Trends in Plant Science

Special Issue: Unravelling the Secrets of the Rhizosphere

Review

Metabolomics in the Rhizosphere: Tapping into Belowground Chemical Communication

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The rhizosphere is densely populated with a variety of organisms. Interactions between roots and rhizosphere community members are mostly achieved via chemical communication. Root exudates contain an array of primary and secondary plant metabolites that can attract, deter, or kill belowground insect herbivores, nematodes, and microbes, and inhibit competing plants. Metabolomics of root exudates can potentially help us to better understand this chemical dialogue. The main limitations are the proper sampling of the exudate, the sensitivity of the metabolomics platforms, and the multivariate data analysis to identify causal relations. Novel technologies may help to generate a spatially explicit metabolome of the root and its exudates at a scale that is relevant for the rhizosphere community.

Communication in the Rhizosphere

By nature, soil communities are extremely diverse. As a consequence, the rhizosphere is populated with myriad organisms, including nematodes, fungi, bacteria, and arthropod herbivores (Figure 1) [1]. Each of these organisms, alone and in combination, may interact with the plant. Given that other forms of communication are not feasible belowground, soil organisms mostly rely on chemical **communication** (see Glossary). Indeed, plants secrete a large array of primary and secondary plant metabolites into the rhizosphere to facilitate interactions with their biotic and abiotic environment. Even the **root exudate** of a small plant species, such as arabidopsis (*Arabidopsis thaliana*), may contain over 100 different metabolites [2]. Recent advances in untargeted **metabolomics** allow us to detect and, to some extent, identify increasingly more of the compounds that are secreted by plants and the organisms interacting with them in the rhizosphere. Reliable quantification and identification will require well-designed experimental set-ups and an answer to the question of which conditions should be applied during exudate collection [3]. Moreover, the experimental designs should be such that they provide a realistic insight into what happens under field conditions.

Here, we review recent advances in metabolomics and the identification of compounds exuded into the rhizosphere, and what is known about their role in the interaction between plants and soil biota. We focus on insect herbivores, nematodes, and beneficial microbes, because the role of exudates in defence against microbial pathogens was recently reviewed elsewhere [4,5]. We specifically focus on the role of plant secondary metabolites because they often have a critical role in species-specific communication between interacting organisms [6]. Finally, we discuss

Trends

Metabolomics is becoming accepted as a tool for the (untargeted) analysis of metabolites in root exudates.

The importance of the rhizosphere microbiome for the functioning of plants is becoming increasingly clear.

Statistical analysis and genetic mapping are used to establish relations between metabolites and (other) traits.

The increased understanding of metabolic engineering in plants will allow the critical assessment of the role of individual compounds in plant–rhizosphere communication.

Novel chemical-analytical platforms, such as laser-assisted electrospray ionisation (LAESI), will allow for spatial metabolomics on the scale of roots and interacting organisms.

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 http://dx.doi.org/10.1016/j.tplants.2016.01.008

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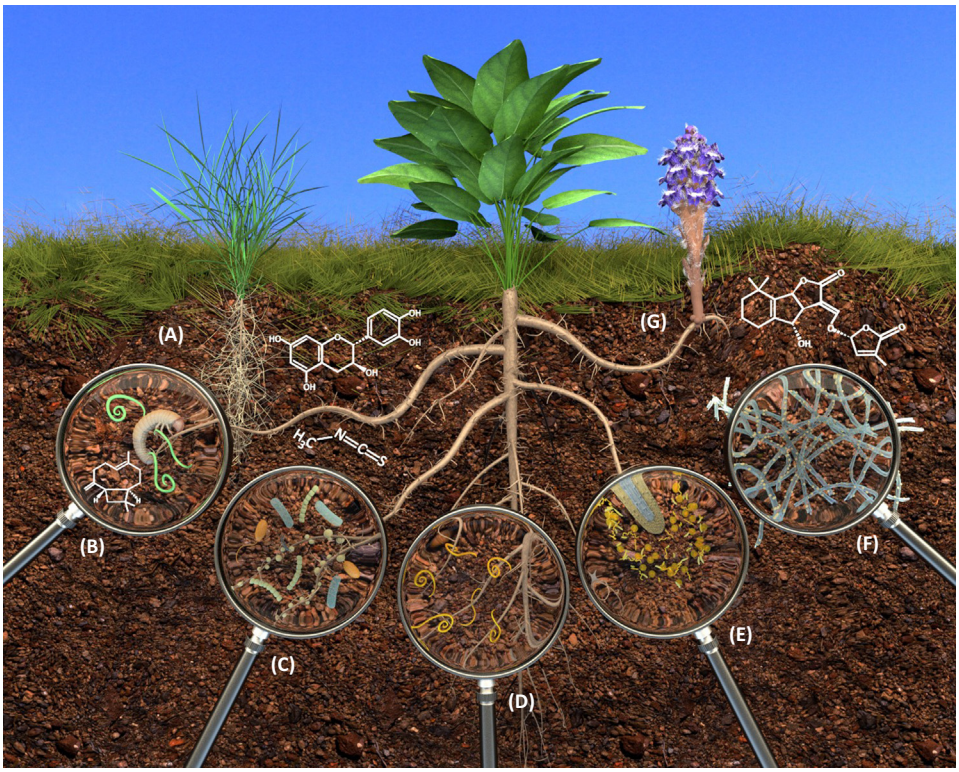

Figure 1. Belowground Chemical Communication of Plants with other Organisms. (A) Plants exude phenolic compounds to inhibit the germination or growth of other plants (allelopathy); (B) insect larvae feeding on the roots induce the production of volatiles, such as sulfur-containing compounds or the terpene (*E*)-β-caryophyllene, attracting entomopathogenic nematodes; (C) the root exudate of plants affects colonisation by rhizosphere bacteria and initiates the colonisation and root nodule formation by *Rhizobia*; (D) root exudates induce hatching of cyst nematodes and attract juvenile nematodes towards the root; (E) the root cap at the tip of the roots is the site of most active exudation; (F) strigolactones in the root exudate induce hyphal branching in arbuscular mycorrhizal fungi, a process required for colonisation; and (G) the germination of parasitic plants is induced by strigolactones in the root exudate of their host.

challenges and opportunities with regard to exudate collection and analysis, data analysis, and the interpretation of the ecological role of the secreted metabolites.

Metabolomics of Root Exudates

It has long been acknowledged that root exudates constitute a significant source of organic carbon in the soil; up to 50% of the total plant photosynthetic production may be exuded into the rhizosphere. A primary function of root exudates is to mobilise recalcitrant and limiting nutrients, such as phosphorous (P), and to detoxify heavy metals by the excretion of organic acids. Therefore, the analysis of exudates is often limited to organic acids and other primary compounds, such as sugars [7,8]. However, the recent development of broad-spectrum and highly sensitive (nano- to picomolar range) **metabolomics platforms** now potentially allows a more comprehensive view on exudate composition.

The principal aim of metabolomics is to analyse as many compounds as possible in a single run. However, the analytical platform chosen greatly determines the classes of compound that can be detected. When studying rhizosphere interactions mediated by volatiles, **gas chromatography-mass spectrometry** (GC-MS) is the platform of choice. By contrast, water-soluble

secondary metabolites in exudates, such as phenolics or flavonoids, need to be analysed on **liquid chromatography (LC) platforms** or **nuclear magnetic resonance** (^{1}H-NMR) [9]. If there is no prior knowledge of the type of molecules present or when comprehensively profiling an exudate, a combination of platforms may be used (e.g., [10,11]). Initially, untargeted analyses merely yield 'fingerprints' providing information about differences in composition between samples. Visualisation techniques, such as heat maps and clustering, can be used to highlight the differences [12]. Only after applying statistical analysis, including multivariate analysis, such as principal component analysis (PCA) and as partial least squares-discriminant analysis (PLS-DA), compounds or **features** that 'make the difference' between samples can be pinpointed. Pivotal metabolites can be further identified, either by applying MS/MS on peaks in the extract, or by isolating the compound for additional structural analysis using NMR. Isolated compounds can also be used for bioassays in which their activity is experimentally assessed. Care must be taken that such bioassays are conducted with biologically relevant and realistic concentrations, which could be assessed by *in situ* measurements in the rhizosphere, although this comes with some challenges [13].

Metabolomics has been successfully applied to profile root exudates of several species (Table 1). Not surprisingly, organic acids, amino acids, and sugars are often found in the exudate or

Table 1. Numbers of Compounds and Compound Classes Identified in Root Exudates by Using Untargeted Metabolomics[a]

Plant Species	Type of Metabolomics Platform Used	Plants Grown On	Number of Peaks/ Features Reported	Number of Compounds Identified[b]	Compound Classes	Refs
Arabidopsis thaliana	LC-ToF-MS	Liquid MS medium	1137 features	103 (42)	AA, nucleosides, dipeptides, GLS bdp, phytohormones, phenylpropanoids, fatty acid derivatives	[2]
Beta vulgaris	LC-MS	Hydroponics	Total not reported, ≥67 m/z signals	2	Citramalic acid, salicylic acid	[7]
Eperua falcata	LC-ToF-MS	Field sample	94 peaks	4 (3)	Flavonoids (chalcone, flavone)	[72]
Heracleum mantegazzianum	LC-ToF-MS	Aeroponics	Total not reported	15 (1)	AA, dipeptides, C18 oxylipins, manonylglycosides	[14]
Medicago trunculata	GC-MS	1% agar/ water	612 features[c]	38	AA, OA, sugars, beta-alanine, urea	[11]
	LC-ToF-MS	1% agar/ water	4343 features[c]	32[d]	Phenolics, saponins/ sapogenins	[11]
Solanum lycopersicum	^{1}H-NMR	Sterile sand	23 peaks	8	AA, OA, sugar-polyalcohols	[10]
	LC-MS	Sterile sand	20 000 m/z signals	0	–	[10]
Zea mays	LC-ToF-MS	Sand with soil top layer	40 features	7	1,3-Benzoxazin-4-one derivatives	[22]

[a]Abbreviations: AA, amino acids; GLS bdp, glucosinolate breakdown products; OA, organic acids; MS medium, Murashige and Skoog growth medium; SPME, solid phase microextraction.
[b]Number of compounds that were identified or classified as indicated by the authors in the publication. Values in parentheses in the same column indicate the number of compounds identified with the use of authenticated standard compounds.
[c]Data reported and shared online compliant with Metabolomics Standard Initiative (MSI; http://msi-workgroups.sourceforge.net/).
[d]Level of identification reported compliant with MSI.

Glossary

Communication: a process by which information is exchanged between organisms. In the case of chemical communication, this is achieved via common chemical signals.
Electron spray ionisation (ESI): the most common method for both LC and GC platforms to ionise molecules before they enter a mass spectrometer. The potential over the ESI nozzle determines whether the analytes are protonated (M+, positive mode) or deprotonated (M–, negative mode). Given that molecules differ in their propensity to accept or lose a proton, the spectrum of metabolites that is recorded is increased by analysing the same sample in positive and negative mode.
Feature: a cluster of spectrometric signals belonging to one, as yet unidentified, metabolite. The clustering is based on algorithms identifying the characteristics, such as retention time or intensity, of the signals as being similar enough to be derived from the same parent molecule. This assignment is seldom perfect; therefore, there may be more features than actual metabolites in one sample.
Gas chromatography-mass spectrometry (GC-MS): used to analyse volatile compounds (boiling point 20–350 °C) and primary metabolites (after derivatisation to make them volatile).
Liquid chromatography-mass spectrometry (LC-MS): generally applied to analyse polar and semipolar, nonvolatile compounds, such as glucosinolates, alkaloids, flavonoids, and other phenolics. Sensitivity is in the nanomolar range.
Metabolomics: the untargeted analysis of as many metabolites as possible in one sample.
Multivariate analyses (MVA): statistical procedures to analyses data sets for which the numbers of variables in a sample are larger than the number of replicates, such as metabolomics or transcriptomics data sets. The models can be either unsupervised (no *a priori*-defined grouping factors enter the model), such as principal component analysis (PCA), or supervised, such as partial least squares–discriminant analysis (PLS-DA). Next to a visual representation of the differences in metabolomic profiles, MVA models

extracts thereof (Table 1). It might be that these primary metabolites are lacking from some of the exudate profiles because they are rapidly degraded under nonsterile conditions [3]. In addition, root exudates contain a variety of secondary metabolites. Phenolic compounds, such as phenylpropanoids and flavonoids, are most commonly reported. The model legume *Medicago trunculata* also secretes saponins from its roots, whereas glucosinolate breakdown products, typical for Brassicaceae, were found in arabidopsis exudates (Table 1). Most interestingly, three studies report the presence phytohormones or related signalling compounds, such as salicylic acid [7], jasmonic acid derivatives [2], and oxylipins [14], in the exudate. These hormones may have a role in either defence against pathogens, or, in the case of jasmonic acid and related oxylipins, in organ development and abiotic stress responses [4,15]. Strigolactones, another class of plant hormones that are secreted by plant roots, are discussed below.

Additionally, roots produce a range of (semi-)volatile organic compounds (VOCs), including terpenoids, sulfides, (thio)cyanides, and thiophenes [16–19]. The VOCs that are produced by roots or rhizomes have recently been reviewed elsewhere [20]. VOC profiles are also analysed on metabolomics platforms, especially GC-MS. Whereas it is evident that the somatic tissues of (damaged) roots emit VOCs [13,17,21], it is less clear whether they are also released in the exudates produced by the root cap, where the plant intensively interacts with microbes and nematodes. Sulfur-containing volatiles that result from glucosinolate catabolism were identified in both the exudate and emerging from the main root [2,17]. By contrast, the sesquiterpene (*E*)-β-caryophyllene is emitted from maize (*Zea mays*) roots upon herbivore damage [21], but is not found in the exudate [22,23]. This may be due to the low water solubility of *(E)*-β-caryophyllene and the propensity to use analytical platforms (LC and NMR) that are more suitable for polar compounds (Table 1).

identify which variables (features or compounds) contribute the most to the observed differences.

Nuclear magnetic resonance (^{1}H-NMR): provides information on the arrangement of the hydrogen atoms, whereas ^{13}C-NMR provides information on the carbon atoms in a molecule. For metabolomics, NMR is generally only used to analyse polar compounds. It is less sensitive than MS-based techniques (micromolar range), but more robust with regard to reproducibility and identification.

Root exudate: the total of molecules actively or passively released by plant roots into the soil or any other medium surrounding the roots.

Time-of-flight mass spectrometry (ToF-MS): may be interfaced with GC or LC platforms. It allows the assessment of the exact mass (ppm range) of a molecule or fragment thereof, which improves the identification of compounds.

Chemical Communication in the Rhizosphere

Insect Herbivores and Their Natural Enemies

Being heterotrophic, insect herbivores are completely dependent on localising a suitable host. Aboveground chemical-ecological studies have shown that insect host choice is driven by physical parameters, such as leaf colour and shape, wax layers and trichomes, as well as chemical parameters, including VOC profiles and the metabolome of the plant. For belowground herbivore–plant interactions, chemical cues are also important. Over longer distances (centimetres), soil herbivores most likely use root volatiles to locate their host. Originally, CO_2, a product of root respiration, was coined as a critical host location cue for belowground herbivores [24]. However, recent studies have pinpointed more specific VOCs as cues, whereby CO_2 may serve as a general 'background' odour [25]. This is illustrated by the fact that larvae of the root-feeding generalist *Melolontha melolontha* have at least three olfactory and seven gustatory sensilla on their antennae and palps. These sensilla respond to a range of volatile and nonvolatile metabolites, including terpenoids, alcohols, acetates, amines, organic acids, and CO_2 [26]. In choice tests, the larvae used these compounds for selecting host plants over a distance of several centimetres [27]. Similarly, larvae of the corn root feeder *Diabrotica virgifera virgifera* can locate roots from centimetres away based on the emissions of the volatile *(E)*-β-caryophyllene (Figure 1) [21,23]. As soon as they have located their host, they use concentrations of nonvolatile 1,4-benzoxazin-3-ones to select the most nutritious root class [23].

Root-emitted VOCs also serve as cues for natural enemies of root herbivores. For example, cabbage root fly (*Delia radicum*) feeding increases the emissions of dimethyldisulfide (DMDS) from the root [17]. DMDS is known to attract ground-dwelling predatory beetles that feed on these larvae, thus reducing herbivore pressure on the plant [28]. Similarly, entomopathogenic nematodes (EPN) use plant volatiles, such as DMDS and terpenes emitted from herbivore-damaged roots, as cues [16,18,29]. A recent study showed that there is an interesting interaction between the emission of (*E*)-β-caryophyllene and root architecture [30]. The addition

of the volatile strongly increased the likelihood that EPNs found their hosts on highly branched roots, more so than on simple unbranched roots [30]. Water-soluble root cap exudates from uninfested plants attracted EPN, indicating that nonvolatile compounds may also serve as arresting cues for natural enemies [31]. However, there is little information on whether root herbivory changes the exudate composition similar to induced VOC profiles.

Phytophagous Nematodes

Nematodes are one of the most ubiquitous members of the rhizosphere mesofauna. Many nematodes are phytophagous, feeding in or on the roots of plants, either as free-living mobile nematodes or as endoparasites that establish a feeding site in the root. In cyst nematodes of the genera *Globodera* and *Heterodera*, the adult females swell up after fertilisation, each containing hundreds of eggs [32]. The eggs can survive for up to 20 years inside these cysts and will hatch in the presence of exudates secreted by the roots of host plants. After hatching, these nematodes can only survive for short periods of time if they do not find a host, on which they totally depend for survival and reproduction. The hatching of cyst nematodes displays a certain degree of host specificity, mediated through differences in the structure of the hatching stimulants, which have been identified in soybean (*Glycinus max*; glycinoeclepin A) [33], kidneybean (*Phaseolus vulgaris*; glycinoeclepin B and C) [34], and tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*; solanoeclepin A) [35]. These eclepins are all triterpenoids and seem to have the same biosynthetic origin.

Juvenile nematodes can only penetrate the root between the tip and the root hair zone [1]. Exudates are excreted close to this zone and, thus, nematodes may use these to localise suitable feeding sites. Potato root exudates indeed attract juveniles of *Globodera pallida* [36]. However, root cap border cells and exudates also contain various defensive compounds [4]. Crown daisy (*Chrysanthemum coronarium*) roots exude lauric acid that, when intercropped, reduces infestation of tomato roots with root knot nematodes (RKNs). Bioassays showed that, at low concentrations (0.5–2 mM), lauric acid attracts RKNs even though 50% may die [37]. At 4 mM, lauric acid is lethal and juveniles are repelled. The nonlinear effect of lauric acid on attraction and lethality at lower concentrations correlated with the expression of *Mi-flp-18*, a motor neuron gene, which was maximal at intermediate concentrations [37]. Similarly, pea root exudates immobilised phytopathogenic nematodes as well as EPN, but the compounds responsible remain unidentified [31,38]. Interestingly, the levels of defence compounds in root exudates may increase upon nematode infection or in the presence of arbuscular mycorrhizal (AM) fungi. The concentration of aucubin and catapol, two defensive iridoid glycosides, increased in *Plantago lanceolata* root exudates when plants were exposed to nematodes [39]. Exudates of mycorrhizal tomato roots reduced the propensity of RKN to penetrate due to lower nematode mobility [40].

Plant–Plant Communication

Root exudates, especially secondary metabolites therein, have an important role in plant–plant interactions. It has been experimentally assessed that arabidopsis plants actively change root allocation patterns under inter- or intraspecific competition. Both growth towards and away from the neighbour have been observed, suggesting that this process is not solely driven by nutrient concentrations, but by specific exudate cues (Figure 1) [41]. Root exudates may also inhibit the germination and growth of other plant species. Such allelopathic effects may contribute to the invasiveness of exotic species, such as giant hogweed (*Heracleum mantegazzianum*). Metabolomics was used to study (genetic) variation in *H. mantegazzianum* exudate composition, which varied between accessions and correlated with reduced lettuce seed germination. Fifteen compounds were tentatively identified as being responsible [14]. A more targeted, genetic approach showed that momilactones in rice root exudates elicited allelopathic effects. Lettuce and grass seedlings grown with rice mutants lacking momilactone production germinated faster

and grew better than those grown with wild-type plants [42]. Remarkably, plants may even recognise the level of relatedness of their neighbour based on exudates. *Deschampsia caespitosa* plants allocated more roots to patches where exudates of unrelated individuals were added than to patches with 'related' exudates or control solutions. This was interpreted as competitive root proliferation towards nonsibling individuals [43]. However, it is still unknown how exudate compounds are perceived by plants, because specific receptors have not been identified yet. Consequently, it was postulated that allelopathic effects are indirect and influence the rhizosphere microbiome of the competitor rather than the plant itself [44,45].

The interaction between plants and root parasitic plants is another intriguing example of belowground plant–plant communication. *Striga* spp. and related *Alectra, Orobanche*, and *Phelipanche* spp. (all Orobranchaceae) are heterotrophic root parasitic plants that are completely dependent on a host for survival and reproduction. Hence, timing of germination is crucial. The majority of these species use strigolactones as host cues [46], which are produced by the plant to establish a symbiotic relation with AM fungi (Figure 1). Under P limitation, the production and release of strigolactones into the rhizosphere are increased to attract more AM fungi [47,48]. Apparently, root parasitic plants have evolved the capacity to use strigolactones, a signal not intended for them but for AM fungi, as a reliable host cue. Since their discovery, additional roles for strigolactones have been discovered and, based on these, they are now considered to act as plant hormones [46].

Plant–Microbe Interactions

The rhizosphere microbiome has a strong effect on plant health by facilitating nutrient acquisition and helping plants to tolerate abiotic stresses [49,50]. Several beneficial microorganisms stimulate growth or strengthen the defences of the plant against pathogens and insects [51,52]. The rhizosphere microbiome associated with a certain plant genotype is likely determined by several factors, such as the type of substrate and the endophytic and soil microbial community present or added. Root architecture [53] and other genetically determined plant traits also affect the rhizosphere microbiome [49]. Experimental evidence to support a genotype effect is still scarce, even though statistically significant differences in the microbiome between different arabidopsis ecotypes were found [54]. The exact mechanisms by which plants shape their microbiome are as yet unknown, but differences in the metabolite blends that plants excrete into the soil are likely an important factor [49,55,56].

Effect of Growth Medium on Exudate Collection

Soil is the natural substrate in which roots are growing and, therefore, is often used to grow plants for root exudate collection. However, it is known that metabolites may adsorb to particles in the growth substrate, for example to common soil minerals, such as goethite [57]. It is easier to collect exudates in hydroponics and the variation caused by microorganisms and the soil matrix can also be better controlled. Aeroponics is not used frequently but allows easy collection of root exudates under high oxygen availability [58]. A complicating factor when sampling root exudates is that the rhizosphere is usually colonised by microorganisms, many of which feed on exudate metabolites. Moreover, other environmental factors, such as pH, soil type, oxygen status, soil temperature, and nutrient availability, also influence root exudation [59,60] (reviewed in [61]). Hence, the metabolite composition that will be measured depends on the biotic and abiotic environment to which the roots are subjected. The effect of the rhizosphere microbiome can be so strong that organic acids could not be detected in tomato exudates of plants growing in hydroponics under nonsterile conditions. However, exudate samples collected from sterile roots contained appreciable levels of various organic acids, such as acetic acid, malic acid, α-ketoglutaric acid, oxalic acid, and citric acid [3]. Thus, it is important to standardise root exudate collection protocols because the conditions the roots are exposed to have a strong effect the outcome of the metabolomics analyses. To exclude the strong effect that microorganisms have

on the root exudate, sterilisation agents or sterile cultivation systems are used (reviewed in [3,61]). By contrast, for a study on the exudation of metabolites under natural conditions, an experimental lab set-up that includes the effect of soil microbes on exudate composition would be desirable. A system in which roots of soil-grown plants are split off into a clean compartment for exudate sampling, such as developed elsewhere [62], could then be a suitable alternative (see also Oburger and Schmidt, this issue).

Concluding Remarks: Challenges and Opportunities

The major challenge in rhizosphere metabolomics is to decide on what constitutes a representative sample of a root exudate (see Outstanding Questions). The answer depends on the scope of the study. Ecologists may be more interested in the exudate composition resulting from the interactions with rhizosphere biota, whereas plant physiologists may be more interested in unmodified, native exudates. Obtaining a native root exudate is challenging, because it is difficult to avoid the presence of microorganisms in the rhizosphere (but see [2,3]). However, in the presence of an active microbiome, certain metabolites that were present in the exudate and may have (had) an effect on rhizosphere processes may have disappeared due to microbial degradation. In addition, the exudate composition may change when the microbiome changes during the experiment. Root architecture and exudation patterns are also affected by the media in which plants are grown. This makes it questionable whether exudates obtained on agar or hydroponics are sufficiently representative of the exudates of soil-grown plants.

The sensitivity of current metabolomics platforms constitutes another constraint showing that metabolomics cannot resolve all rhizosphere signalling relations (see Outstanding Questions). Some signalling compounds are active in picomolar concentrations and can only be detected using targeted analysis, such as triple-quad LC-MS [63,64]. However, untargeted LC-MS analysis is rapidly becoming more sensitive. This will allow for an increasing number of compounds to be detected in root exudates, including novel signalling molecules. However, it will also complicate the identification of the relevant compounds from the larger data sets that will be produced. A multitude of **multivariate analyses** (MVA) models have been developed for identifying which features in metabolomics data sets contribute significantly to the observed effect. However, the chemical identification of these features is not trivial, and a single compound cannot always explain the whole effect. Thus, studies aiming to elucidate the role of exudate compounds in rhizosphere interactions cannot stop at metabolomics profiling. The use of genetic variation in combination with metabolomics can help to further elucidate the regulation and biological significance of root exudates. Several studies revealed that there is genetic variation in the concentration and composition of root exudate metabolites [61]. This variation can be used for genetic mapping, as was demonstrated for strigolactone concentration in root exudates of rice [65]. Combined mapping of metabolites and other traits, such as abiotic or biotic stress tolerance or the microbiome composition, could identify additional relations between these traits. Bioassay-driven fractionation may be another approach to identify active exudate metabolites [66]. Mutants and transgenic lines with altered production of specific metabolites suspected of having a signalling role are valuable tools to assess the effect of a single metabolite in an exudate [42,45]. For example, overexpression of a gene encoding the enzyme (*E*)-β-caryophyllene synthase in an American maize line that normally does not produce this sesquiterpene, resulted in constitutive emission of (*E*)-β-caryophyllene into the rhizosphere, which resulted in enhanced attraction of an EPN and decreased corn rootworm damage [67].

Another technical challenge is to track the temporal and spatial dynamics of the exudate metabolome at a scale that is relevant for rhizosphere organisms. Even within one genotype, exudation rates and exudate composition change with plant development and in response to biotic and abiotic factors [7,23,39]. For root volatiles, online techniques, such as proton transfer reaction (PTR)-MS, may be used to record temporal dynamics [17,68,69]. Recently developed

atmospheric pressure MS imaging (MSI) technologies, such as laser-assisted ESI (LAESI) or laser desorption/ionisation MSI (LDI-MSI), allow for 'spatial explicit metabolomics' and *in situ* analyses of plant compounds without elaborate sample preparation [70]. LDI-MS was used to show that nematicidal phenylphenalenones accumulate at lesions formed in banana roots attacked by the nematode *Radopholus similis* at a resolution of 10 μm. LDI-MSI also showed that these compounds are taken up by nematodes and form droplets inside the 20 μm-wide bodies of the nematodes [71]. Similar techniques could easily be applied to root tips and root caps because they are well-defined zones with a limited number of cell layers (see Outstanding Questions).

Making optimal use of novel technologies may also help us overcome the final challenge, that is, to determine which of the many exudate metabolites are meaningful signals. This knowledge is of absolute importance if we truly aim to achieve a better understanding of the ecological role as well as agricultural significance of plant–environment interactions in the context of soil biodiversity. Transcriptomics analyses of plant, nematode, or microbial species, or even the entire rhizosphere microbiome (metatranscriptome) may reveal which genes are activated in response to a particular exudate compound [37]. Insertion of reporter genes in interacting organisms will show when and where in the organism specific genes are expressed, even if a typical rhizosphere microbiome contains many uncultivatable species [55]. With all these new tools at hand, we expect that we will be able to tap deeper into belowground chemical communications and shine more light into the 'black box' of rhizosphere interactions.

Outstanding Questions

How can we sample root exudates in such a way that it is a realistic representation of what is produced under natural conditions? Samples taken from plants grown in sterile hydroponics or agar systems may only provide an overview of what undamaged roots produce under 'ideal' conditions. Exudates sampled from soil or field-grown plants are likely modified by the rhizobiome.

How can we identify which compounds in the exudate metabolome are the most relevant for particular rhizosphere interactions? MVA should identify reliably which metabolites are responsible for the observed effect and, therefore, should be selected for further testing.

Which experimental set-ups and technologies can best be used to assess the effect of exudates or exudate compounds on interacting organisms in the rhizosphere? Visualisation and analytical technologies should allow spatial resolution at the scale of the rhizobiome.

How can we perform *in situ* metabolomics in the rhizosphere? When designing bioassays to test ecological effects of isolated metabolites, the realistic concentrations to be used must be assessed first. Temporal changes in the concentrations should also be captured with temporally resolved or online measurements.

Acknowledgments

This review greatly benefitted from discussions at the Rhizophere 4 conference held from 21–25 June, 2015 in Maastricht, The Netherlands. We particularly acknowledge the speakers in the 'Metabolomics' session and participants of the Roundtable discussion on root exudate collection chaired by Angela Straathof. N.M.vD. gratefully acknowledges the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118). H.B. acknowledges funding from the Netherlands Organization for Scientific Research (NWO) (VICI grant 865.06.002) and the European Research Council (Advanced grant CHEMCOMRHIZO).

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