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RESEARCH ARTICLE

Plant phenology influences rhizosphere microbial community and is accelerated by serpentine microorganisms in *Plantago erecta*

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One sentence summary: Support for the role of locally adapted microorganisms in impacting plant phenology.

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ABSTRACT

Serpentine soils are drought-prone and rich in heavy metals, and plants growing on serpentine soils host distinct microbial communities that may affect plant survival and phenotype. However, whether the rhizosphere communities of plants from different soil chemistries are initially distinct or diverge over time may help us understand drivers of microbial community structure and function in stressful soils. Here, we test the hypothesis that rhizosphere microbial communities will converge over time (plant development), independent of soil chemistry and microbial source. We grew *Plantago erecta* in serpentine or nonserpentine soil, with serpentine or nonserpentine microbes and tracked plant growth and root phenotypes. We used 16S rRNA gene barcoding to compare bacterial species composition at seedling, vegetative, early- and late-flowering phases. Plant phenotype and rhizosphere bacterial communities were mainly structured by soil type, with minor contributions by plant development, microbe source and their interactions. Serpentine microorganisms promoted early flowering in plants on nonserpentine soils. Despite strong effects of soil chemistry, the convergence in bacterial community composition across development demonstrates the importance of the plant–microbe interactions in shaping microbial assembly processes across soil types.

Keywords: serpentine; plant–microbe interaction; rhizosphere; plant development; *Plantago erecta*

INTRODUCTION

Plant–microbe associations occur on a small scale, but can impact global patterns, including plant and microbial biodiversity (Cui and He 2009; Ravichandran and Thangavelu 2017; Kandlikar *et al.* 2019). Plants associate with distinct microbial communities that can benefit plants by enhancing nutrient acquisition (Emami *et al.* 2018; Fei *et al.* 2020) and protection against pathogens (De Curtis *et al.* 2010; Akhtar, Siddiqui and Wiemken 2011). These associations generate plant–soil feedbacks that can influence plant community structure (Van Der Heijden *et al.* 2006). Plant–microbe associations have also been explored for their ability to impact the phenotypes of agricultural plants (Gouda *et al.* 2018). As a result, microbial amendments are being developed for their ability to influence plant yield (Murgese *et al.* 2020) and stress tolerance (Orlandini *et al.* 2014; Kwak *et al.* 2018). However, the extent to which soil community members establish in the rhizosphere, and when during a plant's development, remain poorly understood and may affect the efficacy of microbial amendments.

Soil chemistry and plant species both influence the composition of rhizosphere microbial communities (Haichar *et al.* 2008;

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Berg and Smalla 2009). Plant development, or phenology, has been shown to correlate with distinct microbial associations. For example, seedlings of *Arabidopsis thaliana* showed distinct microbial communities from later-phase plants (Chaparro, Badri and Vivanco 2014). In *Oryza sativa*, rhizosphere microbial communities are dynamic during vegetative growth and can represent particular life phases (Edwards *et al.* 2018). One mechanism for plant effects on rhizosphere communities is through the rhizodeposition of root exudates, which can change over time and correlates with distinct rhizosphere microbial communities observed at each phase of plant development (Chaparro *et al.* 2013; Zhalnina *et al.* 2018). However, whether the same plant species assemble microbial communities similarly in distinct soil backgrounds remains unexplored.

Changes in plant phenology has severe implications for plant reproduction. If a plant flowers earlier than expected, pollinator mismatch can occur (Kudo and Cooper 2019), which can decrease the reproductive success of pollinator-dependent plants (Rodríguez-Pérez and Traveset 2016). Differences in phenology can also be an indicator of ecotypic variation and local adaptation (Parker *et al.* 2017). Earlier phenology has also been shown to be a consequence of climate change and biodiversity loss (Wolf, Zavaleta and Selmants 2017); however, other factors can contribute to changes in phenology and it is worth exploring microbial-mediated changes to plant development.

Serpentine soils are characterized by low water-holding capacity, elevated concentrations of heavy metals including nickel, low concentrations of essential plant nutrients and high Mg-to-Ca ratios (O'Dell, James and Richards 2006). These characteristics are partially responsible for the low plant productivity and endemism observed on serpentine soils (Anacker 2014). While most plants cannot grow on serpentine soils and other plants can only grow on serpentine soil, serpentine-indifferent plants are able to thrive on serpentine soils and compete on nonserpentine soils (Safford, Viers and Harrison 2005). Serpentine-indifferent plants, with their ability to grow both on and off serpentine soils, are an excellent tool with which to study how soil chemistry influences microbial composition and phenology (Igwe and Vannette 2019). In addition, by utilizing soil treatments with nonadapted microorganisms we can understand how phenology is influenced by different microbial communities.

Abiotic and biotic factors including soil chemistry and soil moisture have also been shown to influence plant phenology; for example, plants growing on serpentine and drought-prone soils have generally been shown to flower sooner than those growing on nonserpentine and non-droughted soils (Sherrard and Maherali 2006; Wright, Stanton and Scherson 2006; Rossington, Yost and Ritter 2018; Sakaguchi *et al.* 2019), but this is not always the case (Schneider 2017). Therefore, an additional goal of our experiments was to investigate the abiotic vs biotic control on phenology, especially as it relates to flowering time.

In this study, we aimed to answer the following broad ecological question: Do plant rhizosphere microbial communities grown in disparate soil chemistries converge or diverge over time? More specifically, we test the hypothesis that soil chemistry influences how microbial communities change over plant development. Previous research with flowering serpentine-indifferent plants on serpentine and nonserpentine soils showed that the rhizosphere microbial communities were similar; therefore, we predict that rhizosphere microbial communities associated with serpentine-indifferent plants growing on serpentine and nonserpentine soils will become more similar as the plant develops. We also hypothesize that serpentine components introduced to nonserpentine soils, including addition of the heavy metal nickel or simulated drought, will change microbial communities and plant characteristics to be similar to those of live serpentine soils. For example, if soil chemistry is the major driver of flowering time, then we can expect that treatments with the same soil origin, regardless of microbial community, will not significantly differ in phenology. If the microbial community influences plant phenology to a greater extent than soil chemistry, we can expect to see significant differences in plant development in soil treatments with nonadapted microorganisms relative to soil treatments with adapted microbes. It is important to understand the relative influence of these factors on plant phenology because the reproductive success of an individual plant and the plant community structure is directly related to plant phenology (Fenner 1998; Rodríguez-Pérez and Traveset 2016; Hidalgo-Triana and Pérez-Latorre 2018).

METHODS

Study system and soil collection

Soils were collected from McLaughlin Natural Reserve in June 2018 from three serpentine and three nonserpentine sites (Sites 1, 2 and 3 from Igwe and Vannette 2019). McLaughlin Natural Reserve is characterized by a Mediterranean climate with hot and dry summers from April to October. Gallon-sized plastic bags of soil were collected every 5 m across a 20 m transect at each site at an average depth of 10 cm. These soils were placed on ice in the field and then in 18-gallon/68-liter plastic containers at 4°C until the start of the experiment in July 2018. We used *Plantago erecta* (serpentine affinity mean = 1.0), which is common in serpentine and nonserpentine sites locally (Safford, Viers and Harrison 2005). Seeds used in the experiment were purchased from S&S Seeds in 2016 (Carpinteria, CA) after field-collected seeds germinated poorly.

Growth chamber experiment

We conducted an experiment to examine how background soil chemistry and soil microbial community jointly influence plant growth and microbial community assembly in the rhizosphere. All soils included an autoclaved soil background (serpentine or nonserpentine), to which live (unautoclaved) soils from either serpentine or nonserpentine soils were added at ~16% (v/w) to create the microbial amendments (Farrer and Suding 2016; Calderón *et al.* 2017). Soils were autoclaved at 120°C at 15 psi for two 30-min periods with 24 h between sterilizations (Ishaq *et al.* 2017). Using this method, four factorial treatments were created: autoclaved serpentine soil with serpentine microbes (S+Sm), autoclaved nonserpentine soil with nonserpentine microbes (NS+NSm), autoclaved serpentine soil with nonserpentine microbes (S+NSm) and autoclaved nonserpentine soil with serpentine microbes (NS+Sm). In addition, to explore which dimensions of serpentine soils shape plant–microbe–soil interactions (Wright, Stanton and Scherson 2006), we either amended some NS+Sm treatments with nickel (final concentration of 25 ppm; NS+Sm+Ni), or grew plants in conditions simulating drought stress (NS+Sm+Drought).

Plantago erecta seeds were added to D16 Deepots (volume: 16 in^3 and 262 mL; Stuewe and Sons, Inc., Tangent, OR) containing ~100 g of soil in one of six soil chemistries above. Plants were grown in a growth chamber under 12:12 light/dark regime at 20°C at the UC Davis Environmental Horticulture Greenhouse Complex and were grown to senescence, with 15 replicates per

treatment and 3 non-planted controls per treatment. The simulated drought soil treatment was watered until the soil was saturated once a week while all other soil treatments were watered daily with DI water. Leaf number and plant height were recorded weekly until senescence. Plants in each treatment were harvested at seedling, vegetative, early- and late-flowering phases, and a random subset ($N = 6$) was used for rhizosphere soil collection, microbial DNA extraction and 16S rRNA gene sequencing. Plants were classified as 'seedlings' upon emergence from the soil. When true leaves were present, plants were classified as 'vegetative'. 'Early flowering' was characterized by shoot development and the presence of an undeveloped terminal protuberance. Once the plant began to bloom, the plant was characterized as 'late flowering'. Once the plant became brittle to the touch, they were classified as 'senescing'. For two treatments (NS+Sm+Ni) and (NS+Sm+Drought), only seedling and late-flowering phases were harvested due to limitation in growth chamber space.

Rhizosphere soil collection

For each harvested plant, roots were shaken to remove loosely adhering soil. An ethanol-sterilized razor was used to separate the stem from the roots. Aboveground plants were dried at 80°C for 48 h and then weighed.

Roots were separated from rhizosphere soils. Briefly, roots were sonicated in 0.9% NaCl/0.01% Tween 80 (v/v) solution for 180 s to remove the tightly adhering soil particles (Barillot *et al.* 2013). Centrifuge tubes containing NaCl/Tween (without roots) were then centrifuged for 20 min at 4°C at 3234 × *g*. The pellet was frozen at −20°C until DNA extraction using a Zymo fecal/soil DNA extraction kit according to enclosed directions (Zymo Research, Irvine, CA). After sonication, root samples were stored in 50% ethanol solution until root image analysis.

DNA extraction was confirmed using a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA, USA) and then samples were submitted to the Centre for Comparative Genomics and Evolutionary Bioinformatics Integrated Microbiome Resource at Dalhousie University for amplification and sequencing. The V6–V8 subregion of the 16S SSU rRNA was amplified using B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) primers (Comeau *et al.* 2011). We chose the bacteria-specific V6–V8 subregion based on the protocol from Integrated Microbiome Resource (IMR) (imr.bio/protocols.html). These primers excluded more chloroplast, eukarya and mitochondria DNA than the other available primers. DNA was amplified using Phusion High-Fidelity DNA polymerase (NEB) and MiSeq (300+300 bp PE) for final amplicon lengths that were 508 bp. Raw sequences are archived at www.ncbi.nlm.nih.gov/sra/PRJNA623253.

Root imaging

To analyze root length, volume, surface area and diameter, samples were scanned using WinRHIZO optical scanner and software (Regent Instruments Inc., Canada). Each root sample was imaged individually by laying them flat onto a tray containing 50% ethanol to cover the entire root. Tangled roots were carefully separated with forceps and any roots broken off were also imaged. Before root parameters were measured, any residual soil particles and foreign root fragments scanned by the imaging software were eliminated from the selected root image. To do this, the entire root was selected by drawing a box around the imaged root. Next, foreign particles were excluded from analysis by selecting *Regions—Exclusion Regions* and drawing a box around each individual target region. Once this step was completed, root parameters including root length, root diameter, root surface area and root volume were measured by selecting *Image—Image with Analysis*.

Bioinformatics

Amplicon sequence variants (ASVs) from 16S rRNA gene amplicons were identified using DADA2 (v1.7.2) (Callahan *et al.* 2016a). Briefly, paired-end fastq files were processed by filtering and truncating forward reads at position 250 and reverse reads at position 200. Sequences were dereplicated, merged and error-corrected according to code archived on Dryad. Chimeras were removed, and the taxonomy assigned using the SILVA database (v128) (Quast *et al.* 2012; Yilmaz *et al.* 2014; Glöckner *et al.* 2017). A phylogenetic tree based on 16S sequences was created using the DECIPHER package (v2.8.1) in R to perform multistep alignment and phangorn (v2.4.0) to construct the tree using neighbor joining (Wright, Stanton and Scherson 2006; Schliep 2011). The sequence table, taxonomy and metadata were combined into a phyloseq object and used for further analysis (phyloseq v1.30.0) (McMurdie and Holmes 2013; Callahan *et al.* 2016b). Mitochondrial and chloroplast sequences as well as any sequences that were not assigned to bacteria were removed from the ASV table.

Statistical analysis

To visualize the relative abundance of each phylum, ASVs were aggregated to the phylum level and taxa representing <2% of relative abundance were filtered out. To determine effect of soil treatment and plant developmental phase on alpha diversity of rhizosphere communities, Shannon diversity was calculated on the full dataset using the estimate_richness function in the phyloseq package (1.30.0) and used as a response variable in an analysis of variance (ANOVA) with plant developmental phase, soil chemistry (S, NS, NS+Sm+Ni and NS+Sm+D) and microbe source (S or NS) as predictors. Shannon index was used because it accounts for both abundance and evenness in samples (Kaisermann *et al.* 2017).

To examine differences in rhizosphere bacterial species composition due to soil chemistry, microbe source and plant developmental phases, Bray–Curtis dissimilarities were calculated and visualized using nonmetric multidimensional analysis. To determine which predictors were associated with variation in rhizosphere bacterial composition, we used the 'adonis' function from the vegan package with Bray–Curtis dissimilarities as the response variable and plant developmental phase, soil chemistry and microbe source as predictors. To test for differences in multivariate dispersion among rhizosphere communities, the 'betadisper' function from the vegan (v2.5.3) package was used (Oksanen *et al.* 2019) with soil chemistry, plant developmental phase and microbe source as predictors.

To determine the effects of soil treatments on plant growth, each plant trait (leaf number, plant height, root length, root diameter, root surface area and root volume) was analyzed using a general linearized model with soil chemistry, microbe source and plant developmental phase as the predictor and differences between group means were identified using likelihood ratio tests. Tukey's Honestly Significant Differences (Tukey's HSD) was used as a post-hoc test to identify differences among groups.

To determine the effects of soil treatments on time to flowering, survival analysis was conducted on binary flowering data

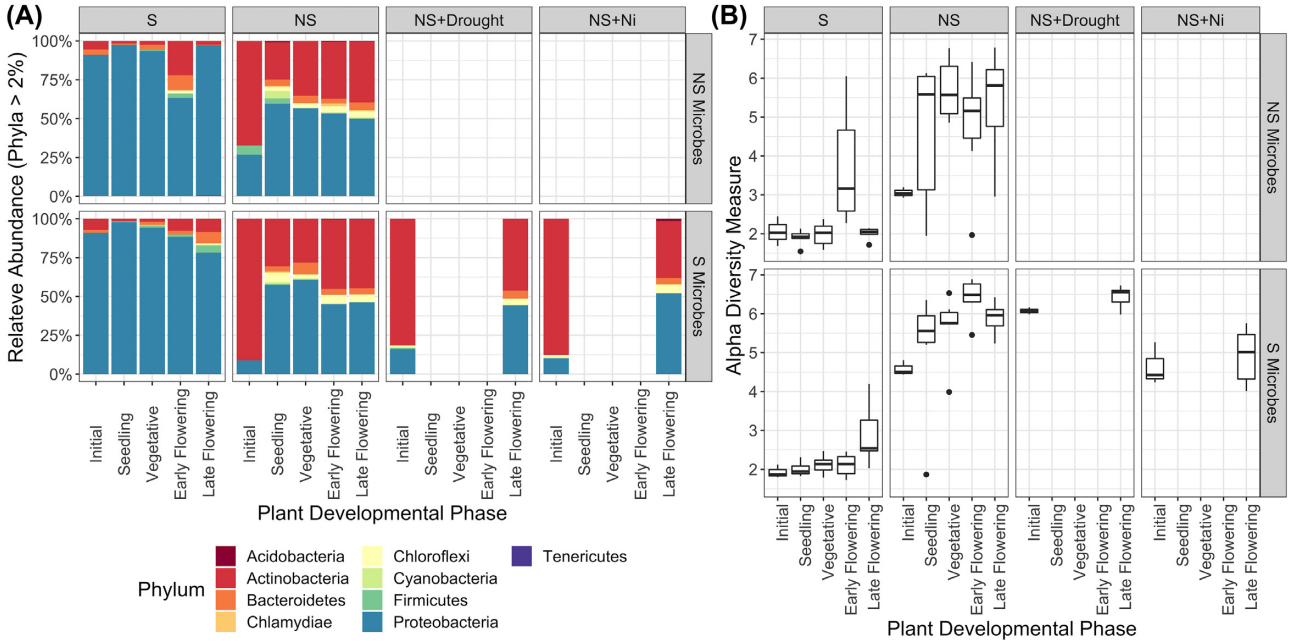

Figure 1. Relative abundance and alpha diversity of bacterial community composition on roots of *P. erecta* across soil treatments and plant phenology. **(A)** Bacterial phyla with a relative abundance of at least 2% are visualized in a bar graph facetted by plant developmental phase and soil treatment (S = Serpentine and NS = Nonserpentine). **(B)** Shannon diversity is significantly different between soil chemistries ($F_{3,100} = 124.05$, $P < 0.001$) and plant developmental phase ($F_{4,100} = 5.74$, $P < 0.001$), but not microbe source ($F_{1,100} = 2.64$, $P = 0.11$). There was a significant interaction between soil chemistry and microbe source ($F_{1,100} = 6.80$, $P = 0.011$) as well as soil chemistry, microbe source and plant developmental phase ($F_{5,100} = 4.08$, $P = 0.004$).

(yes/no) using Kaplan–Meier curves and a Cox proportional hazards regression model ('coxph') on a survival object ('Surv') in the survival package (v2.42.6) to describe the soil treatment impacts the probability of flowering over time (Therneau 2015). The time to event (late flowering) was measured in days from the onset of seedling phase. The model provides a hazard ratio (HR) where an HR > 1 indicates an increased likelihood of development, while an HR < 1 indicates a decreased likelihood of development. Differences in the HR were visualized using 'ggforest' in the survminer package (v0.4.3) (Kassambara and Kosinski 2018).

To determine whether the relative abundance of bacteria ASVs differed among plant developmental phases or soil chemistries, differential abundance analysis using DESeq2 (1.26.0) was used with soil chemistry as the predictor. DESeq2 analysis was conducted with all soil treatments for the seedling and late-flowering plant developmental phase and another analysis was conducted with S+Sm, NS+NSm, S+NSm and NS+Sm for all plant developmental phases to represent the experimental design.

RESULTS

After quality filtering and removal of nontarget sequences, we recovered 1 608 688 reads (average 9353 reads per sample) that were grouped into 23 894 amplicon sequence variants. Sampling curves within most samples were saturating (NS+NSm, NS+Sm+Ni, NS+Sm+D) indicating a robust sampling of the microbial diversity associated with individual plants while serpentine soils did not fully saturate (S+Sm, S+NSm) (Figs S1–S6, Supporting Information). To compare among samples, count data were normalized by relative abundance in a sample.

Soil treatment and plant developmental phase influence species richness and community similarity

Bacteria from the phyla Proteobacteria or Acidobacteria comprised nearly 90% of reads from most samples depending on soil chemistry (Fig. 1A). Alpha diversity differed among soil chemistries (Fig. 1B; $F_{3,100} = 124.05$, $P < 0.001$) and plant developmental phase ($F_{4,100} = 5.74$, $P = <0.001$), but not microbe source ($F_{1,100} = 2.64$, $P = 0.107$). There was a significant interaction between soil chemistry and microbe source ($F_{1,100} = 6.80$, $P = 0.011$), as well as soil chemistry, microbe source and plant developmental phase ($F_{5,100} = 4.08$, $P = 0.004$) on bacterial composition. The serpentine soil treatments (S+Sm and S+NSm) had lower alpha diversity at all time points compared with all treatments with nonserpentine soils (NS+Sm, NS+Sm+Ni, NS+Sm+D). The treatment with simulated drought (NS+Sm+D) treatment had higher species richness than either the live nonserpentine treatment (NS+NSm) or the treatment with nickel added (NS+Sm+Ni).

Bacterial community composition in the rhizosphere varied with soil chemistry and plant developmental phase, with surprisingly minimal contribution from the microbial source (Fig. 2; Table 1). Variability in bacterial communities among plants (beta diversity) was associated with soil chemistry (Betadisper: $F_{3,128} = 93.67$, $P = 0.001$) and plant developmental phases (Betadisper: $F_{4,127} = 5.79$, $P = 0.004$), but only weakly with microbe source (Betadisper: $F_{1,130} = 3.42$, $P = 0.068$). Microbial communities from the seedling phase were less variable than all other plant developmental phases, but there were no significant differences between the variability of other phases. Both serpentine soil treatments were less variable than that of either nonserpentine soil treatment.

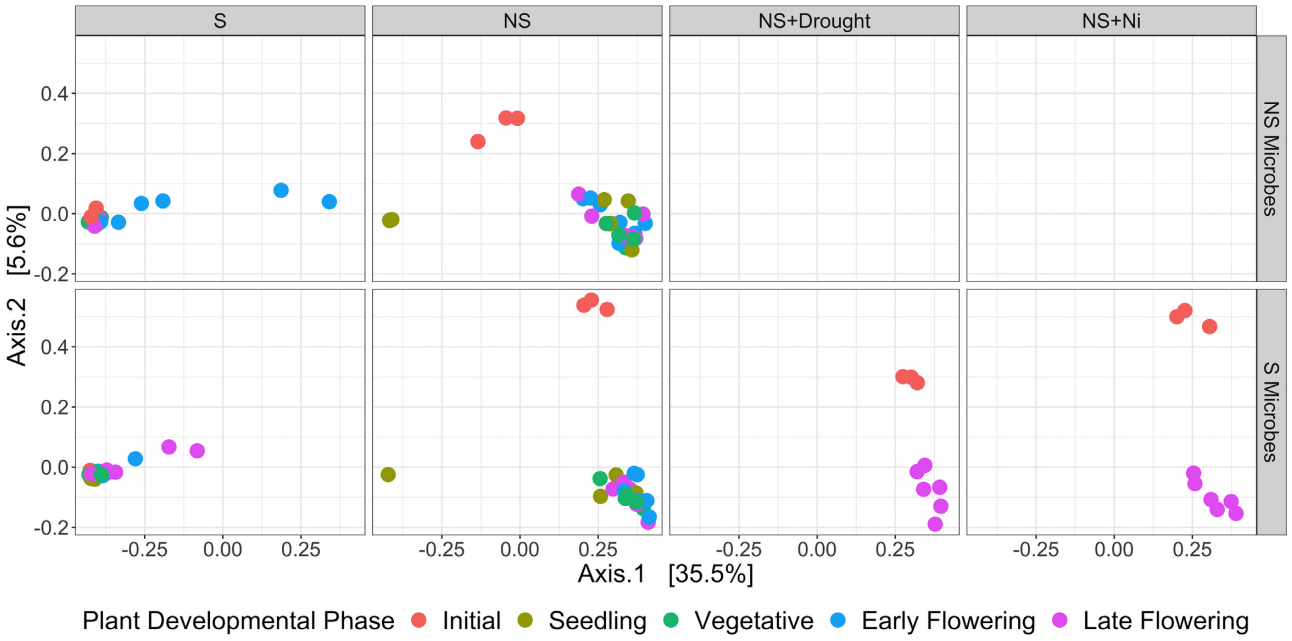

Figure 2. Principal Coordinates Analysis (PCoA) of *P. erecta* rhizosphere bacterial communities across soil treatments and plant phenology using Bray–Curtis dissimilarity. Point color indicates plant developmental phase and panels indicate distinct soil treatments.

Table 1. Statistical analysis of microbial community dissimilarity using ANOVA. The predictors, degrees of freedom (df), number of samples (*N*), *F*-value (*F*), variation (R^2) and *P*-value, are listed.

Predictor	df	N	F	R^2	P
Soil type	3	100	22.23	0.304	0.001
Developmental phase	4	100	3.21	0.059	0.001
Microbe source	1	100	3.24	0.015	0.008
Soil type*Developmental phase	6	100	2.21	0.06	0.001
Microbe source*Developmental phase	4	100	1.36	0.025	0.089
Soil type*Microbe source	1	100	3.04	0.014	0.011
Soil type*Microbe source*Developmental phase	4	100	1.58	0.029	0.031

Plant growth responses to soil chemistry and microbial source

In general, the height, leaf number, aboveground dried biomass, root length, root diameter, root surface area and root volume were all impacted by soil chemistries (Figs 3 and 4; Tables 2 and 3). The interaction between plant developmental phase and microbe source as well as soil chemistry and microbe source also influenced these plant traits. Microbe source, alone, only significantly influenced root diameter.

Serpentine microbes alter plant vegetative and flowering phenology

Cox proportional hazards regression models showed differences in plant progression through developmental phases among treatments (Table 4). Plants associated with serpentine microbes reached the post-seedling vegetative phase and flowered earlier than those associated with nonserpentine microorganisms (Table 4).

Bacterial taxa

DESeq2 identified taxa that were differentially abundant according to soil type (Fig. 5; Figs S7 and S8, Supporting Information). *Solirubrobacter*, *Lactobacillus* and *Methylobacterium* were genera that were of particular interest. *Solirubrobacter* were present in all treatments with nonserpentine soils and absent in both treatments with serpentine soils. *Lactobacillus* were present in both serpentine soil treatments. *Methylobacterium* were most abundant in the NS+NSm, NS+Sm and S+Sm treatments.

DISCUSSION

The role of rhizosphere microbes in plant health is increasingly recognized, but efforts to manage or alter rhizosphere composition require understanding the relative importance of soil chemistry, microbial species pools and plant development in assembly processes. In comparing serpentine to nonserpentine soils and microbial sources, we found that soil chemistry exerts that strongest influence on microbial community composition, with more minor changes with plant phenology and with microbial source. Plant phenology was also impacted by the interaction between soil chemistry and microbe source with plants growing in nonserpentine soils with serpentine microbes having an accelerated vegetative and flowering phenology.

Threats to *P. erecta* populations include climate change, human development and invasive species (Weiss 1999). These factors can all influence flowering phenology, which can have an

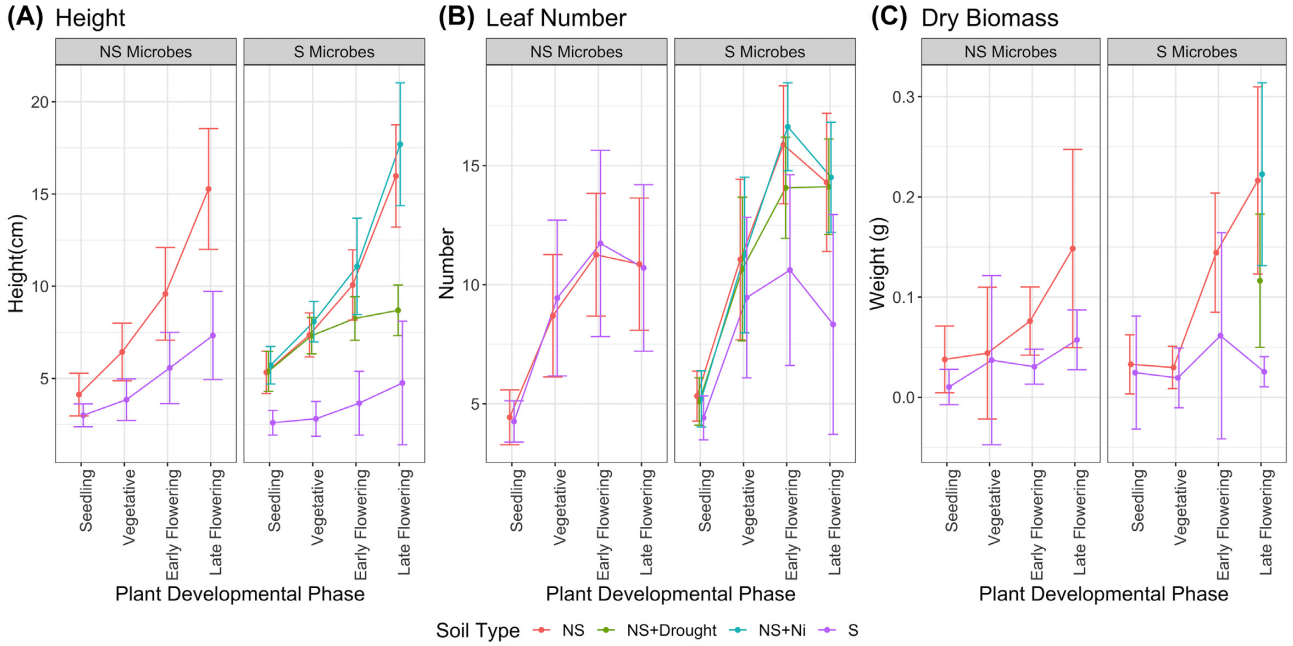

Figure 3. Growth traits of *P. erecta* vary among soil treatments and plant developmental phase. Points indicate mean ± 1SD and points from soil treatments are connected, showing there is a significant difference between **(A)** height and **(B)** leaf number between soil treatment and plant developmental phase. Dry biomass **(C)** was significantly different between plant developmental phase, but not soil treatment.

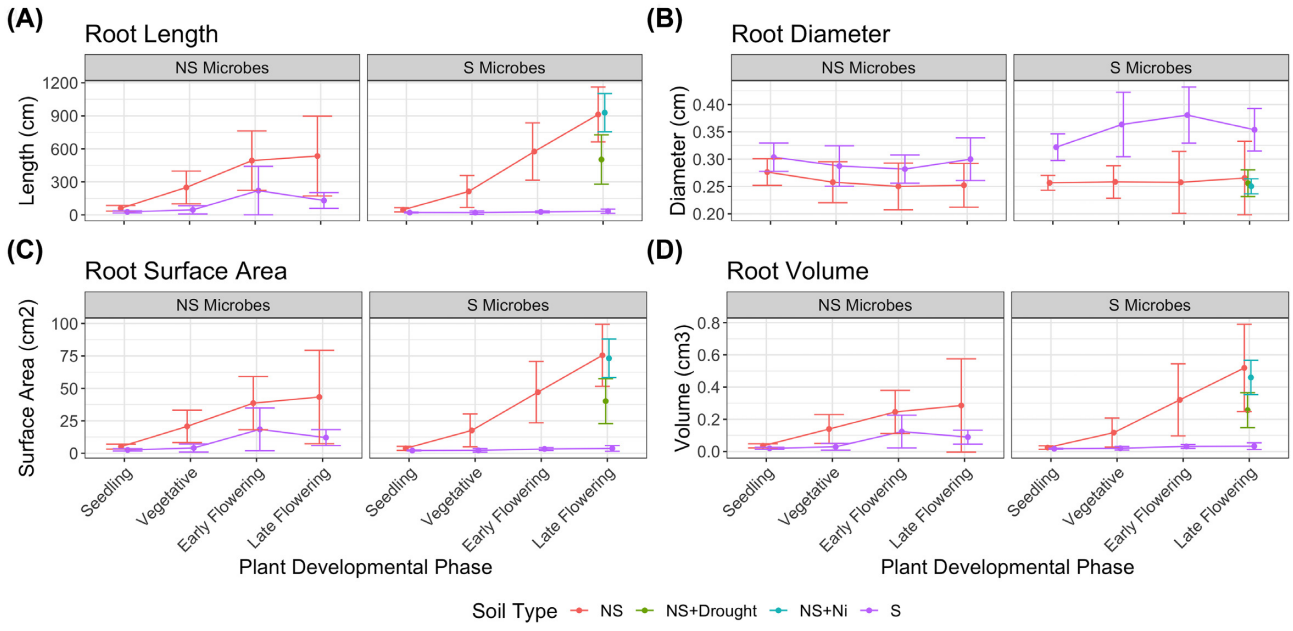

Figure 4. Root growth traits vary among soil treatments and plant developmental phase. Mean value for root traits and standard deviation show that **(A)** root length was significantly different across soil treatment and plant developmental phase. Root diameter **(B)** was different between soil treatments, but not plant developmental phase. Root surface area **(C)** and root volume **(D)** showed significant differences between plant developmental phases and soil treatments. The interaction between soil treatment and plant developmental phase influenced all root metrics.

impact on life-history traits and population dynamics (Dorji *et al.* 2013; Yang *et al.* 2020). In serpentine grasslands, invasive species alter microbial communities so that the soil environment is more hospitable to the invaders (Batten, Scow and Espeland 2008; Hodge and Fitter 2013; LaForgia, Kang and Ettinger 2021). Plant-induced changes to the soil microbial community can impact plant phenology if changes in microbial communities accelerate plant development, as we showed. This has further implications for populations of pollinators that rely on the plant through various life-stages. Changes in plant phenology can impact the range of plants in environments similar to that of *P. erecta* (Benning *et al.* 2019), so how the distribution of *P. erecta* will be impacted by shifts in phenology will be worth further investigation.

Table 2. Statistical results for aboveground plant metrics. Linear mixed-effects model was used to determine the impact of various predictors on plant height, leaf number and dry biomass. The tray where plants were grown was used as a random variable.

			Plant height		Leaf number		Dry biomass	
Predictor	df	N	X^2	P	X^2	P	X^2	P
Soil chemistry	3	3372	4890.83	<0.0001	78.44	<0.0001	60.40	<0.0001
Developmental phase	3	3372	7012.10	<0.0001	3064.16	<0.0001	79.84	<0.0001
Microbe source	1	3372	2.58	0.108	137.12	<0.0001	2.45	0.11784
Soil chemistry*Developmental phase	9	3372	1294.27	<0.0001	57.13	<0.0001	35.69	<0.0001
Microbe source*Developmental phase	3	3372	40.84	<0.0001	20.45	0.0001	8.81	0.03193
Soil chemistry*Microbe source	1	3372	296.64	<0.0001	142.12	<0.0001	2.34	0.12583
Soil chemistry*Microbe source*Developmental phase	3	3372	11.79	0.008	53.86	<0.0001	6.72	0.08134

Table 3. Statistical results for belowground plant metrics. Linear mixed-effects model was used to determine the impact of various predictors on root length, diameter, surface area and volume. The tray where plants were grown was used as a random variable.

			Root length		Root diameter		Root surface area		Root volume	
Predictor	df	N	X^2	P	X^2	P	X^2	P	X^2	P
Soil chemistry	3	242	197.34	<0.0001	145.73	<0.0001	154.25	<0.0001	89.19	<0.0001
Developmental phase	3	242	160.15	<0.0001	0.24	0.971	140.32	<0.0001	94.90	<0.0001
Microbe source	1	242	1.40	0.235	22.25	<0.0001	1.66	0.198	1.86	0.173
Soil chemistry*Developmental phase	3	242	79.24	<0.0001	3.46	0.326	66.09	<0.0001	42.79	<0.0001
Microbe source*Developmental phase	3	242	10.75	0.013	9.81	0.02	9.60	0.022	6.88	0.076
Soil chemistry*Microbe source	1	242	9.65	0.002	29.46	<0.0001	8.62	0.003	5.97	0.015
Soil chemistry*Microbe source*Developmental phase	3	242	17.88	0.001	3.50	0.321	16.63	0.001	12.64	0.005

Table 4. Cox proportional hazards model to determine differences in *P. erecta* phenology. Cox proportional hazards model was used to determine the likelihood of *P. erecta* reaching a particular plant development phase in distinct soil types. An HR = 1 indicates the treatment was used as a reference to which other treatments were compared. An HR > 1 indicates an increased likelihood of development, while an HR < 1 indicates a decreased likelihood of development. For example, *P. erecta* grown in Serp+NSmic is 3.1 times more likely to reach the flowering phase than those grown in live serpentine soil (Serp). Values in parentheses are the confidence intervals for the HR and '*' indicates *P*-value ≤ 0.001.

	Plant developmental phase		
Soil type	Vegetative	Early flowering	Late flowering
S+Sm	1	1	1
NS+NSm	1.38 (0.94–2.0)	1.7 (0.99–3.1)	1.3 (0.68–2.6)
S+NSm	0.84 (0.56–1.2)	1.2 (0.65–2.1)	1 (0.52–2.1)
NS+Sm	6.23* (4.03–9.6)	4.2* (2.36–7.6)	3.1* (1.56–6.1)
NS+Sm+Ni	7.45* (4.02–13.8)	6.3* (3.02–16.1)	2.1 (0.99–4.5)
NS+Sm+D	4.74* (2.61–8.6)	9.9* (4.78–20.3)	30.9* (11.52–83.0)

Soil chemistry and plant developmental phase both significantly impact microbial diversity and community composition

Here, rhizosphere alpha diversity generally increased with plant developmental phase and was generally higher when plants were grown in nonserpentine soils where plants grew larger. Previous research found no or minimal difference in bacterial alpha diversity between serpentine and nonserpentine soils (Oline 2006; Igwe and Vannette 2019). Consistent with our previous research, plants grown on nonserpentine soils showed increased alpha diversity, suggesting that plant growth rather than the diversity of microbes in the species pool is more important in determining bacterial diversity in the rhizosphere. This may be due to accumulation of microbes simply due to the amount of time the plants spent in soil (Dombrowski *et al.* 2017) or changes in the amount or type of exudates deposited in the rhizosphere (Chaparro *et al.* 2013; Zhalnina *et al.* 2018).

The largest change between microbial composition occurred between the start of the experiment and the seedling phase. After the seedling phase, the microbial community composition stabilized. This occurrence is in line with previous research in rice that showed similar results (Edwards *et al.* 2018). Some microbial communities in the serpentine soil treatments at the flowering phases shift to look more similar to those associated with nonserpentine *P. erecta*. Therefore, some convergence is occurring; however, in this experiment, soil chemistry contributed more to the observed beta diversity in the microbial community than plant developmental phase, even at later phases.

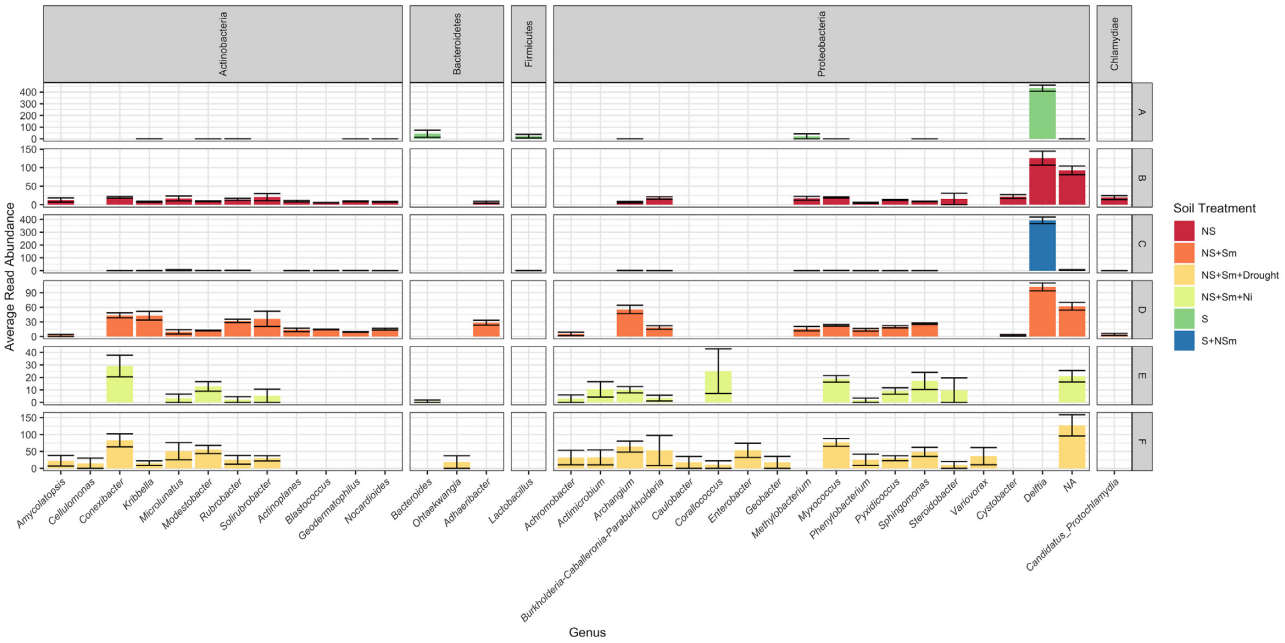

Figure 5. Differentially abundant genera across soil treatments. DESEq2 analysis showing ASVs that were differentially abundant between soil treatments (FDR < 0.01). Bacterial genus is on the x-axis and relative average read abundance on the y-axis. Colors represent soil treatments (A = serpentine soil and serpentine microbes, B = nonserpentine soil and nonserpentine microbes, C = serpentine soil and nonserpentine microbes, D = nonserpentine soil and serpentine microbes, E = nonserpentine soil and serpentine microbes and nickel stress, F = nonserpentine soil and serpentine microbes and drought stress). Bars represent means ± 1SE.

Factors that mimic the serpentine syndrome (e.g. nickel and water stress) did not significantly impact the alpha or beta diversity of the bacterial diversity in the rhizosphere. Nickel or water stress may not be the most important drivers of bacterial community composition. For soils at McLaughlin, pH and the concentrations of magnesium, calcium and potassium were some features that made serpentine and nonserpentine soils distinct. Future research can elucidate the relative influence of each of these properties on structuring the bacterial community in the rhizosphere of serpentine-indifferent plants.

Various mechanisms could contribute to the observed results. For example, the presence of DNA from dead cells that saturated sequencing efforts relative to new DNA. The soil could also exert a selective pressure that is stronger than that of *P. erecta* rhizosphere. Alternatively, it is possible that some serpentine microbes grow well in nonserpentine soils and vice versa. It has been shown that serpentine and nonserpentine soils can host the same microbes with varying accessory genomes (Porter *et al.* 2017). Shotgun sequencing or whole genome sequencing could identify if the same microbes with distinct genotypes grew in reciprocal soil chemistries. By exuding carbon compounds, phenolic acids and amino acids, plants can enhance the growth of specific beneficial or pathogenic members of the soil microbial community, which can enhance plant growth in some cases (Paterson *et al.* 2007). Root exudates change over the course of plant development where younger plants exude more sugars while older plants exude more complex carbon compounds. Root exudate profiles could also differ between plants grown in serpentine and nonserpentine soils and this, in turn, could affect microbial diversity and activity. Characterizing root exudation of *P. erecta* over plant development and correlating the results with changes in microbial community composition can provide greater insight into the role of dynamic plant exudation on survival in serpentine and nonserpentine soils.

Our study cannot disentangle the possible mechanisms that contribute to the observed results. We sampled plants at distinct development phases irrespective of soil residence time, which is an experimental design that considers that plant development phase was shown to influence the rhizosphere microbial community separately from chronological age (Edwards *et al.* 2018). In addition, our study was performed in a growth chamber, preventing the opportunity for microbial immigration from the soil, which could affect microbial diversity and composition in the field.

Soil microbial community and soil chemistry influence time to vegetative growth and flowering

Our reciprocal transplants revealed that serpentine microorganisms, when in nonserpentine soils, accelerate vegetative, early flowering and flowering phenology (Table 4). The importance of the microbial community for time to flowering has been previously demonstrated in *Boechera stricta*, a wild *Arabidopsis* relative (Wagner *et al.* 2014) and *Ipomoea purpurea* (Chaney and Baucom 2020). Drought-adapted microbes accelerated flowering in *Brassica* when compared with non-drought-adapted microbes (Lau and Lennon 2012). A few mechanisms for microbial effects on phenology have been proposed including nutrient availability, production of plant hormones or their precursors, or by exacerbating stress. However, flowering was delayed in the presence of serpentine microbes grown in nonserpentine soils (Table 4), which have been previously demonstrated to be more nutrient-rich than serpentine soils (Brady, Kruckeberg and Bradshaw Jr 2005), suggesting another mechanism may underlie microbial effects in this experiment. It may be that microbes produce plant hormones such as indole acetic acid (IAA), which plays a significant role in flowering time. Nitrogen can be converted to tryptophan and then to IAA and increases time to flowering (Lu *et al.* 2018). Alternatively, flowering time has been shown to be

impacted by biotic stress (Kazan and Lyons 2016). It is possible that introducing nonadapted microorganisms to nonserpentine soils may constitute a biotic stressor that can induce changes in plant phenology.

Thirty-four genera across five phyla were shown to be differentially abundant between soil treatments by DESeq2 analysis. Of particular interest are *Solirubrobacter* that was only detected in nonserpentine soil treatments, *Lactobacillus* that characterized serpentine soil treatments and *Methylobacterium* that was most abundant in NS+NSm, S+Sm and NS+Sm treatments. *Solirubrobacter* are Gram-positive, nonmotile bacteria that have been identified and isolated from bulk soil, rhizosphere and endosphere environments (Albuquerque and Da Costa 2014; Wei *et al*. 2014). In general, it has been shown to associate with high soil quality (Gravuer and Eskelinen 2017; Lopez *et al*. 2017; Sánchez-Marañón *et al*. 2017). Nonserpentine soils are generally more nutrient-rich than serpentine soils and this may influence the abundance of *Solirubrobacter* observed in the nonserpentine soil treatments. *Lactobacillus* are lactic acid bacteria (LAB) that are Gram-positive and microaerophilic. Plasmids comprise up to 4.8% of LAB total gene content and are important for growth in the diverse, yet specific environments where these bacteria are found (Makarova *et al*. 2006). *Lactobacillus* have been shown to be metallotolerant and have the ability to bind heavy metals and protect against metal-induced oxidative stress (Li *et al*. 2017; Liu *et al*. 2019; Barman, Jha and Bhattacharjee 2020). Their abundance in serpentine soil treatments may reflect these phenotypic properties as serpentine soils have high concentrations of heavy metals. *Enterobacter*, which was most abundant in the NS+Sm+Drought treatment, has been shown to have plant growth-promoting properties such as phosphorus solubility and ACC deaminase activity (Danish *et al*. 2020). Its application to *Sorghum bicolor* (L.) Moench increased the plants root architecture and ability to tolerate stress (Govindasamy *et al*. 2020). *Methylobacterium* (order *Rhizobiales*) belong to the same family as *Microvirga*, which has previously been shown to associate with legumes and nonlegumes on serpentine soils (Igwe and Vannette 2019). In addition members of *Methylobacterium* can produce auxins and induce root nodulation (Kelly, McDonald and Wood 2014) and can promote plant growth through the production of ACC deaminase (Belimov *et al*. 2019; Sharma, Chandra and Sharma 2021).

Serpentine soils increase root diameter, but have no impact on other plant growth metrics

Plantago erecta grown in serpentine soils were shorter, and generally smaller than those grown in nonserpentine soils as has been documented previously (O'Dell and Rajakaruna 2004; Kayama *et al*. 2005). Root length, surface area and volume were smallest in serpentine soils while root diameter was the largest in this soil chemistry aligning with previous work that demonstrated that heavy-metal tolerant species of *Arabidopsis arenosa* and *Arabidopsis halleri* have thicker roots than the heavy-metal sensitive *A. thaliana* (Staňová *et al*. 2012). Although all *P. erecta* growing in nonserpentine soils were larger than those in serpentine soils, only the plants in the NS+Sm soil treatment flowered sooner relative to the live serpentine (S+Sm) treatment. Collectively, differences in plant size and vegetative and flowering phenology between *P. erecta* on serpentine or nonserpentine soils are important for a plant's life history (Metcalf *et al*. 2019). Plants that flower, set seed and then die (i.e. monocarpic plants) are those that generally flower at the size that will ensure the best reproductive success (Metcalf, Rose and Rees 2003). Continued research could determine how local adaptation of microbial communities influence reproductive success in plants growing in serpentine and nonserpentine soils.

Phosphate deficiency produces plants that increase lateral root production over primary root production (López-Bucio, Cruz-Ramírez and Herrera-Estrella 2003). Plant growth-promoting bacteria are one mechanism by which plants can access nutrients and defend against pathogenic bacteria (Glick 2012). A few direct plant growth-promoting methods that would be important in serpentine soils include phosphorus solubilization, metal chelation and the production of extra-polymeric substances. Together, these traits would increase nutrient availability, decrease metal availability and increase the water-holding capacity of the soil for the plant. Still, the ability of the microbes to confer benefits to plants growing on serpentine soils is dependent on local adaption of the microbes to serpentine (Porter *et al*. 2016, 2019; Rúa *et al*. 2016). Root diameter was the only plant trait that was larger in serpentine soils relative to nonserpentine soils and removing microbes that were locally adapted to serpentine soils removed this advantage. Conversely, replacing microbes that were locally adapted to nonserpentine with microbes that were locally adapted to serpentine contributed to plants that flowered sooner than other treatments.

CONCLUSIONS

The root-associated microbial communities of *P. erecta* grown in serpentine and nonserpentine soils with adapted or nonadapted microorganisms have differing alpha and beta diversities. Notably, *P. erecta* grown in nonserpentine soils with serpentine microorganisms experienced accelerated vegetative and flowering phenology as they entered the vegetative, early- and late-flowering phases before any plants that were grown in live serpentine soils (S+Sm) or live nonserpentine soil (NS+NSm). Above- and belowground development on *P. erecta* on serpentine soil treatments were less than those grown on nonserpentine soil treatments. Overall, our results support a role of locally adapted microorganisms in impacting plant phenology despite minimal effects on other measurable aspects of plant phenotype.

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SUPPLEMENTARY DATA

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