Root2Res

Root phenotyping and genetic improvement for rotational crops resilient to environmental change

Deliverable 2.3

Phenotyping toolbox - Rhizosphere

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Deliverable D2.3 comprises a list of phenotyping tools suitable for quantifying rhizosphere traits, such as root exudate and rhizosphere microbiome traits. This list will be compiled and added to the phenotyping toolbox. For complicated traits, this will involve the identification and validation of proxies suitable for large scale phenotyping (T2.2).

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	DEM	Demonstrator, pilot, prototype, plan designs	
	DEC	Websites, patents filing, press & media actions, videos, etc.	
	DATA	Data sets, microdata, etc.	
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1. Introduction

1.1. Aim

The aim of this deliverable is to provide a list of phenotyping tools suitable for assessing various rhizosphere traits, such as root exudation and rhizosphere microbiome traits, to support the development of more resilient crop cultivars capable of mitigating the destabilizing impacts of climate change. In addition to a list of phenotyping tools for root architectural traits (D2.1), we compile here a novel toolbox for phenotyping rhizosphere processes. At the moment, the majority of tools available are applicable to small and potentially medium-scale experimental conditions mostly in controlled environments (involving a handful of tested genotypes, soils or environmental conditions). However, during this project, we hope to identify suitable proxies for complex rhizosphere traits that can then be applied in large scale phenotyping (controlled conditions and field trials) (T2.2).

1.2. Background and scope

Belowground traits not only comprise root architectural, but also rhizosphere related traits. The rhizosphere is characterized by dynamic interactions between roots and the surrounding soil, whereby soil physicochemical properties as well as microbial communities are influenced by living roots (York et al., 2016). The rhizosphere can therefore be considered as a part of an extended root phenotype. The root associated microbiome and the resulting cross-talk between plants and microbes are considered to play a crucial role in plant growth performance, especially under changing climatic conditions. Root exudates (i.e. organic compounds released by plant roots into the surrounding soil) are believed to play a crucial role here as they drive a cascade of feedback loops between roots, soil and microbes driving a range of soil and rhizosphere processes (Bais et al., 2006).

The potential role of the rhizosphere as a part of an extended root phenotype has received increasing attention in recent years (Oburger et al., 2022; Williams & de Vries, 2020). However, phenotyping difficulties of complex rhizosphere traits still impede their consideration in applied plant breeding. Screening of easier assessable aboveground traits as proxies for belowground traits may facilitate root and rhizosphere phenotyping. For instance, as rooting depth determines access to water, this trait is linked via water uptake and transpiration with leaf canopy temperature as an aboveground trait. Hence, non-invasive screening of canopy temperature possibly represents a proxy for root depth, since cooler leaves indicate an improved access to water due to deeper roots (Li et al., 2019). In this context, canopy temperature was successfully used to estimate root biomass in wheat (Lopes & Reynolds, 2010).

In order to address promising rhizosphere traits for an improved climate resilience to breeders, geneticists and agronomists, we present here a phenotyping toolbox with cutting-edge methods and proxies as a novel approach.

2. Toolbox for phenotyping rhizosphere traits

2.1. Experimental system

A suitable experimental system is a prerequisite for phenotyping the rhizosphere. The chosen experimental system determines not only plant growth and the expression of rhizosphere traits, but also suitable sampling strategies and subsequent analysis procedures. The unpredictability of field conditions (climatic conditions, pests, etc.) on the one hand, and artifacts (e.g., restricted rooting volume in pots, limited substrate availabilities, etc.) occurring in controlled environment (CE) experiments on the other hand complicate the selection of a suitable growth system. As an example, soil-less hydroponic or aeroponic culture systems are frequently used in CE conditions as they allow operator-defined nutrient availability, as well as easy phenotyping of root growth (Bucher, 2006) and root exudation (Oburger & Jones, 2018). However, these systems lack the interaction with the soil matrix and the microbiome and are therefore unsuitable systems for phenotyping rhizosphere processes. Certain experimental systems, like the combined use of rhizoboxes with a root exudate collecting tool (Oburger et al., 2013), are complicated to implement. They are restricted to CE experiments and only suitable to investigate a small number of plants and treatments. This toolbox (summarised in Table 1)focuses therefore on soil-based CE experimental systems as well as field conditions, potentially allowing phenotyping of rhizosphere traits of a medium or large number of species/ genotypes.

2.2. Rhizosphere soil sampling

The rhizosphere is defined as the volume of soil affected by living roots. Consequently, the rhizosphere reaches as far as any change by the root in bulk soil can be observed (Darrah, 1991). However, in experiments, the rhizosphere is often operationally defined by the selected sampling approach. Here, we focus on rhizosphere soil sampling strategies that are potentially suitable for medium- or large-scale phenotyping. This involves previous excavation of the entire root system in a defined or undefined soil volume (shovelomics) in the field or by removing the rooted soil block from a growth container in CE conditions. Shaking off the bulk soil from the roots after excavation represents an easy and common protocol to separate bulk from rhizosphere soil (Barillot et al., 2013). Rhizosphere soil is then defined operationally by the amount of remaining soil adhering to the roots, an operational definition coinciding with the concept of the rhizosheath (Brown et al., 2017). Comparing the amount of adhering rhizosphere soil (i.e. rhizosheath, being defined as the mass of soil still attached to the roots after gently shaking off the bulk soil) from different genotypes, allows us to obtain information about potential differences in size and strength of the rhizosheath (Brown et al., 2017). However, environmental factors, like soil moisture, will also affect rhizosheath mass which either requires controlling or at least monitoring of soil moisture conditions or which restricts the suitability of this sampling approach to CE conditions. Rhizosheath size and strength could potentially serve as a simply proxy for other, more complex rhizosphere traits (e.g., total C exudation, degree of mycorrhization, etc.), however, so far this still remains to be tested in the course of this project. After freeing the roots from the bulk soil, rhizosphere/rhizosheath soil is collected manually from the root surface by brushing off or by washing off in water or an electrolyte solution (Figure 1).

Although the proposed approach does not contemplate spatial information, e.g., distance from the root, but rather binding strength of the soil to the root surface which is affected by soil moisture (Vetterlein et al., 2021), it represents a widely established sampling strategy enabling large scale phenotype and comparisons with a large body of the scientific literature.

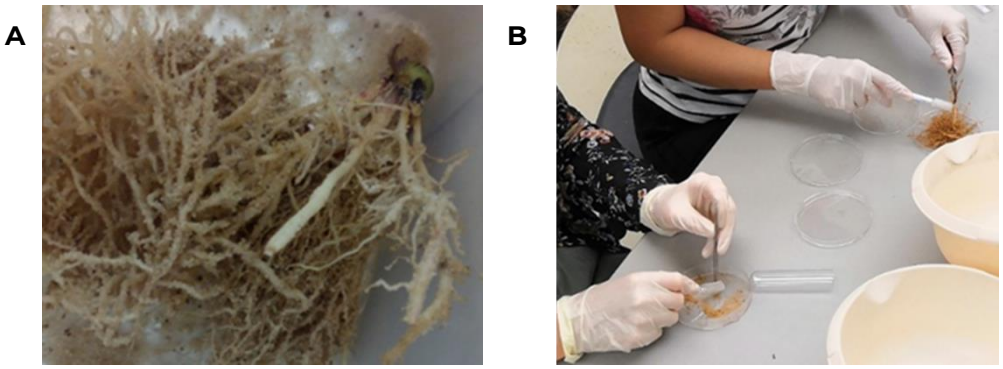

Figure 1 Remaining rhizosphere soil attached to the root surface after the bulk soil was shaken off (A). Collection of remaining rhizosphere soil by brushing it manually off the roots (B) (Source: Oburger).

Other rhizosphere soil sampling approaches do not require a predefinition of the rhizosphere as they are capable of providing information about spatial gradients. Sampling rhizosphere at a well-defined distance to the root is possible with rhizoboxes (Wenzel et al., 2001) and also in their combination with suction cups (Dessureault-Rompré et al., 2006). Plant growth in rhizotrons enables the use of 2D imaging techniques to investigate spatial distribution of rhizosphere non-destructively (Neumann et al., 2009). More sophisticated experimental setups exist that when combined with imaging/isotope labelling techniques, like e.g., X-ray CT, zymography, allow the observation of roots and rhizosphere processes either planar or in 3D (Lippold et al., 2023), while delivering the exact position of an extracted sample/image/gradient in the whole root system. However, these sampling approaches are best suited for in-depth, mechanistic studies with small numbers of investigated genotypes and treatments and therefore are outside the scope of this toolbox for medium- to large-scale phenotyping.

2.3. Rhizosphere microbiome traits

Rhizosphere microbiome traits involve the microbiological component of the rhizosphere. These microbial communities are important because they provide extended functional traits to plants related to health and growth, contributing eventually to the ecosystem functioning (Trivedi et al., 2020). In Root2Res, the microbiome traits are focused on the bacterial, archaea and fungal communities.

These rhizosphere microbial communities are distinct from adjacent microhabitats (Bulgarelli et al., 2013). For instance, while the rhizosphere microbiota communities exhibit a reduced level of diversity compared with the microbiota of the bulk soil, the former manifests a higher degree of taxonomic diversity when compared to the microbiome inhabiting the root system or endosphere. Hence, the correct separation of these microhabitats (with their very distinct microbial traits) is critical during sampling. For instance, as described in the previous section, shaking off the root

system enables the extraction of rhizosphere- from bulk soil, whereas root tissues *per se* represent the endosphere-enriched samples (see summarised in Table 1).

The microbiota is not randomly assembled in the rhizosphere, instead this assembly is determined by the root ontogeny (Rüger et al., 2021), the environment (Alegria Terrazas et al., 2016), the host plant genetics (Escudero-Martinez & Bulgarelli, 2019) and even the influence of other members of the microbiome such as mycorrhizal fungi (Wang et al., 2023). Understanding the factors that dictate these assemblies, dynamics and function of the microbiota is essential to harness the microbiota and its full potential (Schlaeppi & Bulgarelli, 2015). In Root2Res CE experiments, different soil types (environment) and different genotypes of each of the crops (plant genetics) were included to assess their impact over the plant performance on different microbial traits described in the following sections.

2.3.1. Community composition

Microbial community composition traits are measured by sequencing targeted phylogenetic markers corresponding to the different microbial groups i.e., the nuclear ribosomal internal transcribed spacer (ITS) sequences for the fungal, and 16S ribosomal RNA gene for the bacterial and archaeal communities, respectively (Table 1).

Root2Res further aims to investigate arbuscular mycorrhizal fungi (AMF), which are known for their symbiotic abilities and are of great relevance to agriculture (Rillig et al., 2019). We use both, qPCR to quantify the abundance of AMF (Corona Ramírez et al., 2023) and metabarcoding to characterize changes in community composition (Schlaeppi et al., 2016) (see Root2Res Practice Abstract #1).

The targeted sequencing with phylogenetic markers allows for the determination of ecological indexes recapitulating abundance and composition of the microbial communities under investigation. The analysis of the community composition deploys metrics such as beta-diversity, alpha-diversity while abundances of individual members of the communities, often expressed as sequencing reads assigned to given taxa, is compared using multivariate approaches (McMurdie & Holmes, 2013). This information can then be used to weigh the contribution of independent variables, i.e., microhabitat, plant genotype and soil type, to microbiome diversification.

- Beta-diversity is a measure of similarity or dissimilarity between samples. This metric links the overall taxonomic pattern of the community to environmental features, for example microhabitats, soil types or genotypes (Knight et al., 2018).
- Alpha-diversity quantifies the taxonomic diversity within individual samples, and this can be compared across sample groups (Willis, 2019). This metric is based on the number and relative abundance of taxa. The most common alpha diversity indexes are Observed (richness or number of taxa), Chao (richness and abundance) (Chao, 1984), and Shannon (richness and evenness) (Spellerberg & Fedor, 2003). For example, the number of observed taxa can be compared within individual treatments (i.e., microhabitats, soil types or genotypes).

Comparative relative abundances compare samples with varying relative sequencing read counts for each individual taxon. If in the previous ecological indexes calculations differences between groups were observed, this type of analysis will reveal what specific taxa is underpinning such differences.

2.3.2. Morphological observation

Root nodulation describes a biological process occurring in legumes (such as faba bean) in which a symbiotic relationship is established between the plant and specific nitrogen-fixing bacteria known as rhizobia (Poole et al., 2018). This process is essential for improving the legume's ability to obtain nitrogen, an essential nutrient required for its growth. Nodulation can be assessed by counting the number of nodules and measuring the fresh weight per nodule by visual examination of the roots. The number of nodules consists of a combination of exact numbers and of an estimation of number when nodules number are more than 50.

Microscopy approaches are used for phenotyping mycorrhizal associations. A modified method enables objective estimation of vesicular-arbuscular mycorrhizal root colonization. Estimation of colonization is done by the inspection of intersections between microscope eyepiece crosshair and a root part containing mycorrhizal arbuscules (McGonigle et al., 1990)

2.3.3. Functional traits (plant growth promoting hormones, N fix, etc.)

Microbial functional targeted enrichment sequencing is a novel technique that combines microbiology and genomics used to selectively amplify and study functional genes within complex microbial communities. Shotgun metagenomics, or the non-targeted sequencing of the entire microbial community, provides a wealth of microbial functional information (Quince et al., 2017). Community composition associated with biogeochemical cycling is often at a low abundance compared to other soil microbes which requires a significant sequencing depth for accurate estimation of these communities. Hence, obtaining a meaningful ecological interpretation is difficult, due to the costs associated with high throughput sequencing. Furthermore, the technology is still in the early stages of development, thus the bioinformatic analysis of the data output is work intensive. Therefore, the selective enrichment of microbial functional genes will enable a detailed characterization of the diversity of key functional microbial traits (Siljanen et al., 2022).

Changes in the abundance of functional microbial communities in response to plant genotype or climatic factors can also be assessed using qPCR approaches (Garcia et al., 2020; Müller et al., 2015). qPCR techniques are highly specific, sensitive, reproducible and have a wide detection range. Relative to shotgun sequencing approaches they are low cost/high throughput and do not require intensive downstream bioinformatics associated with shotgun sequencing. Microbial communities in soil drive a range of nutrient cycles and underpin essential processes including carbon sequestration and plant nutrient provision (Bhattacharyya et al., 2022; O'Neill et al., 2022). In contrast to approaches utilised to quantify the overall microbial community (where phylogenetic markers are employed), genetic markers specific for a given target gene of interest are quantified to assess the abundance of

functional microbial communities. This allows for assessment of functions that are widely distributed across microbial groups as well as those that are phylogenetically conserved. A range of genetic targets, and associated primer sets have been developed for genes involved in important nutrient cycling processes for e.g., AmoA for nitrification, nosZ for nitrous oxide reduction and phoD in mineralisation of organic P (Duff et al., 2022). In Root2Res, targets associated with the N, P and C cycle will be assessed.

2.4. Geochemical and physical soil traits

Besides plant roots and microorganisms, the soil is also involved as a further actor in the complex rhizosphere interplay. While physical and chemical soil characterization can also help to unravel rhizosphere processes, thorough soil analysis is typically only carried out with bulk soil samples to characterize the plant's environmental growth conditions.

Chemical analysis can include measurement of soil pH, cation exchange capacity (CEC), iron-oxides, and organic carbon. Also, availability of plant nutrients can be assessed using a range of different chemical extraction procedures targeting different plant nutrients. Physical analysis can comprise assessment of soil texture, soil bulk density and water retention of the soil. Soil texture refers to the proportion of clay, silt and sand sized particles as the mineral fraction of the soil. It determines the pore network as well as sorption sites, subsequently water and nutrient fluctuation in the rhizosphere (Jarvis, 2007).

Soil parameters are affected by root activity. For example, root induced structural soil changes affect the pore network and subsequently water and nutrient transport dynamics (Phalempin et al., 2021). However, considering the workload involved in physicochemical soil characterization, we consider rhizosphere pH to be the most applicable rhizosphere phenotyping parameter in medium- to large scale experiments. Rhizosphere soil pH can be determined in a simple water or electrolyte extract by an electrode (ISO 10390:2021 Soil, treated biowaste and sludge – Determination of pH).

When applying destructive sampling techniques (see section 2.2), rhizosphere soil samples are then either air dried, homogenised and sieved (< 2 mm) as preparation before chemical and physical analysis or directly processed as field moist samples, with the latter being our preferred procedure when determining rhizosphere soil pH. The amount of rhizosphere soil obtained by the sampling techniques is usually limited. Hence, not all available soil analyses can be taken into account. A careful selection of suitable analyses is necessary.

2.5. Root exudation sampling and analysis

Root exudates are organic compounds released by plant roots into the surrounding soil. They comprise non-volatile primary and secondary plant metabolites which include low molecular weight metabolites (e.g., sugars, organic anions) as well as high molecular weight metabolites (e.g., enzymes, cell wall components) (Oburger & Jones, 2018). Rhizosphere processes arise when living roots influence the surrounding soil and the associated microbial communities. Root exudates are here of central importance as they mediate the cross-talk between plant roots, soil and microbiome.

For instance, released root exudates can serve as an energy source and signal molecules for microorganisms improving their capacity of colonising the root-soil interface (Canarini et al., 2019a; Jacoby et al., 2021). In return, rhizosphere microorganisms are able to enhance plant nutrient acquisition (Carvalhais et al., 2011) or biotic stress tolerance (Mohanram & Kumar, 2019) which potentially feedback to an improved plant climate resilience. Phenotyping rhizosphere traits consequently requires the characterization of root exudates as this will enable us to better understand exudate-driven rhizosphere processes, especially concerning the feedback-loops between plants and microbes, which is still very poorly understood. An important pre-requisite for characterizing both root exudates and the rhizosphere microbiome, are sampling approaches that allow the collection of rhizosphere soil for microbiome analysis and of exudates from intact roots.

2.5.1. Root exudation sampling from soil grown plants

Sampling root exudates from soil grown plants is highly challenging as the soil (sorption processes) and the rhizosphere microbiome (decomposition) quickly alter the exuded metabolite quantity and quality (Oburger and Jones, 2018). Most soil-based exudate sampling approaches are complex mechanistic methods only applicable for small scale phenotyping. These methods involve plant growth in rhizoboxes combined with a root exudate collection tool (Oburger et al., 2013), plant growth in rhizotrons with subsequent use of specific collection filters (like anion exchange membrane strips) on single root segments (Shi et al., 2011), or cuvette-hydroponic-sampling currently used for collecting root exudates from tree roots (Phillips et al., 2008). Those methods enable non-destructive and repeated root exudate sampling but are not suitable for medium to large scale phenotyping, in both CE and field conditions.

2.5.2. Soil-hydroponic-hybrid approach

The soil-hydroponic-hybrid approach represents an operationally simpler and quicker root exudate sampling method than the ones mentioned above and is therefore also suitable for medium-scale phenotyping. This approach is based on plants grown in soil (pots, rhizoboxes or field), followed by excavation and careful root washing for soil removal, and subsequent exudate collection in a hydroponic setup (Oburger & Jones, 2018), Santangeli et al. 2023, submitted). While some root damage cannot be avoided during the root washing procedure, damaged cell contents can be captured in a pre-sampling step. A pre-sampling step also allows capture of any osmotic adjustment reactions of the roots to the environmental change from soil to water. Despite the sudden change in environment, root cell metabolite contents and therefore also exuded metabolites can be expected to reflect soil growth conditions if root washing and the exudate collection period is kept short (in total not more than 4-5 hours). Moreover, the application of a microbial activity inhibitor to the exudate sampling solution supresses microbial degradation of collected root metabolites. While the soil-hydroponic hybrid technique is far from perfect, we consider it the most applicable and ecologically relevant approach suitable for medium-scale root exudate phenotyping in CE experiments or in the field.

2.5.3. Combined exudation and microbiome sampling from the same plant

Sampling root exudates and rhizosphere soil from the same plant is highly challenging, as exudation sampling requires an intact root system still attached to the shoot while rhizosphere soil is typically collected in a destructive manner (see section 2.2). Exudate sampling requires a gentle removal of all attached soil via washing in order to reduce root damage (see soil-hydroponic-hybrid approach 2.5.2). No separation of bulk- and rhizosphere soil is usually done here. Hence, sampling rhizosphere soil for subsequent microbiome as well as root exudates analysis in one joint experiment demands compromises. Within this project, we tested different approaches for combined exudation and microbiome sampling from the same plant to be suitable for medium-scale rhizosphere trait phenotyping (Figure 3 and Annex 1). All methods tested were generally similar, however bulk soil removal and/or rhizosphere soil collection differed. Bulk soil was either manually shaken off from the roots ("dry bulk soil removal", Figure 2A) or washed off ("wet bulk soil removal", Figure 2B) as in the original soil-hydroponic hybrid approach. After "dry bulk soil removal", the remaining rhizosphere soil attached to the roots was washed off and collected in water (see section 2.2) or removed from the roots by vortexing them for 30 s in a phosphate-buffered saline (PBS) solution. Washed roots of the intact plant were then transferred in the hydroponics for root exudation sampling (see section 2.5.2). When working with the "wet bulk soil removal" approach (Figure 2B), bulk- and rhizosphere soil were washed off both and collected separately, with the washing procedure being stopped when only a thin soil layer attached to roots remains. At this point, roots were repeatedly dipped into a fresh solution (distilled water) to collect the rhizosphere soil (Figure 3). This approach is gentler than shaking off the soil and better suited for clayey soils that produced considerable root damage during dry bulk soil removal (i.e., shaking). However, the distinction between bulk- and rhizosphere soil during wet bulk soil removal can be less precise and more operator dependent.

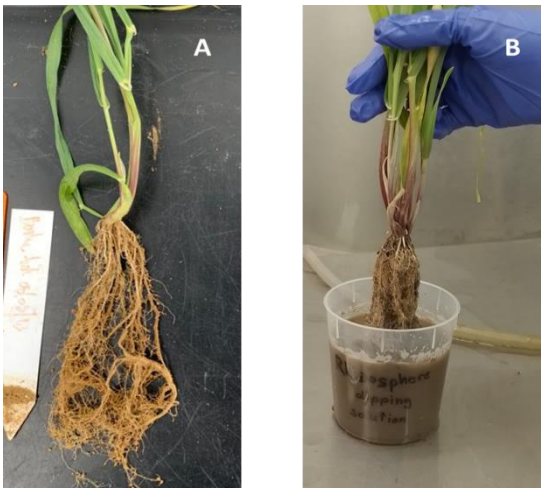

Figure 2 A: Remaining rhizosphere soil after shaking off dry bulk soil: "dry approach" B: After washing off bulk soil, rhizosphere is washed off and collected in a pot: "wet approach" (Cicala & Escudero-Martinez, 2023).

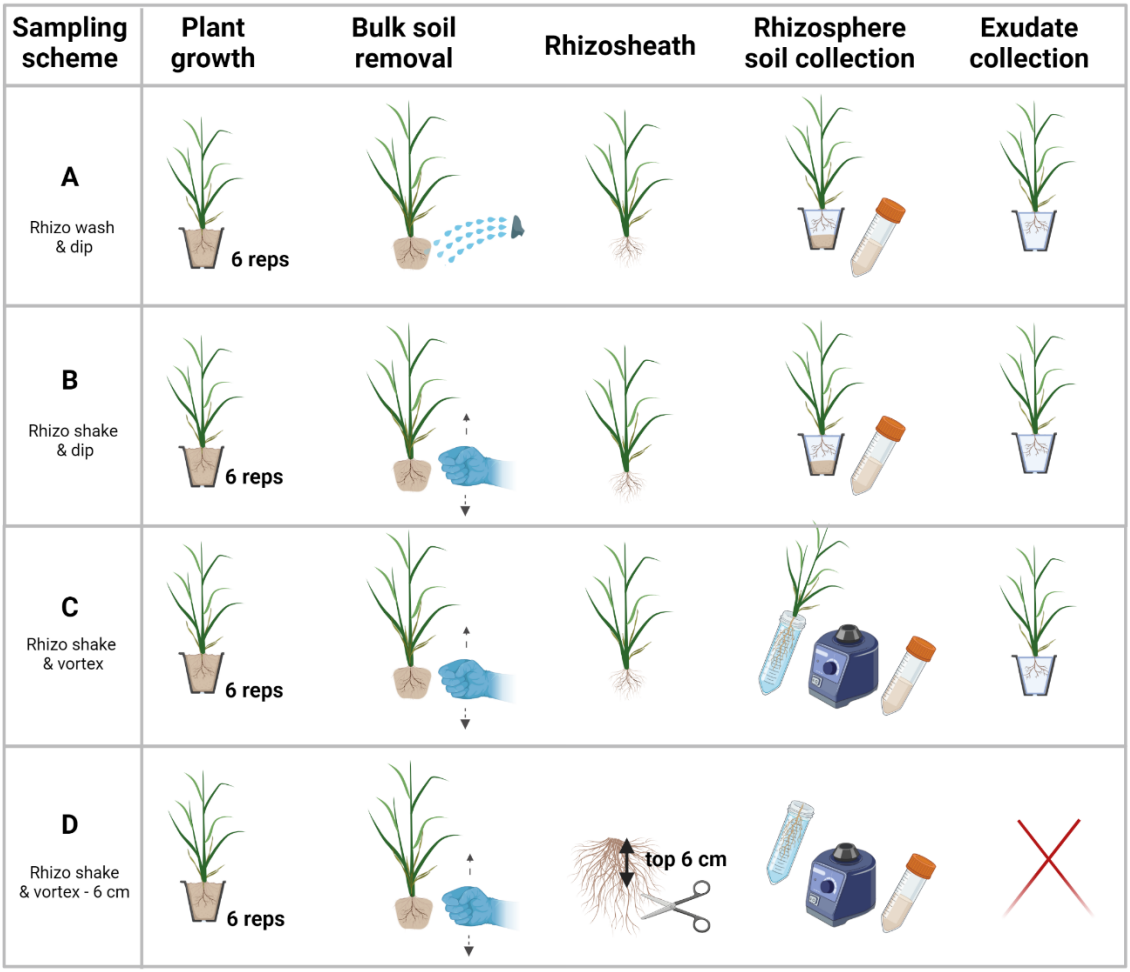

Figure 3. Experimental scheme of different root washing and rhizosphere soil sampling approaches tested. (A) Bulk soil is gently rinsed off until roots are only covered with a thin layer of soil, rhizosphere soil is collected by repeated dipping into vial filled with HQ water. Suspension is centrifuged, supernatant discarded and collected rhizosphere soil is stored at -80°C for microbiome analysis. (B) Bulk soil is gently shaken off, rhizosphere soil is collected via the root dipping approach described in (A). (C) Bulk soil is gently shaken off, roots with attached rhizosheath are placed in a centrifugation vial containing PBS and vortexed for 30s, rhizosphere soil is then collected after centrifugation as described in (A). In approach A, B, C roots were then subjected to exudation sampling for 3 h in HQ water containing 5 mg L-1 Micropur as microbial activity inhibitor. (D) Bulk soil is gently shaken off, roots and shoots are separated, and the top 6 cm of the root system are placed in a centrifugation vial with PBS and vortexed for 30s, rhizosphere soil is then collected after centrifugation as described in (A).

Preliminary results revealed that total dissolved C exudation was not affected by the different bulk soil removal and rhizosphere soil sampling approaches (Figure 4), the microbial diversity within the rhizosphere samples showed a significant reduction compared with the unplanted soil (Figure 5), and that about 78% of bacterial and archaeal (16S) reads were shared across the different root pre-treatment and rhizosphere soil sampling approaches (Figure 6). This demonstrates that all three tested approaches to jointly sample rhizosphere soil for microbiome analysis and root exudates from the same plant produce comparable results. However, root system size might significantly differ depending on species, plant growth period and growth

conditions. Hence vortexing of the root system in a buffer solution only works for plants with small root systems, limiting the applicability of this technique. Furthermore, additional tests demonstrated that especially heavy soils with a high clay and silt content, dry bulk soil removal by gentle shaking can lead to a significant loss of root biomass, as roots are broken off together with silt and clay rich aggregates (Figure 7).

Finding the right choice for sampling exudates together with microbiome traits depends on the research focus, and is strongly driven by soil texture, as lighter, sandy soils can be shaken off more easily with less root damage than heavier clayey soils. Ultimately, the developed combined sampling of exudates and microbiome allows to increase throughput and is can therefore be recommended for medium-scale rhizosphere phenotyping.

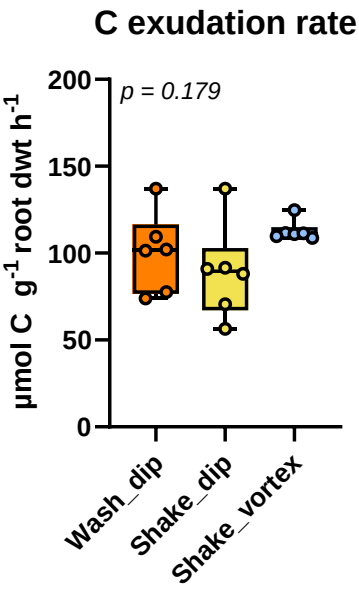

Figure 4. Total dissolved carbon (C) exudation rate (µmol C g^{-1} root dry weight h^{-1}) of barley (Hordeum vulgare Barke) grown for 5 weeks in a sandy soil depending on root pre-treatment approach., n=6, ANOVA, Tukey, p < 0.05.

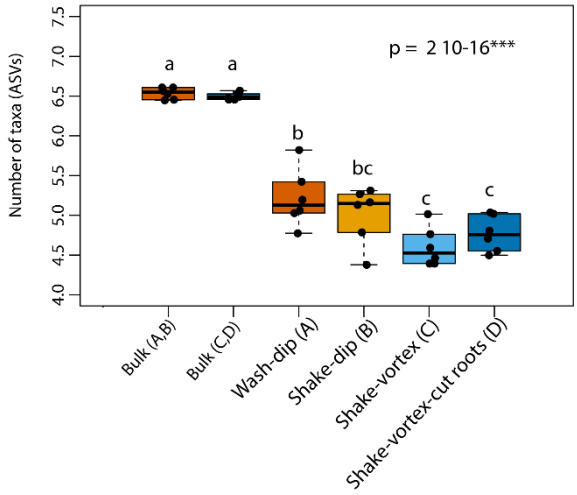

Figure 5 Boxplot representing the alpha-diversity index evenness (Shannon), unplanted soil controls (Bulk A, B) for dip methods, unplanted soil controls (Bulk C, D) for the vortexing methods and methods A, B, C, and D. In each panel, individual dots depict individual biological replicates. Empty dots are outliers. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the box denotes the median. Letters indicate significant differences following ANOVA and post-hoc Tukey test.

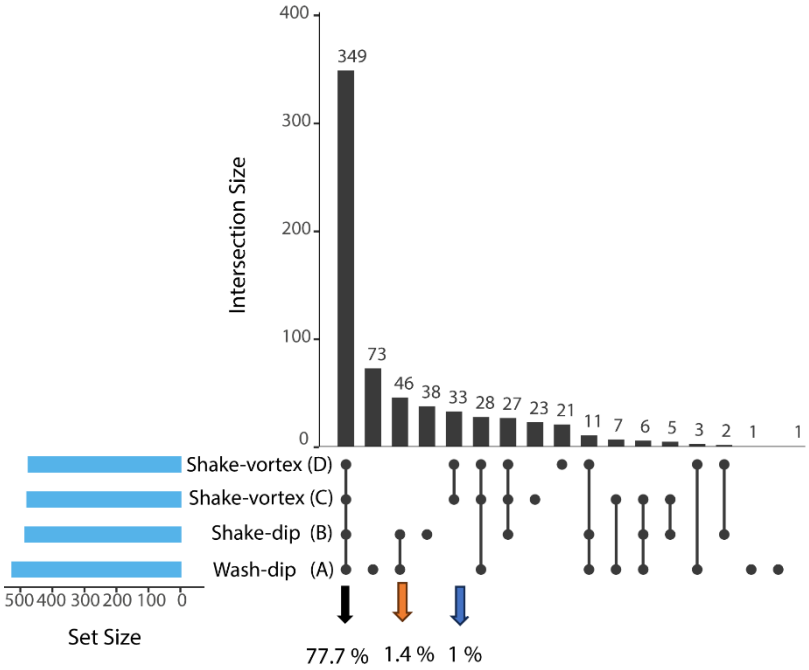

Figure 6 UpSetR plot of Taxa (ASVs) simultaneously enriched in pair-wise comparisons retrieved from the different rhizosphere sampling methods (A, B, C, and D). Vertical bars denote the number of ASVs enriched shared or unique for each comparison, while the horizontal bars refers the number of ASVs enriched in the indicated rhizosphere sampling method. ASVs differentially enriched at individual p-values < 0.05, Wald Test, FDR corrected.

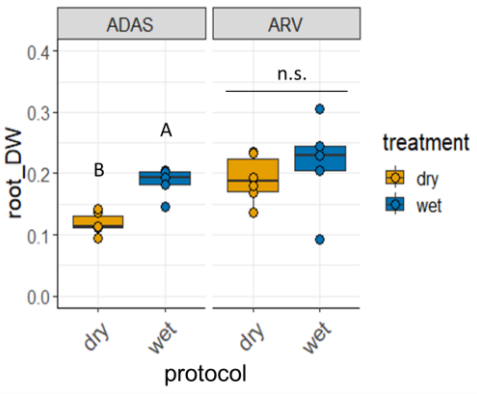

Figure 7 Effect of dry and wet bulk soil removal on root biomass of barley grown for 5 weeks either in a silty clay loam (ARV) or a clay loam (ADAS) soil. Welch Two Sample t-test, p<0.05, n=4.

2.5.4. Root exudate analysis

Root exudates comprise a wide range of different organic compounds (Badri & Vivanco, 2009; Canarini et al., 2019b) which makes their analysis challenging. However, thanks to the recent developments in analytical instrumentation, a comprehensive toolbox for exudate analysis is now available (see sections 2.5.5-2.5.7 and Table 1) to tackle the complex diversity of plant exuded compounds. When working with the soil-hydroponic-hybrid sampling approach, sample preparation like pre-concentration (e.g., by lyophilization) or filtration are generally required prior to exudate analysis.

2.5.5. Total carbon and nitrogen analysis

Total carbon (C) and total nitrogen (N) exudation rates (per unit root biomass or root surface area or root length, with the latter 2 units being recommended to use when comparing different genotypes) are of great importance to phenotype the overall quantity of exudates released into the rhizosphere. Information about changes in total C released with plant development and under changing environmental conditions will contribute to a better understanding of the role of rhizosphere processes in plant resilience (De Vries et al., 2020). In order to consider the general trend of C exudation, relation of targeted compounds (see section 2.5.6) to the total C exudation is crucial. Two relevant analytical approaches exist to measure total C and N exudation which involves the commonly used TOC (total organic carbon) analyser, as well as a quick and inexpensive spectrophotometric method (for total C only) (Oburger et al., 2022).

2.5.6. Targeted compound analysis

Targeted analysis focuses on known compounds or compound classes, however until now only a relatively small fraction of total compound diversity has been identified (Oburger, Staudinger, et al., 2022). Targeted exudate analysis was frequently performed in the past and it offers valuable quantitative information about individual compounds or compound classes exuded by roots. Targeted analysis uses standard analytical approaches like ion chromatography, high pressure liquid chromatography, as well as gas or liquid chromatography coupled with tandem mass spectrometry. In addition to the above, more sophisticated analytical techniques, spectrophotometric

analysis represents a simple and comparatively cheap approach to measure individual compound classes and is therefore still an important tool for exudate analysis, especially in medium to largescale exudate phenotyping. Specific assays for the relevant compound classes exist. This includes a spectrofluorometric assay for amino acids (Jones et al., 2002), an anthrone colorimetric assay for carbohydrates (Hansen & Møller, 1975), and the Folin-Ciocalteau assay for phenols (Ainsworth & Gillespie, 2007). Relating compound specific results to total C exudation (see section 2.5.5) allows the estimation of the relative contribution (%) of targeted compound to the total amount of C released.

2.5.7. Non-targeted metabolomics

Non-targeted metabolomic analysis represents a cutting-edge approach to provide qualitative information about the entire metabolite composition released by roots. Due to its high sensitivity and wide range of covered metabolites, mass spectrometry has become the technique of choice in many metabolomics studies (Fuhrer and Zamboni 2015). Mass spectrometry-based metabolite analysis generally either follows chromatographic (gas (GC) or liquid chromatography (LC)) or electrophoretic separation (capillary electrophoresis) of the sample or can be achieved after direct infusion, particularly in combination with a high-resolution mass spectrometer (Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) (Shulaev, 2006). Nuclear magnetic resonance (NMR) has also been applied in metabolomics studies. While being robust with regard to reproducibility and identification, the major limitation of NMR for comprehensive metabolite fingerprinting is its relatively low sensitivity (van Dam & Bouwmeester, 2016), rendering it unsuitable for the analysis of large numbers of low-abundance metabolites as often present in root exudation samples. Consequently, it is mostly used in combination with other MS-based techniques in the context of root exudation studies (Escudero et al., 2014; Fan et al., 2001).

While non-targeted metabolomic analysis is of great value to shed light onto the metabolite diversity of exudates, compound identification remains a major bottleneck as data evaluation is extremely time-consuming. Available data bases used for compound identification to date only allow the identification of about 10–30% of analysed features (Frémont et al., 2022; Herz et al., 2018; van Dam & Bouwmeester, 2016). Nevertheless, depending on the depth of data evaluation and availability of financial recourses, non-targeted metabolomics can be highly informative analytical tool in medium scale exudate phenotyping. Combining of all presented root exudation analysis (see section 2.5.5 for total C and section 2.5.6 for targeted analysis) consequently delivers both, quantitative and qualitative information and ultimately contributes to a holistic phenotyping of root exudation.

Table 1 *List of phenotyping tools suitable for field- and CE conditions to quantify rhizosphere traits (exudate and microbiome).*

Rhizosphere related traits				Tools for phenotyping			
Category	**Type**		**Rhizosphere trait**	**Rhizosphere sampling**			**Rhizosphere analysis**
microbiome traits	endosphere	community composition	bacterial abundance	excavation	shaking off bulk soil	root washing	16s rRNA + qPCR
			fungal abundance	excavation	shaking off bulk soil	root washing	ITS rRNA + qPCR
			diversity of bacterial communities	excavation	shaking off bulk soil	root washing	16s rRNA + sequencing
			diversity of fungal communities	excavation	shaking off bulk soil	root washing	ITS rRNA + sequencing
			mycorrhizal abundance	excavation	shaking off bulk soil	root washing	qPCR
			diversity of mycorrhizal communities	excavation	shaking off bulk soil	root washing	sequencing
		morphological observation	mycorrhizal associations	excavation	shaking off bulk soil	root washing	staining and microscopy, molecular techniques
			nodulation (legumes)	excavation	shaking off bulk soil	root washing	microscopy
		targeted enrichment	functional microbial communities	excavation	shaking off bulk soil	root washing	shotgun sequencing, qPCR
	rhizosphere	community composition	bacterial abundance	excavation	shaking off bulk soil		16s rRNA + qPCR
			fungal abundance	excavation	shaking off bulk soil		ITS rRNA + qPCR
			diversity of bacterial communities	excavation	shaking off bulk soil		16s rRNA + sequencing
			diversity of fungal communities	excavation	shaking off bulk soil		ITS rRNA + sequencing
			mycorrhizal abundance	excavation	shaking off bulk soil		qPCR

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Rhizosphere related traits				Tools for phenotyping			
			diversity of mycorrhizal communities	excavation	shaking off bulk soil		sequencing
		morphological observation	mycorrhizal associations	excavation	shaking off bulk soil		staining and microscopy, molecular techniques
			nodulation (legumes)	excavation	shaking off bulk soil		microscopy
		targeted enrichment	functional microbial communities	excavation	shaking off bulk soil		shotgun sequencing, qPCR
	bulk soil	community composition	bacterial abundance	excavation			16s rRNA + qPCR
			fungal abundance	excavation			ITS rRNA + qPCR
			diversity of bacterial communities	excavation			16s rRNA + sequencing
			diversity of fungal communities	excavation			ITS rRNA + sequencing
			mycorrhizal abundance	excavation			qPCR
			diversity of mycorrhizal communities	excavation			sequencing
		morphological observation	mycorrhizal associations	excavation			staining and microscopy, molecular techniques
			nodulation (legumes)	excavation			microscopy
		targeted enrichment	functional microbial communities	excavation			shotgun sequencing, qPCR
root exudate traits	exudates	targeted	total C exudation rate	excavation	root washing	hydroponic exudate collection	liquid TOC analyser spectrophotometric assays
			total N exudation rate	excavation	root washing	hydroponic exudate collection	liquid TOC analyser spectrophotometric assays

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Rhizosphere related traits				Tools for phenotyping			
			carbohydrate exudation rate	excavation	root washing	hydroponic exudate collection	spectrophotometric assays
			amino acid exudation rate	excavation	root washing	hydroponic exudate collection	spectrophotometric assays
			phenol exudation rate	excavation	root washing	hydroponic exudate collection	spectrophotometric assays
		non-targeted	exudation of non-targeted metabolites	excavation	root washing	hydroponic exudate collection	LC-MS based non-targeted metabolomics

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3. Outlook

Despite significant advances in the past decades, rhizosphere processes involving the interplay of root, soil and microbiota still largely embody a black box for plant and soil scientist. Hard-to-reach belowground traits require novel but also proven tools to elucidate the complicated cross-talk within the rhizosphere. Thanks to significant progress in analytical techniques, several new tools for analysing microbiome traits as well as root exudates were developed in recent years. For example, higher throughput and lower costs due to advances in sequencing techniques (see qPCR approach in section 2.3.3) drive the progress in phenotyping microbiome traits. With the latest establishment of non-targeted metabolomics in exudation research, the entire metabolite composition released by roots is now assessable. In combination with existing tools, holistic phenotyping of root exudates becomes possible. Other developments, like the spectrometric method to determine total C exudation, improve the throughput in exudation research (Oburger, Staudinger, et al., 2022). However, great constraints remain in sampling these rhizosphere traits, despite the remarkable progress in analytical techniques. In fact, there is no medium- to large-scale sampling approach available to collect root exudates flawlessly nor to obtain unaltered rhizosphere soil samples (e.g., for describing spatio-temporal occurrence of the microbiome). However, good compromises between improved applicability and limited rhizosphere disturbance have already been tested (WP2 T2 – see section 2.5.3 Combined exudation and microbiome sampling from the same plant, and preliminary protocol in annexes). Nevertheless, new ideas for, ideally, non-destructive phenotyping tools are needed. Using aboveground traits as proxies for root and rhizosphere traits seems tempting here, especially when taking advantage of the remote sensing technologies. Proxies for remote sensing would particularly benefit in the field environment, increasing phenotyping throughput significantly. However, aboveground as well as belowground traits are affected by a wide range of environmental factors, especially in the field. Hence, correlations between aboveground and belowground traits are hampered. It remains therefore questionable how significant conclusions based on aboveground traits are in regards to root and rhizosphere traits. Yet, only a few studies investigating proxies for belowground traits exist which is why Root2Res will aim to assess their potential role in improved root and rhizosphere phenotyping.

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Root2Res

Root phenotyping and genetic improvement for rotational crops resilient to environmental change

ANNEXES

Funded by the European Union

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5. Annex 1: Protocol for combined exudation and microbiome sampling from the same plant

5.1. Background

The combined exudation and microbiome sampling from the same plant increases throughput and is therefore a recommended tool for medium-scale rhizosphere phenotyping. We tested and confirmed two suitable approaches for the combined sampling of root exudates and microbiome. These two methods are similar, except for the bulk soil removal which is either washed off the roots (A "wet approach") or manually shaken off the roots (B "dry approach") (Figure A 1).

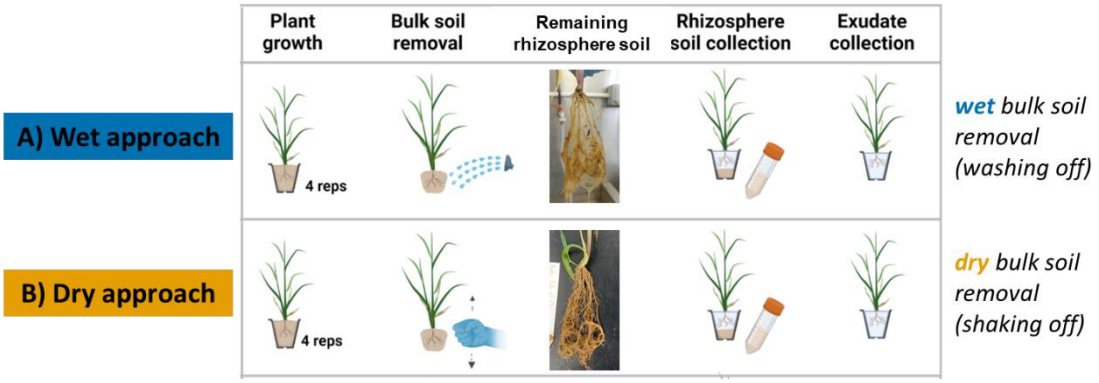

Figure A 1. Two approaches to combined sampling of root exudates and microbiome.

When working with the "wet bulk soil removal approach", bulk- and rhizosphere soil are washed off both and collected separately, with the washing procedure being stopped when only a thin soil layer attached to roots remains. This soil layer is then considered as the rhizosphere fraction which is collected by dipping the roots repeatedly into a bucket filled with a fresh solution (distilled water or electrolyte solution).

In contrast, no water is used for washing off the bulk soil from the roots during the "dry approach". Instead, bulk soil removal is done dry by shaking off the bulk soil carefully until only a thin soil layer attached to the roots remains (this layer is also defined as rhizosheath (Brown et al., 2017)). Collection of this rhizosphere fraction is done as for the wet approach.

The "wet approach" is gentler than shaking off the bulk soil. However, the distinction between bulk- and rhizosphere soil during the "wet approach" is less precise and more operator-dependent than during the "dry approach". Finding the right approach for sampling exudates together with microbiome traits depends on the research focus, but also on the soil texture in the experiment, since lighter sandy soils can be shaken off easier with less root damage than heavier clayey soils.

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5.2. Material needed (for each sample)

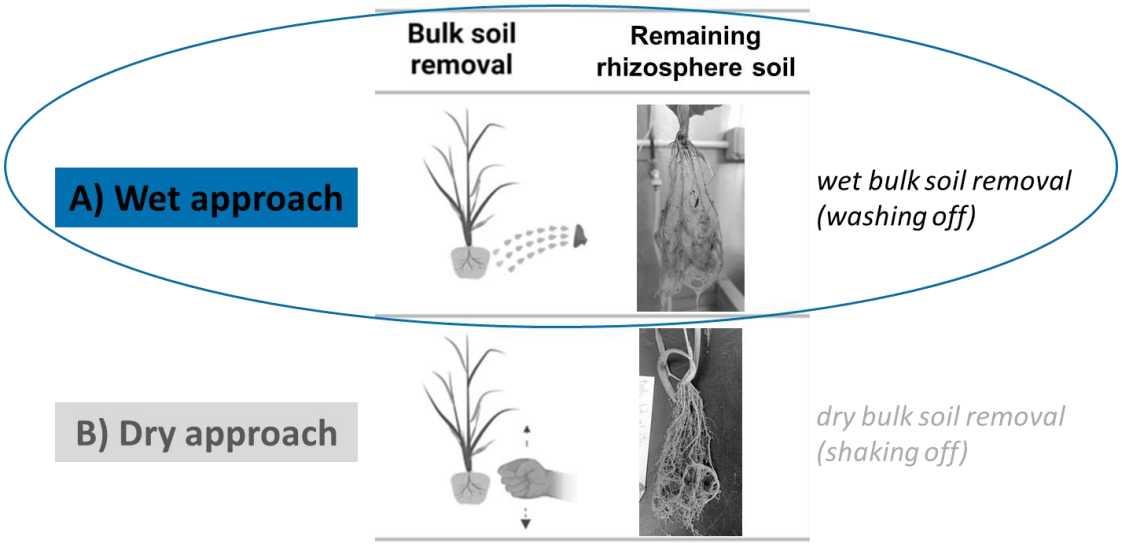
Rhizosphere soil collection

- experimental design: include additional unplanted pots as only bulk soil samples (will be treated similar during experiment: e.g., watering, fertilization)
- running water for root washing
- 1x 10 L basin for washing off the bulk soil
- 250 mL (volume used for 5 week old barley plants, volume will depend on respective root system size) distilled water in a small bucket for collecting rhizosphere soil (dipping)
- 1x 50 mL falcon tube
- Icebox or cold room (keeping rhizospheres)
- 70 % EtOH (cleaning the spatula for bulk soil)
- spatula (bulk soil)
- centrifuge
- liquid nitrogen

Exudate collection (based on soil-hydroponic-hybrid approach)

- sampling solution: Milli-Q water + 5 mg L^{-1} Micropure Classic (Roth Katadyn)
  - to be prepared 2 h before sampling exudates
- bucket volume for collecting root exudates depends on root system size, root (g dwt) to sampling solution volume ratio (L) should be in the range of 2-4
  - e.g.: 250 mL plastic bucket for barley, faba bean and sweet potato (after 5 weeks growth)
  - e.g.: 400 mL plastic bucket for potato (after 5 weeks growth)
- 2x same bucket for previous fine washing and osmotic adjustment
- 1x aluminium foil square (30cmx30cm)
- 20 mL syringes
- 3x 0.2 acetate filters for syringes
- 50 mL tubes for collecting filtered exudates

5.3. Wet bulk soil removal

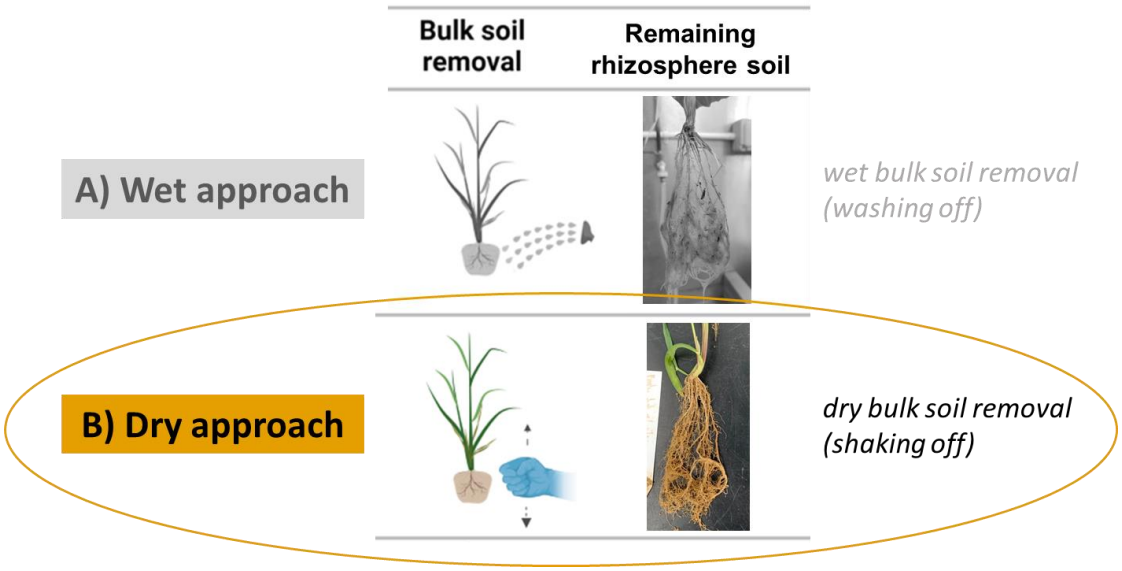


1. remove rooted soil block from pots gently while keeping the entire plant intact
2. soak soil block in the basin pre-filled with distilled water (if not available use tap water)
3. gently free roots from soil by moving the plant inside the bucket (it is important to avoid root damage as much as possible)
4. take roots out to observe how much soil is still attached: wash bulk soil off until roots are covered by a thin soil layer (= remaining rhizosphere soil)
5. when reaching this point, start dipping roots gently and repeatedly in a new bucket filled with approx. 250 mL distilled water (volume depends on root size) until roots are as clean as possible
6. place the bucket with the rhizosphere slurry in the cold room until centrifugation
7. after the rhizosphere fraction has been collected, another gentle root rinsing cycle needs to be done to get rid of the “last” soil attached (remaining soil particles will interfere with exudation results)

Bulk soil sampling:

- bulk soil washed off the roots is not considered for further analysis and thus discarded
- extra unplanted pots (with the same treatment as the pots with plants) represent bulk soil sample
- from the unplanted soil pots: with the help of a spatula remove a portion of soil corresponding to the area explored by the roots. Avoiding the upper layer of the pot as usually will be covered with other autotrophic organisms (e.g., moss)
- place the bulk soil in the container with distilled water

5.4. Dry bulk soil removal



1. remove rooted soil block from pots gently while keeping the entire plant intact
2. begin to remove bulk soil: hold plant in on hand while carefully crumbling bulk soil off the rooted soil block you are holding in the other hand
3. after most of the bulk soil has been removed, vigorously shake the intact root against your palm hand (it is important here to avoid as much as possible root damage)
4. the tight soil attached to the roots represents the rhizosphere fraction (also defined as rhizosheath)
5. when reaching this point, start dipping roots gently and repeatedly in a new bucket filled with approx. 250 ml distilled water (depends on root size) until roots are as clean as possible
6. place the bucket with the rhizosphere slurry in the cold room until centrifugation
7. after the rhizosphere fraction has been collected, another gentle root rinsing cycle needs to be done to get rid of the “last” soil attached (remaining soil particles will interfere with exudation results)

Bulk soil sampling:

- same procedure as for the “wet approach” (see description on previous page)

5.5. Rhizosphere soil collection

1. take the buckets containing the rhizosphere fraction and the bulk soil controls out of the cold room, and centrifuge the sample in 50 mL tubes at 1,500gx for 15 minutes
2. after centrifugation decant the supernatant
3. repeat the previous step with the rest of the rhizosphere sample
4. flash-freeze the rhizosphere samples in liquid nitrogen and store at -70°C

5.6. Exudate collection

A guideline for adjusting the correct sampling volume to collect root exudates

- *Root dry weight (g) / sampling volume (L) = 3±1*

1. prepare Container1 with distilled water, Container2 with random volume of Micropure solution, and Container3 with exact sampling volume of Micropure solution on a table close to each other (to facilitate the transfer of intact plants from container to container)
2. 1st wash in distilled water: once the roots are clean (see point 7), place the entire plant in 250 mL bucket (or 400 ml bucket for potato) filled with distilled water (random volume) = Container1
3. 2nd wash in Micropure solution (Milli-Q water + 5 mg L^{-1} Micropure): once the roots are ultra-clean, transfer the entire plant in 250 mL (or 400mL) buckets filled with the Micropure sampling solution (random volume) – wait for 3 min = Container2
4. prepare 3rd wash Micropure solution: while plants stay for 3 min in Container2, empty the distilled water in Container1 and refill it also with Micropure solution (similar volume than Container2)
5. 3rd wash Micropure solution: transfer plants from Container2 to Container1 – wait for 3 min
6. remove plants from osmotic adjustment buckets (Container1 & Container2), gently place roots for 1-2 seconds on tissue paper that was previously spread on the work desk (fresh tissue for each plant) to remove excess Micropure solution
7. in order to finally collect root exudates, transfer the roots into a third bucket containing the sampling solution with an exact known volume (use the guideline mentioned above to measure volumes like 125-275 mL barley; 250 mL faba bean; 250 mL potatoes; 125 mL sweet potatoes) = Container3
8. wrap Container3 in aluminum foil and take it back to the glasshouse, leave samples for 3 h and record the exact sampling start and end

9. after 3 h of exudate collection, carefully remove plants from the Micropure sampling solution and place them back into the empty Container2
10. filter the exudate solution with pre-rinsed 0.2 µm cellulose acetate syringe filter (OE 66, Whatman, UK) and collect it in 50 mL vials (aliquoting recommended)
    - pre-rinse filter with 2-5 mL Micropure sampling solution and discard a few drops to avoid C contamination
    - exchange filters 2-3 per sample and use a fresh syringe for each sample
11. record the weight of filtered exudate solution and store it at -20°C for later analysis