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Short Communication

An approach for broad molecular imaging of the root-soil interface via indirect matrix-assisted laser desorption/ionization mass spectrometry

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ABSTRACT

Understanding rhizospheric processes is limited by the need for imaging complex molecular transformations at relevant spatial scales within the root soil continuum. Here, we demonstrate a method to enable this analysis by first extracting organic compounds from the rhizosphere onto a PVDF membrane while maintaining their 2D distribution and then imaging the distribution of chemical compounds using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). This approach permitted us to visualize and identify compounds on the root surface and presumed root exudates in the rhizosphere. Within a 1.8 cm × 0.6 cm sampling area of a switchgrass rhizosphere, we could observe at least four chemically distinct zones. Using high performance Fourier transform ion cyclotron MS, we were able to accurately annotate numerous molecules co-localized to each of these zones.

The rhizosphere is a highly dynamic environment, hosting numerous biogeochemical processes linked to root growth, plant-derived carbon inputs, and microbial activity within small spatial zones surrounding plant roots (Philippot et al., 2013). Molecular composition within the rhizosphere is commonly studied using bulk analysis-based mass spectrometry approaches, which can identify a breadth of molecules with great sensitivity and accuracy (White et al., 2017). However, these methods are unable to provide accurate spatial distribution of analytes within a sample.

Emerging techniques in mass spectrometry imaging (MSI) can provide spatial localization of biomolecules, but utilizing MSI methods for *in situ* rhizosphere analyses is largely confounded by the inability to probe these samples directly (due to interference from soil) and/or in maintaining the spatial organization of the molecular constituents (Clode et al., 2009; Jones et al., 2013; Debois et al., 2014; Velickovic and Anderton, 2017; Velickovic et al., 2019a, 2019b). Instead, imaging of organic molecules linked to rhizosphere processes is commonly performed by isolating individual components, where, for example, roots are removed from the soil, washed, and sectioned before MSI analysis (Velickovic et al., 2019a, 2019b) or are grown in soil-free media (e.g. agar) compatible with MSI techniques (Debois et al., 2014). Recent advances in high-spatial resolution secondary ion MSI enable the extensive visualization of nutrient use and transport in the rhizosphere (Nunez et al., 2017; Vidal et al., 2018), but still require cross-linking fixatives which limits the ability to identify metabolite chemical structures.

Here, we demonstrate a novel method that spatially transfers rhizosphere-related compounds to a membrane amenable to matrix-assisted laser desorption/ionization (MALDI) MSI. This indirect imaging approach was adapted from analyses of other challenging samples like skin (Prideaux et al., 2007), leaf surfaces (Li et al., 2011), and lipids separated by thin layer chromatography (Goto-Inoue et al., 2008). We grew switchgrass in 15.2 cm × 20.3 cm x 0.95 cm black high-density polyethylene rhizoboxes with removable side panels (Fig. 1). After 8 weeks of plant growth, we removed a side panel from the rhizobox and installed a prewetted polyvinylidene fluoride (PVDF) membrane against the soil/root interface, then reattached the plexiglass cover while taking care to limit any movement of the membrane during the blotting period (48 h). Immediately after removal from the rhizobox, the PVDF membrane was mounted on a 384-well MALDI target plate (Bruker Daltonics) using double-sided copper tape and fully secured to the plate by rolling the membrane flat using a 20 mL scintillation vial. Soil particles were removed by blowing a stream of nitrogen over membrane. MALDI matrix (organic compounds that enable ionization of analytes) application was performed using a TM-Sprayer (HTX Technologies), where 40 mg/mL of 2,5-dihydroxybenzoic acid (DHB) in 50% MeOH was sprayed with 16 passes at 50 μL/min, 80 °C, a spray spacing of 3 mm, and a spray velocity of 1200 mm/min. MSI was performed on a 15 T Fourier transform ion cyclotron resonance (FTICR)-

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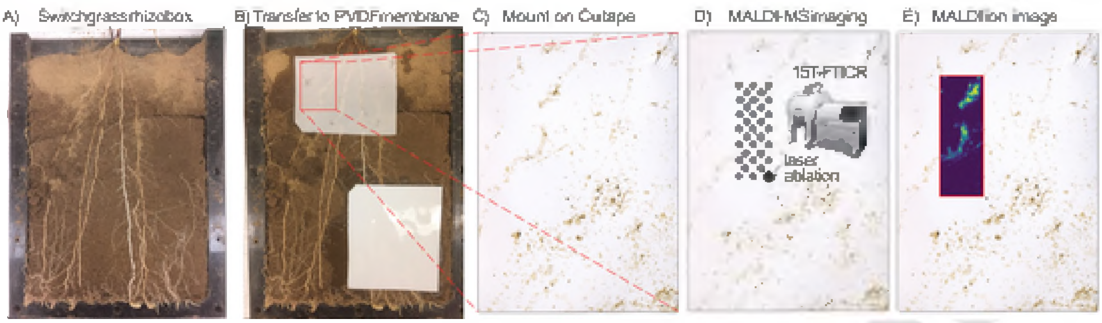

Fig. 1. Example workflow for indirect MALDI-MSI analysis of the root-soil interface. Here, A) Cave-in-Rock switchgrass (*Panicum virgatum* L. from the USDA National Plant Germplasm System) was grown in high-density polyethylene rhizoboxes with removable side panels containing soil (sandy loam Alfisol harvested from plots under continuous switchgrass cultivation for over a decade and sieved at 4 mm) from Kellogg Biological Station, Michigan, USA. Plants were grown in a Conviron walk-in growth chamber (model no. GR48) at 24 °C with 50% humidity during the day and 18 °C with 40% humidity at night (16 h light, 8 h dark) B) Eight weeks after planting, roots were blotted by placing pre-wetted membranes against the soil-root interface for 48 h. The membranes were then removed from the soil, and C) were taped to a steel MALDI target plate using double-sided adhesive copper tape. D) Imaging of the imprinted membrane using MALDI-15 T-FTICR-MS, where the gray spots illustrate ablation areas (ca 30 μm diameter) from laser probing (using 100 μm step size). E) Representative MALDI-MS ion image showing the spatial distribution of kinetin, a plant hormone.

MS (SolariX, Bruker Daltonics), equipped with a SmartBeam II laser source (355 nm, 2 kHz), in positive ion mode using 200 shots/pixel and a 100 μm pitch between pixels. The instrument was operated to collect ions with *m/z* 92-1000 at a mass resolution of ~130,000 at *m/z* 400. To complement the results obtained with MALDI-MSI, multiple membrane pieces were extracted in methanol:water (80:20) and subsequently subjected to LC-MS analysis (Rivas-Ubach et al., 2019).

The ultrahigh mass resolution of the 15 T-FTICR-MS enabled us to annotate exact molecular formulas of ions ablated from the PVDF membrane, and orthogonal LC-MS analysis of bulk membrane pieces assisted us in confident molecular identification of a subset of those ions (Fig. 2 and Supplementary Table 1). These results show we were able to transfer and detect a broad repertoire of small organic compounds from living roots and the surrounding soil. In addition to chemical identification, the combination of membrane blotting and MALDI-based analysis revealed heterogeneous spatial distribution of these compounds in the rhizosphere (Fig. 2A–B). Based on the observed spatial distributions, using SCILS MS imaging processing software, we could define and classify four different groups of rhizosphere related molecules in the example 1.8 cm × 0.6 cm rooting sample we analyzed (Fig. 2C).

The chemically distinct areas that strongly co-localized with the entire root zone (Pearson's correlation coefficient > 0.4) contained the most abundant group of molecules (zones in magenta, Fig. 2C). These molecules include various secondary metabolites, purine and pyrimidine metabolites, and phytohormones (Kuzyakov and Blagodatskaya, 2015). Their spatial distribution suggests a plant origin or production by active rhizosphere microorganisms. The second group comprises of nitrosoglutathione, harman, methyl hydroxy ferulate, and urocanate; these areas occupy small hot spots of ~1 mm × 2 mm ellipse-like zones in proximity (<1 mm) to the main root (zones in yellow, Fig. 2C). Notably, nitrosoglutathione is an abundant S-nitrosothiol in plant cells and serves as a mobile reservoir of nitric oxide (NO) (Kailasam et al., 2018), so this localization may denote intense NO bioactivity in these rhizosphere microregions. Similarly, concentrated urocanate signal in the same areas shows intense histidine catabolism (Bender, 2012). For the third group (zones in green, Fig. 2C), we were only able to annotate arginine. This group was distributed in relatively large islets far away (>2 mm) from any visible root imprint. Arginine is a nitrogen storage compound (Winter et al., 2015) that can be readily visualized in the plant tissues by MALDI-MSI (Walker et al., 2016; Velickovic et al., 2018a, 2018b). However, arginine localization in this sample suggests that it is originated from sources in the surrounding soil rather than the plant root. Lastly, distributed hot spots of ~2–12 imaging pixels in size (areas of 100 μm × 200 μm–300 μm × 400 μm, respectively) comprise the fourth group (zones in cyan, Fig. 2C), and reflect the distribution of carnitine and glutamine. Based on their wide distribution within the sample, we hypothesize that these molecules are not plant exudates, but rather reveal the localization of abundant microbial aggregates within the rhizosphere. This is supported by numerous studies that shows carnitine metabolism by soil microbiome (Meadows and Wargo, 2015).

LC-metabolomic data (as presented in Supplementary information) shows we are also able to spatially transfer, from the rhizosphere to membrane, molecules that are commonly detected in bulk soil analyses (e.g., sugars and organic acids) (White et al., 2017). Howevr, our current limitation to detect these molecules *in situ* by MSI is reflected in the incompatibility of the surface chemistry of the blotting membrane with negative ionization mode analysis, which is generally better for detecting these compounds.

In summary, our findings demonstrate the potential to elucidate the spatial distribution of multiple classes of organic compounds within rhizosphere systems. Although certain common compounds in soils (i.e. sugars, organic acids) were not imaged, we anticipate that further studies utilizing different membrane materials, matrices, and MSI conditions will adequately capture the richness of organic molecules within the rhizosphere. In this work, we focused on an actively growing root with lower lignification than more mature root areas, but a notable advantage of this approach over other rhizosphere chemical imaging methodologies (Jones et al., 2013; Kaiser et al., 2015; Vidal et al., 2018) is that it allows spatiotemporal molecular mapping of living and developing roots in the rhizosphere since a fresh membrane can be used at different stages of root growth. Lastly, our approach offers a path for moving beyond bulk-omic analyses, providing a deeper understanding of rhizosphere processes on the submillimeter scale.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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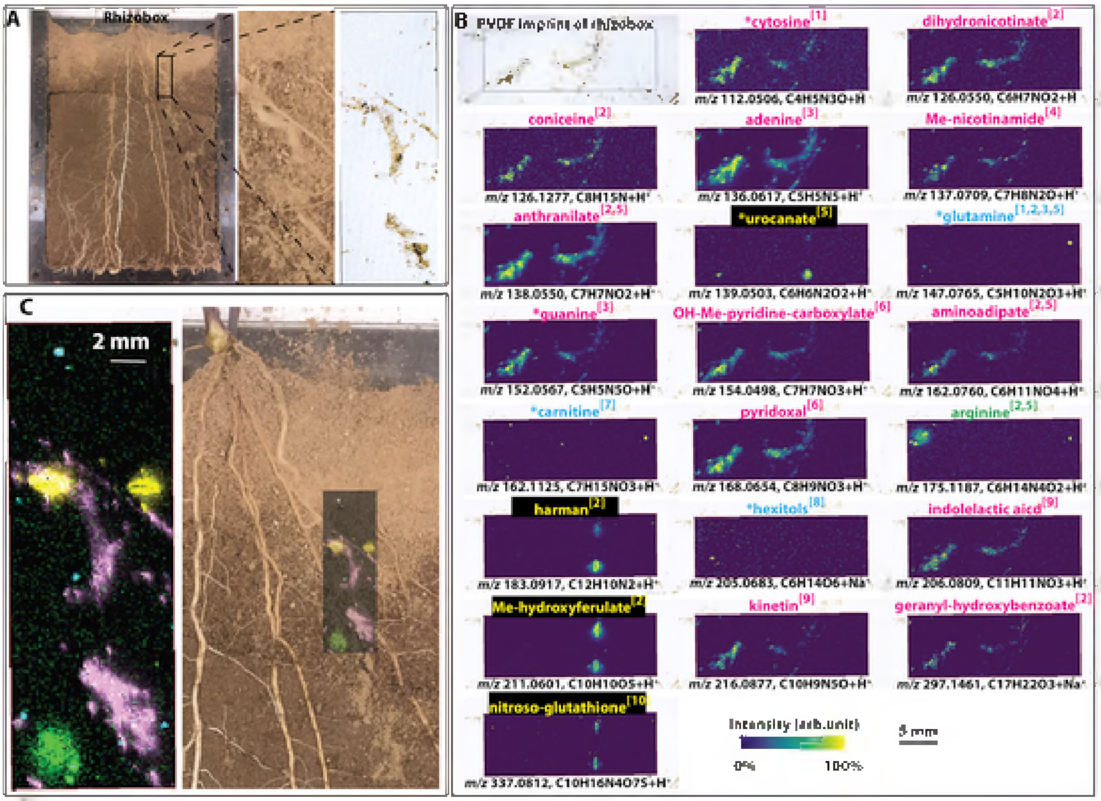

Fig. 2. Indirect MALDI-FTICR-MS imaging of the rhizosphere. A) Black rectangle shows position of analyzed area in rhizobox (left), root-soil interface (middle), root-soil interface imprinted on PVDF membrane (right). B) Ion images and putative molecular annotations of species detected by MALDI-FTICR-MSI of an imprinted rhizosphere on a PVDF membrane (top left, black box indicates area of analysis). Note that the ion images are displayed after 90^0 clockwise rotation compared to panel A. The number ascribed to each molecular annotation represents the major metabolic pathway where the molecule is found as either a reactant, product, or intermediate, specifically: [1] pyrimidine metabolism; [2] biosynthesis of secondary metabolites; [3] purine metabolism; [4] nicotinate and nicotinamide metabolism; [5] biosynthesis of amino acids; [6] Vitamin B6 metabolism; [7] thermogenesis; [8] fructose and mannose metabolism; [9] phytohormone; or [10] NO reservoir. The identity of ions labeled by "*" was confirmed by LC-MS analysis of PVDF membrane extracts using an internal database. All ion images were acquired with a 100 μm step size. C) Distribution of four chemically different areas observed in MALDI-FTICR-MSI of the rhizobox imprinted on PVDF membrane represented by different colors. These four areas co-localize with specific anatomical or molecular features. Specifically, areas containing chemical features that correlate with the root can be observed in magenta, whereas chemical features that correlate with nitrosogluthathione, arginine, and carnitine are observed in yellow, green, and cyan areas, respectively. The far right image shows localization of the 4 chemical zones in the rhizobox. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.107804.

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