

New methods for new questions about rhizosphere/plant root interactions

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New methods for new questions about rhizosphere/plant root interactions

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Abstract In this opinion paper we review recent methodological developments underpinning the study of roots, the rhizosphere and interactions affecting soil functions, and explore new understanding resulting from these advances. We focus on methods that have improved our understanding of rhizodeposition, rhizosphere enzymatic processes and root growth, water and nutrient acquisition at several levels. Finally, we suggest that the future will require new methods that continue to overcome the difficulties posed by the opacity of soil, can scale results spatially and temporally, and integrate multiple aspects of rhizosphere processes simultaneously.

Keywords Enzymology · Rhizosphere · Rhizodeposition · Root methods

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Introduction

The root/soil interface is highly complex and dynamic, and its study is bedevilled by the fact that soil is opaque and any disturbance alters the environment leading, in turn, to modifications to plant growth, microbial composition and rhizospheric processes. During the 30 years that Hans Lambers has been Editor in Chief of Plant and Soil, methods for studying roots and the rhizosphere have advanced substantially. Initially, methodological advances tended to focus on either the study of roots and their responses to soil properties or the composition and activity of microbes and other organisms in proximity to roots. More recently, attention has increasingly focussed on methods that allow characterisation of the multiple processes occurring at the rhizosphere/root interface. Ideally, such methods need to encompass the whole plant and soil system, be non-invasive and non-destructive at levels that are realistic, resolvable and meaningful for the processes being studied, and deliver information in three dimensions and in real-time to resolve spatial and temporal dynamics (for examples see reviews by Oburger and Schmidt (2016), York et al. (2016) and Vetterlein et al. (2020)).

New methods generally arise as a consequence of two interacting processes. First, scientific curiosity and specific questions drive the quest for a means of answering them. For example, determining how much of a crop's photosynthate supports root growth

and the acquisition of nutrients and water drove the early development of soil coring and root washing methods to measure root dry matter (Mengel and Barber 1974; Gregory et al. 1978). Similarly, questions about the distribution of roots in soil led to the adoption of trench profiling, radioactive tracer techniques, minirhizotron and core break technologies with a focus on root length (e.g. Majdi and Persson 1997). Second, advances in physics and biology provide new technologies that facilitate better means of answering old questions and stimulate the study of previously unanswerable questions. For example, ground penetrating radar and x-ray computed tomography both allow insights into the distribution of roots in soil, but the latter also allows study of root interactions with pores and particles (Schmidt et al. 2012). Similarly, the emergence of new questions about nutrient cycling for sustainable crop production and sequestration of soil carbon to mitigate climate change, the concept of soil as a holobiont (Finlay et al. 2020) and the role of roots as a key element of soil formation and functioning (Gregory 2022) have been spurred by the development and application of synchrotron techniques, high-throughput genomic pipelines and metabolomic technologies, and stable isotope methods.

The aims of this opinion paper are to: i) review recent methodological developments underpinning the study of roots, the rhizosphere and interactions affecting soil functions; ii) explore new understanding resulting from these methodological advances; and iii) suggest remaining issues for which new approaches are urgently required and the technologies that might facilitate answers.

Recent methodological developments

Root detection

Cabal et al. (2021) characterised techniques to identify roots of specific plants growing in stands or mixed communities as being either extraction, observation or inference detection methods. The current state and recent advances in these methods are outlined in Table 1.

In addition to these techniques, rhizoboxes are widely used in laboratory and glasshouse studies to give measures of roots and their activities (e.g.

George et al. 2002). This method is still widely used to assess root activities such as enzyme reactions and biochemical changes, but has the disadvantage that only a planar surface is viewed and root growth against a surface or mesh may introduce artefacts. In the laboratory, root observation has been transformed by the development of magnetic resonance imaging and x-ray computed tomography (Mooney et al. 2012) which have also imaged root/soil processes including root/soil contact, gap formation and the development of pores (Schmidt et al. 2012; Carminati et al. 2013; Tracy et al. 2015; Helliwell et al. 2017; Perelman et al. 2020). Radiation sources and detectors now allow μm resolution and new software permits root tracing through soil volumes (Phalempin et al. 2021).

Characterisation of rhizodeposition and impacts on microbial communities

A key driver of plant-microbe interactions is the release of organic compounds from living roots (rhizodeposition), delivering diverse substrate sources to the rhizosphere, impacting microbial species composition and the magnitude of biogeochemical cycling (Kuzakov and Blagodatskaya 2015). More recently, there has been rapid progress in understanding of functional consequences of these interactions, driven particularly in the context of research priorities to achieve sustainable food production and climate change mitigation/ adaptation (Jones and Hinsinger 2008; Philippot et al. 2013). These advances have been facilitated by rapid development of powerful approaches to characterise microbial communities in association with roots (high-throughput and lower costs of sequencing technologies for metagenomics, Schläeppli and Bulgarelli 2015), functional assays of microbial processes (isotopic tracing and transcriptomics, Nkongolo and Narendrula-Kotha 2020) and the increasing resolution of analytical methods to provide comprehensive quantification of plant and microbial metabolites (metabolomics, Kellogg and Kang 2020).

Observing the root-soil interface

Use of high-powered X-ray beams has allowed in-situ measurement of the intimate relationship between roots and soil including interactions between root

Table 1 Field methods for detecting plant roots

Means of detection		Current state	Issues to be resolved	References
Extraction	Method			
	Trenching and washed soil cores	Few advances in last two decades, but better drilling machines, washing techniques and optical detection and software have made data easier to collect. Such techniques have been adopted as one of the only means of studying deep roots	Disadvantages of the methods are widely understood	Smit et al. 2000 for summaries. Maeght et al. 2013 for deep roots
Observation	Minirhizotrons	Used with digital cameras these have advanced the study of fine roots especially root longevity and turnover.	Roots growing against a solid surface can produce artefacts; sequential coring and ingrowth cores are used to complement this method.	Rewald and Ephrath 2013; Arnaud et al. 2019.
	Tomography using electromagnetic waves	Ground-penetrating radar (GPR) has been successfully used to map tree roots non-invasively, to estimate root biomass and to characterise phenotypic variation in root traits	Accuracy is affected by root diameter, proximity to other roots and rock fragments, soil depth, root and soil water content and organic litter meaning that it requires careful interpretation	Butnor et al. 2003; Wasson et al. 2020; Lombardi et al. 2021; Cabal et al. 2021; Tanikawa et al. 2021
Inference	Electrical methods	Electrical capacitance has the longest history of use and has been widely used in hydroponics and pot experiments with wet soils	Determination of root traits and architecture is still problematic with species- and soil moisture-specific calibration required	Wu et al. 2017; Cabal et al. 2021; Cseresnyés et al. 2020; Gu et al. 2021

hairs and rhizosphere microporosity and soil water (Fig. 1; Keyes et al. 2013; Koebernick et al. 2017).

However, such high resolution (μm) is accompanied by very small (mm) sample size leading to growing

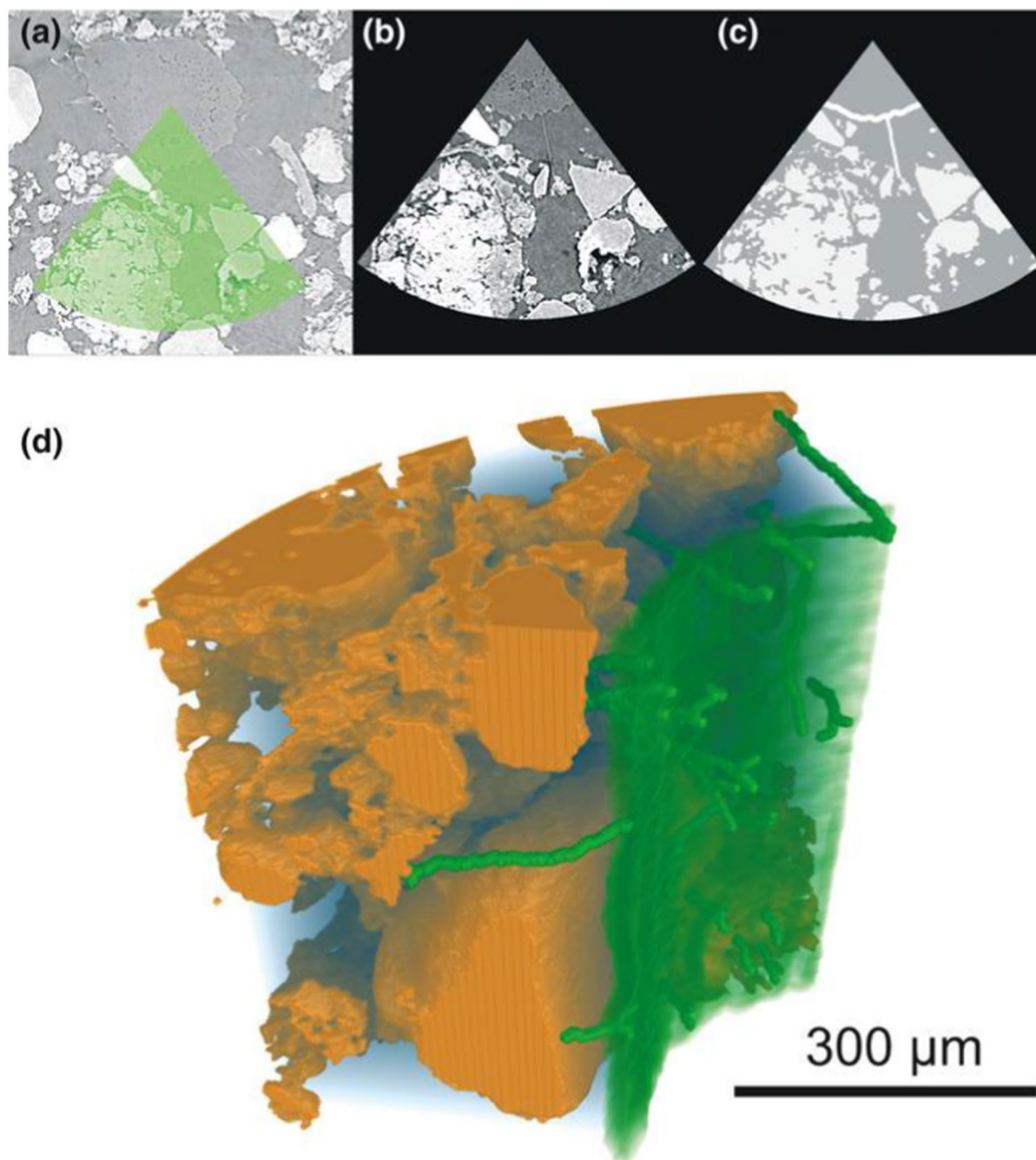


Fig. 1 High resolution synchrotron imaging of wheat root hairs growing in soil: Region selection and classification for rhizosphere simulation from synchrotron data. (a) A segment was defined, with centreline aligned with the centre axis of the root, (b) All other voxels are removed, leaving only the defined

segment, (c) root hairs, soil, fluid and root surface regions are individually defined using different discrete grey-levels, (d) a volume mesh is generated, with root hairs, soil, root surface and water defined separately. (Used with permission from Keyes et al. (2013))

environments that are unrealistic of natural ecosystems. Moreover, throughput is limited and the cost of sample processing and analysis is high. Other synchrotron techniques such as XANES (K-edge x-ray absorption near-edge structure spectroscopy) can observe the presence, concentration, and speciation of elements at the root soil interface providing a potentially powerful technique for understanding the dynamics of nutrients (Gillespie et al. 2009). Combined use of XANES and X-ray CT has shown the impact of soil compaction in the rhizosphere on the availability and speciation of elements (van Veelen et al. 2020). Similarly, NanoSims (nano-scale secondary ion mass spectroscopy) measures the presence of a range of elements at fine resolution, using measurement of secondary ionisation (Oburger and Schmidt 2016; Clode et al. 2009).

Elemental speciation and distributions in the rhizosphere have also been assessed using Scanning Electron Microscopy (SEM) with Energy-dispersive X-ray analysis (SEM-EDX), Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS), and confocal Raman spectroscopy (μ -Raman; Bandara et al. 2021). These techniques can resolve the difference between element distribution between root and soil, within pore space, root hair zones and zones around and within active microorganisms (Oburger and Schmidt 2016; Kilburn et al. 2010). However, these techniques are destructive, and the preparation steps render samples unavailable for further analysis.

In contrast, neutron radiography, while also being used for phenotyping roots systems (Wasson et al. 2020) has been used to measure non-destructively the dynamics of water at the root soil interface (Carminati et al. 2010; Holz et al. 2018).

New understanding resulting from methodological advances

New and improved methods have enhanced our understanding of aspects of root/soil interactions, although there is still a pressing need to link mechanistically the many chemical, physical and biological processes occurring at different spatial and temporal levels (Vetterlein et al. 2020). Here we focus on advances in understanding aspects of rhizodeposition, enzymatic processes and water and nutrient acquisition.

Rhizodeposition

Quantification and chemical characterisation of rhizodeposition in natural soils has been, and remains challenging, as the release of organic compounds from roots is affected by microbial communities, and rhizodeposits are rapidly transformed by microbes in soil (Paterson et al. 2009). Labelling plant assimilate with isotopic tracers (^{13}C , ^{14}C) provides a means of differentiating root-derived C from that cycling through native SOM pools, including microbial biomass and dissolved organic carbon (DOC), and when this is done under steady-state conditions (e.g. continuous, uniform $^{13}\text{CO}_2$ labelling), this allows quantitative partitioning of plant- and SOM-derived sources to soil pools and fluxes (Paterson et al. 2009). This has provided new understanding of plant-mediated impacts on microbial communities and their functions (e.g. priming effects, Kuzyakov 2010), leading to the suggestion that plant-microbe interactions affecting soil processes may be a route to management directed to promotion of beneficial functions for crop productivity and sustainable soil health.

The recent rapid development of molecular methods and associated bioinformatic approaches to characterise microbial community composition has facilitated recognition that both plant species and genotype can have significant influences on rhizosphere microbial community selection and development. This has led to the concept of plant-specific microbiomes, and that roots in combination with associated microbial communities should be considered as ‘holobionts’ affecting ecosystem processes and interactions with the environment (Vandenkoornhuyse et al. 2015). Initially, genotype-specific microbiome selection was demonstrated for *Arabidopsis thaliana*, and the generality of this has now been demonstrated for a range of species, including crop plants (Kumawat et al. 2022). Further, it is now recognised that microbiome structure varies along root axes and as a function of root type/ age, driven by differences in exudation, nutrient uptake, predation and release of signalling compounds (Bonkowski et al. 2021). However, significant challenges remain in establishing the root traits responsible for regulating specificity of microbial community selection and the functional consequences of distinct microbiomes for soil processes, such as biogeochemical cycling. In large part, uncertainty over the functional consequences of microbiome

selection is a result of the very large diversity of rhizosphere microbial communities; that understanding of the genetic bases of microbial functions is incomplete (Young 2016); and that at any point in time, a large proportion of the microbial community is inactive (Joergensen and Wichern 2018). These considerations mean that it is imperative that characterisation of plant microbiome structure is combined with measures of microbial functions to gain mechanistic understanding of plant-specific impacts on soil processes (Sokol et al. 2022).

Identification of microbial community components active in utilisation of root-derived C (interactome) can be established through $^{13}\text{CO}_2$ -labelling and tracing of rhizodeposits into microbial biomarkers and nucleic acids (stable-isotope probing [SIP], Radajewski et al. 2000; Paterson et al. 2007). For example, phospholipid fatty acid (PLFA) analysis provides relatively coarse-resolution of microbial community structure but has the advantage that compound-specific isotope ratio mass spectrometry (IRMS) is a highly sensitive analytical approach for quantification of incorporation of plant-derived C (Paterson et al. 2009). In contrast, tracing ^{13}C into DNA fractions (Radajewski et al. 2000) has much higher taxonomic resolution, but is constrained by the requirement to physically separate labelled fractions via isopycnic centrifugation. An analogous approach is to target RNA rather than DNA, which has the advantage that it can be related to overall (rRNA) or function-specific (mRNA) activity of microbial populations. There is great potential in combining such SIP approaches with concurrent measurement of specific biogeochemical functions (e.g. nutrient cycling fluxes), providing opportunities to identify key components of microbial communities responsible for plant-mediated impacts on soil functions.

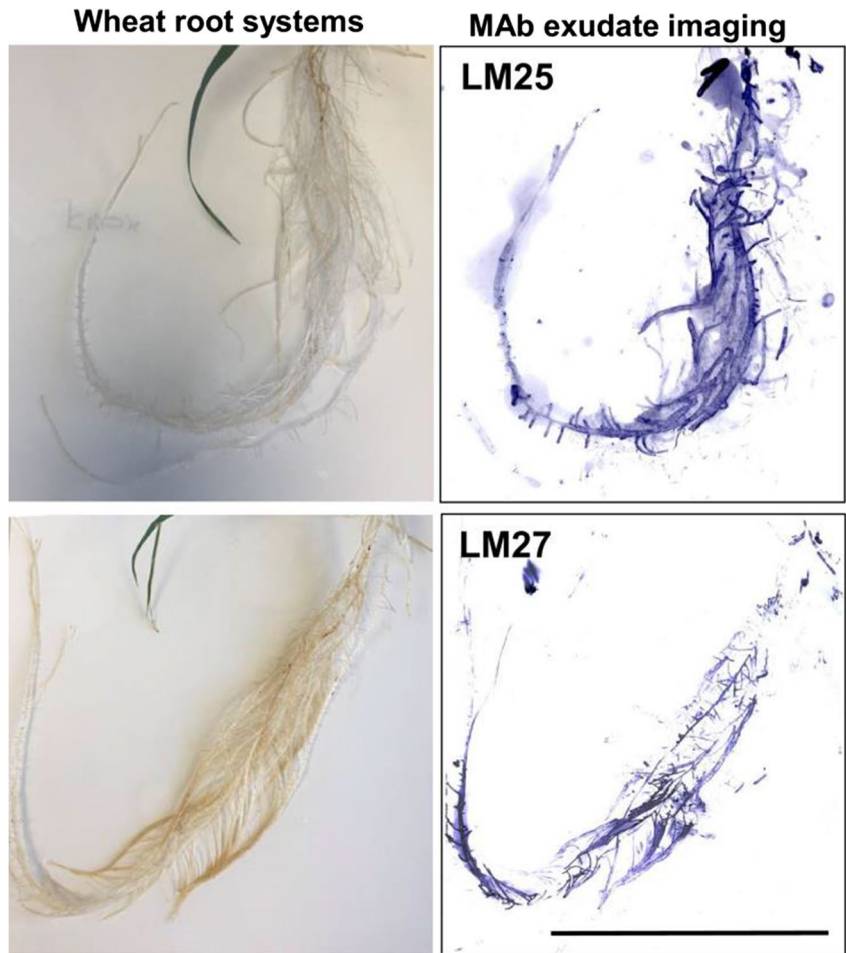
Advances in analytical chemistry and approaches such as metabolic flux analysis, to trace C-flow through biochemical pathways (Dijkstra et al. 2011), have greatly increased the resolution at which rhizodeposition composition and microbial C-processing in the rhizosphere can be resolved. Metabolomics, defined as the non-targeted, comprehensive analysis of metabolites in biological systems, has increased the understanding of root exudate composition, mobilisation of SOM into DOC, and microbial products in the rhizosphere, identifying molecules of low abundance that may have key

roles in communication and elicitation of functions (signalling compounds, Cotton et al. 2019). Targeted immunofluorescence techniques to image the distribution of particular polysaccharide exudates around roots (Fig. 2) have increased understanding of the role exudates play in modifying soil physical conditions. Furthermore, use of position-specific ^{13}C -labelled compounds provide opportunities to characterise microbial metabolic pathways in soil, including important community attributes such as carbon use efficiency (Apostel et al. 2013; Geyer et al. 2019). These methods have particularly strong potential to resolve the mechanistic bases of root-soil interactions when combined with metagenomic, transcriptomic, proteomic and bioinformatic pipelines in systems analysis, but requires co-development of novel statistical approaches to robustly identify significant relationships.

Enzymatic processes

Developments in zymography, where membranes impregnated with enzyme substrates are placed (with a diffusive barrier separating soil and membrane) on the soil surface at the root soil interface capture an enzyme imprint of the rhizosphere in 2D (Razavi et al. 2019). A key advantage of this technique is that a sequence of imprints can be captured and correlated with other 2D imaging techniques to build a comprehensive picture of dynamics and functionality (Fig. 3). For example, such studies have demonstrated the importance of root hairs in the distribution of phosphatase enzymes in the rhizosphere (Giles et al. 2018). When coupled with microbial molecular (Liu et al. 2021b) and imaging approaches such as FISH (Fluorescent In-situ Hybridisation; Spohn et al. 2015), the timing and longevity of hot spots and hot moments of microbial activity in the rhizosphere can be defined (Song et al. 2019). Similarly, when zymography is combined with planar optodes (which measure the spatial distribution of pH [Ma et al. 2019, 2021]) and redox or DGT (Diffusive Gradient in Thin films) optodes (which measure depletion of nutrients from the rhizosphere [Hummel et al. 2021; Fang et al. 2021]) information about the impact of the enzymes on soil chemical properties and vice versa can be elucidated.

Fig. 2 Immunoblotting analysis of root exuded polysaccharides (glycan epitopes) at the wheat root surface: Imaging of glycan epitopes in high-molecular-weight (HMW) exudates released from large wheat roots grown hydroponically for 2 weeks and then lain on sheets of nitrocellulose for 10 min prior to probing with monoclonal antibodies (MAbs). Reproduced with permission from Galloway et al (2020)



Water and nutrient acquisition

Extraction and observation of roots in the field has increased knowledge of the depth of rooting of native species (Canadell et al. 1996) and the role of deep roots in water and nutrient acquisition by crops. For example, White and Kirkegaard (2010) observed that while wheat roots penetrated to 1.6 m, 30–40% were clumped in pores and cracks in surface layers increasing to 85–100% below 60 cm where 44% of roots were in pores occupied by at least three other roots. These observations fed into models exploring the effects of seasonal rainfall distribution, and deeper and denser root systems on water use and demonstrated the multi-faceted nature of water use by rainfed crops (Lilley and Kirkegaard 2011). Similarly, extraction of roots coupled with soil chemical analysis has demonstrated that differences in crop rooting patterns can be

exploited to improve nitrogen use efficiency (Thorup-Kristensen 2006). Deep-rooted and ‘catch’ crops can recover nitrate leached during the growing season from cereal crops and raise nutrients such as potassium and phosphorus from subsoil to topsoil benefiting both yields and the wider environment (Thorup-Kristensen et al. 2020; Han et al. 2021).

X-ray CT imaging has led to insights into the processes affecting root water uptake and the influence of physical changes in the rhizosphere. Growing roots release mucilage and change the bulk density of the soil adjacent to the root thereby altering both the water retention characteristics and hydraulic conductivity of the rhizosphere (Moradi et al. 2011; Ahmed et al. 2014; Carminati et al. 2016). Several studies have shown decreased bulk density in the immediate vicinity of roots (e.g. Landl et al. 2021) but others have indicated an increase (e.g. Koebernick et al.

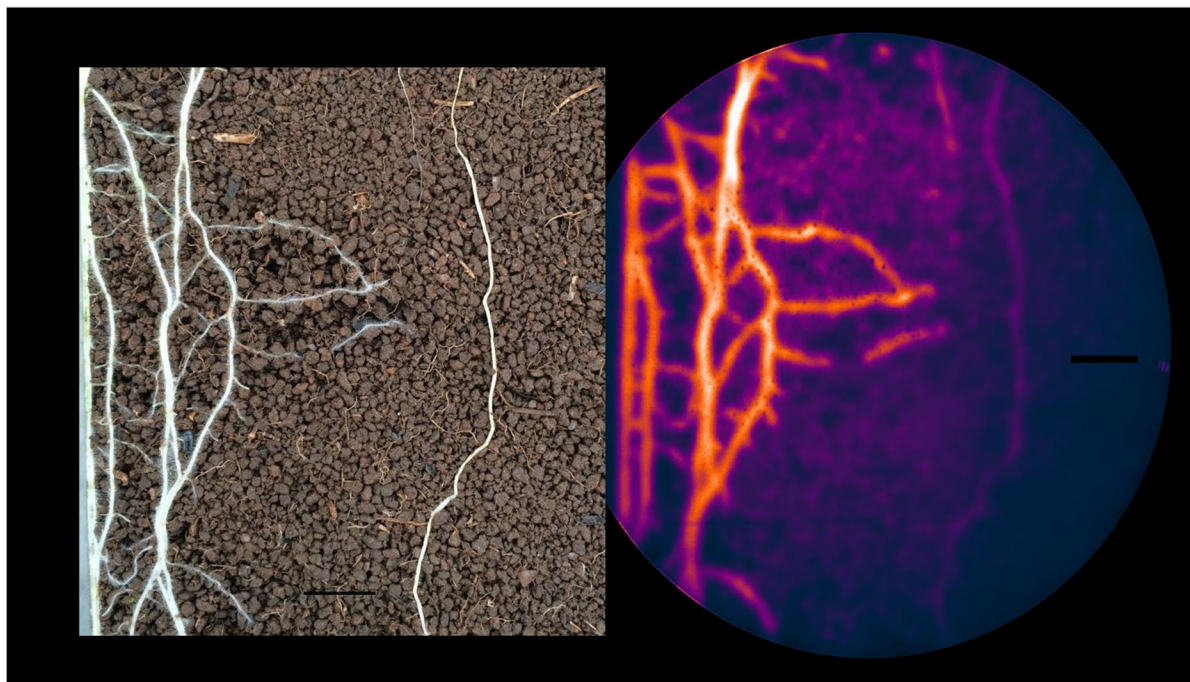


Fig. 3 Zymography image of root exuded acid phosphatase from barley and medicago roots: Comparison between a visible light image and a zymography image taken on the window of roots of barley (*Hordeum vulgare* L.) and barrel clover (*Med-*

icago truncatula L.) growing on a thin layer of soil between two panes of Perspex. Image shows much greater acid phosphatase activity associated with the barley roots. Bar = 1 cm

2017). Decreased density could result from the loose packing of incompressible mineral particles displaced by the root but if particle displacement is constrained for whatever reason, then bulk density will increase because the volume occupied by the root is not matched by a decreased pore volume of the surrounding soil. When both mucilage and bulk density effects were allowed for, modelling showed that the rate of water uptake was reduced but duration was increased (Landl et al. 2021).

Spatial and temporal dynamics of element availability in the rhizosphere captures using DGT have been quantified using laser ablation ICP-MS; the laser destructively samples the imprint and transfers the material into a spectrometer for quantification (Santner et al. 2012; Fang et al. 2021; Bilyera et al. 2022). This combined technique also permits measurements of elements on, and in, live tissue such as roots and biological materials in the rhizosphere, allowing the potential production of maps of element distribution, or the rhizosphere ionome, at the soil root interface (Zaeem et al. 2021). Recently, this approach has measured the

dynamics of nano-sized fertiliser particles (Szameitat et al. 2021) and the impact of liming on nutrient availability (Smolders et al. 2020) in the rhizosphere and may assist the development of improved fertiliser practices. Such approaches when coupled with novel phenotyping tools used for screening crop genotype populations for root ion uptake and respiration (Griffiths and York 2020; Griffiths et al. 2021; Guo et al. 2021) will have profound affects on our ability to select crop genotypes that are best able to utilise the homogeneously distributed resources in the rhizosphere.

What is still required

We have described methods currently used, but the future must focus on methods that provide an integrated understanding of the chemical, physical and biological changes in the rhizosphere and their consequences for plant growth. Here we focus on three issues that limit progress and need to be resolved to realise this ambition: the opacity of soil,

spatiotemporal scaling and integration of data and knowledge.

Opacity

Roots have been widely referred to as ‘The Hidden Half’. X-ray CT has allowed visualisation of seedling roots in small soil samples in the laboratory, but the issue remains in studying root systems in the field. There is an urgent need for a non-destructive field technique.

Even in laboratory studies, many of the techniques (including neutron radiography) require a simplification of the growing system into two dimensions, by growing plants in thin layers of soil between plates. This requirement is generally a response to the need to get easy access to the root surface soil interface and can also be achieved in field soils using root access windows (Neumann et al. 2009). A method that usefully overcomes issues of both opacity and 2-D is the combination of light sheet microscopy with fluorescent labelled plants and microorganisms grown in transparent ‘soil’ (Fig. 4; Liu et al. 2021a; Jones et al. 2021). This novel development has allowed observation of the dynamics of microbial colonization of roots including dynamic waves of microbial growth at the root soil interface. It has shown previously unseen extremely dynamic hot spots and hot moments associated with root growth. Of course, a limitation is that although the transparent ‘soil’ has been shown to behave like true soil in many respects, it is still an artificial system.

Spatiotemporal scaling

While great progress has been made in characterising the mechanistic bases of plant-soil interactions, scaling these to the level of ecosystem processes remains a significant interdisciplinary challenge (Schnepf et al. 2022). The recognised heterogeneity of soil properties and processes, over different scales of space and time, mean that quantitative translation of rates of processes at the rhizosphere scale to landscapes, or indeed globally, is highly complex (Vetterlein et al. 2020). A consequence of this is that modelling approaches, for example applied to the soil carbon cycle, have tended to greatly simplify the complexity of plant-soil interactions, favouring approaches that use environmental parameters as drivers of biologically-mediated processes in linear first-order models. However, many biological

processes are characterised by non-linear functions (e.g. Michaelis-Menten enzyme kinetics), and rhizosphere research has consistently demonstrated the importance of biological diversity and context-specificity in rhizosphere functions. Theoretical advances in quantifying the consequences of non-linearity and heterogeneity of soil processes for upscaling have been made (e.g. Wilson and Gerber 2021), allowing some progress on these issues. In addition, the use of established field-to-catchment scale instrumented sites (experimental platforms) facilitate study of processes at different scales (e.g. combined use of soil flux chambers and eddy-covariance towers), while satellite remote sensing is increasingly a means to infer soil properties and plant growth at global scales. Such approaches are also supported by the increasing availability of molecular microbial data for soils across the globe. These provide an invaluable resource with which to identify patterns of community structures across scales and are a potentially powerful means to relate biogeochemical processes with the agents that mediate them (Vereecken et al. 2016).

Integration of methods

Many of the key global challenges facing society, including climate change, agricultural sustainability, food security and the biodiversity crisis all require understanding of the dynamic zone where roots and soil interact. They are also influenced by extremely complex systems where interactions, integration and competition between mechanisms, individuals and trophic levels are paramount. Methods and techniques are required which can capture this complexity and identify the most important interactions. We can no longer rely on reductionist approaches where the belief that understanding of one plant, one process, one microorganism or one gene will resolve these complex problems. This suggests the types of methods we need in the future. Besides dealing with opacity and spatial and temporal variation in the system, our opinion is that the prime need is to integrate methods to study simultaneously multiple processes and their interactions. We have described several powerful methods for imaging, measuring and quantifying processes at the root soil interface. In combination, many of these techniques could become extremely powerful. Such combinations are starting to be used and have already provided insightful observations. For example, the combination of

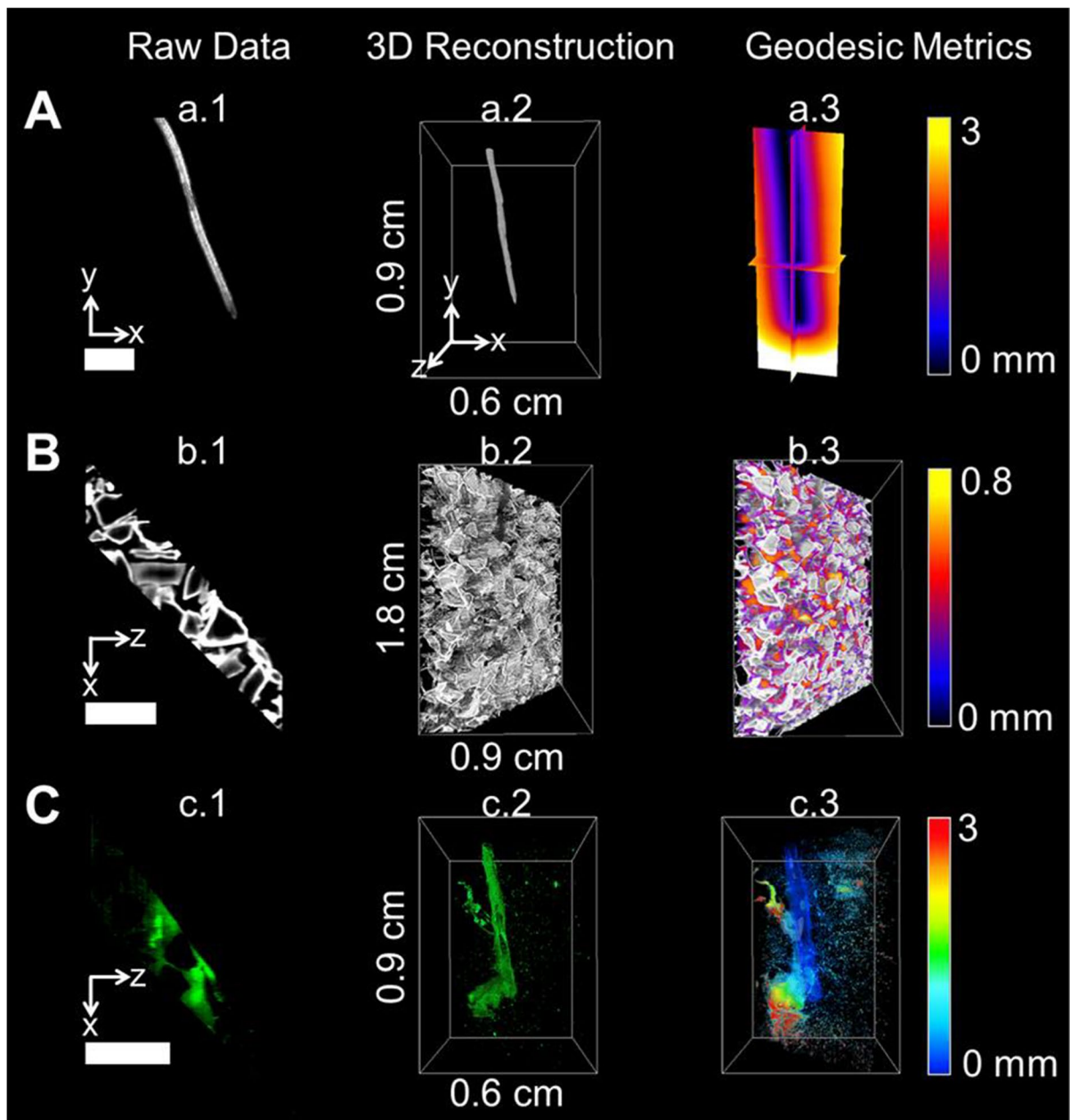


Fig. 4 Quantification of root-soil-bacteria interactions using transparent soil and light sheet microscopy. Image data from lettuce root (**A**), transparent soil particles (**B**), and GFP-labelled *Bacillus subtilis* (**C**). Processing of the data follows 3 steps. Raw data is acquired from the microscope (left). Cross sections are assembled into volume data through stitching and

stacking (middle). Image processing is subsequently performed to quantify temporal and spatial patterns of biological activity in the pore space (right). The metrics obtained from the data include distance from the root surface (**A**), pore size (**B**) and bacterial cell density (**C**). The scale bar represents a distance of 2 mm. (Used with permission from Liu et al. 2021a, b)

NanoSims and pulse-chase isotope labelling has helped resolve the dynamics of nutrient uptake and transfer of rhizosphere bacteria (Clode et al. 2009) and mycorrhizal

fungi (Kaiser et al. 2015). This hybrid technique is now being termed NanoSIP (Pett-Ridge and Weber 2022). Similarly, the combination of several element imaging

techniques correlated with FISH have been shown to resolve the relationships between the rhizosphere microbiome and its chemical environment (Bandara et al. 2021). We earlier showed that the combined methods of DGT and ICP-MS are powerful, but further combination of these techniques with laser ablation, planar optodes, zymography and single cell transcription analysis opens the possibility for comparison of multiple chemical and biological parameters on the same sample. In the not-too-distant future, it should be possible to generate maps of the allied transcriptional response of the microbiome and root cells at the root soil interface at the same resolution as the distribution of the ionome. With such approaches, the opportunity will arise to contribute significant understanding in responding to the global grand challenges facing our society.

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Data availability No data was generated in the production of this manuscript.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to declare.

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