrient concentration identified *T. dicoccoides*, *A. tauschii*, *T. monococcum*, and *T. boeticum* Boiss. as the most promising sources for improving Fe and Zn grain concentration (Cakmak et al., 2000; Monasterio and Graham, 2000). Large scale screening of available wheat genetic resources at CIMMYT identified einkorn wheat, wild emmer wheat, and landraces with high amounts of Zn and Fe in grain (Cakmak et al., 2000; Ortiz-Monasterio et al., 2007). The available genetic variation in wild emmer (*T. dicoccoides*), *T. spelta*, *T. dicoccum* species is being used to develop nutrient-enriched wheat germplasm. The stocks (*T. turgidum* ssp. *dicoccum*/*A. tauschii*) are also being used for genetic biofortification of Zn and Fe by CIMMYT's wheat breeding program (Ortiz-Monasterio et al., 2007; Morgounov et al., 2007). Recently, evaluation of a representative subset of Mexican and Iranian landraces under Zn-enriched soil conditions in Cd. Obregon, Mexico, showed more than a twofold variation for Zn (40–96 mg/kg) and Fe (27–56 mg/kg; Figure 3). A major locus affecting Zn and Fe concentration, *Gpc-B1* (250 kb-loci), was mapped, and found to encode a NAC transcription factor (NAM-B1) that accelerates senescence and increases nutrient remobilization from leaves to grain (Uauy et al., 2006; Distelfeld et al., 2007). Interestingly, the favorable allele of *Gpc-B1* is from *T. dicoccoides* and all modern tetraploid and hexaploid wheats possess a non-functional allele of NAM-B1, indicating that the NAM-B1 function was lost during domestication.

TABLE 2 | Examples of resistance genes for diseases and pests from rye (*Secale cereale*).

Diseases	Gene	Description	Germplasm
Leaf rust (<i>Puccinia triticina</i>)	Lr26	1BL.1RS	Petkus rye; Kavkaz and Veery wheat derived
	Lr25	4BS.4BL-2RL	Transec
	Lr45	2AS-2RS.2RL	RL6144
Stripe Rust (<i>Puccinia striiformis</i> var. <i>striiformis</i>)	Yr9	1BL.1RS	Petkus rye; Kavkaz and Veery wheat derives
	YrCN17 [†]	1BL.1RS	R14, Chuan-nong 17
	YrR212 [†]	1BL.1RS	R212
Stem rust (<i>Puccinia graminis</i> f. sp. <i>tritici</i>)	Sr31	1BL.1RS	Petkus rye; Kavkaz and Veery wheat derives
	Sr50/SrR	1BL.1RS	Imperial rye derives
	Sr1RS ^{Amigo}	1AL.1RS	Amigo wheat
	Sr27	3AL.3RS	WRT238
Powdery mildew (<i>B. graminis</i> f. sp. <i>tritici</i>)	Pm8	1BL.1RS	Petkus rye; Kavkaz and Veery wheat derives
	Pm17; allelic to Pm8	1AL.1RS	Insave rye derives; Amigo wheat derives
	Pm7	4BS.4BL-2RL	Transec
	Pm20	6BS.6RL	WGRC28
Greenbug (<i>Schizaphis graminum</i>)	Gb2	1AL.1RS	Insave rye, Amigo wheat derives
	Gb6	1AL.1RS	Insave rye, GRS1201
Diuraphis noxia	Dn7	1BL.1RS	94M370 wheat
Hessian fly (<i>Mayetiola destructor</i>)	H21	2BS.2RL	KS85HF 011-5
	H25	4BS.4BL-6RL	Balbo rye; 88HF16 wheat
Aceria tosicell	CmC3	1AL.1RS	Amigo wheat
Cereal cyst nematode (<i>Heterodera avenae</i>)	CreR	6DS.6RL	T-701 triticale derives

[†]Temporary designation.

Friebe et al. (1996) and McIntosh et al. (2013).

**FIGURE 3 | Genetic diversity for grain Zinc and Iron in Mexican and Iranian landrace collections.**

Apart from micronutrients, wheat grain is also a good source of other beneficial nutrients which could be targeted by breeding programs to improve the nutritional quality of wheat based products. Grain bran is particularly rich in dietary fiber, vitamins (folic acid), and phytochemicals, which have been associated with a protective role for many chronic diseases including cardiovascular diseases and type 2 diabetes (Jacobs et al., 1999; Liu et al., 1999; de Munter et al., 2007). The HEALTHGRAIN cereal diversity screening project reported diversity for dietary fiber and phytochemicals in the wheat primary gene pool. The levels of dietary fiber ranged from 11.5 to 18.3% of dry matter, and more specifically the content of water extractable arabinoxylans (an important source of soluble dietary fiber, which is more readily fermentable in the colon than insoluble one) ranged from 0.3 to 0.85% in bran and from 0.3 to 1.4% in flour (Gebruers et al.,

2008; Ward et al., 2008; Kariluto et al., 2010). Various research projects are currently ongoing to screen for genetic variability of the bioactive compounds (Di Silvestro et al., 2012; Giambanelli et al., 2013; Laddomada et al., 2016). High heritability for some of these compounds such as tocots, sterols and arabinoxylan fiber (Shewry et al., 2010) and available genetic diversity increases the chances of utilizing the variation for improving nutritional quality in wheat.

The vast catalog of products prepared from wheat requires genetic variation in traits related to grain composition as well. Exploring novel genetic variation could improve processing and end-use quality. Grain proteins are one of the important components that influence end-use quality. Studies have reported higher grain protein content in landraces than in modern wheat (Rodriguez-Quijano et al., 1994; Dotlaci et al., 2010) which means landraces and wild relatives could be a potential source to improve protein content. In fact, as mentioned above *GPC-B1* (also called *NAM-B1*), the first gene identified for grain protein content variation was transferred from a wild emmer accession (*T. dicoccoides*) to modern durum wheat background (Avivi, 1978; Joppa and Cantrell, 1990; Joppa et al., 1997). While grain protein content is important, gluten quality is equally important. Gluten, an essential component of dough, is a complex protein network formed mainly by two kinds of proteins, monomeric gliadins and polymeric glutenins, which in turn are divided into high molecular weight glutenins (HMWGs) and low molecular weight glutenins (LMWGs). Although there is allelic variation in modern wheat for the gene *Glu-1* encoding HMWGs, use of diversity in the Triticeae pool could potentially contribute to improve processing quality (Xu et al., 2010; Rasheed et al.,

2014). The Wheat Gene Catalog currently describes 26 alleles for *Glu-A1*, 56 for *Glu-B1*, 24 for *Glu-D1*, 55 for *Glu-A3*, 32 for *Glu-B3*, and 16 for *Glu-D3* (McIntosh et al., 2013). Several of those alleles have been detected in modern wheat ancestors and wild relatives, such as *Glu-B1q* in emmer (Vallega and Waines, 1987), *Glu-B1be* in wild emmer (Xu et al., 2004), *Glu-D1n* in spelt (Caballero et al., 2001), or *Glu-D1bf* in *A. Tauschii* (Gianibelli et al., 2001). Genetic resources (for example *T. urartu* or *T. monococcum*) can be utilized to introgress *Glu-A1* $x+y$ or y active subunits (always silenced in modern durum and bread wheat, respectively), which will lead to new variations (Alvarez et al., 2009). Recently, a novel allele HMW glutenin allele was identified from *A. longissima* Schweir and Muschl through the use of a Chinese Spring substitution line CS-1S(1B) that could potentially improve dough and breadmaking quality (Wang et al., 2013).

Other important quality traits such as grain hardness or starch properties are also influenced by diverse proteins and therefore genes. Puroindolines a and b (PINA, PINB), encoded by the genes *Pina-D1* and *Pinb-D1*, are responsible for grain hardness (Morris, 2002). Wild alleles of *Pina-D1a* and *Pinb-D1a* are linked to soft grain texture, though several alleles for both *Pin-D1* genes have been associated with harder grain in modern wheat (Giroux and Morris, 1997, 1998; Lillemo and Morris, 2000; Ikeda et al., 2010), landraces (Chen et al., 2005, 2007; Ayala et al., 2013) and wild relatives (Massa et al., 2004; Guzmán et al., 2012; Cuesta et al., 2013). It is interesting to note that some of these alleles have been associated with differences in quality traits other than grain hardness (Brites et al., 2008; Tanaka et al., 2008; Chen et al., 2013). While knowledge on the diverse sources for genes to improve end-use quality is available, utilization of the diversity within the breeding programs is not prevalent.

HARNESSING DIVERSITY IN WHEAT

Traditional Breeding Approaches

The success of breeding to introgress beneficial genomic regions into wheat is conditioned by the relatedness between the species (Friebe et al., 1996). Mujeeb-Kazi and Wang (1995) identified certain key requirements for introgression, (1) the genome constitution of the donor species; (2) the genomic relationship between the donor and recipient species; (3) chromosomal location of the loci of interest; (4) whether the gene(s) of interest can be expressed in the recipient species; and (5) whether gene transfer has any negative effect on the recipient species. For instance, introgression can be achieved by direct hybridization, homologous recombination, backcrossing and selection if the donor species belongs to the primary gene pool, e.g., hexaploid landraces, cultivated tetraploids (*T. turgidum*), wild emmer wheats (*T. dicoccoides*) or diploids *T. monococcum* and *A. Tauschii*. If the donor species belongs to the secondary gene pool (e.g., polyploid *Aegilops* and *Triticum* species, and the S-genome species of the genus *Aegilops*) homologous recombination is possible if the loci of interest are transferred in homologous chromosomes. For species belonging to the tertiary gene pool (e.g., *Elymus* species), gene transfer can be achieved

by exploiting the centric breakage-fusion of univalents, induced homoeology and radiation treatment to induce chromosome breaks (Friebe et al., 1996; Feuillet et al., 2008). Synthetic hexaploid wheats carry novel variation for tolerance/resistance to abiotic and biotic stresses but are usually poor in agronomic performance. While they are used for transferring useful genetic variation into common wheat, typically one or two backcrosses to elite germplasm followed by selection are required to identify lines with superior performance.

Although such introgressions can be of benefit to wheat, the donor sources often negatively impact previously selected adaptation traits in the recipient germplasm because alien chromatin is usually incorporated as large blocks that may carry alleles associated with undesirable agronomic characteristics. Depending on the wheat genetic background, the rye source and the type of abiotic stress factors, studies have shown that rye transferred into wheat may have both positive and negative effects on wheat performance. Monneveux et al. (2003) reported that depending on the wheat background, 1BL.1RS translocations can negatively impact yield under rainfed conditions and heat stress. However, in general, under non-stressed conditions, 1RS confers higher yield regardless of which wheat chromosome (1A, 1B, or 1D) it is translocated into (Kim et al., 2004). On the other hand, the position of 1RS in the wheat genome can negatively affect baking quality, thus genotypes with 1AL.1RS and 1DL.1RS are preferred over genotypes with 1BL.1RS (Graybosch et al., 1993; Kim et al., 2005). Traditional breeding methods such as repeated backcrossing and selection of desirable genotypes often require extensive efforts and are time consuming. However, with the new advances in phenotyping, QTL mapping, and genetic modification, along with sequencing technologies are expected to improve the precision and speed of alien introgression (Jacobsen and Schouten, 2007; Tiwari et al., 2014).

MODERN BREEDING APPROACHES

High Throughput Phenotyping

Phenotypic characterization is important prior to the efficient utilization of genetic diversity. Most phenotypic traits, heading time, photoperiodic responses and vernalization responses are explained by the germplasm's geographical origin (Kato and Yokoyama, 1991; Cavanagh et al., 2013). Phenotyping for agronomic traits, response to disease and pests and other adaptive traits is crucial for the introduction of new allelic variation in breeding programs. Targeted characterization of germplasm panels such as the Focused Identification of Germplasm Strategy (FIGS), developed based on agro-ecological data enables identification of specific adaptive traits within the genetic resources. For instance, Reynolds et al. (2015) applied FIGS set to evaluate landraces, and found that those from heat and drought stressed regions had 40% greater biomass under heat and drought compared to modern varieties.

Greenhouse based automated phenotyping platforms using robotics and sensor imaging are being used for data acquisition in different crops by a number of institutes globally (e.g., IPK Gatersleben, Germany and The Plant Accelerator, Adelaide,

Australia). Though, the high operational cost of such high throughput phenotyping platforms limits their large-scale use in breeding programs. Recent developments in remote sensing and high throughput phenotyping technologies allow characterization of a large number of germplasm in a short amount of time. Spectral imagery can be utilized to measure normalized difference vegetation index (NDVI), canopy temperature, hydration status, and pigment composition (Honsdorf et al., 2014; Rahaman et al., 2015; Reynolds et al., 2015). These spectral indices have already been linked to ground based measurements of yield, biomass, and adaptation (Reynolds et al., 1994). Availability of high-resolution cameras has made it possible to focus on phenotypic characterization at the plot level. For instance, spectral indices estimated by using low level airborne remote sensing showed significant association with those collected at ground level (Tattaris et al., 2013). Along with advances in statistical modeling methods, it is possible to predict plant performances in the field, based on the information obtained from high-throughput phenotyping. Such technologies could be used for characterization of the diverse germplasm pools to identify potential sources for tolerance/resistance to abiotic and biotic stresses.

Genome Wide Association Mapping and Marker Assisted Backcrossing

The use of molecular markers for identifying functional genes and genome wide association studies (GWAS) can greatly facilitate the introgression process. GWAS studies on landraces and wild relatives of wheat have identified quantitative trait loci (QTL) associated with morphological traits in normal irrigated, heat and drought environments and with disease resistance (Kertho et al., 2015; Liu et al., 2015; Sukumaran et al., 2015). If large effect QTL exist for traits of interest and the favorable alleles originate from exotic sources, then marker assisted backcrossing (MABC) can be used to more rapidly introgress such alleles into elite backgrounds compared to conventional backcrossing (Hillel et al., 1990; Tanksley and Nelson, 1996).

Marker assisted backcrossing involves selecting of favorable alleles using QTL linked markers during each backcrossing generation. To reduce the number of backcrossing generations required to recover the recurrent parent genome, markers distributed across the genome can be used to select individuals with the favorable donor QTL and the highest proportion of recurrent parent genome (Young and Tanksley, 1989; Hillel et al., 1990, Hospital et al., 1992). This approach, referred to as MABC with foreground and background selection, can be highly effective with availability of gene based markers and markers tightly linked to QTL determine (Ellis et al., 2014). This approach has been suggested for improving a wide range of traits conferred by large effect genes, including rust resistance genes in wheat. If QTL positions are uncertain (such is the case of positions inferred by QTL mapping studies), then flanking markers located several centimorgans on either side of the QTL are needed to ensure the QTL is not lost during backcrossing (Visscher et al., 1996). This may be problematic if there is linkage drag associated with the QTL, and large flanking segment

may inevitably be introgressed. Fine-mapping or cloning the QTL to develop closely linked or functional markers would be ideal for backcross introgression from exotic germplasm. Unfortunately, in wheat, fine mapping and cloning can take several years.

In addition to certainty of QTL positions and availability of tightly linked markers, the number of targeted QTL is another factor that should be considered before attempting MABC. The proportion of single MABC progeny containing donor alleles at all QTL is 0.5^n , where n is the number of QTL and assuming QTL are unlinked, and the position of the QTL is known with certainty. For example, to introgress of 5 QTL, approximately 3% of the progeny can be expected to contain all favorable alleles; thus 145 progeny would be required obtain one individual with all three alleles with a 1% risk of failure. Reducing linkage drag when introgressing multiple QTL can be hastened dramatically when using background selection to identify the desired recombinants. However, the probability of observing the desired recombinants remains low, and several generations of backcrossing may ultimately be needed. To introgress multiple QTL, a QTL pyramiding scheme where QTL are first introgressed in the desired background singly and then combined would be more efficient (Hospital and Charsosset, 1997). An algorithm for designing optimal gene or QTL pyramiding schemes was presented by Servin et al. (2004).

Marker assisted backcrossing is being applied at CIMMYT to improve grain Zn and Fe concentrations. Various studies have reported QTL for high grain Fe and Zn concentrations on chromosomes 1A, 2A, 2B, 3D, 4B, 6A, 6B, and 7A in different species of diploid, tetraploid, and hexaploid wheat (Peleg et al., 2009; Tiwari et al., 2009; Xu et al., 2012; Hao et al., 2014; Srinivasa et al., 2014). A recombinant inbred line (RIL) population developed from the cross between 'PBW343' and 'Kenya Swara' was used to identify QTL and markers associated with Zn. Two novel large effect QTL on chromosomes 2B and 3A were successfully converted into usable form for marker assisted introgression of this QTL in to an elite background. During the 2014–2015 crop season, selected RILs that showed significantly enhanced Zn compared to either of the parental lines PBW 343 or Kenya Swara was used to transfer the QTL of interest using foreground selection. This strategy will serve to move desirable alleles rapidly and precisely into the adapted background.

Genomic Selection

When the number of QTL is large, MABC and pyramiding schemes may not be feasible. Phenotypic selection is currently the most reliable and widely used method for introgressing of favorable alleles from an exotic, non-adapted parent. GS techniques can also be applied to increase the rate of genetic gain in populations derived from exotic and elite parents. As reviewed by Lorenz et al. (2011), GS is a marker assisted breeding method in which genome wide markers and phenotypes from a reference population are used to train a prediction model. That prediction model is then used to predict breeding values based only on their genome-wide marker data. GS is more effective than MAS or marker assisted recurrent selection for polygenic traits (Bernardo and Yu, 2007). To achieve good prediction accuracy, it

is important that the model training population be representative of the selection candidates that are to be predicted (Hayes et al., 2009; Pszczola et al., 2012).

If exotic parents are used in the breeding program, then an existing model training population will not be effective for predicting the progeny of these crosses. If one is to use GS to select among progeny from an exotic by elite cross, then a subset of the progeny will need to be phenotyped for model training. That prediction model could then be used for a few generations of recurrent selection within the bi-parental population. If the objective is to backcross favorable alleles from the exotic parent into an elite background, then GS can be used to identify the backcross progeny to cross to the recurrent parent. A simulation study by Bernardo (2016) found that the most effective GS backcrossing approach to introgress QTL from an exotic into an elite background was to train the GS model using F2 progeny and then apply that model during multiple generations of backcrossing. This approach led to a greater selection compared to phenotypic selection or selection based on QTL linked markers alone.

If crossing with exotic parents without backcrossing or within family recurrent selection, then it would be best to refrain from GS among families where one of the parents is exotic or exotic-derived until a sufficient number of progeny and other relatives descending from the exotic have been phenotyped. GS for allele introgressions has not yet been attempted in wheat; however, the use of GS in breeding with elite germplasm has shown significant potential. There are at least 29 studies on GS in wheat that have been published. Two studies (Heffner et al., 2011a,b) showed the potential of this approach to predict end-use quality traits of soft bread wheat germplasm, and obtained promising results, although forward prediction of quality traits was not carried out. A 5-years study conducted at CIMMYT with elite breeding lines for flour quality reported forward prediction accuracies of 0.68 and 0.49 for aleveograph W and loaf volume respectively (Battenfield et al., 2016). In another GS study a cross-validation of genomic predictions revealed moderately high predictability for grain Zn(0.5) and Fe(0.6) (Velu et al., 2016). Several cross validation studies have assessed the potential to use GS for improving disease resistance (Ornella et al., 2012; Rutkoski et al., 2012, 2015; Daetwyler et al., 2014; Arruda et al., 2015; Mirdita et al., 2015) and for grain yield (Crossa et al., 2010; Poland et al., 2012; Dawson et al., 2013) in wheat.

Next Generation Approaches

The concept of cisgenesis was defined by Schouten et al. (2006) as the transfer of genes within the gene pool of sexually compatible species of same genus. Though similar to classical breeding, this approach has the potential to overcome its two major limitations. Cisgenesis can be used to hasten the transfer of targeted genes between related species and can avoid linkage drag associated with classical breeding. The strategy can also be used to improve traits with limited natural allelic variation in the gene pool. Higher expression of the traits can be obtained by re-introducing the gene with its own promoter and terminator or expression levels can be lowered through silencing constructs (Holme et al., 2013). In the case of wheat, cisgenic transfers are limited within

the *Triticum* genus, though the availability of tritcale, a hybrid between rye and wheat, and hybrids between barley and wheat, opens up new opportunities for cisgenesis between two divergent sexually compatible gene pools, *Triticum–Secale* and *Triticum–Hordeum* (Holme et al., 2013). There are a few of examples of the use of cisgenesis in cereals. The HMW glutenin subunit 1Dy10 associated with superior bread-making quality is present in hexaploid bread wheat but absent in durum wheat. Cisgenesis was used to transfer the 1Dy10 HMW glutenin gene from bread wheat to durum wheat (Gadaleta et al., 2008). Further work is ongoing to improve phytase activity in barley (Kerr et al., 2010) and drought tolerance in ryegrass (Bajaj et al., 2008).

With evolution of technologies, introgression of multiple genes as cassettes through cisgenesis is also being explored. Extensive time and effort is required to transfer multiple genes from genetically diverse sources in to cultivated varieties, requiring multiple backcrosses and selection against undesirable traits. The development of gene cassettes could potentially solve the issues related to sexual incompatibility, linkage drag and introgression from other genera. Wulff and Moscou (2014) described it to be equivalent to the wheat-rye translocation (1BL:1RS) which harbors different genes for disease resistance. Ellis et al. (2014) suggested constructing gene cassettes with multiple resistance genes combined resistance against the three rust diseases will result in durable resistance in wheat. The ability to produce cassettes will allow combining genes that cannot be selected in a normal breeding processes or introgress genes linked in repulsion; this will lead to rapid introgression into cultivars. Though the new technologies show great potential, they have several limitations. Both the cisgenesis and gene cassettes approaches will require genome-editing technologies that are still under development. Furthermore, there may be issues with gene suppression or loss of gene expression due to host gene interaction. For example, Hurni et al. (2014) observed that the powdery mildew gene *Pm3* in wheat suppresses its ortholog *Pm8* transferred to wheat from diploid rye due to interactions of encoded proteins, thus limiting transfer of multiple genes for resistance. Finally, government regulations and acceptance within the scientific and social community will drive the application of these technologies in wheat breeding.

CONCLUSION

The rich genetic diversity available in wheat is a source of numerous novel alleles for grain yield, disease resistance and tolerance to abiotic stress. While scientists realized the importance of genetic diversity decades ago, there is still a huge gap in characterization of the available genetic resources and their utilization in breeding programs. Over the years traditional breeding strategies have successfully incorporated novel alleles into elite germplasm, which has had significant impacts on production globally. A recent example is the development and release of biofortified wheat 'Zinc Shakti (Chitra)', developed by introgressing synthetic hexaploid (*A. tauschii* background) with elite germplasm, which has 40% higher grain Zn (Velu et al., 2015). Technologies such as GWAS and MABC are currently

being used to explore the diversity and incorporate novel alleles into elite lines, though the lack of well-characterized genes and their closely linked markers impedes the process. GS and cisgenesis are promising technologies that could help harness large numbers of favorable exotic alleles and subsequently transfer them to elite backgrounds. Initiatives for genotyping and phenotyping of genetic resources through the gene banks are required to harness diversity efficiently and utilize in the breeding for improved wheat varieties.

AUTHOR CONTRIBUTIONS

RS: invited author; contributed to genetic diversity in grain yield and disease resistance, breeding approaches for harnessing diversity; SM: corresponding author; Tasks: compiling the review manuscript and contribution to following topics, genetic diversity for grain yield, climate resilience, traditional breeding

approaches, high throughput phenotyping and cisgenesis; JR: harnessing genetic diversity through Genome wide association studies, marker assisted backcrossing, and genomic selection; GV: genetic diversity for Zinc and Iron and breeding approaches for Biofortified wheat; PS: genetic diversity for disease resistance (other than Wheat rust); XH: genetic diversity for disease resistance (other than Wheat rust); LC-H: genetic diversity for pest resistance, grain yield and breeding approaches; CG: genetic diversity for grain composition and end-use quality; SB: genetic diversity for rust resistance (Stem rust, yellow rust); CL: genetic diversity for rust resistance (Yellow rust, leaf rust).

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New Genetic Insights into Pearl Millet Diversity As Revealed by Characterization of Early- and Late-Flowering Landraces from Senegal

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Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a staple food and a drought-tolerant cereal well adapted to Sub-Saharan Africa agro-ecosystems. An important diversity of pearl millet landraces has been widely conserved by farmers and therefore could help coping with climate changes and contribute to future food security. Hence, characterizing its genetic diversity and population structure can contribute to better assist breeding programs for a sustainable agricultural productivity enhancement. Toward this goal, a comprehensive panel of 404 accessions were used that correspond to 12 improved varieties, 306 early flowering and 86 late-flowering cultivated landraces from Senegal. Twelve highly polymorphic SSR markers were used to study diversity and population structure. Two genes, *PgMADS11* and *PgPHYC*, were genotyped to assess their association to flowering phenotypic difference in landraces. Results indicate a large diversity and untapped potential of Senegalese pearl millet germplasm as well as a genetic differentiation between early- and late-flowering landraces. Further, a fine-scale genetic difference of *PgPHYC* and *PgMADS11* (SNP and indel, respectively) and co-variation of their alleles with flowering time were found among landraces. These findings highlight new genetic insights of pearl millet useful to define heterotic populations for breeding, genomic association panel, or crosses for trait-specific mapping.

Keywords: millet, *Pennisetum glaucum*, flowering, genetic diversity, SSR, *PgMADS11*, *PgPHYC*

INTRODUCTION

Low agricultural productivity causes food insecurity and malnutrition in Sub-Saharan Africa (SSA). This is due to climate variability, increased growth and needs of worldwide population, water demand, intensive exploitation of natural resources and environmental degradation. Therefore, better uses of natural resources could help overcoming some of these constraints and greatly contribute to improving productivity.

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a major staple food for many SSA and Asian countries. Pearl millet is highly allogamous and mainly a rainfed crop, covering a wide range of different ecological zones and production systems. However, yield is low and variable, rarely reaching 1000 kg/ha. This is mainly explained by the limited exploitation of genetic resources and availability of improved varieties, but also by low soil fertility, drought, heat and highly variable rainfall (Waddington et al., 2010). Moreover, production is threatened by downy mildew disease, striga parasitic weed and predation by insects (Waddington et al., 2010). Characterization of crops genetic resources is a prerequisite to build up a breeding program for sustainable productivity enhancement.

Senegal is one of the top 10 pearl millet producers in the world (FAOSTAT, 2013), where farmers distinguish two main types of cultivars based on growth duration. Cultivars, called *Souna*, are sensitive to photoperiod, with a short cycle between 65 and 90 days and adapted to low (350–600 mm) rainfall regions. Cultivars, called *Sanio*, are a less photoperiod-sensitive type than *Souna*, with a long cycle between 120 and 150 days, and are adapted to high (900–1200 mm) rainfall regions. Nationwide, *Souna* type occupies nearly half the area sown to cereals (51%) while *Sanio* type, mainly cultivated in the South, represents about 15% of total millet production (ANSD, 2014).

Investigating genetic diversity and patterns of early- and late-flowering landraces is very important since flowering cycles and photoperiod sensitivity play a crucial role in the adaptation to climatic conditions. It is assumed a direct effect of selection for earliness associated with climate variations (Vigouroux et al., 2011). Genome scans and genetic association mapping have identified several genes tightly linked to adaptive traits of pearl millet in semi-arid areas. A SNP in the *Phytochrome C* locus (*PgPHYC*) and an indel variation in *PgMADS11* gene were associated with flowering time variation, annual rainfall and spike length of pearl millet (Saïdou et al., 2009; Mariac et al., 2011; Vigouroux et al., 2011). From framers point of view, distinction between early- and late-flowering landraces is very clear but it is not always associated with clear genetic differentiation (Dussert et al., 2015). Flowering time in pearl millet is derived from a common domestication event and a strong gene flow between early- and late-flowering landraces is observed (Dussert et al., 2015). The level of gene flow would depend on cycle overlapping, agricultural practices and spatial distribution (Mariac et al., 2006b; Allinne et al., 2007; Lakis et al., 2012).

Because there is a critical need for adapting local agriculture to harsher future conditions, landraces and improved varieties adaptation will mostly rely on standing genetic variation available within the cultivated compartment. Recently, phenotyping

(Sy et al., 2015) and genotyping by sequencing (Hu et al., 2015) studies were carried out on a set of Senegalese pearl millet landraces. Sampling was restricted to only one agro-ecological area of Senegal, the Groundnut Basin, and included only nine so-called intermediate-flowering landraces (flower between 75 to 100 days after sowing). Based on phenology, head architecture and grain color, these accessions were classified into three cultivar groups, indicating a morphological diversity between early flowering landraces (Sy et al., 2015). Using 83,875 single nucleotide polymorphisms (SNPs) on the same set of accessions in addition to 252 global accessions, a higher genetic diversity was observed in Senegal accessions compared to millet accessions in India, South and Western Africa (Hu et al., 2015). Any local structure was evidenced, therefore studies of loci that control the cycle length would be necessary to assess more accurately the evolution of cultivated millet varieties (Dussert et al., 2015).

Here, a fine scale sampling strategy and genetic characterization are described that differentiate early- and late-flowering landraces of Senegalese pearl millet. Using highly polymorphic SSRs markers, genetic diversity and population structure of the landraces was assessed. Allelic diversity of *PgPHYC* and *PgMADS11* genes, both linked with flowering time variation and rainfall, was further investigated (Saïdou et al., 2009; Mariac et al., 2011; Vigouroux et al., 2011).

MATERIALS AND METHODS

Plant Materials

Collects were done in 1992 and 1994 in the main areas of millet production in the Groundnut Basin as previously described (Sy et al., 2015). Geographical coordinates of these accessions were partially retrieved (88%) by using village names. Additional collects were done in 2010 and 2014 to cover pearl millet production areas, except the city of Dakar and the eastern-south area where the Niokolo-Koba Wildlife Park is located. Geographical coordinates of these new accessions were recorded using a GPS. As our focus is on local landraces, villages near major roads or markets were avoided. In total, 392 accessions were collected from 316 villages, i.e., 1.24 accessions per village on average. A panel of 404 accessions was analyzed including 12 improved varieties bred locally and widely used by farmers, 306 early flowering landraces (252 villages) and 86 late-flowering landraces (74 villages). Among the 316 villages, 10 villages were sampled with both early- and late-flowering landraces (Supplementary Table S1). Cycles were recorded following farmer interviews.

DNA Extraction and SSR Genotyping

Five seeds per accession were grown in the greenhouse 3–4 weeks according to sampling date. About 200 mg of leaf sample from one individual per accession were collected and DNA extraction was carried out using the previously described protocol (Mariac et al., 2006a). Twelve highly polymorphic microsatellites distributed throughout the pearl millet genome were used (Supplementary Table S2). These markers have been previously described (Allouis et al., 2001; Qi et al., 2001;

Budak et al., 2003; Mariac et al., 2006a). PCR reactions were performed using the Multiplex PCR Kit (Qiagen, Inc) following the recommended protocol. PCR were conducted using a thermal cycler TC-Plus (TECHNE): pre-denaturation of 95°C for 15 min then 35 cycles consisting of a denaturation step at 94°C for 30 s, annealing at 55°C for 90 s, elongation at 72°C for 60 s and a final extension at 60°C for 30 min). Four positive and four negative controls were repeated on each PCR plate. Samples were genotyped on an ABI 3130 Prism® (Applied Biosystems®) and read with Genemapper™ software (version 3.7; Applied Biosystems®).

Genetic Diversity and Population Structure Analyses

Genetic diversity, heterozygosities (expected and observed) and F-statistics were calculated using Genalex 6.5 (Peakall and Smouse, 2012). For genetic structure, a principal component analysis (PCA) using the package ade4 (Thioulouse et al., 1997) implemented in R software (R Development Core Team, 2008) was first performed. Then, population structure was investigated using STRUCTURE software 2.3.3 (Pritchard et al., 2000). Analysis was performed with the admixture model (Falush et al., 2003) with K ancestral populations ranging from 1 to 6. We used 500,000 iterations and a burn-in period of 100,000, 10 runs for each K-value were performed. The values for the number of clusters (K) were assessed according to Evanno et al. (2005) by the ($D\Delta K$) criterion and the log-likelihood ($\ln P(D|K)$) plot. Individuals were assigned to a cluster if their ancestry was higher than 70%, $q \geq 0.7$.

For spatial analysis of genetic variability, a total of 367 geo-referenced accessions including 281 early- and 86 late-flowering landraces were used. Spatial principal component analysis (sPCA) was performed using the *adegenet* package (Jombart, 2008) with R software. The spatial genetic structure was assessed using spatial autocorrelation analyses of kinship coefficients between individuals (Loiselle et al., 1995) following the standard procedure (Vekemans and Hardy, 2004) implemented in SPAGeDi version 1.2 (Hardy and Vekemans, 2002). Mean multilocus kinship coefficient values, F_{ij} , i.e., genetic similarity between individuals i and j relative to the mean genetic similarity between random individuals in the sample, were regressed on both the linear (d_{ij}) and the logarithmic ($\ln(d_{ij})$) spatial distance between individuals. This distance was calculated as the Euclidian distances using spatial coordinates. The regression slopes b_d and b_{Ld} were jointly assessed. Standard errors for the kinship coefficients were estimated using a jackknife procedure over all loci. We tested the significance of the kinship coefficients and the regression slopes b_d and b_{Ld} estimates by comparing the observed values to those obtained after 10,000 random permutations.

PgPHYC and PgMADS11 Genotyping

Polymorphisms in both genes were associated with flowering time variation (Saïdou et al., 2009; Mariac et al., 2011; Vigouroux et al., 2011). The panel of accessions was genotyped with *PgPHYC* (Acc numbers FN376885–FN377564) (Vigouroux et al., 2011)

and *PgMADS11* (Acc numbers FN552468–FN552522) (Mariac et al., 2011) to test the allelic differences in genotype frequencies between early- and late-flowering landraces.

For *PgPHYC*, a polymorphism at the 5' of the gene was assessed. A C/G SNP at that position is cleaved by PvuII restriction enzyme and therefore accessions scored as C/C, G/G and C/G according to their digestion pattern (Saïdou et al., 2009).

For *PgMADS11*, an indel polymorphism of 24 bp was assessed as previously reported (Mariac et al., 2011).

Logistic regressions between genotypes, genetic cluster, latitude and longitude for each gene were further performed.

RESULTS

Genetic Diversity of Senegalese Germplasm

The germplasm collected through this study is the most comprehensive sampling to date of landraces from Senegal (Figure 1). Genotyping data revealed a total of 101 alleles with an average of 8.4 alleles per locus (Supplementary Table S2 and Figures S1–S3). The final data set contained only 1.8% of missing data. High levels of genetic diversity characterize both groups (Table 1). Early flowering landraces presented the highest level of genetic diversity as measured by observed $H_{Obs} = 0.481$ or expected heterozygosity, $H_{Exp} = 0.567$. In contrast, improved varieties showed the lowest, with $H_{Obs} = 0.391$. For early flowering landraces, only one locus (PSMP2249) was found not to be at Hardy-Weinberg equilibrium (HWE). Two loci were not at HWE (PSMP2249 and PSMP2246) in late-flowering landraces. Groups showed low levels of inbreeding with F_{IS} values of 0.160, 0.128, and 0.258 for early and late landraces and improved varieties, respectively. Furthermore, early-, late-flowering landraces and improved varieties present 22, 8 and 1 private alleles, respectively. Low F_{ST} differentiation was found between improved varieties and early flowering landraces (0.004), between early- and late-flowering landraces (0.052) and between late-flowering landraces and improved varieties (0.063).

Population Genetic Structure

Bayesian clustering analyses showed a clear structure between early- and late-flowering landraces (Figure 2). The value of the Evanno criterion (ΔK) was the highest for $K = 2$ (Supplementary Figure S1), supporting the evidence of two major clusters. A total of 89% of the early flowering landraces were assigned to a single cluster, and 90% of the late-flowering landraces were assigned to the other cluster. Thirty-four accessions of early- and nine of late-flowering landraces were misassigned ($q < 0.7$), but showed intermediate ancestries. For the 10 villages where both early- and late-flowering landraces were sampled, all accessions of similar phenotype were assigned to their respective cluster.

Similar results were found with PCA showing a clear distinction between early- and late-flowering landraces (Figure 3). The two first principal components explained, respectively, 5.2 and 2.8% of the inertia. PSMP2247 and PSMP2202 showed the highest contribution to the PC1. Two alleles, PSMP2247-199 and PSMP2202-146, showed

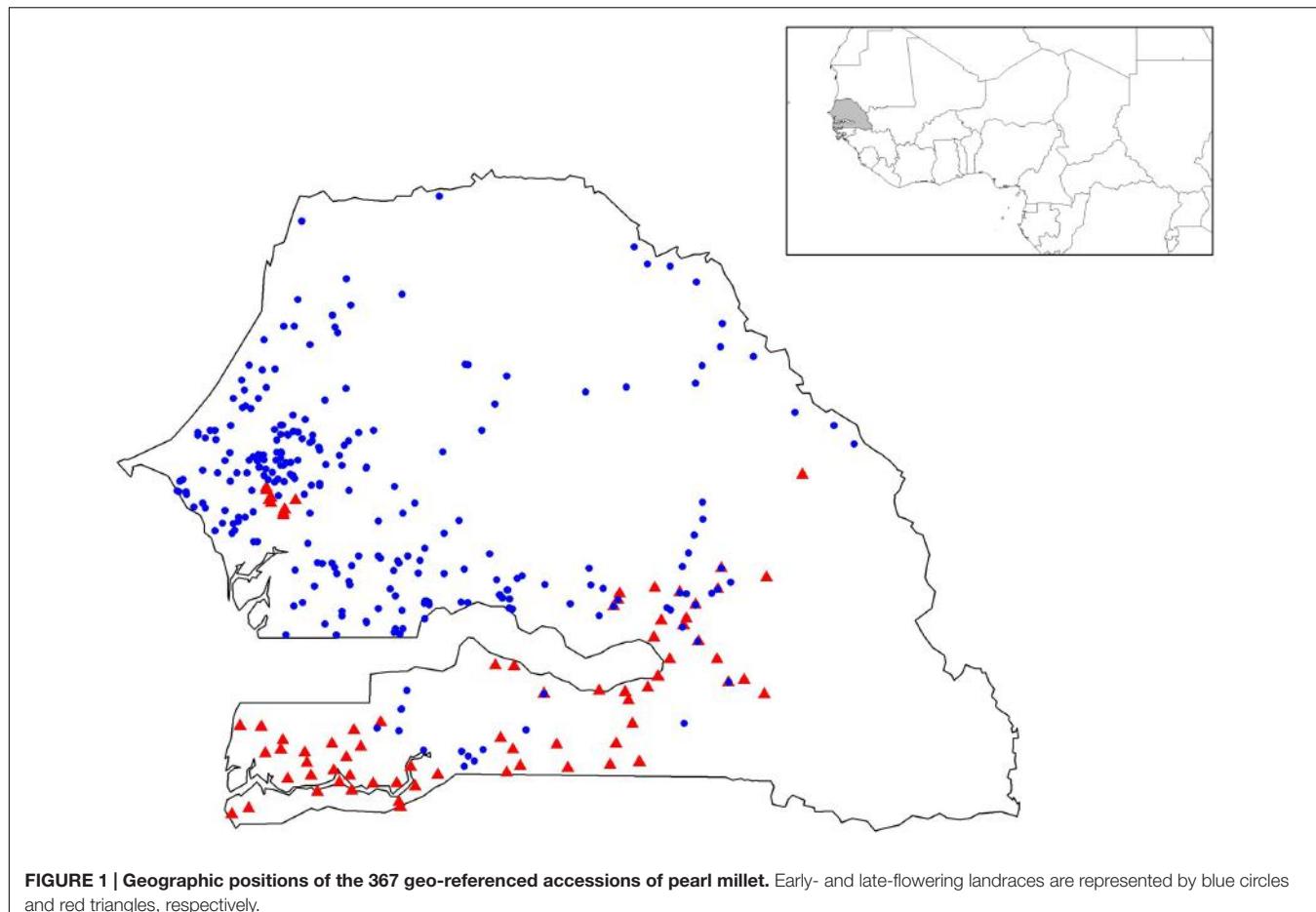


TABLE 1 | Genetic diversity statistics for improved varieties, early- and late-flowering landraces.

	Nb	Na	H_{Obs}	H_{Exp}	F_{IS}
Improved varieties	12	4.4	0.350	0.513	0.258
Early flowering landraces	306	7.7	0.446	0.542	0.160
Late-flowering landraces	86	5.8	0.434	0.494	0.128

Nb, number of accessions; Na, mean number of alleles, H_{Obs} , observed heterozygosity; H_{Exp} , expected heterozygosity; F_{IS} , inbreeding coefficient.

high frequencies in late-flowering landraces (0.92 and 0.83, respectively). However, removing these two loci did not affect PCA results. Finally, any genetic differentiation was found between improved and early accessions.

Spatial Analysis of Genetic Variability

Spatial principal component analysis revealed a more cryptic genetic structure (**Figure 4**). Global structures, i.e., large spatial scale, were significant (*p-value* = 0.0001) with the first principal component, showing a high autocorrelation (Morran's I = 0.50). In contrast, local structures were not significant (*p-value* = 0.77). The first axis of the sPCA identified two clusters. Considering spatial information, early flowering landraces from Southern Senegal showed a more admixed pattern than observed with

STRUCTURE, while no differences were observed considering late-flowering landraces. Bayesian and multivariate approaches confirm genetic assignments for late-flowering landraces from Central Senegal.

The pattern of isolation by distance (IBD) for early- and late-flowering landraces was investigated. Low IBD slopes were obtained for early flowering ($b_E = -2.41E-05$) and late-flowering ($b_L = -9.89E-05$) landraces (**Figure 5**). Similar results were found for logarithmic distances. This suggests no significant pattern of isolation-by-distance.

PgPHYC and PgMADS11 Allele Diversity

Landraces were genotyped for both PgPHYC and PgMADS11 alleles to assess genetic diversity in relation to flowering time (**Table 2** and Supplementary Figures S4, S5). Only early landraces carried early flowering allele (G) at the PgPHYC locus leading to a significant difference in genotypes frequencies (*F*-test, $p = 0.006$). For PgMADS11, a significant difference in genotypes frequencies (*F*-test, $p = 0.004$) was observed with early flowering landraces having higher frequency of the allele (363 bp fragment). A significant correlation between allele G from PgPHYC with latitude (*p-value* = 0.0348) was observed. However, significance disappeared when taking into consideration genetic clustering ($q \leq 0.7$). For

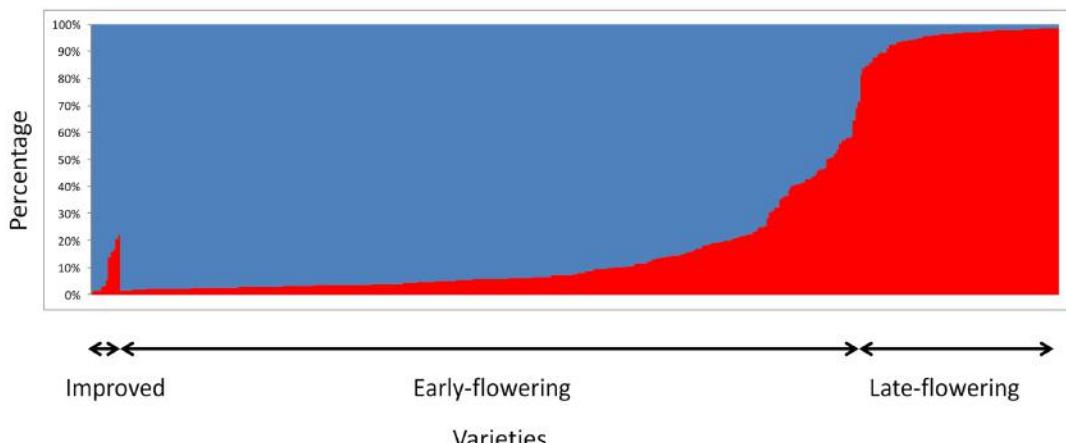


FIGURE 2 | STRUCTURE results for $K = 2$ based on 404 accessions analyzed with 12 SSRs. Each bar represents an individual. It shows the proportion of genome belonging to each of the two genetic groups (blue for early flowering accessions and red for late-flowering accessions) identified with STRUCTURE.

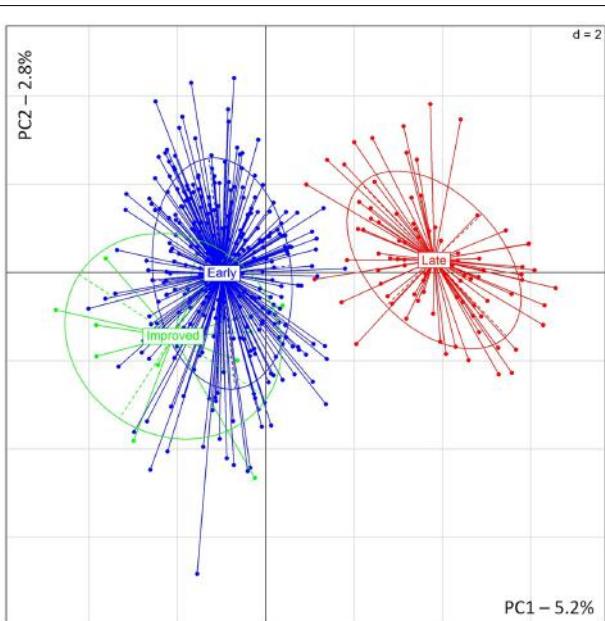


FIGURE 3 | PCA results obtained for 12 improved, 306 early- and 86 late-flowering landraces accessions.

PgMADS11, data reveal a significant correlation with longitude (p -value < 0.001) even when considering genetic clustering in the mode.

DISCUSSION

Large and Untapped Diversity in Senegalese Germplasm

In this study, a relatively high genetic diversity (H_{Exp} : 0.516) of pearl millet germplasm of Senegal is reported. Previous

studies have reported a higher (H_{Exp} : 0.69) (Stich et al., 2010) and a lower ($H_{Exp} = 0.49$) genetic diversity in Niger pearl millet (Mariac et al., 2006a). These differences are due to the number of SSRs used. In the meanwhile, a similar number of alleles per locus (6 alleles per locus for Senegal vs. 6.2 alleles per locus for Niger) was observed. Comparing heterozygosity levels, observation showed a higher coefficient ($F_{IS} = 0.30$) in Niger germplasm than in Senegal germplasm ($F_{IS} = 0.18$). However, these results are consistent with data from others studies carried out on accessions across SSA regions and India (Oumar et al., 2008; Dussert et al., 2015; Hu et al., 2015). Pearl millet shows a high genetic diversity that can be explained by its strong outcrossing rate (75%) and the still on-going gene flow with its wild relative (Mariac et al., 2006a; Lewis, 2010). This high genetic diversity is in line with its high phenotyping diversity observed in Senegal (Sy et al., 2015) as in Western Africa (Pucher et al., 2015). Together, these findings highlight the untapped potential of Senegalese pearl millet germplasm for breeding.

Genetic proximity between early flowering varieties and improved varieties highlight a history of breeding programs and agricultural practices. In Senegal, few breeding programs have been undertaken on pearl millet but all focused on reducing the flowering cycle and were “population” varieties whose parental seeds were collected from local landraces. In addition, farmers still grow improved varieties jointly with landraces in their field, increasing gene flow and thus genetic proximity.

Genetic Structure of Early- and Late-Flowering Landraces

Bayesian results clearly help identifying genetic structure associated with early- and late-flowering landraces. This genetic structure was partly explained by the geographic distribution of landraces as shown through the sPCA analysis, making it difficult to entirely disentangle spatial and genetic structure. More

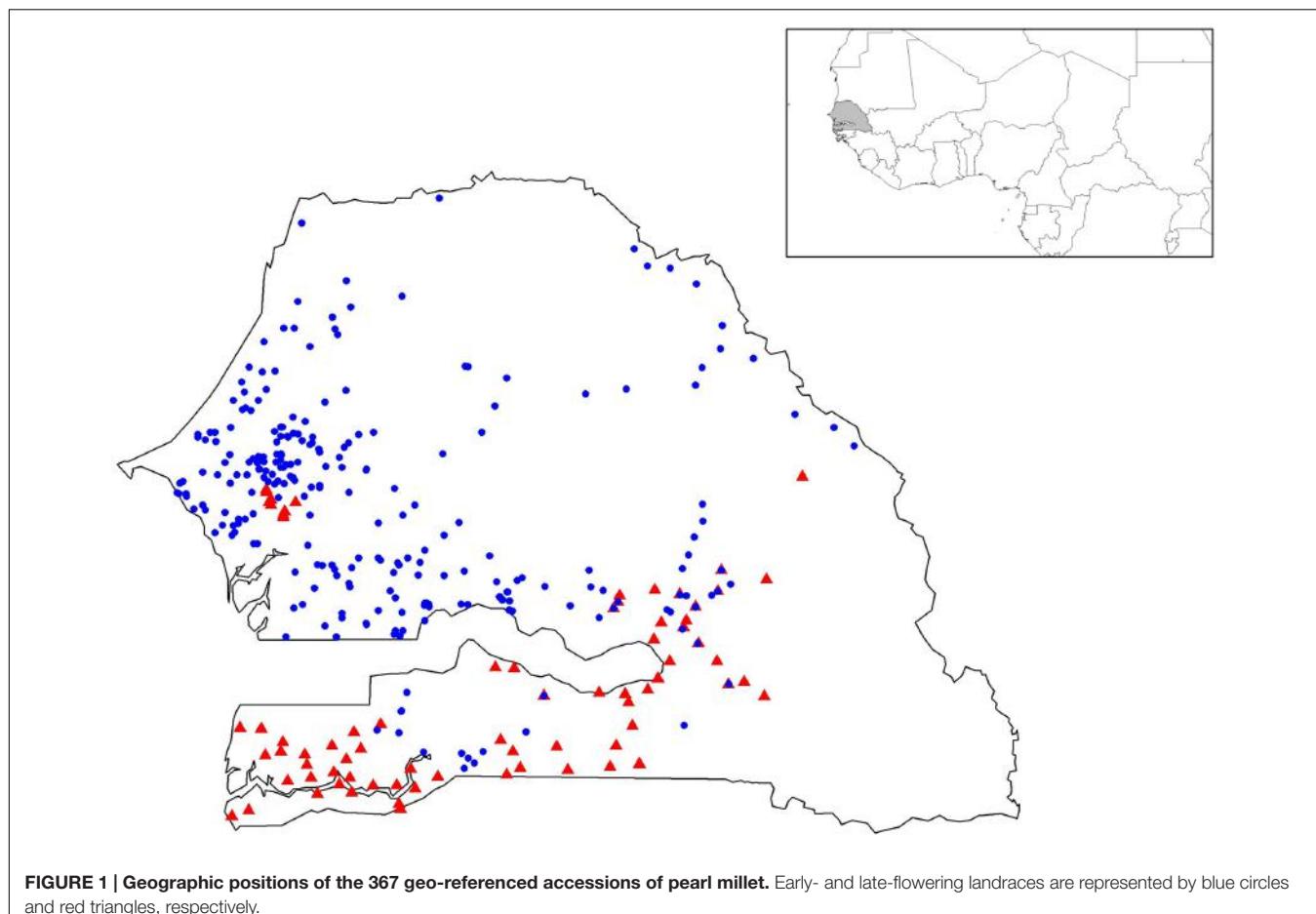


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TABLE 2 | Genotypes counts for improved varieties, early- and late-flowering landraces with q -values ≤ 0.7 .

	<i>PgPHYC</i> alleles			<i>PgMADS11</i> alleles		
	C/C	C/G	G/G	363/363	363/387	387/387
Improved varieties	8	3	1	1	2	9
Early flowering landraces	239	21	0	18	65	185
Late-flowering landraces	79	0	0	2	16	62

contrast in admixture patterns might have been obtained with a higher number of SSR markers. However, this was compensated by our sampling effort and we were able to reveal a clear structure in Senegal landraces where other studies failed with a higher number of markers (Hu et al., 2015).

At a local scale, more contrasted admixture patterns were observed. In Central Senegal, clear differentiation between early- and late-flowering landraces was found. In contrast, more admixed patterns were observed in the Southern Senegal. The results might be related to the agricultural practices of the farmers, such as spatial cropping and seed circulation (Mariac et al., 2006b; Lakis et al., 2012; Kouakou et al., 2013).

Our data indicated two distinct genetic clusters in Senegalese pearl millet germplasm. In addition, comparing early- and late-flowering landraces, we found a slight genetic differentiation between the two groups of 0.052, which is of the same magnitude (0.053) found in Niger (Lakis et al., 2012). A single domestication event led to early- and late-flowering landraces, partly explaining the limited genetic differentiation observed at a regional scale (Dussert et al., 2015).

Flowering Traits Diversity

Photoperiod-sensitivity of pearl millet landraces and thus variation of flowering cycle constitute a key response for adaptation to future climate conditions (Sultan et al., 2013). Indeed, pearl millet landraces from Niger show reduced flowering cycle associated with drought episodes from 1976 to 2003 (Vigouroux et al., 2011). This reduction was correlated with changes in allele frequencies for *PgPHYC*. Correlation between allele frequencies and rainfall were also found with *PgMADS11* (Saïdou et al., 2009; Mariac et al., 2011). Our data showed significant genetic differences for both genes with early flowering landraces enriched in precocity alleles.

Flowering time has been correlated with latitude, early flowering landraces being grown in northern latitude where environmental conditions are more arid (Haussmann et al., 2006; Pucher et al., 2015). In our study, the latitude effect was confounded with spatial genetic structure for *PgPHYC*. On the other hand, a correlation with longitude was found for *PgMADS11*. Further investigation would be needed to fully address this correlation. In any case, the use of flowering genes *PgPHYC* and *PgMADS11* in marker assisted-selection programs presents some interest.

Challenges for Adaptation of Pearl Millet and Breeding Strategies

Sub-Saharan Africa recorded long dry spells in the 1970s and 1980s that led to breeding for short cycles improved varieties. Indeed, pearl millet breeding programs were predominantly built on restricted genetic resources of early flowering landraces. Strong differentiation between early- and late-flowering landraces from Senegal suggests the existence of an important gene pool that has not been exploited yet. The high genetic diversity could explain the wider range of pearl millet adaptation to dry areas and this potential may further contribute to breeding programs in response to the specific needs or target areas (Kouressy et al., 2004). For instance, a key strategy to cope against climate changes within SSA agrosystems is to tap into diversity of flowering time (Haussmann et al., 2012; Sultan et al., 2013) and resilience (Prieto et al., 2015).

Analyses of allelic variation of *PgPHYC* and *PgMADS11* indicate fine-scale genetic difference (SNP and indel, respectively) among individuals and/or genotypes. Knowing that responses to photoperiod and rainfall were genetically associated with both genes, implication could be their use to detect/track climate adaptive changes to environment variations. For example, earliness of flowering and latitude correlation observed in early landraces support assumption that a direct effect of selection for that trait which is associated with climate variations such as photoperiod and rainfall. Both traits are key targets in selection for millet genotypes to be cultivated in rainfed areas.

CONCLUSION

The genetic diversity and population structure of Senegalese pearl millet landraces were assessed using a large panel of accessions and a limited number of SSRs markers. Results highlight a high genetic diversity and an untapped potential of the germplasm. However, two clusters were clearly distinguished as revealed by differentiation between early- and late-flowering landraces. Further, genetic difference and allelic co-variation in flowering genes *PgPHYC* and *PgMADS11* were found among individuals. These findings give new insights into Senegalese pearl millet germplasm and are promising for developing new cultivars and heterotic groups that can be used to breed synthetic and hybrid varieties with higher degrees of heterozygosity in order to intensify yield production under harsh semiarid environments.

AUTHOR CONTRIBUTIONS

NK, AF, DD, MG, YV, and AB designed the study. OD, AF, MG, MP, HT, BD, and OS collected samples. OD, MC, MP, and LZ performed DNA extraction, PCR and sequencing. AB, CB-S, and OD performed the genetic analyses. OD, AB, CB-S, DD, and NK drafted the manuscript. All authors contributed to the final version.

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Using Ancient Traits to Convert Soil Health into Crop Yield: Impact of Selection on Maize Root and Rhizosphere Function

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The effect of domestication and modern breeding on aboveground traits in maize (*Zea mays*) has been well-characterized, but the impact on root systems and the rhizosphere remain unclear. The transition from wild ecosystems to modern agriculture has focused on selecting traits that yielded the largest aboveground production with increasing levels of crop management and nutrient inputs. Root morphology, anatomy, and ecophysiological processes may have been affected by the substantial environmental and genetic shifts associated with this transition. As a result, root and rhizosphere traits that allow more efficient foraging and uptake in lower synthetic input environments might have been lost. The development of modern maize has led to a shift in microbiome community composition, but questions remain as to the dynamics and drivers of this change during maize evolution and its implications for resource acquisition and agroecosystem functioning under different management practices. Better understanding of how domestication and breeding affected root and rhizosphere microbial traits could inform breeding strategies, facilitate the sourcing of favorable alleles, and open new frontiers to improve resource use efficiency through greater integration of root development and ecophysiology with agroecosystem functioning.

Keywords: crop breeding, domestication, maize (*Zea mays*), microbiome, resource acquisition, rhizosphere, roots, soil health

INTRODUCTION

Since its origin in the Balsas river valley of present-day Mexico 10,000 years ago, maize has undergone dramatic changes in shoot development and physiology as early agriculturists and modern breeders selected for greater yield response to increasingly managed agroecosystems (Harlan et al., 1973). Teosinte (*Zea mays* ssp. *parviglumis*), the ancestor of modern maize, originates from a mountainous environment with seasonal nutrient fluxes and high interspecific competition with diverse deciduous trees, grasses, and annual dicots (Gaudin et al., 2011b). After domestication around human settlements in fertile alluvial river banks, early maize varieties spread to other parts of Americas, where landraces were cultivated in traditional *milpa* agricultural systems (maize-bean-squash intercropping; Zizumbo-Villarreal and Colunga-GarciaMarín, 2010). Subsequent innovations following the industrial revolution such as mechanized tillage and the replacement of crop residues and organic inputs with synthetic fertilizers altered the agricultural

landscape substantially, creating the homogeneous, nutrient-rich, high-intraspecific-competition environment seen in present-day monocultures and short rotations (**Figure 1**).

Here we argue that directed selection pressure for yield and aboveground traits during maize evolution coupled with shifts toward high-input, high-density selection environments may have inadvertently altered root system development and ecophysiological functioning. Thus, both host-genotype-driven changes in the ability of maize to recruit and respond to microbial interactions and environment-driven selection pressure on integrated plant and microbial functions may have altered coevolution of the microbiome (Kiers and Denison, 2014; Vandenkoornhuyse et al., 2015; **Figure 1**). Root and rhizosphere interactions have traditionally been neglected in discussions of maize domestication and breeding, despite their importance for plant fitness and productivity at lower input levels. The transition from wild ecosystems to modern maize monocultures may also have altered the ability of roots to dynamically respond to changes in resource availability, cope with stress and rely on microbial interactions in the rhizosphere to cycle and acquire soil resources (Wissuwa et al., 2009; Zancarini et al., 2012), which are essential functions in biologically-based and low input systems.

While past intensification of agriculture dramatically increased crop yields, future increases in productivity required for a growing population must come at a lower environmental cost. For instance, low nutrient use efficiencies

and subsequent nutrient losses, especially of nitrogen (N) and phosphorus (P), contribute to eutrophication, climate forcing, and loss of biodiversity, with serious impacts on human and ecosystem health (Robertson and Vitousek, 2009). Further, climate change will cause more variability in precipitation and temperature (Kirtman et al., 2013) with consequences for crop growth, nutrient cycling, and yields (Lobell et al., 2014). Shifting to more biologically-based or lower input cropping systems shows promise for sustaining or increasing yields while reducing environmental costs and also increasing resilience to extreme events (Bommarco et al., 2013). But if crops are not well-adapted to these new agro-environments, then yield potential may not be fully realized.

Investigating the extent and significance of inadvertent changes belowground during the course of artificial selection is highly relevant to support crop breeders in developing maize varieties able to take full advantage of microbial interactions and high rates of nutrient cycling created by soil-health building management practices. While development and plasticity of root system architecture and physiological traits enable foraging and uptake of soil resources, rhizosphere ecology facilitates plant resource acquisition through synergisms with microbes and exudate production (**Figure 2**). As such, roots and rhizosphere interactions could prove key to developing sustainable maize production systems (Bishopp and Lynch, 2015).

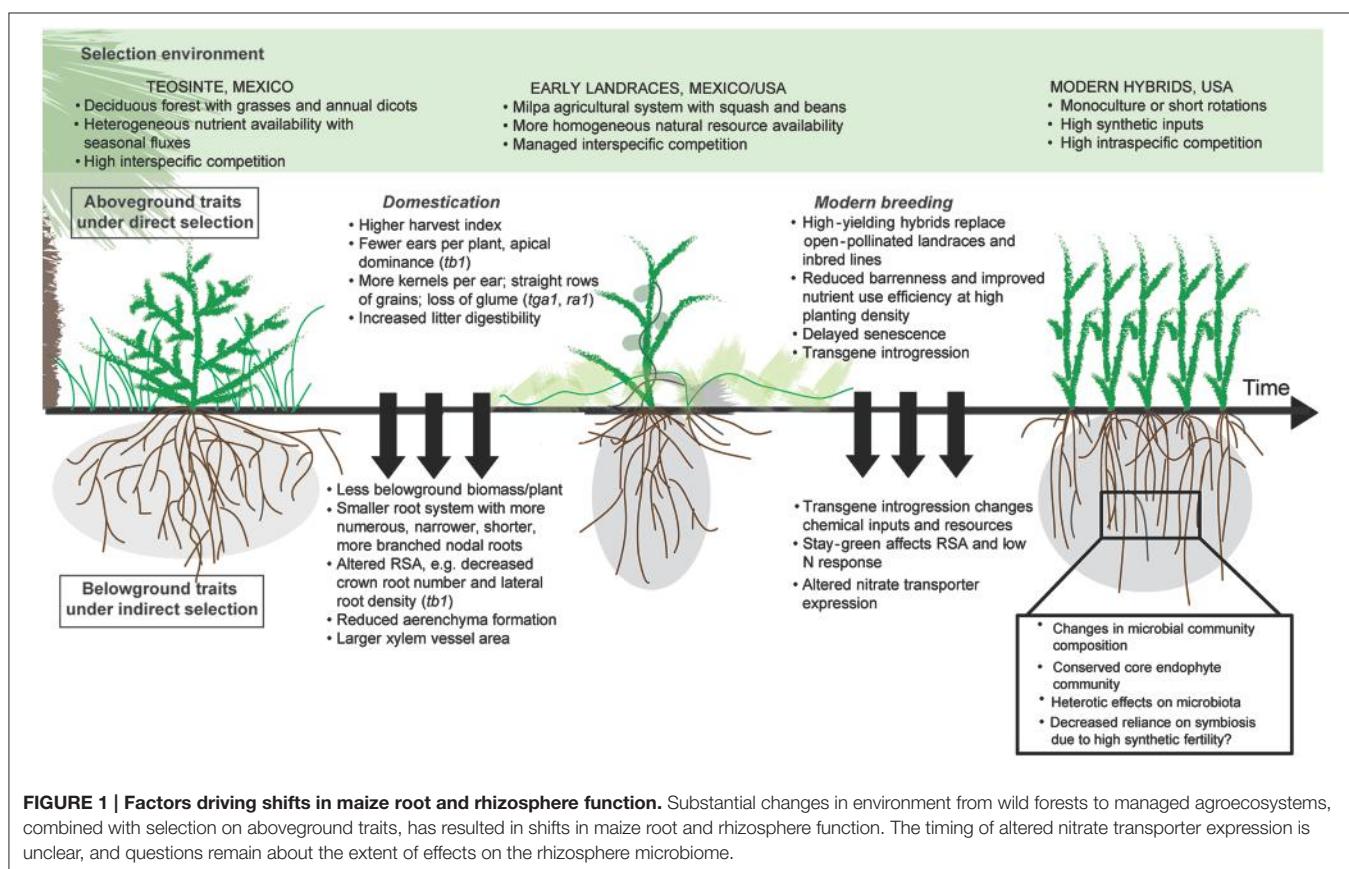


FIGURE 1 | Factors driving shifts in maize root and rhizosphere function. Substantial changes in environment from wild forests to managed agroecosystems, combined with selection on aboveground traits, has resulted in shifts in maize root and rhizosphere function. The timing of altered nitrate transporter expression is unclear, and questions remain about the extent of effects on the rhizosphere microbiome.

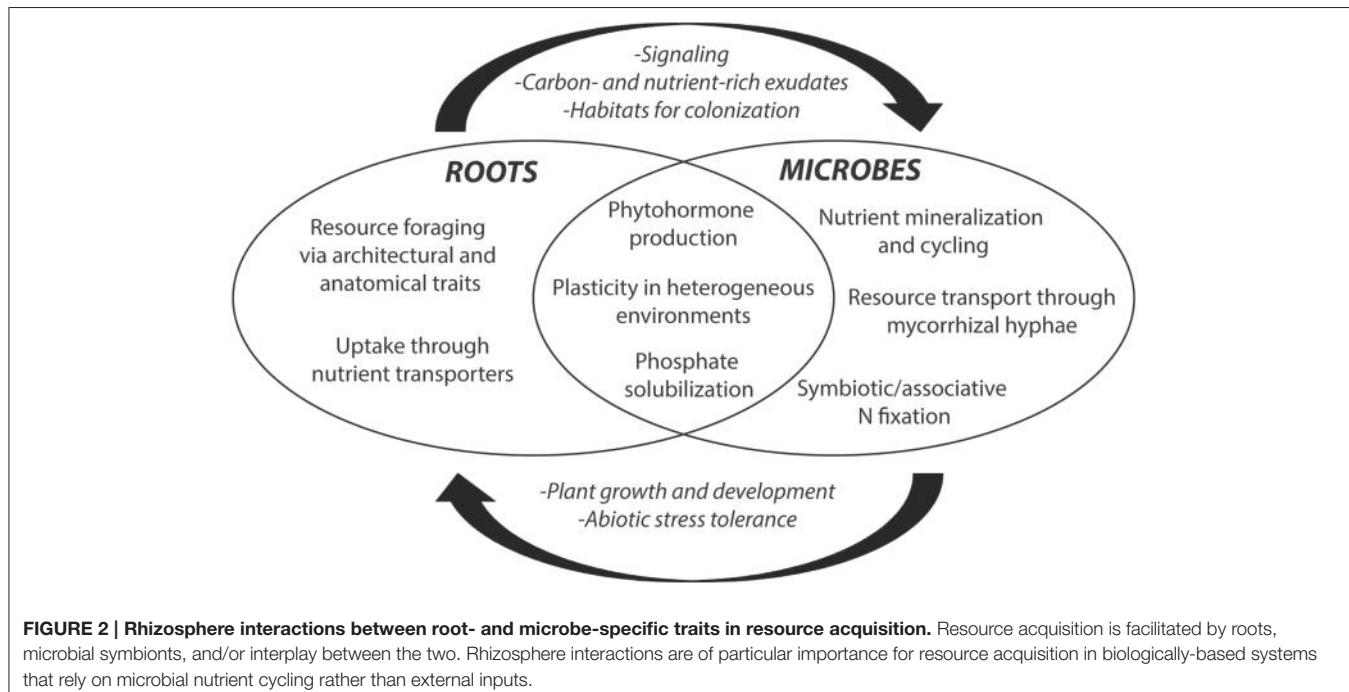


FIGURE 2 | Rhizosphere interactions between root- and microbe-specific traits in resource acquisition. Resource acquisition is facilitated by roots, microbial symbionts, and/or interplay between the two. Rhizosphere interactions are of particular importance for resource acquisition in biologically-based systems that rely on microbial nutrient cycling rather than external inputs.

This review examines scientific evidences and potential drivers of changes in maize root morphology, anatomy, and physiology from teosinte through early landraces to modern hybrids and considers their functional significance for resource acquisition. We discuss current knowledge of human selection-driven shifts in maize rhizosphere ecology in light of the underlying plant-driven (G), environment-driven (E), and genotype-by-environment ($G \times E$) mechanisms and highlight research gaps to be addressed in the future.

HAVE SHOOT AND ROOT TRAITS CO-EVOLVED?

Morphological Traits

The suite of traits selected during the domestication of crop wild relatives to increase yield or facilitate agronomic cultivation are collectively described as “domestication syndrome” (Hammer, 1984). Domestication traits commonly include enhanced fruit production, altered vegetative shoot morphology, and changes in secondary metabolites (e.g., decreased bitterness; Meyer et al., 2012). In cereals, domestication has led to increased seed size and number, increased apical dominance, changes in photoperiodicity, loss of seed dormancy, changes in grain composition, and loss of seed shattering (Harlan et al., 1973; Gross and Olsen, 2010; Abbo et al., 2014).

Aboveground morphological differences between teosinte and modern maize are striking. Teosinte has clusters of small ears in the axils of multiple leaves per stem, whereas modern cultivars show increased biomass and apical dominance with one ear per node and fewer than two ears per stem. Maize ears contain hundreds of large, naked kernels as compared to the few, small,

glume-encased kernels of teosinte ears (Harlan et al., 1973). These changes are mostly attributed to the domestication genes *teosinte branched1* (*tb1*) and *barren stalk1* (*ba1*) (Doebley et al., 1997; Gallavotti et al., 2004; Hufford et al., 2007, 2012), controlling vegetative meristem development, *teosinte glume architecture1* (*tga1*), accounting for the naked kernels (Dorweiler et al., 1993; Wang et al., 2005), and *ramosa1* (*ra1*), controlling kernel row regularity (Dempewolf, 2010; Sigmon and Vollbrecht, 2010).

While root traits were likely not under intentional selection during domestication, plants usually respond to changes in shoot size by compensatory changes in root growth and architecture (Gaudin et al., 2014), perhaps to maintain balance between resource sinks and source tissues. As such, root morphology may have been altered indirectly by selection for higher harvest index and related traits such as apical dominance. In comparison to early landraces, teosinte (*ssp parviflora*) has fewer seminal roots, possibly related to smaller seed size, but a greater number of narrower, shorter, more branched nodal roots, which may be beneficial for early P acquisition (Burton et al., 2013). Domestication studies have also shown common genetic control of above- and belowground morphology as decrease in *tb1* function in modern maize restores the teosinte phenotype both above and belowground, resulting in a larger root system with numerous and highly branched crown roots (Gaudin et al., 2014). Because of their impact on sink strength and nutrient demand, other known domestication genes may also show correlated belowground effects.

Following domestication, human selection for desirable traits has continued to affect maize shoot and root morphology over centuries of landrace cultivation and decades of inbred and hybrid breeding. Desirable improvement traits have included tolerance to higher planting densities (Duvick, 2005) as well

as abiotic stresses such as drought and heat (Tollenaar and Lee, 2002; Campos et al., 2006), the introduction of delayed senescence (stay-green) (Lee and Tollenaar, 2007), and resistance to biotic stresses such as insect herbivory and weed competition via introgression of transgenes (**Box 1**). Although R:S ratio remained conserved across 9000 years of breeding and selection (Gaudin et al., 2011a), modern stay-green hybrids have greater total root length and deeper roots than their non-stay-green counterparts (Ning et al., 2014). As a result of breeding in inorganic nutrient-saturated and homogeneous environments with high intraspecific competition, the root systems of more recent maize cultivars have shallower root angles, fewer nodal roots, and greater distance from nodal roots to lateral branching, potentially enhancing deep resource foraging and minimizing root system overlap (York et al., 2015). The tradeoffs of increased investment in deep roots should be investigated, for example to determine whether corresponding decreases in the lateral roots that are preferentially colonized by arbuscular mycorrhizal (AM) fungi (Gutjahr and Paszkowski, 2013) have affected benefits of AM colonization. Collectively, these reports suggest that domestication and breeding may have decreased topsoil foraging ability and mycorrhizal colonization sites of single plants while increasing exploration of deeper soil layers by plant populations.

Anatomical Developments

Comparisons between teosinte, landraces and modern varieties have revealed major differences in root anatomical traits involved in resource acquisition and transport. Teosinte has higher rates of cortical aerenchyma formation, which can reduce the metabolic cost of roots under stressed conditions, and greater phenotypic variation for this trait than landraces (Burton et al., 2013). Modern varieties have lower aerenchyma plasticity as compared to teosinte, which forms aerenchyma constitutively under non-stressed conditions as well as in response to stress (Mano et al., 2007). This suggests that anatomical traits mediating adaptation

to drought and nutrient-limited conditions (Lynch, 2007) may have been selected against in the irrigated, fertilized environment of modern maize agroecosystems. However, larger total xylem vessel area (XVA) in landraces and modern cultivars as compared to teosinte (Burton et al., 2013) may provide quicker transport of water and nutrients in higher-resource environments to meet the demands of a larger shoot system, perhaps at the cost of increased cavitation under drought stress (Tyree et al., 1994). Since XVA alone is not necessarily related to hydraulic conductivity (Smith et al., 2013), further analysis is needed to determine if the increase in total XVA is formed by larger-diameter vessels, which would increase flow rates (Tyree and Ewers, 1991). Newer modern hybrids have smaller but more numerous xylem vessels as compared to older modern hybrids (York et al., 2015), but vessel size in teosinte and landraces remains to be measured.

Root Physiological Attributes

Along with shifts in architectural and anatomical traits, root physiological activity may have been altered to accompany increases in crop N and water demand during the growing season (Antonieta et al., 2015) to support more vigorous vegetative growth, increased sink capacity during grain filling (Lee and Tollenaar, 2007), and delayed senescence. For instance, two stay-green hybrids had higher N uptake during grain filling (He et al., 2003) and a larger kernel response to N availability than their non-stay-green counterparts (Antonieta et al., 2015). However, scarce information is available on how kinetics and regulation of water and nutrient uptake changed during maize evolution. Productivity gains seen in newer hybrids result in part from increased N (York et al., 2015) acquisition and water (Reyes et al., 2015) use efficiencies compared to earlier varieties; however, evidence conflicts as to whether water and total N uptake has increased over time (Hammer et al., 2009; Nagore et al., 2014; Reyes et al., 2015). Resistance to N stress, as measured by no effect of low N availability on root or shoot biomass, in two teosintes was attributed in part to differential

BOX 1 | TRANSGENES AND THE MAIZE RHIZOSPHERE

Introgression of genetic material from other species has contributed substantially to modern maize productivity and as such deserves attention here despite being a fundamentally different form of genetic modification than traditional breeding. Modern commercial maize hybrids possess up to eight stacked transgenes, most frequently conferring tolerance to the herbicides glyphosate or glufosinate or resistance to insect pests such as corn rootworm, coleopterans, and corn root borer (Dunwell, 2014). Although a detailed impact assessment of these transgenes on the rhizosphere is outside the scope of this review, the topic deserves attention in a discussion of modern maize.

Transgenes are predicted to affect the rhizosphere microbiome primarily through chemical inputs and altered resource provisioning. Introgression of herbicide tolerance, found in almost all modern hybrids (Dunwell, 2014), is accompanied by inputs of the corresponding chemical. Glyphosate alters rhizobacterial community composition and increases the prevalence of pathogenic *Fusarium* (Kremer and Means, 2009), but appears to have less far-reaching impacts than an alternative pre-emergence herbicide containing acetochlor and terbutylazine (Barriuso et al., 2010). However, another study found no effect of glyphosate-resistant maize or glyphosate application on denitrifying bacteria or the fungal community in the rhizosphere (Hart et al., 2009). *Bt* maize affects rhizosphere resource availability. *Bt* maize differs in lignin content and root exudate composition from its non-transgenic counterpart (Saxena et al., 1999; Saxena and Stotzky, 2000, 2001). However, greenhouse and field studies have shown no difference in population size, metabolic profile, or genetic diversity of rhizobacteria (Brusetti et al., 2004; Fang et al., 2005; Icoz et al., 2008; Prischl et al., 2012; Bumunang and Babalola, 2014) although genetic analysis has found some differences (Brusetti et al., 2004). Ecological functions such as nutrient cycling may nonetheless be affected, given that *Bt* introgression influences abundances of archaea and bacteria involved in N metabolism (Cotta et al., 2014). Mycorrhizal community composition (Tan et al., 2011) and spore density (Cheeke et al., 2014), but not colonization potential (Tan et al., 2011), appear to be affected by host *Bt* status.

Transgenes have improved maize productivity and recent studies have introgressed genes improving abiotic stress tolerance, nutrient use efficiency, and nutritional quality of maize (Dunwell, 2014). However, given the potential for transgenes to affect the rhizosphere microbiome and its vital role in ecological function through altered inputs and resource quality, new transgenes should be carefully evaluated for rhizosphere impacts in addition to existing risk assessments.

regulation of genes involved in N assimilation and metabolism as compared to five modern maize lines, although regulation of three N metabolism genes was consistent across all seven lines (Han et al., 2015). Teosinte possesses orthologs of four of the seven modern maize nitrate transporter genes, but upregulates expression of *ZmNrt2.3*, involved in the high-affinity system, twice as much as modern maize under low N conditions (Gaudin et al., 2011b). These physiological attributes may have changed the kinetics of N uptake, particularly under conditions of low soil NO_3^- levels that are common in biologically-based systems, making modern maize better-suited to high inorganic N environments with a corresponding decrease in adaptability to low-input agroecosystems (Ruzicka et al., 2012). Possible changes in ammonium transporters and assimilation should be further studied, as ammonium may be a more significant source of N in lower-input or biologically based soils (Burger and Jackson, 2003).

Root Plasticity

Root plasticity allows plants to adjust root system architecture in response to changing resource availability. Selection pressure on root phenotypic plasticity is particularly relevant to breeding for rhizosphere traits involved in nutrient use efficiency. Schlichting (1989) proposed that the evolution of plasticity would be favored by heterogeneous environments, but it does not appear that the converse has occurred. Homogeneous breeding conditions have not triggered a loss of plasticity and modern maize remains capable of a plastic response to heterogeneous nitrogen supply (Yu et al., 2014), although the particular mechanisms of this plastic response have changed. Teosinte responds to low-nutrient environments and shade by decreasing shoot tillering to reduce nutrient requirements, whereas modern maize appears to have lost this compensatory mechanism (Gaudin et al., 2011b). However, while teosinte reduces crown root number (CRN) through tillering plasticity under low N stress, modern maize achieves CRN reductions by other means. Other root plasticity strategies likewise differ between teosinte and maize despite a conserved overall response to low-N stress (Gaudin et al., 2011b). Root phenotypic plasticity has important ecological consequences for plant-plant competitive interactions in heterogeneous nutrient environments (Miner et al., 2005); the impact of plasticity on plant-microbe interactions under variable nutrient conditions represents an intriguing future area of study.

Potential tradeoffs between root traits (i.e., acquisition of resources with dissimilar distributions in the soil, possible vulnerability to stresses, costs of investment) (Lynch, 2007) must be considered to determine how the suite of changes observed in modern maize affect performance under different conditions.

HOW DID MAIZE EVOLUTION AFFECT RHIZOSPHERE ECOLOGY?

Rhizosphere functioning is shaped by the combined influence of host genotype (G), soil environment as affected by agroecosystem management and inherent soil characteristics (E), and their

interaction ($G \times E$). Shifts in these determinants during the course of maize evolution resulted in profoundly different selection environments that may have altered ecological functions of the rhizosphere, with consequences for foraging and acquisition of soil resources (Figure 2).

Impact of Directed Selection

Maize genotypes differ in recruitment ability, resource provision, and responsiveness to beneficial rhizosphere microorganisms (Kaeplinger et al., 2000; Picard et al., 2008; Willmann et al., 2013). This variation in microbe-related traits has a significant heritable component (Peiffer et al., 2013) that could have been acted upon indirectly during selection for aboveground traits of agronomic interest.

Studies comparing teosinte and maize in a single environment suggest a core microbiome has been maintained through domestication, although some rhizobacterial associations may have been lost (Szoboszlay et al., 2015). Strains of diazotrophic *Burkholderia* sp., an abundant genus in the maize rhizosphere, have been isolated from both maize and teosintes grown on an indigenous maize field (Estrada et al., 2002). Similarly, a significant fraction of endophytic bacteria found in teosinte were shown to be conserved in modern maize, and no decrease in endophyte diversity was observed for modern maize when cultivated on indigenous soil (Johnston-Monje and Raizada, 2011; Johnston-Monje et al., 2014). However, rhizosphere bacterial and fungal abundance and activity differed between a teosinte and two modern *Zea mays* varieties, with teosinte having significantly higher bacterial abundance, diversity, and decreased activity of the soil N-cycling enzyme N-acetylglucosaminidase (Szoboszlay et al., 2015).

Domestication also appears to have affected microbiomes of other agronomically important crop species, further demonstrating the potential for human selection to alter host mediation of rhizosphere community structure. The rhizosphere bacterial community of a wild ancestor of beet, *Beta vulgaris* ssp. *maritimus*, has higher diversity, greater resistance to abiotic stress, and a lower proportion of isolates with anti-phytopathogenic activity than that of modern sugar beet (Zachow et al., 2014). The microbiome of domesticated barley (*Hordeum vulgare*) differs from that of its wild ancestors in function as well as diversity, with genes affecting host-microbe interactions showing evidence of positive selection (Bulgarelli et al., 2015). Older and modern cultivars of lettuce (*Lactuca sativa*) have higher rhizobacterial diversity than their wild ancestor *L. serriola*, but diversity indices do not differ significantly between *L. sativa* cultivars (Cardinale et al., 2015).

The impact of modern breeding in a fertile environment on plant-driven rhizosphere determinants has also been observed through “common garden” studies of microbial communities among older and newer maize varieties. Inbred lines from five genetic groups of maize created by human selection were found to support different rhizobacterial communities, especially with regard to the *Burkholderia* genus, but differences were not correlated with genetic distance of the host (Bouffaud et al., 2012). In a subsequent study, however, rhizobacterial community

shifts were correlated with phylogenetic distance between maize genotypes (Bouffaoud et al., 2014).

The introduction of high-yielding hybrids has had significant effects on the rhizosphere, perhaps because hybrids differ in root traits and exudate production from their inbred parents. Compared to their inbred parents, hybrids generally support more auxin-producing rhizobacteria (Picard and Bosco, 2005); more genetically diverse *Pseudomonas* populations (Picard and Bosco, 2005) and more antibiotic-producing isolates (Picard et al., 2004); stimulate antibiotic production and nitrogen fixation earlier (Picard et al., 2008); and are better at selecting elite rhizobacterial strains (Picard and Bosco, 2006). Heterotic effects have also been studied in AM fungi. A hybrid and one of its parental inbred lines were able to select unique AM fungal communities, whereas the other parental inbred line was not (Picard et al., 2008), suggesting selection of AM fungal strains is controlled by dominant inheritance rather than heterosis. In another study, however, modern hybrids had significantly higher AM colonization than inbreds or landraces (An et al., 2010).

Plants can also affect rhizosphere microbes through changes in provisioning to the rhizosphere (Figure 1). Selecting for high harvest index may have increased aboveground biomass at the expense of exudate quantity and quality, as net rhizodeposited carbon is related to belowground biomass (Amos and Walters, 2006). Altered root traits may likewise have led to changes in the amount, rate, and decomposability of rhizodeposits (i.e., belowground C inputs from root turnover, mucilage, sloughed root debris, exudates), which could affect stimulation of SOM decomposition (i.e., rhizosphere priming) (Kuzyakov, 2002) and subsequent N mineralization (Dijkstra et al., 2009), as well as microbial richness and/or diversity (Bakker et al., 2012). Selective pressure for shifts in microbiome-level metabolism of organic compounds may have been imposed by a decrease in lignin content and lower lignin:N ratio in residues of modern crop varieties as compared to wild ancestors (García-Palacios et al., 2013).

Transgenic approaches to crop improvement have also resulted in substantial changes to rhizosphere inputs (Saxena et al., 1999; Saxena and Stotzky, 2000, 2001); while not strictly the result of breeding, the ubiquity of transgenes in contemporary maize represents a significant alteration of host genotypes that may have had corresponding impacts on the rhizosphere and microbiome (Box 1). Whether changes in resource provision have indeed resulted in altered soil and rhizosphere nutrient cycling patterns remains to be investigated.

In addition to plant-driven effects on the microbiome, changes in plant responsiveness to microbes might have occurred during breeding in high input environments, since plants may derive little benefit from directing C to symbionts in these conditions. If modern maize had an impaired ability to capitalize on beneficial associations for resource acquisition, then growth and yields in low input or biologically-based cropping systems could be compromised. Studies assessing responsiveness to microbial associations in teosinte, early landraces, and modern varieties have attempted to clarify whether domestication and breeding have altered the significance of microbial symbionts in resource acquisition, with conflicting results. No evidence of decreased

mycorrhizal responsiveness was found in a comparison of three newer and three older maize cultivars under low-P conditions (Khalil et al., 1994). However, mycorrhizal inoculation caused variable responses in older cultivars, ranging from no effect to 400% higher growth, whereas newer cultivars responded uniformly with higher growth. Similarly, a meta-analysis of 320 crop genotypes found no evidence of decreasing ability to benefit from mycorrhizal fungi over time, with newer cultivars generally less intensively colonized but more responsive (Lehmann et al., 2012). However, mycorrhizal dependence in wheat (*Triticum aestivum*) tends to be higher in landraces than either wild ancestors or modern cultivars (Herrick et al., 1993) and mycorrhizal responsiveness was lower in modern than older wheat varieties (Zhu et al., 2001). This may indicate that breeding has selected against this trait, perhaps because it was inversely related to phosphorus utilization efficiency (PUTE; Zhu et al., 2001).

Shifts from Natural to Increasingly Managed Soil Environment

Soil origin and type appear to be more significant than plant genotype in determining the microbial community structure of the maize rhizosphere (Dalmastrì et al., 1999; Gomes et al., 2015) since the rhizosphere microbiome is recruited from bulk soil. However, environmental effects remain poorly understood in comparison to plant-mediated effects, despite the potential for this knowledge to inform agricultural management that creates favorable conditions for biologically-based resource acquisition.

Environmental changes during domestication such as shifting geographic distribution, changes in soil fertility from natural to managed environments, and agricultural cultivation practices influence rhizosphere microbial communities (Pérez Jaramillo et al., 2015). Higher synthetic nutrient inputs and resource homogeneity may have caused the loss of root traits (Milla et al., 2015) and microbial interactions (Wissuwa et al., 2009) that aid in resource acquisition under conditions of lower inorganic nutrient availability. Management practices such as tillage, fertilization, and bare fallows also disrupt the evolutionary stability of mycorrhizal symbioses (Herrick et al., 1996; Lekberg and Koide, 2005), potentially leading to decreased cooperativity over time (Duhamel and Vandenkoornhuyse, 2013). Quantifying the benefit of microbial associations for maize and teosinte genotypes in wild ecosystems and modern agroecosystems would elucidate whether environmental changes have affected maize-microbe interactions.

Evolution of Genotype × Environment Interactions

$G \times E$ interactions can pose a challenge for breeders and evolutionary studies alike, causing a genotype selected for desirable traits in a favorable trial environment to be poorly suited to variable or suboptimal field conditions (Ceccarelli, 1994). Studying the effect of domestication and breeding on $G \times E$ interactions requires the evaluation of multiple genotypes in distinct environments, a study design more frequently employed in nutrient use efficiency studies than microbiome analyses.

Mycorrhizal responsiveness showed G × E interactions in four Chinese maize cultivars released between the 1950s and 2008, with the newest cultivar responding positively to colonization regardless of soil P and older cultivars responding neutrally or in a soil-P-dependent manner (Chu et al., 2013). These results suggest that mycorrhizal colonization and responsiveness have co-evolved with other plant improvement traits, although too few genotypes were evaluated to determine whether this is a general trend. Continuing to integrate analyses of microbial responsiveness into resource use efficiency studies will provide useful information on how GxE interactions may affect microbe-mediated resource acquisition pathways.

Phenotypic integration of root and microbial traits may determine the magnitude of G × E-driven evolutionary change in plant-microbe associations (Murren, 2012). Coordination and reciprocal influence between roots and microorganisms are well-characterized, but whether this extends to co-variation over evolutionary time is less clear. Coordinated evolution, i.e., a high level of integration, is predicted to lead to more efficient functioning (Murren, 2012), but may occur to a lesser extent in an environment of artificial selection (Milla et al., 2014). If root and microbe traits are highly integrated, the selective pressures imposed by a heterogeneous, high-nutrient environment may result in evolution toward a rhizosphere where both root and microbe traits are maladapted to low-input systems. In contrast, if root and microbe traits are distinct modules, evolution toward maladaptation to low-nutrient conditions could occur in one module while the other remains unaffected and potentially capable of compensating for lost nutrient acquisition ability.

WHAT ARE THE IMPLICATIONS FOR AGRICULTURAL SUSTAINABILITY?

Ever-growing demand for limited natural resources, spurred by population growth and climate change, as well as the high environmental costs associated with conventional agriculture systems requires shifting to management strategies and matched crop genotypes that promote biologically-based resource acquisition over synthetic inputs. Capitalizing on ecological

functions naturally present in the rhizosphere, a hot spot of root-microbe interactions, can improve maize productivity in low-input or biologically-based systems and enhance agricultural sustainability in a resource-scarce future.

Recent research has focused on the importance of soil health to agroecosystems (Altieri and Nicholls, 2003), but has failed to account for the central role of the host plant in converting soil health into yield. Rhizosphere microorganisms can reduce the need for external inputs by aiding in the acquisition of scarce resources, but the consequences of microbial community shifts for nutrient cycling and acquisition have been relatively neglected. For instance, understanding whether taxa involved in N cycling and N fixation have been lost or retained and whether any losses have been compensated through functional redundancy would affect not only crop N availability but also rates of N loss from the agroecosystem (Jackson et al., 2012). Similarly, changes in taxa involved in rapid organic matter decomposition or bioavailability of nutrients could affect resource acquisition. Clarifying the genetic basis for loss or gain of microbial traits could pave the way to breeding cultivars that facilitate beneficial microbiomes, thus obviating the need for inoculation to introduce favorable species. Even if changes in microbial community composition are limited, maize genotypes that support high microbial activity and organic matter priming, i.e., where host plant exudates and other rhizodeposits stimulate microbe-mediated nutrient cycling through high exudate quantity and quality, could increase nutrient cycling and utilization of soil resources. Thus, genetic variation in rhizosphere traits must be better characterized and represents a prime target for breeding resource-efficient cultivars.

Modern hybrids show evidence of decreased adaptation to environments of heterogeneous, scarce resource availability, but are better equipped for the acquisition of deep or mobile nutrients (perhaps beneficial under drought conditions) and tolerance of high planting density. Pinpointing the loss of favorable traits on an evolutionary timeline can help identify germplasm for use in creating new, resource-efficient varieties suited to low-input systems. For instance, QTL mapping of teosinte × maize crosses has been used to increase aerenchyma

BOX 2 | KNOWLEDGE GAPS AND RESEARCH NEEDS

- *Timeline:* How do the roots and rhizosphere microbiomes of early landraces differ from those of teosinte? Root system comparisons of teosinte and modern maize and sequencing studies of older and newer maize cultivars have neglected landraces. Comparisons of root architecture, anatomy, physiology, and ecology along an evolutionary gradient could allow the appearance or loss of beneficial traits and microbial species to be pinpointed.
- *Resources:* How has changed altered plant belowground resource provisioning affected metabolic processes and nutrient cycling in the soil? Metabolomics studies of teosinte, landraces, and modern varieties could provide clues.
- *Responsiveness:* Has maize responsiveness to microbial associations decreased over time? Inoculation studies commonly determine the response of a single maize genotype, but assessing host benefit from microbial associations across an evolutionary gradient could reveal whether responsiveness has declined.
- *Signaling:* How have root exudates and signaling molecules changed? Plant and microbial signaling compounds should be compared across maize and teosinte genotypes; manipulation of phytomicrobiome signaling has been proposed as a strategy to enhance agricultural sustainability (Quiza et al., 2015).
- *Functioning:* What is the functional significance of known changes in community composition for C and N turnover in the rhizosphere? Has plant ability to capitalize on soil health (i.e., sustainable nutrient sources provided by a soil with high microbial activity and diversity) decreased as a result of compositional changes, or has it been maintained by functional redundancy in the microbiome? An extracellular enzyme involved in N cycling differed between maize phylogenetic groups in one study (Szoboszlay et al., 2015), but more detail is needed on nutrient cycling effects and the groups responsible. Sequencing studies can provide only hypotheses based on previous information about the functions performed by a given taxon in isolation, but overlook functional redundancy and interactions. Meta-transcriptomics, meta-proteomics, meta-metabolomics, and novel isotope labeling approaches (Vandenkoornhuyse et al., 2007) could identify key active species and illuminate their functional roles.

formation (Mano et al., 2007) and similar methods could be used to introgress beneficial allele sources within teosinte and landrace germplasm for increased root hair length or high-affinity ammonium or nitrate transporter expression. Breeding for resource efficiency should be conducted under low-input conditions or where nutrients are supplied from organic sources, so that selection for cultivars able to maintain yields with limited or organic inputs is more efficient (Weber et al., 2012).

Although progress has been made in describing changes in architectural, anatomical, and physiological root traits, as well as microbial community shifts, significant research gaps remain (**Box 2**). Understanding how human selection has affected root traits and rhizosphere interactions can reintroduce allelic diversity tied to beneficial root traits and microbial associations and inform breeding and management practices that promote biologically-based resource acquisition (Wissuwa et al., 2009).

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Characterization of Pearl Millet Root Architecture and Anatomy Reveals Three Types of Lateral Roots

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Pearl millet plays an important role for food security in arid regions of Africa and India. Nevertheless, it is considered an orphan crop as it lags far behind other cereals in terms of genetic improvement efforts. Breeding pearl millet varieties with improved root traits promises to deliver benefits in water and nutrient acquisition. Here, we characterize early pearl millet root system development using several different root phenotyping approaches that include rhizotrons and microCT. We report that early stage pearl millet root system development is characterized by a fast growing primary root that quickly colonizes deeper soil horizons. We also describe root anatomical studies that revealed three distinct types of lateral roots that form on both primary roots and crown roots. Finally, we detected significant variation for two root architectural traits, primary root length and lateral root density, in pearl millet inbred lines. This study provides the basis for subsequent genetic experiments to identify loci associated with interesting early root development traits in this important cereal.

Keywords: lateral root, root growth, metaxylem, root architecture, breeding

INTRODUCTION

In Africa, most of the recent increase in agricultural production has been due to the expansion of cultivated lands rather than an increase in yields (Bationo et al., 2007). Moreover, several climate models predict that global changes may reduce the potential productivity of cereals (Berg et al., 2013). For example, millets potential productivity is predicted to decrease by 6% in the driest cultivated regions. In order to achieve future food security in Africa, it is therefore necessary to improve crop productivity through breeding and improved agricultural practices.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is the sixth most important cereal grain in the world (Food and Agriculture Organization of the United Nation [FAO], 2014). It accounts for 6% of the total cereal production in Africa, and 14% in West Africa alone (Food and Agriculture Organization of the United Nation [FAO], 2014). Pearl millet grain is a significant

source of micronutrients such as iron and zinc with contents higher than those in other cereals (Souci et al., 2000). Both in sub-Saharan Africa and India, it potentially represents one of the cheapest food sources of these micronutrients and proteins when compared with other cereals and vegetables. In addition, pearl millet is well adapted to dry climates and is mostly grown in areas with limited agronomic potential characterized by low rainfall, in the 200–500 mm range, and marginal soils (Guigaz, 2002). These facts make millet an important food staple over much of the African continent, especially in the semi-arid areas of the Western Sahel where other crops tend to fail because of inadequate rainfall and poor soil conditions. Thus pearl millet is an important cereal in arid and semi-arid regions where it contributes to food security and is expected to have an increased importance in the future adaptation of agriculture to climate change in sub-Saharan Africa.

Despite its importance, pearl millet is considered as an orphan crop because it has received very little support from science, industry and politics while other crops such as wheat, rice, or maize were subjected to intense efforts of genetic and agronomic improvement. As a result, it lags behind sorghum and far behind the other major cereals in its genetic improvement. Its average grain yields barely reach 900 kg/ha, compared to 1500 kg/ha for sorghum (Food and Agriculture Organization of the United Nation [FAO], 2014). Moreover, production has increased by only 0.7% a year in West Africa during the last two decades, the lowest growth rate of any food crop in the region and far less than the population's growth rate of nearly 3% per year (United Nations Statistics Division, 2016). However, its untapped genetic potential is vast and could be used to improve pearl millet tolerance to some environmental factors that are the main limitations to its growth potential. For instance, pearl millet is mostly grown in marginal soils such as sandy soils in Western Sahel where low water and nutrient (particularly phosphate) availability are major limiting factors. Moreover, root establishment in poor soil is essential to ensure efficient use of available water.

The importance of root architecture for water and nutrient acquisition has been well documented in both monocots and dicots, and could be successfully used for root trait-targeted genetic improvement. For example, targeted modifications of root architecture in pea to increase P acquisition efficiency were achieved (Lynch, 2011). Pearl millet is a monocot species displaying a fibrous root system in which different categories of roots can contribute to a various extent in root system growth, branching and tropism dynamics as well as to water transport. Importantly, substantial differences in root traits were reported for eight pearl millet varieties grown in soil in Niger (Brück et al., 2003) indicating a potential genetic diversity that could be used for breeding and selecting new varieties with improved root systems. However, the detailed structure and dynamics of pearl millet root system has not been described and very little is known about root growth and anatomy.

Here, we analyzed root architecture during the early phase of pearl millet development. Furthermore, we identified and characterized the anatomy of the different root types. Finally, we compared two root development parameters in 16 pearl

millet inbred lines and show that there is a large diversity of phenotypes that could be exploited in later breeding studies.

MATERIALS AND METHODS

Plant Material

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] inbred lines (Saïdou et al., 2009) originating from Indian, West and Central African landraces were used in this study. Seeds were surface sterilized with 5% hypochlorous acid for 2 min, rinsed three times in sterile water, then immersed in 70% ethanol for 2 min, rinsed three times again and kept for 10 min in sterile water. Seeds were put in Petri dishes containing wet filter paper for 24 h in the dark at 30°C for germination. The age of the plants are given in DAG (days after germination), i.e., the number of days from the date of seed-transfer onto the filter paper for germination.

Root Phenotyping

For analysis of root development, rhizotrons were built according to Neufeld et al. (1989). They were composed of a 400 mm × 700 mm × 20 mm aluminum frame, and, from rear to front, a 5 mm extruded polystyrene layer, a 20 mm layer of substrate, a cellulose acetate tissue layer (40 µm mesh) and a 5 mm plexiglass (Figure 1A). In this system, the root system grows in two dimensions between the fabric and the plexiglass (Figure 1B). The cellulose acetate was chosen because it is both non-deformable, preventing roots to grow through (this was confirmed at harvest), and allows roots to remain hydrated. The water content of the substrate was evaluated at the onset of the experiment and later maintained above stressful threshold by daily weighing the rhizotrons and watering from the top. The substrate used was composed of 30% fine clay, 25% peat fibers, 5% blond peat, and 40% frozen black peat (Klasmann–Deilmann France SARL). The average SWC (Soil Water Content) of the substrate was 56% (w:w). At 1 DAG, one germinated seedling (displaying a primary root of about 1 cm long) was transferred to the top of each rhizotron, in a layer of wet sphagnum. This layer was permanently maintained wet in order to prevent the seedlings from drying out during the early stages of growth. The plants were placed in a 1 m² growth room with a 14 h photoperiod, a temperature of 28°C/24°C during days/nights and a VPD of 1.5 kPa. From the second day of growth onward, rhizotrons were scanned (Epson Expression 10000XL) every day at a fixed time at a resolution of 600 DPI. Root system outlines were then extracted using SmartRoot (Lobet et al., 2011). These outlines comprised information on all root lengths, branching position and angle for every scan.

For high-throughput root phenotyping, a paper-based system was used (Figure 1C) according to Atkinson et al. (2015). One DAG-old seedlings were transferred into pouches and then maintained in a growth room with a 14 h photoperiod (28°C during day and 24°C during night). Pictures of the root system were taken every 2 days for 6 days with a D5100 DSLR camera (Nikon) at a resolution of 16 M pixels. The camera was fixed on a holder to maintain the same distance between the lens and each

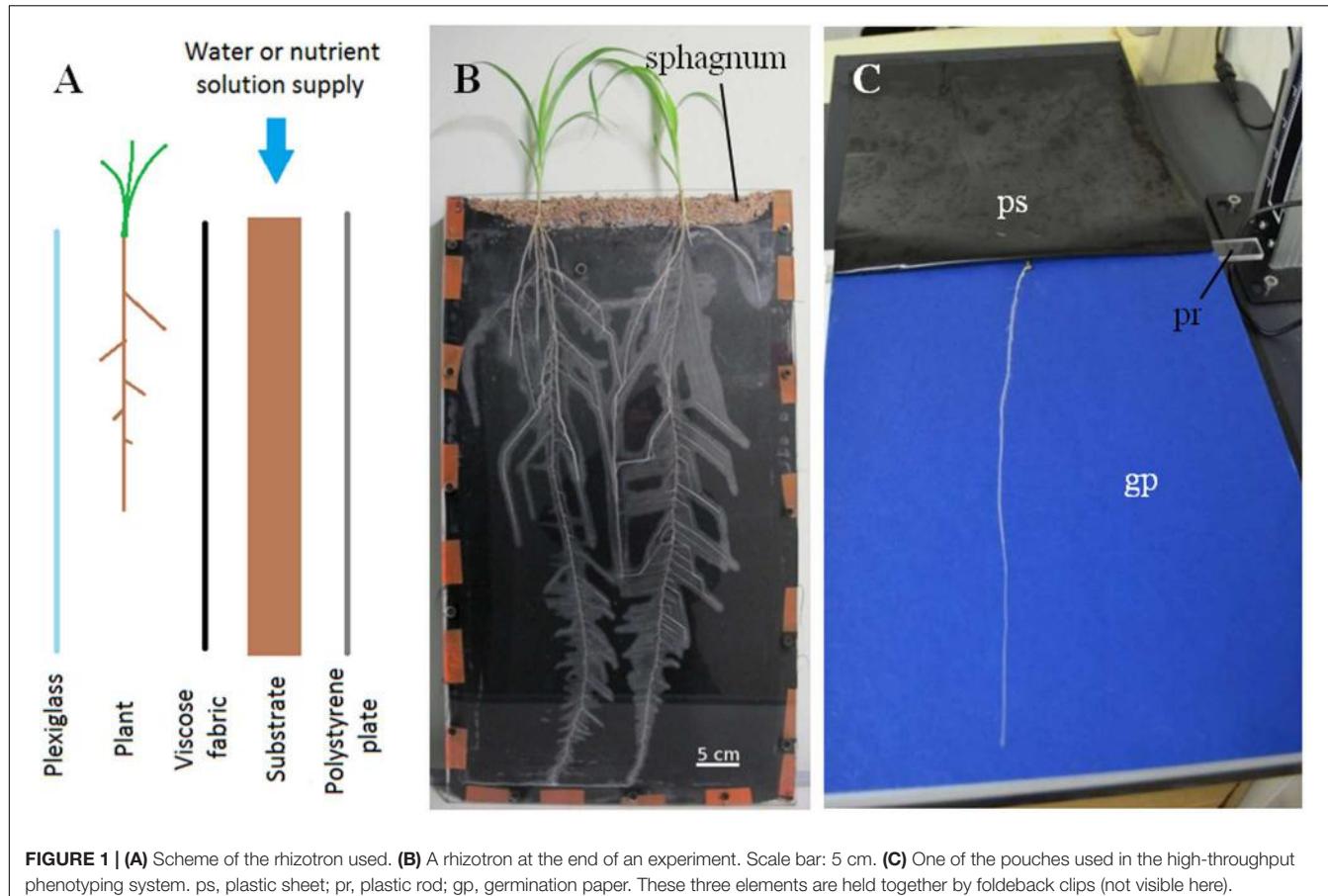


FIGURE 1 | (A) Scheme of the rhizotron used. **(B)** A rhizotron at the end of an experiment. Scale bar: 5 cm. **(C)** One of the pouches used in the high-throughput phenotyping system. ps, plastic sheet; pr, plastic rod; gp, germination paper. These three elements are held together by foldeback clips (not visible here).

root system. At 6 DAG, the root tip of the “fastest-growing” plants reached the bottom of the pouches. The experiment was repeated four times independently. Root traits (primary root length, lateral root density along the primary root and number of crown roots) were extracted using RootNav (Pound et al., 2013).

Root Sections and Microscopy

One DAG-old seedlings were transferred in a hydroponic system containing quarter strength Hoagland medium (Hoagland and Arnon, 1950) or put on the top of seed germination paper (Anchor Paper Company, USA) rolled on itself with the base immersed in distilled water (Hetz et al., 1996). The plants were kept in a growth chamber (12 h photoperiod, a temperature of 27°C and an hygrometry of 60%) for 10–20 days. For sections of fresh material, 1-cm long samples were collected at the root apex and every 5 cm along the root and were embedded in agarose blocks (3% v/v in water) before sectioning, as described in Lartaud et al. (2014). The sampling positions were recorded. Transverse root sections (thickness 60 μm) were obtained using a HM 650 V vibratome (Microm) and observed directly under the epifluorescence microscope. Some section were stained with Safranin and Alcian blue (FASGA, Tolivia and Tolivia, 1987).

For thin sections, samples were fixed and dehydrated as described by Scheres et al. (1994). Samples were then embedded in Technovit 7100 resin (Heraeus Kulzer) according to the

manufacturer's instructions. Thin longitudinal sections (5 μm) were produced with a HM355S microtome (Microm). Sections were stained for 15 min in aqueous 0.01% toluidine blue (pH = 6,8) solution and mounted in Clearium Mountant (Surgipath). Sections were visualized using a Leitz DMRB epifluorescence microscope [objectives used: 10 \times , numerical aperture (NA) = 0,3; 20 \times , NA = 0,5; 40 \times , NA = 0,75]. Pictures were taken using a Retiga SRV FAST 1394 camera (QImaging) and the QCapture Pro7 software (QImaging). Vessel dimensions were measured using ImageJ.

X-Ray Microcomputed Tomography

Plants were transferred to pots (50 mm diameter and 120 mm height) containing “Newport Series Loamy Sand” soil [sand 83.2%, silt 4.7%, and clay 12.1%; organic matter 2.93%; pH = 7.13; Nitrate = 5.48 mg.L⁻¹; Phosphorus = Defra index of 3 (29.65 mg kg⁻¹)] 1 DAG. Plants were maintained throughout the experiment at a soil water content of ~26% (w:w), which corresponds to 75% of field capacity. The SWC was monitored daily by weighing the pots. Plants were scanned with a v|tome| x M scanner (Phoenix/GE Systems), with a maximum energy of 240 kV, four times over an 18 days period (4, 8, 14, and 18 DAG) to image the root structure. Root systems were segmented manually from the image stacks using the VGStudio Max software (Volume Graphics GmbH).

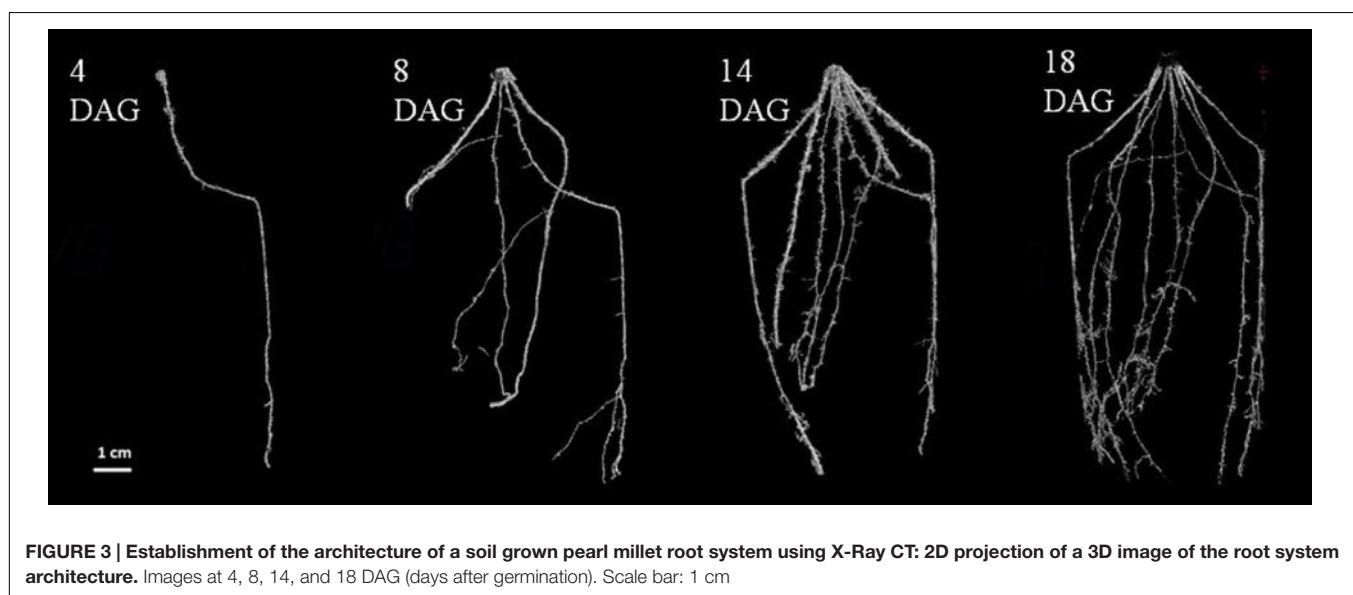
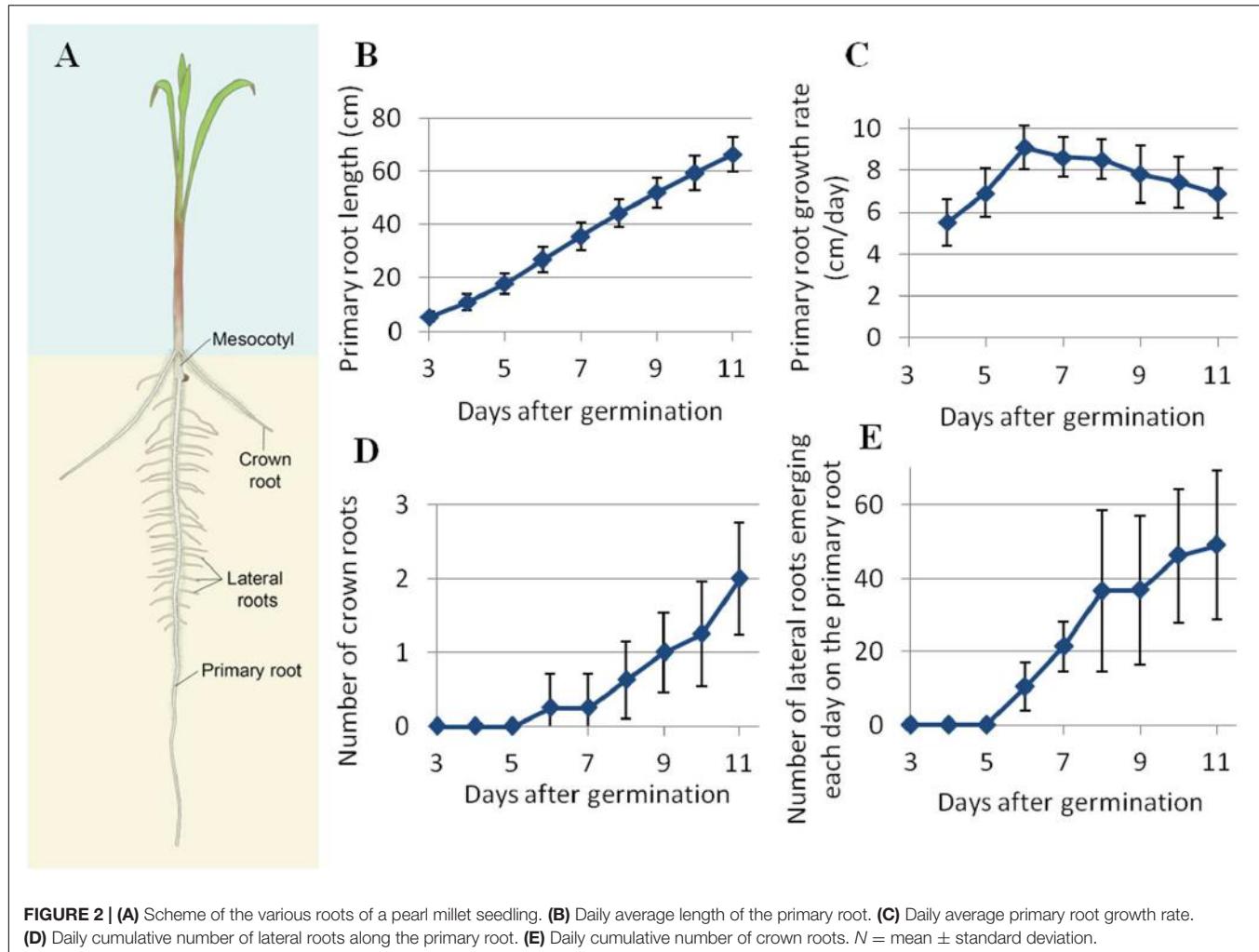


TABLE 1 | Anatomical features of the different root types in pearl millet.

Root type	Root diameter (μm)	Stele diameter (μm)	# Metaxylem vessels	Metaxylem vessel diameter (μm)	<i>n</i>
Primary root	429 \pm 103 ^{ab}	181 \pm 34 ^b	1	58 \pm 11 ^a	10
Crown root	517 \pm 76 ^a	229 \pm 54 ^a	3	56 \pm 9 ^a	8
LR type 1	112 \pm 27 ^d	32 \pm 8 ^e	0	NA	14
LR type 2	264 \pm 22 ^c	74 \pm 9 ^d	1	16 \pm 2 ^b	7
LR type 3	367 \pm 66 ^b	145 \pm 16 ^c	1	50 \pm 6 ^a	12

Mean and standard deviation of all sections. Letters correspond to groups formed by Tukey's Honest Significant Difference test ($\alpha = 0.05$). *n*, sample size.

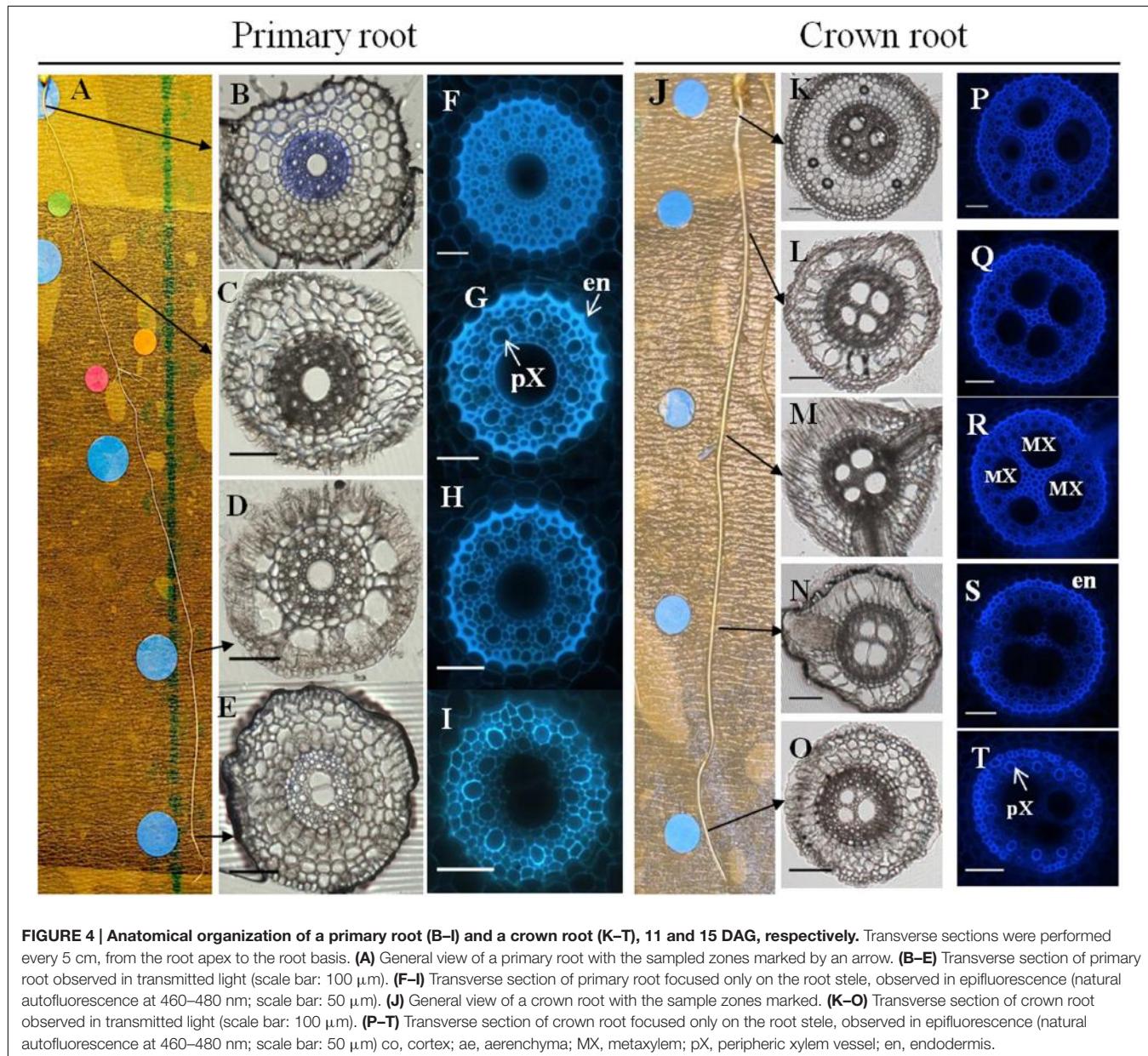


FIGURE 4 | Anatomical organization of a primary root (B–I) and a crown root (K–T), 11 and 15 DAG, respectively. Transverse sections were performed every 5 cm, from the root apex to the root basis. (A) General view of a primary root with the sampled zones marked by an arrow. (B–E) Transverse section of primary root observed in transmitted light (scale bar: 100 μm). (F–I) Transverse section of primary root focused only on the root stele, observed in epifluorescence (natural autofluorescence at 460–480 nm; scale bar: 50 μm). (J) General view of a crown root with the sample zones marked. (K–O) Transverse section of crown root observed in transmitted light (scale bar: 100 μm). (P–T) Transverse section of crown root focused only on the root stele, observed in epifluorescence (natural autofluorescence at 460–480 nm; scale bar: 50 μm). co, cortex; ae, aerenchyma; MX, metaxylem; px, peripheric xylem vessel; en, endodermis.

Statistical Analyses and Heritability Estimates

Statistical analyses were performed using R (R Development Core Team, 2008). An analysis of variance was performed to detect

an effect of the line on the variability of the different root traits measured. When an effect was detected, a Tukey's HSD (Honest Significant Difference) test was used to group lines of homogeneous means for the trait of interest.

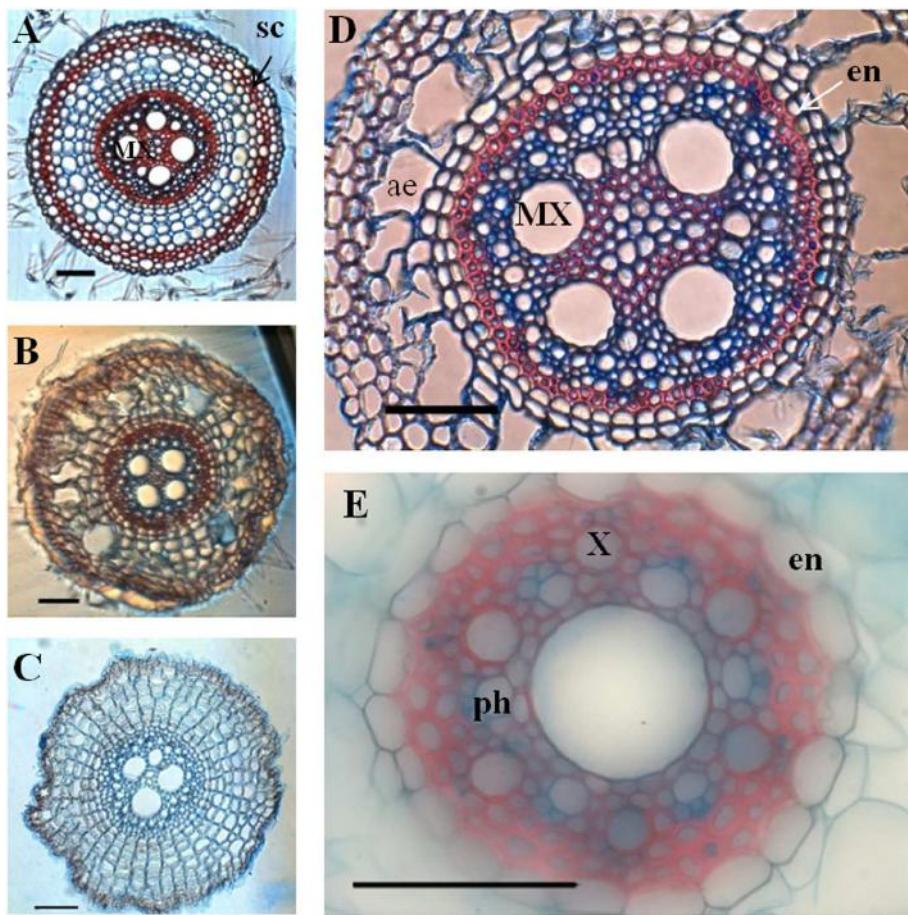


FIGURE 5 | Transverse section of crown roots and primary root stained with FASGA. Sections were performed at various level along the roots axis. **(A–C)** Transverse section of crown root, after FASGA staining. **(D)** Transverse section of a crown root after FASGA staining, focus on the stele. **(E)** Transverse section of primary root after FASGA staining, focused on the stele (scale bar: 100 μm). sc, schlerechyma; en, endodermis; X, xylem vessel; MX, metaxylem vessel; ph, phloem vessel; ae, aerenchyma.

Broad sense heritability was computed by dividing the variance associated with line with the total variance of the character (variance associated with line + environmental variance + residual variance).

Average seed weight for each line was evaluated and a Spearman's rank correlation coefficient was computed to detect a putative correlation between seed weight and root trait.

RESULTS

Early Development of Pearl Millet Root System

The emergence and development of different roots in pearl millet seedling was studied in different growth conditions. Different roots observed at early stage are named according to the nomenclature presented in **Figure 2A**, based on the nomenclature used for maize root systems (Hochholdinger and Tuberosa, 2009). The first root to emerge from the seed, initially called the radicle, is then called the primary root. A small

segment, called the mesocotyl, links the seed and the base of the shoot. At later stages of development, crown roots emerge from the base of the shoot. Branches that appear on the primary or crown roots are called lateral roots. The lateral roots can branch themselves, these ramifications being called secondary lateral roots.

The developmental dynamics of the root system was studied more finely on pearl millet line LCICMB1 (line 109 of the panel). In all of the plants that we analyzed in rhizotrons ($n = 28$), the early root system of pearl millet was made up of a single primary root that has emerged from the seed 12 to 24 h after seed rehydration. This primary root grew vertically at an increasing rate during the first 6 DAG, reaching a maximum of 9.1 cm day $^{-1}$ (**Figures 2B,C**). After that date, the primary root growth rate slightly slows down, but remains *ca.* 7 cm day $^{-1}$ at 11 DAG (**Figure 2C**). The average primary root length at 11 DAG was 66.3 cm (**Figure 2B**). Crown roots and lateral roots started to emerge, respectively, from the shoot base and on the primary root at 6 DAG. The average number of crown roots per plant is shown in **Figure 2D**. Crown roots started to emerge 6 DAG and were in

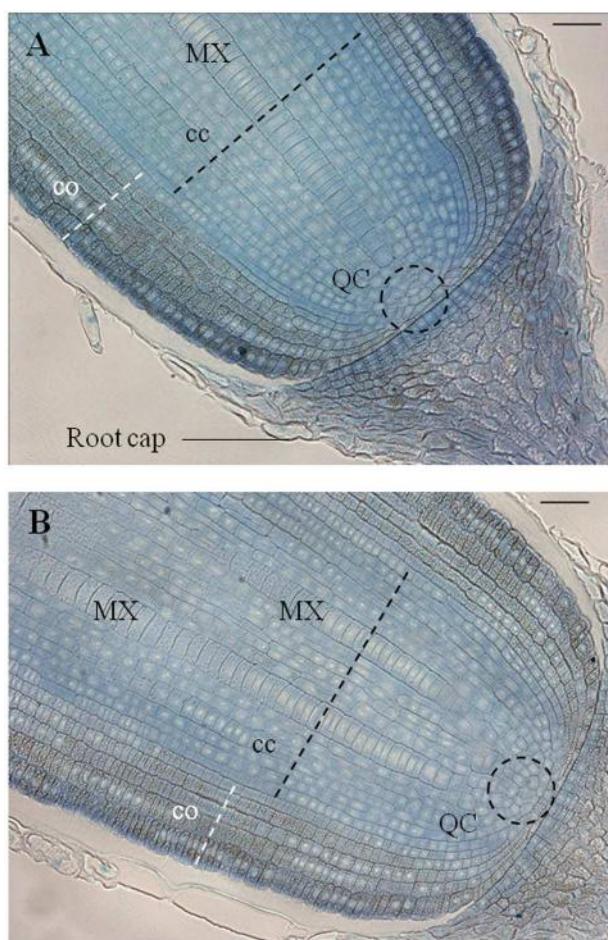


FIGURE 6 | Anatomical organization of primary root and crown apices observed on a longitudinal section, stained with toluidine blue, sampled 5 DAG. (A) Longitudinal section of a primary root apex. **(B)** Longitudinal section of a crown root apex. QC, quiescent center; cc, central cylinder; co, cortex; MX, metaxylem vessel (scale bar: 100 μm).

average two per plant at the end of the experiment. This number is quite low and this experiment only captured the very beginning of crown root emergence period. Average crown root growth rate was 3.7 cm day^{-1} . The number of lateral roots emerging each day on the primary root is shown on **Figure 2E**. Lateral roots started to emerge on the primary root 6 DAG. Their emergence rhythm increased until the end of the experiment, quickly up to 8 DAG and then slowly between 8 and 11 DAG. Lateral root density on the primary root was $4.2 \text{ roots cm}^{-1}$. Lateral root growth rates were heterogeneous, reaching up to 3 cm day^{-1} . Interestingly, crown roots and lateral roots started to appear at 6 DAG, when primary root growth rate reached its maximum, and correlates with the emergence of the third leaf.

Early root development was also analyzed in 3D in soil using micro-computed x-ray tomography (**Figure 3**). LCICMB1 plants were grown in small soil columns (5 cm diameter \times 12 cm high) and scanned at 4, 8, 14, and 18 DAG. As in the rhizotrons, only primary root was visible at 4 DAG and crown and lateral roots

could be detected from 8 DAG onward. This indicated that these roots emerged between 4 and 8 DAG, but the time resolution was too rough to identify a precise emergence date. However, this time interval is consistent with their emergence time observed in rhizotron, of 6 DAG. This observation therefore supports the hypothesis that rhizotrons provide a realistic assessment of root architecture development in natural conditions. The 3D images also gave us information about the organization of the different roots in space. The primary root, first to emerge, grew nearly vertically into the soil volume. On the contrary, crown roots grew at an angle of between 20° and 40° to vertical. This angle appeared conserved for the first centimeters of crown root growth, but the small diameter of the pots scanned constraining root growth to just a few centimeters after emergence, did not allow us to check whether this angle could be maintained. Crown root emergence sites were distributed regularly in space around the stem base.

Hence, early root system development in pearl millet is characterized by a fast growing primary root that quickly colonizes deeper soil horizons, while lateral and crown roots only start to emerge 6 DAG.

Anatomy of the Different Root Types

We next analyzed the cellular organization of primary, crown and lateral roots of young pearl millet plants (LCICMB1 line) grown on germination paper or in hydroponics. Root fragments were harvested at different positions along the root and transverse sections were obtained using a vibratome. As root characteristics did not vary strongly in the zone we sampled (Supplementary Figure 1 for example of stele diameter) we considered all the samples we had to define the anatomical features of the different root types (**Table 1**).

Primary roots were characterized by a large diameter metaxylem vessel located at the center of the stele (**Figure 4**). Their ground tissue contained 3–5 layers of cortical cells. Aerenchyma differentiation was observed in mature parts of the root. Crown roots were thicker than primary roots with a significantly larger stele that contained 2–5 (three in most cases) large metaxylem vessels separated by parenchyma cells (**Figure 4, Table 1**). They also showed 3–5 layers of cortical cells and aerenchyma. In both cases, cell wall autofluorescence was lower in the stele close to the root tip and increased particularly in the endodermis as the root matures, presumably because of cell wall lignification and suberization accompanying caspary strip formation.

In order to localize secondary deposition (lignin or suberin) in the cell wall, we performed FASGA staining on transverse sections of primary and crown roots (**Figure 5**). The formation of a typical horseshoe-shaped Caspary strip could be visualized in the endodermis of both primary and crown roots as they differentiated. In addition, the FASGA staining revealed six xylem poles, alternating with six phloem poles in the primary root (**Figure 5E**), while we observed 12–16 xylem poles in crown roots (**Figure 5D**). Mature parts of crown roots displayed a sclerenchyma, surrounded by a hypodermis and a rhizodermis (**Figure 5A**).

Longitudinal sections (5 μm) through the primary root meristem revealed a closed meristem organization with cell files

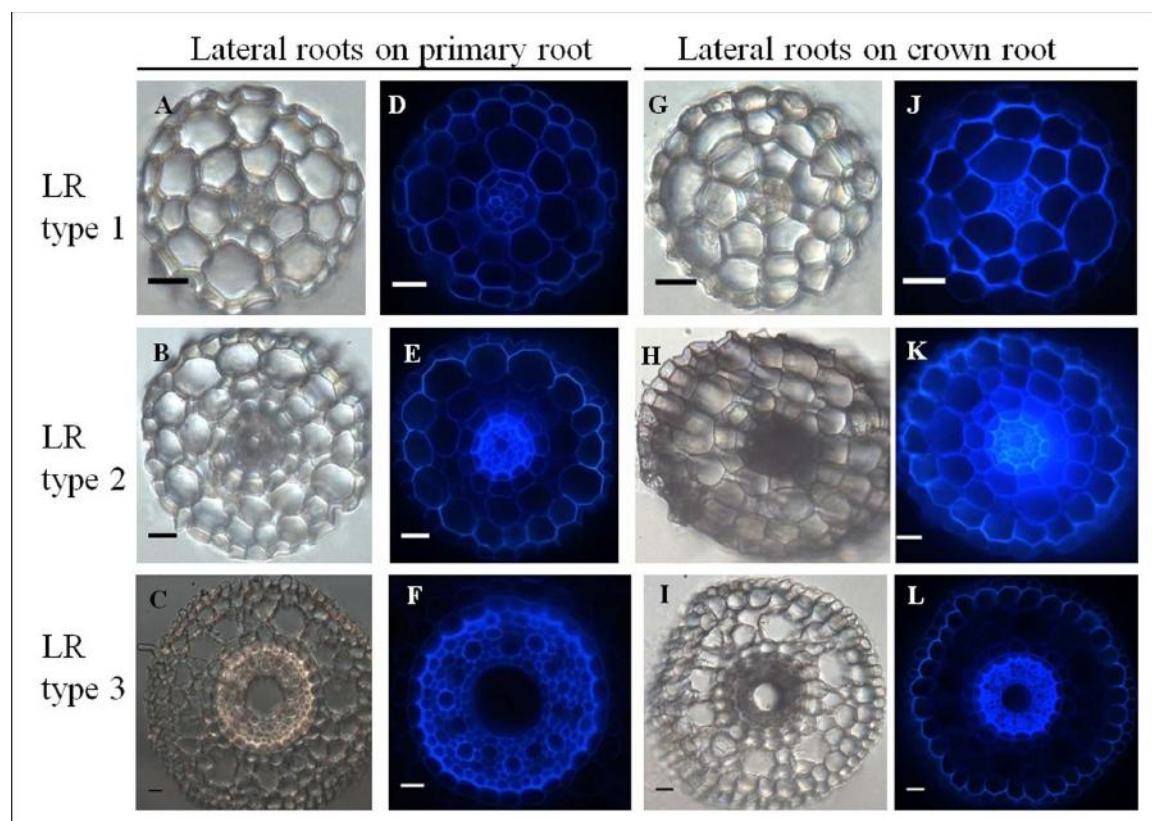


FIGURE 7 | Comparative anatomical organization of lateral roots (left: transmitted light, right: autofluorescence). (A–F) Lateral root emerging from primary root. Picture F only shows the root stele. (G–L) Lateral root emerging from crown root. Three root types are identified, independent of the mother root: LR type 1: small root diameter and no metaxylem (**A,D,G,J**), LR type 2: medium root diameter and small diameter metaxylem vessel, (**B,E,H,K**), LR type 3: large root diameter and large diameter central metaxylem vessel: (**C,F,I,L**). Scale bar: 20 μ m.

converging to a small group of cells whose location and size are consistent with those of quiescent center cells (Figure 6A). The metaxylem differentiated and expanded radially close to the putative initial cells. Cortex parenchyma cells accumulate metabolites, possibly starch grains, but further investigation is needed to identify the nature of this deposit. Longitudinal sections through the crown root meristem showed a similar closed meristem organization with a larger stele (Figure 6B).

Transverse sections through first order lateral roots ($n = 33$) branching from either primary or crown roots revealed distinct organizations. Interestingly, lateral roots could be classified into three types based on their anatomy (Figure 7, Table 1). Type 1 lateral roots are very thin (68–140 μ m diameter) with an anatomy characterized by a diarch (two protoxylem poles) stele without any central metaxylem vessel. Ground tissues include an endodermis, a bi-layered cortex, and epidermis, but neither sclerenchyma nor aerenchyma (Figures 7A,D,G,J). Type 2 lateral roots have a medium diameter (235–291 μ m), show one small (16 μ m diameter in average) metaxylem vessel and three layers of cortical cells. Like type 1, type 2 lateral roots have no sclerenchyma or aerenchyma (Figures 7B,E,H,K). Finally, type 3 lateral root exhibit the largest diameter (328–440 μ m similar to primary root) and the same organization as primary roots,

independently of the root from which they emerge (i.e., primary root or crown root; Figures 7C,F,I,L). Hence our anatomical studies have revealed that there are three distinct types of lateral roots that form on both the primary root and crown roots in pearl millet.

Diversity in Pearl Millet Root Development

We next addressed whether there was significant variation in pearl millet root architecture. We selected 16 lines from a panel of pearl millet inbred lines (Saïdou et al., 2009). As our objective was to maximize diversity, these lines were sampled to represent the whole diversity observed in the phylogenetic tree of 90 inbred lines (Saïdou et al., 2009), taking also into account a sufficient seed set availability and good germination rate. We analyzed the root system of these plants using a germination-paper-based phenotyping platform (Atkinson et al., 2015).

We observed large variation in primary root growth and lateral root density along the primary root among the individuals screened of this panel (Figure 8). In both cases, a significant part of this variability was explained by the genetic line variable (ANOVA $p < 0.01$). The lines could be separated into groups of

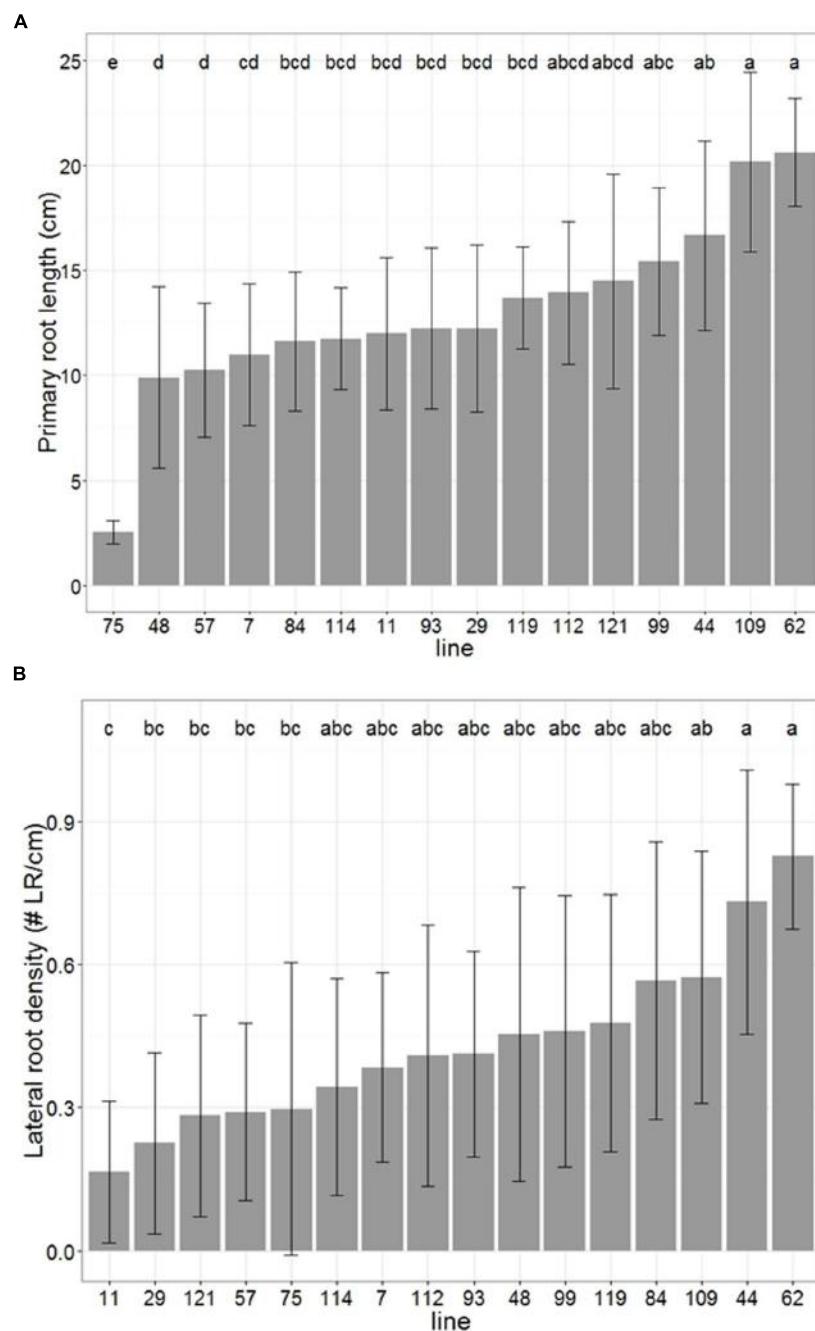


FIGURE 8 | High throughput pearl millet root phenotyping: distribution of primary root length (A) and lateral root density (B) among 16 pearl millet from a panel of inbred lines covering a large genetic diversity. Error bars represent standard deviation, letters represent Tukey's HSD groups.

homogeneous means with a Tukey's HSD test. For primary root length, the group identification showed some clear outliers with especially large or small values, associated with a group of lines with intermediate and quite homogeneous values (**Figure 8A**). For lateral root density, no clear outlier was observed, the values for all the lines forming a rather smooth continuum between small and large values (**Figure 8B**). The broad-sense heritability was equal to 0.72 for primary root length and to 0.34 for

lateral root density. We tested whether the variability in early primary root growth was due to differences in available seed reserves (Supplementary Figure 2) by computing the Spearman's rank correlation coefficient between average seed weight and primary root length for each line. The Spearman's rank coefficient correlation was equal to 0.22. This value was not significantly different to zero ($p = 0.21$), indicating that no correlation could be found between seed weights and primary root length in

our experiments. As seed mainly contains reserves, this result suggests that the differences we observed are not simply due to available reserves.

DISCUSSION

Here, we analyzed root system architecture at early stages of the pearl millet life cycle. We named the different roots following the current standards in terms of monocotyledonous root nomenclature (Hochholdinger et al., 2004). One striking feature of early pearl millet root development is the very rapid emergence and vertical growth of the primary root (7 cm day^{-1} in our experimental conditions) compared to other cereals (3 cm day^{-1} for maize and wheat; Pritchard et al., 1987; Pahlavanian and Silk, 1988; Muller et al., 1998). In contrast, root branching started relatively late after seedling germination (6 DAG). The X-ray CT experiment confirmed this global dynamics of early root system formation. Traditionally, pearl millet is sown at the very start of the rainy season. As it was domesticated in Sahel (Oumar et al., 2008) and is mostly grown in areas characterized by light soils with a low carbon content and water retention capacity, we hypothesize that the observed developmental pattern can be favorable to the rapid colonization of deep soil horizons that retain some water. This might therefore be an important adaptive strategy to deal with early drought stress. The observed anatomy of pearl millet roots is consistent with those found in other cereals such as rice (Rebouillat et al., 2009), wheat, barley and triticale (Watt et al., 2008), or maize (Hochholdinger and Tuberosa, 2009). A striking difference between the different root types comes from the number of central metaxylem vessels: one (or two) in the primary root, always more than two in the crown roots, including the root emerging from the scutellar and coleoptile node. Interestingly, our analyses identified three different lateral root types on the basis of their diameter and radial anatomy. Variation in lateral root anatomy has been reported in other cereals, with numbers of distinct types varying from two in rice (Rebouillat et al., 2009) to five in wheat (Watt et al., 2008). Recently, a more detailed characterization of cortex cell layers present in rice lateral roots revealed that three types of lateral roots exist in rice (Henry et al., 2016). These anatomical distinctions share similar features across species, the smallest root type having a very simple organization, with only two (or three) xylem vessels and no aerenchyma, and the bigger type having an organization similar to a primary root. One can hypothesize that these different lateral root types have different roles: type 1 lateral roots may be involved in the exploitation of resources close to the root whilst type 3 lateral root could be involved in the branching of the root system and the exploration of new soil volumes. The role of type 2 lateral roots is still unclear. Nevertheless, the functional relevance of these differences in anatomy needs to be explored. Similarly, it will be interesting to unravel how these different lateral roots develop and how their formation is controlled by environmental factors. Whilst the molecular mechanism controlling lateral root development has been extensively studied in the model plant *Arabidopsis thaliana* (see Lavenus et al., 2013, for review), how these mechanisms are

modified to form different types of lateral roots in Monocots is completely unknown.

Root phenotyping of different pearl millet inbred lines revealed a high variability for two root traits within the panel, consistent with an earlier study (Brück et al., 2003). Here, we showed that this variability was also visible *in vitro* at a very early stage of growth (6 DAG). This finding together with the high heritability of the primary root length could be exploited to identify the genetic determinants of primary root growth, a potentially beneficial root trait for pearl millet early establishment. For instance, screening of natural variability of the primary root length have been done at the cellular level in *Arabidopsis thaliana* and led to the identification of a root meristem regulator gene (Meijón et al., 2014). Beside, it will be interesting to exploit the large diversity we observed for primary root growth to test the adaptive value of this character for early drought stress tolerance.

CONCLUSION

Our analysis opens the way to dissecting the genetic determinants controlling key root phenes and the characterization of their impact on yield and stress tolerance in pearl millet.

AUTHOR CONTRIBUTIONS

SP, PG, DW, J-LV, YV, YG, BM, and LL designed the study. SP, FG, DM, ML, SG, BMO, JA, MNB, LL performed the experiments. SP, ML, SG, BMO, MJB, DW, J-LV, YG, BM, and LL analyzed the data. SP, J-LV, YG, and LL wrote the paper. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00829>

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Nutritional and Sensory Evaluation of Injera Prepared from tef and *Eragrostis curvula* (Schrad.) Nees. Flours with Sorghum Blends

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Injera is a fermented, sour bread consumed as a staple food in Eritrea and Ethiopia. The bread can be prepared from various cereals but tef [*Eragrostis tef* (Zucc.) Trotter] is the most preferred ingredient. This study assessed the acceptability of injera prepared using grains of a closely related but underutilized grass, *Eragrostis curvula* (Schrad.) Nees. The nutritive value of the grains was compared and the sensory attributes of injera made from flours of tef (control) and *E. curvula*, each combined with 0, 5, and 10% of sorghum flour, were assessed using a tasting panel. Nutrient analysis showed that *E. curvula* contains more than double the amount of crude protein found in tef. *E. curvula* also contains higher fat, dietary fiber and mineral nutrients than tef. Injera made of *E. tef* and *E. curvula* flours showed non-significant differences in taste, texture, appearance and overall acceptability. This suggest that *E. curvula* has the potential to serve as a novel source of gluten-free flour for human consumption. Agronomically viewed, growing *E. curvula* could be more advantageous for smallholder farmers on marginal lands because the species is a perennial that can produce a seed harvest twice a year, unlike tef, which is annual crop. It also tolerates acidic soils better than tef.

Keywords: *Eragrostis curvula*, injera, sensory evaluation, tef, underutilized crops

INTRODUCTION

The challenges facing global food security due to increasing population, increasing pressure on finite land, and water resources and climate change calls for new and innovative solutions (Mabhaudhi et al., 2016a). This has led to suggestion that neglected and underutilized crops could be developed as alternatives to the current staple crops (Hammer and Heller, 1998; Mayes et al., 2012; Mabhaudhi et al., 2016b), especially under the arid and semi-arid conditions of sub-Saharan Africa (SSA; Chivenge et al., 2015). The emerging impetus to promote underutilized crops is mostly associated with their being an integral sub-set of agrobiodiversity, suitability to marginal production environments (Mabhaudhi et al., 2016a,b), often with a high nutritional value (Mabhaudhi et al., 2016a), attributes that can be used to promote food and nutrition security in marginal production areas.

In the course of human history, an estimated 7000 plant species have been cultivated for consumption at some point (FAO, 1998). However, humanity now relies primarily on maize, wheat, rice and soybean for protein and energy needs. Restoring diversity to cropping systems will be essential to achieving global food security (Hammer and Heller, 1998; Mayes et al., 2012; Mabhaudhi et al., 2016a) and building resilience to climate change (Padulosi et al., 2011; Chivenge et al., 2015; Mabhaudhi et al., 2016b). It will also contribute to increased levels of genetic diversity within cropping systems by breeding crops that can be cultivated under unfavorable conditions, such as drought, salinity, flooding, poor soils and extreme temperatures (Delgado et al., 2011; Mayes et al., 2012). However, despite reports of such potential, underutilized crops still remain under-researched, and the major crops continue to dominate agricultural landscapes.

Eragrostis is one of the largest and most widely distributed grass genera, with more than 350 species, adapted to a wide range of habitats (van den Borre and Watson, 1994). Although tef [*Eragrostis tef* (Zucc.) Trotter] is the only fully-domesticated species (Purseglove, 1976), many *Eragrostis* species have been harvested from the wild for millennia as valuable sources of grain (Brink and Belay, 2006). Oral history indicates that the seeds of some wild *Eragrostis* species such as *E. curvula*, *E. cilianensis*, *E. ciliaris*, *E. cylindrica* Hochets, *E. gangetica* (Roxb.) Steud., *E. termula*, and *E. annulata* have been collected as a famine food in Africa (Duke, 1983; National Research Council, 1996; Brink and Belay, 2006). The seeds of *E. curvula* and *E. plana* have been used in making bread and beer (van Wyk and Gericke, 2000; Fish, 2003). Collectively, these accounts suggest that these underutilized wild *Eragrostis* species have the potential to contribute to the mix of food sources more than they currently do. Comparative studies on the morphological and cytological relationship of tef with other wild *Eragrostis* species suggest that these taxa could serve as a useful source of genes for the improvement of tef (Jones et al., 1978). Biochemical assessment of the relationship of tef and the wild *Eragrostis* species also showed many similarities (Bekele and Lester, 1981).

While tef may be of major importance in Ethiopia and Eritrea where it is the major source of flour used for preparing injera, it remains underutilized outside of these countries. There is a dearth of information on the agronomy, eco-physiology and nutritional value of these wild *Eragrostis* species. Currently, it is unknown whether wild *Eragrostis* species would offer adequate supplies of quality protein, mineral, fat and energy to local communities collecting the seeds of these species. If these underutilized *Eragrostis* species have any nutritional or agronomic advantages over conventional crops, then they could be used to diversify the global food basket, which would increase resilience in the global food system (Hammer and Heller, 1998; Mayes et al., 2012; Mabhaudhi et al., 2016a). Moreover, and in-depth knowledge of this wild species of the genus could provide an untapped reservoir of genetic diversity that could be used to improve tef.

The objective of this study was therefore to: (i) assess the nutritive value of *E. curvula* (seed/flour) in comparison to that of tef; (ii) assess the possibility of using *E. curvula* flour for the

production of an acceptable quality injera; and (iii) to assess the overall acceptability of the new injera product through analysis of its sensory properties.

MATERIALS AND METHODS

Plant Materials

Seeds (10 kg of grains) of *E. tef* (cultivar SA-Brown) and *E. curvula* (cultivar Ermelo) were purchased from McDonalds Seeds (Pty) Ltd, Pietermaritzburg, South Africa. The grains were independently stone-milled to a fine powder using Junior Mills (Pty) Ltd in Bloemfontein, South Africa. The flour was sieved to pass through a 0.05 mm mesh sieve and stored in an air tight container until used. Part of the flour was used for analyzing the chemical composition of the grains. For purposes of comparison, a sorghum-based flour (Mabele Meal) was purchased from a local market in Pietermaritzburg, South Africa. Combining sorghum flour with tef flour has been shown to improve the sensory attributes of injera (Egli et al., 2004; Yetneberk et al., 2005).

Preparation of the Blends

Eragrostis tef and *E. curvula* flours were separately mixed with various proportions of sorghum flour (0, 5, and 10%; Table 1). The six blends were replicated three times each, yielding a total of 18 blended grain flour samples for injera baking. The blends were labeled with alphabets for identification and were kept in dry shelves at 25°C in the laboratory until used.

Dough Making, Fermentation, and Injera Preparation

Injera is made by mixing a cereal (e.g., tef, sorghum, barely, and blends thereof) flour with water to make a dough, and then triggering a fermentation process by inoculating the dough with *ersho*, a starter culture, left over from a previous fermentation. The starter culture is typically added at a ratio of 1:1.6 (w/v; Yetneberk et al., 2004; Baye et al., 2013). The fermentation usually lasts 2–3 days (depending on weather conditions), after which the dough is thinned into a batter before baking on an open platter.

In this study, injera was prepared as described by Yetneberk et al. (2004). The 18 flour blends (Table 1) were placed separately in 2 L ice-cream containers and subsequently made into dough by soaking in 500–600 mL of tap-water depending on the total weight of the blends. The dough was kept at room temperature (25°C) for 96 h. Fermentation was initiated by adding an appropriate volume of *ersho* into each container holding the blended flour. At the end of the fermentation process, the pH of the dough was measured using a glass electrode attached to a Horiba B-712 pH meter (Horiba Ltd, Kyoto Japan). Subsequently, the liquid layer that typically forms over the dough was gently poured off, leaving a semisolid dough.

After fermentation, 10% of the fermented dough was thinned with 100 mL of water and cooked in 200 mL of boiling water for 1 min. The gelatinized batter was cooled to ≈45°C at room temperature and added back to the fermenting dough. About 200 g of the fermented batter was poured in a circular manner onto a 45-cm diameter hot clay griddle, covered, and baked for

TABLE 1 | Composition of the six blends of flour prepared for making injera from tef flour and *E. curvula* flour combined with sorghum flour.

Samples	<i>E. tef</i>			Samples	<i>E. curvula</i>		
	<i>E. tef</i> flour (g)	Sorghum flour (g)	Sorghum addition rate (%)		<i>E. curvula</i> flour (g)	Sorghum flour (g)	Sorghum addition rate (%)
A1	250	0	0	B1	250	0	0
A2	250	12.5	5	B2	250	12.5	5
A3	250	25	10	B3	250	25	10

approximate 2 min. The baked injera was then removed and kept in an airtight container.

Sensory Evaluation

In order to determine consumer acceptability of injera prepared using *E. tef* and *E. curvula*, a sensory evaluation was conducted. A semi-trained panel, consisting of 10 panelists (men and women) who regularly consume injera as their staple food, was selected following the criterion described by Stone and Sidel (2004). It was believed that this panel can provide a technical judgment of acceptability useful to predict potential consumer preference. The panelists were provided with the randomly sequenced 18 samples (6 blends replicated 3 times each) for testing. They were asked to evaluate the products for taste, texture (mouth feel), appearance (eye size, honeycomb structure of the top surface of the injera) and overall acceptability. In this study color as a sensory parameter was excluded due to the close similarity of the products in color (Figure 1). All the samples were presented to panelists in a flat tray at ambient temperature (about 25°C) 2–4 h after baking. Since the panelists were not fully-trained, and to make the evaluation process consistent, a simple 5-point hedonic scale (questioner) was used, where 5 was extremely positive (like) and 1 extremely negative (dislike) for each sensory attribute. The panelists were provided with water to rinse their mouths after tasting each sample.

Nutrient Analysis

The determination of protein, fat and fiber was carried out using the Dumas method (dry combustion) on the Leco TruMac™ instrument (2010 LECO Corporation, Saint Joseph, Michigan, USA). This involved a total combustion of the matrix under oxygen. The gases produced were reduced by copper and then dried, while the CO₂ was trapped. The nitrogen was then quantified using a universal detector. Mineral analysis was conducted using a Hunter apparatus (HCL extraction on the ICP), similar to that used for soil analysis.

Statistical Analysis

Data on the chemical composition of tef and *E. curvula* was subjected to a student *t*-test comparison using GenStat® (17th edition, VSN International, UK). The non-parametric data collected on taste, texture (mouth-feel), appearance and overall acceptability was analyzed using the Kruskal-Wallis H non-parametric test procedure. Means were compared using the non-parametric Mann-Whitney *U*-test procedure.

RESULTS AND DISCUSSION

Chemical Composition of *E. tef* and *E. curvula* Grains

The chemical composition of *E. tef* and *E. curvula* grains is presented in Table 2. Compared to *E. tef*, the seeds of *E. curvula* contained significantly (*P* < 0.001) higher levels of fat, ash, ADF (acid detergent fiber), NDF (natural detergent fiber) and more than double the amount of crude protein (18.47 ± 0.05 g/100 g sample). The measured values were higher than those measured in staple cereal crops such as rice, wheat, maize and sorghum (Moreno et al., 2014). The results of the current study were consistent with earlier reports (Jansen et al., 1962; Bekele and Lester, 1981). Bekele and Lester (1981) studied the variation in protein and amino acid composition both within and between 11 accessions tef varieties and 10 accessions of wild *Eragrostis* species, including *E. curvula*. They found that the wild species of *Eragrostis* had higher levels of protein and certain amino acids than the domesticated tef. This confirms that *E. curvula* is a nutritionally valid alternative to tef and other major cereal staples. The seeds of *E. curvula* also contained higher levels of minerals such as Zn, Cu, K, and Fe than *E. tef*. Higher levels of dietary fiber were also detectable in *E. curvula*, though it is unknown how such an amount of fiber would influence protein digestibility.

The flour of *E. tef* is gaining popularity in the Western World because of its attractive nutritional profile and its gluten-free nature. It can be used for the therapeutic treatment of patients with celiac disease (Moreno et al., 2014). However, grains of the pasture species, *E. curvula*, have superior levels of crude protein, dietary fiber, and minerals (Table 2). Therefore, cultivation and consumption of grains of *E. curvula* (also gluten-free) could be promoted in marginal production areas to contribute to food and nutrition security in these areas. In addition, its flour could also be promoted as an alternative to current gluten-free flour products.

Sensory Evaluation

Sensory evaluation is defined as the examination of a product (e.g., foods and beverages) through the evaluation of the attributes traceable by one or more of the five human senses—taste, smell, touch, sight, and hearing (Piana et al., 2004). It is used in food science to objectively analyse food quality. In many cases, it is an indispensable tool because it allows for the objective determination of whether or not consumers will accept a novel food product. Previous studies (Jansen et al., 1962; Bekele and

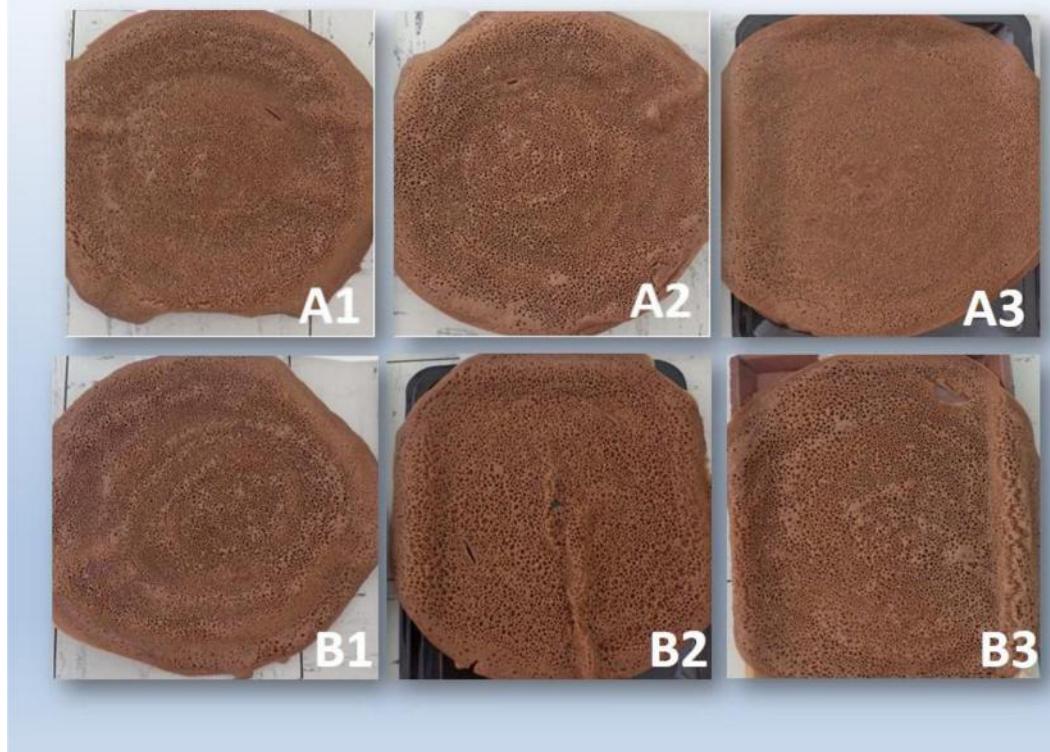


FIGURE 1 | The six injera products prepared from *E. tef* and *E. curvula* flours combined with 0, 5, and 10% of sorghum flour (Mable Meal). (A1), tef + 0% sorghum; (A2), tef + 5% sorghum; (A3), tef + 10% sorghum; (B1), *E. curvula* + 0% sorghum; (B2), *E. curvula* + 5% sorghum; (B3), *E. curvula* + 10% sorghum.

TABLE 2 | Nutrient analysis of whole grains of *Eragrostis tef* (cultivar SA-brown) and *E. curvula* (cultivar Ermelo) (On 100% dry basis).

Composition	Unit	<i>E. tef</i>	<i>E. curvula</i>	Unpaired <i>t</i> -test and <i>p</i> -value
Ash	g/100g	2.49 ± 0.03	3.18 ± 0.03	<i>t</i> (df = 8) <i>t</i> = -16.9; <i>P</i> = 0.000**
Fat	g/100g	2.64 ± 0.02	2.83 ± 0.05	<i>t</i> (df = 8) <i>t</i> = -3.48; <i>P</i> = 0.008*
ADF	g/100g	7.50 ± 0.64	18.34 ± 0.10	<i>t</i> (df = 8) <i>t</i> = -16.8; <i>P</i> = 0.000**
NDF	g/100 g	11.78 ± 0.57	24.30 ± 0.29	<i>t</i> (df = 8) <i>t</i> = -19.5; <i>P</i> = 0.000**
Crude-protein	g/100g	8.28 ± 0.04	18.47 ± 0.05	<i>t</i> (df = 8) <i>t</i> = -171; <i>P</i> = 0.000**
Ca	mg/100g	0.19 ± 0.04	0.22 ± 0.00	<i>t</i> (df = 8) <i>t</i> = -0.842; <i>P</i> = 0.424NS
Mg	mg/100g	354.18 ± 1.16	115.00 ± 0.63	<i>t</i> (df = 8) <i>t</i> = -26.0; <i>P</i> = 0.000 **
K	mg/100 g	0.42 ± 0.06	0.584 ± 0.03	<i>t</i> (df = 8) <i>t</i> = -20.2; <i>P</i> = 0.000**
Na %	mg/100 g	0.01 ± 0.00	0.00 ± 0.00	<i>t</i> (df = 8) <i>t</i> = 1; <i>P</i> = 0.347NS
K/Ca+Mg	mg/100 g	0.44 ± 0.02	0.48 ± 0.00	<i>t</i> (df = 8) <i>t</i> = -2.12; <i>P</i> = 0.067NS
P	mg/100 g	0.42 ± 0.00	0.41 ± 0.05	<i>t</i> (df = 8) <i>t</i> = -0.11; <i>P</i> = 1.00NS
Zn	mg/100 g	37.30 ± 0.81	51.43 ± 0.07	<i>t</i> (df = 8) <i>t</i> = -17.0; <i>P</i> = 0.00**
Cu	mg/100 g	4.27 ± 0.08	10.02 ± 0.14	<i>t</i> (df = 8) <i>t</i> = -21.2; <i>P</i> = 0.00**
Mn	mg/100 g	354.18 ± 1.17	114.94 ± 0.64	<i>t</i> (df = 8) <i>t</i> = 193; <i>P</i> = 0.00**
Fe	mg/100 g	50.78 ± 1.08	84.53 ± 0.93	<i>t</i> (df = 8) <i>t</i> = -21.8; <i>P</i> = 0.00**

Moisture % of samples as received by laboratory. NPN is non-protein nitrogen. ADFN and NDFN are calculated as nitrogen. Nitrogen % is calculated by dividing Protein by 6.25. The K/Ca + Mg should not be more than 2. Data on 100% dry matter basis.

*Significant difference at 5% level of significance.

**Highly significant difference at 1% level of significance.

TABLE 3 | Distribution of responses on a hedonic scale of 1–5 (bad to good), with resulting statistical indices for the six injera blends testing for taste, texture, appearance, and general acceptance.

	Assigned value	Frequency of responses					
		<i>E. tef</i>		<i>E. curvula</i>			
Injera varieties	A1	A2	A3	B1	B2	B3	
pH	3.8	4.1	4.0	3.9	3.8	3.8	
FOR TASTE							
Dislike very much	1	0	0	0	0	0	
Dislike moderately	2	0	0	1	1	2	
Neither like nor dislike	3	1	1	2	2	1	
Like moderately	4	6	2	4	2	6	
Like very much	5	3	7	3	5	1	
Total responses	10	10	10	10	10	10	
Mean rating	4.2 ^b	4.6 ^a	3.9 ^{cd}	4.1 ^{bc}	3.6 ^e	3.8 ^{de}	
SE	1.14	1.30	0.71	0.84	1.05	0.71	
% "Like" responses	90	90	70	70	70	70	
FOR TEXTURE							
Dislike very much	1	0	0	0	0	0	
Dislike moderately	2	0	0	0	0	1	
Neither like nor dislike	3	1	0	1	1	0	
Like moderately	4	4	1	6	4	8	
Like very much	5	5	9	3	5	1	
Total responses	10	10	10	10	10	10	
Mean rating	4.4 ^b	4.9 ^a	4.2 ^b	4.4 ^b	3.9 ^c	3.9 ^c	
SE							
% "Like" responses	90	100	90	90	90	70	
FOR APPEARANCE AND COLOR							
Dislike very much	1	0	0	0	0	0	
Dislike moderately	2	0	0	0	0	1	
Neither like nor dislike	3	0	0	0	0	3	
Like moderately	4	6	1	4	2	5	
Like very much	5	4	9	6	8	1	
Total responses	10	10	10	10	10	10	
Mean rating	4.4 ^{bc}	5.0 ^a	4.8 ^{ab}	4.8 ^{ab}	3.8 ^d	4.1 ^{cd}	
SE							
% "Like" responses	100	100	100	100	60	80	
FOR GENERAL ACCEPTANCE							
Dislike very much	1	0	0	0	0	0	
Dislike moderately	2	0	0	0	0	0	
Neither like nor dislike	3	0	0	0	0	2	
Like moderately	4	5	0	2	2	6	
Like very much	5	5	10	8	8	2	
Total responses	10	10	10	10	10	10	
Mean rating	4.5 ^b	5.0 ^a	4.8 ^{ab}	4.8 ^{ab}	4.0 ^c	4.6 ^{ab}	
SE							
% "Like" responses	100	100	100	100	80	100	

The injera were; tef (A1 = 0%, A2 = 5%, and A3 = 10% sorghum flour added) and *Eragrostis curvula* (B1 = 0%, B2 = 5%, and B3 = 10% sorghum flour added).

^{a–e}Mean rating values in the same row with shared letter(s) are not statistically different according to Duncan's multiple range test at 5% level of significance.

Lester, 1981) that report on the nutritional value of *E. tef* and *E. curvula* did not evaluate its acceptability. This information is important for the successful promotion of *E. curvula* as a healthy alternative in the diets of people.

TABLE 4 | Chi-square values comparing the six flour blends using the Kruskal–Wallis non-parametric test and Pair-wise comparison of the six injera using the Mann–Whitney *U* statistics for the four sensory parameters, Taste, Texture, Appearance, and Overall Acceptability.

No.	Pairs	Taste	Texture	Appearance	Overall acceptability
1	A1 and A2	19.00*	25.00 ns	20.00*	25.00 ns
2	A1 and A3	49.5 ns	45.00 ns	30.00 ns	35.00 ns
3	A1 and B1	40.5 ns	34.00 ns	30.00 ns	35.00 ns
4	A1 and B2	37.00 ns	36.00 ns	31.00 ns	30.00 ns
5	A1 and B3	41.5 ns	36.50 ns	37.00 ns	45.00 ns
6	A2 and A3	27.00 ns	30.00 ns	40.00 ns	40.00 ns
7	A2 and B1	37.00 ns	40.00 ns	40.00 ns	40.00 ns
8	A2 and B2	12.00*	10.00*	10.00*	10.00*
9	A2 and B3	17.00*	10.00*	10.00*	30.00 ns
10	A3 and B1	43.00 ns	39.00 ns	50.00 ns	50.00 ns
11	A3 and B2	39.5 ns	31.50 ns	17.00*	18.00*
12	A3 and B3	43.00 ns	32.00 ns	19.00*	40.00 ns
13	B1 and B2	32.5 ns	19.00*	17.00*	18.00*
14	B1 and B3	36.00 ns	19.00*	19.00*	40.00 ns
15	B2 and B3	46.50 ns	49.5 ns	41.50 ns	26.00 ns
Chi-Square		11.63	18.46	24.93	19.45
Df		5	5	5	5
Significance level		0.040	0.020	0.000	0.002

*Denotes significant difference and ns, non-significant difference both at 5% level of significance.

In the current study, a panel of 10 judges was used to describe the degree of consumer acceptance and satisfaction to the injera prepared using different combinations of *E. tef* and *E. curvula* flour, combined with sorghum flour. The taste of injera is associated with the sweet, sour and bitter sensations triggered in the mouth by contact with the injera. The sensory responses of the tasting panel to the injera prepared from six different blends of flours of sorghum, tef and *E. curvula* are provided in **Table 3**. Pair-wise comparisons of the products are given in **Table 4**. Out of the six injera samples, Sample A2 (tef + 5% sorghum) was the most preferred taste, scoring a 90% positive (like) response and the highest mean rating 4.6 followed by injera of Sample A1 (tef + 0% sorghum added) with a mean rating of 4.2. Pair-wise comparison between and among the injera prepared from tef (A1) and all the *E. curvula* flours (B1, B2, and B3) showed non-significant differences in taste. This implies that injera prepared using *E. curvula* flour tasted the same as the traditional injera prepared from tef flour.

The taste of Sample A3 (tef + 10% sorghum) was not significantly different from the taste of all the other injera prepared from *E. curvula* flour (B1, B2, and B3), and 70% of the panelist liked the taste of Samples A3, B1, B2, and B3. The dough of the six injera blends showed no significant difference in acidity. Combining tef flour with 5% sorghum flour significantly improved the taste and appearance of injera. By contrast, combining *E. curvula* flour with 5 and 10% sorghum flour caused a significant negative impact on the texture and appearance of injera (**Table 4**).

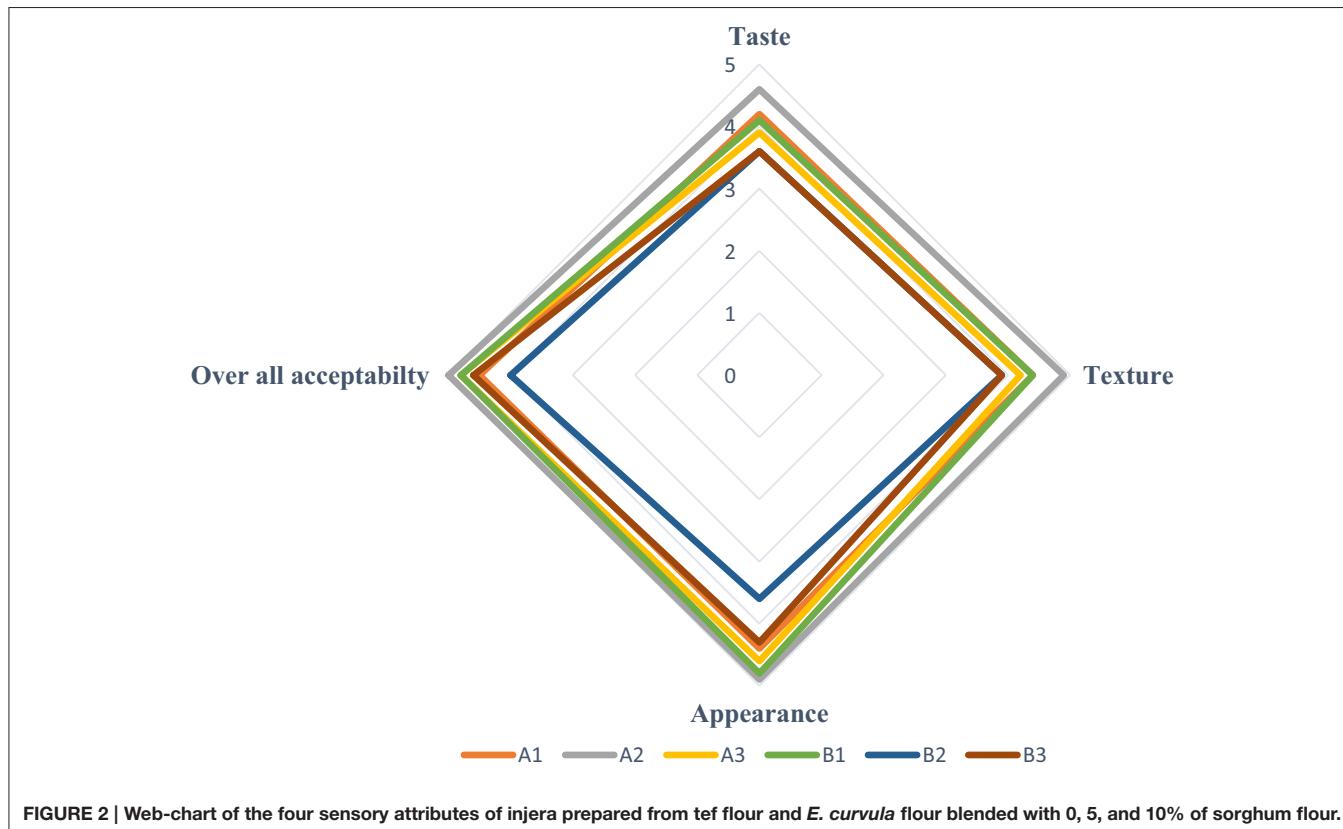


FIGURE 2 | Web-chart of the four sensory attributes of injera prepared from tef flour and *E. curvula* flour blended with 0, 5, and 10% of sorghum flour.

Texture is another important parameter often used to measure the quality of breads. It is determined by touch and refers to the degree of fluffiness, roughness, smoothness, hardness or softness. Out of the six samples, Sample A2 (tef + 5% sorghum added) was the most liked for texture, scoring a 100% positive (like) response, with the highest mean rating of 4.9, followed by Samples A1 (tef + 0% sorghum), A3 (tef + 10% sorghum), and B1 (*E. curvula* + 0% sorghum). 90% of the panelists liked the textures of injera of Samples A1, A3, B1, and B2. The lowest positive response was the injera of Sample B3 (*E. curvula* + 10% sorghum). Compared to the positive Control (Sample A1, tef flour with no sorghum added), the injera prepared from *E. curvula* flour with no sorghum added (B1) showed no significant difference in texture (Table 3) and were as likable as the Control. This indicates that the flour of *E. curvula* can be used to produce a well-textured injera. However, adding sorghum flour significantly decreased the quality of the texture of the injera made from *E. curvula* flour.

The appearance of injera is one of the most important parameters, which refers to the quality of the eyes (cells) of the honeycomb-like structure of the top surface of injera formed during cooking due to escaping CO₂ bubbles (Yetneberk et al., 2005). The color of injera also affects the appearance of the injera in relation to its aesthetic appeal. In areas where injera is consumed as a staple food, (Eritrea and Ethiopia), people prefer their injera be white in color (Gebrekidan and GhebreHiwot, 1982). In this study, all the injera prepared from *E. curvula* and tef (cultivar SA-brown) flours were brown in color (Figure 1). The sensory test showed significant differences ($P < 0.001$) in

the appearances of the samples. The injera of Samples A2, A3, and B1 were the most preferred samples, followed by A1 and B6. The most interesting result was that injera prepared from 100% of *E. curvula* flour (Sample B1) was highly rated for its appearances similar to a classic injera. Even when blended with 5 and 10% sorghum flour, the appearance of injera prepared from *E. curvula* flour was liked by 60 and 80% of the panelists, respectively (Figure 1; Table 4).

Overall acceptability refers to the combinations of evaluations by consumers or panelists of a product. In this experiment, results showed that there was a statistically significant difference ($P < 0.001$) in the overall acceptability of the six injera samples. Injera of Samples A2, A3, B1, and B3 were the most acceptable followed by that of Sample A1. Similarly, 100% of the panelists liked all the injera prepared from tef (A1, A2, and A3). One hundred percentage of the panelists also accepted the overall qualities of injera prepared from two *E. curvula* flours (B1 and B3). However, Sample B2 (*E. curvula* + 5% sorghum) produced the least acceptable product, scoring a mean rating of 4.0 and an 80% overall acceptability (Table 4).

Taking all sensory attributes into account, though there was a statistically significant difference among samples, all blends scored a mean rating well above average (Table 3) which is an indicative of the goodness as products (Figure 2). The most preferred injera was produced from tef flour combined with 5% sorghum flour (Sample A2). However, the injera prepared from the flour of *E. curvula* also produced an excellent quality injera, especially when *E. curvula* flour with no sorghum was used

(Sample B1). Apart from sorghum grains, grains of other crops may also be tried by blending in different proportion to prepare value added products from *E. curvula*.

CONCLUSIONS

The present study revealed that grains of *E. curvula* contain high levels of protein, dietary fiber and minerals such as Fe and Mg, and that these values were substantially higher than tef and most other cereals. The injera (breads) made from flour of *E. curvula* had positive sensory attributes (taste, texture, appearance and overall acceptability) similar to those of the traditional injera made using tef flour. These findings suggest that beyond its current use as a pasture crop, flour from the grain of *E. curvula* could serve as an alternative source of food to produce high quality injera for human consumption, and could become a valued gluten-free flour globally. It is also possible that the grains of *E. curvula* could serve as a raw material for other food products such as porridge, biscuits, muffins, beer and beverages. Given that *E. curvula* is a drought resistant perennial grass compared to tef (annual grass), and given its hardiness and tolerance of acid soils, there is scope to promote its cultivation and utilization in semi-arid areas of sub-Saharan Africa where other major cereal staples do not perform well. Even in Ethiopia

and Eritrea, it could be grown in regions with severe soil acidity that limits tef production. However, further studies are required to evaluate the nutritional qualities, health benefits and grain productivity of *E. curvula* as necessary steps toward developing it as a productive, nutritious grain crop that can be grown under semi-arid conditions.

AUTHOR CONTRIBUTIONS

The idea of testing the grains of *Eragrostis curvula* for injera making was first conceived by KK. Afterwards, ML, SH, and HG designed and carried out a series of experiments. The manuscript was prepared by HG and TM. The manuscript was edited by all authors.

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Biological Implications in Cassava for the Production of Amylose-Free Starch: Impact on Root Yield and Related Traits

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Cassava (*Manihot esculenta*, Crantz) is an important food security crop, but it is becoming an important raw material for different industrial applications. Cassava is the second most important source of starch worldwide. Novel starch properties are of interest to the starch industry, and one them is the recently identified amylose-free (waxy) cassava starch. Waxy mutants have been found in different crops and have been often associated with a yield penalty. There are ongoing efforts to develop commercial cassava varieties with amylose-free starch. However, little information is available regarding the biological and agronomic implications of starch mutations in cassava, nor in other root and tuber crops. In this study, siblings from eight full-sib families, segregating for the waxy trait, were used to determine if the mutation has implications for yield, dry matter content (DMC) and harvest index in cassava. A total of 87 waxy and 87 wild-type starch genotypes from the eight families were used in the study. The only significant effect of starch type was on DMC ($p < 0.01$), with waxy clones having a 0.8% lower content than their wild type counterparts. There was no effect of starch type on fresh root yield (FRY), adjusted FRY and harvest index. It is not clear if lower DMC is a pleiotropic effect of the waxy starch mutation or else the result of linked genes introgressed along with the mutation. It is expected that commercial waxy cassava varieties will have competitive FRYs but special efforts will be required to attain adequate DMCs. This study contributes to the limited knowledge available of the impact of starch mutations on the agronomic performance of root and tuber crops.

Keywords: waxy starch, yield penalty, cassava markets, economic impact, root and tuber crops

INTRODUCTION

Cassava (*Manihot esculenta*, Crantz) is an important source of food calories in sub-Saharan Africa, fulfilling a critical role as a food security crop (Haggblade et al., 2012). Its roots are also one of the most important sources of commercial starch in tropical and subtropical countries (Moorthy, 2004). In fact, the crop is the second most important source of starch worldwide, after maize, and the most traded one (Stapleton, 2012). The global export of cassava starch and flour in 2014 amounted to 8.5 million tons (Food and Agriculture Organization [FAO], 2015). In South and South-East Asia, starch export has been one of the drivers of cassava expansion and 40% of the total

cassava production is used for starch extraction (Onwueme, 2002; Fuglie et al., 2006). Applications of cassava starch (also known as tapioca) is found in the textile and pharmaceutical industry and within food manufacturing, for which it is well suited since it has a bland taste and produces a clear paste (Jobling, 2004; Fuglie et al., 2006; Food and Agriculture Organization [FAO], 2015).

Amylose-free (or waxy) starch phenotype was first identified in maize germplasm from China (Collins, 1909; Collins and Kempton, 1914) and few years later in the segregating progeny from a landrace (Sanford's White Flint) in Connecticut (Mangelsdorf, 1924). The recessive nature of this trait was also reported about a century ago (Collins and Kempton, 1914; Mangelsdorf, 1924; Kiesslbach and Petersen, 1926). During the Second World War the United States could not import tapioca from Thailand. The industry searched for alternatives to the cassava starch it could no longer import and found that functional properties of waxy maize starch resembled more closely (than wild type maize) those of tapioca. The industry of waxy maize was thus borne (Fergason, 2001). Spontaneous waxy starch mutations have been found in barley, wheat, rice, potato and sorghum (Wang et al., 1995; Graybosch, 1998; McPherson and Jane, 1999; Song and Jane, 2000). Low amylose genotypes of yam have also been identified (Pérez et al., 2011). Compared to their wild-type counterparts, amylose-free starches generally show a higher swelling power, higher peak viscosity, clearer pastes and a better freeze-thaw stability, which are highly desirable properties in many food applications (Jobling, 2004).

In spite of the relevance of cassava for the starch industry, no commercial cassava varieties offering special functional properties has ever been released. Amylose content has profound effect on starch functional properties in different crops as stated above. The average starch content of the cassava accessions in the FAO cassava germplasm collection is 84.5% (on a dry root basis) and the average amylose content is 20.7%, ranging between 15.2 and 26.5% (Sánchez et al., 2009). It was only in 2006 that a cassava mutant lacking amylose in the starch was discovered at the International Center for Tropical Agriculture (CIAT). The discovery came as result of self-pollinations of a large number of cassava genotypes, conducted to bring forward low-frequency recessive traits in the crop (Ceballos et al., 2007). Recently new sources of amylose-free cassava have been identified (Morante et al., 2016). The development of varieties with waxy starch had long been an objective for the cassava community. It was envisioned that waxy varieties could better fit the needs for specific uses and, therefore, entail higher selling prices of the roots and/or strengthen markets for cassava. In other words, a commercial variety with amylose-free starch would benefit both, farmers and processors. The waxy starch trait is so important that successful transgenic approaches to down-regulate *GBSSI* have also been conducted (Raemakers et al., 2003, 2005; Zhao et al., 2011).

The lack of amylose in waxy genotypes has been shown to be the result of a recessive mutation in the *wx* locus encoding granule-bound starch synthase I (*GBSSI*), the protein responsible for the elongation of amylose in the starch granule (Fukunaga et al., 2002; Larkin and Park, 2003; Ceballos et al., 2007; McIntyre et al., 2008). The full-length sequence of the cassava

GBSSI gene has been determined (Aiemnaka et al., 2012) and single-nucleotide amplified polymorphism (SNAP) markers has been developed to differentiate waxy (*wx wx*) from non-waxy heterozygous (*Wx wx*) and non-waxy homozygous (*Wx Wx*) genotypes. In most cases waxy starches lack completely amylose. However, in some cases small amount of amylose (<5%) can be found in certain waxy mutations in maize (Andres and Basciollo, cited by Fergason, 2001). In the case of cassava spontaneous mutations in cassava (Ceballos et al., 2007; Morante et al., 2016) have no amylose, whereas transgenic lines vary from about 2–10% of amylose (Zhao et al., 2011; Koehorst-van Putten et al., 2012).

Since the discovery of the waxy starch mutation in cassava several studies have been conducted to characterize its functional properties (Ceballos et al., 2007; Sánchez et al., 2010; Rolland-Sabaté et al., 2012, 2013). Compared to other waxy and non-waxy starches, waxy cassava starch has been shown to have improved freeze-thaw stability (Raemakers et al., 2005; Sánchez et al., 2010; Dufour et al., 2013) as also reported in potato (Jobling et al., 2002). In addition, waxy cassava starch was reported to have a clearer paste and a higher peak viscosity than other waxy and non-waxy starches, with the exception of potato (Sánchez et al., 2010). The starch content of the first non-transgenic waxy cassava genotype (AM 206-5) was shown to be comparable to that of two wild-type starch varieties (Ceballos et al., 2007).

The advantages of the *in planta* versus *in vitro* modification of starch functional properties has been already reported (Slattery et al., 2000; Davis et al., 2003). Waxy starches from root and tuber crops (e.g., cassava and potato) offer the advantage of clearer gels, bland or neutral flavor and taste (Koehorst-van Putten et al., 2012) and higher viscosities and different gel textures (Sánchez et al., 2010) compared with those from cereals. It is undeniable that waxy cassava starch offers enough advantages for the industry. Consequently, projects to develop non-transgenic commercial cassava varieties producing amylose-free starch have been initiated (Aiemnaka et al., 2012) with the support of the private sector. This is the first time ever that the starch industry invests in cassava breeding and it is an evidence of the interest generated by this waxy starch. However, it is not clear what the biological impact is when a cassava plant produces amylose-free starch. For a commercial variety and the entire value chain to be successful, the advantages derived from the special properties of the waxy starch must not be counterbalanced by potential agronomic disadvantages of the varieties producing it. The objective of this study, therefore, was to determine the effect on cassava yield and yield components of the waxy trait in cassava (Karlström, 2015).

MATERIALS AND METHODS

Parental Material and Population Development

This study took advantage of eight full sib families (same male and female progenitor) segregating for the mutation at the *GBSSI* locus. The families were developed from *F*₁ plants which were obtained from crosses between the first identified source of waxy starch (AM 206-5) and eight elite varieties with good

TABLE 1 | Description of family size, number of selected plants and distribution within sets for each of the eight families.

Family	Selected (waxy + wild type)	Set 1	Set 2	Set 3
GM 5458	10+10	3	3	4
GM 5466	15+15	5	5	5
GM 5507	8+8	3	3	2
GM 5536	8+8	3	3	2
GM 5615	8+8	3	3	2
GM 5619	8+8	3	3	2
GM 5672	15+15	5	5	5
GM 5722	15+15	5	5	5
Total	87+87	30	30	27

agronomic characteristics adapted to the main cassava growing environments in Colombia (except highlands). The F₁ plants (heterozygous for the waxy allele) from unrelated families were crossed to produce a pseudo-F₂ generation. **Table 1** describes the full-sib families from this F₂ generation. Starch type was identified by screening the roots with the reliable iodine staining technique first reported by Weatherwax (1922). Waxy roots are typically stained reddish-brown by the iodine solution, whereas wild type starch roots stain dark-blue. Homozygote (*Wx Wx*) and heterozygote (*Wx wx*) wild type plants were not distinguished in the study.

The number of genotypes in each family varied and, therefore, each family was represented by a different number of genotypes (**Table 1**). However, for each family, an equal number of waxy

and wild type genotypes were chosen (87 genotypes per type of starch). As seen in **Figure 1**, family GM 5619 (used as an example) had 38 genotypes, with 15 of them producing waxy starch and the remaining 23 with amylose-containing starch. A total of eight genotypes among the group of the 15 producing waxy starch were randomly chosen. Similarly, eight genotypes with wild type starch out of the 23 found in this family were randomly picked. It is assumed that each of the two groups of eight genotypes are random samples of the genetic diversity of this family, with the exception that one group produced waxy starch and the other group produced wild type starch. A similar approach was taken for the seven remaining families.

Field Evaluation

The effects of the waxy trait on yield and yield components were evaluated based on data from a field-trial conducted at CIAT experimental station, Palmira, Valle del Cauca, Colombia. Soils at this station are fine-silty, mixed, isohyperthermic Aquic Hapludol (Duque-Vargas et al., 1994). Standard cultural practices were used for this evaluation, providing irrigation when necessary, and keeping the main pest problems (particularly the whitefly *Aleurotrachellus socialis* and different mite species) under control.

Experimental Design

A special field design was used for a truly randomized distribution of the plants and genotypes involved in the study. Instead of planting all genotypes from a certain family together, they were more or less equally split into three different sets (**Table 1**). Each set had a combination of about 1/3 of the

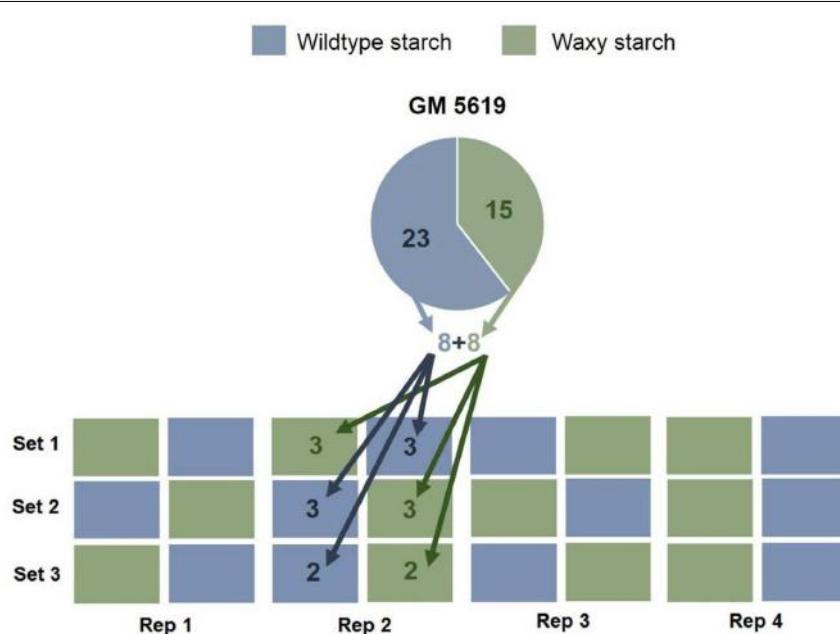


FIGURE 1 | Illustration of how the genotypes from each of the eight families were used to obtain random and representative samples. Family GM 5619 had 23 and 15 genotypes producing wildtype and waxy starch, respectively. Eight waxy and eight wild-type genotypes were randomly chosen and allocated into three sets (3+3+2 = 8 genotypes). Each set was then replicated four times. The waxy and wildtype starch genotypes from the other seven families were similarly distributed for a total of 30 plants in each plot.

genotypes from each of the eight families and were all planted simultaneously in the same field. Each set contained a total of 30 waxy and 30 wild type genotypes from the same F₂ families except set 3 which only had 27 genotypes of each starch type (**Figure 1**). Therefore, three additional genotypes were added to the third set as filling (they were not included in the analysis of results). The three sets of genotypes were replicated in four blocks. In other words, the same genotype was cloned and replicated four times (one plant per genotype and block). This added up to a total of 348 waxy and 348 wildtype plants ($87 \times 4 = 348$). In each plot with 30 plants, planting was done in five rows with six plants per row. The plants were harvested, as usual by hand, at 11 months after planting.

The data collected at harvest were fresh root yield (FRY), harvest index (HIN, root weight/total weight of biomass including roots) and dry matter content (DMC). HIN is usually used as an indirect yield indicator in cassava after reports of a higher correlation between HIN in single row trials and FRY performance in replicated plots in multi-location trials than the correlation between root yields in the same type of trials (Kawano et al., 1978). Because of the volume and weight of a total of 30 plants at harvest, measurements were done on a total per row within each plot. Results from the six rows in each plot were then combined for further analysis. Data from individual genotypes, therefore, were not available. FRY was measured by bulking the harvested roots from the five plants of each row and then adding the weights across the six rows from each plot. The FRY per hectare ($t \text{ ha}^{-1}$) was then estimated taking into account the area of each plot. Roots with symptoms of rotting were discarded and not included in the measurement. When plants were missing an adjustment for missing plants was used in the estimation of FRY (Ad.FRY; Pérez et al., 2010). HIN was also estimated by bulking the weight of roots and above ground biomass from the plants of each row. Averages for each plot were then calculated. DMC was estimated using the gravimetric method (Kawano et al., 1978; Toro and Cañas, 2012) by bulking the roots from each row, with two replicated measurements per row. The two measurements per row were then used to calculate the average DMC across the six rows in each plot. Therefore a total of 12 independent estimations for DMC among the 30 plants in each plot were used for the average DMC.

Statistical Analysis

The main question to answer by this study is the biological impact of the waxy starch mutation in key agronomic traits.

Although there is genetic variation among the 87 genotypes (within each type of starch), analysis was done on the bulked data. Individual genotypes within each starch group were randomly chosen. Genotypes representing each group are considered to be random samples of the genetic variation within each family. The only difference being the production of waxy or amylose-containing starch. Although variation among individual genotypes is relevant, the main objective of this study focused on the impact of the mutation across different genotypes. The data for each plot (bulked across rows) was used for the analysis of variance. Because of the field design the statistical analysis were conducted with the sets nested within replicate effects and starch type nested within set effects. All sources of variation in the analysis of variance were considered random, except for the type of starch. Statistical analysis was made using the SAS Software and the Proc GLM procedure (SAS, 2008).

RESULTS

Plant germination and growth was satisfactory. Environmental conditions were drier than normal. Since irrigation was available when necessary, plants did not suffer drought stress but grew under slightly higher than normal average temperatures and lower relative moisture in the air. Averages for FRY, HIN and DMC of the waxy genotypes and their wild type sibling counterparts are presented in **Table 2**. Starch type was confirmed using the efficient and reliable iodine test (**Figure 2**).

Starch type (waxy versus wild type) had no significant effect on FRY, with similar results when yields were adjusted to take missing plants into consideration. Only few plants were missing in the entire experiment and, therefore, an agreement between the two ways to assess FRY was expected. The only significant effect on FRY and Ad.FRY was for the replication source of variation ($p \leq 0.05$). Likewise, there was no significant effect of starch type on HIN, while both replication and set within replications had highly significant ($p \leq 0.01$) effects (**Table 3**).

There was a highly significant effect ($p \leq 0.01$), on the other hand, of starch type on DMC. In fact this is the only case where starch type had a significant effect in any variable. Average DMC in waxy starch genotypes was 32.8%, whereas in wild type starch genotypes the average was 33.6%. The difference of 0.8% is small, but nonetheless, highly significant from a statistical point of view. Replication and sets within replication also showed highly significant ($p \leq 0.01$) effects on DMC (**Tables 2 and 3**).

TABLE 2 | Means and standard deviations of FRY, adjusted FRY, DMC, and HI for the waxy and wild type starch cassava full-siblings.

Starch type	FRY ($t \text{ ha}^{-1}$)	Ad.FRY ($t \text{ ha}^{-1}$)	DMC (%)	HIN (0–1)
Waxy	7.24 (± 2.40)	7.57 (± 2.58)	32.8 (± 1.49)	0.41 (± 0.08)
Wild-type	7.40 (± 2.73)	7.67 (± 3.61)	33.6 (± 1.61)	0.38 (± 0.07)
LSD _{0.05}	0.91	0.94	0.46	0.02
LSD _{0.01}	1.21	1.25	0.61	0.03

Also included is the least significant difference (LSD) at the 0.05 and 0.01 level for the analysis of variance.



FIGURE 2 | Illustration of the staining technique based on an iodine solution used to identify roots with waxy starch (staining reddish) or amylose-containing wild type starch (staining blue).

TABLE 3 | Mean squares from the analysis of variance for FRY, adjusted FRY, DMC, and HI in eight full sibling families segregating for the waxy trait.

Source of variation	df	FRY	Ad.FRY	DMC	HIN
Replicate	3	24.4*	26.8*	7.8**	0.056**
Set (Replicate)	8	9.2	10.3	6.2**	0.013**
Starch type (Set* Replicate)	12	2.9	3.7	6.1**	0.005
Error	96	6.3	6.7	1.6	0.004
Coefficient of variation	—	34.2	34.1	3.8	16.4

* , **Significant at the 0.05 and 0.01 probability level, respectively.

DISCUSSION

The present evaluation aims at assessing the biological implication for cassava (without any previous selection) to produce roots with waxy starch. The average performance of the waxy clones compared with their wild type counterparts is useful only for understanding the biological impact of the mutation. Results from this study suggest that selected clones with waxy starch will be competitive regarding FRY. Results for DMC, on the other hand, raised some concerns since predictions indicate lower DMC on waxy genotypes. Data from individual genotypes was not taken because the main interest focused on the differences in the average performance of waxy versus

wild-type genotypes. Therefore, results from this study are not able to determine if individual clone(s) producing amylose-free starch can or cannot reach competitive levels of DMC as well. This distinction is important because it is outstanding individual (waxy) cassava clones which will be grown commercially by farmers, not the entire full sib families, but the study was not designed to address this issue.

The most advanced program to develop waxy commercial cassava varieties (in Thailand) faced problems selecting progenies with DMC comparable with those of commercial varieties, whereas for FRY results were comparable for both types of starch (Rodjananidpichet et al., 2012). Preliminary results from the ongoing work to develop amylose-free commercial cassava

varieties in Colombia also points to difficulties in achieving the levels of DMC observed in commercial checks (data not presented). However, differences in DMC comparing commercial checks and selected waxy starch clones were as large as 3–4% (Rodjanaridpichet et al., 2012). The present study, on the other hand, reports differences in DMC of less than 1%. Although, the penalty on DMC is relatively small (**Table 2**), it could potentially become a quantitatively important economic loss in large scale processing of (waxy) cassava starch. However, yield penalty (through a reduced DMC) should not be as large as those reported in the first batch of waxy cassava clones reported by Rodjanaridpichet and co-workers in 2012. Results from this study suggest that the next selection cycle of breeding for waxy cassava starch should focus particularly in identifying materials with higher DMC.

Results from Thailand and Colombia compared preliminary selections of waxy cassava varieties (out of less than 3000 initial genotypes) with the best commercial varieties available (which had been selected after many years of evaluation and arose after testing 10s of 1000s of genotypes). They are, therefore unfair comparisons which would tend to overestimate the actual effect for a cassava plant producing roots with waxy starch. Perhaps the most relevant study to assess the biological impact of waxy starch in cassava is the ongoing work to evaluate waxy transgenic cassava developed at Wageningen University (Koehorst-van Putten et al., 2012). The Indonesian cultivar *Adira 4* was transformed using the antisense approach to generate versions of the same genotype but with amylose contents ranging from 2 to 10%. The advantage of that study is that basically the same genotype producing contrasting starch types were compared. The comparison of non-transformed versus transformed versions of the cultivar indicated that there was no major difference for FRY. This agrees with results in Thailand and Colombia to develop commercial waxy varieties based on the spontaneous mutation as well as with the results presented in this study. Unfortunately, however, the authors (Koehorst-van Putten et al., 2012) did not report on DMC in that research article.

Results with waxy cassava agree, to some extent, with those reported for maize and other cereals. Waxy maize hybrids had higher moisture at harvest (22.7 versus 21.2%) and lower yields (9.33 versus 9.66 t ha⁻¹) than the normal starch counterparts (Ferguson, 2001). Similarly, Oscarsson et al. (1998) found that waxy barley varieties were among the lowest yielding and with lowest starch content within a group of 10 varieties. Rooney et al. (2005) reported a 17% reduction in yield in waxy sorghum compared to non-waxy counterparts derived from the same population, while Jampala et al. (2012) found no effect of starch

type on grain yield in a sorghum population segregating for the waxy trait. In a study of waxy wheat lines, they were shown to have lower flour yields but the same grain yield as commercial checks (Graybosch et al., 2003).

It is not clear if the low DMC in waxy starch cassava genotypes is related to the biosynthesis of amylopectin in these materials (e.g., pleiotropic effect) or else if the original mutation at the GBSS locus is linked to other loci influencing DMC. If the latter hypothesis is correct, then further sexual crosses among amylose-free cassava genotypes should increase the chances of breaking the eventual linkage between waxy starch and lower-than-desirable DMC. This study provides an unbiased estimate of the biological impact of the waxy starch mutation in cassava without any previous selection: a reduction of less than 1% in DMC. The larger penalty observed in ongoing breeding work (3–4%) may be explained, as suggested above, by the size of populations used for selecting waxy starch versus wild types.

As it is the case waxy maize grains and their derived starch (Elbehri and Paarlberg, 2003), it can be expected that waxy cassava will be sold for a premium price, thereby economically compensating the eventual lower DMC in the roots. The Global Cassava Development Strategy (Food and Agriculture Organization/International Fund for Agriculture Development [FAO/IFAD], 2000) identified the need to strengthen markets for cassava products as a key strategy to realize the impact that this crop can have in the livelihood of millions of people. It is envisioned that developing competitive commercial varieties with amylose-free starch will strengthen the already thriving market for cassava starch, which ultimately helps not only the industry but farmers as well.

AUTHOR CONTRIBUTIONS

AK conducted the basic research and took field data as part of her M.Sc dissertation work. She also contributed with data handling and analysis; SS and FC are the assistants of the cassava breeding program and contributed with the original planting of the experiment and continue with further work on the subject; NM is the assistant in charge of making the crosses in the cassava breeding program. All sexual seed used to generate the materials evaluated in this study were produced by his team; DD is an expert in food technology. Many articles describing the functional properties of waxy cassava were co-authored by him; HC is the senior cassava breeder at CIAT and was involved in this project from the very beginning by asking the question that this research attempted to answer. He also contributed with the field design.

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From the Lab to the Farm: An Industrial Perspective of Plant Beneficial Microorganisms

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Any successful strategy aimed at enhancing crop productivity with microbial products ultimately relies on the ability to scale at regional to global levels. Microorganisms that show promise in the lab may lack key characteristics for widespread adoption in sustainable and productive agricultural systems. This paper provides an overview of critical considerations involved with taking a strain from discovery to the farmer's field. In addition, we review some of the most effective microbial products on the market today, explore the reasons for their success and outline some of the major challenges involved in industrial production and commercialization of beneficial strains for widespread agricultural application. General processes associated with commercializing viable microbial products are discussed in two broad categories, biofertility inoculants and biocontrol products. Specifically, we address what farmers desire in potential microbial products, how mode of action informs decisions on product applications, the influence of variation in laboratory and field study data, challenges with scaling for mass production, and the importance of consistent efficacy, product stability and quality. In order to make a significant impact on global sustainable agriculture, the implementation of plant beneficial microorganisms will require a more seamless transition between laboratory and farm application. Early attention to the challenges presented here will improve the likelihood of developing effective microbial products to improve crop yields, decrease disease severity, and help to feed an increasingly hungry planet.

Keywords: biofertility, biocontrol, commercialization, agricultural products, food security

INTRODUCTION

The alarm cry of impending global food shortages is not new. Over the centuries figures such as Tertullian, Townsend, Malthus, and Ehrlich (Hardin, 1998; Alexandratos and Bruinsma, 2012) have warned of dire consequences of the inability of Earth's capacity to sustain its growing population (Ehrlich and Ehrlich, 1990). Each time, crisis has been averted due to technological advances in plant breeding, fertilization, crop protection and agronomic management. For example, over the past 50 years the human population of our planet has doubled, and the need for increased food production was met by the application of new technologies, such as the discovery of the Haber-Bosch process (Erisman et al., 2008), and agronomic management strategies. Although they contributed to staving widespread famine and saving billions of lives, novel and complementary solutions are needed to continue to improve crop yield. As we face our

next challenge, it is critical that we continue to discover new sustainable cropping system solutions to produce more with fewer resources.

By the year 2050, the global population is expected to reach 9.6 billion which has been estimated as our planet's maximum capacity (Wilson, 2003). This increase in population will require at least double our current agricultural production, despite the challenges with current resource requirements and a decline in arable land (Bruinsma, 2009). Similar to the green revolution, in order to ensure global food security for a growing population we need to devise enhanced cropping systems that maximize productivity while minimizing the resources required. In most agricultural lands, maximizing yield requires additional inputs to maintain productivity and crop yields. These additions include both phosphorus and nitrogen as fertilizer, as well as pesticides that help control invasive weeds, pathogens and insects. Farmers could benefit from new sustainable products to boost or maintain yields, often under increasing environmental stresses (Baulcombe et al., 2009). While chemistries and trait development remain critical in developing stress tolerance and pathogen resistance programs of agriculture, the application of microbial products is now considered a valuable addition to precision agriculture (Berg, 2009; Bhattacharyya and Jha, 2012).

Microbial products have been used commercially in global agriculture for over 120 years (Nobbe and Hiltner, 1896; Deaker et al., 2004), but have recently received increased attention. There are currently over 149 registered microbial strains for agricultural products (Copping, 2009). A recent special publication by the American Society for Microbiology suggested that microbes may be, at least in part, a sustainable solution to increasing agricultural production and outlined current shortcomings of microbes in helping to feed the world (Reid and Greene, 2013). The market for commercial biofertility inoculant and biocontrol products in 2012 was valued at over \$1 billion US dollars (USD) and is expected to exceed \$7 billion USD by 2019, increasing at a double digit compound annual growth rate (CAGR) between 2013 and 2019 (Transparency Market Research, 2014). Major growth drivers include growing consumer interest in organic crops, reducing synthetic products, and the economic potential in emerging markets such as China (Transparency Market Research, 2014). Despite the benefits and potential of agricultural microbial products, a recent spotlight on plant yield promoting bacteria pointed out that "The scientific literature abounds with many potentially highly useful strains that did not appear on the commercial market" (Bashan et al., 2014). In a 30 year span ending in 2002, an estimated 72% of biocontrol business ventures failed (Glare et al., 2012). Most often, failures result from underestimating costs associated with developing and marketing microbial products (CPL, 2006, Biopesticides). The incongruence between effective microbial strains and successful agricultural products suggests a need to address obstacles that may not be anticipated.

Microbes will certainly play a role in revolutionizing agriculture over the next several decades to help meet the demands of a growing population. Promising agricultural products include organisms that increase crop yield through enhanced nutrient uptake by plants (inoculants), and organisms

that reduce crop loss due to pests (biocontrol). While timely and extremely valuable, the American Society for Microbiology report (Reid and Greene, 2013) focuses primarily on what Bashan et al. (2014) call the 'research facility' side of product development and omits important characteristics of the 'industry' role. This review provides an industrial perspective on the current state of these types of microbial products. Also, in an effort to help maximize the number of strains that make a practical impact on agriculture, some of the challenges involved with taking a successful laboratory strain and making a viable commercial product are discussed.

BIOFERTILITY INOCULANTS

Deployment of microbes to enhance crop productivity by boosting the availability of key nutrients is a concept widely referred to as biofertility. Biofertility inoculants as defined above is not a new concept, and the commercial application of inoculants dates from the launch of a bacterial product for legumes called "Nitrogin" by Nobbe and Hiltner (1896) and Sahoo et al. (2013). In the late 1940s, Timonin (1948) disclosed bacterial products termed "Alnit" to augment the productivity of non-legume crops. The market for commercial biofertility inoculants in 2012 was valued at \$440 million USD and is expected to exceed \$1 billion USD by 2019, growing at a CAGR of 13% between 2013 and 2019 (Transparency Market Research, 2014).

The most limiting soil nutrients for plant growth are nitrogen and phosphorus (Schachtman et al., 1998). Although many soils contain ample quantities of these nutrients, most are not readily accessible for plant growth (Rai, 2006). Consequently, microbial products have been developed to increase the availability of nitrogen or phosphorus to crops (Vance, 2001), thereby maximizing the efficient, sustainable use of nutrients.

Nitrogen-Fixing Microbes

Nitrogen is the most critical nutrient for plant growth, and perhaps the most recognizable example of biofertility inoculants are the rhizobia which fix atmospheric nitrogen in nodules of legume crops. This diverse group of bacteria comprises some of the most intensely investigated microbes owing to their value as inoculants. Despite their taxonomic diversity, all rhizobia establish symbiotic interactions with their host plant via highly conserved mechanisms which have been reviewed extensively (Alexander, 1984; Weidner et al., 2003; Zahran, 2009; Terpolilli et al., 2012). Legume crops are grown on an estimated 250 million hectares globally and fix roughly 90 million metric tons of atmospheric nitrogen annually (Zahran, 2009).

Effective rhizobial products exhibit high rates of nitrogen fixation and compete successfully with less efficient indigenous rhizobia populations to colonize and form nodules on target host plants. Successful commercial production of rhizobia required the ability to produce the organisms in large quantities and enable a long-term shelf life. Unfortunately, many microbial products fall short in the latter specification leading to overall poor performance in the field. In the 1980s and 1990s many rhizobial

products showed poor efficacy (Catroux et al., 2001). However, over the past decade both quality standards and performance of these products have improved substantially, and several marketed products have been shown to affect consistent improvements in yields of legume crops. Nitrogen-fixing products sold today contain substantially higher numbers of viable organisms per gram than those from earlier decades. Additionally, improved product formulations have resulted in enhanced stability (Grooms, 2008). In tests with inoculated soybeans, Beuerlein (2008) reported yield improvements averaging approximately 120 kg per hectare.

Soybeans contain 37–45% protein by weight, and thus, a 3600 kg ha⁻¹ crop requires 136 kg of nitrogen (Beuerlein, 2008). To illustrate the impact of rhizobial products on soybean yields, products sold by the Monsanto BioAg Alliance (Optimize[®]) (Monsanto BioAg Alliance, 2015e), BASF (Vault[®]), ABM (ExcalibreTM), and MycoGoldTM are discussed. In addition to live *Bradyrhizobium* cells, Optimize[®] for soybeans contains lipochitooligosaccharide, a molecule that enhances the soil microbial environment¹. Seeds treated with Optimize[®] consistently show an increase in yield over untreated controls (Figure 1). Similarly, Vault[®] is a seed treatment consisting of *Bradyrhizobium* and a patented rhizobial enhancer (BASF-Corporation, 2015). ExcalibreSATM is a blend of Bradyrhizobia², and MycoGoldTM blends *Bradyrhizobium* with biostimulants and other microbes³. The use of bioinoculants on soybean crops consistently provides a 4:1 return on investment.

In addition to the nodule-forming rhizobia which establish nitrogen-fixing symbioses in legumes, there are numerous species of non-legume nitrogen fixing bacteria that associate with agriculturally important crops (Rai, 2006). Among these, members of the genera *Azospirillum* MicroAZ-STTM (TerraMax⁴), and Mazospirflo-2 (Soilgro; Owen et al., 2015), *Azotobacter* Bio-NTM (Agriculture Solutions⁵), and *Gluconacetobacter* have attracted interest, because they are root-colonizing and exhibit the potential to transfer fixed nitrogen to the plants with which they associate. Non-legume nitrogen fixing bacteria have been shown to increase yield of various crops including sunflower, carrot, oak, sugar beet, sugar cane, tomato, eggplant, pepper, cotton, wheat, and rice (Bashan et al., 1988; Bashan and Holguin, 1997). In a review summarizing 20 years of global field trials, Okon and Labandera-Gonzales (1994) reported that in 60–70% of the trials, inoculation with various *Azospirillum* strains increased crop yields by 5–30%. Another extensive, multi-year study conducted by Diaz-Zorita et al. (2012) showed that on-seed inoculation increased wheat and maize yields by 244 kg ha⁻¹ (3.9 bu ac⁻¹) and 514 kg ha⁻¹ (8.2 bu ac⁻¹), respectively. In addition to nitrogen fixation, some *Azospirillum* species are capable of producing plant growth-promoting compounds which may play a role in their

mode of action (Okon et al., 2015). Non-leguminous nitrogen fixing bacteria also manifest other plant-beneficial traits such as remediation of soils polluted with heavy metals (Ullah et al., 2015) and confer enhanced tolerance in plants to abiotic stresses such as drought (Vargas et al., 2014).

Phosphate Solubilizing Microbes

Compared with other soil nutrients, phosphorus is the least mobile and is usually in a relatively unavailable form for plant uptake. Next to nitrogen, this nutrient is the second most important nutrient in crop production and is traditionally applied in the form of chemical fertilizers or manure. The world's supply of rock phosphate is expected to be largely depleted in the next few decades (Gilbert, 2009; Scholz et al., 2013). With China, India, and the US as the major users of rock phosphate and 70% of known deposits located in China, Russia, Morocco, and the US, the long term sustainability of current phosphate resources is debated. To ensure the most efficient use of limited supplies of rock phosphate fertilizer and circumvent future shortages, phosphorus-solubilizing microorganisms have been developed to enhance the nutrition of crops in a sustainable manner.

Ironically, the total amount of phosphorus in soils may be high, but it is usually present in forms that are unavailable for plant growth. These comprise both organic and inorganic pools, of which 20–80% can be found in organic forms that include phytic acid (inositol hexaphosphate) as a major component (Richardson, 1994). The largest fraction of inorganic phosphate in soil resides in complexes with metals (particularly Ca, Al, and Fe) (Richardson, 2001). Soil microbes that liberate phosphate from organic and inorganic pools have been promoted as products that effectively mobilize phosphate from poorly available sources in soil and reduce the application of rock phosphate fertilizer. Products such as these are expected to show rapid commercial growth over the next few years. While the genetic and biochemical components underlying the mechanisms of phosphate-liberation by these organisms have not been as extensively studied as nitrogen fixation, excretion of organic acids and synthesis of phosphate-scavenging enzymes such as phytases have been implicated in their modes of action (Richardson, 2001).

Pools of insoluble phosphate in metal complexes can be made available to plants through the action of phosphorus-solubilizing microorganisms. Improved crop yields resulting from the application of phosphorus-solubilizing organisms in the field have been reported (Pradhan and Sukla, 2005), notably *Bacillus* (Symbion-P[®]) and *Pseudomonas* among bacterial genera, and *Aspergillus* and *Penicillium* are among the most important fungal taxa. In comparing characteristics of phosphorus-solubilizing bacteria and fungi, it has been reported that fungi exhibit greater solubilizing activity than bacteria (Nahas, 1996). *Penicillium bilaiae* is a fungus present in the commercial product Jumpstart[®] marketed by the Monsanto BioAg Alliance (2015a). The organism solubilizes soil phosphorus by a mechanism that involves secretion of citric and oxalic acids (Cunningham and Kuiack, 1992). A recent publication by Leggett et al. (2015) summarized the findings of a large multi-year field study to assess the yield responses of maize to inoculation with JumpStart[®]. Rigorous statistical analyses of both large and small test plots

¹http://www.monsantobioag.com/global/us/Products/Documents/Labels/Optimize_200_LiquidSoybean_Extended_Label.pdf

²<http://www.abm1st.com/crops-products/soybeans/excalibre-sa/>

³<http://www.mycogold.com/>

⁴<http://www.terramaxag.com/products/micro-az-st-dry/>

⁵<http://www.agriculturesolutions.ca/bio-n-azotobacter-inoculants>

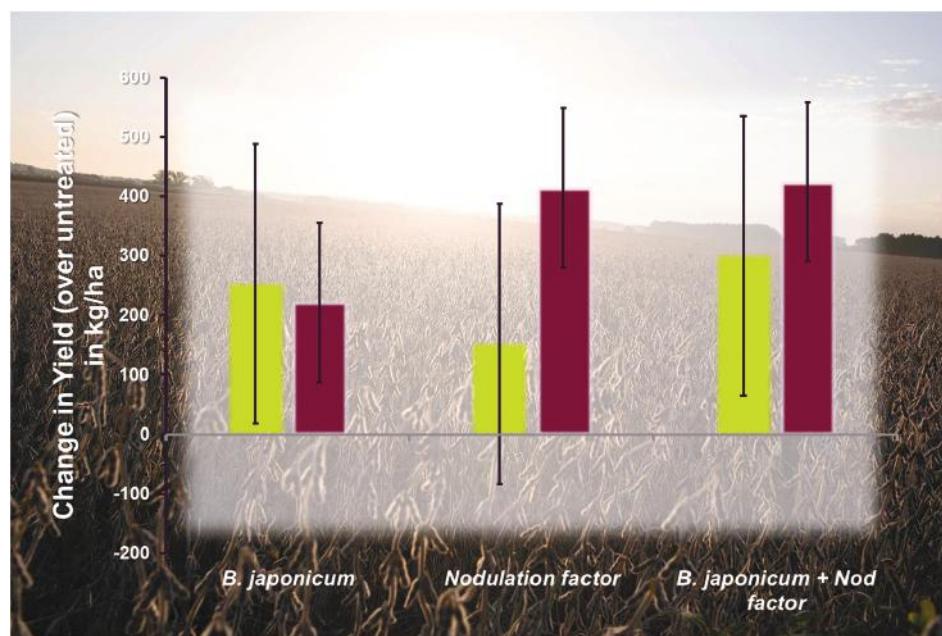


FIGURE 1 | Performance of *Bradyrhizobium japonicum*, a nodulating factor (LCO), and the combination of *B. japonicum* and nodulating factor (Optimize®) in field trials. Field trials occurred in pristine soil (no previous soy; violet), and soils with previous soy crops (green). Error bars represent least significant difference at 95% (adapted from Smith et al., 2015).

revealed significant yield increases in 66 of 92 (72%) small plots and 295 of 369 (80%) large plots (Table 1). These results strongly suggest a significant impact on maize yields as a result of the fungus *P. bilaiae*. The lack of successful commercial phosphate-solubilizing inoculants has been noted (Leggett et al., 2001) and attributed to plant or environmental incompatibility.

Another group of phosphate solubilizing microorganisms are arbuscular mycorrhizal fungi (AMF) that are able to form a network of hyphae that interact with the plant roots to improve nutrient transport and protect the plant against pathogens and some forms of abiotic stress (Porcel et al., 2012; Hodge and Storer, 2015). Most of the vascular plants on Earth form an association with AMF (Smith and Read, 2008); they are ubiquitous and ecologically important for soil health. Within the AMF, the most widely used products in agriculture usually belong to the phylum Glomeromycota (Owen et al., 2015) and have been shown to increase P uptake. Some of the examples of AMF products include Mycormax® (JH Biotech⁶), BEI (BioOrganics^{TM7}), BioGrow Endo (Mycorrhizal Applications⁸), and VAM (Microbesmart⁹).

Products Containing Multiple Biofertility Microbes

Interestingly, a few commercial products have emerged that take advantage of combining different biofertility products. One such

TABLE 1 | Summary of small and large plot field trials to measure maize yield response to inoculation with the phosphorus-solubilizing fungus *Penicillium bilaiae* (adapted from Leggett et al., 2015).

Trials	Sample size, n	Yield increase (kg/ha ± SE)	Increase %
Small plot	92	169 ± 2.8	1.8
Large plot	92 369	326 ± 1.6	3.5

product, marketed under the trade name QuickRoots®, is sold by the Monsanto BioAg Alliance (2015b). This product contains a patented combination of the *Bacillus amyloliquefaciens* and the filamentous fungus *Trichoderma virens* (Monsanto BioAg Alliance, 2015c,d). Both of these organisms are known to liberate bound phosphate making this nutrient more available to plant roots (Fan et al., 2011; Akладиос и Abbas, 2012; Molla et al., 2012; Lamdan et al., 2015), and the combination purportedly imparts increased availability of nitrogen, phosphorus and potassium in soil resulting in expanded root volume for enhanced yield potential¹⁰. Field trial data with QuickRoots® applied to corn shows a positive yield ranging from 220 to 500 kg ha⁻¹ increase representing a 2:1 to 5:1 return on investment (Figure 2)¹⁰. Lastly, the combination of these two organisms may also enhance favorable interactions of plant roots with mycorrhizal fungi in the soil (Johnson, 2013, 2015). Other examples of mixed products include Excalibre-SA (ABM), which combines *Trichoderma* with *Bradyrhizobium* for soy¹¹, and

⁶<http://jhbiotech.com/docs/Flyer-Mycormax.pdf>

⁷<https://bio-organics.com/product/endomycorrhizal-inoculant/>

⁸<http://mycorrhizae.com>

⁹<http://www.microbesmart.com.au/index.php/what-is-vam>

¹⁰<http://www.monsantobioag.com/global/us/harvest/pages/corn.aspx>

¹¹<http://www.abm1st.com/crops-products/soybeans/excalibre-sa/>

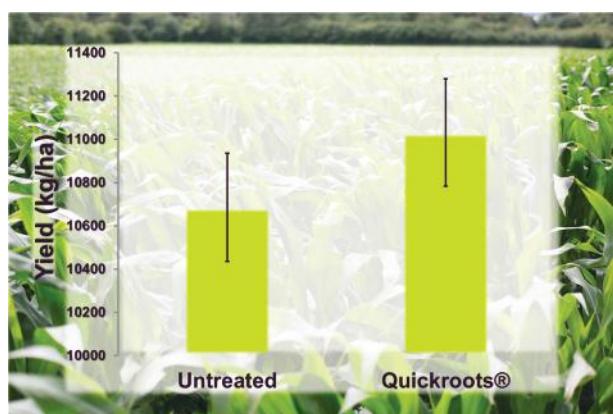


FIGURE 2 | Performance of QuickRoots® (*Bacillus amyloliquefaciens* plus *Trichoderma virens*) product compared with untreated corn seeds in small plot trials ($N = 104$). Error bars represent Standard Error. Yield values are significantly different (two-tailed t -test < 0.001).

BioGrow Endo (Mycorrhizal Applications) combines AMF and *Trichoderma*¹².

BIOCONTROL ORGANISMS

Plant diseases and pests are among the largest contributors to crop losses worldwide, with an estimated 27–42% in production systems and potential losses of 48–83% in the absence of crop protection (Oerke and Dehne, 2004). The use of biological organisms to control plant disease (biocontrol) could potentially augment the use of synthetic pesticides (e.g., residue and resistance management). Despite clear enthusiasm around the potential for biocontrol microbes, challenges still exist in efficacy, field performance, and cost. In this section, the role of biocontrol in plant pest management from an industry perspective is addressed. We focus on both the scientific and production strategies necessary to bring biocontrol products to market, and highlight a few examples of commercially available biocontrol strains.

Biocontrol research has received a lot of attention in recent years, and there are many well documented examples of biocontrol microbial activity in scientific literature (Glare et al., 2012; Junaid et al., 2013; Bardin et al., 2015; Pelizza et al., 2015), however, synthetic pesticides still dominate the commercial market (Elad, 2003). Only an estimated 3.5% of the global pesticide market is represented by biocontrol products (valued at 1.6 billion USD in 2009) (Lehr, 2010). In North America and Western Europe, biocontrol markets have been estimated to be \$594.2 million in 2009 and \$1.09 billion in 2015 (Frost and Sullivan, 2009). Although challenged by issues of performance and cost, it is clear that the biocontrol market is growing rapidly. Estimates have proposed a 15.6% CAGR, resulting in over 7% global market shares in 2014 (Lehr, 2010; Glare et al., 2012). Regardless of how size estimates are made, all indications point

to continued growth for the biocontrol market, well beyond that predicted for the synthetic pesticide market (Glare et al., 2012).

Historically, early sales within the biocontrol market consisted predominately of a single product type containing *Bacillus thuringiensis* (Bt) targeted against lepidopterans (e.g., cabbage worms and gypsy moth). In 1990, over 90% of biocontrol sales corresponded to Bt-related products, with a total market of approximately \$120 million USD (Rodgers, 1993), although other biocontrol products such as entomopathogenic nematodes have played a key role (Shapiro-Ilan and Gaugler, 2002). After 2 decades, the estimated total sales for microbial-based biocontrols was close to \$400 million USD with just over 50% of sales corresponding to Bt-related products (Glare et al., 2012). The geographical distribution of biocontrol sales has changed dramatically over the last two decades to cover a broader global market and a greater number of agricultural crops (Rodgers, 1993; Glare et al., 2012). These trends suggest that the geography, market sectors, major arable crops, and diversity of microbial strains all continue to expand. Major drivers for growth in biocontrol use include growing consumer interest for products in emerging markets such as China and India.

Broad adoption of biocontrol products into mainstream agriculture requires advances in technology, increased understanding of the biology and ecology of active organisms, and cost effective, efficacious products. Industry concerns generally focus on production, formulation, and delivery when commercializing a biocontrol product (Fravel, 2005). In addition to these attributes, industry must consider aspects of product registration, intellectual property, and an understanding growers needs. Finally, aspects of efficacy, persistence, and mode of action (biology) must be considered when developing an effective biocontrol product.

Biology of Biocontrol

Biocontrol agents are broadly classified as preparations either derived-from or containing living microorganisms that can prevent or suppress pests like pathogens, insects, and weeds. Biocontrol agents can include living microbes (bacteria, fungi, nematodes, viruses and protozoa), bioactive compounds such as secondary metabolites (e.g., spinosads and avermectins), or naturally derived material such as plant extracts (Kiehnck, 2007; van Lenteren, 2012). Pest damage prevention by biocontrol agents is based on several mechanisms that may involve antibiosis, competition for space and nutrients, mycoparasitism, enzymatic activity, and induced resistance (Lo, 1998). These modes of action are certainly not exclusive, and biocontrol agents likely enlist a combination of activities when counteracting disease.

As previously mentioned, industrial application of biocontrol microbes will require a deeper understanding of the biology of the microbe, the targeted pest or pathogen, and interactions with host plants, other microbes, and the environment. Drivers of microbe communities in the rhizosphere, for example, involve soil type and plant genotype (Berg and Smalla, 2009; de Bruijn, 2013), whereas the phyllosphere microbiome is influenced by plant genotype and environmental factors like humidity, ultraviolet light, and geographic location (Vorholt, 2012; Rastogi et al.,

¹²<http://mycorrhizae.com/>

2013). Understanding these ecological differences is critical when making decisions about product development and commercial application. In an illustration of abiotic effects, biocontrol efficacy by nonpathogenic *Fusarium oxysporum* was significantly affected by both temperature and light (Larkin and Fravel, 2002). In another example of biological complexity, Erlacher et al. (2014) found that shifts in lettuce microbe communities caused by pathogen infection (*Rhizoctonia solani*) were offset by the biocontrol agent *Bacillus amyloliquefaciens* FZB42. Such selective compensation of pathogen impact by a biocontrol strain suggests a novel mode of action and highlights the complexity of biocontrol within a plant–microbe ecosystem. Understanding how biocontrol microorganisms interact with one another represents another biological challenge for product development. Co-inoculation of *Trichoderma viride* strain GB7 and *Serratia plymuthica* strain 3Re4-18 resulted in greater biocontrol efficacy against *R. solani* in lettuce, compared to application of single strains (Grosch et al., 2012). However, combined biocontrol application also had a more pronounced impact on the microbial community structure at large (Grosch et al., 2012). These studies highlight the complex and fluid interactions between plant, pathogen, biocontrol agent, microbe community, and the environment. To commercialize effective biocontrol microbes as products, industries will need to invest in fundamental and early development research surrounding these biological questions. This will require deeper partnerships within industry as well as greater communication with academic (public and private) and government research organizations.

Screening for Biocontrol

Commercialization of a successful biocontrol product ultimately depends on the availability and isolation of candidate microbes. This screening process involves isolation from a particular environment and early trials to characterize a microbe's biocontrol capability. While no single screening method is optimal for all biocontrol endeavors, a logical strategy should be followed based upon the pathosystem (plant-pathogen-environment) of interest (Fravel, 2005). For example, finding biocontrol agents against foliar-specific pathogens would likely require screening microbes that can colonize the phyllosphere. Culturing phyllosphere-associated microbes from tomato (Enya et al., 2007) and wheat (Yoshida et al., 2012) has resulted in the identification of potential biocontrol microorganisms for foliar pathogens. Likewise, screening for biocontrol against post-harvest diseases would require identifying microbes that effectively protect the harvested crop (Janisiewicz and Korsten, 2002).

Successful candidate identification starts with a suitable population of microbes to be evaluated. While screening processes are becoming more robust and generating higher throughput, less than 1% of candidate microbes make successful products (Bailey and Falk, 2011). The generation of large microbe collections, both through targeted and broad sampling techniques, is required for identifying biocontrol candidates. One example, TrichobankTM, is a fungal culture collection of >2000 isolates of 21 *Trichoderma* spp. (Stewart et al., 2010). This collection has been successfully used to develop biocontrol

agents like SentinelTM for the control of gray mold of grapes caused by *Botrytis cinerea*. In the case of microbe databases like TrichobankTM, information on the isolates within the collection is matched to a desired set of biocontrol capabilities based on pathogen targets, host plants, mode of action, and environmental niche (Glare et al., 2012). This subset of selected isolates is then subjected to a series of standardized bioassays to establish biocontrol efficacy and field performance capability. It is worth noting here that biocontrol efficacy in a field setting is key for adoption and implementation of microbial products. While many biocontrol agents were identified and/or validated through *in vitro* screens, caution should be taken when assuming correlation between *in vitro* inhibition and field performance (Burr et al., 1996; Milus and Rothrock, 1997; Fravel, 2005). Screening strategies can follow varied approaches, but the desired outcome is the same in identifying efficacious, environmentally safe, and cost-effective biocontrol agents (Köhl et al., 2011; Ravensberg, 2011).

SUCCESS OF A PRODUCT

The success of agricultural microbial products, whether biofertility or biocontrol, is rarely due to just one attribute, but instead is generally due to a number of factors (Ravensberg, 2011). Gelernter and Lomer (2000) suggest a framework for evaluating successful biocontrol products, but here we improve on these criteria to include all microbial products. Aside from technical efficacy, or the ability to improve yield or reduce crop damage, successful products meet two or more of the following conditions.

Efficacy

The most important factor for a successful product is the ability to increase or protect yield. This is obviously the most important goal and a given factor in combination with other factors mentioned below for overall product success. However, efficacy in the laboratory and/or greenhouse does not always translate to field success (Nicot et al., 2011). Whipps (2001) stated “The key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in soil and root environment is required to predict the conditions under which biocontrol can be achieved.” Nicot et al. (2011) suggests that the success gap between lab and field efficacy can be improved by understanding of in-field mode of action. Although they specifically refer to biocontrol microbes, this same principle applies to biofertility products as well. Efficacy data that do not account for ecological interactions in a complex microbe-plant field ecosystem including at least some of the factors discussed below risks failure (Fravel, 2005). In many cases biocontrol microbial products are included as a part of an integrated pest management program (Chandler et al., 2011).

In addition to in field-efficacy, there are often efficacy challenges that arise with scaling production for widespread distribution. Some of the challenges described by Takors (2012) include the genetic stability of the strain and the impact of

mutation, viruses, and phase variation, as well as other chemical and physical factors associated with going from bench-top to industrial scale bioreactors.

Versatility

Plants may recruit specific microbes based on their development and environment, responding to stresses or nutrient availability (Smalla et al., 2006; Hartmann et al., 2009). The effectiveness of microbial strains colonizing the plant are impacted by a number of biotic and abiotic factors (Barea, 2015), including the previous cropping history (Berg and Smalla, 2009; Peiffer et al., 2013), suggesting that microbial compositions of the soil are modulated by changes in cropping practices. The ability to colonize is also impacted by genotype of the plant, showing variations in community structure between variants in the same species (Siciliano and Germida, 1999; Briones et al., 2002). Specific plant exudates in the form of volatile organic compounds, carbon sources or organic acids encourage colonization and growth of a relatively narrow group of organisms (Sloan and Lebeis, 2015). For example, studies on *Arabidopsis* demonstrate not only bacteria-specific responses to targeted exudates like malic acid (Rudrappa et al., 2008), but also community responses over time due to development stage of the plant (Chaparro et al., 2014). In addition to targeted strains and temporal development of colonization, strains must also associate with the appropriate root architecture of the plant, whether by interaction with receptors on the surface of the roots or by maintenance of cell numbers within the rhizosphere influenced by the plant (Compañt et al., 2010). These factors allow for selective colonization of specific microorganisms and promote diversity of the community to fit the functional needs of the plant (Mendes et al., 2015). Improving microbial support for a crop requires understanding the needs of the plant, in combination with the composition of the soil and the surrounding communities to best determine the products that will benefit the crop. However, it should be noted that there is often an ecological trade-off when selecting for a specific trait in a microbial strain. For example Ehinger et al. (2014) explored the relationship between *Bradyrhizobium* and either specialized or generalized hosts and found a trade-off in host range and efficacy. Conversely, selecting for or developing strains that are specialists and highly effective in desired traits such as biocontrol or host interaction can result in loss of fitness (Kassen, 2002).

Biocontrol microbial strains are often highly targeted to specific species of pests (Nicot et al., 2011), so farmers may need to apply different products to control multiple pest species. Relevant narrowed spectrum, short-lasting, slowing-kill microbial based products are big hurdles for successful product commercialization. For example, the fungus *Colletotrichum gloeosporioides* f. sp. *malvae* was discovered to cause seedling blight on round-leaved mallow plants being grown in weed control trials in Saskatchewan (Harding and Raizada, 2015). However, the diversity of weeds in the field combined with its narrow host range have limited its usage in the market. Since ecological interactions are so important to in-field efficacy, organisms that have greater versatility will have improved efficacy over a number of different field conditions. This versatility includes interaction with different hosts and different pathogens

and will be an ongoing process as pathogens continuously evolve to circumvent plant defenses and overcome biocontrol mechanisms (Brockhurst and Koskella, 2013; Zhan et al., 2014).

Practicality

Another important factor in the success of an inoculant or biocontrol product is practicality for both the producer and the consumer. The product must ideally have a low barrier to adoption and be compatible with the farmer's equipment and production practices.

Mass production of the microbe responsible for improving crop yield is one of the prime requirements for commercialization (Moosavi and Zare, 2015). *Pasteuria* is a good case study of a product that in the past was not very practical from an industrial perspective due to difficulties with mass production. *Pasteuria* was originally described from water fleas over 100 years ago, however, cultivation efforts were unsuccessful (Metchnikoff, 1888). Nearly two decades later, Cobb (1906) discovered these organisms infecting a nematode. *Pasteuria* species are able to effectively parasitize different developmental stages of nematodes (Chen and Dickson, 1998), but for over a century commercialization of *Pasteuria* was limited due to the inability to mass produce spores as a product. *Pasteuria penetrans* is an obligate parasite of *Meloidogyne* species, which are obligate plant parasites (Davies, 2009). Until recently, harvesting spores for commercial product required extracting spores from infected nematodes extracted from infected plants and was not an ideal system for mass production. It is currently a commercial product Clarivar® (Syngenta®¹³).

Many farmers perceive inoculants and biocontrol microbial products as more costly and less effective than traditional agrochemicals. For example, microbial biocontrol strains are not always a quick acting option: they often work by suppressing pest populations through slower processes rather than killing on contact which may allow crop damage to continue for some amount of time. In some cases, to use biocontrol strains effectively, growers need to identify and know a great deal about the lifecycle of the pest or pathogen they are trying to control and understand the timing and appropriate conditions for application of the product. More outreach is needed between industrial or technical specialists and the agricultural community to help growers accustomed to broad-spectrum agrochemicals integrate inoculants and biocontrol microbial products into their cropping systems.

Delivery

Appropriate formulation is required for a high quality product. Since microbial products are often stored under less than optimum conditions (e.g., high temperature, light exposure, high humidity), they must have an extended shelf life and the microorganism needs to be either robust or well protected to be able to survive under harsh conditions. Good formulation will also provide optimal conditions to enhance microorganism life on roots or on leaves to obtain optimal benefits after

¹³<http://www.syngentacropprotection.com/clariva-complete-beans-seed-treatment>

application to the target plants. To be widely adopted by farmers, an inoculant or biocontrol product must be cost effective and easy to apply, ensuring that the microorganisms are delivered to the target plant in the most appropriate manner and form. Formulation of inoculants and biocontrol products is a crucial issue but little research has been conducted on this subject. For some strains, particularly gram positive spore formers, formulation and long-term stability methods are much more developed than for gram negative strains. A literature survey by Xavier et al. (2004) showed that since the 1980s, most rhizobial research focused on the bacterial genetics and physiology and less than 1% of research articles on rhizobia have focused on formulation aspects of products. However, there is a real need for improved formulations of products, to create and commercialize new microbial products that will be more effective, stable, and higher quality to meet farmers' needs.

Formulation of products by adding compounds to active ingredients can improve field performance, shelf life, and stability (Warrior et al., 2002; Leggett et al., 2011; Ravensberg, 2011), ultimately reducing variability. Formulation allows for several functional goals including safety, effective application, and enhanced persistence (Ravensberg, 2011). A lack of published research in this area is likely indicative of protection through intellectual property, like trade secrets, which is often necessary to protect investments in product development. Industry investments in current and future technologies will be critical in formulating novel products. One example of formulation utility is around microbes that do not form spores (e.g., gram negatives) or microbes that are highly sensitive to desiccation and temperature extremes. *Serratia entomophila* is the active ingredient in BioShield®, an insect biocontrol agent (Glare et al., 2012). New formulation techniques have reportedly allowed for stabilization of BioShield® to extend shelf life to more than 6 months without loss of viability (Swaminathan and Jackson, 2011). In addition, formulation additives like diluents and oils have been used successfully for *Metarhizium acridium* products, enhancing fungal spore attachment and infection in target insects (Hunter, 2010).

The microbial ecology of biocontrol agents has been shown to indicate whether they are rhizosphere or phyllosphere competent (Kamilova et al., 2005; Bruck, 2010; Vorholt, 2012). Formulation technologies can therefore be used to improve delivery, colonization, germination, and establishment of microbes in those particular zones. Seed coating with microbes can provide an inexpensive option for targeted delivery, but improvements still need to be made in coating materials, microbe and chemistry compatibility and application technology, especially when considering the diverse requirements of biological organisms (Glare et al., 2012). One of these requirements is water availability, which can have profound influence on survival of bio-products (Connick et al., 1996). Dry or desiccated products weigh less, are more cost effective to ship, and have a lower risk of contamination. This type of formulation may be amenable to microbes that produce stable storage structures like spores, but non-spore producers likely require different formulation strategies.

Closely tied to formulation parameters is the actual delivery system used to apply beneficial microbes in an agriculture setting. A delivery system targeting precise timing and specific sites can greatly improve both bioinoculant and biocontrol product efficacy, persistence, and cost-effectiveness. Delivery presents a major challenge to industry in part because it requires mass production, formulation, and application of biocontrol microbes and/or their bioactive compounds (Ravensberg, 2011; Glare et al., 2012). As previously mentioned, the biology of the microbe may dictate the best avenue for delivery, leading to decisions of application site (e.g., seed, foliar, root) and timing. Researchers are looking beyond traditional seed coats or foliar sprays and investigating aspects of timing and treatment location. Varied spray schedules of *Trichoderma* biocontrol strains were used to control gray mold and anthracnose in strawberries (Freeman et al., 2004). Continuous application of the *Pseudomonas putida* in low concentrations through irrigation water resulted in soil populations similar to a single application at a 10-fold higher concentration (Steddom and Menge, 2001). This suggests that targeted delivery systems (site and timing) can result in field efficacy in a cost-saving manner.

Persistence

Some of the issues associated with failure of microbial products involve the timing of the application of the product in the field (Chutia et al., 2007). Microbial products tend to act on more specific targets and have a shorter shelf and sometimes active life than chemical fertilizer/pesticides (van Lenteren, 2012). The combination of selectivity of the microbial strain to host or target and lack of persistence often results in inconsistent field data (Nicot et al., 2011). For example, Bt toxin proteins are degraded very quickly when they are exposed to sunlight. Bt-based microbial products often need multiple applications and result in high cost. In other cases, the efficacy of a product presents a tradeoff between immediate short-lived impact and persistence in the environment (Barea, 2015). The persistence of strains varies greatly in the environment. Some strains such as *Trichoderma harzianum* and *Bacillus amyloliquefaciens* FZB42 decrease below detectable limits within a few weeks of application (Papavizas, 1982; Kröber et al., 2014), whereas other strains such as *Rhizobium phaseoli* and *Bradyrhizobium japonicum* will persist indefinitely, but at a lower abundance than is required for efficacy (Robert and Schmidt, 1983; Narożna et al., 2015). Some products may be formulated to successfully enable persistence of the product long enough to show activity due to compatibility between a strain and the environment if they can occupy a niche or colonize before competitors show up (Verbruggen et al., 2012) impacting community assembly in the rhizosphere (Nemergut et al., 2013). In cases where the biological product does not readily colonize the rhizo/phyllosphere, compatibility and niche space in the environment will severely impact efficacy.

Commercial Viability

High cost associated with production is another obstacle for success of developing a biological product. For example, AMF products generally contain spores, colonized roots, hyphae segments, or a mixture of the three (Dalpé and Monreal, 2004),

and a wide range of carriers can be used (peat, compost, vermiculite, perlite, sand). Because AMF are obligate symbionts in nature, their proliferation and high-scale production require more specific skills and infrastructure. First attempts in AMF cultures used the pot-culture methods. Colonized root segments or spores of well-known AMF species are used to inoculate young seeds in a fresh sterile substrate. Plants are grown in pots, bags, or beds and the AMF colonize roots and substrate as the host develops, leading to a high concentration of AMF spores and colonized roots. Spores and roots obtained can then be used for commercial product preparation or to inoculate a new batch of sterile substrate. This kind of production method faced some difficulties such as uniformity of spores from batch to batch, production space requirements, and quality variation. In addition to production costs and return on investment for farmers, economic aspects of agricultural microbial products include market size and value (Nicot et al., 2011).

Regulations

Regulatory frameworks and product registrations are used worldwide to guide the commercial development of microbial products. When developing new microbial products, the requisite regulatory framework varies by country, the product's characteristics, and its intended usage. These national and international regulations must be taken into account during every part of the product development cycle, including its earliest stages, as certain regulations also outline where natural microbes can and cannot be harvested. Interestingly, the regulations pertaining to inoculants and biocontrol strains, while similar, may differ in certain parts of the world. Nevertheless, regulatory cycles for the development of new bioinoculants and biocontrol products are generally streamlined and well-articulated. As a result, microbial products are an appealing and cost-effective choice when taking an integrated, systems-level approach toward crop productivity and agricultural pest management.

CONCLUSION

Microbial products to improve crop yields and health are readily available commercially, and their quality as well as efficacy has improved considerably over the past decade. The field performance of these products continues to be enhanced as major agricultural companies commit substantial research revenues to discovery and development of new products. Determining

the appropriate microbial products for the functional needs of each crop will require input from both farmers and researchers. Soil type, microbiome, environmental conditions, pest presence and cropping system are all factors that could influence the benefit that a microbe may provide. The crop being planted is another key consideration, as many plants colonized by specific bacteria are unable to maintain high populations when other crops are planted. Further exploration into the mechanisms and specificity of plant growth promotion from key microorganisms will refine their specific use and maximize the potential inherent in the microbiome of plants and soils. In this regard, recently published studies (Agler et al., 2016; van der Heijden and Hartmann, 2016) revealed that the complex, interconnected microbial communities associated with plants harbor discrete keystone species, termed "microbial hubs" that play a critical role in mediating communications between the plant and its microbiome. Clearly, the ability to influence these functions for more efficacious biofertility and biocontrol applications is an area that will receive much attention.

Increased understanding of the impact microorganisms play in the growth and development of crops is key to future development of microbial products. In-depth studies into the effects of consortia and bacterial community structure on crop development will continue to expand our knowledge of the necessary effects the microbial community has on plants. Further examination of responses between target crops and microbes will better determine the specific signals that recruit or prevent colonizing microorganisms of critical food crops. These areas of research will result in a better understanding of the complex associations between the microbes in the soil and critical crops, a necessary step in providing farmers the tools necessary to continue feeding the planet in a sustainable manner.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Developmental Peculiarities and Seed-Borne Endophytes in Quinoa: Omnipresent, Robust Bacilli Contribute to Plant Fitness

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Among potential climate change-adapted crops for future agriculture, quinoa (*Chenopodium quinoa*), a facultative halophyte plant with exceptional nutritional properties, stands out as a prime candidate. This work examined how quinoa deals with extreme situations during seed rehydration. Quinoa distinguishes itself from other plants in multiple ways. It germinates within minutes, even under extremely hostile conditions. Broken seeds/split embryos are able to regenerate. Furthermore, quinoa seedlings are resurrection-competent. These peculiarities became in part explainable upon discovery of seed-borne microorganisms. 100% of quinoa seeds, from different sources, are inhabited by diverse members of the genus *Bacillus*. These endophytes are motile and reside in all seedling organs, indicating vertical transmission. Owing to their high catalase activities and superoxide contents the bacteria potentially manipulate the host's redox status. Superoxide-driven cell expansion enables quinoa to overcome a critical period in development, seedling establishment. Quinoa's immediate confrontation with "foreign" reactive oxygen species and bacterial elicitors likely induces a naturally primed state, enabling plants to withstand extreme situations. The endophytic bacteria, which are cultivable and highly robust themselves, have high potential for application in agriculture, food (amylase) and cosmetics (catalase) industry. This work also discusses the potential of transferring quinoa's microbiome to improve stress resistance in other plant species.

Keywords: abiotic stress, *Bacillus*, *Chenopodium quinoa* (quinoa), germination, reactive oxygen species (ROS), seed-borne endophyte

INTRODUCTION

Plant Stress and Seed Germination

The progressive salinization and desertification of land due to climate change will inevitably affect the soil microbiome, soil fauna as well as vegetation. Thus, there is a man-made selection pressure for multi-tolerant extremophiles. Saline and dry soils are poorly accessible for agricultural cultivation. Unlike other environmental factors such as drought, heat, or high light which may influence plant growth at various developmental stages, soil composition, and moisture determines whether seeds give rise to viable plants at all. Drought and high salinity provoke overlapping responses in plants (recently reviewed in e.g., Huang et al., 2012; Golldack et al., 2014). Both stresses are perceived by plant cells as deprivation of water. The lower

water potential in saline soil reduces the water availability for the plant. High NaCl concentrations trigger a hyperosmotic shock, which is accompanied by the accumulation of reactive oxygen species (ROS). Similarities also exist in plant adaptation strategies toward high salinity and heavy metal stress (Bose et al., 2014). For this reason halophytes are widely advocated for phytoremediation purposes (Lutts and Lefevre, 2015).

Reactive oxygen species are essential for cellular and long-distance signaling. The role of ROS, including hydrogen peroxide, superoxide and hydroxyl radical, is two-sided (Pitzschke et al., 2006; Bailly et al., 2008). On the one hand, they are critical signaling molecules. On the other hand, ROS at excessive amounts lead to oxidative damage to diverse biomolecules, and ultimately to cell death. To some extent, plants can evade toxic ROS accumulation through ROS-scavenging molecules and enzymes (Mittler, 2002).

Studies in diverse plant species have provided evidence that responses to numerous abiotic and biotic stresses involve ROS production and activation of mitogen-activated protein kinases (MAPKs). MAPKs act both up- and downstream of ROS (Asai and Yoshioka, 2008; Pitzschke and Hirt, 2009), and deficiency or excessive activation is associated with severe developmental defects or abnormal stress phenotypes (Andreasson and Ellis, 2010). Besides mediating stress responses, MAPKs and ROS are also important coordinators of germination. According to a current model, H₂O₂ induces a MAPK-dependent decrease in the contents of abscisic acid (a germination-inhibiting hormone). In addition, H₂O₂ triggers carbonylation of seed storage proteins, favoring their mobilization (Barba-Espin et al., 2011, 2012). Moderate H₂O₂ applications improve germination performance (Barba-Espin et al., 2011), and they can also prevent viability loss in partially desiccated pea seedlings (Roach and Krammer, 2011).

Seed imbibition entails a large increase in ROS contents. For successful germination they must be kept within a certain range that allows ROS signaling. ROS levels above or below the 'oxidative window for germination' block development (Bailly et al., 2008). Owing to their ability to react with polyphenols/carbohydrates, ROS also participate in cell wall loosening, which is critical for cell expansion, and thus, plant growth (Tenhaken, 2014).

Furthermore, ROS are essential for priming-mediated resistance, stress acclimation as well as rapid systemic acquired acclimation (Baxter et al., 2014). All these processes rely on the plant's ability to generate superoxide; accomplished by membrane-bound NADPH oxidases. These enzymes are also crucial for seed germination (Ishibashi et al., 2010; Krammer et al., 2010; Liu et al., 2012a; Singh et al., 2014). Similar membrane-bound enzymes for extracellular superoxide production have recently been discovered in heterotrophic bacteria (Diaz et al., 2013).

Bacteria in Seed Germination and Plant Development

In their natural habitats, plants are surrounded by myriads of microorganisms that can potentially enter and colonize plant tissues. Most plants become colonized during development.

In fact, to be inhabited by microorganisms is rather the rule than the exception and numerous associations between plants and pathogenic or symbiotic fungi or bacteria have been explored (Partida-Martinez and Heil, 2011). The rhizosphere represents an important entry point. Soil-borne bacteria, attracted by root exudates, can have a huge impact on plant development. A striking example is the pathogen *Agrobacterium tumefaciens*, which manipulates hormone balances, represses defense mechanisms, and provokes genetic reprogramming in infected host plants; culminating in the growth of tumor-like structures crown-galls (Gelvin, 2012; Pitzschke, 2013). By contrast, rhizobia have growth- and health-promoting effects; they fix nitrogen and mobilize nutrients. Through, biosynthesis of diverse volatile organic compounds microbes cannot only communicate with each, but also with their hosts. Both plant growth- and health-promoting effects are known from certain rhizobacteria (Ryu et al., 2004; Zhang et al., 2007).

Although the role of seed endophytic bacteria still is underestimated, such associations could be beneficial for germination and seedling establishment (Truyens et al., 2015). Seed maturation involves accumulation of starch and a drastic decline in water content. Environmental conditions for plant colonizers thus change substantially, and only endophytes able to withstand high osmotic pressure will be successful seed inhabitants. Characteristic features of some seed-borne bacteria known so far comprise endospore formation, amylase and phytase activity to mobilize starch and phosphorus, respectively, and cell motility to migrate into the seeds before they harden (Johnston-Monje and Raizada, 2011; Truyens et al., 2015). This motility is a main criterion for vertical transmission. In rice, wheatgrass, and maize, respectively, the same bacteria species could be isolated from seeds of consecutive generations (Mukhopadhyay et al., 1996; Johnston-Monje and Raizada, 2011; Liu et al., 2012b; Ringelberg et al., 2012; Gagne-Bourque et al., 2013).

Effects of Seed-Borne Bacteria on their Hosts

Despite their beneficial effects on plant health and development and the respective potential for application, seed-borne endophytic bacteria are still largely unexplored. Major effects of seed endophytes on their hosts comprise promotion of growth and protection from stress (reviewed in Truyens et al., 2015). The underlying mechanisms are largely elusive. Growth-promoting effects have been ascribed to seed bacteria from rice (Hardoim et al., 2012), cactus (Puente et al., 2009), and tomato (Xu et al., 2014). In rice, seed endophytes protect young roots from colonization by soil-borne pathogens (Bacilio-Jiménez et al., 2001). Interestingly, besides such antagonistic potential against phytopathogens, seed endophytic bacteria can also alleviate symptoms triggered by abiotic stress. In tobacco, seed endophytes (*Pseudomonas* sp., *Enterobacter* sp.) reduce cadmium phytotoxicity (Mastretta et al., 2009). Long-term cultivation on heavy metal-contaminated soils changes the endophyte composition in seeds of the perennial grass *Agrostis capillaris*. Because selected Cd-resistant seed bacteria (*Bacillus*

sp., *Pantoea* sp.) were found to increase Cd uptake when re-inoculated into Cd-exposed hosts, they hold promise for phytoremediation purposes (Truyens et al., 2014).

Enhanced seed germination arising from seed-borne microorganisms has been related to secretion of bioactive secondary metabolites, or to production of ACC deaminase, which lowers the level of the stress hormone ethylene (*Bacillus/tomato*; Glick, 2014; Xu et al., 2014).

Quinoa

The pseudo-cereal quinoa is found natively in the Andean region, where it grows at >4000 m above-sea-level. Quinoa is the traditional crop in South America. In the recent past quinoa has witnessed increasing popularity globally, due to its exceptional nutritional properties. The seeds are gluten-free and rich in minerals, proteins, and vitamins (Ruales and Nair, 1992; Vega-Galvez et al., 2010). Quinoa cultivation is spreading worldwide¹. Being a facultative halophyte, quinoa is able to cope with high levels of salinity and drought stress. Some varieties even accept sea water for irrigation (Adolf et al., 2013). Among potential climate change-adapted crops for future agriculture, quinoa therefore stands out as a prime candidate.

The current work on quinoa germination under non-stress and extreme stress conditions discloses some very unusual plant habits. In the course of experiments, seed-borne endophytes were discovered and their potential contribution to quinoa's phenotypic peculiarities assessed.

MATERIALS AND METHODS

Plant Material, Growth- and Incubation Conditions

Organic seeds, of the white cultivar 'Real,' harvested in Bolivia, were purchased from two independent suppliers (al natura, Austria and Ziegler-Naturprodukte, Germany).

Seeds (app. 20, 10; or single seeds, respectively) were placed into 12-, 24-well or 96-well plates containing sterile imbibition liquid. Alternatively, they were sown onto moist filter paper or YPD agar. Plates were incubated for various periods. All imbibition solutions were freshly prepared before each experiment. Special care was taken to avoid any cross-contamination (single-use equipment). Seeds were considered as germination when their radicles had protruded the seed coat. All experiments, incubations (including bacterial cultivation) and stainings were conducted at 22–24°C.

Tests of Plant Material for the Presence of Microorganisms

Seed powder was prepared using a mill (Retsch; Germany). Seedlings were surface-sterilized by incubation in 70% ethanol (two times à 5 min, shaking), followed by three washes in distilled water. Seedlings were either macerated (using sterile micro pistils) or cut into segments (with single-use blades) and subsequently incubated on YPD agar.

¹<http://www.fao.org/quinoa/en/>

Histochemical Detection of Hydrogen Peroxide

Seeds were directly incubated in water containing 1 mg/ml DAB (diluted from a 50-fold stock in water pH 3.8). Alternatively, DAB was added 4 h after seed imbibition in water. Formation of brown precipitates was documented by photography. Experiments were repeated several times (>5).

Histochemical Detection of Superoxide

Seeds were imbibed in water containing 0.2 mM NBT (NBT itself had no influence on germination). Alternatively, 4-days-old seedlings were incubated in NBT solution. Consistent data were obtained from three independent experiments, and also when using Hepes- or Tris-buffered solution (pH 6.6–7.5) instead of water. Superoxide contents in microbial material were assessed in a similar way, using 0.2 mM NBT solutions.

Catalase Tests

The formation of bubbles in the presence of exogenous H₂O₂ was considered as a (non-quantitative) readout for catalase activity. Seeds were imbibed directly in H₂O₂ solution (0.1, 1 mM). For catalase tests with microorganisms, H₂O₂ solution was added to colony material collected from seed surfaces or YPD agar. Bubble formation/foaming was documented by photography.

Microscopy

Microbes proliferating on quinoa seeds were resuspended in water and analyzed using a Leica Linux20 microscope attached to a camera.

PCR and Taxonomic Classification

DNA was isolated from microorganisms (proliferating on seeds or YPD agar) using a previously published method for genomic DNA extraction from yeast (Lööke et al., 2011). Alternatively, microbial material was used as a template directly ("colony PCR"). To obtain sequence information from single colonies, dilutions of microbial suspensions were plated on YPD agar. After PCR (conditions: 95°C 5 min, 35 cycles of [95°C 15 s, 50°C 30 s, 70°C 40 s], 70°C 5 min), reaction products were separated on 1x TAE/1% agarose gels and visualized with Midori Green. Primers for DNA sequencing (Microsynth, Switzerland) were 16S_27F (5'-AGAGTTTGATCMTGGCTCAG-3'), or 16S_1492R (5'-RGYTACCTGTTACGACTT-3'), respectively. Presence of fungal DNA was tested with PCR primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

The MultiAlin tool² was used for sequence alignments. Phylogenograms were subsequently generated using the ClustalW2 tool³.

Detection of Total Protein Profiles

Single colonies, obtained from dilutions of microorganisms proliferating on seeds, were cultivated on YPD agar. Cell material

²<http://multalin.toulouse.inra.fr/>

³<http://www.ebi.ac.uk/>

was re-suspended in 1x SDS-loading dye (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 0.01% bromophenol blue), denatured at 95°C for 5 min, and separated by electrophoresis using 10% SDS-polyacrylamide gels. Gels were subsequently stained with Coomassie Blue R-250. GenBank accession numbers for 16S rDNA sequences are: KU510076-KU510083.

RESULTS

Rapid Germination and Early Stress Insensitivity

The initial motivation of this work was to study how an extreme halophyte handles extreme situations at the very onset of its life cycle. To this end, seeds were imbibed in water, or in 200, 300, or 400 mM NaCl. Seed swelling, concurrent with uptake of the surrounding liquids, happened within few minutes. Radicles protruded in the majority of seeds after 30 min; and over the next 2 h roots had clearly elongated (>1 mm). Numerous repeats of the experiment, conducted over a 12-months-period, re-confirmed these observations. Consistently, germination rates were 70–80% both in water and in saline solutions. It was a general rule that seeds showing no radicle emergence within the first 4 h would never germinate. As a comparison, germination was also monitored in amaranth. Quinoa and amaranth share a similar seed architecture (Pal et al., 1990; Prego et al., 1998), and both belong to the Amaranthaceae family. Upon imbibition, amaranth seed swelling resembled that of quinoa, both in terms of duration and relative liquid volume absorbed. However, thereafter amaranth could not keep pace with quinoa. In line with previous work by others (Aufhammer et al., 1998), amaranth radicle emergence only became visible after 3 days (Supplementary Figure S1).

Quinoa's germination behavior is not only peculiar in terms of timing, but also in terms of stress sensitivity: NaCl had no adverse effect during this early developmental phase. In fact, NaCl (up to 300 mM) rather seemed to accelerate radicle emergence. However, saline solutions did impair seedling development later on (Supplementary Figure S2), suggesting that the treatments *per se* were truly challenging to quinoa. It is a general conception that conditional seed dormancy is a strategy enabling plants to keep the highly sensitive embryo protected until conditions become more favorable. Compared to other developmental stages, plants are very vulnerable during germination. Accordingly, environmental adversities normally block development at the very beginning, i.e., at the germination stage. Likewise, in a given plant species, stress-tolerant ecotypes, transgenics, or mutants frequently exhibit partial insensitivity to the inhibitory effects of stress agents on radicle protrusion AND post-germination development (Carvalho et al., 2010; Duarte et al., 2013; Pitzschke et al., 2014). Quinoa's unusual germination performance could be applicable to NaCl stress only, or be a more general habit. To test these options, quinoa seeds were challenged with several abiotic stressors at purposely high concentrations that are normally toxic to plants. Surprisingly, imbibition solutions containing 20 mM Pb₂NO₃, 20 mM CuSO₄, or 2 mM CdCl₂ still enabled

radicle emergence and initial axis growth (Supplementary Figure S3). In fact, germination even occurred in 10% methanol and isopropanol (not shown), conditions that are certainly unacceptable for plant development and reproduction. What is more, quinoa seeds accepted YPD agar as a growth medium (Figure 4; Supplementary Figures S7 and S8). YPD, consisting of yeast extract, peptone and dextrose, represents a rich cocktail of microbial elicitors. Because excessive elicitation of plant immune responses (e.g., ROS production) usually means death, one would consider YPD as highly unsuitable for plants. Accordingly, there was no sign of germination on YPD agar in (viable) seeds of other plant species (amaranth, sesame, millet) over a 14-days observation period.

These observations pointed to a more general and transient “blindness” of quinoa to hostile environments. Though the underlying mechanism remains elusive at this stage, several scenarios can be envisaged: (i) The developmental program is switched on merely “physically,” i.e., by seed swelling and mechanical receptors. Seeds lack sufficient amounts of germination-inhibiting substances; or the embryo is insensitive to such inhibitors. The respective stress signals and receptors are established only *after* radicle protrusion. (ii) Similar to other conditional dormancy-competent plants, quinoa is principally capable of producing and sensing inhibitory compounds. However, these substances are unstable or the respective receptors are temporarily blocked. (iii) Germination may be “enforced” by signals/mechanisms that override any germination-inhibitory program.

Gas-Producing Activities in Rehydrated Seeds

On the surfaces from imbibed seeds, particularly at the radicle exit site, a progressive release of air bubbles was recognizable. Bubble formation started 1–5 min after seed submergence (in water or saline solution) and ceased after ~30 min (Figure 1a). A simple explanation would be that air, which had been trapped in dry seeds, elapsed through cracks in the seed surface. However, this is very unlikely because bubbles continued to elapse after completion of seed swelling. The volume of gas released from each seed was disproportionately large compared to seed volume, i.e., seeds lack the respective “storage capacity.” Furthermore, killed seeds (30 min 90°C prior to imbibition) generated no bubbles. These observations rather pointed to enzymatic activities; and catalase, which uses H₂O₂ to generate water and oxygen, was the suspect enzyme. Its activities are knowingly high in extracts from dry quinoa seeds (Pitzschke et al., 2015), and in-gel activity assays confirmed this (not shown). If “bubbling” indeed resulted from catalase-mediated oxygen formation one would expect a certain pool of the enzyme's substrate in seeds. Consequently, histochemical stainings were conducted using diaminobenzidine (DAB), which forms brown precipitates in the presence of endogenous H₂O₂ and peroxidases. Particularly intensive browning, indicative of local H₂O₂ accumulation, appeared on the radicle exit point. Once testa rupture was accomplished, the major site of DAB precipitation shifted to the tip of the growing radicle (Figure 2).

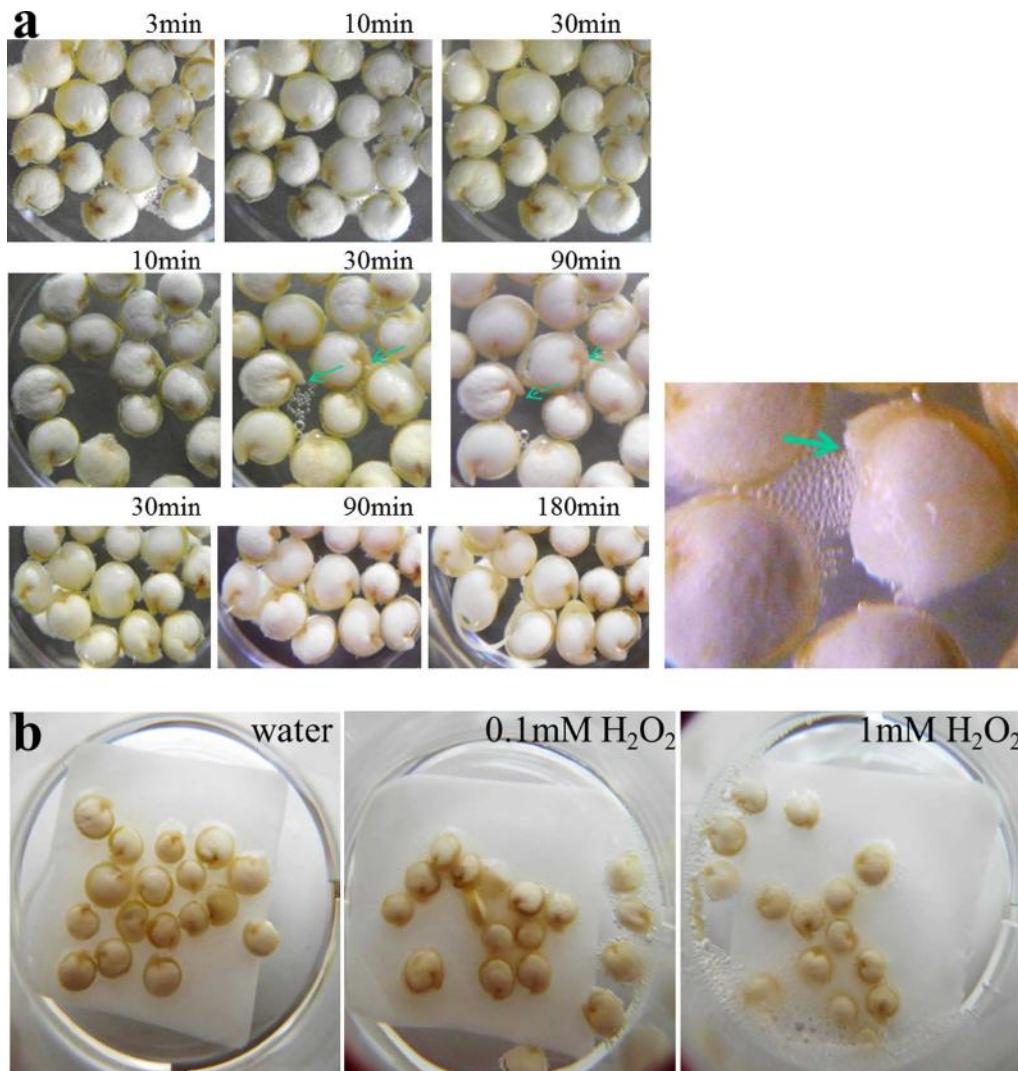


FIGURE 1 | Early germination and bubble-releasing activities in quinoa. **(a)** Seeds were placed in water ($t = 0$), and development was monitored in three independent time series experiments as indicated. Representative images are shown. Note seed swelling between the 10- and 30-min time point (middle panel). Radicles during and after the gas-generating phase are indicated by lined and dashed arrows, respectively. Right: close-up view of seedling showing intense air production. The arrow points to the emerging radicle tip. **(b)** Exogenous H_2O_2 intensifies air bubble release from seeds. Seeds were imbibed in water, 0.1 mM H_2O_2 or 1 mM H_2O_2 for 10 min.

If bubbling derived from catalase activity, and if the enzyme's substrate in seeds was below-saturation levels one would expect exogenous H_2O_2 to intensify gas formation. Seeds were therefore soaked in water, or in dilutions of H_2O_2 . H_2O_2 -treated seeds showed clearly elevated bubbling activities. The effect was stronger in 1 mM H_2O_2 as compared to 0.1 mM H_2O_2 , indicating that 0.1 mM was still below the enzyme's saturation limit (Figure 1b). Subsequent experiments using a Clarke electrode (oxygen detection) removed any doubt concerning the identity of the arising gas (not shown).

Resurrection-Competence

Given the known overlap between plant responses to saline and dry conditions, quinoa seedling performance was examined

under extreme drought stress. Seeds were placed on moist paper (to allow germination) and allowed to air-dry completely. Dried seedlings had a crumpled appearance and weighted ~10% less than completely untreated seeds. Surprisingly, upon re-wetting the fully dehydrated seedlings readily resumed growth (Figure 3).

Regeneration from Broken Seeds – the “Planarian Phenomenon”

Details on quinoa seed architecture have been reported recently (Lopez-Fernandez and Maldonado, 2013). The seed resembles a swollen disk in which the embryo forms a ring embracing the perisperm. Therefore, any vertical cut would not only cleave the perisperm in two. It would also disrupt the embryo and separate essential plant organs. Surprisingly, seed cutting did



FIGURE 2 | Histochemical detection of H_2O_2 in germinating quinoa.
Seeds were placed for 20 h directly into DAB (1 mg/ml in water) directly (**left**) or after 4 h imbibition in water (**right**). Brown precipitates indicate the presence of hydrogen peroxide. Some heterogeneity exists in H_2O_2 contents and distribution; therefore data of three independent staining experiments are shown.

not abort embryo axes growth in quinoa. Instead, there were leaves developing from both halves of a split seed. They had a healthy green color, indicative of photosynthetic activity. Transfer from YPD to ‘regular’ plant medium ($1/2$ MS, 1% agar; **Figure 4**) further facilitated plant growth. To my knowledge, damaged seeds/embryos of no other plant, including Amaranth (same seed architecture) have this capability. For cell culturing and callus propagation plant cells can of course originate from various explants (Moscariello et al., 2013), but there is no *ad hoc* differentiation from broken seeds. In its ability to re-generate quinoa rather resembles planarians (Reddien and Sanchez Alvarado, 2004; Shomrat and Levin, 2013).

Seed-Borne Microorganisms

Under high humidity conditions (wet filter paper in covered Petri dishes), the previously smooth surfaces of seeds became covered by whitish “bumps.” First appearing after 2 days of incubation these grew appreciably within few hours, indicative of microbial proliferation. At days 3–4, individual seeds became

interconnected by a net-like structure (**Figure 5a**). Though still recognizable as such, seed(ling)s fully collapsed upon gentle touch with a tooth pick. With respect to texture and color, seed coat and seed interior became indistinguishable from the soft and whitish microbial mass that was piling up in the surrounding area. Any attempts (e.g., sterile plastics, paper, water from independent sources, change of equipment) to prevent seed colonization under the described incubation conditions failed, a mere “contamination problem” therefore appeared unlikely. More plausibly, the microorganisms originated from the seed interior, as their proliferation was unaffected by seed surface disinfection treatments. Harsh conditions (seed washes in >5% sodium hypochloride for >5 min) proved to be unsuitable, as they blocked germination ability.

Until here experiments had been conducted on pools of 10–30 seeds. In a given sample, microbes released from a single seed can potentially spread and grow on all remaining seeds (“cross-contamination”). To clarify which proportion of seeds actually hosted the microorganisms, seeds were placed one-by-one into separate wells of a 96-well microtiter plate containing sterile water. After 4 days of incubation microbial proliferation was recognizable in each sample. Noteworthy, microbes also emerged from seeds of independent suppliers/batches, as well as on a red quinoa cultivar (**Figure 5a**, right). Their presence was therefore not a seed batch- or storage-related phenomenon, which would have respectively only limited scientific impact. Unfortunately, such constitutive colonization impedes comparative studies between microbe-free vs. microbe-containing quinoa.

To gain insight into this novel plant-microbe association, the microbial partner was characterized in more detail and its potential to account for the observed quinoa growth phenomena was examined.

Endophytic Lifestyle and Indications of Movement within the Host

Microbial softening of plant tissue might be indicative of a pathogenic lifestyle. However, this encroachment only occurred under high-humidity conditions (details see Supplementary data file). Seedlings grown in a wet environment failed to reach far enough into the air. Their stems and leaves remained close to the ground, where they became easily “caught” by the towering mass of microbes. A wet environment, in turn, facilitated colony growth. In contrast, low humidity conditions (seeds plated drily on solid plant medium or slightly moistened filter paper) – which better reflect quinoa’s natural habitat – gave rise to normal-looking healthy seedlings (**Figure 5b**).

To assess presence of microorganisms in healthy and symptom-free plants, surface-sterilized individual seedlings were macerated and the resultant liquid examined. Alternatively, root, stem, and cotyledons were separated by cuts with single-use blades (to exclude cross-contamination). With the intention to support growth of any potentially existing microbial organism, plant materials were placed onto YPD agar. In macerated material, colonies appeared after 1 day. After 3 days, colonies also emerged from cut surfaces of seedling segments. They subsequently spread on/around roots, stems as well as cotyledons. This strongly indicates seed-borne microbes to be able to move



FIGURE 3 | Quinoa seedlings are resurrection-competent. Seeds were placed on moist paper tissue to enable germination. After 24 h they had dried-out completely (left). The right photo was taken 20 h after addition of water to fully dehydrated seedlings. Scale bar: 5 cm.

within the plant. Microbial presence *per se* is harmless to quinoa, as plants lack signs of infection or other developmental impairments. The microbes do not kill their host and *vice versa*, they can be regarded as endophytes. Endophytes are “microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects” (Bacon et al., 2000).

Knowledge about the existence of an endogenous microbial partner makes quinoa’s peculiarities (rapid germination, early and transient stress insensitivity, bubble release) appear in a new light. Unfortunately, there is no seed material available for studying quinoa in a microbe-free context; and attempts to generate such material (cultivation with antibiotics) have proved unsuccessful. Seed-borne endophytes, by contrast, proliferate externally and are cultivable on YPD agar in the absence of plant material. One may thus at least examine their potential contribution to the observed host phenomena.

Reactive Oxygen Species in Host and Microbes

General Consideration

The process of germination involves seed rehydration and emergence of the radicle through the seed coat. It has been shown that embryonic axes growth results from cell elongation rather

than cell division (Gimeno-Gilles et al., 2009; Weitbrecht et al., 2011). In part, this is a turgor-driven process, but cell expansion also requires relaxation, i.e., loosening, of the cell walls. Owing to their ability to react with cell wall components, ROS – primarily the short-lived forms, superoxide and hydroxyl radical – play a critical role here. ROS (H_2O_2) occurring at moderate levels are used by peroxidases for cross-linking phenolic compounds and glycoproteins causing cell wall stiffening. Stronger oxidative conditions, however, favor formation of superoxide and hydroxyl radical, which directly react with cell wall polymers, causing cell wall loosening (Tenhaken, 2014). Quinoa’s rapid germination and initial plant growth could thus theoretically be attributable to elevated ROS contents. Critical amounts of ROS, in turn, may derive from plant and/or microbial activities. As long they are sufficiently close to the cell wall, these molecules should be equally capable of driving cell expansion, and thus, organ growth. The microbe’s ROS-generating capacity was therefore examined, employing histochemical stainings with nitroblue tetrazolium (NBT), which reacts with superoxide to form blue formazane precipitates.

Superoxide Contents in Quinoa

To monitor superoxide generation during quinoa germination, seeds were directly imbibed in water containing 0.2 mM NBT.

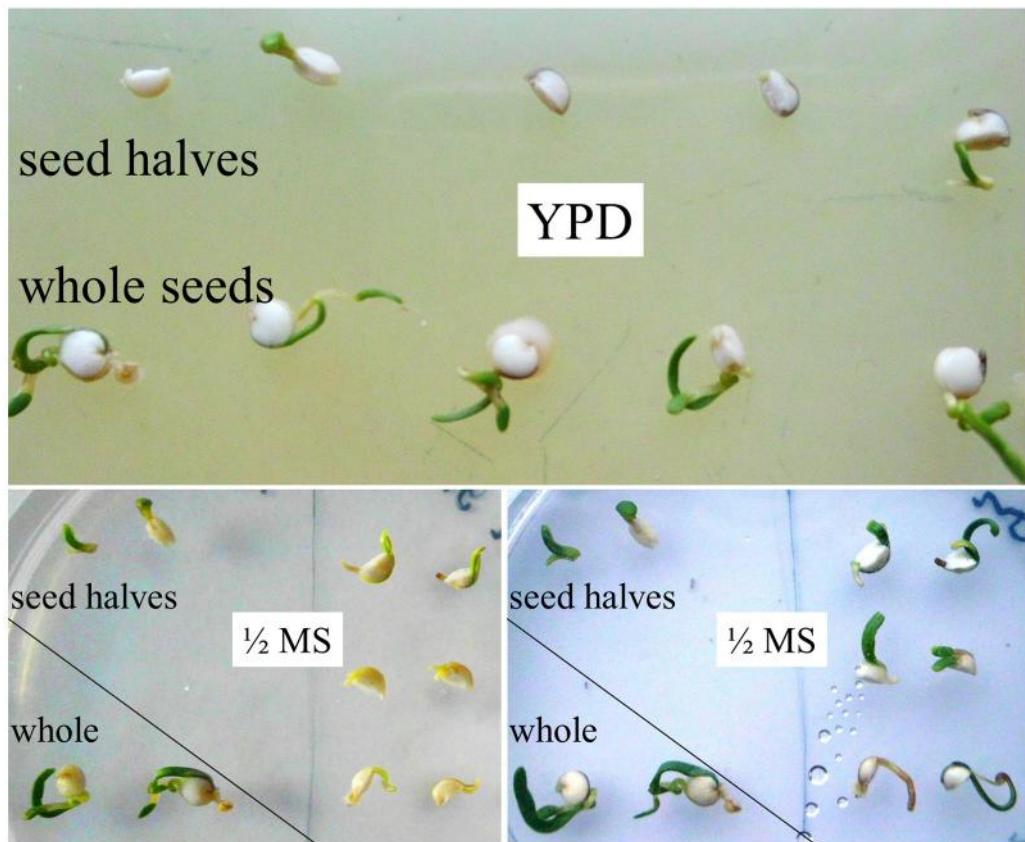


FIGURE 4 | Regeneration ability of split seeds/embryos. Intact seeds and seeds split in halves were incubated on YPD agar for 6 days. Instead of releasing/stimulating microbial colonization, seed halves start to grow. Both halves of the same seed have re-generating capacity. Images were taken directly and 20 h after transfer of seed halves from YPD to $\frac{1}{2}$ MS agar (placed pairwise).

Approximately 3 h after imbibition distinct patches of blue appeared. As a comparison, among the fastest accumulators reported so far, *Vigna radiata*, forms visible precipitates only after 10 h of seed imbibition in NBT (Singh et al., 2014). In quinoa, blue color intensity was particularly intense in the distal root zone, suggesting this to be the major site of elongation. Notable amounts of superoxide also occurred in cotyledons, especially when apical axis expansion had contributed to testa rupture (**Figure 6a**). Seeds that failed to germinate produced no color. Superoxide production may therefore be considered as marker for successful germination; and accordingly, NBT stainings as a simple methodology for assessing seed vigor in quinoa. The data corroborate the view that superoxide generation is essential for seed germination and associated growth (Ishibashi et al., 2010; Kranner et al., 2010; Liu et al., 2012a; Singh et al., 2014).

Superoxide was also visualized in healthy 4-days-old quinoa seedlings lacking obvious signs of colonization. Upon seedlings submergence in NBT solution some patches of blue had formed after 1–2 h (**Figure 6b**). Contrasting the situation in imbibed seeds (see above), color intensity remained weak also after prolonged incubation, and there were no distinct zones of blue. Thus, despite hosting microbial cells and the respective elicitors, quinoa seedlings apparently maintain a “normal” redox

balance. Superoxide accumulation is most pronounced during cell expansion-driven growth, i.e., germination and the early development.

Microbes have High Superoxide Levels

Next, superoxide contents were investigated in quinoa’s microbial partner, using heavily colonized seed(ling)s (grown on wet filter paper or YPD). Upon addition of NBT solution, samples turned blue within seconds, reaching maximum color intensity (dark-blue) after 5 min (**Figure 6c**). In their function as cell wall-loosening agents, ROS released from seed-borne microbes may thus assist radicle protrusion and cell expansion. Under conditions unfavorable to plant development, microbial ROS likely facilitate tissue softening, thus making nutrients available for proliferation. Considering that superoxide and its even more aggressive side-product OH⁻ are destructive to DNA, proteins and other biomolecules, its accumulation seen here, even under non-challenging conditions, appears unusually high. However, extracellular ROS generation – and respective limited intracellular damage – had recently been discovered in bacteria (Diaz et al., 2013).

Although quinoa endophytes are strong ROS-generators and knowingly present inside symptom-free plants (see above),



FIGURE 5 | Humidity-dependent microbial encroachment on quinoa. **(a)** Seeds were incubated under sterile and high-humidity conditions and photographed after 4 days. White quinoa seeds (left, middle) are from two independent seed batches; one is shown at a higher resolution. **(b)** Seeds grown on agar medium or slightly moistened filter paper develop normally.

superoxide levels were low in 4-days-old seedlings (Figure 6b). As a likely explanation, healthy plants carry few microbial cells only. It should also be pointed out that microbial superoxide production is strongest during proliferation (Figure 6d). Inside living host tissues the microbes likely proliferate much less “aggressively,” as compared to outside.

Microbial Catalase Activities as Likely Cause of Bubble Release from Seeds

Catalases are wide-spread among living organism (Klotz et al., 1997). Against this background I examined a possible involvement of endophytes in “seed bubbling”. When freshly grown colony material was immersed in H₂O₂ solution, bubble formation started immediately. The foam that built up within seconds points to strong microbial catalase activities. Observations were largely identical with colonies proliferating on seeds or YPD medium (Supplementary Figure S4).

Quinoa Endophytes belong to the Genus *Bacillus*

The final question was which type(s) of microorganisms inhabited quinoa seeds and thus could account for the

developmental phenotypes observed. Despite strong similarities in colony morphology to some yeast strains growth (Honigberg, 2011) (Supplementary Figure S5), microscopy and PCR-based analyses revealed exclusive presence of bacterial populations. To get an idea on population diversity, I used DNA-homology-based classification and protein profiling analyses. Microscopy of microbial populations revealed certain differences with respect to cell shape (roundish vs. stretched) and motility (slow, rapid, or extremely rapid). Cells had the tendency to aggregate, and cell sizes (600–700 nm width) spoke for bacterial rather than fungal origin (Supplementary Figure S6). To nevertheless assess the possibility of co-existing fungi among the bacterial cells, PCR experiments were conducted using standard primers (fungITS1/ITS4) for the amplification of fungal ribosomal RNA genes. Reactions were set up in parallel with primers for bacterial ribosomal RNA (bact16S_27f/1492r).

Template material originated from colonies that had been proliferating on imbibed seeds. More precisely, these were two independent microbial communities for each of two seed batches. Consistent with results from microscopy, fungal DNA-directed primers yielded no detectable product. Bacterial 16S rDNA-targeting primers generated products of the expected

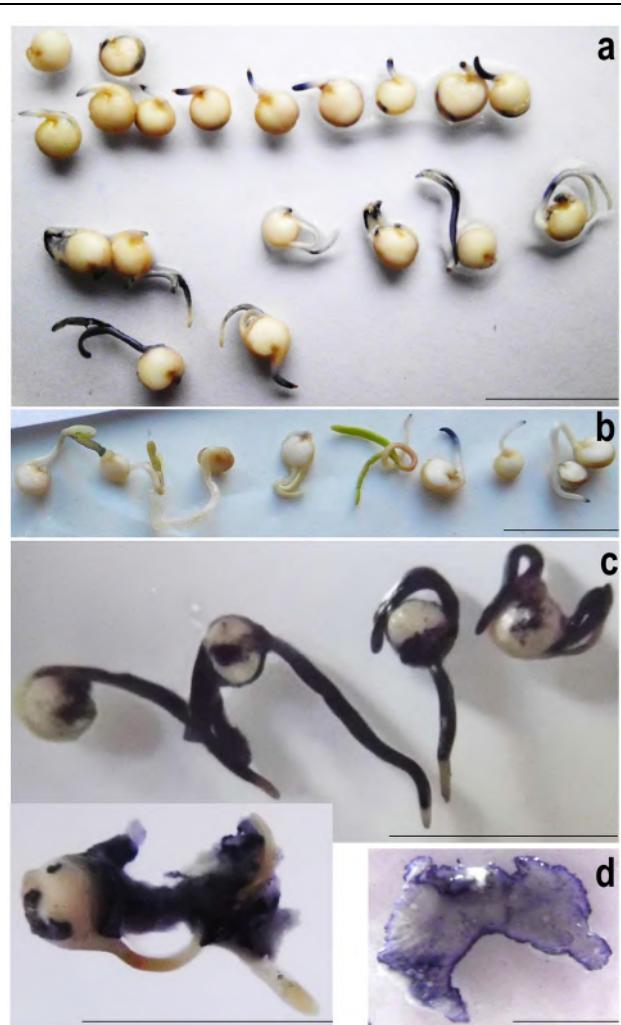


FIGURE 6 | Superoxide detection in quinoa (a,b) and seed-borne microorganisms (c,d). (a) Quinoa seeds were imbibed in water containing 0.2 mM NBT for 12 h, arranged according to their developmental stage and photographed. Note the lack of superoxide generation in non-germinating individuals (top; 1 representative shown). (b) NBT stainings of 4-days-old seedlings grown on damp filter paper. Experiments were repeated at least three times; one representative is shown. (c) Quinoa seedlings heavily colonized by endogenous microbes were incubated in NBT solution for 10 min. The seeds had been germinated on filter paper under high-humidity conditions for three (top) or four (bottom) days. (d) Microbial colonies proliferating on YPD agar were stained in NBT solution and photographed after 2 min. Note that color intensity is particularly high at the border, i.e., growing zone. Scale bar: 1 cm.

size (1.5 kb) in all four templates. Based on their 16S rDNA sequence homology quinoa endophytes belong to the genus *Bacillus*. Notably, heterogeneity existed within and between individual DNA samples, even though microbial template material originated from the same seed batch or even the same seed. This suggested co-existence of multiple bacterial isolates. Consequently, single colonies (obtained via diluting and subculturing) were used for 16S rDNA sequence analysis, homology search, and alignments (Figure 7a; Supplementary Table S1,

GenBank submissions). All samples turned out to represent the genus *Bacillus*, and closest homologs were *Bacillus subtilis*, *B. amyloliquefaciens*, *B. methylotrophicus*, or *B. tequilensis*.

To further explore microbial population diversity, I used comparative protein profile analysis. Such approach had proven useful in a similar context in maize (Figueiredo et al., 2009). In their protein patterns, individual colonies originating from a single seed (Figure 7b) clearly differed from each other. One may interpret this pattern heterogeneity as a first indication of strain-specific sub-tasks. Notably, all colonies examined (>50) had high superoxide contents. They also consistently showed catalase activity; consistent with the homology-based classification as *bacilli* (Jedrzejas and Huang, 2003).

It is evident that the here-presented collection of sequences and protein profiles is not exhaustive. Quinoa's microbiome in its entirety can only be elucidated using high-throughput techniques (e.g., NGS). The current data suggest co-existence of several microorganisms in quinoa, most/all of which are *bacilli*.

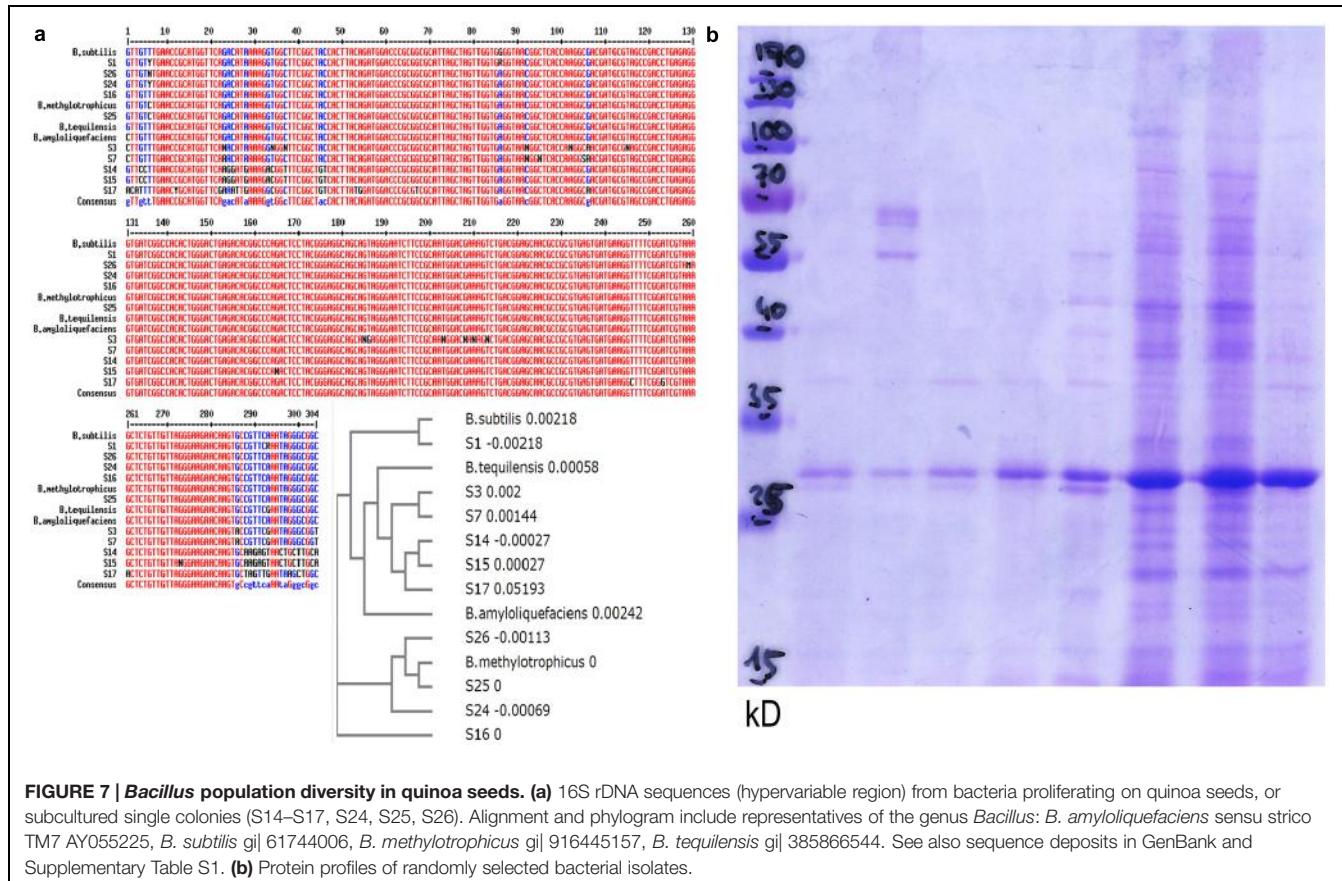
More precise statements on the identity of quinoa seed-borne microbes cannot be made at this stage. As highlighted previously, there are “difficulties of purely 16S rDNA-based taxonomy, emphasizing the need to interpret the massive amounts of molecular data from environmental sequencing projects in a bacterial ecology framework” (Maughan and Van der Auwera, 2011). Future work will therefore employ other marker genes for a more detailed taxonomic classification; accompanied by further biochemical characterization.

DISCUSSION

Quinoa demonstrates in multiple ways that our knowledge on plant habits is anything but complete. Most of us would presumably have considered some phenomena as “very odd” or “simply impossible.” Quinoa distinguishes itself from other plants in terms of germination time (rapid) and flexibility (hostile/artificial environments tolerated), seedling growth (fast) and bubble release during early imbibition. In addition, broken seeds/split embryos are able to regenerate, and seedlings can revive from an air-dry state. The latter ability knowingly exists only in a small heterogeneous group of so-called resurrection plants (Dinakar and Bartels, 2013). Quinoa would thus be the first crop plant with resurrection-competence. Ongoing research – of obvious practical relevance – shall determine until which developmental stage quinoa holds its ability to revive.

Endophytic bacteria belonging to the genus *Bacillus* were found to reside in 100% of seeds, from different batches and cultivars. It is tempting to speculate that the association is obligate for the host. Because these bacilli are mobile and omnipresent their vertical transmission is very likely. It remains to be seen whether (seed-originating) microbes can enter plants anew, i.e., via roots. This would enable them to make their way into previously “naive” plants and resultant progenies.

The fact that under low/moderate humidity bacteria stay within the plant may explain why they remained unnoticed so far. Under conditions found in quinoa's natural habitat (the Andes; arid climate), both plant and microbe may benefit



from this endophytic association. Bacilli can persist as spores over a long time (Gest and Mandelstam, 1987). Seeds appear as an ideal means for storage but also for distribution. Cells exiting from germinating seedlings are potential starting material for colonization of a (new) soil environment. To pioneer new grounds the endophyte must permit host survival (seed production).

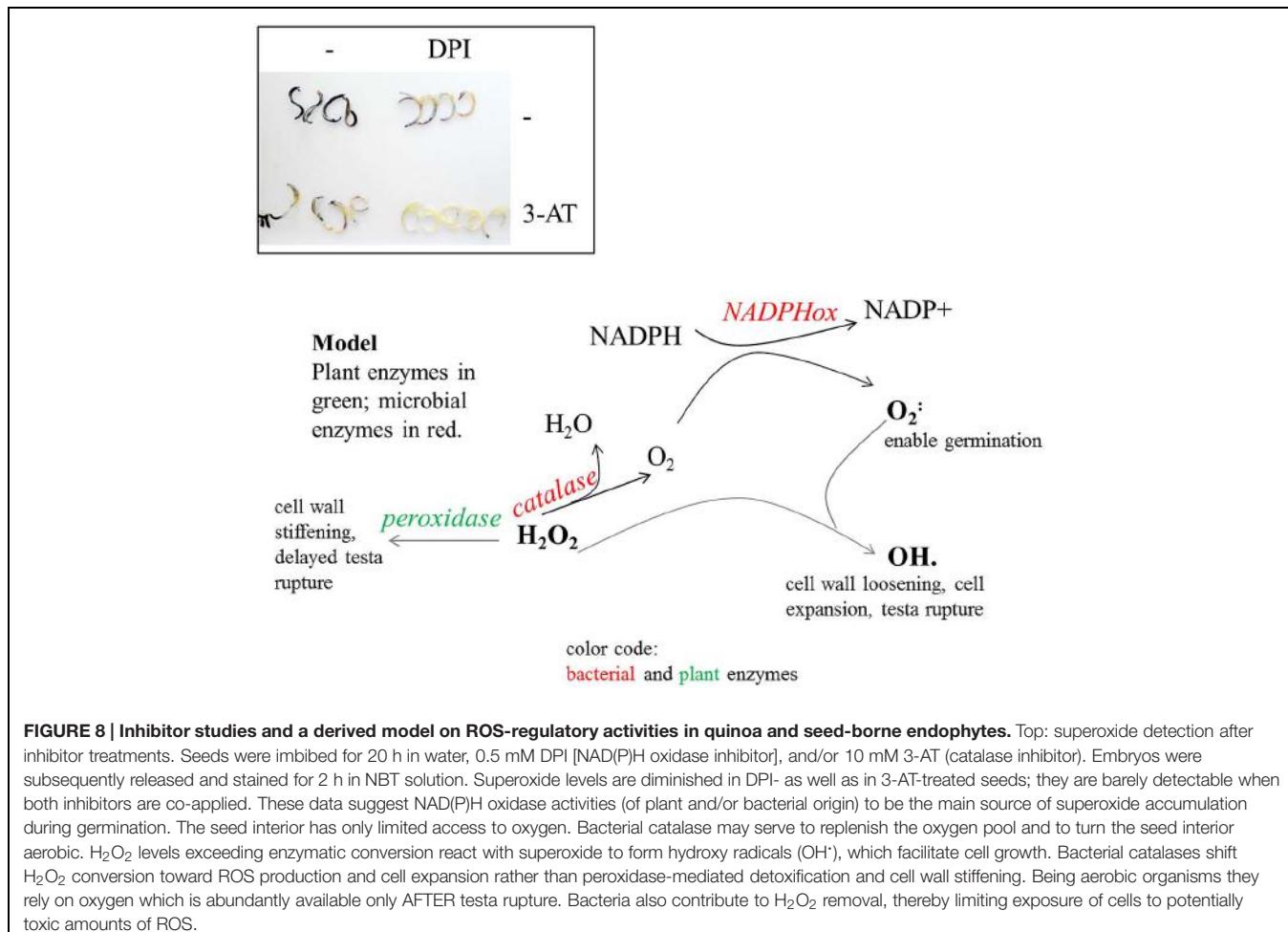
Under high-humidity conditions quinoa seedlings performed rather poorly. The microbial partners made the best of the situation by exploring its host as a mere energy source. Seed coat softening owing to bacterial activities enabled them to access nutrients also from outside. Soon, plant material became covered under layers of microbes. This seems an efficient for microbial proliferation, but it is not a sustainable strategy because it means death of the host before seed production. How long the bacilli can survive and form spores outside their host remains elusive. Cells are cultivable on YPD (Supplementary Figure S5), but in nature (soil) nutrients are rather scarce. What is more, quinoa's natural habitat is hostile, not only for plants. If microbes want to survive and proliferate, they must withstand the same demanding environment as their host. At least for high-salinity stress this is indeed the case.

The general impression was that quinoa is “forced” to grow, irrespective of its environment. Though final proof is lacking (see below), a model can be proposed based on the current data: owing to rapid cell elongation, stimulated by bacterial

superoxide, quinoa overcomes the most sensitive stage in plant development within short time (Figure 8, model). It is a general conception that plants withstand stresses better when encountered later in development. Its exceptional resurrection-competence makes quinoa even more flexible. It remains to be seen whether quinoa endophytes have additional, i.e., redox-independent mechanisms, to manipulate host growth. Notably, members of the genus *Bacillus* are known to produce plant growth-promoting substances (Chen et al., 2007; Madhaiyan et al., 2010).

The endophytic association *may* protect quinoa from other – potentially pathogenic – microorganisms, thereby ensuring seed viability and plant health. This presumption derives from the known ability of plant endophytes to produce antimicrobial substances (Mousa and Raizada, 2015) and the virtual absence of other microorganisms in/outside quinoa seeds. Irrespective of seed surface sterilization, fungi or obviously different bacteria emerged only rarely (<1%), even when seeds were incubated on sugar-containing medium, YPD- or LB-agar. Resuspended seed powder gave no rise to any colony growth (>14 days incubation on YPD or LB).

A very instructive review article compiling reports on bacterial seed endophytes made clear that to harbor a single bacterial strain is rather the exception. Among the 25 host plant species known so far, 19 are inhabited by multiple endophytes, including Actinobacteria, Bacteroidetes, Firmicutes,



and Proteobacteria. Proteobacteria (particularly gamma-) are the most predominant ones isolated from a wide variety of plants. Quinoa adds an economically important crop to the list of host plants. Its endophytic population exhibits important features for seed colonizers (reviewed in Truyens et al., 2015). These include amylase activity to utilize starch and resume growth after long-term survival in dry seeds, cell mobility to migrate within the plant and to enter seeds before seed hardening, and the ability to withstand high osmotic pressure arising from accumulation of starch and water loss during seed maturation.

Indications for Quinoa endophyte amylase activities are their ability to fully soften quinoa seeds, whose major part, the centrally located perisperm, consists of starch-enriched dead cells (Lopez-Fernandez and Maldonado, 2013) as well as sequence homology to *B. amyloliquefaciens*. Furthermore, quinoa endophytes accept starch as a sole energy source (AP, unpublished).

Cell motility is high. (In fact it was barely possible to get a sharp microscopy image). Being able to form spores (as observed by microscopy), quinoa-derived bacteria are well-prepared for long-term survival and osmotic pressure. They also exhibit high stress resistance in their vegetative stage, as concluded from the rapid encroachment on seeds imbibed in high-salinity solutions.

In addition, quinoa endophytes withstand other potentially toxic adversities, as they also proliferated on CdCl₂-treated seeds. Because quinoa itself displays high tolerance toward heavy metal stress the potential of this novel plant-endophyte-association for phytoremediation deserves further attention.

It will be interesting to see whether *all* quinoa plants growing worldwide harbor seed endophytes, and whether the microbiome in highly salt-tolerant quinoa cultivars differs from that in less tolerant cultivars. Such specificity would indicate that plants owe their robustness to an ingenious combination of bacteria. An attractive hypothesis arising from these considerations is: are bacterial strains from highly tolerant quinoa cultivars transferable and effective, i.e., tolerance-enhancing, in less resistant cultivars?

An intriguing question concerns the microbe's capability to enter species other than quinoa. Not necessarily would other hosts benefit from such association. To stay healthy, the host must be able to actively maintain its redox balance (i.e., flexibly adjust its own ROS producing and scavenging activities) and/or tolerate major fluctuations in ROS concentrations. In other words, quinoa and its endophyte may be a perfect match, but the same endophyte in a different plant might be a disaster. Ongoing research therefore involves cocultivation of several plant species with quinoa endophytes, assessment of disease symptoms and

stress performances (AP, unpublished). It remains to be seen whether the bacteria can establish themselves in these plants. As highlighted very recently, inoculation of microbiomes into gnotobiotic hosts holds huge potential to improve plant health (Mueller and Sachs, 2015). A major bottleneck for such approach, cultivability, would not be a hurdle for quinoa endophytes.

As microbial cells are present in seeds already, they can potentially active the plant's immune system from the very beginning on. Flagellin and other bacterial elicitors, undoubtedly present also on the surface of quinoa endophytes, can be perceived by respective MAMP (microbe-associated molecular patterns) receptors of the host cells. It is tempting to speculate that signaling pathways, e.g., those involving MAPKs (Pitzschke et al., 2009), are activated to turn the plant into a state of alert. In other words, endophyte-hosting seeds are 'naturally' primed. As a result, plants arising from such seeds may tolerate stress better as compared to non-inhabited seeds. Quinoa endophytes do not just 'reside' in plant tissue, but move within the plant and are metabolically active. Time-dependent changes in MAMP identity and composition are therefore likely to occur; and new MAMPs can activate other host receptors. In the long run, numerous stress signaling pathways should become activated and mediate host resistance to multiple stresses. In this context it is worth mentioning a recent review (Wiesel et al., 2014), which highlights the potential of biological elicitors for crop protection. One reason for the high salinity tolerance in quinoa plants is their low stomatal density (Shabala et al., 2012). Because (at least in *Arabidopsis*) elicitor-inducible MAPK proteins have a second function in stomatal pattern control (Wang et al., 2007; Pitzschke, 2015), an indirect contribution of quinoa to limit water loss appears possible.

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Leading scientist in the field believe that 'quinoa has some unique as yet unidentified features' which account for its outstanding salt tolerance (Adolf et al., 2013). In the current work several findings and considerations suggest endophytic partners to be one of these features, and to account for the observed phenotypes. For an ultimate proof one would have to cure quinoa from its endophytes and then use naïve seeds for re-inoculation. This is not trivial because harsh sterilization procedures prevent seed germination, antibiotics severely affect seedling growth, and plants develop poorly in autoclaved soil.

AUTHOR CONTRIBUTIONS

AP discovered quinoa endophytes, designed and conducted all experiments and wrote the paper. Helpful comments/assistance by others are listed under "acknowledgments."

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00002>

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Application of Plant-Growth-Promoting Fungi *Trichoderma longibrachiatum* T6 Enhances Tolerance of Wheat to Salt Stress through Improvement of Antioxidative Defense System and Gene Expression

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Soil salinity is a serious problem worldwide that reduces agricultural productivity. *Trichoderma longibrachiatum* T6 (T6) has been shown to promote wheat growth and induce plant resistance to parasitic nematodes, but whether the plant-growth-promoting fungi T6 can enhance plant tolerance to salt stress is unknown. Here, we determined the effect of plant-growth-promoting fungi T6 on wheat seedlings' growth and development under salt stress, and investigated the role of T6 in inducing the resistance to NaCl stress at physiological, biochemical, and molecular levels. Wheat seedlings were inoculated with the strain of T6 and then compared with non-inoculated controls. Shoot height, root length, and shoot and root weights were measured on 15 days old wheat seedlings grown either under 150 mM NaCl or in a controlled setting without any NaCl. A number of colonies were re-isolated from the roots of wheat seedlings under salt stress. The relative water content in the leaves and roots, chlorophyll content, and root activity were significantly increased, and the accumulation of proline content in leaves was markedly accelerated with the plant growth parameters, but the content of leaf malondialdehyde under saline condition was significantly decreased. The antioxidant enzymes-superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in wheat seedlings were increased by 29, 39, and 19%, respectively, with the application of the strain of T6 under salt stress; the relative expression of SOD, POD, and CAT genes in these wheat seedlings were significantly up-regulated. Our results indicated that the strain of T6 ameliorated the adverse effects significantly, protecting the seedlings from salt stress during their growth period. The possible mechanisms by which T6 suppresses the negative effect of NaCl stress on wheat seedling growth may be due to the improvement of the antioxidative defense system and gene expression in the stressed wheat plants.

Keywords: *Trichoderma longibrachiatum* T6, wheat seedling, salt stress, plant-growth-promoting, antioxidative defense system and gene expression

INTRODUCTION

Salt stress is one of the major abiotic stresses that affects plant growth, development, and crop yield (Ma et al., 2012; Rivero et al., 2014). Wheat (*Triticum aestivum*), the most important cereal crop in the world, is considered to be salt sensitive (Tian et al., 2015). Grown under salt conditions, wheat plants often produce a significantly low grain yield with poor quality. Studies have shown that salt stress can induce several morphological, physiological, and metabolic responses of plants, which causes ROS stress and osmotic stress in plants, leading to increased peroxidation of lipid and antioxidant enzyme inactivation (Garg and Manchanda, 2009). Also, plants grown under salt stress conditions usually synthesize several kinds of soluble compounds including soluble sugars and proteins, which may help adjust osmoticum, retain cell turgor, and stabilize cell structures (Bartels and Sunkar, 2005).

At the present time, about 6% of the arable land on the earth is salt affected, especially in arid and semiarid regions (Bui, 2013). This seriously threatens global agricultural sustainability and food security. Thus, it is critically important to develop effective and practical techniques to alleviate the negative effects of salt stress on plant growth and development. Conventional breeding and transgenic technology have been used to develop new cultivars with improved salt tolerant traits, but breeding salt tolerance has not been successful (Phang et al., 2008). The long breeding cycle and low breeding efficiency for the quantitative trait presents challenges. Transgenic technology has the ability to incorporate salt tolerant genes in new plant materials (Sairam and Tyagi, 2004; Sahi et al., 2006; Lu et al., 2007), but the effectiveness has been low and also enveloped in controversy (Zou et al., 2015). Furthermore, gene loss, high cost, and other regulatory issues are the main bottlenecks for commercial transgenic plants use (Glick, 2007). A newer attempt is to apply exogenous compounds to decrease the negative effect of abiotic stress; this technique has been shown to increase plant tolerance to salt stress, such as using oligochitosan (Ma et al., 2012), nitric oxide and calcium nitrate (Tian et al., 2015), chitooligosaccharides (Zou et al., 2015), and jasmonic acid (Qiu et al., 2014) in wheat, as well as gibberlllic acid and calcium chloride in linseed (*Linum usitatissimum*; Khan et al., 2010), and ascorbic acid in broad bean (*Vicia faba*; Younis et al., 2010). These exogenous compounds have been shown to improve the salt tolerance of plants, but the exact physiological mechanisms are unknown. A new, innovative technique that has attracted a great deal of attention in recent years, is to use plant-growth-promoting bacteria and fungi to induce plant resistance to abiotic stress. It is an effective approach for enhancing plant tolerance to salt stress and this approach may play a role in the development of sustainable agricultural systems. *Trichoderma* spp. is one of

the important groups of rhizosphere microorganisms, which can impart some beneficial effects on promoting plant growth and development (Harman et al., 2004; Qi and Zhao, 2013). The *Trichoderma* species have also been known to be used by plants as biological control agents for controlling different species of plant fungus diseases for decades (Harman et al., 2004). Mastouri et al. (2010) have reported that *Trichoderma afroharzianum* T22 can enhance tomato (*Solanum lycopersicum*) seed germination under biotic and abiotic stresses, alleviating oxidative damage in osmotic stressed seedlings. However, the underlying mechanisms responsible for the alleviation of oxidative damage remain to be explored. Little information is available regarding the potential and possible mechanisms of plant-growth-promoting fungi T6 in enhancing the tolerance of wheat to salt stress.

Our previous studies show that *Trichoderma longibrachiatum* has a higher potential of parasitic and lethal effects against *Heterodera avenae* (Zhang et al., 2014b), but its effects on wheat are fairly high in promoting plant growth and nematode control (Zhang et al., 2014a). However, the previous studies failed to determine the possible mechanism of T6 enhancing the tolerance of wheat to salt stress. Therefore, the objectives of the present study were to (i) evaluate the effect of the strain of T6 on wheat growth under various levels of salt stress, and (ii) explore the possible mechanism of T6 in response to salt stress at physiological, biochemical, and molecular levels.

MATERIALS AND METHODS

Experiments were carried out at the Pratacultural Engineering Laboratory of Gansu Province. The replicated experiment was firstly conducted in 2014, and in order to obtain solid results, the same and entire experiment was repeated for a second run in 2015.

Fungal Inoculum Preparation

The salt tolerance strain of T6 was obtained from the Laboratory of Plant Pathology, Gansu Agricultural University. The conidia suspension of T6 was prepared according to the method of Zhang et al. (2014b). Final suspension of 1.0×10^8 conidia per ml were prepared and stored at 4°C .

Plant Material and Treatment Conditions

All the experiments were conducted with wheat (cv. Yongliang 4). Consistent sizes of wheat seeds were surface-sterilized with a 1% NaOCl solution for 10 min, and then thoroughly washed with distilled water six times over. Wheat seeds were soaked in T6 or sterilized distilled water overnight for 12 h, and then transferred to Petri dishes with two layers of moist gauze for germination at 25°C for 24 h in the dark. Fifty germinated wheat seeds were planted in each transparent box (12 by 12 by 5 cm) which was filled with water agar containing 0 and 150 mM NaCl (control and NaCl, respectively) and were grown in an incubator at a day/night cycle of 16/8 h. The germinated seeds grown in transparent boxes were cultured in an incubator with a relative humidity (RH) of 65% and a light intensity of $600 \text{ mol m}^{-2} \text{ s}^{-1}$. Thus, the experiments were designed for four groups, which included a

Abbreviations: AN, acid ninhydrin; ASA, aqueous sulfosalicylic acid; BSA, bovine serum albumin; CAT, catalase; CFUs, colony-forming units; GAA, glacial acetic acid; H₂O₂, hydrogen peroxide; MDA, malondialdehyde; PBS, phosphate buffer solution; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TTC, triphenyl tetrazolium chloride; T6, *Trichoderma longibrachiatum* T6.

control (neither treated with T6 nor 150 mM NaCl solution), a negative control with 150 mM NaCl stress, a positive control with T6 treatment, and a stressed group T6-NaCl (treated with T6 and 150 mM NaCl). Each treatment was repeated six times.

Number of Colonies in Wheat Root

Fifteen days after being treated with 150 mM NaCl and the suspension of T6, number of colonies in wheat root was assessed and recorded. The ability of T6 to colonize and grow in association with wheat roots were assessed by determining final T6 densities. Root colonization was assessed following an established protocol (Zhang et al., 2015), where 1 g of surface-sterilized sub-samples of air-dried chopped roots was crushed in 9 ml of sterile water with antibiotics (50 mg^{-1} of streptomycin sulfate) with a sterilized pestle and mortar. The root suspension (10^{-3}) was plated onto each of six 9-cm diameter Petri dishes [containing *Trichoderma* medium E (TME); Papavizas and Lumsden, 1982] and incubated in the growth incubator at 25°C for 72 h. The number of T6 density per g of air-dried roots was counted from clear CFU forming on medium after dilution plating of the root suspension. Each treatment was repeated six times.

Growth Parameters

Wheat seedlings were harvested 15 days after NaCl treatment. Shoots and roots of wheat seedlings were separated and washed with distilled water three times, and then dried and weighed. Root length and weight were determined immediately after being grown for 15 days. For the determination of dry weight, all the samples of wheat seedling shoots and roots were oven-dried at 105°C for 30 min, and then kept at 80°C to obtain a constant weight and were then weighed. Each treatment and control was repeated six times. Relative water content (RWC) of the shoots and roots were recorded by the method of Tian et al. (2015).

$$\text{RWC (\%)} = (\text{FW} - \text{DW})/\text{FW} \times 100$$

Where RWC represents relative water content, FW represents fresh weight, and DW represents dry weight.

Chlorophyll and Proline Content

Chlorophyll was extracted with 80% (v/v) cold acetone from all leaf segments (200 mg), which were frozen at -20°C after 15 days of 150 mM NaCl treatment. The content of chlorophyll a, chlorophyll b, and total chlorophyll in wheat seedling leaves were determined spectrophotometrically according to the method of Lichtenthaler (1987).

Proline content of leaves was determined following the procedure of Bates et al. (1973). After 15 days of NaCl treatment, 0.5 g of fresh wheat seedling leaf samples were homogenized with 10 ml of 3% ASA. After that, 2 ml of AN and 2 ml of GAA were added to 2 ml of the extract and mixed for 1 h at 100°C . The reaction was then stopped by using an ice bath. The reaction mixture was extracted with 4 ml toluene. The absorbance of fraction with toluene aspirated from liquid phase was measured at 520 nm. Proline concentration was determined by following a calibration curve and expressed as micromoles proline per gram of fresh weight. Each treatment was repeated for six times.

Soluble Sugar and Protein Content

All the collected leaf samples from the treatment and control were washed with distilled water three times and cut into small pieces to determine the content of soluble sugar and protein in wheat seedling leaves. Thereafter, the small pieces of wheat seedling leaves were dried, weighed, and placed separately in glass vials which contained 10 ml of 80% (v/v) ethanol, and then placed in a water bath heated at 60°C for 30 min. The filtered extracts were diluted with 80% (v/v) of ethanol to get a total volume of 20 ml. Soluble sugar concentration in the extract was determined by comparison with a standard curve using the criterion of glucose, as described by Giannakoula et al. (2008). Soluble protein content was carried out according to the method described by Bradford (1976). The coomassie brilliant blue G-250 reagent with BSA was regarded as a standard to determine the content of soluble protein in wheat seedling leaves.

Root Activity and Lipid Peroxidation Degree

Root activity was determined by TTC, as described by method with some modifications (Zhang et al., 2014a). Wheat seedling roots were washed, and then excised at 2 cm in length from the root tips 15 days after NaCl application. Root tips were dried with filter paper and homogenized with liquid nitrogen in an ice cold mortar and pestle. The reaction mixture consisted of 0.5 g samples of root tips, 5 ml of PBS (pH 7.0), and 5 ml of 0.4% TTC in a beaker, with root tips fully immersed in the solution for 1 h at 37°C , then immediately mixed with 2 ml of 1 M sulfuric acid to stop the reaction. The red extraction was moved into a tube making the total volume 10 ml using ethyl acetate. Thereafter, the extraction was added and vortexed for 30 s and centrifuged (1,000 rpm, 5 min). The extraction was measured at 485 nm against a blank of ethyl acetate. The root activity of wheat seedlings was determined by measuring the activity of dehydrogenase, which present the function to reduce the chemical TTC. The analysis was repeated six times.

The concentration of MDA, a product of lipid peroxidation, was assessed by the method of TBA. The contents of MDA were determined following the method of Madhava Rao and Sresty (2000). Samples of fresh leaves (0.5 g) were homogenized in 10 ml of 0.1% (w/v) TCA. Then, 4 ml of 0.5% (w/v) TBA containing 20% (w/v) TCA was added to 1 ml aliquot of supernatant. The absorbance of the supernatant was recorded at 532 and 600 nm and MDA content was expressed as $1 \mu\text{mol MDA g}^{-1} \text{ FW}$. The content of TBARS was determined as described by Hodges et al. (1999). The concentration of TBARS was calculated based on the absorbance at 532 and 600 nm. All the treatments and control were repeated six times.

Antioxidant Enzymes Activities

Antioxidant enzymes were extracted at 4°C using 0.5 g tissue from the fresh samples of wheat seedling leaves after 15 days of NaCl treatment. Fresh samples were homogenized with 5 ml of extraction buffer, which contained 0.2 mM EDTA, 0.1 M phosphate buffer (pH 7.8) and 2% polyvinylpyrrolidone. Extracts were centrifuged at 10,000 rpm for 15 min, and the supernatants

were used for determining the activities of antioxidant enzymes. All the analysis was repeated six times.

Superoxide dismutase activity was measured as described by the method of Costa et al. (2002). One unit of SOD activity was defined as the amount of crude enzyme extract that inhibits the reduction of β -nitro blue tetrazolium chloride by the rate of 50% at 560 nm in the spectrophotometer.

Peroxidase activity was assayed by determining the increase of absorbance at 470 nm with guaiacol as the substrate (Kochba et al., 1977). The concentration of protein in the extracts was calculated and carried out as the method described by Lowry et al. (1951).

Catalase activity was assayed according to the method of Cakmak and Horst (1991) with some modifications. The activity of CAT was determined by calculating the decline decomposition of H_2O_2 in absorbance at 240 nm.

Extraction of Total RNA and Analysis of Gene Expression by Quantitative Real Time Reverse Transcriptase-PCR (qRT-PCR)

Total RNA was extracted from different treatment of the wheat seedlings leaves (0.2 g) by using PureLink[®]RNA Mini Kit (Tiangen Biotechnology, Beijing, China). The quality of total RNA was quantified by the UV spectrophotometer. First-strand cDNA was synthesized by Revert Aid TM First Strand cDNA Synthesis Kit (Tiangen Biotechnology, Beijing, China). The qRT-PCR was performed in a 20 μ l reaction volume tube using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) according to the method described previously by Li et al. (2013) and Qiu et al. (2013). Specific primers for SOD, POD, and CAT genes, and the internal control tubulin gene were used to amplify amplicons specific for wheat seedlings. Specific primers were designed according to wheat EST sequences of candidate proteins available in NCBI (Qiu et al., 2014; Zou et al., 2015), and the DNA sequences of specific primers are provided in Table 1. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. Gene expression was counted and expressed relative to the expression levels of an internal reference gene actin in each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

The data was subject to one-way ANOVA using the SPSS package (SPSS V16.0, SPSS, Inc., Chicago, IL, USA). Treatment effects were determined using Duncan's multiple range test and the significances were expressed at $P < 0.05$.

RESULTS

Plant Growth and Relative Water Content

Fifteen days after NaCl treatment, the wheat seedling height (Figure 1A) and root length (Figure 1B) were significantly

TABLE 1 | DNA sequences of qRT-PCR primers for determining the antioxidant gene expressed in wheat seedlings under salt stress.

Gene names	Accession number	Premiers sequence (5'-3')
Actin	AB181991	Forward: CTCTGACAATTCGGCTCA Reverse: ACACGCTTCCTCATGCTATCC
SOD	JQ613154.1	Forward: CATTGTCGATAGCCAGATTCCCTT Reverse: AGTCTCCACCAGCATTCCAGTA
POD	X53675.1	Forward: CAGCCCTGTAGCCAACATAAA Reverse: GCACCTCCACGACTGCTTTG
CAT	GU984379.1	Forward: TTTGATGGGAGTCTGTGCTTGTG Reverse: ACGGTGAGGGAGTTGTCGTTGTT

reduced compared to the control, while the application of the plant-growth-promoting fungi T6 significantly increased wheat seedling height and root length, compared to the NaCl stress treatment. Compared to the control, T6 promoted wheat seedling growth after 15 days without NaCl treatment.

The plant-growth-promoting fungi T6 showed a great ability to colonize the roots of wheat seedlings. Compared to the control and NaCl treatments alone, colonies of T6 were re-isolated from the wheat roots, regardless of whether or not the wheat seeds were soaked with the suspension of T6 under salt stress (Table 2). In contrast, there were no colonies re-isolated from the roots in the control or NaCl treatments alone. These observations indicated that the strain of T6 had the ability to colonize the roots of wheat seedlings under salt stress.

The concentration of 150 mM of NaCl stress decreased wheat seedling growth. The shoot height and root length decreased by 17 and 16%, respectively, compare to the control (Table 2). The shoot and root fresh weights decreased by 33 and 26%, and dry weights decreased by 15 and 19%, respectively, compared to the control (Table 3). However, compared to NaCl-stressed plants, the shoot height and root length increased by 15 and 34%, respectively, in plants after being treated with T6 under salt stress (Table 2). Also, the shoot and root fresh weights increased by 23 and 22%, and dry weights increased by 10 and 24%, respectively (Table 3).

Compared to the control, the RWC of shoots and roots were lower for the plants treated with 150 mM of NaCl stress alone, but inversely, the application of T6 significantly increased the water content in wheat shoots and roots (Table 3).

Chlorophyll and Proline Content

Chlorophyll a, chlorophyll b, and total chlorophyll contents were decreased by 15, 17, and 15%, respectively, when treated with 150 mM of NaCl, compared to the control. However, the leaf chlorophyll a, b, and total chlorophyll contents in NaCl-stressed wheat seedlings were reversed to a similar level as the control, after being treated with T6. Leaf chlorophyll contents were lower in the plants under salt stress without T6. Also, compared to the control, the values of chlorophyll a, b, and total chlorophyll contents were increased significantly with the application of T6 without salt stress (Table 4).

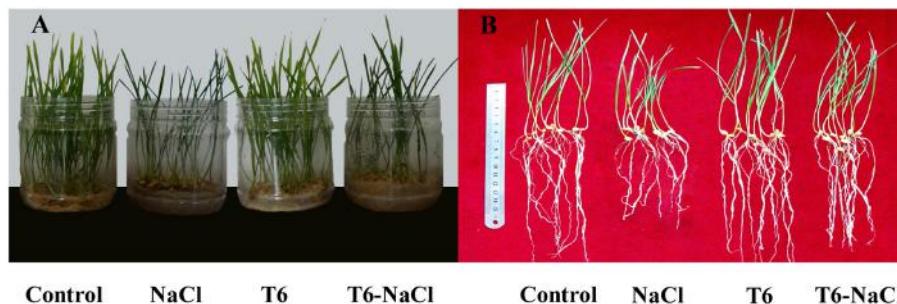


FIGURE 1 | Effect of salt stress and *Trichoderma longibrachiatum* T6 treatments on (A) wheat seedling growth and (B) wheat root length. The treatment names are detailed in the footnote of **Table 2**.

TABLE 2 | Effects of *Trichoderma longibrachiatum* T6 on number of colonies in wheat roots and plant growth traits under salt stress.

Treatments	Colony densities [CFU g ⁻¹ of root ($\times 10^5$)]	Plant growth parameters	
		Plant height (cm plant ⁻¹)	Root length (cm plant ⁻¹)
Control	0.00 ± 0.00 c	13.30 ± 0.50 b	12.14 ± 0.78 bc
NaCl	0.00 ± 0.00 c	11.03 ± 0.58 c	10.18 ± 0.59 c
T6	5.40 ± 0.47 a	15.23 ± 0.59 a	14.34 ± 0.49 a
T6-NaCl	4.46 ± 0.36 b	12.70 ± 0.61 bc	13.62 ± 0.64 ab

Mean ± standard error of replicates in a column followed by different letters are significantly different based on Duncan's multiple range test at $P < 0.05$ ($n = 12$). Control represents wheat seedlings grown under normal condition; NaCl represents wheat seedlings stressed under 150 mM NaCl; T6 represents wheat seeds pretreated with T6 for 12 h before planting without NaCl treatment; T6-NaCl represents wheat seeds pretreated with T6 for 12 h before planting and then stressed under 150 mM of NaCl.

Compared to the control, the proline content was significantly increased in wheat seedling leaves, after being treated with 150 mM of NaCl solution or with the T6 strain. The highest increase of proline was presented in wheat plants pretreated with T6 under 150 mM NaCl stress, which increased by 35% in leaves with NaCl treatment for 15 days, compared to NaCl-stressed plants (Table 4).

Soluble Sugar and Protein Content

Application of T6 increased the soluble sugar and protein contents in the wheat seedlings grown under salt stress or non-saline stress, compared to the control. However, the content of soluble sugar and protein in wheat seedlings significantly decreased after the treatment of NaCl alone (Table 4). In the wheat leaves, the content of soluble sugar and protein decreased by 15 and 18% after the NaCl treatment alone compared to the control, but they increased by 41 and 46% when treated with T6 alone, and increased by 31 and 35%, respectively, when treated with T6 in combination with 150 mM of NaCl, compare to the NaCl-stressed plants. These results showed the application of T6 increased the contents of soluble sugar and protein in wheat seedlings, with the effect on the soluble protein content being greater than the effect on soluble sugar content (Table 4).

Root Activity and Lipid Peroxidation Degree Detection

The root activity of wheat seedlings significantly decreased under salt stress. The root activity decreased by 20% in wheat seedlings treated with 150 mM of NaCl, while the root activity significantly increased after the seedlings were treated with T6 or T6 plus 150 mM of NaCl. T6 had a high effect on the root activity whether the wheat seedlings were under salt stress or non-saline stress (Table 5).

Malondialdehyde, a product of lipid peroxidation, is generally regarded as an indicator of free radical damage to cell membranes caused by oxidative stress. Wheat seedlings inoculated with 150 mM of NaCl and without T6 increased the MDA content in leaves by 50%, compared to the control. However, the seedlings inoculated with the strain of T6 had high efficiency in decreasing the content of MDA under salt stress, compared to NaCl-stressed plants. Thus, the content of MDA decreased by 45 and 15% in T6 alone and T6 plus NaCl treated plants, respectively, compared to NaCl-stressed plants (Table 5).

Antioxidant Enzymes Detection

Salt stress significantly induced and increased the antioxidant enzymes activities in wheat seedlings, including the activities of SOD, POD, and CAT (Figure 2). Also, the activities of SOD, POD, and CAT were significantly increased after being treated with T6 under salt stress or non-saline stress, compared to the control and the NaCl-stressed plants. Moreover, the application of T6 significantly increased the activities of SOD (Figure 2A), POD (Figure 2B), and CAT (Figure 2C) either in the control group or in the NaCl-stressed wheat seedling leaves; in the control, these values increased by 13, 12, and 14%, respectively; in the NaCl-stressed plants, these values increased by 29, 39, and 19%, respectively, compared to the NaCl treatment alone. The activities of SOD, POD, and CAT were greater with the addition of T6 in the NaCl-stressed plants, compared to the control plants with T6. The activity of CAT in seedlings treated with T6 alone did not differ from seedlings treated with T6 plus NaCl stress treatment, whereas the activities of SOD and POD differed significantly between the two treatments.

TABLE 3 | Effects of *T. longibrachiatum* T6 on wheat seedling weight and relative water content under salt stress.

Treatments	Wheat shoot			Wheat root		
	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)	Relative water content (%)	Fresh weight (g plant ⁻¹)	Dry weight (g plant ⁻¹)	Relative water content (%)
Control	0.271 ± 0.02 b	0.048 ± 0.003 ab	82.16 ± 0.53 a	0.121 ± 0.006 b	0.021 ± 0.001 b	82.61 ± 0.67 ab
NaCl	0.182 ± 0.01 d	0.041 ± 0.001 b	77.75 ± 0.76 c	0.089 ± 0.003 c	0.017 ± 0.002 c	80.65 ± 0.47 b
T6	0.299 ± 0.02 a	0.054 ± 0.004 a	82.00 ± 0.47 a	0.136 ± 0.004 a	0.023 ± 0.002 a	83.01 ± 0.59 a
T6-NaCl	0.224 ± 0.01 c	0.045 ± 0.002 ab	80.08 ± 0.30 b	0.109 ± 0.006 b	0.021 ± 0.001 b	81.27 ± 0.64 ab

Mean ± standard error of replicates in a column followed by different letters are significantly different based on Duncan's multiple range test at $P < 0.05$ ($n = 12$). The treatment names are detailed in the footnote of Table 2.

TABLE 4 | Effects of *T. longibrachiatum* T6 on the contents of chlorophyll, proline, soluble sugar, and protein in wheat seedlings under salt stress.

Treatments	Chlorophyll a content (mg g ⁻¹)	Chlorophyll b content (mg g ⁻¹)	Total chlorophyll content (mg g ⁻¹)
Effect on chlorophyll			
Control	1.44 ± 0.09 b	0.48 ± 0.06 c	1.92 ± 0.15 b
NaCl	1.23 ± 0.13 c	0.40 ± 0.11 c	1.63 ± 0.11 c
T6	1.59 ± 0.12 a	0.56 ± 0.08 a	2.16 ± 0.20 a
T6-NaCl	1.42 ± 0.16 b	0.54 ± 0.10 b	1.96 ± 0.21 b
Treatments	Proline (μmol g ⁻¹ FW)	Soluble sugar (mg g ⁻¹)	Soluble protein (mg g ⁻¹)
Effect on proline, soluble sugar, and protein			
Control	15.23 ± 0.29 c	20.58 ± 0.42 c	15.58 ± 0.41 b
NaCl	20.40 ± 0.44 b	17.44 ± 0.35 d	12.74 ± 0.64 c
T6	22.17 ± 0.93 b	24.54 ± 0.67 a	18.56 ± 0.62 a
T6-NaCl	27.63 ± 1.05 a	22.85 ± 0.40 b	17.25 ± 0.84 ab

Data are mean ± standard error of replicates in a column followed by different letters are significantly different at $P < 0.05$ ($n = 12$), based on Duncan's multiple range test using one-way ANOVA. The treatment names are detailed in the footnote of Table 2.

The Level of SOD, POD, and CAT Gene Expression

Compared to the control plants, there were higher levels of SOD, POD, and CAT gene expression in wheat seedlings after being induced by NaCl stress (Figure 3). With the T6 treatment, the gene expression of the SOD (Figure 3A), POD (Figure 3B), and CAT (Figure 3C) were significantly up-regulated whether or not the wheat seedlings were treated with NaCl stress, compared to the control. In contrast, there were no significant differences in the expression levels of the SOD genes in the treatment of the NaCl stress alone or T6 alone (Figure 3A).

DISCUSSION

Previous studies have demonstrated that a high level of salinity is one of the major environmental stress factors that causes biochemical alterations in plants, limits plant growth, and decreases plant productivity (Allakhverdiev et al., 2000; Mahmood et al., 2012). *Trichoderma* species are one of the most

TABLE 5 | Effect of *T. longibrachiatum* T6 on the root activity and MDA content of wheat seedlings under salt stress.

Treatments	Root activity (μg g ⁻¹ h ⁻¹)	MDA content (μmol g ⁻¹ FW)
Control	225.44 ± 6.45 b	16.79 ± 0.31 c
NaCl	179.56 ± 8.71 c	25.12 ± 1.12 a
T6	259.84 ± 8.11 a	13.87 ± 1.15 c
T6-NaCl	242.46 ± 10.43 ab	21.37 ± 0.52 b

Data are mean ± standard error of replicates in a column followed by different letters are significantly different at $P < 0.05$ ($n = 12$), based on Duncan's multiple range test using one-way ANOVA. The treatment names are detailed in the footnote of Table 2.

versatile opportunistic plant symbionts which can colonize plant roots (Brotman et al., 2013). These symbionts are well-known for their remarkable interactions with host plants and their ability to induce broad-spectrum resistance to plant pathogens (Naseby et al., 2000; Yedidia et al., 2003; Harman et al., 2004). Although, the plant-growth-promoting capability of *Trichoderma* spp. has been previously reported, there is little information concerning plants' systemic responses induced by T6 under salt stress conditions. Our results demonstrated that the NaCl treatment significantly inhibited wheat seedling growth and development after 15 days and the effect was alleviated substantially with the application of T6. To the best of our knowledge, the present study is the first to discover the role of plant-growth-promoting fungi T6 in enhancing the tolerance of wheat seedlings to salt stress. Also, our study determined the possible mechanism of how plant-growth-promoting fungi T6 alleviated the negative effect of NaCl stress in wheat seedlings. The use of T6 enhanced the tolerance of wheat seedlings to salt stress at physiological and molecular levels.

Shoresh et al. (2010) reported that host roots colonized by *Trichoderma* strains enhanced whole-plant tolerance to biotic and abiotic stresses. The enhancement was indicated by increased plants root growth and nutritional status (Harman et al., 2000), and induced systemic resistance to diseases (Harman et al., 2004). In the present study, we found that T6 has a great ability to colonize the roots of wheat seedlings under salt stress, which significantly improved wheat seedlings growth and development under salt stress. Bae et al. (2009) showed that cacao (*Theobroma cacao*) seedlings which were colonized by *Trichoderma hamatum* isolate DIS 219b enhanced seedling growth and development. In

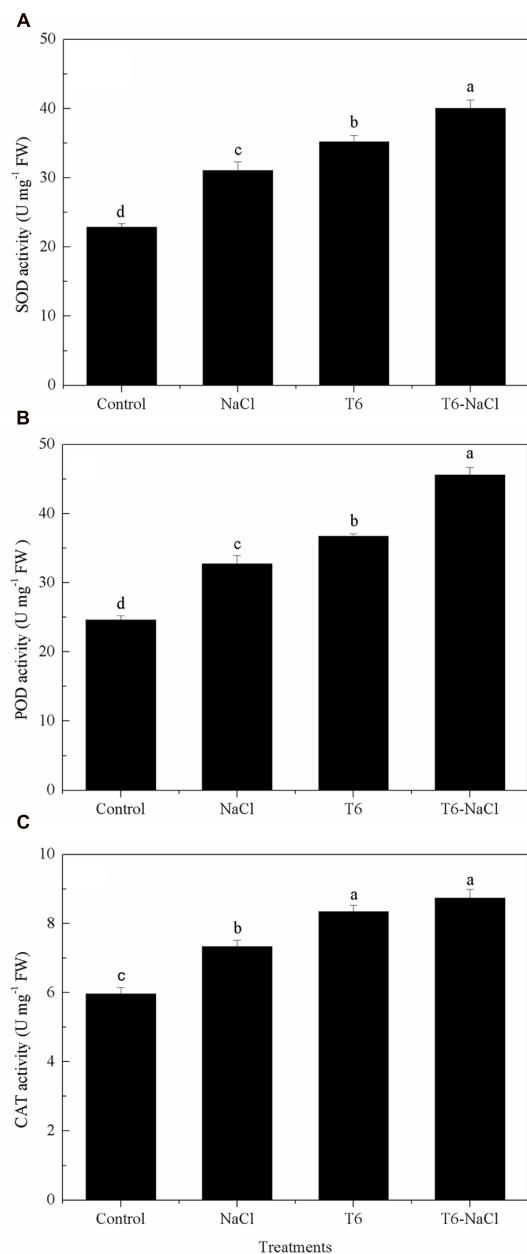


FIGURE 2 | Effect of *T. longibrachiatum* T6 treatment on the activity of (A) SOD, (B) POD, and (C) CAT in the leaves of wheat seedlings under salt stress. Small bars represent the standard errors of the means ($n = 12$). Different lowercase letters indicate significant differences at $P < 0.05$ in Duncan's multiple range test using one-way ANOVA. The treatment names are detailed in the footnote of **Table 2**.

a similar study, Adams et al. (2007) found that the plant saplings grown with *T. afroharzianum* T22 produced more biomass than non-inoculated controls in metal contaminated soil. Our findings are supported by a number of previous observations where *Trichoderma* spp. has the ability to colonize plant roots, establish symbiotic relationships with a wide range of host plants, and promote plant growth and development (Shoresh et al.,

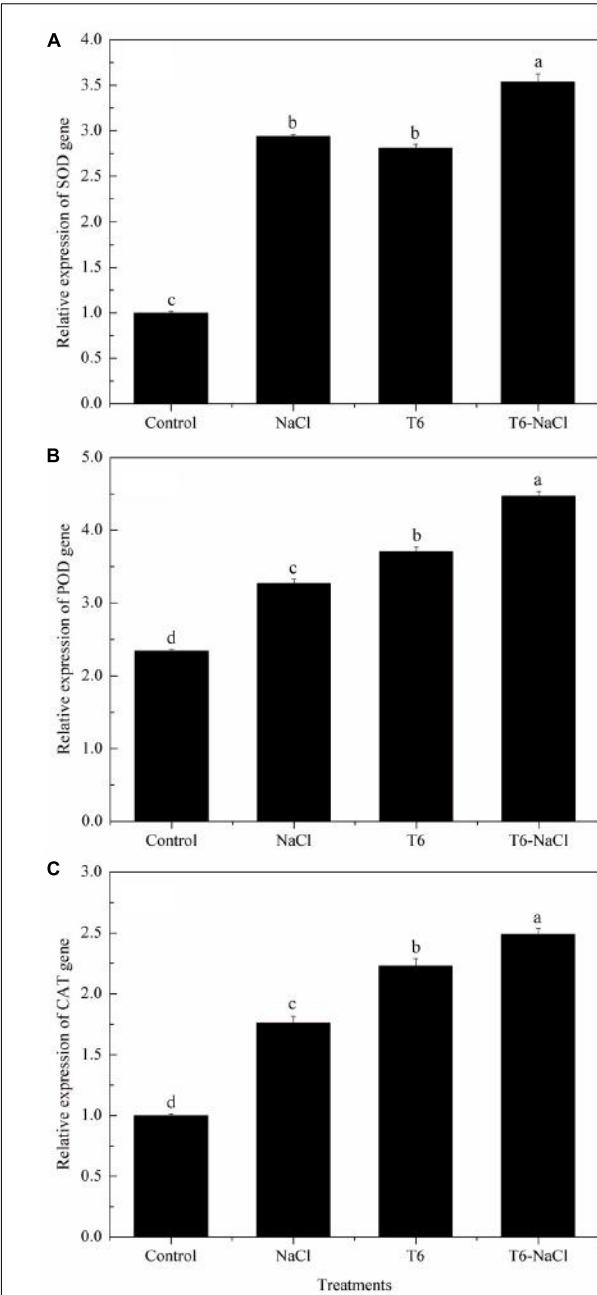


FIGURE 3 | Effect of *T. longibrachiatum* T6 on the genes of (A) SOD, (B) POD, and (C) CAT expression in the leaves of wheat seedlings under salt stress. Small bars represent the standard errors of the means ($n = 12$). Different lowercase letters indicate significant differences at $P < 0.05$ in Duncan's multiple range test using one-way ANOVA. The treatment names are detailed in the footnote of **Table 2**.

2010; Harman, 2011). Moreover, similar results were reported that *Trichoderma parareesei* increased the tomato lateral root development and growth promotion under salt stress conditions (Rubio et al., 2014).

Chlorophyll content is widely used as an indicator of abiotic tolerance in plants. Singh and Gautam (2013) reported that plants

exposed to salinity stressful environments decreased chlorophyll concentration, leading to overall growth retardation. In the present study, we found that NaCl-induced stress significantly decreased chlorophyll content in wheat seedlings, but chlorophyll content was reversed back to a similar level as the control in wheat seedlings treated with T6. Similar results were reported in NaCl-stressed soybean (*Glycine max*) and cotton (*Gossypium spp.*) seedlings by other researchers (Simaei et al., 2011; Liu et al., 2014). Tariq et al. (2011) found the oxidation of chlorophyll and chloroplast pigments, as well as the instability of the pigment protein complex in salt stress conditions were the possible reasons for the decrease of chlorophyll content in salinity-stressed wheat seedling leaves. However, we discovered that the content of chlorophyll significantly increased in wheat seedlings with the application of T6 whether or not the seedlings were subjected to salt stress. The latter application may inhibit the production and accumulation of ROS in plant tissue. Similar results were reported in NaCl-treated cucumber (*Cucumis sativus*) seedlings (Qi et al., 2012).

Plant roots are critical for plant growth and development which is attributed to their function and importance in absorption of nutrients and water from soil (Qi et al., 2012). We found NaCl stress had a high effect on the wheat root growth and development, and that salt treatment significantly decreased the root activity. However, the root activity was significantly increased when the plant-growth-promoting fungi T6 was applied to the wheat seedlings either under salt stress or non-saline conditions. A similar phenomenon was found in our previous studies where the strain of *T. longibrachiatum* significantly increased the root activity after wheat seedlings were infected with *H. avenae*, a plant parasitic nematode (Zhang et al., 2014a).

In the present study, the content of proline was increased in wheat seedling grown with NaCl alone, compared to the control. The results from the previous research indicated that the increased level of proline in plants under salt stress condition may have been due to the activation of proline biosynthesis which enhances protein turnover (Khan et al., 2010). Proline is an important nitrogen source that is available for plant recovery from environmental stress and restoration of growth (Trotel et al., 1996), and it can act as an osmolyte that reduces the osmotic potential of the cell and the uptake of toxic ions (Woodward and Bennett, 2005). Thus, proline plays a predominant role in protecting plants from osmotic stress (Khan et al., 2010). An added value from the present study is that the use of the plant-growth-promoting fungi T6 can significantly increase the proline content in wheat seedlings under salt or non-saline stress. Alleviating effects of oligochitosan on salt stress-induced oxidative damage in wheat leaves might be related to its regulation roles in proline levels. In addition, the results from our study indicated that the content of MDA significantly increased in NaCl-stressed wheat seedlings in comparison to the control plants, which is consistent with the findings of Seckin et al. (2008) in wheat. Thereafter, the content of MDA significantly decreased after wheat seeds were soaked in the suspension of T6 before NaCl stress. Taken together, our results are consistent with data from Rawat et al. (2011), who demonstrated that wheat seed

biopriming with salinity-tolerant isolates of *T. harzianum* Th-14, Th-19, and Th-13 reduced the accumulation of MDA content, whereas, it increased the proline content in wheat seedlings under both salt and non-saline conditions.

It is widely accepted that osmosis molecules, including soluble sugars and proteins, are important indicators in response to abiotic stress (Azevedo-Neto et al., 2006). The increased accumulation of glucose and sucrose in plants usually indicates a highly protective mechanism against oxidative damage caused by high salinity in the plant environment (Murakeozy et al., 2003; Bartels and Sunkar, 2005). However, most previous studies determined the physiologic role of soluble sugars and utilization by plants. We found that T6 had a highly significant effect on the content of soluble sugar and protein in wheat seedlings either under salt stress or non-saline conditions.

In plants, the overproduction of ROS is considered a biochemical change under salt stress (Younis et al., 2010), which is the most important factor responsible for NaCl-induced damage to macromolecules and plants cellular structures (Özdemir et al., 2004). To alleviate the damage associated with the overproduction of ROS, plants have naturally developed a wide range of enzymatic defense mechanisms to detoxify free radicals and thereby help protect themselves from destructive oxidative damage (Parida et al., 2004; Li et al., 2011). One of the important protective mechanisms in plants is the enzymatic antioxidant system, which involves the simultaneous action of a number of enzymes including SOD, POD, and CAT (Ma et al., 2012). The findings from the present study demonstrate that NaCl stress induced plants produce a higher level of SOD, POD, and CAT activity in wheat seedlings than the control samples. However, the use of T6 increased SOD, POD, and CAT activity in wheat seedlings regardless of salt concentration, which was in accordance with the findings of Ahmad et al. (2015), who demonstrated that the role of *T. harzianum* in Indian mustard (*Brassica juncea*) was to mitigate NaCl stress by an antioxidative defense system. Our results suggested that the coordination of POD and CAT activity along with SOD activity played a central protective role in the O_2^- and H_2O_2 scavenging process in wheat seedlings treated with T6. Also, we suggested that the strain of T6 had better O_2^- and H_2O_2 scavenging ability than the control in protecting the plants from oxidative damage.

Some plant-beneficial fungi *Trichoderma* species can induce profound impacts or changes in different species of plant gene expression under biotic and abiotic stresses (Mastouri et al., 2010). Increased SOD activity in stressed plants may be attributed to the significantly increased level of ROS, which causes an increase of gene expression responsible for encoding the activity of SOD (Bowler et al., 1992; Shekhawat et al., 2010). Therefore, in the present study, we firstly determined the effect T6 might have had on the expression of antioxidant enzyme genes (SOD, POD, and CAT). We found that the change in expression for the genes of SOD, POD, and CAT had resulted in increased transcription levels which were in accordance with the increase of SOD, POD, and CAT activity; these increases coincided with the trend of the activities of the corresponding enzymes. A number of previous studies have also demonstrated that there is a higher level of gene family expression for genes involved in plant

protection against abiotic stresses (Bailey et al., 2006; Alfano et al., 2007) in *Trichoderma* spp. pretreated plants. However, there are no in-depth studies on specific gene expression of wheat seedlings treated with plant-growth-promoting fungi T6. Meanwhile, we have confirmed that ROS generation under salt stress is followed by increased transcription and activity of ROS-scavenging enzymes in T6-challenged wheat plants, which indicated that the important role of ROS was detoxifying cellular survival and regulating plant acclimation (Miller et al., 2010). In addition, ROS also served as a critical signaling molecule in cell proliferation and survival. Similar results have been reported that salinity is one of the environment factors that can change the normal homeostasis of plant cells, and causes an increased production of ROS within plants. The ROS molecule functions as a toxic by-product of stress metabolism and is important in signaling transduction molecules in response to salt stress (Miller et al., 2010).

CONCLUSION

In summary, our results indicated that the strain of plant-growth-promoting fungi T6 has a remarkable effect on alleviating the adverse effects of salt stress on wheat seedling growth and development. Multiple tests employed in the study allowed us to explore the possible mechanisms at a physiological and molecular level in which T6 provides the ability of alleviating the suppression effect of salt stress. The mechanisms may include (i) T6 increasing the activity of antioxidative defense system

in wheat seedlings to resist salt stress, and (ii) enhancing the relative levels of antioxidant gene expression in the stressed plants. However, there are some issues that need to be addressed in future studies, such as the efficacy of the strain of plant-growth-promoting fungi T6 interactions with other plant species and other abiotic stresses. More detailed studies may be necessary to determine which compound plays the signaling role in T6 that induces systemic changes in the expression of encoding antioxidant enzymes and genes.

AUTHOR CONTRIBUTIONS

SZ and YG conceived and designed the experiments with the help of BX. SZ performed most of the salt treatment experiments and prepared the wheat RNA samples. YG and SZ performed qRT-PCR and analyzed the data, with the help of BX. SZ and YG wrote the manuscript. SZ, YG, and BX revised and approved the final manuscript.

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Deciphering *Staphylococcus sciuri* SAT-17 Mediated Anti-oxidative Defense Mechanisms and Growth Modulations in Salt Stressed Maize (*Zea mays L.*)

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Soil salinity severely affects plant nutrient use efficiency and is a worldwide constraint for sustainable crop production. Plant growth-promoting rhizobacteria, with inherent salinity tolerance, are able to enhance plant growth and productivity by inducing modulations in various metabolic pathways. In the present study, we reported the isolation and characterization of a salt-tolerant rhizobacterium from Kallar grass [*Leptochloa fusca* (L.) Kunth]. Sequencing of the 16S rRNA gene revealed its lineage to *Staphylococcus sciuri* and it was named as SAT-17. The strain exhibited substantial potential of phosphate solubilization as well as indole-3-acetic acid production (up to 2 M NaCl) and 1-aminocyclopropane-1-carboxylic acid deaminase activity (up to 1.5 M NaCl). Inoculation of a rifampicin-resistant derivative of the SAT-17 with maize, in the absence of salt stress, induced a significant increase in plant biomass together with decreased reactive oxygen species and increased activity of cellular antioxidant enzymes. The derivative strain also significantly accumulated nutrients in roots and shoots, and enhanced chlorophyll and protein contents in comparison with non-inoculated plants. Similar positive effects were observed in the presence of salt stress, although the effect was more prominent at 75 mM in comparison to higher NaCl level (150 mM). The strain survived in the rhizosphere up to 30 days at an optimal population density (ca. 1×10^6 CFU ml $^{-1}$). It was concluded that *S. sciuri* strain SAT-17 alleviated maize plants from salt-induced cellular oxidative damage and enhanced growth. Further field experiments should be conducted, considering SAT-17 as a potential bio-fertilizer, to draw parallels between PGPR inoculation, elemental mobility patterns, crop growth and productivity in salt-stressed semi-arid and arid regions.

Keywords: antioxidants, biofertilizer, reactive oxygen species, salinity, *Staphylococcus sciuri*, *Zea mays*

INTRODUCTION

The world has experienced an exponential increase in population within the last few decades leading to a reduced availability of quality food. The problem is worsened due to an increase in the salinization of agricultural lands (Ghafoor et al., 2002). High levels of soluble salt in soil cause deleterious effects on germination, seedling vigor, crop establishment, plant metabolism and

reproductive growth (Zhu et al., 2004), which contribute to a reduced yield of agronomically important crops. Exposure of plants to high salinity stress inhibits water uptake by roots and also induces osmotic shock, which modulates cell division, cell expansion and stomatal closure (Flowers, 2004). Long-term exposure to salts causes the increased uptake of Na^+ together with a decrease in the uptake of Ca^{2+} and K^+ (Yildirim et al., 2006). Nutritional imbalances/deficiencies result in the senescence of leaves, reducing photosynthetic area necessary to maintain the optimum growth. In addition, uptake and accumulation of Cl^- may disrupt photosynthetic function through the inhibition of nitrate reductase activity (Xu et al., 1999). Once the capacity of cells to store salts is exhausted, salts build up in the intercellular space, which results in cell dehydration and death (Sheldon et al., 2004). Moreover, at higher salinity, plants ultimately die due to reduced leaf production and expansion rates caused by oxidative damage (Kravchik and Bernstein, 2013). In Pakistan, 8.6 million hectares of arable land is saline and high yields of crops are usually not acquired because a great amount of time and energy is annually spent on reclamation strategies.

A possible strategy to cope with the low productivity of saline lands is microbial assisted amelioration of salt-induced damage (Dodd and Pérez-Alfocea, 2012). Among soil microbiota, plant growth-promoting rhizobacteria (PGPR) are potential candidates that are capable of colonizing the rhizosphere, penetrating the roots and triggering plant salinity-tolerance mechanisms (Tank and Saraf, 2010; Sheng et al., 2011). They influence plant physiology by releasing growth regulators, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, enhanced soil phosphate solubilization and up-regulating the conserved salinity responsive mechanisms (Rajput et al., 2013; Kim et al., 2014). Many PGPR with inherent salt-tolerance have been isolated, characterized and applied to plants to increase crop productivity in saline regions (Rajput et al., 2013; Ahmad et al., 2014). There is evidence that PGPR regulate hormonal status (Sahoo et al., 2014) and initiate antioxidant defense mechanisms in plants exposed to high salt stress (Islam et al., 2015). Members of the *Staphylococcus* genus have been isolated from diverse environments and characterized as having salt-tolerance potential (Roohi et al., 2012; Nanjani and Soni, 2014). It has been reported that *Staphylococcus* mitigated the deleterious effects of salinity in radish (Yildirim et al., 2008), sweet cherry (Zhou et al., 2015) and strawberry (Karlidag et al., 2013). Sagar et al. (2012) reported that *Staphylococcus arlettae* strain Cr11 promoted plant growth via the reduction of hexavalent chromium.

Besides up-regulating stress responsive factors, PGPR also enhance the mobilization of fixed nutrients in salt-affected soils (Paul and Lade, 2014). A major soil-fixed nutrient is phosphorus (P) which is bound to cations (Ca^{2+} , Al^{2+} , Fe^{2+}) and thus remains unavailable to plants (Bhattacharyya and Jha, 2012). Farmers apply phosphate-based fertilizers where a very limited amount is used by the plants and a large amount of fertilizers are converted into insoluble complexes in the soil (Zaidi et al., 2009). The excessive use of fertilizers adversely affects the environment as these are a potential source of environmental contamination (Savci, 2012). Moreover, phosphate fertilizers

often leach from the soil and cause the eutrophication of surface and groundwater sources (Sharpley, 1999; He et al., 2003). Alternatively, a trend toward the use of slow-release phosphate (rock phosphate) fertilizers has been reported (Duponnois et al., 2005). Furthermore, efforts have also been made to explore PGPR as fertilizer supplements with the objective of substantially reducing the use of synthetic fertilizers (Hanif et al., 2015; Shahid et al., 2015). The issues of low nutrient availability and oxidative damage in saline lands severely affect the growth and physiology of Maize (*Zea mays* L.), which is one of the important domesticated cereal crops grown widely throughout the world. It is a nutritious rich source of human food and animal feed, and also provides raw material for industrial products.

The present work was designed to explore the potential of a salt-tolerant PGPR strain *S. sciuri* SAT-17 to boost maize growth under saline environments. To the best of our knowledge, this is the first report elucidating the physiological and phenotypic responses of maize after inoculation with a phyto-beneficial *S. sciuri* strain. The study will raise attention toward the establishment of long-term programs involving PGPR-based bioformulations for the efficient utilization of salt-affected soils.

MATERIALS AND METHODS

Sampling Site and Bacterial Isolation

The roots and rhizospheric soil surrounding Kallar grass [*Leptochloa fusca* (L.) Kunth] was collected from salt rich fields located at/near Pakka Anna ($31^{\circ}13'60\text{ N}$ and $72^{\circ}48'0\text{ E}$), Punjab, Pakistan. The samples were transported to the laboratory in sterilized polythene bags. The roots were shaken gently in sterile distilled water to remove the loosely adhering soil. One gram of strictly adhered soil was added in 9 mL of 0.85% (w/v) NaCl solution and serially diluted, as described by Somasegaran and Hoben (1994). An amount of 100 μL from three dilutions (10^{-4} , 10^{-5} , and 10^{-6}) was spread on nutrient agar, amended with 8% NaCl, and plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Purification of the culture was achieved through repeated-streaking and pure culture (designated as SAT-17) was stored in 20% (v/v) glycerol at -80°C . Colony morphology, cell shape, motility and Gram's reaction was performed under a light microscope (Olympus, Tokyo, Japan) as described earlier (Vincent, 1970). Catalase (CAT) activity was determined by pouring H_2O_2 on the culture on a glass slide. The physical and chemical analysis of rhizospheric and bulk soil samples was carried out at Ayub Agriculture Research Institute, Faisalabad, Punjab, Pakistan.

Molecular Identification and Phylogenetic Analysis

Total genomic DNA of the isolate "SAT-17" was isolated by the alkaline lysis method (Maniatis et al., 1982), quantified by the NanoDropTM 2000/2000c (Thermo Fisher Scientific, Waltham, MA, USA) and used to amplify the 16S rRNA gene using primers fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rD1 (5' AAGGAGGTGATCCAGCC 3') (Weisburg et al., 1991). The reaction mixture and thermocycler conditions were set

as described earlier by Shahid et al. (2015). Subsequently, the amplicon was cloned in pTZ57R/T and sequencing of the 16S rRNA gene was carried out by Macrogen, South Korea. Trimming of raw sequences, BLASTn analysis and phylogenetic studies were conducted using the methods and software packages described previously by Shahid et al. (2016).

Characterization of Salt-Tolerance and Plant-Beneficial Traits

Salt-Tolerance Studies

The pure culture of SAT-17 was streaked on nutrient agar media with various NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, or 4 M) and the salt-tolerance level was determined by measuring the minimum inhibitory concentration (MIC) of NaCl. Additionally, SAT-17 was re-exposed to NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5 M) in nutrient broth and subjected to the serial dilution method (Somasegaran and Hoben, 1994) to determine the bacterial cell density up to the MIC.

Phosphate Solubilization

A single purified colony of isolate SAT-17 was inoculated in 100 mL Pikovskaya's broth (Pikovskaya, 1948) medium supplemented with different levels of NaCl (0, 0.5, 1, 1.5, 2 or 2.5 M) and incubated at $30 \pm 2^\circ\text{C}$ for 240 h in an orbital shaker (150 rpm). Twenty milliliter of bacterial culture was harvested and centrifuged at 13,000 g for 10 min. The quantitative measurement of phosphate solubilization was performed according to phosphomolybdate blue color method (Murphy and Riley, 1962) using a UV-visible spectrophotometer (Shimadzu UV/VIS, Kyoto, Japan) at 882 nm.

Indole-3-Acetic Acid (IAA) Production

The method described by Gordon and Weber (1951) was used to estimate IAA synthesis potential of the strain SAT-17. A single colony of SAT-17 was inoculated to 100 mL nutrient broth media with (100 mg L^{-1}) or without tryptophan, each amended with NaCl (0, 0.5, 1, 1.5, 2, or 2.5 M). The cultures were grown on an orbital shaker (150 rpm) at $30 \pm 2^\circ\text{C}$ for 48 h. Thereafter, the cultures were harvested and centrifuged at 13,000 g. An amount of 1 mL supernatant was mixed in 2 mL of Salkowisk's reagent. The tubes were kept in the dark for 30 min for color development. Quantification was carried out through a spectrophotometer at 540 nm. The IAA solutions (0, 5, 10, 50, 100, 200, or 500 $\mu\text{g mL}^{-1}$) were used to draw standard curve for comparative measurements.

1-Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase Activity

The ability of isolate SAT-17 to use ACC as a sole nitrogen source was assessed in 5 mL DF salt minimal medium (Penrose and Glick, 2003) containing 3 μL of 0.5 M ACC and supplemented with different concentrations (0, 0.5, 1, 1.5, 2, or 2.5 M) of NaCl. Cultures were grown at $30 \pm 2^\circ\text{C}$ for 24 h in a shaker. To determine ACC deaminase activity, the turbidity of the inoculated cultures was compared to that of the non-inoculated control.

Greenhouse Experiment

Comparative Fitness Studies and Inoculum Preparation

Rifampicin-resistant derivatives of strain SAT-17 (SAT-17_{rif}) were constructed, followed by comparative growth studies with its wild-type (SAT-17_w) as described earlier by Shahid et al. (2012). For inoculum preparation, SAT-17_{rif} was grown up to 10^9 CFU mL^{-1} cell density. The culture was centrifuged at 8,000 g and washed twice with ddH₂O. The cells were re-suspended in equal volume of ddH₂O and diluted to 10^8 CFU mL^{-1} .

Experimental Soil

The soil with textural class clay loam (available P: 8.3 mg kg⁻¹, total N: 0.89 g kg⁻¹, available K: 112 mg kg⁻¹, organic matter 1.6% and pH 7.1) was obtained from the botanical garden of Government College University, Faisalabad, Pakistan. The soil was pre-inoculated by mixing 7 mL of SAT-17_{rif} inoculum per 100 g of soil (inoculated soil) or by mixing 7 mL of ddH₂O per 100 g of soil (non-inoculated soil). A total of 18 pots were filled (each with 600 g soil) where nine pots received the inoculated and nine the non-inoculated soil.

Plant Material and Experimental Design

Maize seeds (FH-992) were surface-sterilized by immersing in 5% (w/v) sodium hypochlorite for 10 min and subsequently washed thrice with ddH₂O. The seeds were submerged in SAT-17_{rif} inoculum and ddH₂O separately for 20 min. The SAT-17_{rif}-inoculated seeds were sown in pots containing inoculated soil, while ddH₂O-dipped seeds were sown in non-inoculated pots. Seed rate was set at eight seeds per pot. The plants were thinned to five plants per pot after seedling emergence. Initially, the pots were irrigated, with a 3 days interval, using canal water. When seedlings emerged, these were watered periodically with equal volumes of half strength Hoagland solution (Arnon and Hoagland, 1940) with 3 NaCl levels, i.e., 0, 75 or 100 mM (Batoor et al., 2013; Kim et al., 2014). The total treatments were named as follows:

Control₀: non-inoculated soil with 0 mM NaCl
 Control₇₅: non-inoculated soil with 75 mM NaCl
 Control₁₅₀: non-inoculated soil with 150 mM NaCl
 SAT-17₀: SAT-17_{rif} inoculated soil with 0 mM NaCl
 SAT-17₇₅: SAT-17_{rif} inoculated soil with 75 mM NaCl
 SAT-17₁₅₀: SAT-17_{rif} inoculated soil with 150 mM NaCl

The experiment was conducted in a greenhouse (day/night temperature 25/20°C, light/dark periods 16/8) with a completely randomized design (CRD) and three replications for each treatment.

Bacterial Recovery and Growth Data

Before seed sowing, three random samples from inoculated and non-inoculated soil were serially diluted (Somasegaran and Hoben, 1994) and spread on nutrient agar plates amended with (50 $\mu\text{g mL}^{-1}$) rifampicin to determine initial soil population density of SAT-17_{rif}. Thereafter, one plant from each inoculated and non-inoculated pot was randomly uprooted, at 10, 20, and 30 days after sowing (DAS) and a survival rate of SAT-17_{rif} was

determined, as described by Shahid et al. (2012). Plant growth attributes (length as well as fresh and dry weights) were recorded for the remaining plants at 30 DAS.

Analysis of Physiological Parameters and Nutrient Acquisition Patterns of Maize Plants

Lipid Peroxidation

The malondialdehyde (MDA) content of plant tissue was determined by thiobarbituric acid (TBA) reaction (Heath and Packer, 1968) to estimate the level of lipid peroxidation. The shoots homogenized with 0.1% trichloroacetic acid (TCA) were centrifuged and the supernatant was added to 20% TCA containing 0.5% TBA. The reaction mixture was heated (100°C) for 30 min and subsequently cooled to stop the reaction. The samples were centrifuged again and the absorbance of the supernatant was measured at 532 nm using Ultrospec 3000 (Biochrom Ltd, Cambridge, England) and adjusted for non-specific absorbance at 600 nm. The extinction coefficient was 155 mM cm⁻¹.

Proline Content

The method of Bates et al. (1973) was used to determine the proline concentrations. Fresh leaves (ca. 0.5 g) were homogenized in sulfo-salicylic acid (3%, w/v) and the filtrate (2 mL) was mixed with 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid. The mixture was incubated at 100°C for 60 min followed by cooling. Toluene (4 mL) was added to the solution and the contents were mixed well. The absorbance of the lower layer (chromophore-containing toluene) was observed spectrophotometrically at 520 nm.

Catalase Activity

Leaf material was homogenized in phosphate buffer (50 mM and pH 7.8) and centrifuged at 10,000 g for 10 min. The CAT activity of the supernatant was measured (Aebi, 1984) where the sample (ca. 100 µL) was mixed with H₂O₂ (0.75 M) and a decrease in absorbance was recorded at 240 nm for 20 s with the Ultrospec 3000 (Biochrom Ltd, Cambridge, England). Extinction coefficient was 0.039 mM cm⁻¹.

Peroxidase (POD) Activity

The protocol described by Chance and Machly (1955) with some modifications was used for the determination of POD activity. The plant samples were ground in phosphate buffer (pH 7.8) and centrifugation was performed at 8,000 g at 25°C. The absorbance of the reaction mixture [(2.7 mL phosphate buffer (pH 5), 0.1 mL Guiacol (20 mM), 0.1 mL H₂O₂ (40 mM) and 0.1 mL plant extract] was measured every 20 s for 2 min at 470 nm in a spectrophotometer. One unit of POD activity was considered as change of absorbance of 0.01 units min⁻¹.

Total Phenolics Content

A 0.5 g of fresh leaf tissue was homogenized in 80% (v/v) acetone solution and centrifuged at 10,000 g for 10 min at 4°C. The supernatant (100 µL) was diluted with 2 mL of water plus 1 mL of Folin–Ciocalteau's phenol reagent. Five mL of 20% (w/v) Na₂CO₃

was then added and the volume was made up to 10 mL with ddH₂O. The absorbance was read at 750 nm and the results were expressed as mg g⁻¹ FW of leaf (Julkunen-Tiitto, 1985) by comparison with standards of known concentrations.

Determination of Plant Nutrient Elements

The plant roots and shoots were oven-dried at 105°C for 24 h. The oven-dried ground plant material (ca. 0.5 g) was taken in digestion flasks containing 5 mL conc. H₂SO₄ (Wolf, 1982). The flasks were incubated overnight at room temperature. A 0.5 mL solution of 35% (v/v) H₂O₂ was poured and the flasks were then placed over a hot plate (350°C). The digestion flasks remained on a hot plate until no fumes were produced. Afterward, these were removed from the hot plate and allowed to cool. The step was repeated until the digestion mixture became fully transparent. The cooled mixtures were then diluted up to 50 mL, filtered and stored at 4°C. The N, P, K, Ca, and Mg contents of the dried shoot and root samples were determined using an atomic absorption spectrophotometer (Hitachi, Model 7JO-8024, Tokyo, Japan) with flame spectrophotometry. To minimize the matrix affect during plant metal analysis, standard reference materials (SRM) and standard solutions were used.

Plant Chlorophyll Content

The method of Arnon (1949) was used for the determination of plant chlorophyll contents. An 80% (v/v) solution of acetone was used for homogenization followed by centrifugation and filtration. The absorbance of the supernatant was recorded using a spectrophotometer at three different wavelengths, i.e., 663, 645, and 480 nm.

Plant Protein Content

Fresh plant material was homogenized, centrifuged (at 10,000 g for 15 min at 4°C) and mixed with Bradford reagent (Bradford, 1976). The mixture was incubated for 15–20 min and the absorbance was measured spectrophotometrically at 595 nm. Total soluble proteins were estimated by comparison with a standard curve of bovine serum albumin (BSA).

Data Analysis

Data for green house experiments were analyzed statistically by analysis of variance (Steel et al., 1997) using Statistix (ver. 8.1) software. The least significant difference test (Fisher's LSD) at 5% probability was used to compare the differences between treatment means. The phylogenetic tree was constructed with MEGA (ver. 6) software (Tamura et al., 2013).

RESULTS

Soil Physicochemical Analysis

The physical and chemical properties of bulk and Kallar grass rhizospheric soil samples collected from Pakka Anna were determined and are presented in Table 1. Soil texture was sandy loam for bulk soil and sandy clay loam for rhizospheric soil. Both soils exhibited high EC and pH but were deficient in organic matter contents; this is an indication of saline nature. The examined soils were also low in total N and available P contents.

TABLE 1 | Physicochemical properties of soil samples from Pakka Anna.

Soil properties	Bulk soil	Rhizospheric soil
Textural class	Sandy loam	Sandy clay loam
Sand (%)	62	61
Silt (%)	23	19
Clay (%)	15	20
Bulk density (mg m^{-3})	1.44	151
EC (dS m^{-1})	8.13	8.11
pH	8.5	7.6
Organic matter (%)	1.80	1.49
Organic C (g kg^{-1})	3.9	4.32
Total N (g kg^{-1})	0.49	0.56
C:N ratio	8	7.7
Available P (mg kg^{-1})	3.4	4.2
Available K (mg kg^{-1})	176	210

Molecular Identification of SAT-17

The isolate was identified as *S. sciuri* SAT-17 on the basis of 16S rRNA gene sequence analysis. BLASTn analysis of 1388 bp sequence (submitted to NCBI GenBank as Acc. # KU672729) of SAT-17 showed 99% sequence identity with *S. sciuri* PS25 (Acc. # KM276789) and *S. sciuri* DHAN01 (Acc. # KT270573) (Supplementary Figure S1). Furthermore, strain SAT-17 was placed in a cluster of *S. sciuri* ATCC 29062^T (Acc. # S83569), *S. sciuri* PS25 (Acc. # KM276789) and *S. sciuri* DHAN01 (Acc. # KT270573) in a phylogenetic tree (Figure 1).

Physiological Characterization

Staphylococcus sciuri SAT-17 was able to grow in culture medium amended with 2.5 M NaCl and this level of *in vitro* salinity stress was considered the MIC for the strain. Moreover, the bacterial cell density ($\text{Log CFU mL}^{-1} 24 \text{ h}^{-1}$) was measured in the following descending order at 0, 0.5, 1, 1.5, and 2 M NaCl concentration: $10.6 \pm 0.7 > 10.5 \pm 0.8 > 9.3 \pm 0.4 > 7.2 \pm 0.5 > 5.4 \pm 0.3 > 2.9 \pm 0.3$ (Table 2).

The tricalcium phosphate (TCP) solubilizing ability of the strain was found to be up to $40.8 \pm 5.2 \mu\text{g mL}^{-1}$ in the presence of 0.5 M salt. This ability decreased together with an increase in salt level and only $7.6 \pm 1.1 \mu\text{g mL}^{-1}$ TCP was solubilized at a concentration of 2 M NaCl. The strain synthesized IAA ($3.2 \pm 0.23 \mu\text{g mL}^{-1}$) up to 2 M NaCl addition in the presence of tryptophan, while in the absence of tryptophan, $11.9 \pm 0.73 \mu\text{g mL}^{-1}$ IAA was measured up to 1.0 M NaCl stress. *S. sciuri* SAT-17 was also found to be positive for *in vitro* ACC deaminase activity with the addition of up to 1.5 M NaCl (Table 2).

Comparative Growth Studies and Bacterial Recovery from Rhizosphere

The wild type strain (SAT-17_w) and its derivative (SAT-17_{rif}) showed similar growth behaviors in plate count method and spectrophotometric OD methods, as presented in Figure 2. The growth of both SAT-17_w and SAT-17_{rif} was found to be optimal (ca. $1 \times 10^{10} \text{ CFU mL}^{-1}$) and a normal bacterial growth curve pattern was obtained after plotting the measurements obtained at different time intervals.

The population density of SAT-17_{rif}, on rifampicin-amended agar plants before seed sowing, was found to be $3.5 \times 10^7 \text{ CFU g}^{-1}$ of soil. The strain survived in the maize rhizosphere up to 30 DAS at an optimal population density of $6 \times 10^6 \text{ CFU g}^{-1}$ soil (in treatment SAT-17₀) and $4.9 \times 10^5 \text{ CFU g}^{-1}$ soil (in treatment SAT-17₇₅). The population density of SAT-17_{rif} decreased to $8.9 \times 10^4 \text{ CFU g}^{-1}$ in the treatment SAT-17₁₅₀. We did not observe any bacterial growth on agar plates for the treatments control₀, control₇₅ and control₁₅₀ (Figure 2C).

Effect of Salt Stress and SAT-17 Inoculation on Maize Growth

In the absence of SAT-17 inoculation, a decrease in root length was observed with increasing salt level. The SAT-17 inoculation resulted in enhanced root length, with a maximum (9.98 cm) in the absence of NaCl. At 75 mM salt stress, the percentage difference between the SAT-17 treated and non-treated plants was 55.9%, where the treated ones exhibited greater root length (8.83 cm). Similarly, at 150 mM salt stress, we observed a percentage difference of 42.6% with higher root length in plants that received the treatment, i.e., SAT-17₁₅₀ (Table 3).

The control plants exhibited a shoot length of 26 cm and the addition of salt reduced the shoot length, significantly at 150 mM (19.2%). The inoculation with SAT-17 increased the shoot length both in the absence (0 mM) and presence (75 or 150 mM) of NaCl. The SAT-17 treatment, in the presence of 75 mM salt, resulted in a 24.6% increase in shoot length compared to the control, while, at this level of stress, there was 31.1% difference in shoot length between SAT-17 treated (29.7 cm shoot length) and non-treated (21.7 cm shoot length) plants (Supplementary Figure S3). The control plants and those grown in the presence of 150 mM salt, without SAT-17 treatment, exhibited 34% difference for the root fresh weight, where a lower weight was recorded in the latter group (3.1 g). The SAT-17 application increased the root FW, irrespective of the level of imposed stress. A similar trend was observed for root DW as well as shoot biomass.

Effect of Salt Stress and SAT-17 Inoculation on Antioxidants

The control plants exhibited a phenolics content of 26.8 mg g^{-1} FW. When no SAT-17 treatment was applied, a reduction of 25 and 31.3% was observed, in comparison to the control, at 75 and 150 mM stress, respectively. When no salt stress was present, the SAT-17 treatment enhanced the content up to 50.3% (40.3 mg g^{-1} FW). Plants grown at a salt level of 75 mM along with SAT-17 treatment exhibited higher phenolic content (37.6 mg g^{-1} FW) compared to plants that received 75 mM salt stress but no SAT-17 inoculation (20.1 mg g^{-1} FW). There was a 42.8% difference between the SAT-17 treated and non-treated plants at 150 mM salt stress, with the former resulting in higher phenolics content (30.1 mg g^{-1} FW). Without SAT-17 application, we observed a 46.2% (at 75 mM salt) and 153% (at 150 mM salt) increase in the MDA content compared to the control plants ($1.75 \text{ nmole/g}^{-1}$ DW). The plants that received SAT-17 treatment, in the absence of salt, exhibited a 13.7% decrease in MDA content. At 75 mM salt

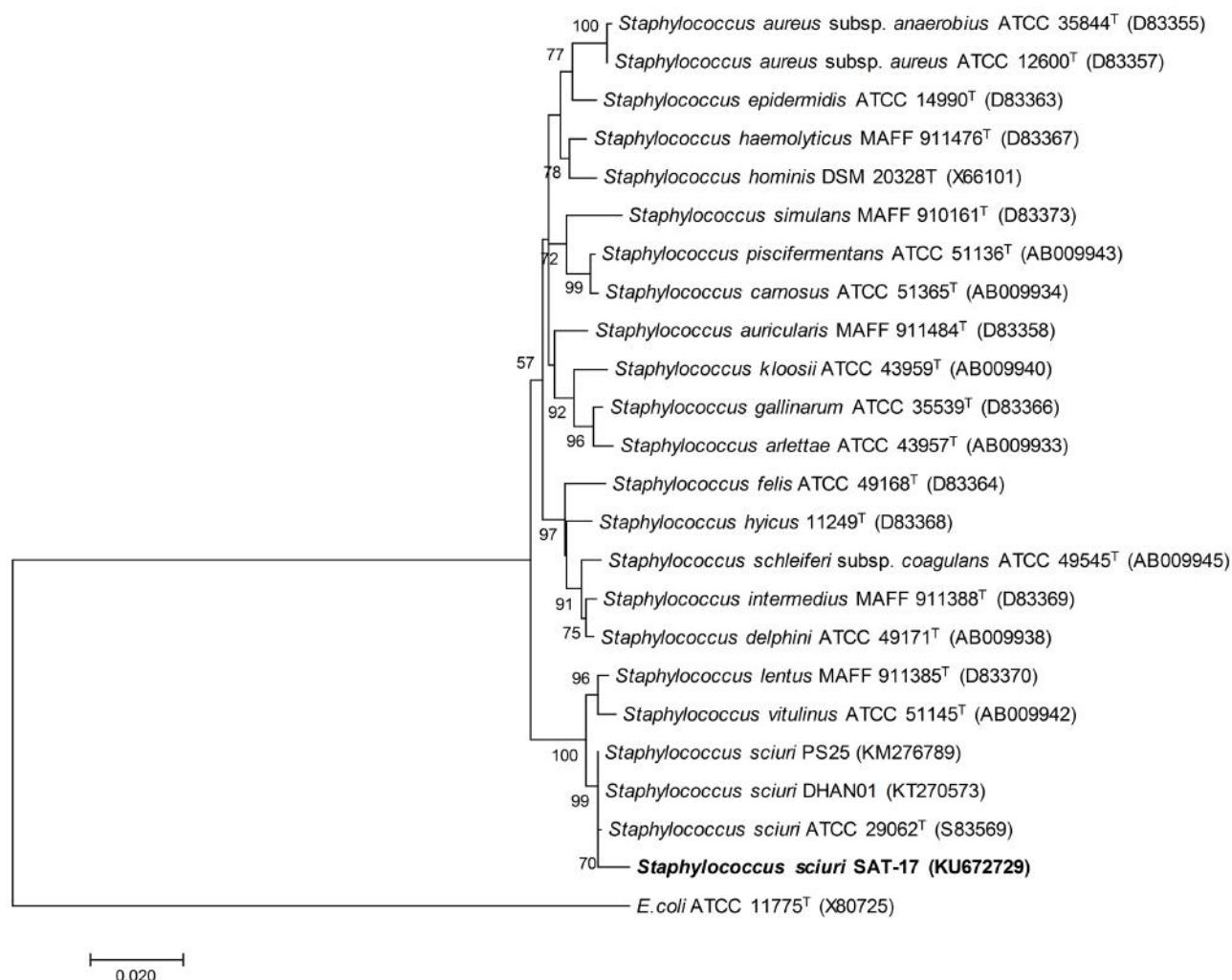


FIGURE 1 | Phylogenetic analysis of *S. sciuri* SAT-17 with type strains of genus *Staphylococcus* and the closest GenBank matches. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.39351674 is shown. The percentages ($\geq 50\%$) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions of 24 nucleotide sequences included were 1st + 2nd + 3rd + Non-coding.

stress, the MDA contents were 61.2% different in SAT-17 treated (1.3 nmole/g^{-1} DW) and non-treated plants ($2.56 \text{ nmole/g}^{-1}$ DW). A similar trend was observed at a salt level of 150 mM, where treated plants exhibited less MDA than the non-treated ones.

An H_2O_2 level of 13.8 ng g^{-1} DW was observed in the control plants. The presence of salt, without SAT-17_{rif} treatment, increased the H_2O_2 level, significantly (26.0%) at 150 mM salt stress. The plants that received 75 mM salt stress exhibited higher H_2O_2 levels (14.2 ng g^{-1} DW) than those which were grown with salt and SAT-17 treatment (12.5 ng g^{-1} DW). A similar trend was observed for plants grown with 150 mM salt stress alone and those that received salt stress as well as SAT-17 treatment. Without SAT-17 treatment, the

maximum proline content ($2.97 \mu\text{g g}^{-1}$ DW) was observed in plants grown with 150 mM salt level, with results that were 10.8 and 8.3% higher than for those plants grown with 0 and 75 mM salt levels, respectively. The application of SAT-17 (at 0 mM salt level) reduced the proline content in comparison to control plants, while at 75 mM, the SAT-17 treatment significantly increased (11.0%) the proline content in comparison to the plants that did not receive the treatment. There were no significant differences in proline content of the plants that received ($2.96 \mu\text{g g}^{-1}$ DW) or did not receive ($2.97 \mu\text{g g}^{-1}$ DW) SAT-17 treatment at the salt level of 150 mM. However, the observed values were significantly higher than those reported in the control plants ($2.68 \mu\text{g g}^{-1}$ DW proline).

TABLE 2 | Physiological characterization of *S. sciuri* SAT-17 under salinity stress.

Parameters	NaCl concentration					
	0 M	0.5 M	1.0 M	1.5 M	2.0 M	2.5 M
Bacterial cell density (Log CFU mL ⁻¹ 24 h ⁻¹)	10.6 (0.7)	10.5 (0.8)	9.3 (0.4)	7.2 (0.5)	5.4 (0.3)	2.9 (0.3)
P solubilization (μg mL ⁻¹)	36.5 (4.7)	40.8 (5.2)	33.2 (3.7)	18.7 (2.5)	7.6 (1.1)	ND
IAA production (With tryptophan)	9.1 (0.49)	11.8 (0.76)	9.9 (0.91)	8.6 (0.51)	3.2 (0.23)	ND
IAA production (Without tryptophan)	16 (0.83)	17.6 (0.64)	11.9 (0.73)	ND	ND	ND
ACC deaminase activity	+	+	+	+	-	-

Each value is the mean of three replicates and standard errors are presented in parentheses. ND, not determined.

Without SAT17_{rif} inoculation, the 75 mM NaCl level increased the CAT activity (32.5 U/mg protein), while the higher stress level (150 mM) resulted in reduced (19.8 U/mg protein) CAT activity in comparison to control plants (28 U/mg protein). The imposition of salt stress (75 or 150 mM) combined with SAT-17 treatment (SAT-17₇₅ and SAT-17₁₅₀) resulted in higher CAT activity compared to the plants that received salt stress but not the SAT-17 application (Control₇₅ and Control₁₅₀). The maximum POD activity (38.2 U/mg protein) was observed in plants grown under 150 mM salt stress without SAT17_{rif} inoculation (control₁₅₀ treatment). This POD activity was 70.5 and 101% higher than the activity observed in control₀ (22.4 U/mg protein) and SAT-17₀ (19.0 U/mg protein) plants, respectively. At 75 mM salt level, we observed a 9.9% difference in POD activity between the SAT-17 treated and non-treated plants. However, at the 150 mM salt level, the SAT-17 treated and non-treated plants exhibited a similar POD activity (Table 4).

Effect of Salt Stress and SAT-17 Inoculation on Maize Nutrient Physiology

The addition of NaCl hindered the uptake of soil N and a decrease in root N content was observed together with an increase in the imposed salt stress. Without SAT-17 treatment, a decrease of 13.6% in N content was observed in plants grown in the presence of 150 mM salt in comparison to control plants (30 mg g⁻¹ DW). The SAT-17 treatment, at 75 mM salt, resulted in increased N content (30.5 mg g⁻¹ DW) in comparison to plants that received only salt stress (Control₇₅). However, at higher salt stress (150 mM), the difference between SAT-17 treated and non-treated plants was non-significant, with the former resulting in 26.1 mg g⁻¹ DW and the latter 25.9 mg g⁻¹ DW root N content. The SAT-17 treatment, in the absence or presence of salt stress, increased the shoot N content where the maximum shoot N content (28.7 mg g⁻¹ DW) was observed in plants grown without salt stress (SAT-17₀). At 75 mM stress, the difference between the SAT-17 treated and non-treated group was 9.64%, where the treated group showed higher shoot N. However, at a salt level of 150 mM, the difference between SAT-17 treated (SAT-17₁₅₀) and non-treated (Control₁₅₀) plants was not significant, with 20.2 mg g⁻¹ DW and 19.6 mg g⁻¹ DW shoot N, respectively (Table 5).

The control plants exhibited 2.83 mg g⁻¹ DW root P content. The addition of salt reduced the P uptake, significantly at the salt

level of 150 mM (1.9 mg g⁻¹ DW root P content). The SAT-17 treatment increased the uptake of P, at 0 and 75 mM NaCl levels, as indicated by higher root P content. At the higher salt level (150 mM), the reduction in P uptake was 2.16 mg g⁻¹ DW and 1.9 mg g⁻¹ DW with and without SAT-17 treatment, respectively. Without SAT-17 inoculation, the imposed salt stress reduced the shoot P content either non-significantly (at 75 mM salt) or significantly (at 150 mM salt) compared to control plants (2.2 mg g⁻¹ DW shoot P content). The SAT-17 treatment increased the shoot P content, significantly, at all of the applied salt levels.

The root K contents were decreased together with an increase in the salt stress level, where 150 mM NaCl resulted in a 35.1% decrease in root K in comparison to the control (12.7 mg g⁻¹ DW). The control plants exhibited 9.8 mg g⁻¹ DW shoot K content. Without SAT-17 treatment, 150 mM salt stress resulted in a 38.7% decrease, while, at this level, the SAT-17 treated plants exhibited only a 14.2% decrease in comparison to control. The effect of strain SAT-17_{rif} inoculation on the uptake and translocation of Ca and Mg was found to be non-significant compared to non-inoculated plants exposed to different salinity levels (data not shown).

The control maize plants showed 24.3 mg g⁻¹ FW total chlorophyll (Chl) content while SAT-17_{rif} inoculation, in the absence of salt, resulted in a 38.1% increase (39.3 mg g⁻¹ FW). The salt stress, without SAT-17 application, resulted in a reduction of maize Chl contents, which was significant (64.1%) at 150 mM. A difference of 42.4% was observed between the treated and non-treated plants grown with 75 mM salt stress, where the former showed a higher Chl content (35.4 mg g⁻¹ FW). A similar trend was observed between the treated and non-treated plants at a salt level of 150 mM.

DISCUSSION

The present work reported the identification and characterization of a PGPR strain isolated from the rhizosphere of a halophytic plant "Kallar grass [*Leptochloa fusca* (L.) Kunth]." The 16 s gene sequence identity (99%) and its clustering with *S. sciuri* ATCC 29062^T (S83569) (Figure 1) confirmed its molecular identity as *S. sciuri* and the strain was designated as SAT-17 (Supplementary Figure S2). Most strains of *S. sciuri* have been reported to form commensal associations with animals (Nemeghaire et al., 2014). Members of the genus *Staphylococcus* can tolerate high salt concentrations (Roohi et al., 2012; Khan et al., 2015) and exhibit

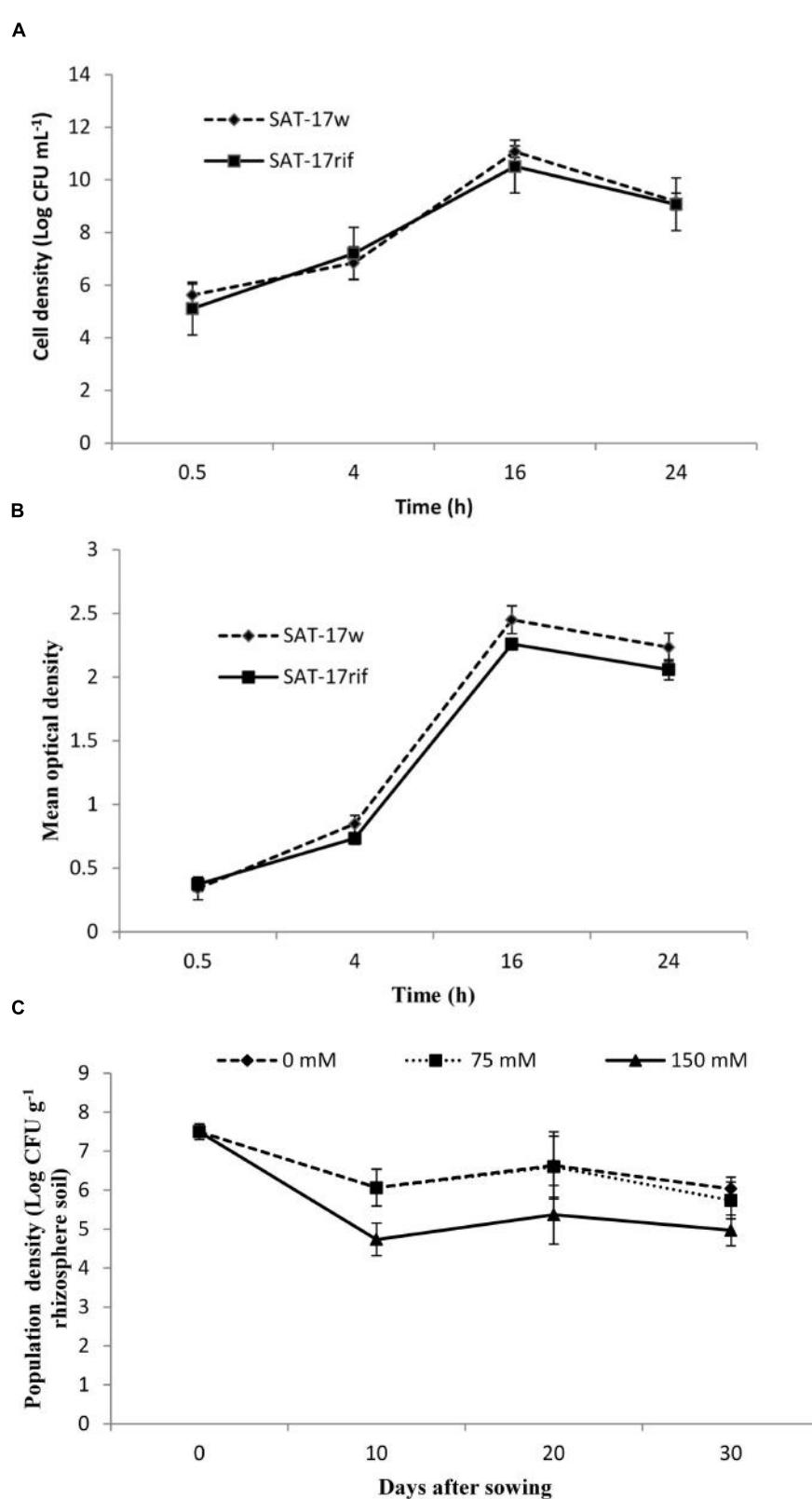


FIGURE 2 | Comparative growth curve of wild-type and rifampicin-resistant derivative of *S. sciuri* SAT-17 constructed with the plate-count method (A) and by measuring OD (B). Population density of *S. sciuri* SAT-17 recovered from maize rhizosphere at different time intervals (C). Error bars represent standard errors ($n = 3$).

TABLE 3 | Effect of *S. sciuri* SAT-17 inoculation on various growth parameters of maize with or without salt stress.

Treatments*	Root length (cm)	Shoot length (cm)	Root FW (g)	Shoot FW (g)	Root DW (g)	Shoot DW (g)
Control ₀	7.87 (0.66) ^{c**}	26 (1.02) ^{cd}	4.73 (0.37) ^d	14.9 (1.05) ^b	1.23 (0.32) ^b	4.03 (0.68) ^b
Control ₇₅	4.97 (0.25) ^e	24.3 (1.52) ^{de}	3.71 (0.44) ^e	11.5 (0.80) ^c	0.97 (0.23) ^b	3.74 (0.54) ^b
Control ₁₅₀	4.37 (0.41) ^e	21.7 (1.15) ^e	3.10 (0.60) ^f	9.8 (1.30) ^c	0.81 (0.18) ^b	3.49 (0.50) ^b
SAT-17 ₀	9.98 (0.55) ^a	31 ± (2) ^a	8.55 (0.18) ^a	18.7 (1.52) ^a	2.31 (0.61) ^a	6.34 (0.87) ^a
SAT-17 ₇₅	8.83 (0.50) ^b	30.3 (2.30) ^{ab}	7.75 (0.31) ^b	19.3 (1.49) ^a	2.26 (0.80) ^a	6.31 (0.60) ^a
SAT-17 ₁₅₀	6.74 (0.36) ^d	29.7 (3.21) ^{bc}	6.18 (0.41) ^c	15.3 (0.57) ^b	1.34 (0.56) ^{ab}	4.50 (0.45) ^b

*Control₀, non-inoculated control without NaCl; Control₇₅, non-inoculated control with 75 mM NaCl; Control₁₅₀, non-inoculated control with 150 mM NaCl; SAT-17₀, inoculation with *S. sciuri* SAT-17 without NaCl; SAT-17₇₅, inoculation with *S. sciuri* SAT-17 with 75 mM NaCl; SAT-17₁₅₀, inoculation with *S. sciuri* SAT-17 with 150 mM NaCl. **Data, analyzed by one-way analysis of variance, are presented as the mean of three replications ($n = 3$) and standard errors are presented in parentheses. Values that differ significantly (Fisher's LSD; $P \leq 0.05$) are presented with different lower-case letters and standard errors are given in parentheses.

TABLE 4 | Oxidative stress responsive species and antioxidants as affected by inoculation of *S. sciuri* SAT-17 at different salinity stress levels.

Treatments*	MDA (nmol g ⁻¹ DW)	H ₂ O ₂ (ng g ⁻¹ DW)	Proline (μg g ⁻¹ DW)	CAT (U mg ⁻¹ protein)	POD (U mg ⁻¹ protein)	Total phenolics (mg g ⁻¹ FW)
Control ₀	1.75 (0.21) ^{c**}	13.8 (1.50) ^b	2.68 (0.45) ^a	28 (2.54) ^c	22.4 (2.59) ^c	26.8 (1.05) ^b
Control ₇₅	2.56 (0.32) ^b	14.2 (0.36) ^b	2.74 (0.39) ^a	32.5 (1.44) ^b	37.1 (3.15) ^a	20.1 (2.24) ^c
Control ₁₅₀	4.43 (0.42) ^a	17.4 (0.75) ^a	2.97 (0.50) ^a	19.8 (2.83) ^d	38.2 (2.34) ^a	18.4 (2.98) ^c
SAT-17 ₀	1.51 (0.12) ^c	10.4 (1.20) ^d	2.59 (0.12) ^a	26.7 (3.95) ^c	19.0 (3.54) ^a	40.3 (2.33) ^a
SAT-17 ₇₅	1.36 (0.31) ^c	12.5 (1.38) ^c	3.08 (0.46) ^a	38.8 (3.80) ^a	33.6 (3.41) ^b	37.6 (1.23) ^a
SAT-17 ₁₅₀	1.77 (0.17) ^c	11.9 (0.99) ^c	2.96 (0.40) ^a	38.9 (4.49) ^a	37.6 (2.18) ^a	30.1 (3.0) ^b

*Control₀, non-inoculated control without NaCl; Control₇₅, non-inoculated control with 75 mM NaCl; Control₁₅₀, non-inoculated control with 150 mM NaCl; SAT-17₀, inoculation with *S. sciuri* SAT-17 without NaCl; SAT-17₇₅, inoculation with *S. sciuri* SAT-17 with 75 mM NaCl; SAT-17₁₅₀, inoculation with *S. sciuri* SAT-17 with 150 mM NaCl. **Data, analyzed by one-way analysis of variance, are presented as the mean of three replications ($n = 3$). Values that differ significantly (Fisher's LSD; $P \leq 0.05$) are presented with different lower-case letters and standard errors are given in parentheses.

plant growth-promoting properties (Yildirim et al., 2008). Zhou et al. (2015) reported the increased growth of sweet cherry plants after inoculation with a PGPR strain *S. sciuri* subspecies *sciuri*, grown in sterilized soil.

The *in vitro* phosphate solubilization ability of the strain was comparable with that reported previously (Oliveira et al., 2009). Solubilization of bound soil phosphate by PGPR triggered soil acidification by the production of organic acids, depending upon the number of carboxylic groups carried. The rhizospheric acidosis promoted the release of cations (Al^{2+} , Fe^{2+} , Ca^{2+}) associated with phosphate, thereby making it available for plant uptake (Mullen, 2005; Trivedi and Sa, 2008). The strain SAT-17 produced a significant amount of IAA up to a salt treatment level of 2 M with the addition of tryptophan (Table 2). Spaepen et al. (2007) reported that IAA is one of the best characterized traits of many PGPR and it is an important phyto-hormone involved in the regulation of plant growth and development. The hormone is also thought to be involved in plant stress responses as a signaling molecule (Spaepen and Vanderleyden, 2011). The SAT-17 also exhibited ACC deaminase activity, a key factor for a PGPR strain to induce stress tolerance in host plants by controlling ethylene concentrations (Glick et al., 2007). Furthermore, the salt treatment level of 2.5 M was found to be the MIC for the isolate SAT-17, making it physiologically more competent than the previously reported PGPR strains.

The rifampicin-resistant derivatives of strain SAT-17 (*S. sciuri* SAT-17_{rif}) were constructed so that the strain could successfully be recovered and identified after inoculation. Rifampicin was

selected as a selectable marker due to the susceptibility of most soil bacteria against rifampicin (Shahid et al., 2012). The comparative growth curves of SAT-17_w and SAT-17_{rif} revealed that the derivative strain was healthy enough to inoculate the maize seedlings. It survived in the rhizosphere, with an optimal population density of 6×10^6 CFU g⁻¹ rhizospheric soil, up to 30 DAS, suggesting that the strain was rhizospherically competent (Shahid et al., 2012). Many PGPR exhibiting *in vitro* beneficial traits have failed to induce positive effects, *in vivo*, due to poor root colonization and antagonism with the native soil microorganisms (Benizri et al., 2001). The SAT-17_{rif} density was initially declined in the soil but later the strain adapted well and survived. Fischer et al. (2010) also reported an initial decrease in the density of the rifampicin-resistant derivative of *Pseudomonas* sp. SF4c in the rhizosphere of wheat which later became stable and induced beneficial effects.

Plant growth-promoting rhizobacteria can relieve plants from the deleterious effects of salinity and enhance plant growth and productivity through a variety of mechanisms (Rajput et al., 2013; Kim et al., 2014; Islam et al., 2015). *S. sciuri* SAT-17_{rif}, when used as an inoculum, significantly promoted the growth and biomass of maize plants grown in the absence and presence of 75 mM salt stress, each compared with the non-inoculated ones (Table 3). Adverse effects of salt stress on plant growth are mainly attributed to limited water uptake as a result of ion osmotic effect, which in turn affect the photosynthetic rate, cell function, nutrient balance and several other metabolic functions (Kumar et al., 2005). In the present study, it seems likely that

TABLE 5 | Effect of *S. sciuri* SAT-17 inoculation on maize nutrient, chlorophyll and protein contents at different levels of salt-stress.

Treatments *	Root N (mg g ⁻¹ DW)	Shoot N (mg g ⁻¹ DW)	Root P (mg g ⁻¹ DW)	Shoot P (mg g ⁻¹ DW)	Root K (mg g ⁻¹ DW)	Shoot K (mg g ⁻¹ DW)	Total chlorophyll (mg g ⁻¹ FW)	Total protein (mg g ⁻¹ FW)
Control ₀	30 (1.20) ^{b**}	26.7 (1.56) ^b	2.83 (0.3) ^b	2.2 (0.30) ^b	12.7 (1) ^a	9.8 (1.74) ^a	24.3 (3.19) ^b	374 (28) ^c
Control ₇₅	27.1 (2.28) ^c	22.7 (2.93) ^d	2.53 (0.55) ^b	2.07 (0.49) ^b	11.0 (0.90) ^b	8.7 (0.80) ^b	23 (3.97) ^b	254 (22) ^d
Control ₁₅₀	25.9 (1.42) ^d	19.6 (1.67) ^e	1.9 (0.15) ^c	0.98 (0.21) ^d	8.23 (0.70) ^d	6.00 (0.62) ^c	14.8 (2.88) ^c	217 (20) ^d
SAT-17 ₀	32.8 (2.47) ^a	28.7 (1.93) ^a	3.50 (0.61) ^a	3.07 (0.55) ^a	13.1 (1.01) ^a	9.9 (0.56) ^a	39.3 (3.99) ^a	501 (32) ^a
SAT-17 ₇₅	30.5 (3.26) ^b	25 (2.43) ^c	3.43 (0.77) ^a	3.06 (0.61) ^a	12.5 (0.66) ^a	10 (1) ^a	35.4 (2.39) ^a	432 (42) ^b
SAT-17 ₁₅₀	26.1 (2.70) ^d	20.2 (1.68) ^e	2.16 (0.40) ^b	1.79 (0.40) ^c	9.2 (1.22) ^c	8.4 (1.90) ^b	28.4 (4.30) ^b	423 (26) ^b

*Control, non-inoculated control without NaCl; Control₇₅, non-inoculated control with 75 mM NaCl; Control₁₅₀, non-inoculated control with 150 mM NaCl; SAT-17₀, inoculation with *S. sciuri* SAT-17 without NaCl; SAT-17₇₅, inoculation with *S. sciuri* SAT-17 with 75 mM NaCl; SAT-17₁₅₀, inoculation with *S. sciuri* SAT-17 with 150 mM NaCl. **Data, analyzed by one-way analysis of variance, are presented as the mean of three replications ($n = 3$). Values that differ significantly (Fisher's LSD, $P \leq 0.05$) are presented with different lower-case letters and standard errors are given in parentheses.

SAT-17_{rif} modulated the plants' ability to uptake water more efficiently, probably by modulating the root system architecture as the root hairs and lateral roots are the main sites for PGPR colonization in members of the family Poaceae (Combes-Meynet et al., 2011; Couillerot et al., 2011). Moreover, PGPR supply may enhance nutrient uptake by stimulating root formation (Yildirim et al., 2011) and inhibiting the salt-ion accumulation (Mayak et al., 2004), which in turn promotes plant growth (Supplementary Figure S3). Furthermore, PGPR inoculation might alter the source-sink relations as *Capsicum annuum* plants, co-inoculated with *Azospirillum brasilense* and *Pantoea dispersa*, exhibited the higher plant dry matter accumulation associated with a higher source activity, stomatal conductance and photosynthesis (del Amor and Cuadra-Crespo, 2012). As the SAT-17 produced optimum IAA *in vitro*, it was suggested that IAA acted as a negative feedback signal to temporarily repress cytokinin synthesis in the roots and their transport to the shoot (Rahayu et al., 2005) leading to increased root elongation. The observed effects on plant biomass may also be attributed to modulated regulation of ethylene, as SAT-17 exhibited significant ACC deaminase activity (Dodd and Pérez-Alfocea, 2012; Kim et al., 2014). Our results coincide well with the findings of Rajput et al. (2013) who reported an increase in the growth and yield of wheat under salt stress conditions after treating the wheat plants with *Planococcus rifetoensis* strain SAL-15. In another study, Ahmad et al. (2014) reported that the integrated use of PGPR, biogas slurry and chemical nitrogen enhanced maize growth and productivity. The PGPR strains *Erwinia persicinus* RA2 (with ACC deaminase activity) and *Bacillus pumilus* WP8 (without ACC deaminase activity) significantly enhanced tomato growth and quality under seawater irrigation (Cha-Um and Kirdmanee, 2009).

In non-inoculated plants, salt stress increased the MDA content and triggered the production of H₂O₂; this is an indication of oxidative damage to membranes, as reported for various crops (Cui and Wang, 2006; Nasraoui-Hajaji et al., 2012). Increased MDA contents at the cellular level resulted in oxidative injury as well as a disruption of nutrient ion balance (Azooy et al., 2009). Ashraf (2010) reported that reduced leaf water potential was observed due to lipid peroxidation and ionic leakage induced by salt stress. Inoculation of maize seedlings with *S. sciuri* SAT-17_{rif} significantly prevented salt-induced lipid peroxidation of membranes, as evidenced by the decreased MDA contents in the present study.

Proline may act as an enzyme protectant and stabilize the structure of other macromolecules (Mahajan and Tuteja, 2005).

In the present study, increased proline accumulation in the inoculated plants alleviated the adverse effects of salt stress. It is well known that proline accumulation and other compatible solutes under stress conditions might help with the osmotic adjustment at a cellular level. PGPR are well known for producing resistance in plants against various stresses like salinity through the enhanced production of antioxidants (Younesi and Moradi, 2014).

The adverse environmental conditions triggered the synthesis of hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and/or hydroxyl (OH⁻) radicals (Shigeoka et al., 2002); all of these ROS

pose potential hazards if they are not detoxified by the plants. The anti-oxidative defense system, comprised of enzymatic and non-enzymatic components, provides a mechanism against the deleterious actions of ROS in plant cells (Ali and Ashraf, 2011). Inoculated maize plants under salinity treatments (75 and 150 mM) showed significantly reduced ROS levels and elevated levels of antioxidant enzymes (CAT, POD), suggesting the induction of oxidative damage repair mechanisms (**Table 4**). Our results were in agreement with earlier findings that PGPR strains under salinity stress environments triggered the stress responsive non-enzymatic (Kim et al., 2014; Islam et al., 2015) and enzymatic anti-oxidative defense systems (comprised of CAT and POD) (Ali and Ashraf, 2011; Masood et al., 2012). The PGPR-induced salt alleviation may be attributed to bacterial exopolysaccharides which adhere to soil Na⁺ and prevent its transfer to shoots/leaves (Ashraf et al., 2004). In addition, PGPR also confer salt-tolerance in plants by the tissue-specific regulation of the Na transporter *HKT1* (Zhang et al., 2008). Under saline conditions, PGPR regulate the enzymes of important metabolic pathways (tricarboxylic acid cycle, glyoxylate cycle, glycolysis) and improved the energetic status of the plant (Sheng et al., 2011), which in turn help to sustain ionic homeostasis by maintaining the Na⁺ exclusion capacity in the roots and delay the incidence of toxic ionic effects (Pérez-Alfocea et al., 2010). Sahoo et al. (2014) reported that *Azotobacter vinelandii* (SRIAz3), isolated from rice rhizosphere, improved rice productivity by inducing stress tolerance and altering plant endogenous hormonal levels.

Salt stress negatively affected the uptake of N, P and K in the roots and shoots of maize, probably due to the increased uptake of Na and Cl ions (**Table 5**). Earlier works reported that K⁺ uptake in tomato plants decreased due to the antagonistic relationship of Na⁺ and K⁺ at the root surface under NaCl stress (Bastias et al., 2010). Furthermore, the root membrane structure and selectivity for ions has also been reported to be negatively affected under salt stress due to the interference of Na⁺ with K⁺ (Grattan and Grieve, 1999). Inoculation of maize seedlings with *S. sciuri* SAT-17_{rif} significantly increased the uptake of N, P, and K⁺ in roots (and translocation to the shoots) compared to the non-inoculated control. *S. sciuri* SAT-17 was a potential phosphate-solubilizing strain; hence it enhanced the mobilization of P from the soil to the roots/shoots. Similarly, the plant growth-promoting effect of the strain favored the maize plants accumulating more macronutrients in the biomass. Our results are in accordance with the findings of Mohamed and Gomaa (2012), who reported a significant increase in macronutrient (N, P, K⁺, Ca²⁺ and Mg²⁺) concentrations in radish when the seeds were inoculated with PGPR (*Bacillus subtilis* and *Pseudomonas fluorescens*). Yildirim et al. (2011) reported that the increased nutrient concentrations in broccoli plants were due to the increased root surface area as well as root exudation. The enhanced root exudation up-regulates microbial activity, leading to increased soil nutrient solubility and, later, higher influx into the plant roots (Adesemoye et al., 2008). The enhanced nutrient uptake by maize roots, after SAT-17 inoculation, may

be attributed to a low rhizospheric pH which increased the bioavailable fraction of cationic nutrients as reported earlier (Abou-Shanab et al., 2006). However, the underlying mechanisms are not yet completely elucidated. A similar trend was also determined for total chlorophyll and protein contents of the plants. Earlier works with PGPR have also been shown to alleviate NaCl stress by increasing the leaf chlorophyll contents. The increase in leaf chlorophyll contents under PGPR inoculation was attributed to the increased nutrient availability (Karlidag et al., 2013). The observed effect is also likely due to root proliferation and enhanced water absorption, which in turn increases the leaf numbers and leaf surface area for photosynthesis. Hence, increased chlorophyll contents, after *S. sciuri* SAT-17_{rif} inoculation, were responsible for the shoot growth enhancement under salt stress which offered new binding sites for nutrient ions. Under salt stress, the protein contents were significantly reduced, probably due to biological degradation, as the toxic ions can activate the mechanism of protein denaturation in maize plants (Gadd and Griffith, 1978). Alterations in protein contents under saline conditions may modulate the enzyme activities responsible for the anti-oxidative defense to cope with salt-mediated production of ROS (Hossain et al., 2012). Inoculation of maize seedlings with *S. sciuri* SAT-17_{rif} significantly increased the protein contents of maize, enabling the plants to synthesize anti-oxidative enzymes and better withstand the salt stress.

CONCLUSION

The salt-tolerant rhizobacterium *S. sciuri* strain SAT-17, characterized in the current study, exhibited substantial phosphate solubilization as well as indole-3-acetic acid production and 1-aminocyclopropane-1-carboxylic acid deaminase activity. The inoculation of maize with SAT-17 improved plant growth alongside decreasing the reactive oxygen species levels and increasing the cellular antioxidant enzyme activities (CAT, POD and proline). Moreover, the inoculation increased the uptake of N, P and K to maintain optimum nutrient, chlorophyll and protein levels in maize plants, thereby preventing drought-induced lipid peroxidation of membranes under salt stress. It has been concluded that the use of *S. sciuri* SAT-17 could serve as an efficient approach for enhancing crop tolerance to salinity in arid and semi-arid regions. This study opens the future directions for researchers to investigate about the genetic mechanism involved in salinity tolerance induction by *S. sciuri* SAT-17 in maize. The study on expression profiling of some stress-responsive genes of maize in response to *S. sciuri* SAT-17 inoculation might be helpful to understand about molecular cross-talk between plant and bacterial strain.

AUTHOR CONTRIBUTIONS

MSA: designed experiments, data analysis, manuscript preparation. MT: designed experiments, manuscript preparation.

MS: designed experiments, manuscript preparation, data analysis. MA: manuscript preparation, data analysis. MTJ: critical revision of manuscript, data analysis. SS: student who practically performed experiments, manuscript preparation. SR: student who practically performed experiments, manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00867>

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Impact of Wheat/Faba Bean Mixed Cropping or Rotation Systems on Soil Microbial Functionalities

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Cropping systems based on carefully designed species mixtures reveal many potential advantages in terms of enhancing crop productivity, reducing pest and diseases, and enhancing ecological services. Associating cereals and legume production either through intercropping or rotations might be a relevant strategy of producing both type of culture, while benefiting from combined nitrogen fixed by the legume through its symbiotic association with nitrogen-fixing bacteria, and from a better use of P and water through mycorrhizal associations. These practices also participate to the diversification of agricultural productions, enabling to secure the regularity of income returns across the seasonal and climatic uncertainties. In this context, we designed a field experiment aiming to estimate the 2 years impact of these practices on wheat yield and on soil microbial activities as estimated through Substrate Induced Respiration method and mycorrhizal soil infectivity (MSI) measurement. It is expected that understanding soil microbial functionalities in response to these agricultural practices might allows to target the best type of combination, in regard to crop productivity. We found that the tested cropping systems largely impacted soil microbial functionalities and MSI. Intercropping gave better results in terms of crop productivity than the rotation practice after two cropping seasons. Benefits resulting from intercrop should be highly linked with changes recorded on soil microbial functionalities.

Keywords: arbuscular mycorrhizal fungi, cropping systems, nutrient uptake efficiency, microbial soil functions, Mediterranean region

INTRODUCTION

The ecological key processes that warrant the productivity and stability of terrestrial ecosystems have been designated as efficient models for sustainable agricultural management. Since it is well known that the functioning and stability of terrestrial ecosystems are dependent to plant biodiversity and species composition (Naeem et al., 1994; Tilman and Downing, 1994; Tilman et al., 1996; Hooper and Vitousek, 1997), mixing plant species in cropping systems reveals many potential advantages under various conditions to contribute to modern and sustainable agriculture

(Vandermeer, 1989). In agroecosystems, multispecies cropping systems may (i) sustain biomass production and decrease the risk of crop failure in unpredictable environments, (ii) rehabilitate disturbed ecosystem services (i.e., water and nutrient cycling), and (iii) decrease risks of invasion, pests, and diseases through enhanced biological control or direct control of pests (Gurr et al., 2003). These cultural practices have often been considered as a practical application of ecological principles related on biodiversity, plant interactions, and other natural regulation mechanisms (Malézieux et al., 2009). Numerous different multispecies cropping systems can be designed by including various criteria such the frequency of land-use rotation, the intensity of intercropping, etc. (Garcia-Barrios, 2003). For instance, the intercropping, defined as the growth of more than one crop species or cultivar simultaneously in the same field during the same growing season (Ofori and Stern, 1987; Hauggaard-Nielsen et al., 2007), enhanced the use efficiency of environmental sources for plant growth resulting in stable yields (Hauggaard-Nielsen et al., 2001; Corre-Hellou and Crozat, 2005; Jensen et al., 2015). It has been also reported that the association of cereals and legumes at the same space and time led to higher yields and improved N (via biological N₂ fixation for the legume) and P nutrition (Li et al., 2005; Betencourt et al., 2012; Latati et al., 2013, 2014).

The positive effects of species diversity in intercropping systems result from two main processes: complementarity and facilitation (Fridley, 2001; Hinsinger et al., 2011) whereas in rotation systems (i.e., legumes/cereals rotation), they occur through indirect feedback interactions (Schnitzer et al., 2011). It has been reported that these biological processes were mainly driven by soil microbe activities (Klironomos, 2002; Tang et al., 2014). For instance, the enhancement of P acquisition in the context of cereal/legume intercrops could occur as a consequence of microbial mediated processes involving soil fungi and bacteria (Wang et al., 2007). Positive feedback often involves changes in abundance and diversity of symbiotic mutualists such as nitrogen-fixing rhizobacteria and mycorrhizal fungi (van der putten et al., 2013). These biological processes have been particularly studied for their impacts on primary production, nutrient retention, and resilience after stress in natural prairie ecosystems (Tilman et al., 1996, 1997) or in natural forest ecosystems (Altieri, 1999). In contrast, few studies have been conducted in agricultural systems to determine the impacts of multispecies cropping systems on microbial soil functionalities and mycorrhizal soil infectivity in relation with crop productivity.

It has been suggested that Faba bean could enable diversification of the agrosystems (Garofalo et al., 2009; Köpke and Nemecek, 2010) and it has been highlighted the importance of the N and P contributed by faba bean in intercropping and rotation systems (Li et al., 2003). However, the Faba bean impacts on soil microbial functionalities in field conditions have been less investigated (Köpke and Nemecek, 2010). In the present study, two cereal/legume systems (intercropping and rotation) were investigated in a field experiment during two growing seasons. The specific aims of this study were (i) to evaluate the agronomic performance of wheat/Faba bean in intercrop and in rotation and (ii) to monitor the impacts of these cultural

practices on the mycorrhizal soil infectivity and on the microbial soil functionalities.

MATERIALS AND METHODS

Field Conditions and Experimental Design

The field experiment was performed in the 2011–2013 growing seasons in the Haouz plain at about 30 km at the East of Marrakech ($31^{\circ}4'60''$ N and $7^{\circ}3'0''$ W, Morocco). Soil chemical properties (0–0.10 m layer) were as follows: pH (H_2O) 7.2; carbon (%) 1.54; nitrogen (%) 0.08, C/N 19.2; Total P ($mg\cdot kg^{-1}$) 502.9 and Olsen P ($mg\cdot kg^{-1}$) 22.1. This soil exhibited a high content of available P presumably non-limiting for plant growth and resulting from large-P fertilizer applications during the last decades, until this part of the field was dedicated to organic farming 1 year before the beginning of this experiment. The regional climate of the experimental site is typical Mediterranean with surface soils regularly undergoing drying-rewetting cycles from the irregular distribution of rainfall. The annual average rainfall was 282 mm, mostly in Autumn/Winter (59%) and in Spring (22%). The dry season is from May to September. The mean air temperature is $17.9^{\circ}C$ in autumn, $12.8^{\circ}C$ in winter, $18.5^{\circ}C$ in spring, and $24.7^{\circ}C$ in summer. Soil, cropped in the previous growing season with durum wheat (*Triticum durum* Desf.), was plowed to a depth of 0.30 m in Summer and then shallowly harrowed to control weeds. No herbicide nor chemical fertilizers were applied. The experimental design had a randomized block design with two factors and four replication blocks. The factor was the cropping system (wheat/faba bean intercropping, rotation, or wheat monoculture). Plots were $3.0\text{ m} \times 3.0\text{ m}$; each main plot was spaced 1.0 m out from the next. The crops were sown in December 2011 and 2012 at a rate of $400\text{ viable seeds m}^{-2}$ in rows 0.18 m apart for wheat and at a rate of $200\text{ kg}\cdot ha^{-1}$ for faba bean. When intercropped, the two species were sown in the same row in order to maximize root proximity and plant-plant interactions. The experimental plot consisted of four rows 3 m long. Weeds were controlled by hand during the experiment. Hence, 3 treatments were examined, namely durum wheat as sole crop (W) and two cropping systems: durum wheat/Faba bean intercrop (WF) and wheat/Faba bean rotation (F+W).

Plant Analyses

Wheat was considered as the main crop and faba bean as an intercrop component. Hence, the expected benefits of the cultural practices on yield productivity were only examined on wheat plants. After one and two growing seasons, at wheat tillering and at the same time, the total number of wheat plant and spike per plot were counted. Then 10 randomly chosen plants in the middle of the plot were harvested. The seeds from each plant were collected, counted, and weighed to determine the dry weight of 1000 seeds (grain yield). These measurements recorded on a plot basis were converted to hectare for statistical analysis. The aerial parts of each plant were then oven dried at $70^{\circ}C$ during 2 weeks and weighed. After drying, shoot tissues were ground,

ashed (500°C), digested in 2 ml HCl 6N and 10 ml HNO_3 N for nitrogen and then analyzed by colorimetry for phosphorus (John, 1970). For nitrogen determination (Kjeldahl method), they were digested in 15 ml H_2SO_4 (36 N) containing 50 g l^{-1} of salicylic acid. Roots from five other randomly chosen plants in the middle of each plot were sampled and root subsamples of about 3 g each were taken. Each subsample was stained with 0.05% trypan blue in lactic acid according to Phillips and Hayman (1970); root colonization by AMF was then measured with the grid intersect method according to Giovannetti and Mosse (1980).

Soil Microbial Analysis

After one and two growing seasons, soil cores (1 kg) were collected at 0- to 20-cm depth in each plot. About 10 soil samples were taken from each plot and pooled together. Soil samples were crushed and passed through a 2-mm sieve. Then hyphal length that is considered as a main component of the mycorrhizal soil infectivity (Kisa et al., 2007) was measured by the filtration-grid-line method on membrane filters according to Jakobsen and Rosendahl (1990). Patterns of *in situ* catabolic potential (ISCP) were designed to assess the functions of soil microbial communities and the microbial functional diversity in soil treatments after one and two growing seasons. The diversity of the catabolic potentials of the total soil bacterial community was evaluated according to Campbell et al. (2003) by a microrespirometry method performed in 96-well microtiter plates. In order to ensure the resumption of microbial activity, sterile distilled water was added to reach 30% of the water-holding capacity and plates were incubated 3 days in the dark at 28°C . Then soil wells received 28 organic substrate solutions (three wells per substrate). Stock solutions for thirteen carbohydrates (D-mannose, D-mannitol, D-trehalose, L-arabinose, D-xylose, D-sucrose, D-galactose, meso-inositol, D-sorbitol, L-rhamnose, L-arabitol, mesoerythriol, D-Glucose), eight carboxylic acids (citric acid, maleic acid, D,L-malic acid, oxalic acid, Na-gluconate, α -ketoglutaric acid, L-ascorbic acid) and seven amino acids (L-asparagine, D,L-valine, L-methionine, L-glutamine, D,L-alanine, D,L-serine, *N*-acetyl-D-Glucosamine) were prepared with distilled water and their concentrations were calculated to lead, respectively, 0.03, 0.04, and 0.004 mmol g^{-1} soil. Basal respiratory activity was calculated in triplicate with distilled water. The colorimetric detection plates were assembled and used according to MicroRespTM (Aberdeen, UK) recommendations. Absorbance was measured at 572 nm with a Tecan infinite M200 reader before substrate spiking (t0) and after 6 h of incubation at 28°C (t6). For each well, absolute respiratory activity was calculated by subtracting the absorbance value at t0 from the value at t6. The average basal respiration value was then subtracted from all the individual substrate respiration values. For each carbon source, this substrate-specific respiratory activity was averaged and the value was finally divided by the sum of all the mean substrate-specific respiratory activities (pi value). The catabolic evenness (E) was calculated to determine the catabolic diversity of soil treatments. It represents the variability of catabolized substrates amongst the range of the targeted substrates and is calculated using the Simpson-Yule index $E = 1/\sum p_i^2$ with $p_i = (\text{respiration response to individual substrates})/(\text{Total respiration activity induced by all substrates for a soil treatment})$; Magurran, 1988).

The catabolic evenness could be used to evaluate the ability of microbial communities to resist against environmental stress or disturbance (Magurran, 1988). Data were calculated for the individual responses to substrates but also for the average responses with carbohydrates, carboxylic acids, and amino acids.

Statistical Analysis

All the data were subjected to a two-way analysis of variance and comparisons among means were made using the Newman-Keuls test ($P < 0.05$). The percentages of the mycorrhizal colonization were transformed by arcsin(sqrt) before the statistical analysis. The relationships between ISCP profiles table (2012 data) and yield variables table (2013 data) were analyzed using Between-Group CO-Inertia Analysis (BGCOIA). BGCOIA is a Co-Inertia Analysis on the two tables of group means obtained after a Between-Group Analysis (BGA, Thioulouse et al., 2012). As a first step, a BGA is therefore computed on the two data sets, considering each treatment as a group. The technical details of BGCOIA are given in Franquet et al. (1995). Examples of use and a comparison with other methods are presented in Thioulouse (2011) in the framework of k-tables data analysis methods. Let g be the number of groups (treatments here). The table of group means for SIR profiles is obtained by computing the means of each substrate within each treatment. This gives a new table, with g rows and p columns (p substrates). The same computations are done for the yield data table, leading to a second new table with g rows and q columns (q yield variables). A Co-Inertia Analysis is then performed on these two new tables. The rows of the initial tables can be projected into this analysis to help interpret the results (Lebart et al., 1984). Computations and graphical displays can be produced with the ade4 package for the R software (Thioulouse and Dray, 2007).

RESULTS

Grain Yield and Biomass Production

After one cropping season, no significant effect on the wheat development has been recorded between the wheat monoculture and the wheat/faba bean intercropping (Table 1). After the second cropping season, crop data evidenced that total biomass yield, spike number, spike dry weight were higher in the intercropping treatment (WF) compared to the wheat monoculture (W) and Faba bean/wheat rotation (W+F) treatments with some enhancements resulting from the intercropping vs. monoculture of +84.2, +24.8, and 122.7%, respectively (Table 1). The thousand-seed weight was significantly higher in the WF and W+F treatments compared to the monoculture (W; Table 1). After the second growing season, the shoot P content was significantly increased in the intercropping and rotation treatments whereas nitrogen enhancement was only recorded in the WF treatment with (Table 1).

TABLE 1 | Effects of cropping systems (W: wheat monoculture; WF: wheat/Faba bean intercropping; W+F: Wheat/Faba bean rotation) on total biomass yield ($\text{kg} \cdot \text{ha}^{-1}$), spike number per ha, spike dry weight ($\text{kg} \cdot \text{ha}^{-1}$), thousand-seed weight (TSW), mineral nutrition, mycorrhizal colonization of durum wheat and on soil catabolic evenness, and standardized average substrate-induced respiration (SIR) responses with each substrate group (carboxylic acids, amino-acids, and carbohydrates) in 2012 and 2013 in the field experiment located in the Haouz plain at about 30 km at the East of Marrakech (Morocco).

	Treatments					
	2012			2013		
	W	WF	W+F	W	WF	W+F
Total biomass yield ($\text{kg} \cdot \text{ha}^{-1}$)	4662 (78.5) ¹ a ²	3897 (49.3) a	—	4434 (66.1) a	8167 (97.3) b	5038 (94.6) a
Spike number per ha ($\times 10^4$)	219.3 (12.2) a	213.7 (24.4) a	—	192.3 (37.5) a	240.1 (10.2) b	179.1 (39.9) a
Spike dry weight ($\text{kg} \cdot \text{ha}^{-1}$)	2290 (12.6) a	2283 (33.4) a	—	2224 (17.1) a	4953 (58.3) b	2661 (41.5) a
Thousand-seed weight (g)	42.7 (2.9) a	41.1 (1.9) a	—	42.7 (2.9) a	53.9 (2.7) b	52.8 (4.5) b
Shoot N content (%)	nd ³	nd ³	—	5.08 (0.31) a	5.55 (0.17) b	4.71 (0.41) a
Shoot P content ($\text{g} \cdot \text{kg}^{-1}$)	nd ³	nd ³	—	6.01 (0.59) a	7.47 (0.38) b	7.56 (0.34) b
Mycorrhizal colonization (%)	46.7 (5.8) a	62.4 (6.4) b	—	54.7 (4.7) a	74.7 (4.8) b	69.9 (3.2) b
Hyphal length (m g^{-1} dry soil)	1.66 (0.08) a	2.85 (0.07) c	2.14 (0.06) b	1.72 (0.07) a	2.98 (0.05) b	2.85 (0.04) b
Catabolic evenness	15.2 (0.83) b	12.3 (0.23) b	17.4 (0.48) c	15.1 (1.13) b	12.7 (1.01) a	13.5 (1.23) ab
Carbohydrates	0.025 (0.01) a	0.029 (0.02) b	0.028 (0.02) b	0.024 (0.02) a	0.021 (0.01) a	0.023 (0.02) a
Amino-acids	0.008 (0.01) a	0.006 (0.01) a	0.008 (0.01) a	0.007 (0.01) a	0.005 (0.01) a	0.006 (0.01) a
Carboxylic acids	0.067 (0.04) a	0.102 (0.07) b	0.060 (0.03) a	0.064 (0.04) a	0.075 (0.05) b	0.078 (0.05) b

¹Standard error. ²For each year, data in the same line followed by the same letter are not significantly different according to the Newman–Keul's test ($p < 0.05$). ³nd, not determined.

Mycorrhizal Soil Infectivity and Soil Microbial Functionalities

The mycorrhizal colonization of wheat root systems was significantly higher in the WF treatment compared to the wheat monoculture after one cropping season (Table 1). After two cropping seasons, wheat root systems were highly colonized by the native mycorrhizal fungi in the WF and W+F treatments compared to the wheat monoculture (Table 1). The development of the mycorrhizal hyphal network was also stimulated in the intercropping and rotation treatments with highest values recorded in the WF treatment after the first growing season (Table 1).

In 2012, the catabolic evenness ranged as follow $\text{W+F} > \text{W} = \text{WF}$ whereas it ranged as $\text{W} > \text{W+F} > \text{WF}$ in 2013 (Table 1). No significant differences were recorded for the average amino-acid induced respiration within the treatments in 2012 and 2013 and for the average carbohydrate induced respiration in 2013 (Table 1). In contrast, the average SIR was significantly higher with carbohydrates in the WF and W+F treatments after the first growing season (Table 1). For the carboxylic acids, highest values were measured in 2012 with the WF treatment and in 2013 with the WF and W+F treatments (Table 1).

After one cropping season, the highest SIRs have been obtained with citric acid, maleic acid, malic acid, oxalic acid, ketoglutaric acid, and ascorbic acid in the WF treatment (Figure 1) whereas and after two cropping seasons, a significant highest SIR response was only recorded with maleic acid in the WF treatment (Figure 2). The BGA permutation test of ISCP profiles in the three treatments (W, W+F, WF) is highly significant ($p \sim 1.10 \cdot 10^{-4}$) in 2012, but not in 2013. The BGA

permutation test for the comparison of SIR profiles in 2012 vs. 2013 is also significant ($p \sim 0.03$). There is no relationship between SIR profiles and yield variables in 2012 or in 2013 (the permutation tests of Co-Inertia Analyses are not significant). But there is a strong relationship between 2012 SIR profiles and 2013 yield variables ($p \sim 0.004$; Figure 3). Samples with higher yields are on the left, they correspond to intercropping (WF), and to high consumption of the following organic substrates: trehalose, glucosamine, glucose, sucrose, and organic acids (Figure 3). Lower yield levels are on the right, they correspond to wheat monoculture (W), and to the following organic substrates: galactose, glutamate, asparagine (Figure 3). On the vertical axis, rotation system (W+F) correspond to intermediate yield levels, and to particular organic substrates: gluconic acid, glutamine, xylose, mannose, mannitol, sorbitol (Figure 3).

DISCUSSION

This study conducted during two growing seasons clearly shows that intercropping could lead to highest performances in terms of crop yield than the rotation and the monoculture after two cropping seasons. Benefits resulting from intercrop seem to be linked with changes recorded on the mycorrhizal soil infectivity and on soil microbial functionalities.

These results are in accordance with previous studies showing that productivity advantages of intercropping may arise from complement use of growth resources such as N and water in either space or time (Akter et al., 2004; Chu et al., 2004). In the same way, intercropping legume and cereals may lead in highest nitrogen content in the cereal grain, improving that quality criterion (Bulson et al., 1997; Gooding et al., 2007).

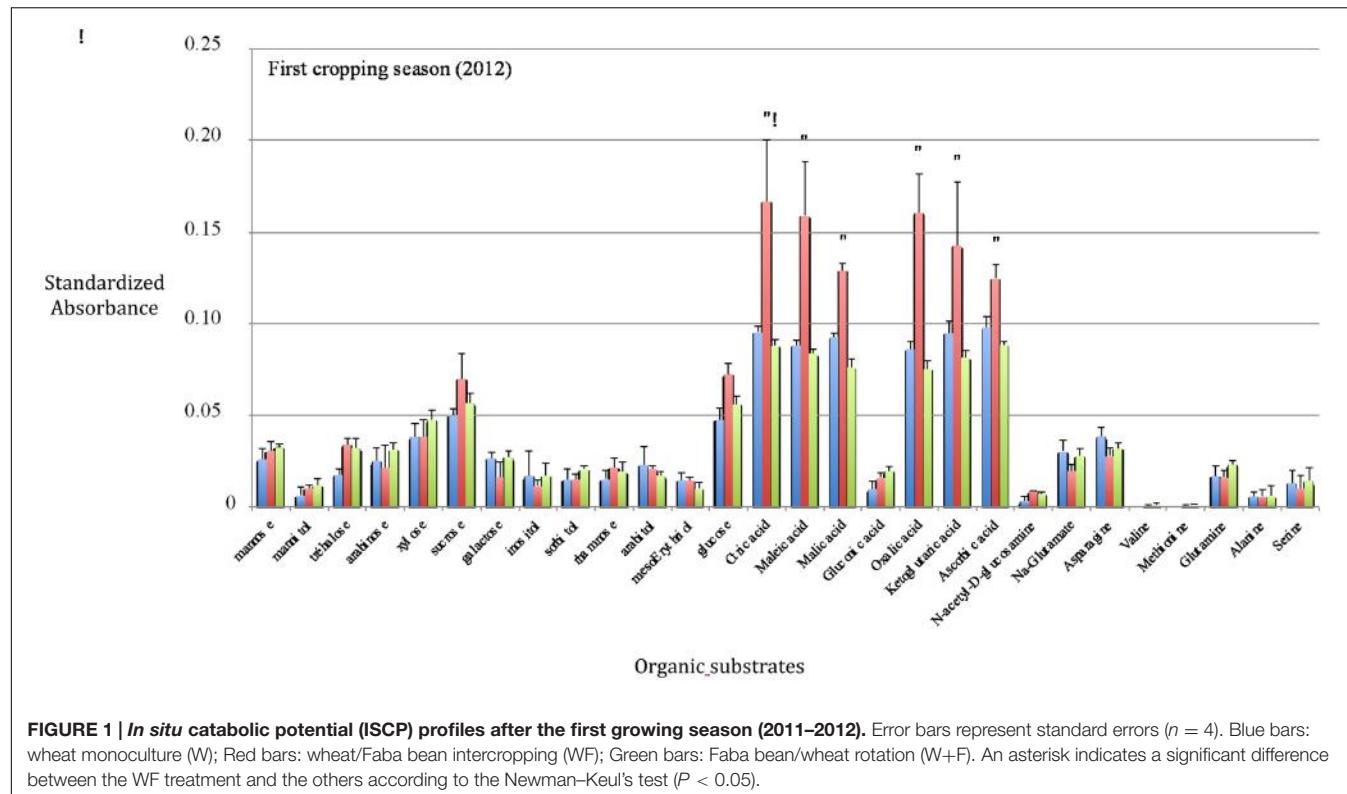


FIGURE 1 | *In situ* catabolic potential (ISCP) profiles after the first growing season (2011–2012). Error bars represent standard errors ($n = 4$). Blue bars: wheat monoculture (W); Red bars: wheat/Faba bean intercropping (WF); Green bars: Faba bean/wheat rotation (W+F). An asterisk indicates a significant difference between the WF treatment and the others according to the Newman–Keul's test ($P < 0.05$).

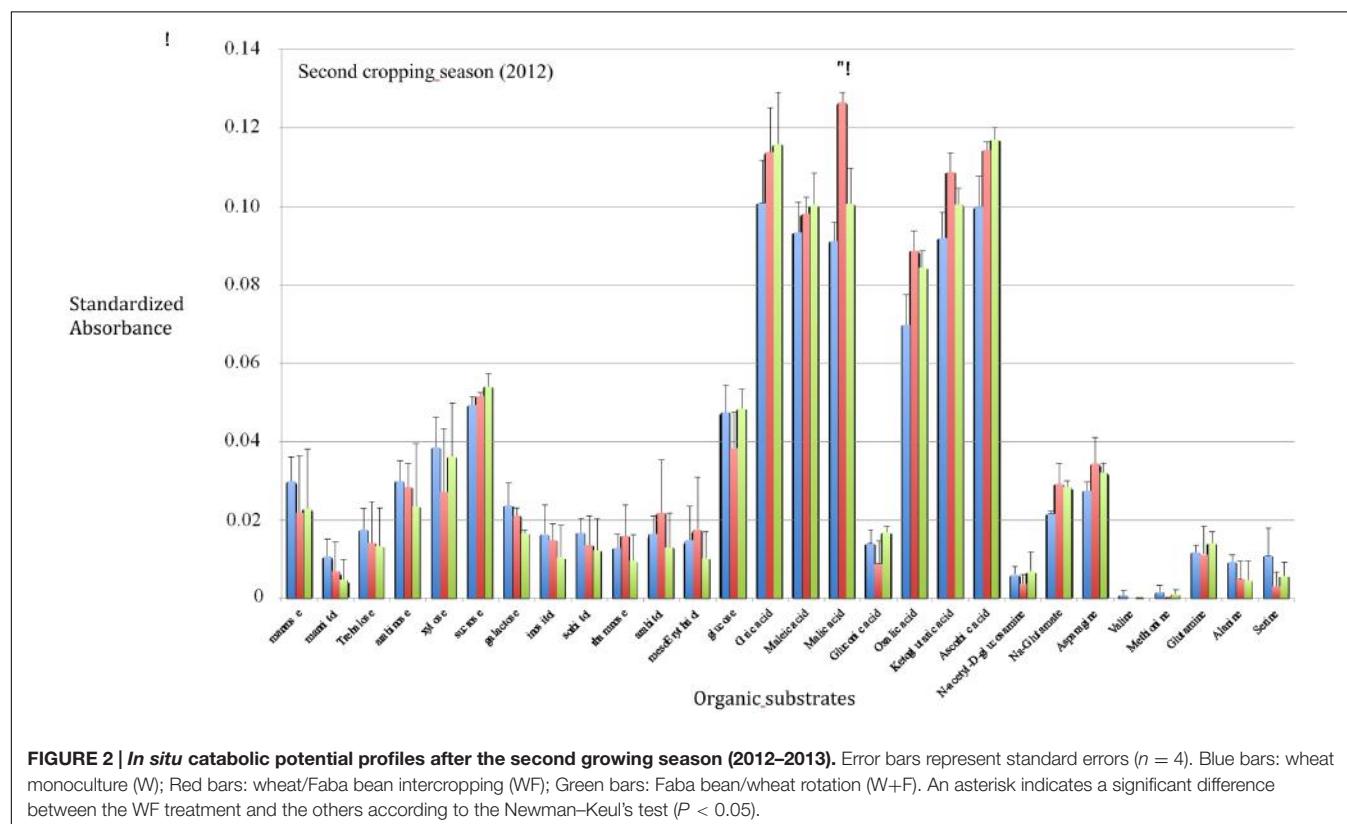


FIGURE 2 | *In situ* catabolic potential profiles after the second growing season (2012–2013). Error bars represent standard errors ($n = 4$). Blue bars: wheat monoculture (W); Red bars: wheat/Faba bean intercropping (WF); Green bars: Faba bean/wheat rotation (W+F). An asterisk indicates a significant difference between the WF treatment and the others according to the Newman–Keul's test ($P < 0.05$).

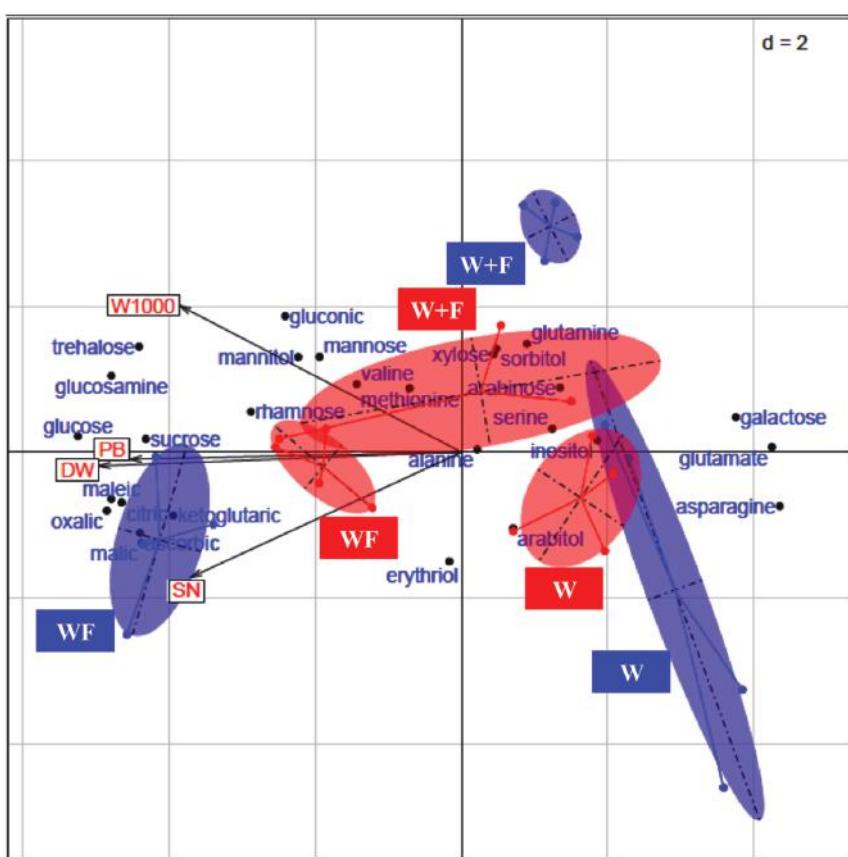


FIGURE 3 | Between-Group CO-Inertia Analysis (BGCOIA) on patterns of ISCP profiles (2012) and yield variables (2013). The three red ellipses represent the samples of the yield variables table in 2013 for the three treatments (W = wheat monoculture, W+F = wheat/Faba bean rotation, WF = wheat/Faba bean intercropping). The four yield variables (in red) are represented by the four arrows pointing to the left (W1000, weight of 1000 grains, PB, plant biomass, DW, dry weight of spikes, SN, spike number). The three blue ellipses represent the samples of the ISCP profiles table in 2012 for the same three cultivation modes. The names of the organic substrates are given in blue.

The importance of above-belowground interactions in both natural and agricultural systems has been highlighted during the last decade (Wolters et al., 2000; Soler et al., 2012; Orrell and Bennett, 2013). Mixing plant species will create new habitats for associated species and more particularly through its impact on the soil microbiota composition (Bartelt-Ryser et al., 2005). In particular changes in plant cover composition alter the composition of Arbuscular Mycorrhizal (AM) fungal communities (Lovelock et al., 2003). AM fungi facilitate plant uptake and transport of less mobile soil nutrients (Jakobsen et al., 2001), enhance drought tolerance (Kaya et al., 2003) and reduce pathogenic infections (Abdallah and Abdel-Fattah, 2000). These fungal symbionts are also involved in the biological mechanisms that influence plant community productivity and plant-plant interactions (Wagg et al., 2011). It has been also reported that an AM fungal diversity increase could relax competition in species network (Bastolla et al., 2009). Although AM fungi have been traditionally believed to be non-host specific in their ability to infect and to promote the host plant growth, the benefits expected from the mycorrhizal symbiosis to enhance the plant host development may highly depend on the particular

species involved (Bever et al., 2001; Jansa et al., 2005). For instance, it is well known that plant species, highly dependent to the mycorrhizal symbiosis for their growth (i.e., legumes) will promote the development of the mycorrhizal fungal growth, spore production, and hyphal network extend (Duponnois et al., 2001). Our results corroborate these previous studies as higher plant development; better mineral nutrition and higher mycorrhizal development were recorded in the intercropping treatment after one growing season.

Interactions of AM fungi with soil microbiota are an important driver of plant growth (Linderman, 1988; Johansson et al., 2004; Artursson et al., 2006). AM symbiosis is known to promote root exudation (Rambelli, 1973), and to influence rhizosphere microbial communities (Johansson et al., 2004). All these interactions constitute a plant root-mycorrhiza-bacteria continuum named “mycorrhizosphere.” In the current study, the soil compartment can be assimilated to the mycorrhizosphere. After one growing season, soil microbial functionalities were highly impacted by the plant cover composition (monoculture vs. intercropping) highlighting the importance of these aboveground-belowground interactions.

The main differences were observed between the treatments with SIR response to carboxylic acids recorded with the intercropping treatment. AM fungi and their associated bacteria may excrete carboxylic acids as well as faba bean (Zhou et al., 2009; Hafidi et al., 2013). These organic compounds could also exert a selective influence on soil microbial communities by enhancing the multiplication of microorganisms able to catabolise organic acids (Ouahmane et al., 2007; Hafidi et al., 2013).

In the current study, a strong relationship between 2012 SIR profiles and 2013 yield variables was recorded. This result showed that intercropping strongly impacted soil microbial functionalities resulting in a positive effect of this cultural practice recorded again in the second cropping year.

CONCLUSION

This field experiment demonstrates that the benefits of intercropping are highly subjected to the mycorrhizal symbiosis establishment and its impact on the soil microflora functionalities. In the present study, intercropping gave better results than the rotation practice in terms of crop productivity. However, this study has been only performed during two growing years and other studies have to be undertaken for more than two growing seasons to evaluate the long-term impact of intercropping compared to that resulting from rotations. These results emphasize the need to develop crop diversity

in agroecosystems and to include the management of AM fungal communities in agro-ecological strategies in order to sustainably maintain the crop productivity. Another point of major interest would be to check, in different situations and over several crop rotations, the predictive validity of the SIR approaches as a bioindicator of soil high productivity with wheat and other cereals. In other words, in the absence of any chemical inputs, are SIR values valid indicators of soil resilience, across several successive legume/crops associations? Limiting SIR substrates to carboxylic acids would be a way to simplify and reduce the cost of such a biotest, as to maintain its reliability.

AUTHOR CONTRIBUTIONS

SW, YP, JT, HS, EB, and RD: These authors contributed to defining the objectives of the experiment, the interpretation of results, and the redaction of the article. TM, KO, CL, AG, and MH: These authors contributed to the implementation of the experiences and proofreading of the article.

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Symbiotic Performance of Diverse *Frankia* Strains on Salt-Stressed *Casuarina glauca* and *Casuarina equisetifolia* Plants

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Symbiotic nitrogen-fixing associations between *Casuarina* trees and the actinobacteria *Frankia* are widely used in agroforestry in particular for salinized land reclamation. The aim of this study was to analyze the effects of salinity on the establishment of the actinorhizal symbiosis between *C. glauca* and two contrasting *Frankia* strains (salt sensitive; Ccl3 vs. salt tolerant; CeD) and the role of these isolates in the salt tolerance of *C. glauca* and *C. equisetifolia* plants. We show that the number of root nodules decreased with increasing salinity levels in both plants inoculated with Ccl3 and CeD. Nodule formation did not occur in seedlings inoculated with Ccl3 and CeD, at NaCl concentrations above 100 and 200 mM, respectively. Salinity also affected the early deformation of plant root hairs and reduced their number and size. In addition, expression of symbiotic marker *Cg12* gene, which codes for a subtilase, was reduced at 50 mM NaCl. These data suggest that the reduction of nodulation in *C. glauca* under salt stress is in part due to inhibition of early mechanisms of infection. We also show that prior inoculation of *C. glauca* and *C. equisetifolia* with *Frankia* strains Ccl3 and CeD significantly improved plant height, dry biomass, chlorophyll and proline contents at all levels of salinity tested, depending on the *Casuarina*-*Frankia* association. There was no correlation between *in vitro* salt tolerance of *Frankia* strains and efficiency *in planta* under salt-stressed conditions. Our results strongly indicate that increased N nutrition, photosynthesis potential and proline accumulation are important factors responsible for salt tolerance of nodulated *C. glauca* and *C. equisetifolia*.

Keywords: salinity, *Frankia*, *Casuarina glauca*, *Casuarina equisetifolia*, root hair deformation, *CgNIN*, *Cg12*, proline

INTRODUCTION

Soil salinization is a major problem worldwide. Indeed, high levels of salt in soil limit crop production and increase the loss of arable land. More than 800 million hectares of land worldwide are salt-affected (Munns and Tester, 2008). By the year 2050, 50% of all arable lands could be affected by salinity (Wang et al., 2003). There is therefore a need to design strategies to rehabilitate salinized areas.

Actinorhizal plants belonging to *Casuarinaceae* family such as *Casuarina glauca* and *C. equisetifolia* are able to grow under saline environments (El-Lakany and Luard, 1983; Girgis et al., 1992; Tani and Sasakawa, 2003). They are fast-growing trees, originated from Australia and Pacific islands, widely used in agroforestry systems for several purposes (Diem and Dommergues, 1990). In many tropical and subtropical countries, *Casuarina* species play a major role in land reclamation, crop protection and as windbreaks (National Research Council, 1984). In Senegal, a green barrier of *C. equisetifolia* was established on the northern Atlantic fringe between Dakar and Saint-Louis to stabilize sand dunes and protect the vegetable and fruit producing so-called “Niayes” area (Maheut and Dommergues, 1961; Mailly et al., 1994). *C. equisetifolia* is also appreciated for source of poles, firewood and charcoal (Diagne et al., 2013; Potgieter et al., 2014). Thus, this family of plants is of high importance for salinized land reclamation.

Casuarina species are pioneer plants, able to colonize poor and degraded lands and increase their fertility (Duhoux and Franche, 2003). Therefore, they promote development of pedogenetic processes leading to the formation of a more suitable microclimate for the installation of other plants species (Moiroud, 1996). This property is mainly due to the tremendous plasticity of their root system allowing them, among other things, to establish a nitrogen-fixing actinorhizal symbiosis with a filamentous soil bacterium called *Frankia*. Nitrogen is one of the main factors limiting plant growth and crop production worldwide, despite being the most abundant element in the atmosphere (80%). Unlike nitrogen-fixing plants, the majority of plant species are unable to directly utilize atmospheric nitrogen and rely on poor nitrogen sources in soils for their nutrition (Santi et al., 2013).

Among *Casuarina* species, *C. glauca* and *C. equisetifolia* display a high salt tolerance (El-Lakany and Luard, 1983). In addition, *C. glauca* is a model tree for basic and fundamental research in actinorhizal symbiosis with the development of many tools including genetic transformation of *C. glauca* and transcriptome analyses (Smouni et al., 2002; Gherbi et al., 2008; Tromas et al., 2012; Svistoonoff et al., 2013, 2014; Diédiou et al., 2014; Champion et al., 2015). They are therefore good models to study the mechanisms involved in tolerance to salt stress in actinorhizal trees. One important question is how salt stress impacts actinorhizal symbioses establishment. The early steps of the infection process leading to the development of root nodules of *Casuarina* tree starts with the induction of root hair curling by *Frankia*, as early as 24 h after inoculation (Callaham et al., 1979; Perrine-Walker et al., 2011). *Frankia* hyphae proceed to penetrate

the host plant through a deformed root hair (Frache et al., 1998) and induce the expression of several plant genes involved in actinorhizal nodule formation and functioning. Among them are the *CgNIN*, encoding a transcriptional factor, expressed at pre-infection stages in root hairs competent for *Frankia* infection (Clavijo et al., 2015) and *Cg12*, encoding a subtilase, whose expression is linked to the infection of root hairs and cortical cells by *Frankia* (Laplaze et al., 2000; Svistoonoff et al., 2003).

Frankia is a genus of soil actinobacteria (Normand et al., 2014). These Gram+, aerobic, heterotrophic bacteria are able to fix nitrogen both under free-living conditions and inside symbiotic root nodule (Simonet et al., 1989). The first pure culture of a *Frankia* strain was isolated from *Comptonia peregrina* nodules (Callaham et al., 1978). Since then, several *Frankia* strains have been isolated from different actinorhizal species (Diem et al., 1982, 1983; Gomaa et al., 2008; Gtari et al., 2015). They are grouped into four major clusters (Normand et al., 1996). *Frankia* strains in cluster 1 form nodules either with members of *Betulaceae* and *Myricaceae* (cluster 1a) or *Casuarinaceae* (cluster 1c). Cluster 2 includes *Frankia* strains able to infect the *Coriariaceae*, *Datiscaceae*, *Rosaceae*, and *Ceanothus* of the *Rhamnaceae*. *Frankia* strains in cluster 3 form effective nodules with the *Myricaceae*, *Rhamnaceae*, *Elaeagnaceae*, and *Gymnostoma* belonging to *Casuarinaceae*. Cluster 4 includes atypical *Frankia* strains, which are non-infective and/or non-nitrogen-fixing. *Casuarina* isolates show contrasting responses in their salt tolerance (Ngom et al., 2016). Indeed, some strains are more tolerant *in vitro* to salt stress than others even though they were isolated from the same host plant (Dawson and Gibson, 1987; Fauzia, 1999; Tani and Sasakawa, 2003; Oshone et al., 2013). Nevertheless, the symbiotic performance under salt stress of diverse *Frankia* strains toward *Casuarinaceae* species remains poorly understood.

In this study we aim to analyze (i) the effects of salinity on the establishment of symbiosis between *C. glauca* and two *Frankia* strains: CcI3 (a salt sensitive strain) vs. CeD (a salt tolerant strain) and (ii) the role of these isolates in salt tolerance in *C. glauca* and *C. equisetifolia*.

MATERIALS AND METHODS

Bacterial Material and Growth Conditions

Two contrasting *Frankia* strains were used in this study. CcI3 strain, whose isolation was reported by Zhang et al. (1984) is sensitive to salt stress while CeD, isolated by Diem et al. (1982) is salt tolerant in our cultivation conditions. Both *Frankia* isolates were grown in liquid BAP medium which contained (at final concentration) 1.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.195 mM FeNaEDTA, 5.6 mM KH_2PO_4 , 3.2 mM K_2HPO_4 , trace elements (H_3BO_4 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Na}_2\text{MOO}_4 \cdot 2\text{H}_2\text{O}$, and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) and vitamins (thiamine-HCL, pyridoxine-HCL, folic acid, Ca panthothenate, nicotinic acid, biotin, and riboflavin) at a final pH of 6.7 (Murry et al., 1984). Sodium propionate (5 mM) and NH_4Cl (5 mM) were used as carbon and nitrogen sources, respectively. For rapid hyphal growth, this nutrient BAP medium was modified and

supplemented with phosphatidyl choline (3.33 g/L) and MES-Tris buffer (0.5 M, pH 6.8; Schwencke, 1991). Cultures were maintained at $28 \pm 1^\circ\text{C}$, in darkness under stirring conditions.

Plant Material, Plant Transformation Growth Conditions

C. glauca seeds (seed lot 15,934, ref. 086-5929) were collected at the Myall Lakes National Park in Australia and provided by the Australian Tree Seed Centre (ATSC, CSIRO). *C. equisetifolia* seeds (seedlot SN/2011/0014/D) were collected in Louga area in Senegal and provided by the National Tree Seed Program (PRONASEF).

For experiments with non-transgenic plants, *C. glauca* and *C. equisetifolia* seeds were germinated under semi axenic conditions in a plastic tray (53.5 × 27.5 cm) containing a sterile mixture of compost (ref EN 12580) and sandy soil (v/v; 120°C, 60 min). They were watered daily with a quarter-strength Hoagland liquid medium (Hoagland and Arnon, 1950) to promote germination and initial growth of the seedlings.

Genetic transformation of *C. glauca* was performed using an *Agrobacterium tumefaciens* strain containing a ProCg12:GFP construct (Svistoonoff et al., 2003). Six independent *C. glauca* transgenic lines were generated as described previously (Smouni et al., 2002). For each transgenic line, GFP expression was analyzed. All plants showed the expression pattern described in Svistoonoff et al. (2003). The ProCg12:GFP line showing the highest expression levels of GFP was clonally propagated as described (Svistoonoff et al., 2010). Similarly for ProCgNIN:GFP, we used the transgenic line previously described (Clavijo et al., 2015) which showed the highest GFP expression.

Effect of Salinity on Nodulation of *C. glauca* Plants

One month after seed germination, *C. glauca* seedlings were uprooted from the soil, gently washed 5 times with distilled water. Seedlings were individually transferred in hydroponic conditions, into Gibson glass tubes filled with a 50 mL liquid BD medium supplemented with KNO_3 (5 mM) as nitrogen source, at pH 6.7 (Broughton and Dilworth, 1971). They were incubated in a growth chamber at $28 \pm 1^\circ\text{C}$ with 16 h day/8 h night photoperiod and a $74 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The BD medium was renewed every 2 weeks to avoid nutrient depletion and pH drift. After 1 month, salt stress was applied gradually through the weekly increment of one concentration of NaCl at 0, 50, 100, 200, 300, 400, and 500 mM. When 500 mM NaCl was reached, the plants were placed in nitrogen free-BD medium before being inoculated separately either with CcI3 or CeD *Frankia* strains.

C. glauca nodulation was performed as described previously (Ngom et al., 2015). Before inoculation, homogenized cells of CcI3 and CeD were suspended in sterile water with a final absorbance of 0.2, measured at $\lambda = 595 \text{ nm}$ for each strain. To establish actinorhizal symbiosis, inoculum of each strain was first brought into contact with the root system for 2 h. Plants were replaced back into Gibson tubes replenished with a 45 mL of nitrogen free-BD medium +5 mL of each bacterial suspension.

Nodulation rate or the percentage of nodulated plants (total number of nodulated plants/total number of inoculated plants × 100) and the mean nodule number (average number of nodules per plant) were followed for about 2 months after inoculation. All experiments were repeated twice and 22 plants were used for each salt treatment per experiment.

Effect of Salinity on *C. glauca* Root Hair Deformation

C. glauca seedlings were placed in hydroponic culture. Salt stress was applied gradually and plants were inoculated separately with either *Frankia* strain CcI3 or *Frankia* strain CeD, as described above. Two days after inoculation, root hair deformation was evaluated through micrographs of small lateral roots acquired with a Micro Publisher 3.3 RTV digital camera (QImaging) and a BX50F microscope (Olympus). For each treatment, five plants were used and three lateral roots were analyzed per plant. A total of 180 lateral roots and 12,217 root hairs were observed. Root hair deformation intensity was evaluated as described in Clavijo et al. (2015). For each micrograph, root hairs were observed and the following scoring was used: 0, no deformation; 1, straight root hair with tip swelling; 2, only one change in growth direction; 3, more than one change in growth direction but no bifurcation; 4, one or more bifurcations. At least two independent experiments were performed.

Analysis of CgNIN and Cg12 Activation under Salinity

Transgenic lines expressing ProCgNIN:GFP or ProCg12:GFP fusions were propagated and grown hydroponically in BD medium as described previously (Svistoonoff et al., 2010). Two NaCl concentrations (0 and 50 mM) were applied for 7 days. Plants were inoculated either with *Frankia* strain CcI3 or CeD, as described above. For each transgenic line, four plants per treatment were used. Activation of ProCgNIN:GFP was monitored 24, 48, and 72 h after inoculation. Activation of ProCg12:GFP was observed 3, 7, and 14 days after inoculation and nodule sections were examined for GFP fluorescence. GFP expression was observed using an AZ100 epifluorescence microscope (Nikon) and a GFP filter.

Effects of Prior Inoculation with *Frankia* on the Salt Tolerance of *C. glauca*

C. glauca seedlings were cultivated in hydroponic conditions and were nodulated with *Frankia* strains CcI3 or CeD, as described above. A batch of 22 uninoculated plants was used as controls. After inoculation, nodule formation was monitored weekly. Twenty-five days after inoculation, all of the plants were nodulated and treatment with NaCl was initiated. Salt stress (0, 50, 100, 200, 300, 400, and 500 mM NaCl) was applied gradually, as described above, to avoid osmotic shock. Morphological and physiological parameters of growth such as length of aerial parts, shoot and root dry weight, chlorophyll, and proline contents were evaluated as described below. Independent experiments were performed twice with 22 plants each treatment per experiment.

Effects of Prior Inoculation with *Frankia* on the Salt Tolerance of *C. equisetifolia*

One month after seed germination, *C. equisetifolia* seedlings were transplanted into plastic bags containing sterile sandy soil (120°C, 1 h). The experiments were conducted in a nethouse (Bel-Air experimental station, 14°44'N–17°30'W, Dakar, Senegal). Seedlings were watered daily and inoculation was applied 1 month after transplantation. Suspension of crushed nodule was used as inoculum. Nodules (20 g) were collected from *C. glauca* plants grown in hydroponic conditions and inoculated separately with *Frankia* strains CcI3 or CeD. Nodules were surface-sterilized with 5% sodium hypochlorite for 20 min then rinsed 3 times in sterile distilled water as described by Ng (1987). Grounded nodules were resuspended in 500 mL sterile distilled water. A 5 mL suspension was added into each bag according to the *Frankia* strain except for uninoculated plants. A batch of 8 plants was used for each treatment. As for *C. glauca*, the establishment of the symbiosis was monitored before gradually applying salt stress (0, 50, 100, 200, 300, 400, and 500 mM NaCl), as described above. Morphological and physiological parameters of growth such as length of aerial parts, shoot and root dry weight, chlorophyll, and proline contents were evaluated as described below.

Growth of Aerial Part and Dry Weight Determination

Length of aerial parts were measured every 2 weeks. Four months after inoculation, plants were harvested. Shoot and root systems were collected, washed in deionized water, surface-wiped with blotting paper, and dried at 70°C for 72 h. The dried biomasses of each samples (*C. glauca* $n = 22$, *C. equisetifolia* $n = 8$ per sample) were weighed separately.

Measurement of Chlorophyll Content

Chlorophyll content was determined using Arnon's method (1949). Fresh leaves (100 mg) were crushed in 10 mL of acetone at 80%. Samples were incubated overnight at 4°C and centrifuged at 6000 g for 10 min. The absorbance of chlorophyll (a) and (b) was measured using a UV-1800 spectrophotometer (UVisco) at $\lambda = 663$ and 645 nm, respectively. Total chlorophyll content (*C. glauca* $n = 5$, *C. equisetifolia* $n = 4$ per sample) was calculated according to Arnon (1949).

Extraction and Measurement of Proline Content

Fresh leaves (100 mg) were crushed in 2 mL of methanol at 40%, and the samples were immersed in a water bath at 85°C for 1 h. After cooling, 1 mL of leaf extract was mixed with 1 mL of ninhydrin at 2.5% and 1 mL of the reaction mixture (48 mL distilled water, 32 mL acetic acid, and 120 mL orthophosphoric acid). A second incubation was done in a water bath at 100°C for 30 min. Samples were cooled on ice, then a 5 mL toluene was added to the mixture. The upper phase was collected after vortexing and dehydrated with anhydrous sodium sulfate. Absorbance of leave samples was measured using a spectrophotometer at $\lambda = 520$ nm, as described by

Monneveux and Nemmar (1986). Proline contents (*C. glauca* $n = 5$, *C. equisetifolia* $n = 4$ per sample) were calculated and determined through a calibration straight graph constructed from a standard range of proline concentrations (Monneveux and Nemmar, 1986).

Acetylene Reduction Assay (ARA)

Nitrogen fixation was measured using the acetylene reduction assay described by Hardy et al. (1973). *C. glauca* plants were placed in tightly closed 150 mL jars. In each jar, 10% of the air (15 mL) was removed and replaced with acetylene. Plants were incubated at 28°C for 3 h. From each jar, 1 mL was withdrawn and assayed for ethylene using a gas chromatograph (Agilent 6850, GC System). Nodules were removed from plant roots and dried at 70°C for 72 h. Nitrogenase activity was calculated per nodule dry weight and expressed as nmols ethylene/nodule (g).

Statistical Analysis

Statistical analyses were performed on dry weight, chlorophyll and proline data. Statistical tests were performed using the XLSTAT 7.2 software. The Student-Newman-Keuls test at $p < 0.05$ was used to evaluate the differences between inoculated and uninoculated plants and between NaCl treatments.

RESULTS

Effects of Salt and Osmotic Stresses on the Growth of *Frankia* Strains CcI3 and CeD

First we analyzed the growth of 8 *Frankia* strains isolated from several *Casuarina* species under saline conditions. All of the strains showed a reduced growth in response to salt treatment (data not shown). Two *Frankia* strains (CcI3 and CeD) were selected on the basis of their different sensitivity to salt and osmotic stresses. As shown in Figure 1, the growth of both *Frankia* isolates was reduced by increasing the NaCl and PEG concentrations in the medium. At 100 and 200 mM NaCl or PEG, the growth of CeD was significantly less impacted than the growth of CcI3 (Figures 1A,B). At high concentrations of NaCl and PEG (300, 400, and 500 mM), no or reduced growth was observed for both strains, with a more pronounced effect in presence of PEG 4000 in the medium. Altogether, our data indicated that CeD is more tolerant to salt and osmotic stresses than CcI3.

Salinity Inhibits *C. glauca* Plant Nodulation

The impact of different NaCl concentrations on the nodulation of *C. glauca* plants by *Frankia* strains CcI3 and CeD was studied (Table 1). In both plants inoculated with *Frankia* CcI3 and CeD, the control plants (0 mM NaCl) had higher mean nodule number and rate of nodulation than NaCl-treated plants. The number of nodules formed increased over time at different rates with more nodules on seedlings inoculated with *Frankia* strain CeD for 63 days. At 50 mM NaCl, the mean nodule number declined by 66.6 and 60.3% in plants inoculated with CcI3 and CeD, respectively, compared to control plants. Nodule formation did not occur in seedlings inoculated with strain CcI3 at NaCl concentrations above 100 mM, whereas some plants inoculated with strain

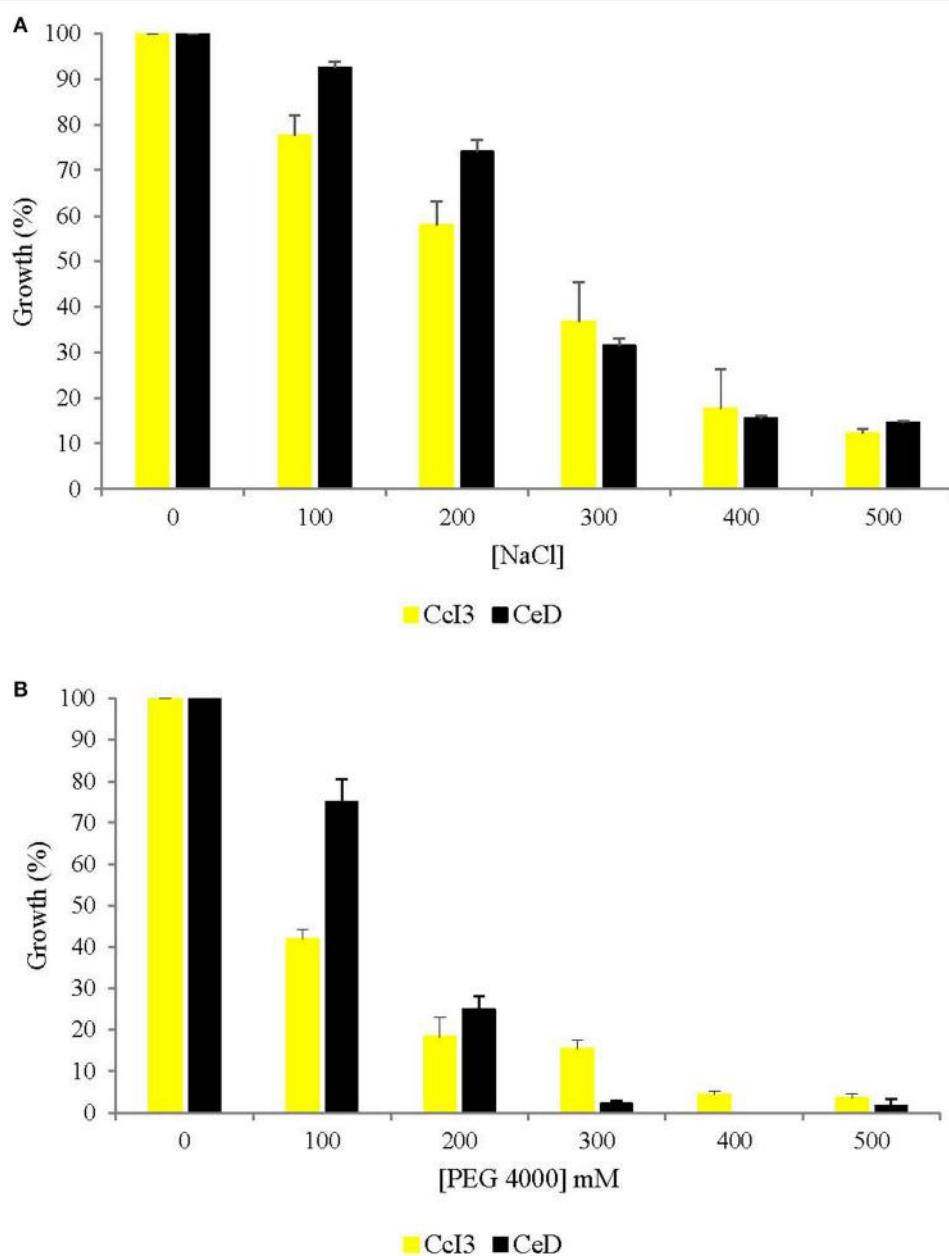


FIGURE 1 | Effect of salt and osmotic stresses on the growth of *Frankia* strains CcI3 and CeD. Cultures were grown under several concentrations of NaCl (A) and Polyethylene Glycol 4000 (B) for 7 days. Growth of each *Frankia* strain was estimated by measuring the turbidity at $\lambda = 595$ nm. The growth of *Frankia* in absence of salt and osmotic stresses (100% growth) was compared with those in the presence of NaCl or PEG 4000. Vertical bars indicate the standard error of mean (2 biological and 8 technical replicates). The absence of error bars indicates that the size of the error does not exceed the size of the symbol.

CeD were still forming nodules at 200 mM NaCl after 49 days of inoculation. Mean nodule number and nodulation rate of seedlings were reduced by increasing the salinity level.

Salinity Severely Affects Root Hair Deformation Response to *Frankia* Inoculation

Because salinity inhibited nodulation, the effects of salt stress during the early stages of the actinorhizal symbiosis

establishment was investigated. Root hair deformation responses in *C. glauca* plants treated with several concentrations of NaCl and inoculated with *Frankia* strains CcI3 and CeD was first analyzed. Regardless of the presence of *Frankia*, salt treatment reduced the number and size of root hairs in both uninoculated and inoculated *C. glauca* plants (Figure 2A). In *C. glauca* plants inoculated with *Frankia* strains CcI3 and CeD, extensive root hair deformation was detected 2 days after inoculation, in small lateral roots of no salt-treated plants. Increased salinity reduced the

TABLE 1 | Effects of several concentrations of NaCl on the nodulation of *C. glauca* plants inoculated with *Frankia* strains Cc13 and CeD.

<i>Frankia</i> strains	NaCl treatments (mM)	Nodulation kinetic (Days after inoculation)							
		14	21	28	35	42	49	56	63
Cc13	0	Nodulation rate (%)	72.7	95.5	100	100	100	100	100
		Mean nodule number	4.4 ± 1.1	9.9 ± 1.7	27.9 ± 3.4	36.1 ± 4.2	40.4 ± 4.4	42.2 ± 4.1	44.1 ± 4.2
	50	Nodulation rate (%)	36.4	59.1	86.4	86.4	90.9	90.9	90.9
		Mean nodule number	0.8 ± 0.3	3 ± 0.8	7.4 ± 1.4	9.6 ± 1.9	10.2 ± 2	11.7 ± 1.9	13.4 ± 2.2
	100	Nodulation rate (%)	4.5	18.2	40.9	40.9	40.9	40.9	40.9
		Mean nodule number	0.05 ± 0.05	0.4 ± 0.2	24 ± 0.9	3.6 ± 1.3	3.7 ± 1.4	4.2 ± 1.6	4.5 ± 1.7
>200	0	Nodulation rate (%)	0	0	0	0	0	0	0
		Mean nodule number	0	0	0	0	0	0	0
	50	Nodulation rate (%)	50	90.9	100	100	100	100	100
		Mean nodule number	2.3 ± 0.7	11 ± 2	28 ± 3.3	37.1 ± 3.3	38.8 ± 3.3	44.8 ± 3.4	48.8 ± 3.5
	100	Nodulation rate (%)	18.2	50	68.2	68.2	81.8	81.8	81.8
		Mean nodule number	0.5 ± 0.3	2.7 ± 0.8	7.1 ± 1.5	12.2 ± 2.5	13.5 ± 2.6	16.7 ± 2.8	18 ± 3
CeD	0	Nodulation rate (%)	9.1	22.7	40.9	40.9	59.1	63.6	68.2
		Mean nodule number	0.05 ± 0.05	0.1 ± 0.1	3 ± 1.2	4.6 ± 1.6	4.7 ± 1.6	7.9 ± 2.3	9.1 ± 2.4
	50	Nodulation rate (%)	4.5	0	0	0	0	9.1	9.1
		Mean nodule number	0	0	0	0	0	0.5 ± 0.3	0.6 ± 0.4
	100	Nodulation rate (%)	0	0	0	0	0	0	0
		Mean nodule number	0	0	0	0	0	0	0
200	0	Nodulation rate (%)	0	0	0	0	0	0	0
		Mean nodule number	0	0	0	0	0	0	0
	200	Nodulation rate (%)	0	0	0	0	0	0	0
		Mean nodule number	0	0	0	0	0	0	0
	>300	Nodulation rate (%)	0	0	0	0	0	0	0
		Mean nodule number	0	0	0	0	0	0	0

Salt stress was first gradually applied then plants were inoculated separately with each *Frankia* strain. Nodulation rate is the percentage of plant nodulated and mean nodule number is the average number of nodule per plant. Values represent the mean ± standard deviation of plants used in each treatment ($n = 22$).

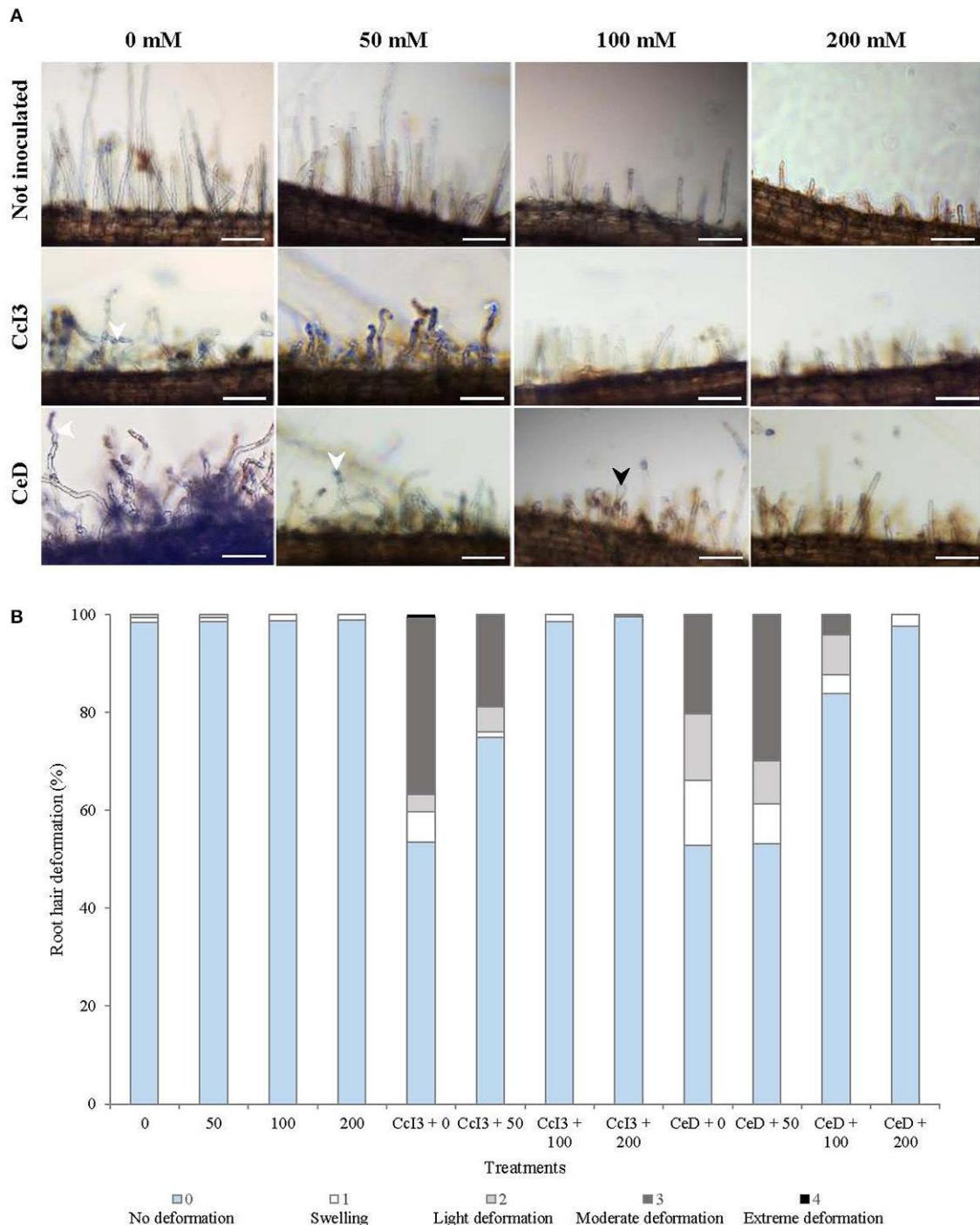


FIGURE 2 | Effect of various levels of NaCl on *C. glauca* root hair deformation. (A) Root hairs under salt-stressed conditions from uninoculated and inoculated *C. glauca* plants, observed 2 days after inoculation. White arrows indicate moderate deformation and black arrows swelling root hairs (Bars, 100 µm). **(B)** Quantification of root hair deformation showing the proportion of deformed root hairs in short lateral roots 2 days after inoculation with *Frankia* strains CcI3 and CeD. For each treatment, 5 plants were used and 3 lateral roots were observed per plant.

amount of deformation in seedlings inoculated by both strains with a more pronounced effect for plants inoculated with CcI3 (Figure 2B). Deformation was particularly low at 200 mM NaCl,

which also showed previously the smallest number of nodules in plants inoculated with CeD and no nodule development in plants inoculated with CcI3. No or few deformation were

TABLE 2 | Effect of salt stress on ProCgNIN and ProCg12 genes activation.

(A)					
Transgenic line	Frankia strains	NaCl treatments (mM)	Hours after inoculation		
			24	48	72
ProCgNIN: GFP	CcI3	0	+	+	+
		50	+	+	+
	CeD	0	+	+	+
		50	+	+	+
(B)			Days after inoculation		
Transgenic line	Frankia strains	NaCl treatments (mM)	3	7	14
ProCg12: GFP	CcI3	0	—	—	+++
		50	—	—	+
	CeD	0	—	—	+++
		50	—	—	+

Activation of ProCgNIN:GFP (A) and ProCg12:GFP (B) genes in presence of 0 and 50 mM of NaCl. Reporter gene expression (GFP) was detected using an epifluorescence microscopy. More sign + indicate more fluorescent spots observed per plant in transgenic lines.

+, gene activation; —, inactivation of gene.

observed on uninoculated plants treated with various levels of NaCl.

Effects of Salinity on CgNIN and Cg12 Expression

To further investigate the effects of salinity during the early stages of the establishment of symbiosis, we studied the impact of salt stress on the expression of two early symbiotic marker genes: CgNIN (Clavijo et al., 2015) and Cg12 (Svistoonoff et al., 2003) using transgenic plants of *C. glauca* expressing ProCgNIN:GFP and ProCg12:GFP. CgNIN gene is a pre-infection marker which is early expressed in root hairs competent for *Frankia* infection (Clavijo et al., 2015) and Cg12, an infection marker associated with root hairs and cortical cells infection by *Frankia* (Svistoonoff et al., 2003).

Observations revealed that ProCgNIN:GFP was activated in both control and 50 mM NaCl treated plants, from 24 to 72 h after inoculation with *Frankia* strains CcI3 and CeD (Table 2A), suggesting that CgNIN expression was not repressed by salt treatment (50 mM NaCl). Expression of ProCg12:GFP was observed 14 days after inoculation in both control and 50 mM NaCl treated plants. A lower number of fluorescent spots per plant were detected in NaCl treated plants (Table 2B), pointing to a possible inhibition of infection and ProCg12 expression by salinity. We were not able to detect any differences regarding the pattern or the intensity of ProCg12 activation in prenodules or nodules when comparing control and NaCl-treated plants, as shown in Figure 3.

Nodulated *C. glauca* and *C. equisetifolia* Plants are More Tolerant to Salt Stress

In addition to *C. glauca*, *C. equisetifolia* was studied because it is the most introduced Casuarina species worldwide for land reclamation and reforestation programs including Senegal

(LADA, 2003; National Research Council, 1984). Furthermore, *C. equisetifolia* is also highly tolerant to salt stress (El-Lakany and Luard, 1983). The effects of prior inoculation with *Frankia* strains CcI3 and CeD on the salt tolerance of *C. glauca* and *C. equisetifolia* was studied to see if these nitrogen-fixing bacteria could be used to increase the salt tolerance of *Casuarina* species. Both *Frankia* strains CcI3 and CeD improved the growth of *C. glauca* at all concentrations of NaCl tested compared to the control plants (Figure 4A). In control plants, growth decreased with increased NaCl concentrations. There was no growth above 50 mM NaCl, 12 weeks after inoculation. Positive effect of inoculation with *Frankia* CcI3 and CeD on plant height started to be observed 4 weeks after inoculation. Plant height increased progressively at all NaCl concentrations in inoculated plants compared to the control, but growth gradually decreased with increasing NaCl concentrations. For instance, an increase by 65.5 and 44.5% was observed in 500 mM NaCl treated plants inoculated with *Frankia* strains CcI3 and CeD, respectively compared to controls. In contrast, in *C. equisetifolia* plants only *Frankia* strain CeD increased plant growth at all NaCl concentrations as compared to the control (Figure 4B).

Inoculation with *Frankia* strains CcI3 and CeD improved *C. glauca* shoot and total biomass significantly in all NaCl treatments compared to control (Table 3A). Compared to control, *Frankia* strains CcI3 and CeD significantly increased root biomass in all NaCl treatments except for 0, 50, and 100 mM salt-treated plants inoculated with CeD. In both control and inoculated plants, root, shoot, and total biomass decreased with increasing salt concentration, but the change was not significant in plants inoculated with strain CeD and in root dry biomass of control plants. On the other hand, only CeD increased significantly shoot and total dry biomass of *C. equisetifolia* plants for some NaCl treatments (0–200 mM) compared to the control and the plants inoculated with CcI3 (Table 3B). As observed for *C. glauca* plants, root, shoot, and total dry biomass decreased

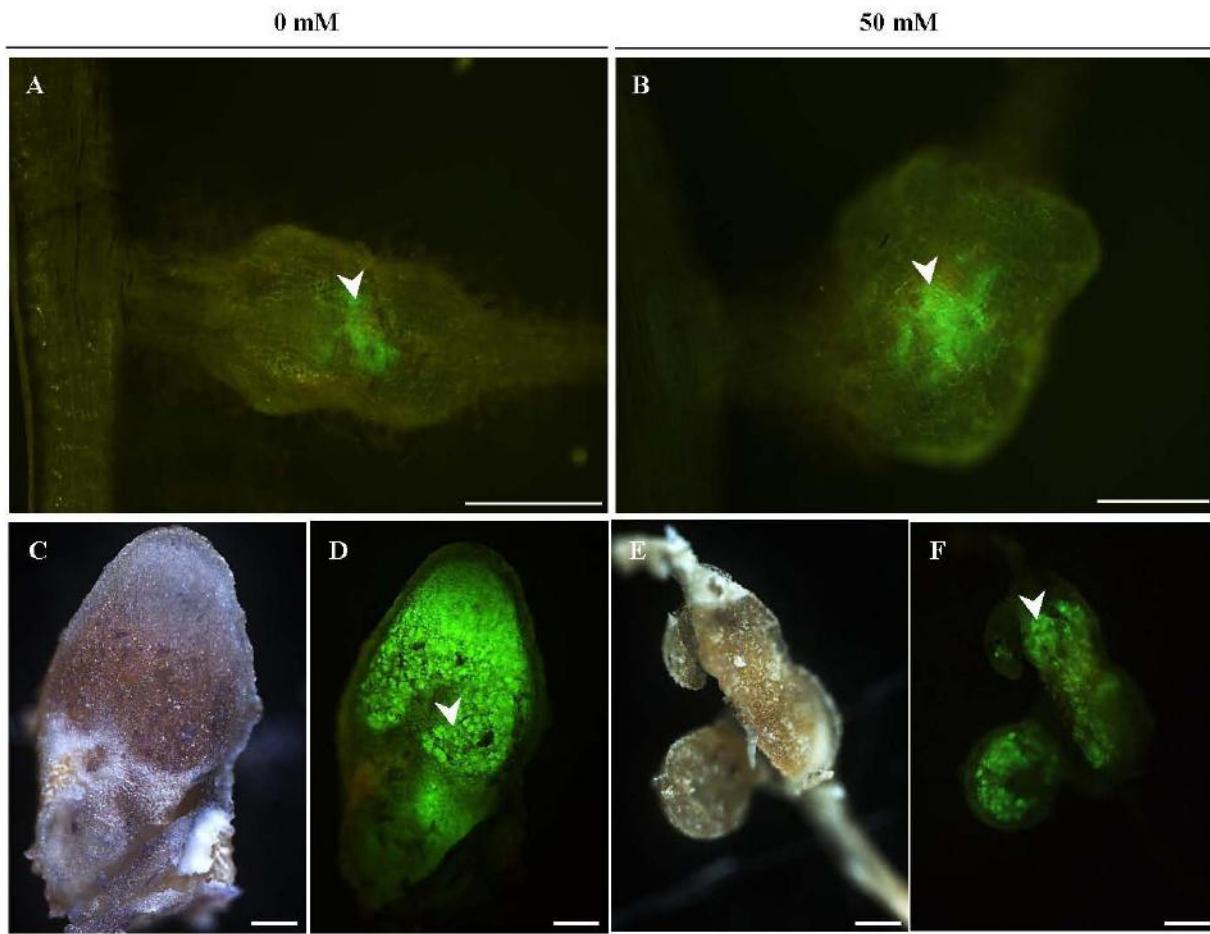


FIGURE 3 | *ProCg12* is active in saline condition during infection by *Frankia*. Salt stress (50 mM NaCl) was first applied then *C. glauca* plants were inoculated separately with *Frankia* strains CcI3 (**A–D**) and CeD (**E,F**). *ProCg12* is activated in prenodules (**A,B**) and nodules (**C–F**) of control and NaCl treated *C. glauca* plants, 14 days after inoculation. (**C–F**) Sections of matures nodules expressing green fluorescent protein (GFP). White arrows indicate reporter gene expression. (**C,E**) Bright field microscopy. (**A,B,D,F**) Epifluorescence microscopy. Bars 100 μ m.

with increasing salt concentration in both control and inoculated plants. The change was significant between NaCl treatments in general and between low (0 and 50 mM NaCl) and high salinity (300 and 500 mM NaCl) in particular.

Chlorophyll and proline contents were determined in order to appreciate physiological state of non-nodulated and nodulated plants in saline conditions. The chlorophyll content (a, b, and total) was significantly increased in *C. glauca* plants inoculated with *Frankia* strains CcI3 and CeD, as compared to control (**Figures 5A–C**). However, there was no significant difference between NaCl treatments in *C. glauca* plants. In *C. equisetifolia* plants, the chlorophyll a was significantly increased by strain CeD only in no salt-treated plants (**Figures 5D–F**). No significant difference was observed between NaCl treatments. However, *Frankia* strain CeD increased the total chlorophyll content in 0, 50, 100, and 200 mM NaCl treated plants, compared to control and plants inoculated with CcI3.

As what was observed in chlorophyll content, there were significant changes in proline content between control and

inoculated plants (**Figure 6**). *Frankia* strains CcI3 and CeD increased the proline content of *C. glauca* at all concentrations of salt tested (**Figure 6A**). Proline content increased with increasing salt concentrations in the control and inoculated plants, but the change was not significant in *C. glauca* plants inoculated with strain CeD. Similarly, in *C. equisetifolia* plants, proline content increased with increasing salinity (**Figure 6B**). There were significant differences between NaCl treatments in both control and plants inoculated with strains CcI3 and CeD. Only *Frankia* strain CeD increased proline contents significantly in 0, 50, and 300 mM NaCl treated plants.

DISCUSSION

Among Casuarina tree species, *C. glauca* and *C. equisetifolia* have been shown to be highly salt-tolerant (Hyland, 1983; Luard and El-Lakany, 1984; Aswathappa and Bachelard, 1986; Van der Moezel et al., 1989) and are widely planted outside of

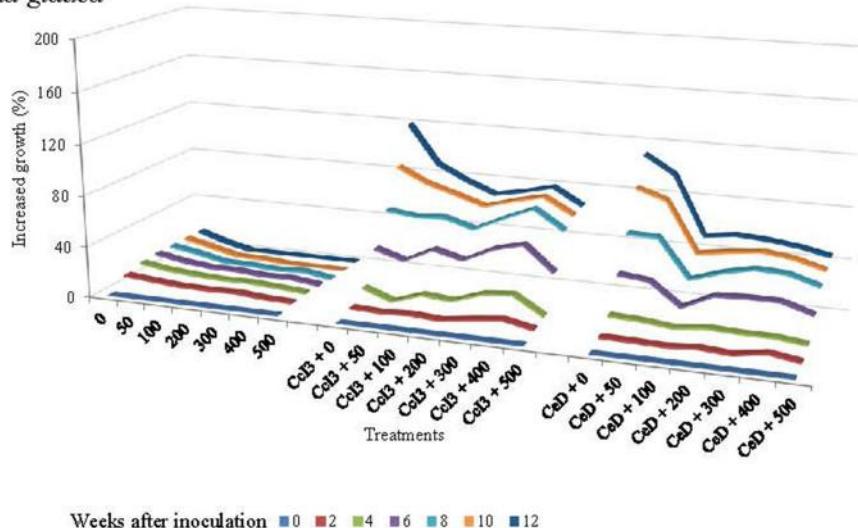
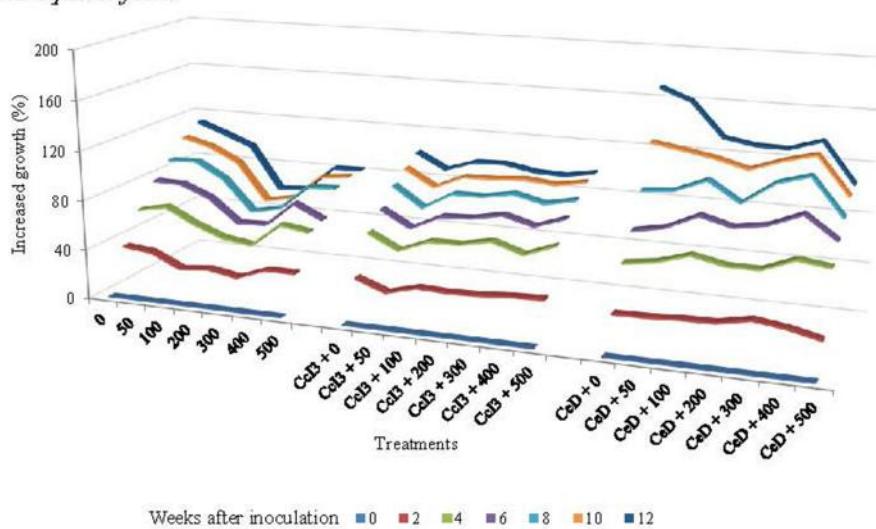
A*Casuarina glauca***B***Casuarina equisetifolia*

FIGURE 4 | Shoot growth of non-nodulated and nodulated *C. glauca* (A) and *C. equisetifolia* (B) plants treated with various salt concentrations. Plants were inoculated separately with *Frankia* strains CcI3 and CeD. Salt stress was applied gradually after the establishment of symbiosis. For each treatment, height growth was measured every 2 weeks from the day of inoculation and the increased growth was calculated from this time (0% of growth). Each value represents the mean of plants used in each treatment (*C. glauca* $n = 22$, *C. equisetifolia* $n = 8$).

their native habitat (National Research Council, 1984). However, salinity could affect plant growth and the establishment of actinorhizal symbiosis which could be thus a limit to salinized land reclamation (Reddell et al., 1986). In this study, we first investigated the effect of salinity on the symbiotic relationship between *C. glauca* and two contrasting *Frankia* strains CcI3 (salt sensitive) and CeD (salt tolerant).

Our results indicate that nodule formation in *C. glauca* is inhibited by salt stress regardless of the salt tolerance of the *Frankia* strain. However, the most salt tolerant strain, CeD, is still able to infect *C. glauca* up to 200 mM NaCl while the salt-sensitive strain CcI3 is not. Nitrogen fixation was not measured in this study, although it has been reported a significant correlation between nodule number per plant in *C. glauca* and

TABLE 3 | Mean comparison of shoot and root dry weight of non-nodulated and nodulated *C. glauca* (A) and *C. equisetifolia* (B) plants treated with several salt concentrations.

(A) <i>C. glauca</i>		Dry weight (g)		
	NaCl treatments (mM)	Control	CcI3	CeD
Shoot	0	0.449 c A	1.322 a A	0.876 b A
	50	0.364 c AB	1.103 a AB	0.800 b A
	100	0.320 c B	1.111 a AB	0.694 b A
	200	0.347 c AB	0.924 a B	0.708 b A
	300	0.330 c B	0.920 a B	0.660 b A
	400	0.291 c B	0.921 a B	0.661 b A
	500	0.244 c B	0.836 a B	0.697 b A
Root	0	0.238 b A	0.422 a A	0.272 b A
	50	0.222 b A	0.359 a AB	0.264 b A
	100	0.204 b A	0.354 a AB	0.241 b A
	200	0.192 b A	0.284 a B	0.258 a A
	300	0.190 b A	0.291 a B	0.259 a A
	400	0.177 c A	0.309 a B	0.230 b A
	500	0.174 b A	0.256 a B	0.272 a A
Total biomass	0	0.687 c A	1.744 a A	1.148 b A
	50	0.586 c AB	1.462 a AB	1.064 b A
	100	0.524 c AB	1.465 a AB	0.935 b A
	200	0.539 c AB	1.208 a BC	0.966 b A
	300	0.520 c AB	1.211 a BC	0.919 b A
	400	0.468 c B	1.230 a BC	0.891 b A
	500	0.418 b B	1.092 a C	0.969 a A
(B) <i>C. equisetifolia</i>		Dry weight (g)		
Shoot	0	2.813 b A	3.220 b A	5.252 a A
	50	2.336 b AB	2.351 b B	3.432 a B
	100	2.159 b ABC	2.106 b BC	2.804 a BC
	200	1.828 b BC	1.882 b CD	2.518 a CD
	300	1.538 a BC	1.790 a CD	1.918 a D
	400	1.531 a BC	1.496 a D	1.838 a D
	500	1.349 a C	1.384 a D	1.696 a D
Root	0	1.136 a A	1.375 a A	1.249 a A
	50	0.730 b B	1.026 ab B	1.170 a A
	100	0.850 a B	0.778 a C	0.940 a B
	200	0.614 a B	0.689 a CD	0.788 a B
	300	0.553 a B	0.595 a CD	0.544 a C
	400	0.595 a B	0.544 a CD	0.503 a C
	500	0.575 a B	0.395 a D	0.464 a C
Total biomass	0	3.949 b A	4.595 b A	6.501 a A
	50	3.066 b B	3.377 b B	4.602 a B
	100	3.009 b BC	2.884 b C	3.744 a BC
	200	2.442 b BC	2.571 b CD	3.306 a CD
	300	2.091 a BC	2.385 a CD	2.462 a DE
	400	2.126 a BC	2.040 a DE	2.341 a DE
	500	1.924 a C	1.779 a E	2.160 a E

Each value represents the mean of plants used in each treatment (*C. glauca* n = 22; *C. equisetifolia* n = 8). For each salt concentration, different lowercase letters (a–c) indicate significant difference between control and plants inoculated separately with *Frankia* CcI3 and CeD. For each condition (control/plants inoculated with each strain), different capital letters (A–E) indicate significant difference between NaCl treatments according to the Student-Newman-Keuls (SNK) test at P < 0.05.

the acetylene reduction activity (ARA) under salt stress (Girgis et al., 1992). A decrease in nodulation under saline conditions has been previously reported for *C. equisetifolia* (Ng, 1987; Tani and Sasakawa, 2003) and *C. obesa* (Reddell et al., 1986) depending on the *Frankia* source, culture conditions and duration of the experiment. Nodulation did not occur in *C. equisetifolia* inoculated with Ceq1 strain and cultured in 500 mM NaCl for 6 weeks (Tani and Sasakawa, 2003), while nodules were formed in *C. equisetifolia* seedlings cultured for 24 weeks at 500 mM NaCl and inoculated with a nodule suspension (Ng, 1987). With *C. obesa*, increased salinity reduced nodule dry weight with both *Casuarina*–*Frankia* associations having a more pronounced effect with one of the inoculum source (Reddell et al., 1986).

The effects of salinity on *C. glauca* nodulation could be due to an inhibition of nodule initiation and/or infection processes. These processes leading to the development of root nodules of *Casuarina* involve various responses such as root hair deformation (Torrey, 1976) and early expression of several genes like *CgNIN* and *Cg12* (Laplaze et al., 2000; Svistoonoff et al., 2003; Clavijo et al., 2015). Extensive deformation of root hairs occurs in the zone of root hair elongation within the first 24 h after inoculation (Torrey, 1976). *Frankia* hyphae infect plants through the intracellular infection pathway in *Casuarina* trees (Callaham et al., 1979; Perrine-Walker et al., 2011). We observed that an increase in salt concentration reduced the percentage of root hairs deformed in *C. glauca* plants, 48 h after inoculation with both strains and with a more pronounced effect in plants inoculated with the salt-sensitive strain CcI3. Root hair deformation is dependent on the production of diffusible signals by *Frankia* (Cérémonie et al., 1999). The observed results might be due to the fact that the salt tolerant strain CeD is able to maintain growth and production of symbiotic factors at higher salt concentration than CcI3. This effect could explain in part the impact of salt on nodule formation. Indeed, there is a positive correlation between the extent of root hair deformation and the number of nodules which subsequently developed (Callaham et al., 1979). In addition, salt stress decreased the number and size of root hairs regardless of the presence of *Frankia*, which could reduce their availability and susceptibility. Therefore, *Frankia* colonization may decrease and the establishment of the symbiosis is thus impaired. A similar reduction in root hair deformation by salt stress was reported in legumes *Vicia faba* (Zahran and Sprent, 1986), *Glycine max* (Tu, 1981), and *Medicago sativa* (Lakshmi-Kumari et al., 1974) in response to rhizobial inoculation. The extent of the deformation depends on the association *Rhizobium*–Legume and was correlated to the number or dry weight of nodules. Morphological symptoms of damage by NaCl such as reduction in the number and size of root hairs was observed in *Medicago sativa* (Lakshmi-Kumari et al., 1974).

CgNIN is a transcription factor which plays a central role in the nodulation of actinorhizal hosts and is induced by diffusible symbiotic signals produced by *Frankia* (Clavijo et al., 2015; Chabaud et al., 2016). *Cg12* is a subtilisin gene isolated from *C. glauca* and its expression is associated with *Frankia* infection (Laplaze et al., 2000; Svistoonoff et al., 2003). In this study, we showed that *CgNIN* was activated in both control and 50 mM NaCl treated plants (Supplementary Figure 1), from 24 to 72 h after inoculation. This effect suggests that the production of

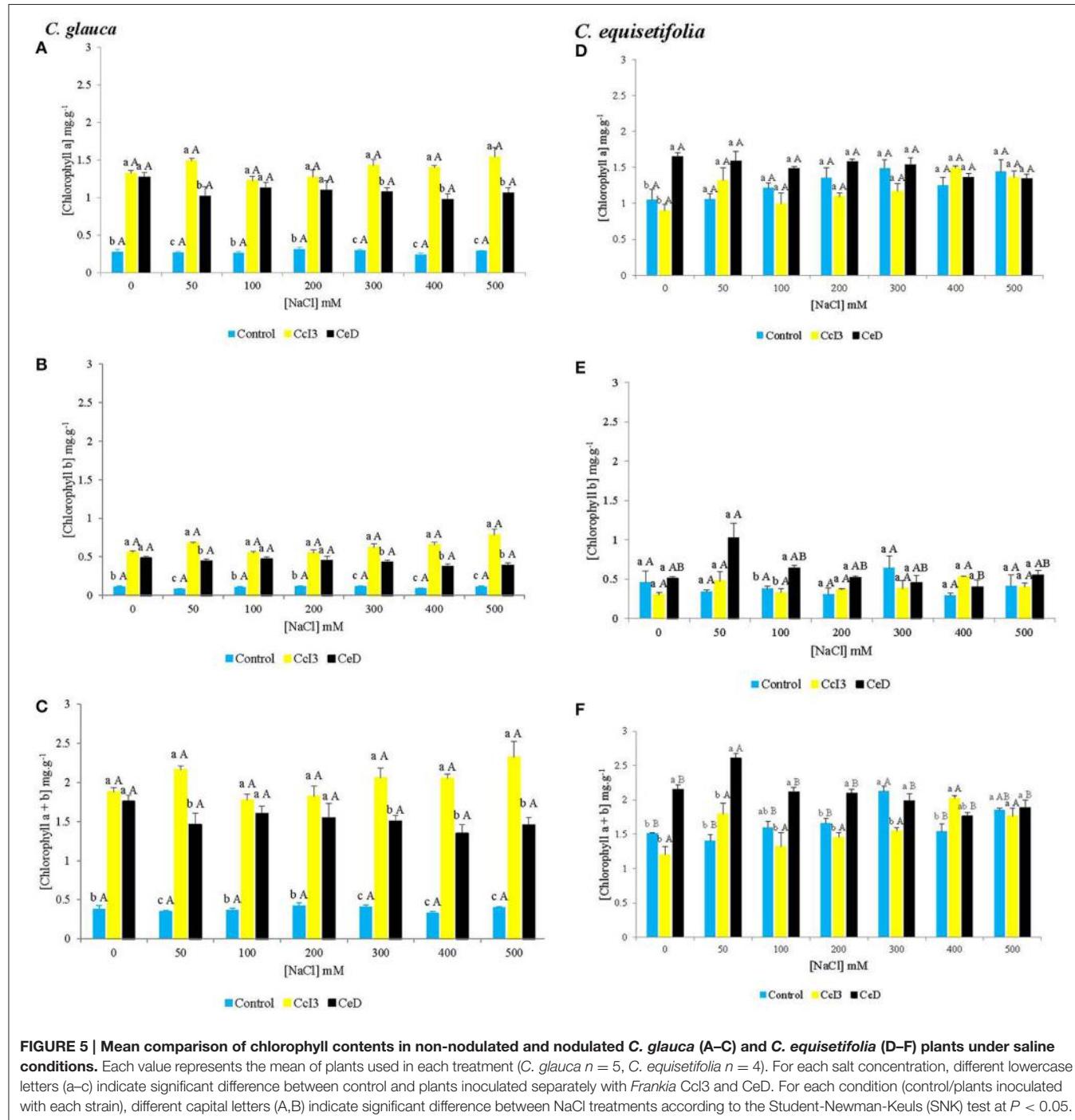


FIGURE 5 | Mean comparison of chlorophyll contents in non-nodulated and nodulated *C. glauca* (A–C) and *C. equisetifolia* (D–F) plants under saline conditions. Each value represents the mean of plants used in each treatment (*C. glauca* $n = 5$, *C. equisetifolia* $n = 4$). For each salt concentration, different lowercase letters (a–c) indicate significant difference between control and plants inoculated separately with *Frankia* CcI3 and CeD. For each condition (control/plants inoculated with each strain), different capital letters (A,B) indicate significant difference between NaCl treatments according to the Student-Newman-Keuls (SNK) test at $P < 0.05$.

symbiotic diffusible signals by *Frankia* or its perception is not perturbed by mild salt stress for both strains. On the other hand, expression of *Cg12* was observed 14 days after inoculation in both treatments with low number of fluorescent spots in NaCl treated plants. This effect suggests *Cg12* expression is negatively affected by salinity that is possibly related to a perturbation of plant cell infection. This result is in accordance with those of Duro et al. (2016) which showed that *Cg12* was down-regulated with increasing salt concentration. However, this study used higher

levels of NaCl (200, 400, and 600 mM) than what we used in this experiment (50 mM).

Altogether, our results indicate that salt stress alters actinorhizal symbiosis formation in *C. glauca*. This effect could be due at least in part to a negative impact of salt stress on the infection process that might be related to a reduction of potential infection sites (root hairs) or reduced perception of infection signals. Furthermore, the salt-tolerant strain CeD is able to infect at higher concentrations of salt than the salt sensitive strain

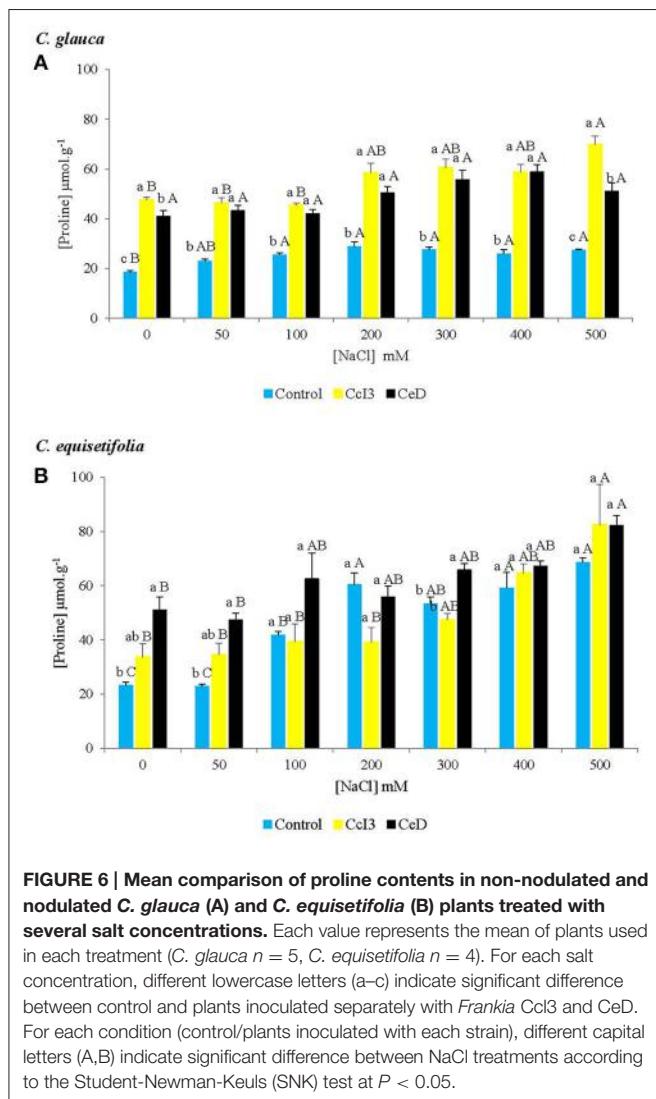


FIGURE 6 | Mean comparison of proline contents in non-nodulated and nodulated *C. glauca* (A) and *C. equisetifolia* (B) plants treated with several salt concentrations. Each value represents the mean of plants used in each treatment (*C. glauca* $n = 5$, *C. equisetifolia* $n = 4$). For each salt concentration, different lowercase letters (a–c) indicate significant difference between control and plants inoculated separately with *Frankia* CcI3 and CeD. For each condition (control/plants inoculated with each strain), different capital letters (A,B) indicate significant difference between NaCl treatments according to the Student-Newman-Keuls (SNK) test at $P < 0.05$.

CcI3. This result indicates that the use of appropriate strains is necessary for efficient nodulation of trees in salinized soils.

The impact of *Frankia* inoculation on salt tolerance in *C. glauca* and *C. equisetifolia* was tested. Our results indicate that inoculation of *C. glauca* and *C. equisetifolia* by *Frankia* strains CcI3 and CeD significantly improved plants growth under salt stress, depending on the specific *Casuarina-Frankia* association. For *C. glauca*, both *Frankia* strains significantly increased plant height, shoot, root and total dry weight at all concentrations of NaCl, as compared to uninoculated plants. This positive effect was more pronounced in plants inoculated with strain CcI3. In contrast, only *Frankia* strain CeD increased *C. equisetifolia* height at all NaCl treatments, and significantly elevated plant shoot, root, and total dry weight from 0 to 200 mM NaCl, as compared to control. These results suggest that the effectiveness of the symbiosis in saline conditions depends on the appropriate *Casuarina-Frankia* association. Indeed, according to Grgis et al. (1992), there is no correlation between *in vitro* salt tolerance of *Frankia* strains and their effectiveness in association with

plants under salt-stressed conditions. However, it is important to emphasize that the experiment with *C. glauca* was conducted in hydroponic conditions, whereas *C. equisetifolia* was grown in soil. The improvement of morphological parameters (height, shoot, root and total dry weight) may be due to the increased N nutrition and photosynthesis potential in *Casuarina* inoculated with *Frankia* compared to the uninoculated controls. This conclusion was supported by our results for chlorophyll (a, b, and a + b) content and nitrogenase activity under saline conditions. Under all NaCl concentrations, chlorophyll content was significantly increased in *C. glauca* plants inoculated with both strains, as compared to control. With *C. equisetifolia*, only CeD increased significantly total chlorophyll content from 0 to 200 mM NaCl. Salinity decreased nitrogenase activity in *C. glauca* (Supplementary Figure 2). However, N₂ fixation occurred even at the highest NaCl concentration (Supplementary Figure 2). This implies that increased N nutrition and potential photosynthesis allow inoculated *Casuarina* plants to grow better than uninoculated controls under saline conditions. These results are in agreement with a previous report showing that the actinorhizal tree *Alnus glutinosa* inoculated with *Frankia* and cultivated in alkaline and saline anthropogenic sediment, had better plant growth, leaf N and chlorophyll a + b content than the control (Oliveira et al., 2005). Several studies have shown that inoculation with selected microsymbionts like *Frankia* can enhance the development of actinorhizal plants and their resistance to other abiotic stresses such as heavy metals and extreme pH and temperature (Reviewed by Ngom et al., 2016). Symbiotic associations with arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria called rhizobia can also enhance plant salinity tolerance, leading to better plant growth and yield, nutrient acquisition and chlorophyll content in several species including *Medicago sativa* (Azcon and El-Atrash, 1997), *Acacia nilotica*, *Leucaena leucocephala*, *Prosopis juliflora* (Bala et al., 1990), *Phaseolus vulgaris* (Dardanelli et al., 2008), and soybean (Elsheikh and Wood, 1995), under saline conditions. The benefits of these microsymbionts in saline environments depend also on the symbiotic associations.

Compatibles solutes or osmolytes such as glycine betaine, mannitol, or proline are accumulated in organisms in response to salt and osmotic stresses (Delauney and Verma, 1993; Wang et al., 2003). They play important roles in maintaining cell turgor and thus the driving gradient for water (Wang et al., 2003). Compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins (Lee et al., 1997; Bohnert and Shen, 1998; McNeil et al., 1999; Diamant et al., 2001). Proline, an amino acid, is the most common osmolyte accumulated under salinity and drought stress in plants (Watanabe et al., 2000; Tani and Sasakawa, 2003). In our study, a significantly higher proline content was observed in all inoculated *C. glauca* plants at all NaCl concentrations, as compared to the control. Significant improvement of proline content was also observed in 0, 50, and 300 NaCl treated *C. equisetifolia* plants inoculated with strain CeD. In both control and inoculated plants, proline content increased with increasing salinity. These results suggest that, in addition to better N nutrition and potential photosynthesis, proline accumulation

adjusts the osmotic pressure and maintain cell homeostasis in inoculated *C. glauca* and *C. equisetifolia* plants, under saline conditions. These results are in agreement with those of Diouf et al. (2005) which showed that inoculation with both *Rhizobium* and AMF induced higher proline content in legumes such as *Acacia auriculiformis* and *Acacia mangium*, compared to uninoculated plants, at all levels of salinity tested (0, 50, and 100 mM NaCl). Proline accumulation under salt stress has been previously described in *C. equisetifolia* seedlings not infected by *Frankia* (Tani and Sasakawa, 2006).

In conclusion, our results strongly indicate that the beneficial effects of *Frankia* inoculation are due to improved N nutrition, photosynthesis potential and proline accumulation in inoculated plants under salt stress conditions. There was no correlation between *in vitro* salt tolerance of *Frankia* strains and efficiency *in planta* in salt stress conditions. Hence, the success of planting *Casuarina* in saline sites will require appropriate salt-tolerant *Casuarina-Frankia* associations that will form an efficient N₂-fixing symbiosis. *In vitro* salt tolerance of *Frankia* strains should be considered if they are introduced in saline soils, otherwise, the screening should be done with both symbiotic partners.

AUTHOR CONTRIBUTIONS

MN conducted some experiments, analyzed the data, interpreted the results, and prepared the manuscript. KG and JF

conducted some experiments and prepared the manuscript. ND, HG, VH, SS conducted some experiments, interpreted the results, and improved the manuscript. RO, LL, and LT interpreted the results and improved the manuscript. AC and MS designed and coordinated the experiments, analyzed the data, interpreted the results, and improved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01331>

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