Special Issue: Unraveling the Secrets of the Rhizosphere

Review

New Methods To Unravel Rhizosphere Processes

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Root-triggered processes (growth, uptake and release of solutes) vary in space and time, and interact with heterogeneous soil microenvironments that provide habitats for (micro)biota on various scales. Despite tremendous progress in method development in the past decades, finding a suitable experimental set-up to investigate processes occurring at the dynamic conjunction of biosphere, hydrosphere, and pedosphere in the close vicinity of active plant roots still represents a major challenge. We discuss recent methodological developments in rhizosphere research with a focus on imaging techniques. We further review established concepts that have been updated with novel techniques, highlighting the need for combinatorial approaches to disentangle rhizosphere processes on relevant scales.

Investigating the Rhizosphere

The conceptual term ‘rhizosphere’ is commonly used to describe the volume of soil influenced by roots but may also be extended to describe root–soil interfaces that can be separated into ecto-rhizosphere (rhizosphere soil), rhizoplane (root surface), and endo-rhizosphere (inner root). Rhizospheres are complex and highly-dynamic environments where an immense number of interactions between roots, minerals, organic compounds, solutes, gases, and (micro)organisms drive the biogeochemical cycling of elements [1]. The interlinked multicomponent complexity of the rhizosphere requires experimental designs and measuring techniques capable of resolving processes on a wide range of scales (cm to sub- μm). Given the spatial resolution needed to investigate rhizosphere dynamics, and the unpredictability of field conditions, most studies seek to simulate field conditions in semi-artificial experimental set-ups. Instrumental limitations (e.g., immobility) also frequently constrain experiments to the laboratory. However, these can be at least partially overcome by analyzing *in situ* collected plant and soil samples (e.g., undisturbed soil columns of various size). We aim here to take a closer look at recently developed laboratory applications including destructive and non-destructive imaging, isotope and ‘metabolic’ techniques, to briefly summarize their main advantages, problems, and applicability in the field and to undisturbed field samples (Table 1), and highlight promising combinatorial approaches (Table 2). Developments in root phenotyping and mathematical modeling are not within the scope of this review. Elaborate reviews and methodological summaries of phenotyping and mathematical modeling as well as of well-established experimental set-ups in rhizosphere research can be found elsewhere [2–6].

Accessing the Rhizosphere and Rhizosphere Traits

Sampling Rhizosphere Soil – Growth Designs

Three major designs of growth containers can be distinguished that enable a wide range of investigations and are frequently combined with new applications. Pot/compartments systems (i) [7–9] provide the simplest differentiation between bulk soil and rhizosphere samples (soil and

Trends

Visualization of 2D and 3D rhizosphere processes via chemical imaging, microbial imaging, and non-invasive imaging has significantly enhanced our knowledge of rhizosphere processes across a range of scales from cm to sub- μm .

The use of stable isotopes in combination with complementary techniques such as mass spectrometry, high-throughput sequencing, nanoscale secondary ion mass spectrometry, and neutron radiography has great potential to unravel mechanisms of uptake, storage, and translocation of carbon and nutrients at plant–microbe–soil interfaces.

The importance of rhizodeposits in shaping the rhizosphere has been increasingly put forward, leading to the development of novel experimental approaches that enable root exudation sampling from soil-grown plants.

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roots). Horizontal [10] or vertical [11,12] root-mat rhizoboxes (ii) offer the possibility of rhizosphere sampling in well-defined distance from the root mat (e.g., using thin sectioning [13]) to investigate biogeochemical gradients. Rhizotrons of various sizes (iii) positioned at a 30–45° angle during plant growth [2] are used for investigations of single roots along the root axis and are usually chosen when 2D imaging techniques are applied (see below). While taking rhizosphere samples enables us to analyze biogeochemical changes induced by roots and (micro)biota *ex situ*, increasing focus in the past years has been on visualizing rhizosphere processes *in situ* as well as on investigating rhizodepositions that trigger the latter.

Sampling Root Exudates

Significant advances in the analytical sector allow us to move from targeted compound analysis to root exudate fingerprinting, thereby providing novel in-depth information on the compound composition in its entire complexity that triggers many rhizosphere processes (for more details see the review paper on metabolomics by Bouwmeester and van Dam in this issue). However, the impact of high-end analytical approaches is determined by our ability to identify relevant compounds as well as by our exudate sampling techniques. To avoid alterations in the exudation profile as a result of sorption to the soil matrix and microbial decomposition, the majority of studies on root exudates have been and still are carried out in hydroponic systems. While the altering effect of non-sterile conditions on the exudation profile is well investigated [14], the few comparisons between soil and nutrient solution culture as a growth medium showed a significant, soil-dependent difference in compound concentrations released by roots [9,15]. Although hydroponic set-ups allow (relatively) simple collection of root exudates, the relevance of obtained results remains questionable with respect to field conditions.

Soil-based exudate sampling approaches targeting the entire root system to date include (i) the ‘quick and dirty’ approach where plants are grown in soil-filled pots followed by careful root washing (soil removal) and hydroponic exudate sampling [9,15]; (ii) rhizobox growth and hydroponic sampling [12]; (iii) a novel method using rhizoboxes in combination with a root exudate-collecting (REC) tool [9,15] where unaltered exudates are repeatedly and non-destructively collected. In addition, (iv) single roots or root segments from plants grown in rhizotrons can be sampled by applying agar sheets, specialized resins, or filter papers (this allows only qualitative and semi-quantitative comparison of compounds [16–18]) or (v) excavation/cuvette-hydroponic-sampling can be applied [19] (see also Table 1). To date a thorough methodological comparison of exudate sampling techniques to evaluate the effect of different experimental set-ups on the quantity and quality of rhizodepositions in the laboratory/greenhouse as well as in the field is still missing.

Sampling Rhizosphere Soil Solution

Micro-suction cups (MSC) are a powerful tool to investigate biogeochemical changes in rhizosphere soil solution at a high spatial resolution ($\mu\text{L}/\text{mm}$) [15,20,21]. However, they are not suited for the collection of unaltered root exudates or exudation rates because immediate interactions with the soil matrix (sorption) as well as microbial activity (the decomposition and release of microbial metabolites) will change compound composition. In a novel approach, microcapillaries coupled with capillary electrophoresis/inductively coupled plasma mass spectrometry (ICP-MS) were successfully used to monitor the effect of organic acids on depleted uranium in the rhizosphere of white lupin on a $\text{nL}/\mu\text{m}$ scale (single-cell sampling and analysis, SiCSA) [22]. Recently, microdialysis has been introduced as a novel method to sample diffusive fluxes of metals [23,24] and nitrogen (N) species [25,26] from soil. While the miniature design enables installation with minimal disturbance and highly localized sampling suitable for rhizosphere dimensions, accurate calibration and deduction of external soil solution concentrations is problematic due to the inhomogeneous physical characteristics of the porous soil matrix [26] (Table 1).

Table 1. Main Advantages and Limitations of Methods Discussed and their Applicability in the Field as well as to Structurally Intact Field Samples

Methods	Scale	Advantages	Limitations	Applicability to Field Samples ^a	Applicability in the Field
<i>Sampling Root Exudates</i>					
Hydroponic growth and sampling	Single root segment – root system	Controlled, easily manipulated conditions (i.e., nutrient/pollutant availability, microbial decomposition); no interaction with the soil matrix	Artificial growth conditions; comparability to soil conditions questionable	No	No
Soil growth – hydroponic sampling	Single root segment – root system	Soil-grown plants combined with a simple exudate collection technique	Potential artifacts due to root damage during excavation and washing	Yes	Yes
Soil growth and sampling Rhizobox/REC	Root system	Non-destructive and repeated sampling; soil growth	Semi-natural growth conditions; complicated set-up; exudate recovery needs to be tested	No	No
<i>Sampling Rhizosphere Soil Solution</i>					
Microsuction cups (MSC)	μl to ml	Sampling of <i>in situ</i> concentrations; wide range of sample volume depending on MSC size	Concentration and sample volume dependent on soil water content; precise positioning difficult; MSC material-dependent artifacts (retention, adsorption)	Yes	Yes
Single-cell sampling and analysis (SiCSA)	nl to μl	Highly localized sampling; sampling in nl range	Slow-throughput; representation and scaling up depend on number of replicates	Yes	No
Microdialysis	μl to ml	Localized sampling; minimal disturbance during <i>in situ</i> installation	Difficult calibration and interpretation of results	Yes	Yes
<i>Investigating Plant–Microbe Interactions</i>					
Amplicon sequencing	Selected groups – whole community	Targeted gene detection; parallel analysis of high sample number	Destructive sampling; spatial information depending on sampling accuracy	Yes	No
Metagenomics	Whole community	Untargeted gene screening; assessment of diversity and functional potential	Limited number of replicates; computational power required	Yes	No
Metatranscriptomics	Whole community	Untargeted gene screening; assessment of gene expression and metabolic pathways	Limited number of replicates; computational power required; comparison to metagenomics data required		
Metaproteomics	Whole community	Untargeted protein screening; assessment of protein expression	Computational power and comparison to metagenomic/transcriptomic data required	Yes	No
Metabolomics	Whole community	Targeted or untargeted metabolite detection; assessment of metabolic pathways	Vast number of metabolites; limited number of replicates; computational power required	Yes	No
<i>Isotopes</i>					
Stable isotope fractionation	Plants, soil, microbial community	No heavy isotope-enriched substrate needed; assessment of the relative importance of specific physiological/biogeochemical reactions	Detection of minimal changes in isotopic ratio; high-end analysis required	Yes	No
Stable isotope enrichment	Plant, soil, microbial community	Target analyte partitioning within and across different biomes	Heavy isotope-enriched substrate and high-end analysis required	Yes	Yes
Stable isotope probing	Selected groups – whole community	Identification and quantification of active microorganisms with specific functions	Heavy isotope-enriched substrate and high level of enrichment	Yes	No

Table 1. (continued)

Methods	Scale	Advantages	Limitations	Applicability to Field Samples ^a	Applicability in the Field
			needed; high-end analysis required		
<i>Isotope Imaging</i>					
NanoSIMS	nm	Highly resolved (nanoscale) and sensitive measurement of isotope distribution	Low number of samples measured on small field of view; expensive; limited accessibility	Yes	No
Neutron radiography	mm to dm	Repeated and non-invasive quantification of water fluxes	Accuracy dependent on soil water content and texture	Yes	No
Phosphor imaging (Autoradiography)	cm to dm	Tracing and visualization of nutrient/pollutants and photosynthates	Laboratory suitable for radioisotope handling or field work permission required; image-blurring with high activity isotopes	Yes	Yes
<i>Chemical Imaging</i>					
DGT	mm to cm	Diffusive-based sampling; spatial distribution of labile solutes; multi-element mapping	Integrated snapshot image, sampled concentration does not directly correspond to soil solution concentration; *sufficient homogeneous soil contact and water content required	(Yes) if*	(Yes) if*
Planar optodes	mm to dm	Diffusive-based sensing; spatial distribution of analyte concentration; repeated measurements over time (time-lapse studies)	Limited number of simultaneously measured analytes (max. 2–3) *sufficient homogeneous soil contact and water content required	(Yes) if*	(Yes) if*
Zymography	mm to dm	Diffusive-based sampling; spatial distribution of enzyme activity	Integrated snapshot image; limited number of simultaneously measured target enzymes; potential image blurring; targets only enzymes in the soil solution phase; *sufficient homogeneous soil contact and water content required	(Yes) if*	(Yes) if*
<i>Microbial Imaging</i>					
FISH	Single microbial cell	Identification; quantification; and visualization of native cells <i>in situ</i>	Fixation (i.e., killing) of microbial cells; interference of autofluorescent plant and soil material	Yes	No
Nafion-GFP	Single microbial cell	Non-destructive detection and visualization of cells <i>in situ</i> and <i>in vivo</i>	Inoculation of genetically modified microbial cells required; artificial substrate	No	No
<i>Non-Invasive Imaging</i>					
X-ray μ CT	nm to dm	Visualization of root and soil features <i>in situ</i> ; variable sample size (up to large soil columns)	Limited sample number; long scan times affecting (soil) biota	Yes	No
Synchrotron tomography	nm to cm	Visualization of root and soil features <i>in situ</i> ; short scan time; high spatial resolution up to nanoscale	Limited sample size and number; expensive; limited accessibility	Yes	No

^aUndisturbed soil cores with or without roots.

Investigating Plant–Microbe Interactions

Rhizosphere microorganisms (archaea, bacteria, fungi) are involved in a myriad of processes that (in)directly affect plant health. Consequently, the study of root-associated microbiomes has received a great deal of attention since high-throughput sequencing (HTS) allowed highly resolved screening (presence, relative abundance, potential activity and function) and monitoring of the spatiotemporal dynamics of rhizosphere microorganisms. In particular, comparative analyses of microbial populations associated with microenvironments such as rhizosphere, rhizoplane, and endosphere are frequently performed that require the recovery of sufficient amounts of inhibitor-free DNA/RNA as well as a thorough evaluation of separation strategies (e.g., washing, sonication [27]). Owing to the increasing amount of studies we briefly highlight the potential of several methodological approaches that have recently been applied in rhizosphere research: (i) amplicon gene sequencing of conserved marker genes (e.g., 16S rRNA) investigates the presence (DNA) and potential activity (reverse-transcribed RNA) of microbial community members [28,29]; (ii) metagenome sequencing of DNA obtains information on the genomic diversity and physiological potential of whole microbial communities [30,31]; (iii) metatranscriptome sequencing of reverse-transcribed mRNA assesses gene expression in relation to reference (meta)genomes, and thus identifies potentially metabolically-active microbes [32,33]. Further techniques of the ‘meta-omics’ consortium do not rely on DNA/RNA sequencing but on state-of-the-art MS of extracted proteins/metabolites: (iv) metaproteomic profiling measures expressed protein abundance, giving information on the functional activity of microbial communities and plants [34,35]; (v) metabolomic profiling detects and quantifies the abundance of molecules involved in metabolic (re)actions of microbial communities and/or plants [36,37]. For more detailed information on HTS and ‘meta-omics’ in rhizosphere research we refer readers to the reviews in this issue on metabolomics (Bouwmeester and van Dam) and the rhizosphere microbiome (Poole *et al.*), as well as to other recent reviews [38,39].

Tracing Rhizosphere Processes with Isotopes

Stable and radioactive isotopes represent exceptional markers to trace the uptake and release of carbon (C), nutrients and pollutants, their storage, and related transfer processes between plants, soil, and associated microorganisms. While the use of radioisotopes in combination with novel methods is mainly limited due to safety constraints, improved analytical instrumentation nowadays allows widespread application and combination of stable isotopes with advanced techniques. Traditional stable isotope experiments require a stable isotope enriched substrate or atmosphere, most commonly ^{13}C or ^{15}N , that is introduced into the plant–soil system to trace its partitioning and fate (e.g., [40]). In addition, biological stable isotope fractionation (based on natural abundance) within plant tissue and/or soil can be used to follow N fixation and assimilation [41], to differentiate between different Fe and Zn acquisition and uptake mechanisms [42,43], and to help in deciphering phosphate transformations using $\delta^{18}\text{O}$ in the soil–plant system [44,45]. The fate of assimilated substrates has been analyzed in bulk plant and soil samples via isotope ratio MS [43,44,46] and in rhizosphere-associated microorganism via stable isotope probing (SIP) of phospholipid fatty acids (PLFA) [47,48] or nucleic acids (DNA/RNA) [49,50]. Novel combinations of SIP with microbiological methods aim to analyze metabolically active microorganisms in more detail via mRNA-SIP [51] or protein-SIP [52]. The recent advent of HTS of extracted and fractionated DNA/RNA samples allows previously unmatched in-depth analysis of metabolically-active cells that have taken up the enriched isotopes, as shown for root exudate-feeding bacteria in the endosphere and rhizosphere of rice [53]. In addition, position-specific labeling [46] and dual-labeling approaches [54] (see also Table 1) have been applied to further elucidate soil organic carbon turnover dynamics. In the future, combinatorial approaches of HTS or ‘meta-omics’ with stable isotope techniques will be necessary to unravel root–microbe interactions because neither amplicon sequencing of transcribed functional genes nor metatranscriptomic/proteomic profiling of genes/proteins necessarily reflect microbial activity and nutrient turnover rates [55,56].

Visualizing Rhizosphere Processes

Isotope Imaging

Bulk measurements of stable isotopes in destructively sampled roots and associated soil (see above) are limited in their spatial resolution and may not identify processes *in situ* that occur on a biologically relevant scale [57]. Recently, nanoscale secondary ion MS (NanoSIMS) has been introduced to rhizosphere research, and this allows next-level imaging of isotope distributions on resolutions beyond individual cells (50–100 nm) [58]. In general, a primary ion beam (Cs^+ , O^-) is focused on soil or root samples to erode the element of interest and to produce secondary ions that are collected according to their atomic mass. The wide range of elements that can be mapped (H, C, N, O, P, Si, S, Fe, and others) at high resolution make the NanoSIMS an indispensable instrument for unraveling plant–microbe interactions in the rhizosphere at a submicron level. The preparation of rhizosphere samples for NanoSIMS analysis typically includes either resin embedding [59] or detachment of microorganisms from soil particles [60], and remains a crucial issue that must be evaluated carefully. Pulse-labeling experiments in combination with NanoSIMS now allow us to trace the uptake, storage, and translocation of stable isotopes, as well as the competition thereof among individual players (plant–microbe resource competition [57,61]). As such, nutrient transfer between arbuscular mycorrhizal fungi (AMF) and host plants was recently visualized *in situ* using NanoSIMS (Figure 1A) [62,63]. The combined use with techniques of microbiology (^{13}C -PLFA, 16S rRNA gene microarray) showed that nutrients translocated by AMF strongly influenced bacterial community structure and substrate use. Very recently, root exudates were shown to accelerate mineralization of rhizosphere carbon by detaching cations from metal-organic complexes [64]. NanoSIMS studies further revealed that bacteria of the same species exhibit a surprising heterogeneity in metabolic rates [65,66], a finding that has implications on all aspects of elemental cycling in the rhizosphere. Despite its great potential, the number of NanoSIMS applications has been mainly limited by the number of instruments worldwide (35 ± 5).

Another recent methodological development is the use of cold neutron radiography (NR) in combination with deuterated water (D_2O) to repeatedly and non-invasively quantify water fluxes in roots and rhizosphere soil. A neutron beam is guided through the samples, and the intensity/attenuation of the transmitted beam behind the sample is detected by a scintillator plate (neutron detector) and then converted into grey-value images by a CCD (charged-coupled device) camera [67]. Comparing different growth media, NR was found to perform best in sand or sandy soil owing to its low concentration of high neutron-absorbing elements, and the minimum detectable root thickness was found to be 0.2 mm [68]. In addition, NR was found to be limited at high and low soil water contents as a result of increased neutron scattering and root dehydration, respectively. Applications of NR with D_2O revealed that water uptake is not uniform along roots, with higher radial fluxes occurring at the proximal than at the distal root segments (Figure 1B) [69]. Furthermore, using NR it was repeatedly shown that, in contrast to previous assumptions, soil water content remains higher in the vicinity of roots during drying events and that the rhizosphere exerts a non-uniform hydrophobicity along the roots during re-wetting [70,71]. Despite not being a novel technique, rhizosphere autoradiography (imaging of radioisotopes) has recently been updated by replacing traditional X-ray films by phosphor-imaging, which is based on photoinduced chemiluminescence and is about two orders of magnitude more sensitive to β - and γ -rays. Consequently, ^{14}C phosphor-imaging has been used to visualize the allocation of photoassimilates into roots and associated rhizosphere soil [72].

Chemical Imaging

Originally developed for solute speciation in aquatic systems, diffusive gradients in thin films (DGT) has also been used to determine nutrient and pollutant availability in soils [73,74]. More recently, DGT has also been adapted for 2D imaging of the distribution of labile chemical species in the rhizosphere. Typically consisting of a polyacrylamide hydrogel layer that contains a binding

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Figure 1. Imaging of Processes at Root-Microbe-Soil Interfaces on Various Scales. (A) NanoSIMS images revealing the uptake of photoassimilated ^{13}C into arbuscular mycorrhizal hyphae colonizing the wheat root cortex. Greyscale $^{12}\text{C}:^{14}\text{N}$ image and corresponding $^{31}\text{P}:^{12}\text{C}$ and $^{13}\text{C}:^{12}\text{C}$ images given in hue saturation intensity color-code show the location and enrichment (blue: low; pink: high) of naturally abundant (^{31}P) and experimentally enriched (^{13}C) stable isotopes; scale bar, 10 μm (adapted from [62]). (B) Time-series of neutron radiographs showing the flow of deuterated water (D_2O) into the proximal parts of lupin roots at night. Brighter colors in lateral roots (i.e., higher $\text{D}_2\text{O}:\text{H}_2\text{O}$ ratio) visible 20 min after labeling indicate that radial transport of D_2O into the taproot was significantly slower than into the lateral roots (adapted, with permission, from John Wiley & Sons [69]). (C) Visualization of O_2 , Fe, As, and Ni in the rhizosphere of wetland rice using a combined optode-DGT sensor. Relatively high O_2 concentrations (pink) correlated with high fluxes of Fe and As mainly around root tips (white), while Ni concentrations were depleted in the vicinity of roots (yellow) (with permission from ACS Publications [88]). (D) Zymograph showing *N*-acetyl-glucosaminidase (chitinase) activity in rooted rhizobox 10 days after lupin shoots were removed. High levels of chitinase (red color) were locally restricted to lateral root tips (adapted, with permission, from Springer Science and Business Media [95]). (E,F) Microbial colonization of rhizoplanes and associated soil particles using confocal scanning laser microscopy. (E) *In situ* visualization of native bacteria (green) on the rhizoplane of rice (red) via CARD-FISH; scale bar, 20 μm (adapted, with permission, from Oxford University Press [101]). (F) *In vivo* detection of

agent overlaid by a protective membrane, this set-up allows analytes to diffuse towards the resin gel where they are immediately bound. Deployed on plant roots grown in soil-filled rhizotons for several hours and subsequently dried and analyzed by laser ablation ICP-MS, DGT is a promising tool to visualize gradients of multiple solutes along the root axis (Figure 1C). Examples for the successful application of DGT imaging are the comparison of phosphate depletion of two *Brassica napus* cultivars differing in P efficiency [75] and the investigation of solute mobilization around white lupin cluster roots [76]. DGT results should only be interpreted as time-averaged flux into the sampler, or as time-averaged concentration at the sampler surface that generally does not correspond to soil pore water concentrations (see also Table 1); nevertheless, with a sample application area size of several cm² and a typical spatial resolution of 300 × 300 μm, DGT allows a relative comparison of concentration/fluxes within and between images [77].

Another promising chemical imaging technique comprises reversible optical sensors (planar optodes) that are capable of real-time measurement of changes in analyte concentrations based on photoluminescence [77]. The planar optode set-up consists of an analyte-specific photoluminescent indicator entrapped in a permeable matrix coated onto a plastic foil which is then, as in DGT, applied to rhizospheres at the surface of rhizotrons. The imaging principle is based on the measurement of fluorescence intensity or phosphorescence lifetime following light excitation (usually by high-power light-emitting diodes, LEDs), with the former being recorded by either CCD or CMOS (complementary metal oxide semiconductors) cameras and the latter using commercially available digital single-lens reflex (DSLR) cameras [77]. Depending on the chemical stability of the sensor (a few weeks up to several months), planar optodes offer the unique opportunity to monitor dynamic changes of the analyte of interest in the vicinity of roots during plant growth *in situ*. The list of sensors applied in rhizosphere research so far includes O₂ [78–81] (Figure 1C), pH [82–85], pCO₂ [86], and NH₄ [87]. Sensors specific for temperature, H₂S, Cl[−], or NO₃[−] have not been applied in terrestrial rhizosphere research to the best of our knowledge. The choice of the sensor needs to be considered carefully because many optode indicators are sensitive to changes of temperature, ionic strength, and pH [77]. The simultaneous application of multiple optode sensors [80], as well as the combination of planar optodes with DGT (Figure 1C) [79,88], neutron radiography, or fluorescent proteins [84], has significantly improved our understanding of plant–soil and plant–plant interaction in the rhizosphere (see also Table 2).

The principle of using agar/agarose gels or filter papers in combination with dye indicators to map rhizosphere traits dates back to the 1980s and 1990s [89–91], and has been adapted and improved over the past decades, including enzyme mapping among others (e.g., [92]). The recently presented zymography technique (Figure 1D; Table 2) deploys enzyme substrate-loaded agarose gel sheet on a soil/root surface to visualize enzyme activities in the rhizosphere. After deployment, enzyme activity is either determined by staining and scanning the remaining substrate (protease and amylase activity [93]) or by measuring the fluorescence of the reaction product by photographing the degree of excitation with UV light (acid and alkaline phosphatase activities [94]; cellulose and chitinase activities [95]). While planar optodes can be used for repeated imaging (reversible sensors) over several days/weeks, DGT and zymography only provide an integrated snapshot image of experimental conditions during the deployment time (Table 1).

GFP-labeled inoculated *Pseudomonas fluorescens* cells (green) on surfaces of a lettuce root (grey) and Nafion particles (red); scale bar, 150 μm (adapted, with permission, from Taylor & Francis Ltd [107]). (G) High-resolution imaging of the wheat rhizosphere via synchrotron tomography showing the rhizoplane with root hairs (green), associated rhizosphere soil (brown), and water-filled pore space (blue) (adapted, with permission, from John Wiley & Sons [110]). (H) Non-invasive monitoring and reconstruction of bean root system architecture development via X-ray μCT. The distribution of mature roots (dark red) at the soil surface correlated with lowest simulated soil water potentials (light green) (adapted from [112]). Abbreviations: CARD-FISH, catalyzed reporter deposition combined with fluorescence *in situ* hybridization; DGT, diffusive gradients in thin films; Nano-SIMS, nanoscale secondary ion mass spectrometry.

Table 2. A Selection of Recent and Promising Method Combinations

Combined Methods	Main Findings	Potential for Future Rhizosphere Research	Ref.
DGT and planar optodes	Oxygen release and acidification by rice roots increased soluble Fe(II), Pb, and As due to slowed Fe(II) oxidation or due to pH-mediated desorption of Fe(II) from freshly formed iron plaque	Simultaneous monitoring of root activity (pH, O ₂ , cations) and its effect on spatial (im)mobilization of nutrients/pollutants in the rhizosphere	[88]
Planar optodes and neutron radiography	Oxygen dynamics and depletion in white lupin rhizosphere are highly sensitive to water content distribution	Investigating the effect of root-induced changes in soil water content on soil O ₂ concentrations	[81]
Planar optodes and GFP	Degree of pH change in maize and bean rhizosphere was less pronounced when roots of both species were interacting than when roots were not growing in the vicinity of each other	Elucidating root–root interaction and its effect on rhizosphere chemistry	[84]
Zymography and FISH	Hotspots of bacterial abundance and phosphatase activity are spatially separated in barley rhizosphere	Verifying/falsifying the correlation of microbial and enzymatic spatial distribution at root–soil interfaces.	[124]
DNA-SIP and HTS	Continuous addition of labile carbon to soil stimulates native C loss and increases soil bacterial diversity	Obtaining highly resolved phylogenetic information on <i>de facto</i> metabolically-active microbes	[54]
X-ray μ CT and qPCR	Accumulation of pathogen DNA in soil induces the reduction of root number, volume, and surface area in wheat and oil-seed rape	Detecting and visualizing the impact of pathogens and PGPR on root health <i>in situ</i>	[123]

Abbreviations: DGT, diffusive gradient in thin films; FISH, fluorescence *in situ* hybridization; GFP, green fluorescent protein; HTS, high-throughput sequencing; SIP, stable isotope probing; PGPR, plant growth promoting rhizobacteria, qPCR, quantitative polymerase chain reaction; X-ray μ CT, X-ray microfocus computed tomography.

Microbial Imaging

Another technique which can provide snapshots of rhizosphere processes driven by microorganisms is fluorescence *in situ* hybridization (FISH). FISH represents a powerful tool that combines the phylogenetic identification of single prokaryotic cells with their quantification, localization, and visualization via fluorescent microscopy [96]. Oligonucleotide probes labeled with a fluorophore that match the 16S rRNA sequence of the microorganism of interest are mainly used to obtain a distinct fluorescent signal per individual microbial cell. Although microbial activity cannot be inferred from signal intensity [97], the *in situ* detection of microbial colonization of root surfaces is of fundamental importance to our understanding of root–microbe interaction and co-existence. Until recently, detection rates suffered from weak signals and high background fluorescence of root tissue (Table 1), making the *in situ* localization of single cells associated with rhizoplanes difficult at best [98,99]. To overcome these limitations a FISH modification drastically increasing signal intensities (catalyzed reporter deposition combined with fluorescence *in situ* hybridization, CARD-FISH [100]) has been introduced to rhizosphere research, and this has allowed the precise localization of native phylogenetic groups of archaea and bacteria on rice rhizoplanes and associated rhizosphere soil (Figure 1E) [101,102]. Even though the number of applications is still limited, CARD-FISH has the potential to help us to monitor microbial presence [103] and related colonization strategies of rhizoplanes and endospheres [103,104], to verify the actual presence of microbial phyla identified as important root colonizers via sequencing techniques [105], and to identify potential hot spots of root–microbe interactions. In addition, a CARD-based approach using gold nanoparticles (Gold-FISH [106]) allowed *in situ* identification and localization of *Rhizobium leguminosarum* cells on rice roots via

scanning electron microscopy. In another novel experimental approach, traditional inoculation with GFP-labeled bacteria was combined with an artificial substrate (Nafion, ‘transparent soil’) to overcome soil opacity and to avoid destructive sampling and chemical fixation (i.e., killing) of microbial cells necessary for FISH (Table 1). Thus, the spatial interaction of *Pseudomonas fluorescens* with roots and ‘soil’ particles was observed non-destructively, *in situ*, and *in vivo* via fluorescence microscopy (Figure 1F) [107].

Non-Invasive Imaging

The potential to characterize root system architecture and associated plant–soil interactions on biologically relevant (sub) μm scales in 3D has made radiation-based techniques such as X-ray microfocus computed tomography (X-ray μCT) or synchrotron tomography (SR-CT) a current focus in rhizosphere research [108]. We only briefly highlight recent applications; more comprehensive reviews on imaging methodologies capable of visualizing abiotic and biotic interactions in the rhizosphere have been published recently [4,108,109]. To obtain rhizosphere data, plants cultivated in soil columns of different diameters (mm to dm) are introduced into the scanning device and irradiated (hours to minutes). The spatial resolution of a scan is a direct result of sample size, in other words the analysis of μm -sized structures requires relatively small samples (approx. 1 cm in diameter). In a recent study, soil tubes 5 mm in width have been used to successfully visualize fine wheat root-hairs colonizing macro-pores *in situ* [110] or infiltrating soil aggregates via synchrotron technology (Figure 1G) [107]. Although SR-CT is regarded as being superior to commercially available μCT in several aspects such as scan time and spatial resolution [111], accessibility and cost make μCT scanners the more frequently applied instrument to study rhizosphere processes. Consequently, X-ray μCT has recently been used to (i) monitor the growth of mature root systems (Figure 1H) [112,113], (ii) study root–soil and root–root interactions [114–116], and (iii) gain insights into hydraulic properties of the rhizosphere [117–119]. Low irradiation doses applied to plant–soil interfaces were reported to neither influence root growth [120] nor microbial cell number, community structure, or potential activity [121,122]. Thus, X-ray μCT may be well-compatible with studies of rhizosphere ecology. The potential inherent in such combinatorial approaches was shown very recently by visualizing plant–pathogen interactions *in situ* at high spatial resolution for the first time [123]. In conclusion, the application of radiation-based imaging holds great potential to falsify or verify some of our conceptions of rhizosphere processes, and thus to disentangle the complexity of plant–microbe interactions.

Concluding Remarks

Advanced techniques to identify, trace, and visualize rhizodeposits and the resulting biogeochemical changes have significantly contributed to our understanding of rhizosphere dynamics. Despite the immense potential of the presented methods, the acquisition of data is often highly laborious and expensive, and is thus restricted to only few time-points and analysis spots. Consequently, critical interpretation of obtained results is indispensable. Regarding visualization techniques, the recent increase of spatial resolution in which rhizosphere processes can now be investigated typically coincides with a decreased field of view (FOV). Therefore, our results and conclusions can easily be biased by the selection of unrepresentative FOVs and an insufficient number of replicates. Upscaling from (sub) μm FOVs to single roots, whole root systems, or even field-scale is another important issue that needs to be more thoroughly addressed. In addition, there is also a strong need for improved image analysis (including adequate statistics) to allow and support quantitative conclusions.

Many of the presented methods are not directly applicable in the field but can be used to analyze (structurally intact) field samples (e.g., amplicon sequencing, ‘meta-omics’, isotope techniques, microbial imaging; see also Table 1) to help understanding potential differences and congruencies of rhizosphere processes under controlled laboratory/greenhouse conditions and in the

Outstanding Questions

How comparable are rhizosphere processes observed in laboratory experiments to data obtained under field conditions?

How can the simulation of field conditions in the laboratory be further optimized to improve the assessment of rhizosphere processes on biologically relevant scales?

How can the applicability of methods to the field or undisturbed field samples be improved?

What are the best combinatorial approaches to disentangle all active participants (not necessarily the most abundant ones) involved in rhizosphere nutrient cycling (plants, fungi, invertebrates, protozoa, bacteria, archaea)?

How can results obtained on (sub) μm scale best be upscaled and/or translated to root-system or landscape scale?

field. However, chemical image techniques in particular struggle with the inhomogeneity of soil surfaces *in situ*, which hampers accurate application in the field or to structurally intact field samples.

While each of the presented techniques on their own provide important new insights, we believe that particularly the combined use of different methodological approaches (see also Table 2 and Outstanding Questions) will be most promising in shedding light onto the great number of yet unrevealed processes at the root–microbe–soil interface.

Acknowledgments

E.O. is currently funded by the Austrian Science Fund (FWF; grant P 25942-N28). H.S. has been supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme.

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