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Plant root exudates and rhizosphere bacterial communities shift with neighbor context

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ABSTRACT

A plant's neighborhood context can alter its interactions with other organisms, but little is known about how these dynamics occur belowground, especially with soil microbes. Microbial communities in rhizosphere soil are influenced by many factors, including abiotic conditions and root-derived signals. In particular, root exudates have strong effects on rhizosphere assembly, respond to changes in abiotic conditions, and help plants interact with neighbors. Therefore, we predicted that root exudates likely play a central role in neighbor-induced shifts in rhizosphere communities. We conducted a greenhouse experiment to test this and determine how the rhizosphere bacterial community of a focal plant, *Panicum virgatum*, changed when beside different neighbors, and whether these shifts were mediated by neighbor-induced changes in root exudation. We found that neighbor altered both focal plant exudates and rhizosphere community, and that changes were largest when the focal plant was beside the most competitive neighbor, *Rudbeckia hirta*, which reduced both focal plant growth and nitrogen uptake. Several factors contributed to neighbor impacts on rhizosphere assembly, including neighbor-induced changes in root exudates during nitrogen-limitation and microbial spillover from roots of larger neighbors. Using an additional soil incubation, we also found that these changes in exudates can have even greater effects on soil nutrients than on microbial assembly. Overall, we show that neighbors influence one another's microbiomes, and highlight neighbor-induced changes in root exudates as one mechanism through which this may occur. This work suggests that rhizosphere assembly may differ in mixed-species communities and thus emphasizes a need for microbiome studies that consider neighborhood context.

1. Introduction

Decades of research show that a plant's neighbors can alter its interactions with other organisms. This ecological concept, referred to as "associational effects", has been primarily studied in aboveground, plant-insect interactions (reviewed by Barbosa et al., 2009; Underwood et al., 2014). However, increasing evidence suggests that plants' belowground neighborhoods are also important (Li et al., 2016; Huang et al., 2018; Kong et al., 2018; Chen et al., 2019). Similar mechanisms that drive aboveground associational effects may also occur belowground, including direct effects of neighbor plants on local abiotic and biotic conditions, changes in plant physiology and chemistry, and interplant signaling. For instance, root exudates transmitted between neighbors can serve as warning signals that stimulate herbivore defenses in a focal plant (Glinwood et al., 2003; Babikova et al., 2013). Still, despite increasing recognition of the role of neighborhood contexts, little is known about their broader role in shaping plants' interactions with soil microbial communities (Howard et al., 2021).

One challenge with identifying the role of neighbors on soil microbiomes is that many studies either focus on isolated plants in pots or, in the case of field studies, ignore neighborhood context. Still, these controlled studies show that plants assemble a species-specific soil

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microbiome near their roots, the rhizosphere (reviewed by Berg and Smalla, 2009; Philippot et al., 2013). Plants also host species-specific rhizosphere communities in field settings (Rosenzweig et al., 2013; Schöps et al., 2020), but in some cases rhizosphere structure changes with neighborhood richness (Bakker et al., 2013; LeBlanc et al., 2015). Therefore, it is unclear if the same mechanisms that mediate rhizosphere assembly in isolated plants are maintained in diverse plant communities. In fact, in many cases, predictions of plant community dynamics fail if they are based on greenhouse studies with isolated plants (Forero et al., 2019), perhaps because the predictions assume that rhizosphere communities do not change with active neighbor interactions.

Only a few studies have investigated how neighborhood context alters rhizosphere assembly (Hausmann and Hawkes, 2009; Bakker et al., 2013; Morris et al., 2013; Hortal et al., 2017; Cavalieri et al., 2020; Mony et al., 2021), and the patterns and mechanisms driving these associational effects vary widely. In some cases, plant neighbors have neutral effects on each other's rhizospheres, such that they maintain their species-specific communities while growing together (Hausmann and Hawkes, 2009). Other times, a focal plant's rhizosphere may begin to resemble that of its neighbor (Hawkes et al., 2006; Hortal et al., 2017). This can occur during strong neighbor competition for nutrients (Hortal et al., 2017), especially if the neighbor roots are overlapping, as occurs in dense grasslands (Vieira et al., 2019). Dense, overlapping root systems may affect rhizosphere assembly through 'microbial spillover', whereby microbes from a larger root system disperse to its neighbor through close root contact. Because rhizosphere assembly is related to host phylogeny (Emmett et al., 2017), it is likely that spillover of novel microbes from a distantly related neighbor would drive greater shifts in a focal plant rhizosphere (Mony et al., 2021). Still, more competitive neighbors do not always overwhelm a focal plant's rhizosphere assembly (Cavalieri et al., 2020). Together, these studies show that diverse neighborhoods affect host rhizosphere assembly through many mechanisms, but that these mechanisms may vary by plant species or other factors, and need to be elucidated.

Neighbor-induced shifts in root-derived metabolites, root exudates, may play a particularly important role in belowground associational effects. Root exudates have been shown to recruit particular microbes to a plant's rhizosphere (De-la-Peña et al., 2008; Zhalnina et al., 2018). In addition to their role in rhizosphere assembly, root exudates also change in response to neighbors (Badri et al., 2012; Kong et al., 2018; Weinhold et al., 2022). Abiotic conditions have similarly strong effects on root exudates. Water and nutrient limitation, each of which may occur with a competitive neighbor, have strong effects on root exudate profiles (Dakora and Phillips, 2017; Gargallo-Garriga et al., 2018; Smercina et al., 2020). Therefore, because root exudates respond to neighbors and environmental cues, and are central to rhizosphere assembly, we hypothesize that neighbor-induced shifts in root exudates also contribute to belowground associational effects.

Here, we use a greenhouse experiment to investigate how plant neighbors alter rhizosphere dynamics. We first hypothesize that the focal plant's rhizosphere bacterial community composition will change when beside different neighbors, consistent with a belowground associational effect. Second, we hypothesize that the bacterial community changes will correlate with neighbor-induced shifts in root exudate profiles. If this is true, we would expect to see an overall correlation between the exudate composition and bacterial communities, as well as repeatable shifts in taxa with the manipulation of key exudates. Third, we hypothesize that strongly competitive neighbors will induce larger shifts in focal plant exudates and rhizosphere bacteria than less competitive neighbors. This is because strong competitors are more likely to induce a physiological response in the focal plant and may also cause greater spillover effects if their root systems are larger.

2. Methods

2.1. Study species

The focal species (*Panicum virgatum* L. *v*ar. Southlow) is a C4, perennial grass native to tallgrass prairies and is also a candidate bioenergy crop (McLaughlin and Kszos, 2005). *P.virgatum* is suggested to associate with beneficial microbial communities that can improve its growth and tolerance of stressful conditions (reviewed by Hestrin et al., 2021). However, it is unknown if these microbial associations change with shifts in *P. virgatum*'s growing context, for instance if it grows in diverse prairies or monocultures for bioenergy. To this end, we studied the effect of neighborhood interactions on *P. virgatum's* rhizosphere community and root exudates. The neighbor species included three perennial prairie species known to co-occur with *P. virgatum*, including *Andropogon gerardii* Vitman (C4 grass), *Koeleria macrantha* Ledeb. (C3 grass), and *Rudbeckia hirta* L. (forb).

2.2. Greenhouse experiment

We carried out a greenhouse experiment at Michigan State University's W.K. Kellogg Biological Station. The focal plant, *P. virgatum,* was exposed to five neighbor treatments: either no neighbor, a single neighbor conspecific, or other prairie species (*A. gerardii, R. hirta,* or *K. macrantha*). Each plant species was also planted in 'monoculture treatments', consisting of each plant neighbored by its conspecific (two plants per pot). Each treatment was replicated five times, with a total of 45 pots (9 treatments x 5 replicates = 45 total pots, see Supplemental Fig. 1 for experimental design).

All seeds were sterilized and grown for five weeks in flats with a light layer of soil inocula before transplanting into the experimental treatments (see supplemental for more details on seedling preparation). After five weeks, we removed the seedlings, rinsed the roots with RO water, and planted them into their neighborhood treatments. The plants were grown in 3.8 L pots (Elite Nursery Classic 300) with a substrate mixture of autoclaved sand, autoclaved vermiculite and field soil inocula (45:45:10). This substrate will be referred to as soil throughout the text, though we recognize that it is an artificial soil mixture. The soil inocula, which was the same used in the germination flats, was collected from a nearby mid-successional grassland at the W.K. Kellogg Biological Station Long-Term Ecological Research Site in southwest Michigan. The soil is a sandy-loam and the field was dominated by *Bromus* sp. grasses. The soil was sieved (4 mm) and kept at 4 °C for ten weeks prior to inoculating the pots. Although the original field soil community likely changed after ten weeks, the purpose of the inoculum was to provide an initial soil community that was not pre-conditioned by any species in the study, not to represent the original field community.

The plants grew for eight weeks in their neighbor treatments with temperatures controlled at a maximum of 26 °C during the day and minimum of 15 °C at night with 14 h of artificial lighting. They were watered with RO water as needed and fertilized twice with ammonium nitrate (equivalent of 46 kg N ha^{-1} pot^{-1}) in 200 mL of half-strength Hoagland's solution (2.5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.024 mM H_3BO_3, 0.004 mM $MnCl_2{\cdot}4H_2O$, 0.102 μM $CuSO_4{\cdot}5H_2O$, 0.382 μM $ZnSO_4{\cdot}7H_2O$, 0.248 μM $Na_2MoO_4{\cdot}2H_2O$, 5.4 μM NaFeEDTA).

2.3. Plant harvest and soil analyses

After eight weeks of growth, we collected plant biomass, rhizosphere soil, homogenized pot-level bulk soil, and focal plant root exudates. Rhizosphere was collected by carefully removing each plant, untangling their roots, and collecting the closely-adhering soil that remained after multiple shakes; the rhizosphere soils were stored at −20 °C for DNA extractions. Though the rhizosphere soil was attached to the focal roots, the fact that the neighbor and focal roots were intertwined likely led to

greater microbial spillover that we could not distinguish from neighborhood effects (see Discussion). All non-focal plants' roots and shoots were dried (55 °C) while the focal plants were left intact and temporarily placed in sterile whirlpacks (Nasco, USA) with 0.05 mM calcium chloride buffer solution prior to root exudate collection (details below). The remaining bulk soil from the pots was homogenized for pot-level nutrient and microbial biomass analyses. Briefly, bulk soils were stored at 4 °C and subsampled for gravimetric soil moisture content analysis (55 °C), and chloroform fumigation and potassium sulfate extractions for microbial biomass and soil nitrate and ammonium analyses (see supplemental for more details on soil analyses).

2.4. Exudate collection

We collected root exudates from the focal plants (n = 25) using a soil-hydroponic-hybrid method (Oburger and Jones, 2018). The benefit of this method is that plants are grown in soil-like conditions with active microbial communities so that exudates are not altered by artificial sterile conditions, but a minor drawback is potential artifacts from root damage during washing (Williams et al., 2021a), as well as microbial excretion and consumption of root exudates during collection. Despite these caveats, this approach is still a common method used to study interactions between root exudates and rhizosphere communities (Vieira et al., 2019; Brisson et al., 2021). After harvesting, the focal plants were placed in buffer solution (0.05 mM calcium chloride, $CaCl_2$), left to recover for three to 6 h, and then the roots were cleaned of residual soil and detached, dead roots. Submerging the roots in fresh solutions prior to exudate collection can help remove metabolites released from damaged tissues (Oburger and Jones, 2018), but a recent study suggests that a recovery period of at least three days is preferred (Williams et al., 2021a). Once all root systems were cleaned, we collected root exudates by submerging the intact plants in flasks with 250 mL of fresh 0.05 mM $CaCl_2$ solution. Flasks were covered with parafilm to reduce airborne contamination and kept in the dark with a foil covering. The flasks, including three no plant controls, were placed on a shaker table with supplemental lights, and the exudate solutions were filter sterilized (0.2 μm) and frozen at −80 °C after 6 h (17 h–23 h). Over the 6 h collection period, it is possible that some metabolites were degraded or consumed by root microbes, as a collection time of less than 4 h is often recommended in non-sterile systems (Oburger and Jones, 2018; Williams et al., 2021a). The focal plant shoots and roots were dried at 60 °C, weighed, ground (Qiagen Tissue Lyser II), and analyzed for total C and N (Costech Elemental Combustion System 4010).

2.5. Exudate analysis

We thawed and subset the frozen root exudate solutions into 50 mL centrifuge tubes for downstream exudate analysis, including quantification of dissolved organic carbon (DOC) and metabolite fingerprint analysis with liquid- and gas-chromatography mass-spectrometry (LC-MS, GC-MS). Thawed exudate solutions were run on a Total Organic Carbon (TOC) analyzer to determine total DOC per sample (Shimadzu TOC-VCPH); two samples were missing from this analysis because there was not enough excess exudate solution. Another 50 mL subset of extracts were lyophilized and sent for LC- and GC-MS analysis. During lyophilization, several tubes cracked, so the initial exudate volumes vary between samples and, therefore, normalized metabolite data are reported.

Lyophilized exudates were prepared for MS analysis by resuspending them in 2 mL of methanol:water (80:20). Tubes were centrifuged and extracts were transferred into 2 mL glass vials, dried down with a centrifugal vacuum evaporator, and resuspended into a final volume of 300 μL. Untargeted LC-MS analyses were performed directly on the extracts. For GC-MS measurements, an aliquot of 200 μL from each exudate sample was dried down into an HPLC vial and chemically derivatized to trimethylsilyl ester before analyses (Kim et al., 2005). The LC-MS and GC-MS files were processed using MZmine 2.37 (Pluskal et al., 2010) and Metabolite Detector 2.5 (Hiller et al., 2009), respectively. LC-MS features were identified using exact mass and retention time from an in-house library of metabolites, corresponding to the second-level of putative identification (Sumner et al., 2007). GC-MS metabolites were identified using a modified version of FiehnLib (Kind et al., 2009) and verified using NIST14 GCMS library. LC- and GC-MS datasets were combined, filtered, and metabolic features within each sample were normalized by the total intensity of chromatograms (details on MS analysis and data filtering described in supplemental methods). Missing data (NAs) were imputed for downstream statistical analyses using the 'MissForest' R package (Stekhoven and Bühlmann, 2012).

2.6. Soil incubation experiment

Due to limitations with correlating omics datasets (Pang et al., 2021; Zancarini et al., 2021), an additional soil incubation was performed to establish a more causal link between changes in root exudates and rhizosphere assembly. We manipulated the relative concentration of malic acid in soil incubations to determine if the bacteria enriched while neighboring *R. hirta* were driven by greater malic acid exudation. Malic acid was exuded more when the focal plant neighbored *R. hirta* (see Results, Supplemental Fig. 5), and it was also the second most abundant metabolite in the exudate solutions, and contributed to shifts in overall bacterial community structure (see Results, Fig. 4B).

We added two exudate solutions (100 μg C g^{-1} dry soil) – high malic acid (75% malic acid, 8.33% citric acid, 8.33% sucrose, 8.33% glucose, n = 5) and low malic acid (25% malic acid, 25% citric acid, 25% sucrose, 25% glucose, n = 5)– along with a water control (n = 3) to soil mesocosms daily over 24 days. Before additions, the exudate solutions were brought to a neutral pH (6.0) with potassium hydroxide (pH probe: Mettler-Toledo, Five Easy Plus), filter sterilized (0.22 μM), divided into weekly aliquots, frozen, and thawed for weekly additions.

The soil mesocosms (237 mL glass mason jar) were filled with the equivalent of 30 g dry soil and raised to 65% water holding capacity (WHC) with autoclaved milli-Q water (0.22 μm). The soils were collected from the same mid-successional grassland used for the greenhouse experiment, albeit two years later, sieved (4 mm) and analyzed for WHC. The jars were wrapped with Breathe-Easy® (Sigma-Aldrich) micropore film to allow gas exchange but prevent airborne contamination, maintained at 55% WHC, and stored in the dark at room temperature (approximately 25 °C). After 24 days, the soils were subsampled for DNA extractions, gravimetric soil moisture content, and chloroform fumigation and potassium sulfate extractions for soil dissolved organic carbon (DOC), total extractable nitrogen (TN), and microbial biomass analyses, following the same procedures detailed previously.

2.7. DNA extraction, Illumina sequencing, and bioinformatics analysis

DNA was extracted from 0.25 g of homogenized soil from the initial soil inocula, greenhouse experiment soils (focal, neighbor plants, and no-plant controls, n = 80) and soil incubation soils (n = 13) using the MoBio PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA). We targeted the bacterial V4 region of the 16S rRNA gene (primers 515f/806r) with MiSeq Illumina (V2) paired-end sequencing, conducted by the Research Technology Support Facility Genomics Core at Michigan State University, East Lansing, Michigan. The reads were quality filtered and clustered into unique operational taxonomic units (OTUs) based on 97% identity using the Silva (version 123) bacterial database at 80% confidence (Quast et al., 2012), and a bacterial phylogenetic tree was created using an iterative maximum-likelihood approach with the 'PASTA' R package (Mirarab et al., 2015).

The library sizes significantly differed by 2.5-fold among greenhouse treatments (all greenhouse & focal samples ANOVA $F_{9,73} = 3.81$, $P < 0.001$); therefore, we rarefied both the greenhouse and incubation dataset to 16,224 reads. To reduce the effect of rare or spurious taxa, we

removed any OTUs not present in at least 10 samples, resulting in 6,221 taxa for the greenhouse dataset and 4,234 in the soil incubation dataset. All bacterial beta- and alpha-diversity metrics were calculated on the rarefied and filtered datasets. See Supplemental Information for more details on sequencing and bioinformatics methods.

2.8. Univariate data analysis

For either experiment, plant and soil characteristics, microbial biomass, and bacterial alpha diversity data were confirmed to meet normality assumptions and analyzed using one-factor analyses of variance (ANOVA) and type 3 sum of squares (Satterthwaite's method), followed by post-hoc pairwise comparisons (Benjamini-Hochberg False Discovery Rate, FDR, $\alpha = 0.05$). Data that did not meet normality assumptions were transformed (soil nitrate, square-root transformed). For the greenhouse experiment, individual one-way ANOVAs were used to determine the effect of treatment on either the focal plant, monoculture, or pot-level responses. Microbial biomass and soil chemistry data were collected and analyzed at the pot-level, representing the shared conditions for both plants in the pot.

We conducted additional ANOVAs with focal plant aboveground biomass as a covariate to account for differences driven by neighbor competition. While the reduction of a focal plant's biomass is a classic definition of competition (Grace, 1995) we also calculated the relative strength of competition using RII for an alternative assessment of neighbor competition (Armas et al., 2004). We paired the five replicates for each treatment for the calculation. Negative RII values indicate that the focal plant is suppressed by its neighbor through competition, with a more negative value indicating stronger competition, and positive values indicating facilitation.

2.9. Multivariate data analysis

Multivariate analyses of the bacterial composition were performed on Weighted-Unifrac distance matrices from the rarefied community and all multivariate analyses of the exudate data were performed on Euclidean distance matrices. Because Weighted Unifrac analyses can bias against rare, less-abundant taxa, we further partitioned the focal plants' bacterial communities to determine if dominant or non-dominant taxa were driving the treatment effects. We defined 'dominant' as the top 10% most abundant taxa across all focal plant samples and the non-dominant taxa as the remaining 90%. The dominant group included 571 taxa and made up 69.6% of the focal plant bacterial reads, while the non-dominant taxa included 5,142 taxa and made up 30.4% of the focal plant bacterial reads.

We evaluated the effect of plant treatment on the bacterial communities and exudate profiles using one-factor permutational multivariate ANOVA tests (permANOVAs, n = 9999 permutations), followed by post-hoc pairwise comparisons with FDR adjustment ($\alpha = 0.05$). Additional permANOVAs with focal plant biomass included as a covariate were used to control for the effect of neighbor plant competition. We identified bacterial genera representative of each treatment in the greenhouse and soil incubation experiments using indicator species and differential abundance analyses. The magnitude and direction of neighborhood effects on rhizosphere assembly was assessed with permANOVAs that compared the community structure of the focal plant with that of its direct plant neighbor, its neighbor species' monoculture, and the focal plant *P. virgatum* monoculture. For this analysis, the OTU abundance of the monoculture plant treatments were averaged into a single value using 'merge_samples' (fun = "mean") in the 'Phyloseq' R package (McMurdie and Holmes, 2013).

To further partition variation in the focal plant root exudates, we used sparse Partial Least Squares Discriminant Analysis (sPLS-DA) to determine which of the identified exudates contributed to the greatest variation in treatments. We then determined how the top ten identified exudates correlated to the soil and plant characteristics using p-adjusted Pearson correlations. We used variance partitioning analysis to identify which of the plant and soil variables had the largest effect on the complete root exudate profiles. We identified three extreme outliers (three times the interquartile range) in the gravimetric soil moisture content data, so these samples were removed from analyses that correlated soil conditions with microbial or exudate data.

Finally, we investigated the relationship between the bacterial and exudate datasets. Because no single correlation technique yields the same result (Weiss et al., 2016), especially when comparing two -omics datasets (Pang et al., 2021), we used multiple statistical approaches to determine if neighbor-induced shifts in the root exudates were correlated with shifts in the bacterial community. First, we performed a principal component analysis on the exudate dataset and Hellinger-transformed bacterial dataset and then evaluated the similarity in the matrices with a Protest analysis. Second, we used variance partitioning analysis to determine how the top ten most abundant identified exudates, neighbor treatment (categorical), and focal plant C:N affected bacterial community composition. Third, we evaluated the effect of the top ten identified exudates, as well as the focal soil and plant characteristics, on bacterial community structure using distance-based redundancy analysis (dbRDA). Lastly, we looked for specific relationships between bacterial genera and the 140 identified exudates using the 'CCREPE' (compositionality corrected by renormalization and permutation package) R package (Schwager et al., 2020). This method outperforms traditional correlation techniques, such as Pearson and Spearman, which are not suitable for compositional data and are known to have high false positive rates for compositional data (Pang et al., 2021).

The statistical program R (version 4.0.5) was used for all analyses and all package and parameter information is detailed in the Supplemental Information *Data Analysis* section. Sequencing pipeline, code, and links for data are available at https://github.com/TaylerUlbrich/NeighID_Switchgrass; raw sequence fastq files can be found on the NCBI repository (Accession number PRJNA773254).

3. Results

3.1. Neighbor identity altered focal plant biomass and soil properties

Overall, plant neighbor altered focal plant biomass, root and shoot C:N, and soil conditions (Table 1, Fig. 1). These properties were most affected when *R. hirta* was a neighbor. *R.* hirta decreased total switchgrass biomass by 70%, was larger than other neighbors (Supplemental Fig. 2A, aboveground biomass: $F_{3,16} = 78.39$, $P < 0.001$; belowground biomass: $F_{3,16} = 16.70$, $P < 0.001$), and was classified as the strongest competitor by the relative strength of competition index ($P = 0.057$, Table 1, Supplemental Fig. 2B). *R. hirta* maintained this large size in a monoculture, where it had 2.2 times greater total biomass than all other species in monoculture (Table 1).

In addition to plant biomass, neighbor effect on focal plant C:N and soil moisture was also most prominent with *R. hirta* (Table 1, Fig. 1B and Supplemental Fig. 3A). Focal plants neighbored by *R. hirta* had 66 and 65% higher root and shoot C:N (respectively) than focal plants in the other neighbor treatments (Fig. 1B). *R. hirta* also used more moisture, as *R. hirta* pots had lower soil moisture when present as a neighbor, and in monoculture (Table 1, Supplemental Fig. 3A). Neighbor treatment had a small effect on soil nitrate (Table 1, Supplemental Fig. 3B), though in monocultures, *K. macrantha* had four times greater soil nitrate than any other plant monoculture (Table 1). There was also clear uptake of soil nitrate during plant growth, as the no plant control had 8.5 times higher soil nitrate than all other treatments.

3.2. The most competitive neighbor had stronger effects on focal plant bacterial community

The most dominant rhizosphere phyla across all plant species were

Table 1
Effect of neighbor treatment on focal plant growth, soil conditions, bacterial community, and root exudates, as well as differences among monoculture treatments in greenhouse experiment. ANOVA and permANOVA results shown; permANOVAs conducted on bacterial community structure (Weighted Unifrac) and root exudate profiles (Euclidean); significant *P* values bolded ($P \leq 0.05$).

	Focal Treatments (Focal plant)		Monoculture Treatments	
	F	*P*	F	*P*
Plant and Soil Variables				
Total biomass (g)	4.01	**0.015**	23.54	**<0.001**
Aboveground biomass (g)	4.65	**0.008**	21.17	**<0.001**
Belowground biomass (g)	2.15	0.112	10.73	**<0.001**
Shoot C:N	10.95	**<0.001**	NA	NA
Root C:N	6.13	**0.002**	NA	NA
Soil moisture (g water g^{-1} dry soil)	6.99	**0.001**	4.35	**0.020**
Soil nitrate (μg NO_3^- g^{-1} dry soil)	3.61	**0.023**	10.76	**<0.001**
RII (aboveground biomass)	3.09	0.057	NA	NA
RII (belowground biomass)	0.53	0.667	NA	NA
RII (total biomass)	2.02	0.151	NA	NA
Bacterial Community and Exudates				
Shannon Diversity	6.16	**0.002**	2.99	**0.044**
Pileau's Evenness	0.79	0.546	1.46	0.24
Chao1	9.96	**<0.001**	3.55	**0.024**
Microbial biomass carbon	2.19	0.107	2.61	0.087
Bacterial community structure (all taxa)	1.35	**0.056; $R^2 = 0.21$**	5.40	**<0.001; $R^2 = 0.31$**
Bacterial community structure (dominant taxa)	1.29	0.099; $R^2 = 0.21$	NA	NA
Bacterial community structure (non-dominant taxa)	1.32	**0.014; $R^2 = 0.21$**	NA	NA
Root Exudates (all)	4.92	**<0.001; $R^2 = 0.50$**	NA	NA

Proteobacteria, Acidobacteria, Verrucomicrobia, Plantomycetes, and Bacteriodetes (37%, 15%, 13%, 9%, 8% relative abundance respectively). Each of the four plant species were associated with differently structured, but not sized, bacterial communities (Table 1). Diversity also differed and was highest in the *P. virgatum* monoculture and lowest in the *K. macrantha* monoculture (Table 1).

Neighbor identity did not affect total microbial biomass carbon, but significantly altered both the diversity and structure of the focal plant's bacterial community (Table 1, Fig. 1C & D). Bacterial Shannon and Chao1 diversity, but not evenness, differed by neighbor treatment; specifically, diversity was lower when switchgrass neighbored *K. macrantha.* These patterns did not change when we controlled for the effect of competition on focal plant aboveground biomass (Supplemental Table 1). Neighbor also affected community structure, explaining 20% of the variation in the focal plant's bacterial community (Table 1), and these shifts were more influenced by the non-dominant taxa than the dominant taxa (permANOVA $P = 0.014$ for non-dominant taxa; permANOVA $P = 0.099$ for dominant taxa; Table 1).

The neighbor-induced shifts in microbial community structure were strongest with *R. hirta*, which was also the most competitive neighbor (see above). Still, when we controlled for neighbor competition (by including focal plant biomass as a covariate), the focal plants' non-dominant taxa differed by neighbor treatment (Supplemental Tables 1 and 2). Compared to the other neighbor treatments, *R. hirta* led to twice as many indicator genera (n = 15) in the focal plant rhizosphere, including *Sphingomicrobium, Zymomonas, Methylotenera, Caulobacter, Methylophilus, Flavobacterium* (Supplemental Table 3 for complete list of indicator genera). Interestingly, the relative abundances of these genera were also greater in the *R. hirta* monoculture compared to the other neighbor monocultures (Supplemental Fig. 4).

We further evaluated how neighbors altered the focal rhizosphere by comparing the focal and neighbor bacterial communities to those in their monocultures (Fig. 2). *A. gerardii* and the focal species (*P.virgatum*) rhizosphere communities were similar to one another when each was grown in monoculture, and they did not alter each other's rhizosphere communities when grown together in a neighborhood, for both dominant and non-dominant communities (Fig. 2A & D). *K. macrantha* and the focal species, on the other hand, had more dissimilar rhizosphere communities, but when in a shared neighborhood, their communities resembled the focal monoculture (Fig. 2B & E). These patterns observed with the *A. gerardii* and *K. macrantha* neighbors did not differ for the dominant versus non-dominant taxa, but they did with *R. hirta.* When

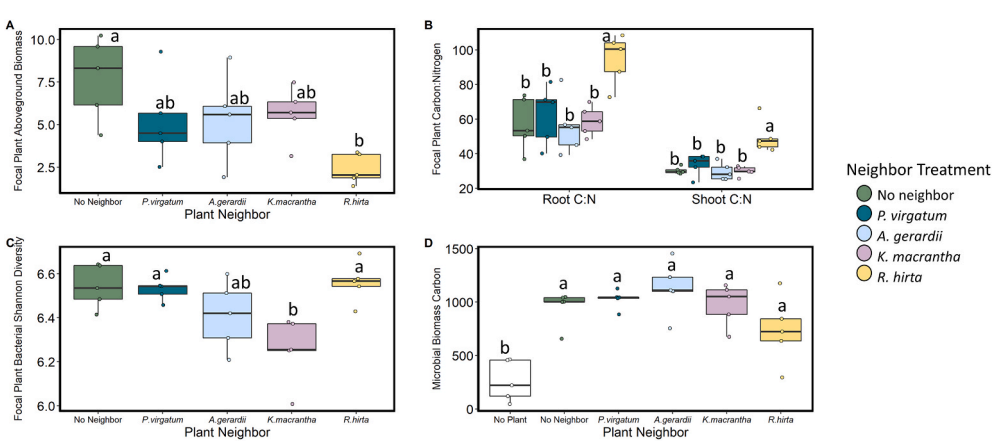

Fig. 1. Neighbor effect on focal plant A) aboveground biomass (g), B) shoot and root carbon:nitrogen content, C) bacterial Shannon diversity, and D) microbial biomass carbon (μg C g-1 dry soil, collected at the pot-level). The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, $P < 0.05$).

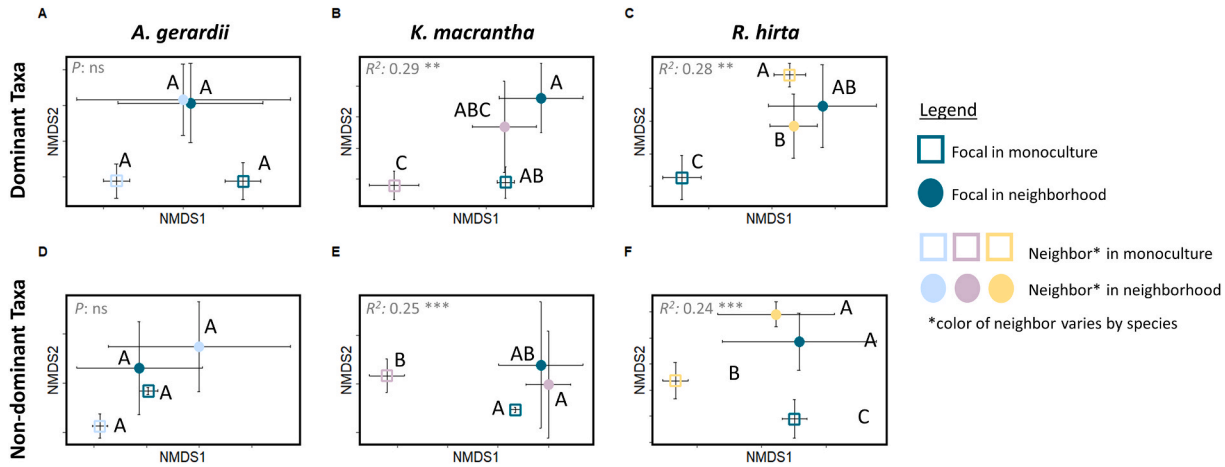

Fig. 2. Nonmetric multidimensional scaling (NMDS) ordination comparing focal (*P. virgatum*) and neighbor plant bacterial communities in shared pot neighborhoods to their respective monoculture treatments (OTU abundance averaged at pot-level). A-C represent dominant taxa (top 10% most abundant) and D-F represent non-dominant taxa (lower 90% abundant). Open squares represent monocultures for either focal plant (dark blue) or neighbor species (*A. gerardii* – light blue, *K. macrantha* – purple, *R. hirta* – yellow); closed circles represent the focal or neighbor species in a shared pot neighborhood. Each centroid is the average of sample replicates (n = 5) and bars indicate ± 1 standard error from the centroid. PermANOVA results presented in top left of each panel (R^2, ns $P > 0.05$, *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$); Different letters denote significant differences among treatments (false discovery rate, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sharing a pot with *R. hirta,* the dominant taxa resembled that of the *R. hirta* monoculture (Fig. 2C), but the non-dominant taxa were distinct from either monoculture species (Fig. 2F). These comparisons also revealed that despite the distinct effect of each neighbor on the focal rhizosphere, there was strong homogenization between the interacting plants: the focal rhizosphere communities did not significantly differ from that of their direct neighbor in a shared pot (permANOVA effect of treatment by plant position: Weighted Unifrac; neighbor effect: $F_{3,39} = 2.21$ $P < 0.001$, $R^2 = 0.16$; shared pot effect: $F_{1,39} = 0.63$, $P = 0.91$; neighbor*pot $F_{3,39} = 0.58$, $P = 0.99$).

3.3. Neighbor identity alters focal plant root exudates

We detected 14,648 unique metabolite features from the root exudates of the focal *P. virgatum* plants (LC-MS and GC-MS), of which 140 of them were putatively identified. The top 10 most abundant identified compounds in the root exudates were quinate, malic acid, qluconic acid, hydropxypyruvate, myo-inositol, fructose, lyxose, shikimic acid, and azelaic acid (metabolite abundance by treatment - Supplemental Fig. 5). Of all unknown and identified exudates, quinate and malic acid were the top two most abundant.

Neighbor identity had a strong effect on the focal plant root exudate profile, but not on the amount of total carbon exuded (total organic carbon: $F_{1,18} = 1.12$, $P = 0.379$). This effect was even stronger than the effect of neighbor on rhizosphere community structure, as neighbor treatment explained 50% of the variation in the focal plant root exudates (Table 1) and 38% when we controlled for variation in focal aboveground biomass (Supplemental Table 1). When neighbored by *R. hirta* and conspecific *P. virgatum* the exudates were most dissimilar from the no neighbor treatment (pairwise *p-values* Supplemental Table 2).

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) further indicated that the *R. hirta* and *K. macrantha* neighbor treatments had the most distinct exudate profiles (Fig. 3A). The first component (PC1) of the sPLS-DA explained 15% of the variation and separated the *R. hirta* neighbor treatment from the other four treatments. Of the top 15 discriminant exudates for PC1, eight of them were most abundant in the *R. hirta* treatment, including fumaric acid, malic acid, stearic acid, cytosine, 1-methylguanosine, hypoxanthine, and sn-glycerol-3-phosphate (Fig. 3B). Component two (PC2) explained 14% of the variation and separated the *K. macrantha* treatment, which had a greater abundance for 12 of the top 15 discriminant exudates for C2 (Fig. 3C).

We used variance partitioning to determine which factors had the largest effect on the root exudate profiles. Neighbor treatment explained the most variation in the exudate profiles (41.3%), followed by the focal plant's aboveground biomass (16.5%), root C:N ratio (9%) and soil moisture content (6.0%). All four variables cumulatively explained 51.5% of the variation in root exudates. The top ten most abundant identified exudates also correlated with these plant and soil factors

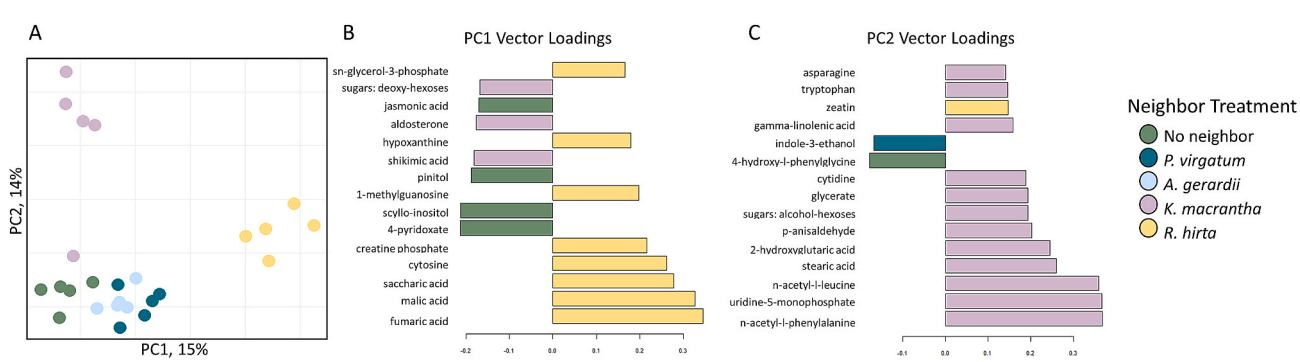

Fig. 3. A) Principal Coordinates Analysis (PCA) of focal plant identified exudates by treatment. Loading vectors from sparse Partial Least Squares Discriminant Analysis (plsDA) for Component 1 (B) and Component 2 (C). Bar colors in B & C indicate which treatment had the highest mean value for each exudate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Supplemental Fig. 6A). Malic acid was exuded significantly more with the *R. hirta* neighbor (14-fold greater peak area with *R. hirta,* Fig. 3B & Supplemental Fig. 5) and had the strongest correlations with these factors (Supplemental Fig. 6A, but the correlations were driven by the significantly lower C:N plant tissue and soil moisture in the *R. hirta* focal treatment (Supplemental Figs. 6B–E).

3.4. Exudates and bacterial communities are correlated at whole community-level

Overall, we found that the focal plant root exudates had stronger correlations with the entire bacterial community than with individual genera. Protest analyses showed that the significant and strong correlation observed with the whole community (Procrustes Protest test on PCA axes, $r = 0.78$, $m^2 = 0.47$, $P = 0.006$), was similar for both the dominant and non-dominant taxa (Dominant taxa: Protest test, $r = 0.80$, $m^2 = 0.45$, $P = 0.009$; Non-dominant: Protest test, $r = 0.71$, $m^2 = 0.54$, $P < 0.001$). The variance partitioning (Fig. 4A) and db-RDA results (Fig. 4B) suggested that the top ten most abundant exudates, specifically malic acid (dbRDA: $R^2 = 0.08$, $P = 0.021$) and stearic acid (dbRDA: $R^2 = 0.06$, $P = 0.113$), drove shifts in the community, and that focal shoot C:N (dbRDA: $R^2 = 0.06$, $P = 0.090$) also played a role. Similar to the Protest results, these two dominant exudates influenced both the dominant and non-dominant taxa, but only the dominant taxa were impacted by focal shoot C:N (db-RDA analyses, Supplemental Table 4). Despite significant correlations at the whole community level, we identified only six significant correlations between individual exudates and bacterial genera. The bacterial genera *Methylophilus* had the highest number of significant correlations with exudates, including malic acid, fumaric acid, and pyruvic aldehyde (*nc correlation metric* > 0.50, *P.adj* < 0.02) (Supplemental Table 5 for complete list).

3.5. Malic acid alters bacterial communities and increases soil carbon and nitrogen

Malic acid was exuded more near *R. hirta* (Fig. 3B and Supplemental Fig. 5) and had a significant impact on the overall community structure (Fig. 4B), but not on individual bacterial genera. Therefore, we used a soil incubation experiment to more causally link malic acid exudation with shifts in bacterial community structure. We found that the concentration of malic acid has a strong effect on bacterial community structure (PermANOVA: $F_{3,17} = 3.83$, $P = 0.04$, $R^2 = 0.32$), with *Brevundimonas, Pedobacter, Pseudoxanthomonas, Pseudospirillum, Hirschia, Flavitalea,* and *Chryseolinea* genera enriched in the high malic acid treatment (Supplemental Table 6 for complete list). However, these same genera did not strongly correlate with malic acid in the greenhouse experiment and, similarly, were not enriched when the focal plant neighbored *R. hirta* (Supplemental Fig. 7).

The malic acid additions also altered soil carbon, nitrogen, and microbial biomass. The high malic acid soils had two times greater dissolved organic carbon ($P < 0.001$, Fig. 5A) and extractable total nitrogen ($P < 0.001$, Fig. 5B) than the low malic acid soils. Microbial biomass C:N ratio was 55% lower in the high malic acid soils ($P = 0.011$), and this was reflected in the microbial biomass N, which was 18% greater in the high malic acid soils ($P = 0.035$, Fig. 5D) while microbial biomass C was marginally lower in the high malic acid soil ($P = 0.087$, Fig. 5C).

4. Discussion

4.1. Neighbors induced species-specific changes in focal plant rhizosphere bacterial community

We found that neighbor identity altered the composition of the focal plant rhizosphere community, offering support for our first hypothesis that associational effects, widely observed aboveground, also occur in soil. The functionally-similar C4 grass neighbor, *A. gerardii*, had a neutral effect on the focal rhizosphere, while the forb neighbor, *R. hirta*, altered both the focal plant's dominant and rare bacterial taxa. These neighbor-specific effects on soil communities have been observed in previous studies (Hausmann and Hawkes, 2009; Mony et al., 2021), and could have several explanations. First, a more functionally-dissimilar neighbor, like *R. hirta* in our study, may introduce novel taxa to a focal plant's existing microbiome. This was also previously observed with mycorrhizal fungal communities (Mony et al., 2021). Differences in growth and competition for resources can also alter the strength of associational effects. This was likely a strong contributing factor in our study, as *R. hirta* was the fastest growing neighbor plant, both in monoculture and neighborhood. *R. hirta's* root system was larger than all other neighbor species, which likely led to greater microbial spillover to the focal rhizosphere (discussed more below). Overall, we cannot distinguish the effect of neighbor growth and competition for resources from their functional dissimilarity, but future studies might experimentally investigate what predicts the strength of belowground associational effects, and why.

4.2. Changes in root exudates correlate with overall bacterial community but not individual genera

Overall root exudate and bacterial community profiles were strongly correlated, suggesting that neighbor-induced changes in exudates may contribute to belowground associational effects, and should be tested for causality in mechanistic studies. In contrast, we observed few strong correlations between specific metabolites and bacterial genera. Thus, we found some support for our second hypothesis. Two highly abundant

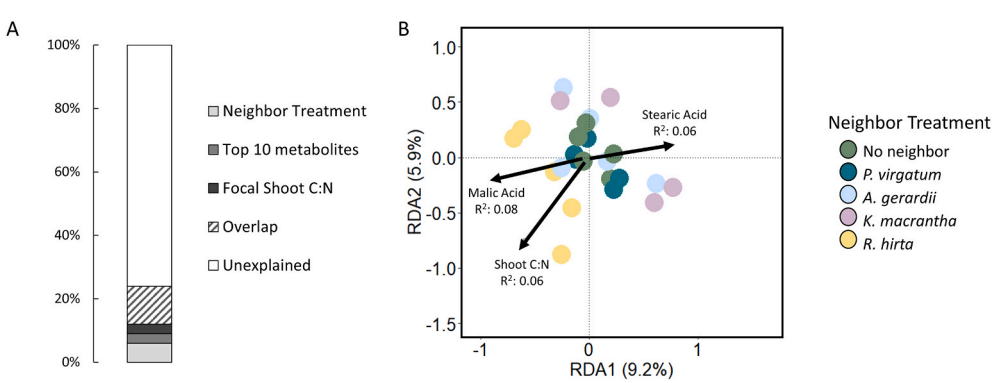

Fig. 4. Variance in the focal plant bacterial community composition (Weighted-Unifrac) explained by exudates, plant and soil factors. A) Variance partitioning analysis depicting the proportions of variance in bacterial community explained by the neighbor treatment (6%), top ten most abundant identified metabolites (3%), and focal shoot C:N (3%). All factors explain 11% of the total variation in bacterial community. B) Distance-based Redundancy Analysis of focal plant rhizosphere bacterial communities by neighbor treatment; arrows indicate plant and soil factors and identified exudates that explain significant variation in the community structure ($P \leq 0.10$). These three variables account for 20% of the variation in the community (constrained proportion); overall dbRDA model statistics: ANOVA $F_{3,20} = 1.64$, $P = 0.008$. Only four replicates shown for *K. macrantha* treatment after removing an extreme outlier from soil moisture data.

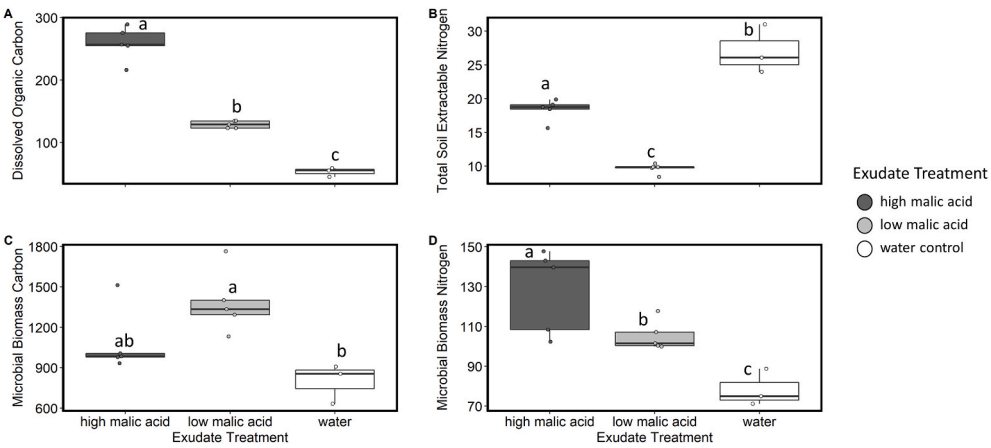

Fig. 5. Effect of malic acid concentration on soil incubation A) dissolved organic carbon (μg C g^{-1} dry soil), B) total soil extractable nitrogen (μg N g-1 dry soil), C) microbial biomass carbon (μg C g-1 dry soil), and D) microbial biomass nitrogen (μg N g-1 dry soil). The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, $P < 0.05$).

exudates in particular, malic acid and stearic acid, correlated with overall shifts in the focal plant's rhizosphere communities, suggesting they play a role in shaping communities. Both compounds are commonly reported root exudates (Aulakh et al., 2001; Liu et al., 2015), and malic acid has been identified as a chemoattractant for beneficial rhizosphere bacteria (Rudrappa et al., 2008; Ling et al., 2011; Jin et al., 2019).

We also cannot ignore several methodological factors that may have affected our ability to test the linkage between exudates and neighbor-induced shifts in bacterial communities. First, because root exudates secreted from root hairs are suggested to have the greatest influence on bacterial communities (Rüger et al., 2021), our sampling of the entire root system may have weakened our ability to detect strong correlations. Second, microbial spillover from neighbor roots may have contributed to shifts in the rhizosphere community. Lastly, it is possible that microbial excretion or consumption of metabolites altered the exudate profiles. But thorough root washing, and the generally higher production of plant-derived versus microbially-derived metabolites per unit volume probably made this only a small factor for highly-abundant metabolites (Williams et al., 2021a). The abundance of malic acid, in particular, can increase with root damage (Williams et al., 2021a), potentially elevating its statistical impact on the microbial community.

When we added malic acid directly to soil in incubations, we also saw changes in microbial communities, but the bacterial genera enriched in the high malic acid incubations were not the same genera correlated with malic acid in the greenhouse experiment. The observed inconsistencies in bacterial enrichment with malic acid are likely driven by artifacts from distinct experimental conditions in the greenhouse and laboratory, including the use of different soil substrates in either experiment. These inconsistencies could also suggest that other factors beyond selection by malic acid are shaping neighbor-induced changes in microbial communities. For instance, we found an unexpected correlation between malic acid exudation and the abundance of the bacterial genera *Methylophilus*. This group consists of facultative methylotrophs that would not utilize malic acid as a primary energy source (Jenkins et al., 1987), and may indicate that other factors, such as shifts in soil nutrients, drove this correlation.

The soil incubation further highlighted that neighbor-induced changes in root exudates may have even more pronounced effects on nutrient cycling, than on bacterial assembly. We found that the greater addition of malic acid increased soil DOC and TN and decreased microbial biomass C:N. While these results may be influenced by differences in sugar content between the high- and low-malic acid treatments, which could alter microbial growth and N-use (Schneckenberger et al., 2008; Cao et al., 2021), our results are consistent with previous studies showing that organic acids stimulate greater release of microbially-available N than sugars (Yuan et al., 2018). Organic acids can stimulate the release of C and N from soil through two mechanisms: first, they can stimulate microbial enzyme production, which then releases mineral-bound nutrients, and second, they can directly liberate organic compounds from mineral soils, making them more available to microbes (Keiluweit et al., 2015; Jilling et al., 2021). These studies used oxalic acid, another commonly exuded organic acid, but we show that malic acid could play a similar role in nutrient mineralization in soils. In sum, the soil incubation suggests that neighbor-induced changes in exudates play an important role in nutrient cycling, and that the correlations between malic acid and bacterial community structure may be driven by the microbes' response to soil N, rather than a direct chemotactic response to the exudate.

4.3. Neighbor effects are greatest during competition and nutrient-limitation

In support of our third hypothesis, we found that neighbor-induced changes in bacterial communities were greatest during strong competition. All neighbors had a competitive effect on focal plant biomass (negative RII), but the forb *R. hirta* caused the greatest reduction in focal plant biomass and had the strongest effect on the rhizosphere community. Patterns in focal plant exudates were similar, but also responded to the less competitive *K. macrantha* neighbor. This suggests that while microbiomes are strongly influenced by characteristics of strong competition, such as nutrient stress and reduction in biomass, other mechanisms contributed to shifts in the exudates, such as aboveground signaling (Li et al., 2020; Kong et al., 2021) or neighbor detection (Biedrzycki and Bais, 2010; Kong et al., 2018). Still, we cannot distinguish how the observed effects on rhizosphere structure are impacted by neighbor identity and competition. *R. hirta* was both the only forb neighbor and the largest neighbor, and caused the greatest reduction in focal plant biomass, tissue N content and soil moisture, and each mechanism may have contributed to its greater effect on focal plants.

Abiotic factors in particular can directly affect microbial community structure (Fierer, 2017; Naylor and Coleman-Derr, 2018) and may also drive indirect, host-mediated shifts in microbiomes by altering root exudates (Smercina et al., 2020; Williams and de Vries 2019). We saw that focal plant C:N explained variation in both the bacterial community and root exudates, suggesting that N competition was particularly important to *R. hirta*'s strong effect on the focal plant. Surprisingly, soil nitrate levels did not differ by treatment, but the drier soil of the *R. hirta* treatment may have reduced focal plant N uptake (Gonzalez-Dugo et al., 2012; Bista et al., 2018). This N and water limitation likely increased exudation of compounds related to plant stress response. For instance, glycerol 3-phosphate (G3P), which was exuded more next to *R. hirta,* is shown to increase in both plants (Shen et al., 2006) and microbes (Albertyn et al., 1994) during osmotic stress. There may even be a link between G3P and the recruitment of beneficial, drought-tolerant

microbes (Xu et al., 2018), as well as host immunity against pathogens (Chanda et al., 2011; Mandal et al., 2011). In this study we did not find strong correlations between G3P and bacterial taxa, suggesting that more studies are needed to elucidate the role that G3P exudation plays under stress and, specifically, if it influences microbial assembly.

P. virgatum also exuded more organic acids (fumaric acid, malic acid, and saccharic acid) while neighboring *R. hirta*, likely due to nutrient limitation. Plants release more organic acids under a variety of nutrient stresses (K+, P, N, Ca^{2+}, Zn^{2+}) (reviewed by Jones, 1998; Panchal et al., 2021), so though we know that N was limited, other nutrients may have also triggered this response. Several recent studies show that our focal plant, *P. virgatum,* exudes more organic acids and fewer carbohydrates in N-limited, sterile, conditions (Smercina et al., 2020), and that these organic acids increase soil DON and N-mineralization, but not biological N-fixation (Liu et al., 2022). Accompanied with our soil incubation results, this shows that organic acids may do more to alleviate plant N stress through physical liberation of minerally-bound N, rather than through recruitment of beneficial microbes, such as free-living N-fixers.

Finally, in addition to *R. hirta's* distinct effect on soil resources, it was also the largest neighbor plant, which may have contributed to its strong effects on the focal plant's rhizosphere community. With a root system that was ten times larger than the other neighbors, *R. hirta's* roots likely had greater overlap and microbial spillover with the focal plant rhizosphere. In fact, the bacterial genera that were more prevalent in the focal plant's rhizosphere near *R. hirta* were also most abundant in the *R. hirta* monoculture (Supplemental Fig. 4), suggesting a role of microbial spillover. Higher exudation rates in grassland forbs than grasses (Williams et al., 2021b) may have also strengthened its effect on the focal plant's rhizosphere. *R. hirta's* effect on the focal plant's non-dominant taxa, however, was not driven by microbial spillover, as the non-dominant taxa represented a novel community distinct from either the *R. hirta* or *P. virgatum* monoculture. This result indicates that mechanisms other than microbial spillover, perhaps shifts in neighbor signaling, drive changes in non-dominant rhizosphere taxa. Lastly, these subtle, but clear shifts in non-dominant taxa may also help explain why neighborhood effects are seldom noticed, yet why some plant pairings have non-additive effects on microbial functions, such as nutrient cycling (Betencourt et al., 2012; Li et al., 2016; Sekaran et al., 2020).

5. Conclusion

In summary, we show that neighbor plants influence one another's rhizosphere assembly, especially during strong neighbor competition. This suggests that studies on isolated plants may not be predictive of rhizosphere assembly in natural conditions. We found evidence for multiple mechanisms contributing to neighbor-induced changes in the rhizosphere bacterial communities. While the exudate profile was strongly correlated to the overall microbiome, suggesting that exudates may play a role, we could not repeat the same taxonomic shifts by manipulating a dominant exudate, and did not identify a causal link between these factors. In fact, the dominant exudate also increased soil N, suggesting that neighbor-induced changes in exudates may have even stronger effects on soil nutrients than microbial assembly. Still, future studies should explore the spatial and temporal scales at which neighbors affect exudates and rhizosphere taxa, as this likely influenced our ability to correlate shifts in taxa and exudates. Overlapping roots and microbial spillover also contributed to the strong neighborhood effects. Future studies with root barriers will help elucidate the relative role of microbial spillover and exudate-mediated microbial assembly on associational effects. Overall, this study highlights that exploring plant-microbial dynamics in mixed-species neighborhoods can help increase our understanding of the mechanisms that drive rhizosphere assembly in nature, as well as improve our ability predict and manage for beneficial microbial interactions.

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.

The authors declare no competing interests.

Code and data are openly available: https://github.com/TaylerUlbrich/NeighID_Switchgrass; Raw bacterial fastq files (NCBI accession number PRJNA773254).

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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.2022.108753.

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