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In Situ Non-Destructive Temporal Measurements of the Rhizosphere Microbiome 'Hot-Spots' Using Metaproteomics

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Abstract: Rhizosphere arguably embodies the most diverse microbial ecosystem on the planet, yet it is largely a functional 'black box' of belowground plant-microbiome interactions. The rhizosphere is the primary site of entry for subsurface injection of fixed carbon (C) into soil with impacts on local to global scale C biogeochemistry and ultimately Earth's climate. While spatial organization of rhizosphere is central to its function, small scale and steep microbial and geochemical gradients within this dynamic region make it easily disrupted by sampling. The significant challenge presented by sampling blocks elucidation of discreet functions, drivers, and interactions within rhizosphere ecosystems. Here, we describe a non-destructive sampling method linked to metaproteomic analysis in order to measure temporal shifts in the microbial composition and function of rhizosphere. A robust, non-destructive method of sampling microbial hotspots within rhizosphere provides an unperturbed window into the elusive functional interactome of this system over time and space.

Keywords: rhizosphere; metaproteomics; hot-spots; metagenomics; in situ; non-destructive; temporal

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1. Introduction

Rhizosphere microbiomes inhabits the narrow (~2–4 mm) zonal interfaces between soil and plant roots, representing the most diverse microbiomes on Earth, containing up to 10^{11} microbial cells and ~30,000 bacterial species per gram of root [1]. The rhizosphere microbiome exists through an interwoven tapestry of bacteria, viruses, archaea, protists, fungi, nematodes, and small arthropods interacting directly with plant roots and each other. Plant functional traits are influenced by the rhizosphere microbiome, which includes impacts on plant metabolism, hormonal pathways, nutrition, stress tolerance (e.g., drought), and enhancement of biosynthetic capacities [2]. The rhizosphere is fundamentally essential for multiple ecosystem processes including recycling and storage of plant fixed C (CO_2 consumed during photosynthesis), water cycling, nitrogen fixation, and nutrient storage [3]. Increased anthropogenic C cycling is manipulating the global climate with resulting differential moisture extremes, rising temperatures, and pathogen migrations that are undoubtedly impacting the function of the rhizosphere microbiome and shifting its overall functional capacity. Warming is also causing increased global soil respiration and the resulting CO_2 fluxes driven by microbial heterotrophic metabolism. The impact of these microbes 'breathing harder' results in net C losses and likely the overall reduction in C storage in soils [4], with resulting direct and indirect impacts on the global food supply chain including, crop production, and primary livestock feed [3]. Possessing spatially and

temporal focused tools for functional assessment will be crucial for forming a predictive understanding of both how the rhizosphere influences C and nutrient cycling and how these impacts may be amplified or dampened by future environmental changes.

Despite the importance of spatial organization within rhizosphere, sampling of intact rhizosphere 'omes' without disruption or destruction remains elusive. While metagenomics, metatranscriptomics, metaproteomics, and metabolomics can provide an integrated 'multi-omic' and 'metaphenomic' view of soil/rhizosphere microbiome [5], these tools are limited to only looking at samples after physical disruption required for analysis, such as soil sieving which alters 'real-time functions,' destroys spatial gradients, and erases spatial specialization of the rhizosphere. Metaproteomics provides a representative measurement of microbiome function with the stability of proteins and helps to elude biases associated with mRNA analysis. However, rhizosphere metaproteomics is not without challenges linked to high diversity, heterogeneity of soil matrices, extraction biases, and limited metagenomic reference databases for these systems [6]. Significant improvements with respect to high-resolution mass spectrometry, extraction [7], increased separation via proteomic fractionation using 2D [8], and metagenomic assembly for proteomic databases [9] have provided higher proteomic resolution, but the ability for metaproteomics to measure the rhizosphere/soil microbiome still relies on disruptive and destructive sampling methods.

Here, for the first time, we describe and demonstrate an integrated temporally resolved sampling and measurement technique of the metaproteome within switchgrass rhizosphere in a non-disruptive/non-destructive manner to bridge high-throughput mass spectrometry with specific physical locations on rhizosphere.

2. Materials and Methods

2.1. Soil and Plant Sampling Set-up

Switchgrass (*Panicum virgatum* L., var. Cave-in-Rock [10]) was grown in rhizoboxes containing soil harvested from a W.K. Kellogg Biological Station (KBS) field plot that has been in continuous *Panicum virgatum* (switchgrass) cultivation since July 2008 [11,12]. The soil is characterized as a Typic Hapludalfs with approximately 43% sand, 40% silt, and 17% clay content [13] and was maintained at 4 °C from harvest until sieving through a 4 mm^2 sieve in preparation for plant growth. The plants were cultured by sowing a single seed into each of triplicate rhizoboxes (dimensions of 1 cm × 20 cm × 15 cm) that had removable opaque sides to enable sampling of soil. Plant cultivation was in a Conviron (Winnipeg, MB, Canada) model number GR48 walk-in growth chamber following the methods of Ilhardt et al. [14]. Briefly, a 16:8 h lighting cycle (1219 µmol s^{-1} m^{-2}) at 24 °C and 60% humidity was used during the light cycle and 18 °C with 50% humidity during the night cycle. Rhizoboxes were placed at roughly a 30° angle offset from vertical to encourage root growth along the sampling pane of the rhizobox and roots were visualized via their auto fluorescence using a Typhoon Laser Scanner 9500 (GE Healthcare, Chicago, IL, USA).

2.2. Proteomics

We performed non-destructive protein extraction (Figure 1) from each rhizotron by removing the side panel and applying a pre-wetted nitrocellulose membrane to the exposed root-rhizosphere soil surface, following the blotting methods of Lin et al. for 15 min [15]. Proteins were transferred from the sample to the membrane surface over a 15 min duration, fixed and stained using Sypro™ Ruby general protein stain, and then visualized using a Typhoon™ FL 9500 (GE Healthcare, Chicago, IL, USA) laser scanner to localize protein (by fluorescence). Manual sampling of small subsections of the membrane was guided by black light illumination of the sample, which enabled us to performed membrane excisions in areas of high protein abundance using a biopsy device (Integra Miltex Biopsy punch, Integra 3335, Integra Life Sciences, Princeton, NJ, USA), which removed 5 mm diameter membrane sections. These section punches from each individual rhizobox and sampling day were combined for a total of 15 samples (3 rhizoboxes; 5 sampling days). We performed trypsin digestion on the membrane then the resulting peptides were analyzed

via the nanoproteomics platform (SNaPP) [16] on a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA, USA). Briefly, peptides were analyzed by injecting the entire 20 µL sample (resulting from the on-membrane trypsin digests) onto an in-house simplified nanoproteomics platform (SNaPP), where by bypassing the immobilized trypsin column, the peptides were trapped and desalted via an on-line SPE column (4 cm length × 150µm inner diameter) packed with 5µm C_{18} particles (Phenomenex) [16]. The peptides were then separated using a 25 cm analytical column with 50 µm I.D. packed with 1.7 µm AQ C_{18} media (Waters, Milford, MA, USA). The eluting peptides were analyzed on a Q-Exactive mass spectrometer (Thermo Scientific). Mass spectra were collected from 400 to 2000 m/z at a resolution of 70 k followed by data dependent HCD MS/MS at a resolution of 17.5 K for the twelve most abundant ions.

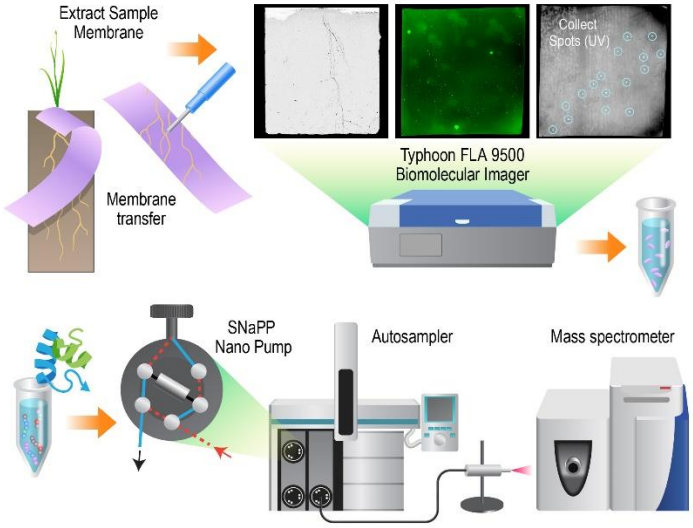

Figure 1. Setup of Rhizobox, sampling, and measurements. A pre-wetted nitrocellulose membrane is placed on an exposed soil surface on the side of a rhizobox and used to non-destructively remove mobile proteins from the sample while preserving their two-dimensional location on the membrane. Spatial distributions of collected proteins were determined using a general protein stain which helps guide excision of small (1 mm diameter) membrane sections used for proteomic analysis. Products of on-membrane trypsin digests were purified and separated using the SNaPP method [16] to minimize loses to increase overall proteomic yields from analysis by mass spectrometry.

A Kellogg Biological Station (KBS) at Michigan State University metaproteomic reference database constructed from the KBS rhizosphere metagenome [17] via ATLAS [9] and 16S amplicons were analyzed as previously described [18]. Mass spectra, peak discovery, and refinement using MS-GF$^+$ were completed as previously described [8]. In order to compare the temporally resolved proteomic analysis, we also performed 16S amplicon as previously described using PowerSOIL DNA extraction, Earth Microbiome PCR methods, and qiime2 [18] and more traditional and bulk scale metaproteomic assessment (using ~5 g of excised soil) following the simultaneous extraction of metabolites, proteins, and lipids MPLEx extraction [7].

3. Results and Discussion

We compared taxonomic identification between the temporally resolved non-disruptive/destructive rhizosphere 'hot-spot' metaproteomics and the more traditional 16S amplicon, bulk proteomic, and metagenomic analyses performed at the end of the experiment. Note, here we refer to hot-spots as the specific locations containing enhanced protein abundance as measured by staining and fluorescence. We resolved nine different phyla of bacteria (Figure 2) between all measurements and elucidated the temporal dynamics of which of these groups were functionally active in the 'hot-spots.' Measurements greater than >30%,

on average, of the results were obtained from groups that were taxonomically unidentified (Figure 2).

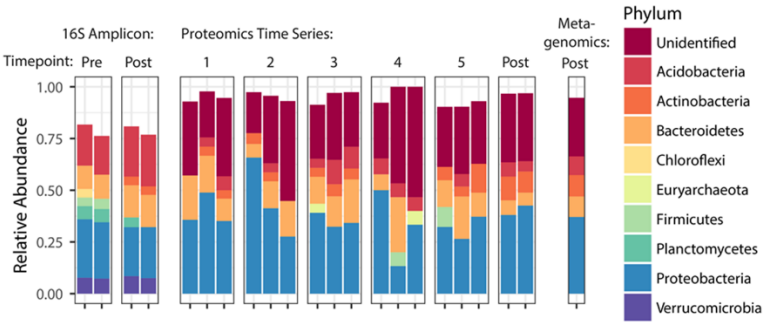

Figure 2. Taxonomic and functional profile of rhizosphere hot-spots. Taxonomy is based on SILVA database for 16S amplicons. The bulk metagenome from KBS, hotspot proteomic time series (1–5 represent days; Pre = beginning of the experiment or day 1; Post = Day 5), and the post (bulk ~5 g) metaproteome are based on RefSeq taxonomy from ATLAS.

We further elucidated the proteins by function, which included both plant and microbial proteins. The plant-specific proteins identified in the hot-spots included ATP-synthases and ribosomal proteins (Table 1). The microbial-specific proteins included kinases, chaperones (Hsp70), polymerases, glyceraldehyde-3-phosphate dehydrogenase, isomerases, pyridoxal phosphate-dependent aminotransferase, and malate dehydrogenase (Table 1). Only fast-growing fastidious members from Gammaproteobacteria and Bacteroidetes such as *Acinetobacter*, *Pseudomonas*, and *Mulicaginibacter* were identified (Table 1). These *Pseudomonas* and *Acinetobacter* (a member of the same order) are strong root colonizers that often form biofilms and limit competing microbes from colonizing as well as provide plant-growth promotion [19].

Table 1. Protein identifications from the 'rhizobox' samples blots. Functions and taxonomic are from RefSeq notation.

Membrane Protein Observed	Observed In (RhizoBox)	Protein Function	Organism
6SS2_k121_2341198_12	B1, B2, C2, B4, B5	Nucleotide-Binding Domain of the sugar kinase/HSP70/ actin superfamily	Bacteria
6SS2_k121_3766132_8	C1,	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily	Bacteria
6SS4_k121_1151881_1	A2,	molecular chaperone DnaK Hsp70 protein	Acinetobacter
6SS4_k121_241109_3	A2,	DNA-directed RNA polymerase subunit alpha	Acinetobacter
6SS4_k121_25570_1	A2,	pyridoxal phosphate-dependent aminotransferase	Acinetobacter
6SS4_k121_444875_3	A2,	malate dehydrogenase	Acinetobacter
6SS4_k121_107562_9	B2,	phosphonate ABC transporter substrate-binding protein	Acinetobacter
6SS2_k121_309756_1	A3, A5, B5	F0F1 ATP synthase subunit alpha	Panicum
6SS2_k121_3149475_2	A3, B5	Ribosomal protein S11	Bacteria
6SS4_k121_315695_2	A3,	glyceraldehyde-3-phosphate dehydrogenase	Chryseobacterium
6SS4_k121_820786_1	A3, A4,	ketol-acid reductoisomerase	Pseudomonas
6SS2_k121_559796_1	A5, B5	ATPase, F1 complex	Panicum
6SS5_k121_1973917_7	B5	nucleoside-diphosphate kinase	Mucilaginibacter

4. Conclusions

Our study provides the first 'proof-of-concept' non-disruptive/non-destructive measurements of taxonomy and functions of the rhizosphere 'hot-spots' of microbial metabolism over temporal space. Coupling of this approach with stable-isotope labeling would help distinguish proteins being generated in real-time by currently growing microbes from residual/relic proteins synthesized before the application of an isotope tracer [20,21]. Activity-based probes and fluorescence in situ hybridization (FISH) could also be used to assay for functional genes or microbial functional guilds within these rhizosphere hot-spots [22,23], but these approaches are also very targeted and cannot provide the broader data coverage of a metaproteomic approach as developed here. We further revealed that these rhizosphere hot-spots can detect similar taxonomic diversity and functions even via small sampling when compared to those predicted by traditional 16S, metagenomics, and bulk shotgun metaproteomics [24], further illustrating why the direct measurement of these dimensions will provide enlightening details of the belowground functional black box.

Author Contributions: J.R. and J.J.M. conceived and designed the experiments. J.R. completed measurements and experiments of metaproteomics. P.D.P. assisted with proteomic analysis. R.A.W.III conducted data analysis, metagenomic assemblies, metagenomic annotation, and cowrote the paper with J.R., C.J.B. conducted 16S data analysis and integration of annotations. All authors contributed to editing and read and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that there are no conflict of interest. RAWIII is the CEO of RAW Molecular Systems (RAW), INC, but no financial, IP, or others from RAW INC were used or contributed to the study.

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