

**Exploratory Project: Rigid nanostructured organic polymer monolith for in situ collection
and analysis of plant metabolites from soil matrices**

FINAL TECHNICAL REPORT

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ABSTRACT:

Plant metabolites released from litter leachates and root exudates enable plants to adapt and survive in a wide range of habitats by facilitating resource foraging and plant-organismal interactions, and could influence soil carbon storage. The biological functions of these plant inputs and the organismal interactions they facilitate in soil are strictly governed by their composition and molecular identity. Our current understanding about the molecular identity of exudates is based on physiological studies that are done in soil-less axenic cultures. On the other hand, ecological studies that rely on isotope labeling to track the fluxes of carbon from plants to soil have found the complexities of soil-microbe matrices as an insurmountable barrier to undertake any meaningful molecular level characterization of plant inputs. Although it is constantly advocated to undertake a molecular level identification of the dynamicity of plant metabolites in soils, the complexity of soil system has thus far prevented any such endeavors.

We developed polymeric probes through in-situ polymerization of poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) to elucidate the chemical environment of the soil to which the plant roots are exposed. Hypercrosslinking of the polymeric probes through an in-situ Friedel-Crafts alkylation significantly increased the surface area and the sorption capacity of the probes. Surface functionalization of the probes using a hybrid approach was also attempted. The efficacy of these probes was tested using batch equilibration. Scanning electron microscopy revealed extensive modification of the surface of the probes through hypercrosslinking. The probes exhibited a lower site specific sorption (slope of Freundlich adsorption isotherm close to unity) and percent recovery of the sorbed compounds from the probes were >70, indicating a predominance of reversible sorption. Further we imparted specificity to this copolymer matrix by using molecular-template imprinting approach for the selective capture of less abundant plant metabolites from a crowded soil/litter leachate.

Our results suggests that i) the root exudate pattern of plants is highly dependent on the nutrient status of the plant, with greater specificity of root exudation occurring in growing medium with low available form of nutrients, ii) the chemical composition of root exudation is a function of the distance of sampling from the source-roots, with the composition of root exudation being more enriched in polar metabolites farther from the source-roots, iii) Further we demonstrated that the compounds present in real root exudates diffuse farther from the source roots than those in artificial root exudates that are traditionally used.

Thus, our project highlights how the soil matrix is instrumental in modifying the chemical composition of root exudates, and highlights that, apart from the plant physiology, the specificity and function of root exudates is also modified by environmental factors.

Award # DE-SC0010832

Program officer: Dr. Daniel B. Stover

PROJECT OVERVIEW AND SPECIFIC GOALS: (proposed)

Project Overview: Release of photosynthates from plant roots and decomposing litter forms a major conduit through which atmospheric carbon dioxide reaches belowground ecosystems. These metabolites mobilize the unavailable nutrients in soil matrices and thus assist in resource foraging in plants; also they form the major source of energy for soil fauna that facilitates the formation and stabilization of carbon in soil, thus regulating the soil carbon sequestration in terrestrial ecosystems. Despite the profound implication of these metabolites in the organismal- and ecosystem-level interactions, the complexities of soil matrices hinder the current efforts to delineate the specific chemical composition of these plant inputs. Such information is vital as the biological functions of these plant inputs and the interactions they facilitate are strictly governed by their composition and molecular identity. To transcend this problem, the project aims to develop new polymeric probes that will be highly effective in the *in-situ* capture of plant metabolites. The polymeric matrix of the probes will be optimized using various monomer combinations that would confer optimal balance between high porosity, high sorption capacity and the versatility of functional group attachments, while maintaining rigidity and reusability of the probes. The probes will be tested in both managed and non-managed ecosystems for the efficient capture of plant metabolites, which then will be characterized using advanced analytical techniques. The proposed probes will be instrumental in gaining a better understanding of the chemical environment in soils to which the roots are exposed to, and would help scientist to measure the real-time fluxes of plant exudates in different ecosystems, which in turn will enable an accurate classification of the labile pools of carbon in biogeochemical models.

Specific Goals:

- 1) Develop polymeric probes with various meso and macro porosity using various polymeric matrices.
- 2) Use post-polymerization monolith modification to graft various functional groups on to the monolith to increase the range of compounds that could be efficiently captured by the probe.
- 3) Use the optimized probes for the capture and characterization of plant root exudates.

ACTIVITIES AND ACCOMPLISHMENTS:

What was accomplished under these goals?

Brief summary of the activities and accomplishments are provided under specific goals:

Goal 1: Experiments to develop polymeric probes with various meso and macro porosity using various polymeric matrices

- The traditional Poly(styrene-divinylbenzene) copolymer matrix were first attempted. But this resulted in very low surface area (Fig. 1).
- Further, we developed polymeric probes by adding chloromethyl-styrene to the above mix and conducting *in-situ* polymerization of poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) in stainless steel tubes (Fig. 1).
- The composition of monomers and porogens, the ratio of monomer to porogen, the temperature and time of polymerization reaction were optimized to develop probes that have an optimal balance of mesopores and macropores (Fig. 2).

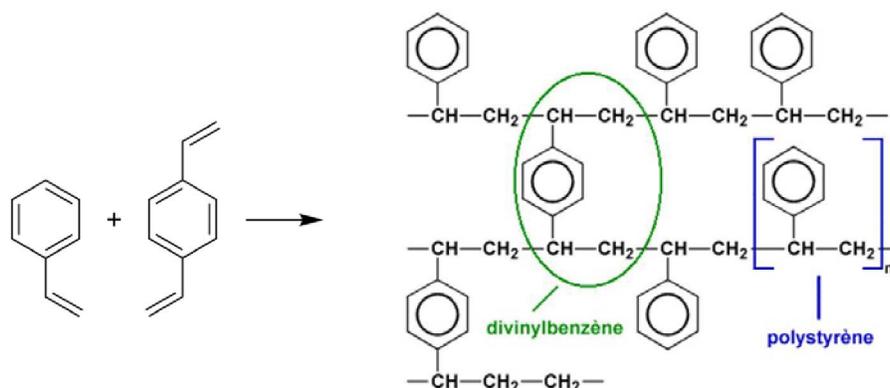


Fig. 1. Traditional poly(styrene-co-divinylbenzene) co-polymer that exhibit modest surface areas in the range of tens of m^2g^{-1}

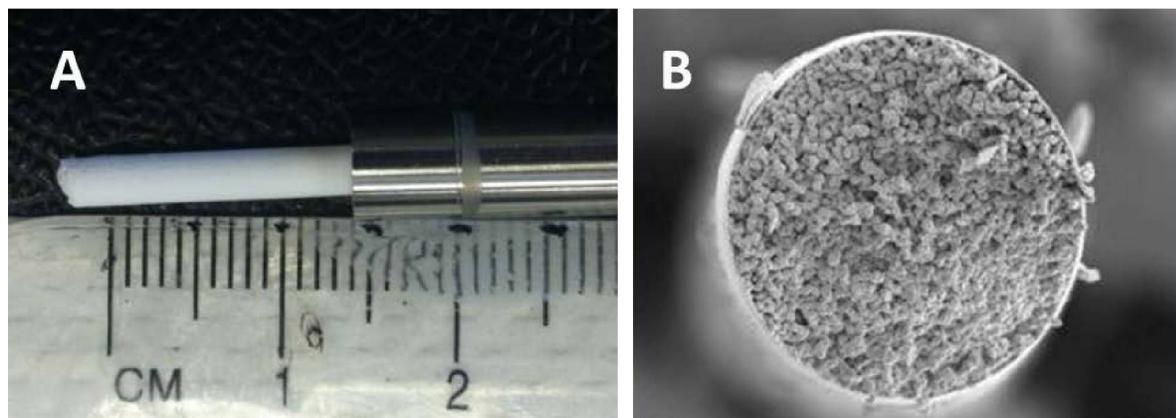


Fig. 2. Polymeric probes molded in 2.1mm stainless steel tubes (A). Cross section of a similar mixture polymerized in a 250μm glass capillary column.

Goal 2: Experiments utilizing post-polymerization monolith modification to increase the range of compounds that could be efficiently captured by the probe.

G2.1) Hyper crosslinking of the polymeric matrix

- The polymeric probes were developed through in-situ polymerization of poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) were swollen in dichloroethane and hypercrosslinked via Friedel-Crafts reaction catalyzed by ferric chloride (Fig. 3).
- The reaction temperature and the duration was altered to change the surface area

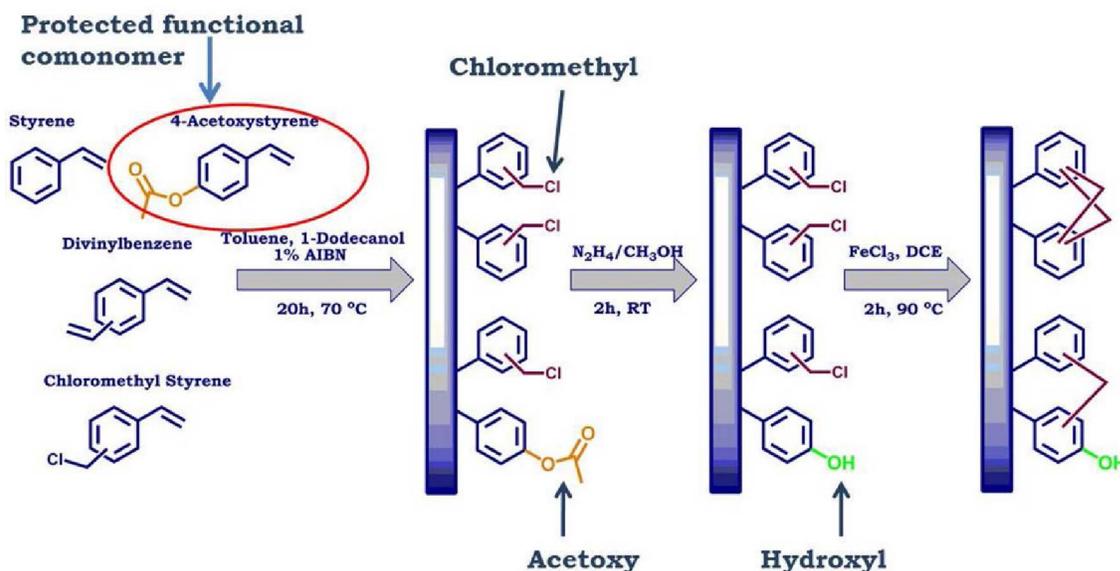


Fig. 3. Simplified scheme showing preparation of hypercrosslinked monoliths with phenolic functionalities. Acetoxystyrene is copolymerized with styrene, vinylbenzyl chloride, and divinylbenzene to produce monoliths with tunable chemistry, which is enabled by the coexistence of both chloromethyl and acetoxyl groups. Deprotection of these monoliths converts the acetoxyl functionalities to phenolic hydroxyl groups, thus changing the polarity of the pore surface. The percentage of hydroxyl groups in the monoliths enables modulation of polarity of the stationary phase, and represents functionalities that are potentially suitable for further modifications

- Hypercrosslinking yielded surface area of over $600 \text{ m}^2 \text{ g}^{-1}$, adsorption based on hydrophobic and π - π interaction
- Surface functionalization of the probes using a hybrid approach was also attempted.
- Scanning electron microscopy revealed extensive modification of the surface of the probes through hypercrosslinking.

- The efficacy of these probes was tested using batch equilibration studies utilizing various plant root exudates that contained compounds with contrasting polarity.
- 70-100 times higher sorption capacity of various plant metabolites on the probe was achieved through hypercrosslinking
- The probes exhibited a lower site specific sorption (slope of Freundlich adsorption isotherm close to unity) and percent recovery of the sorbed compounds from the probes were >70, indicating a predominance of reversible sorption.

G.2.2) Applying polymer coating on to stainless-steel rods

- One of the caveats with the above approach was the low rigidity of the probes with increase in porosity that reduced the lifespan and reusability of the probes. Hence, the above polymeric coating was applied to steel rods.
- Polymeric coating (poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) was applied to the surface of stainless steel rods (200 μm – 1mm diameter) through chemical bonding (Fig. 4).
- The thickness of polymer coating was increased with sequential (10-20) short incubations (5 hr) in fresh polymer mix. The probes were hypercrosslinked after the completion of all polymerization cycles (Fig. 5).

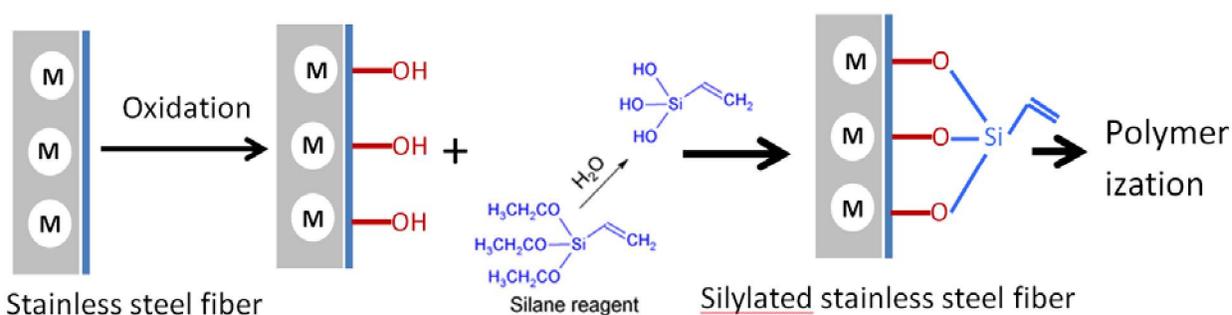


Fig. 4. Simplified scheme of chemical modification of stainless steel rods prior to the in-situ polymerization. The stainless steel surfaces were first subjected to oxidation using an alkaline solution, the hydroxyl-group on the surface of the rod was then silylated using a sililating reagent, which was then subjected to in-situ polymerization as described in Fig. 2.

- Batch equilibration studies were conducted to assess the sorption affinity of the plant metabolites on to the hypercrosslinked probes. The sorption data fitted well to Freundlich isotherm indicating the presence of infinite sorption surface on the hyper-crosslinked monolith. Thus, the above monolith could be used for the efficient capture of compounds that are in high and low abundance.

- As indicated by the Freundlich sorption coefficient (K_F), the hydrophilic glycosides have a lower affinity to the hypercrosslinked monolith, however their respective aglycons exhibited over 160 times sorption affinity on to the monoliths, revealing the potential of hyper-crosslinked monoliths to capture hydrophobic compounds from soil (Fig. 6).
- The higher sorption affinity (solution phase concentration more than 100 times lower than the sorbed concentration) indicates that the hydrophobic compounds would exhibit a relative exhaustive sorption as opposed to equilibrium sorption exhibited by other sorbents including polydimethylsiloxane (PDMS).

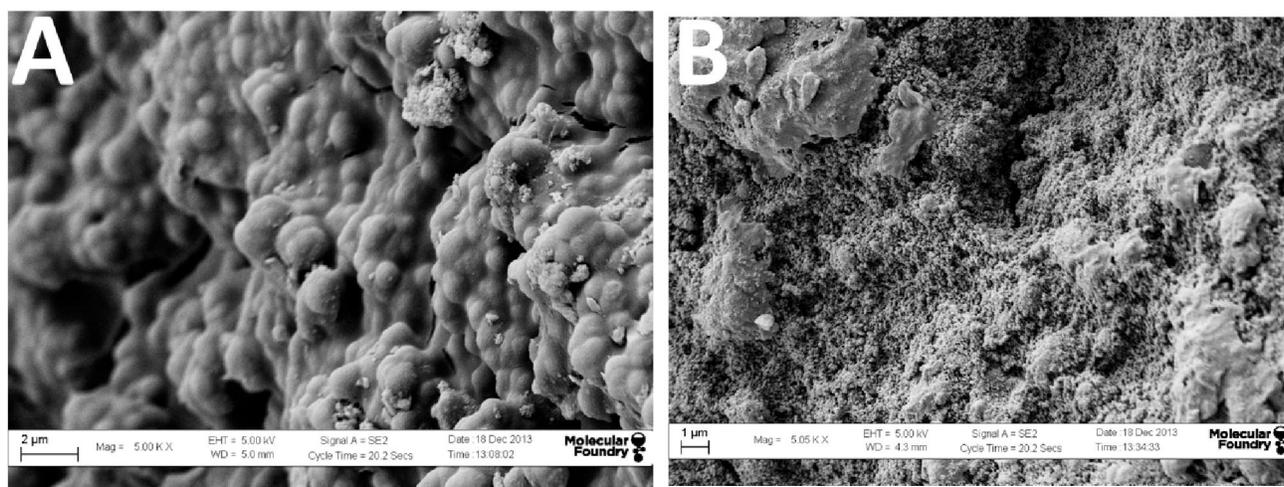


Fig. 5. Scanning electron micrographs of poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) probes coated on to stainless steel rods before (A), and after (B) hypercrosslinking showing the surface modifications. The hypercrosslinking increased the abundance and distribution of pores by immobilizing polymer chains in their solvated state. The coating thickness after 5 consecutive coatings was $\sim 10 \mu\text{m}$.

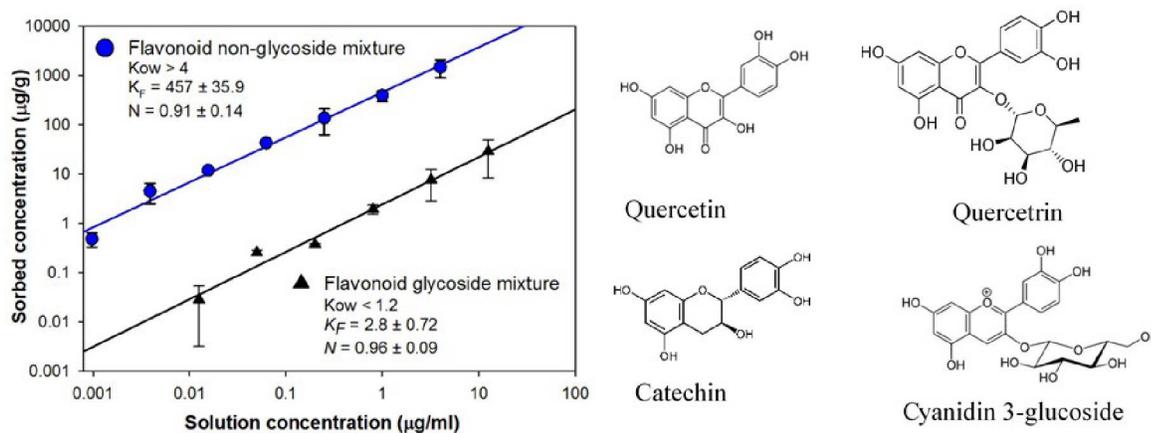


Fig. 6. Freundlich sorption isotherm describing the sorption of two flavonoid glycosides (quercitrin and cyanidin 3-glucoside) and their respective aglycone (quercetin and catechin) present in plant root exudates on to a hypercrosslinked poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) probe after a 20 hr batch equilibration experiment.

G.2.3. Molecular imprinting of the polymeric probes.

One of the caveats of the polymeric probes is the discrimination that it exhibits towards the polar compounds especially in complex soil matrix that is abundant in dissolved organic matter. This discriminates polar compounds from adsorbing on to the probes. We partially corrected this using the molecular template imprinting that increased the specificity of the probes.

- Specificity in trapping the metabolites of interest was imparted to the probes using molecular imprinting approach (Fig. 7).
- Templates of metabolites were copolymerized along with various monomers (methacrylic acid, MAA; or glycidyl methacrylate, GMA), and crosslinkers at different polymerization condition (temperature, type and amount of porogen etc.) to increase the specificity in extracting less abundant compounds from complex matrices.

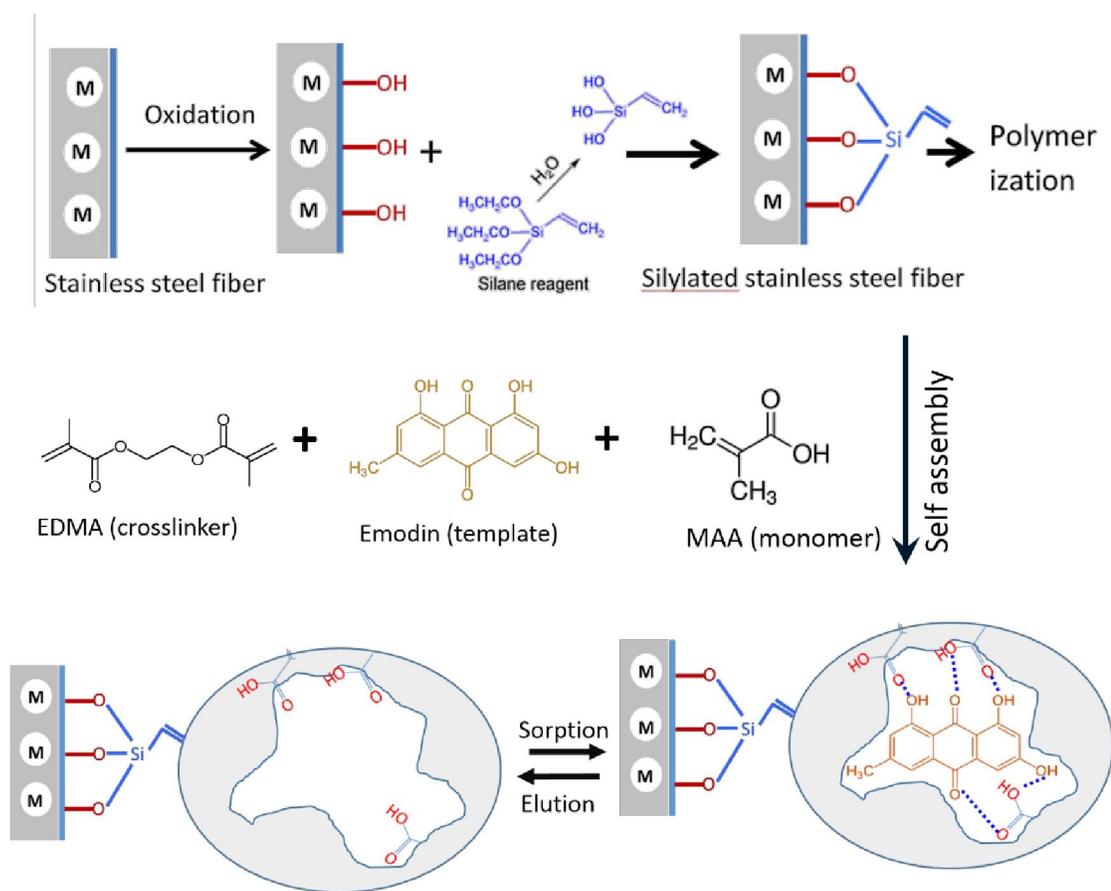


Fig. 7: Schematics of molecularly imprinted polymer probes with emodin (flavonoid found in plant roots) as template

Goal 3: Use the optimized probes for the capture and characterization of plant root exudates.

G.3.1. Root exudate characterization studies.

- Analysis of root exudates using mass spectrometric approach was undertaken to identify the chemical compounds in exudates and how the chemical composition of exudates changes with respect to nutrient deficiency.
- The root exudates of plants were found to be tailored by the overall nutrient status of the plants, with less available form of P (tricalcium phosphate) inducing a higher metabolic cascade than the P-deficient or the full-P treatment (Fig. 8).

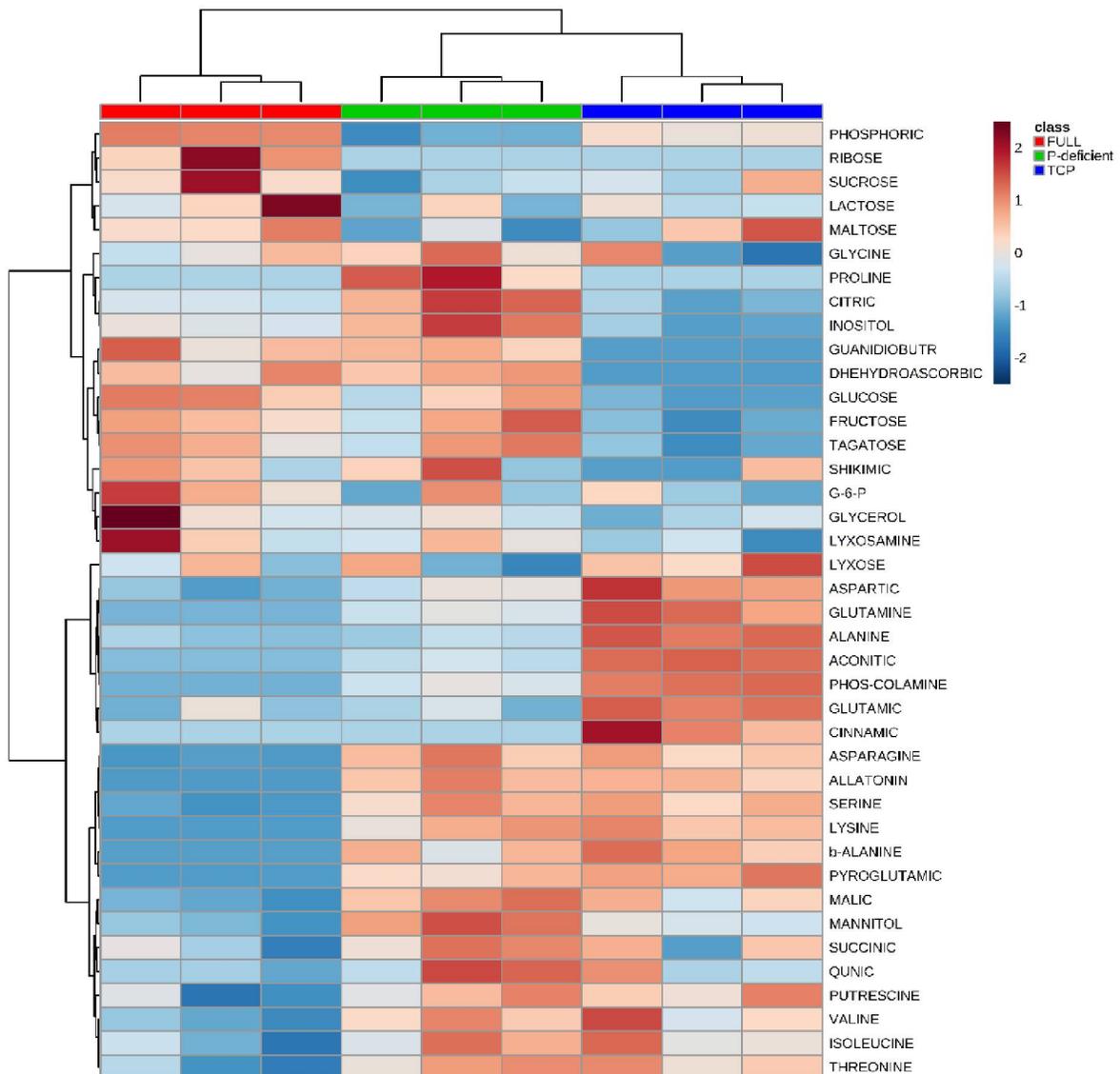


Fig. 8. Heatmap and two-way hierarchical clustering of metabolomic data from roots of *A. virginicus* exposed to different phosphorous (P) treatments (tricalcium phosphate-TCP, full P as KH_2PO_4 , and P-deficient).

G.3.2. In-situ experiment to measure compositional changes in root exudates due to interference from the soil matrix

- We tested the hypothesis that the composition of exudates is a function of distance that the exudate moves from the source root.
- The experiments were setup in 12 x 6 cm soil column (silt-loam) at 80% field capacity, and the exudate mixtures were applied through a stainless steel sparger at the rate of 0.5 ml/hr. The composition of the exudates was sampled at 0.3 mm spatial resolution in concentric circles away from the stainless-steel source.
- The composition of root exudates changed with respect to the distance from the source of the exudates, with the exudates becoming enriched in more polar compounds (tyrosine and flavonoid-glycosides), as the less polar compounds (phenolic acids and flavonoid aglycons) were sorbed to the soil particles (Fig. 9).

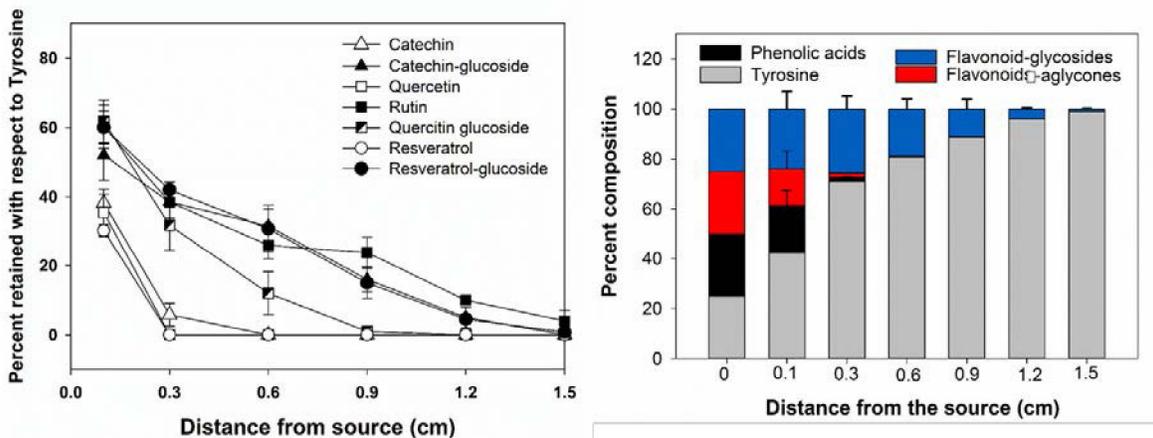


Fig. 9. Spatial patterning of root exudates in soils away from the source of exudation (source = 0 mm)

G.3.3. Diffusion of root exudates as a function of plant nutrient status.

- We tested the hypothesis that the changes in root exudate composition due to nutrient deficiency will influence the extend of diffusion of compounds in the exudates.
- Carbon normalized root exudates of *A. gerardi* was spiked with different plant flavonoid compounds along with tyrosine that acted as a tracer. The diffusion of the flavonoid was measured at 0.2-0.3 mm resolution away from the source.
- Greater diffusion of catechin away from the source was observed when this compound was spiked in the root exudates collected from plants that experienced P deficiency (Fig. 10).

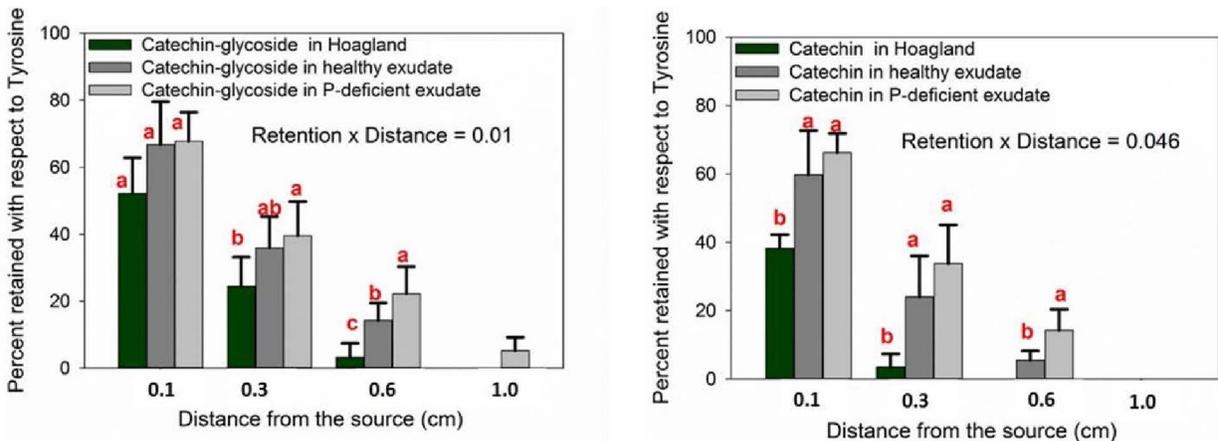


Fig. 10. Relative proportion of catechin (with respect to tyrosine) retained on the surface modified probes placed at varying distance from the source as a function of the exudate matrix (real root exudates [P-deficient plant & P-sufficient plant] and Hoaglands solution without root exudates).

TRAINING AND PROFESSIONAL DEVELOPMENT OPPORTUNITIES

The project trained one graduate student and 2 undergraduate students. The PI collaborated with Lawrence Berkeley National Lab, and Environmental Molecular Science Laboratory, through competitive facility access awards as part of this project. The PI completed initial optimization of the polymer matrix under the supervision of Dr. Frantisek Svec at LBNL. The chemical characterization of plant tissues that was optimized as part of these studies were incorporated in the curriculum of a graduate course that the PI teaches. In addition, the methods that were optimized for this project were utilized for the characterization of plant root tissues.

RESULT DISSEMINATION

To date, we have published one research article in a peer-reviewed journal and one is currently under revision. The results of the project were presented at national meetings of scientific societies (Ecological Society of America).

PRODUCTS:

Peer-reviewed publication:

Wang J., Tharayil N., Chow A.T., Suseela V., Zeng H. 2015. Phenolic profile within the fine root branching orders of an evergreen species highlights a disconnect in root tissue quality predicted by elemental- and molecular-level carbon composition. *New Phytologist*. 206: 1261-1273.

Suseela V, Tharayil N., Pendall E, and Rao A M. Warming and elevated CO₂ alter the suberin chemistry in roots of photosynthetically divergent grass species. *AoB Plants* (In review).

Conference Proceedings and Presentations:

Nishanth Tharayil. Evaluating the pattern and composition of root exudation using improved sampling and analytical techniques. Ecological Society of America Meeting. August 2013

Nishanth Tharayil. Surface-modified polymeric probes for the in-situ capture of plant metabolites from soil matrices. Ecological Society of America Meeting. August 2014

Nishanth Tharayil, Jun-Jian Wang, Colleen M. Iversen, Vidya Suseela, Joanne Childs, Alex T. Chow, Hui Zeng, Richard Norby. Phenolic composition of fine roots is influenced by environments and is disconnected from macro-elemental parameters. Ecological Society of America Meeting. August 2015

Nishanth Tharayil, Fernando Maya, Frantisek Sevc. Surface-modified polymeric probes for the in-situ capture of plant metabolites from soil matrices. Department of Energy's Terrestrial Ecosystem Science (TES) and Subsurface Biogeochemical Research (SBR) program's joint Environmental System Science principal investigator meeting. April. 2014.

Nishanth Tharayil, Fernando Maya, Frantisek Sevc. Surface-modified polymeric probes for the in-situ capture of plant metabolites from soil matrices. Department of Energy's Terrestrial Ecosystem Science (TES) and Subsurface Biogeochemical Research (SBR) program's joint Environmental System Science principal investigator meeting. April. 2015. (yearly progress reported).

Nishanth Tharayil, Jun-Jian Wang, Vidya Suseela, Alex T. Chow, Phenolic profile within the fine root branching orders highlights a disconnect in root tissue quality predicted by elemental- and molecular-level carbon composition. Department of Energy's Terrestrial Ecosystem Science (TES) and Subsurface Biogeochemical Research (SBR) program's joint Environmental System Science principal investigator meeting. April. 2015.

Collaboration with DoE Facilities and Laboratories.

The project team worked with multiple DoE user facilities to support this project, through competitive awards that were independent from this project.

- Rigid nanostructured organic polymer monolith for in situ collection and analysis of plant root exudates from soil matrices. Molecular Foundry, Lawrence Berkeley National Laboratory
- Visualizing the three-dimensional organization of biopolymers within the plant root-matrix to understand the physiology and decomposability of roots. Environmental Molecular Science laboratory.

CONCLUSION

This project resulted in several novel findings that have greater implications for plant nutrient acquisition and soil carbon sequestration.

- Through this project we developed and optimized polymeric probes that are suited for the capture of plant metabolites in soil matrices. Though the probe is currently being utilized for capture of specific compounds through molecular template imprinting approach, it could be further optimized to capture wider range of compounds from soil.
- We show that the root exudate pattern of plants is highly dependent on the nutrient status of the plant, with greater specificity of root exudation occurring in growing medium with low available form of nutrients.
- We show that the chemical composition of root exudation is a function of the distance of sampling from the source-roots, with the composition of root exudation getting more enriched in polar metabolites away from the source-roots.
- Further, we demonstrated that the compounds present in real root exudates diffuse farther from the source roots than those in artificial root exudates that are traditionally used.
- Thus, our study highlights how the soil matrix is instrumental in modifying the chemical composition of root exudates, and underlies that the specificity and function of root exudates is also modified by environmental factors.