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Root Exudation of Specialized Molecules for Plant–Environment Interaction

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Abstract: It has been estimated that between 20 and 40% of the assimilated carbon is diverted to the roots and released in the rhizosphere in form of root exudates. Root exudates thus define a complex mixture of low and high molecular weight compounds, including carbohydrates, amino acids, organic acids, and proteins, but also a broad spectrum of specialized molecules, such as flavonoids, glucosinolates, terpenoids, or alkaloids. Root exudates favour soil mineral nutrition, can bind to soil aggregate and in turn modify soil physico-chemical properties, but also mediate plant–plant, plant–microbe, and plant–animal interactions belowground. With this review, we aim to highlight how chemical ecologists have approached the study of root exudates-mediated interactions between plants and their biotic and abiotic surroundings. We do so by presenting a series of study cases for, on one hand, showcasing different methodologies that have been developed to test the activity of different root exudates, and, on the other hand, to show the broad array of interactions mediated by root exudates. Ultimately, we aim to spur further research and collaborations between chemists and ecologists studying belowground chemically-mediated interactions, so as to tackle essential challenges in terms of food security and climate change in the near future.

Keywords: Behavioural assays · Flavonoids · Hydroponic culture · Root chemistry · Strigolactones, sesquiterpenes · Untargeted metabolomics

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1. Introduction

Broadly speaking, the unique challenge for a plant throughout its lifetime is to maximize carbon and mineral acquisition within a relatively limited area and volume of soil, so to optimize its fitness.[1] This apparently simple function is nonetheless in opposition with the observation that the estimated >300'000 plant species, having colonized virtually every available habitat on Earth, display an incredible amount of morphological and chemical variation, both aboveground[2] as well as belowground.[3] Plants, indeed, organize themselves into forms and functions that depend on the complex combination of growth strategies,[4] structural traits,[5] and chemical composition, including proteins, sugars, and lipids (also referred to as primary metabolites), but more particularly, also including the full spectrum of specialized molecules – described as secondary metabolites.[6] Within the context of plant functional ecology, the chemical diversity of specialized molecules produced by plants is therefore also considered as another axis of functional trait diversity.[7] The plethora of specialized molecules that each plant produces during its entire lifetime which is not directly involved in the primarily metabolism (*i.e.* growth or development), is yet essential for plants' interactions with external biotic and abiotic factors and eventually fitness.[6]

The incredible diversity of specialized molecules (>400'000 described molecules so far, *e.g.* as described in ref. [8]), only rivals the incredibly complex networks of interactions in any given ecosystems.[9] Specialized metabolites are, for example, involved in protecting plants against abiotic stresses,[10] plant–plant interaction such as allelopathy[11] or communication,[12] plant–herbivore interaction,[6] plant–herbivore-predator interaction,[13] plant–pollinator interaction,[14] plant–microbe interaction,[15] plant nutrient acquisition[16] and nutrient cycling.[17] Therefore, whilst other plant functional traits are related to the use of a single resource (*e.g.* C, N, P, or water), the plant specialized metabolomes can be associated with numerous critical functions for plants; such as protection against abiotic[18] and biotic factors,[7b,19] or shaping its direct environment.[20]

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Since chemical ecology has emerged as a fully-fledged discipline for describing and understanding the role of specialized molecules in mediating-plant–environment interaction, most of the work – likely because of the convenience – had focused on the aboveground ecosphere.[21] However, during the past two decades, the focus has also shifted belowground.[22] Accordingly, similar to aboveground tissues, roots permanently release a wealth of compounds in the environment. Concerning the belowground subsystem, the surrounding environment for roots is called the rhizosphere, which is defined as the zone of the soil under the influence of the plants. Such zone of influence can span a few millimetres up to several centimetres away from the root tip depending on how far a molecule can travel the soil matrix. This review addresses how root metabolites exuded in the soil matrix can help plants deal with the constraints of the environments in which they grow. Particularly, we will focus on multiple examples showing how different classes of secondary metabolites exuded by roots to mediate plant–environment interaction can be measured. Finally, we will discuss the upcoming technical challenges for root metabolome studies in relation to climate change and sustainable agriculture.

2. The Chemistry of Root Exudates

Root exudates define a complex mixture of low and high molecular weight compounds, including carbohydrates (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acids), proteins such as exoenzymes, but also the full spectrum of specialized molecules, the so-called secondary metabolites,[6] such as flavonoids, glucosinolates, terpenoids, or alkaloids.[23]

All the molecules exuded in the soil matrix can affect the rhizospheric environment (both biotic and abiotic), and are released in the rhizosphere by passive or active mode. Passively, low molecular weight molecules diffuse through the cell membranes and cytosol of root cells following the inverse concentration gradient that naturally exists between the exterior of the living root tissues and the exterior, the soil.[11,24] In this manner, the soil surrounding the root system naturally accumulates a conspicuous amount of carbon that is used by the soil microbial and animal communities as resource. In fact, it has been estimated that about 40% of the photosynthates synthesized in the plant parts is lost through the root system into the rhizosphere within an hour.[25] Other chemicals that cannot easily diffuse through the cell membranes, such as specific carboxylates (*e.g.* citrate, malate, oxalate), can be exuded using anion channels, mediating the controlled release of these products by roots.[26] Finally, the transport of high-molecular-weight compounds, such as mucilage polysaccharides, or proteins (exoenzymes), needs to be achieved using vesicles.[27] Once in the rhizosphere, root exudates mostly follow four pathways; i) they are adsorbed to the soil particles, in turn changing the physico-chemical properties of the soil,[26] ii) they can undergo biological (mostly microbial) degradation,[28] iii) they facilitate metal and mineral absorption by plants,[29] iv) they mediate changes in the composition and activity of the soil biota surrounding the roots. Within this review, we will predominantly focus our attention on how chemical ecology research has addressed the sampling and the role of root exudates for mediating plant–soil biota interactions. Because of lack of space, we won't discuss the role of root exudates for plant nutrition, such as the release of phytosiderophores for chelating iron or other forms of iron to make it available to plants,[30] but we acknowledge this to be a very important topic, with implications for plant growth under different conditions,[30b] and plant–soil–microbe interactions.[31]

3. Sampling and Analysing Root Exudates

Despite significant progress, there is to date not one unique sampling method for root exudates. The very nature of the rhizosphere indeed makes sampling of root exudates challenging and often requires innovative solutions for developing targeted or untargeted sampling techniques.[24] However, if these limitations are well understood and thoughtfully interpreted, sound data can be collected and may provide key elements in the elucidation of to-date still unravelled links driving roots' interactions with their biotic and abiotic environment. Accordingly, the ecological relevance of the sample needs to be further carefully examined using bioassays specifically designed for studying the root–soil interface. Sampling in natural conditions was, and still is, highly challenging; root structural diversity, minute heterogeneity of the soil matrix, or the fact that in soil, exudates will be immediately altered when released (*e.g.* sorption to soil, microbial mineralization), are only few examples of the challenges met by rhizosphere ecologists. Root exudates can be differentiated based on several different characteristics and properties, but for the purpose of this review, we adopt two main categories; (i) water soluble and (ii) volatile exudates. For both categories, sampling can be done under hydroponic (water soluble) or artificially-created headspace (root volatilome) conditions or *in situ* in mostly undisturbed soil conditions (for both exudate categories). Moreover, sampling of the entire root system, or parts of it, can also be accomplished. Below, we hand-picked a series of examples to illustrate the broad range of techniques using for sampling root exudates. For a full range of current approaches used in the field of research, please refer to recent reviews on the topic.[24,32]

3.1 *Sampling of Water-soluble Root Exudate*

Hydroponic culture-based solution – Sampling root exudates in a watery solution also containing plant nutrients has been used since the 1940s. This method has the advantage to avoid shifts in the exudation profile due to sorption on the soil matrix and microbial interaction (when the hydroponic conditions are sterile). Hydroponic conditions consist of a stagnant or flowing solution, an agar (or other gel-like) matrix, a mist chamber, or percolating systems (perlite/vermiculite, glass beads, transparent soil).[33] Despite the ease of sampling, the use of hydroponic culture for sampling root exudates has the clear disadvantage of poorly mimicking how roots behave and secrete exudates in natural soil conditions.

Soil-based approaches – To approach more realistic conditions, root exudates could be sampled from soil-grown plants, or from hybrid soil/hydroponic systems. For instance, plants can be first grown in soil, at least during the first experimental phase, and later placed in hydroponic conditions (second phase) to collect and further analyse root exudates. The disadvantages of this hybrid method include the damaging of the roots during the washing procedure, or the loss of certain exudates during this same step. Repeated leaching of the soil might prevent the washing step, yet this could alter the interactions between the roots and the soil matrix. In addition, this technique does not allow measurements of gross exudation rates. Lastly, this method is not really appropriate to sample highly labile metabolites, such as amino acids or sugars, which are likely to be quickly assimilated by microorganisms in the environment, or highly charged molecules, which will very likely be retained on soil aggregates. To overcome the latter, it has been proposed to use a leaching solution of 50% methanol and 0.05% formic acid, but the effect of this leaching solution on root integrity remains to be documented. Sampling sections of the root system remains possible *in situ* using hydroponic exudation traps but such approach implies manipulations of the root sections of interest and therefore may cause tissue damage impacting root exudation profiles.

Sampling of mucilage – Mucilage is a polymeric gel often found at the root tip. Despite its 'gluey' nature, mucilage is water soluble. However, once solubilised in water, it loses its physical properties (cannot be restored upon drying). This aspect does not impact chemical analyses of the mucilage composition but has to be taken in account when extrapolating to ecological impacts of the structured mucilage in the soil. Mucilage is classically sampled under hydroponic conditions and soil sampling of this particular root exudate remains to be accomplished.

3.2 *Sampling of the Root Volatilome*

Sampling root exudates with very low water solubility (*i.e.* root volatile organic compounds, rVOCs) is as challenging as collecting soluble exudates. The first identification of an ecologically active rVOC was made by pulverizing root material in liquid nitrogen. The obtained fine powder was then placed in a glass vial, heated up to 40 ºC and finally the headspace was sampled with a solid phase micro-extraction (SPME) fibre.[34] Despite being relatively easy to set up, this sampling method only provides the instantaneous production of rVOCs. Gfeller *et al*.[35] further refined this destructive method to sample rVOCs from intact root material, yet still detached from the stem. Non-destructively, rVOCs have been sampled by pushing purified air in the aboveground headspace and then pumping the air through Super-Q adsorbent filters inserted in the rhizosphere.[36] The use of these active sampling methods allowed the quantification of rVOCs that were actually released by living roots. Subsequently, to sample rVOCs in the field, soil probes were modified to accommodate Super-Q adsorbent in the probe, which was then attached to a pump to aspire root volatiles directly in the probe allowing rVOCs sampling in the field.[37] Such dynamic airflow methods were further adapted to sample rVOCs influencing bacteria behaviour in the rhizosphere[38] or to evaluate rVOCs production in different regions of the rhizosphere.[39]

3.3 *Analysis of Root Exudates*

Classic methods for estimating root exudation is to use isotope (radioactive or stable) based approaches, which allow tracing the movement of photosynthetic carbon within the plant system and exudates. The major limitation of this approach is also related to the damage caused to the roots while separating them from the soil matrix (*e.g.* loss of root and root hairs), resulting in an overestimation of the isotopes of interest present in soil as compared to what is measured from the plant material. Imaging like zymography (visualisation of enzymatic activity on agarose gel) is one of the historical approaches used to analyse root exudate despite presenting several of the same biases as hydroponic culture.

To date, most of the current analyses of root exudates are based on mass spectrometry coupled to liquid or gas chromatography. The current development of these technologies allows non-targeted approaches (metabolite fingerprinting), explaining the increasing number of contributions to the referred literature using non-targeted mass spectrometry (MS) approaches. However, despite of the advancements in MS acquisition techniques, as well as new development in artificial intelligence algorithms for peak detection and deconvolution, and mass spectral library comparison,[7a] we also would like to highlight that the current databases allowing the identification of each molecule, based on the ions-based molecular fingerprints, are still limited, thus allowing for the identification of only a minute fraction of what is obtained in the analysis. Therefore, while the correct identification of the exuded metabolites is key to study their function in the rhizosphere, much effort is still needed to build molecular libraries of root exudates.

4. Root Exudates Mediate Plant–Soil Biota Interactions

By passively or actively releasing root exudates in the rhizosphere, plants indirectly might create a favourable environment for a wealth of microbial and animal diversity, including beneficial microbes, but also pathogens and pests, including bacteria, fungi, nematodes and soil arthropods.[23] In this context, chemical ecology research on underground sub-systems, as mostly done aboveground so far[7a,40] aims to understand the role of specialized molecules in mediating plant–plant, plant–microbe, and plant–animal interaction, *via* both mutualistic and antagonistic interactions.[22a,e,i,41]

4.1 *Plant–Plant Interaction*

Plant–plant interactions have been extensively studied for aboveground tissues, particularly in the context of herbivore-induced volatiles that can be perceived by neighbouring plants and 'warn' them for imminent attack.[42] Belowground, such chemically-mediated plant–plant interaction has received far less attention. For example, it has been argued that when *Vicia faba* (broad bean)[43] or *Phaseolus lunatus* (lima bean)[44] plants are subjected to aphid or spider mite attack, respectively, they release root exudates that induce the release of volatiles in undamaged neighbouring plants, which in turn attracts aphid parasitoids. To date the root exudates mediating these interactions remain to be elucidated. To uncover such chemistry, a straightforward, although labour-intensive, approach would be to perform bioassay-guided fractionation experiments. For instance, plants with or without herbivores could be grown in hydroponic solution. The fractionated (*e.g.* initially using solid-state extraction (SPE) columns, and later fractions collected from liquid chromatography columns) solutions could then be added to healthy plants, and, by using wind tunnels or olfactometers, insect attraction could be monitories as a proxy of plants changing their chemical phenotype in response to a particular root exudate compound or mixture.

On the other hand, more commonly studied belowground negative plant–plant interactions, such as allelopathy, also exist, which involves the release of chemicals from roots that inhibits the performance of neighbouring plants. For instance, the benzoxazinoid DIMBOA, released by maize and several other grasses has allelopathic properties.[*e.g.* 45a,b,c] Hydroxamic acids are generally acid-extracted using ethyl acetate from fresh samples or seedling roots,[46] and their allelopathic activity tested with germination bioassays in Petri dishes.

4.2 *Plant–Animal Interaction*

Soil fauna, composed largely of immature stage of Coleoptera and Diptera, collembolan, mites, and earthworms can help in increasing soil fertility thought the decomposition process, but some can impact plants negatively, since many of the soil animals feed on roots.[47] Since organic matter decompositions can have profound effects on nutrient cycling, in turn favouring plant nutrient acquisition,[48] it might be profitable for plants to exude compounds into the soil to attract decomposers. This remains to our knowledge unexplored and merits further investigation. For instance, such experiments could be performed using belowground olfactometers or thin-layered rhizotrons so to monitor small arthropod larvae or earthworm movement in soils with and without soluble fractions of root exudates. Concerning host location for root feeding insects, already several dozens of compounds in root exudates have been shown to serve as foraging cues for root-feeding insects.[49] For example, using a transparent plastic container where roots were visible, and where the movement of root feeding larvae could be monitored at regular intervals, Robert *et al*.[50] showed that the larvae of the chrysomelid beetles *Diabrotica virgifera virgifera* can exploit benzoxazinoids released by maize roots, to recognise the most nourishing roots.

4.3 *Plant–Microbe Interaction*

A large body of literature is showing that plant–microbe (fungi and bacteria) interactions are largely mediated by root exudates.[*e.g.* 28,51] For instance, the growth and branching of germinating arbuscular mycorrhizae fungal hyphae has been shown to be facilitated by the presence of flavonoids exuded in the soil.[52] Moreover, the carotenoid-derived terpenoids strigolactones have been shown to fuel cell proliferation, spore germination, and branching in a variety of mycorrhizal species.[52,53] For instance, the activity of the ethyl-acetate extracts of *Lotus japonicus* containing strigolactones was demonstrated by measuring the hyphal branching patterns in germinating spores of the arbuscular mycorrhizal fungus *Gigaspora margarita* using standard Petri dishes bioassays.[53] Similarly, flavonoids in root exudates have been shown to help recruit nitrogen fixing and growth promoting bacteria (*e.g.* Rhizobia) within the rhizosphere of several legumes.[54] Finally, specific root exudates might serve to attract plant-growth-promoting bacteria (PGPB) within the rhizosphere.[55] These root exudates might include carbohydrates and amino-acids, including the primary metabolite tryptophan.[56] Recent evidence also shows that benzoxazinoids released by maize roots may also serve as attractants for PGPB.[57]

4.4 *Interaction between Plants and Herbivore-killing Predators and Pathogens*

Plants can also use root exudates to attract predators to the sites where the roots are being attacked by herbivores.[58] For example, using belowground olfactometers or Petri dishes bioassays it was shown that soil-dwelling entomopathogenic nematodes can follow the concentration gradient of a multitude of compounds.[59] Indeed, a combination of behavioural assays and metabolomics analyses has revealed the role for root-produced volatiles in the foraging success of entomopathogenic nematodes.[34,36a,60] In most of these cases, the chemical cues mediating plant–entomopathogenic nematode interactions is of volatile nature. For instance, maize and citrus roots have been shown to release the terpenoids (*E*)-β-caryophyllene, and pregeijerene, respectively.[34,36a,60] Because of the ephemeral nature of these compounds, sampling them directly from the soil matrix has proved to be highly challenging. Therefore, to date, the bulk of the work on the volatile root exudates has been to measure them directly from freshly ground roots placed in headspace vials, and the volatile compounds, sampled using SPME and analysed using gas-chromatography coupled to mass-spectrometry. Some efforts have also been devoted to sample the air directly from the soil[36a] but this requires that the volatile compound is concentrated enough so to travel though the headspace of the soil matrix and readily adsorbed on the sampling polymer (see discussion on this topic above).

5. Future Venues for Root Exudate Research

5.1 *Root Exudates to Increase Crop Yield*

Since it has been discovered that plants influence their environment *via* primary and secondary metabolites, researchers have envisioned the possibility to use this knowledge to enhance crop yield and food security.[61] Such attempts have also involved the use of belowground exudates. For instance, the exudation of phenolics and terpenoids usually results in strong antimicrobial and antiherbivore effects,[41a] or can attract root pest antagonists.[41b] Root cap exudates have shown detrimental effects on root pathogens, or induce quiescence to plant parasitic nematodes.[62] Key root metabolites exudated in the rhizosphere can severely inhibit the host finding behaviour of insect root herbivores,[63] or inhibit the colonization of crop roots by hemiparasites.[64] Using diversified crop management systems, a subsequent crop planting can benefit from the legacies of the previous crops, if the previous crop had produced siderophores to enhance the availability of insoluble Fe in soil.[65] These examples are only a few among many possibilities to use root exudates in pest management and crop health. Nonetheless, while the ecological mechanisms mediated by root exudates hold great promise when tested in controlled conditions, it is recognized that the natural environment is much more complex, making the output of field trials highly variable,[66] and additional research using field trials under different conditions are needed to fully assess the efficacy of root exudates for increasing crop yield.

5.2 *Root Exudates Mediating Key Ecosystem Properties*

It is clear that by releasing exudates in the rhizosphere, plants modify the microbial and rhizosphere communities.[51a] In turn, changes in rhizosphere microbial communities will impact key ecosystem properties such as soil aggregation, nutrient cycling, as well as C and N cycling and greenhouse gas emissions.[56,67] For instance, increasing interest is focusing on the role of biological nitrification inhibitors root exudates, such as different fatty acid derivates, to retain N in soil, and in turn, increase crop yield.[68] Along these lines, a more recent surge of research is aiming to strengthen soil foodwebs, *via* the input of root exudates, which in turn would also ameliorate soil organic matter stabilization.[69] Therefore, despite taking place on a relatively small scale, the cumulative effects of root exudation are of global importance. Thorough estimations of the quality and quantity of root exudates released by plants growing in different ecosystems is thus of primary importance for untangling the complex biogeochemical processes occurring in the rhizosphere and their feedback loops. Accordingly, the effects of root exudates for predicting or mitigating the effects of climate change on ecosystems are now being recognized[*e.g.* 70a,b,c] but further research is needed to understand the context-dependency of root exudation in term of climate, soil and vegetation control.

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