Applied Soil Ecology 131 (2018) 89–98

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Foliar application of Fe resonates to the belowground rhizosphere microbiome in Andean landrace potatoes

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ARTICLE INFO

Keywords:
Fungal community
Iron
Mycorrhizae
Glomus
Rhizosphere
Soil

ABSTRACT

Iron (Fe) is a crucial nutrient for plant growth (e.g. chlorophyll production), and though it is one of the most abundant elements in soil, very low bioavailability can limit plant growth. Studies indicate that many soil bacteria and fungi (e.g. mycorrhizal) play a role in Fe nutrient cycling and plant production, but the evidence for fungal support of plant growth is overwhelmingly correlative and in need of experimental corroboration. An Andean native potato landrace was grown in a greenhouse under Fe limitation and using three levels (Low, Medium, High) of foliar fertilization (FeEDDHA). Application occurred at 45, 60 and 70 days of growth corresponding to periods where Fe limitation is expected to be greatest. The rhizosphere soils were sampled at the flowering stage (80 days). Soil bacterial and fungal communities were examined using high-throughput sequencing of 16S and ITS regions of ribosomal RNA gene, respectively, followed by analysis using Quantitative Insights Into Microbial Ecology (QIIME v1.8). Multivariate data analyses showed that Fe fertilization of leaves significantly ($p < 0.05$) influenced the beta diversity of fungi but not bacterial communities in the rhizosphere. Using our novel approach, it was expected and confirmed that fungal communities would shift and mycorrhizal genera (*Glomus*) would be altered, however, the degree to which community change was observed was more than expected. *Glomeromycota* (~16.3%) related to the family *Gigasporaceae* accounted for 2.8% of OTU and were 2–3 times greater in the rhizosphere of high relative to medium and low Fe conditions. Overall, the results indicate that foliar addition of Fe influences plant Fe and resonates into the root system to affect rhizosphere fungal communities. Potato Fe status thus appears to impact potato root-fungal interactions potentially mediated through mycorrhizal fungi.

1. Introduction

Iron (Fe) limitation can decrease biomass and yield production in crops such as tomato (*Solanum lycopersicum*) and others (Briat et al., 2015). Furthermore, Fe-deficiency anemia has been reported to be a highly prevalent malnutrition problem, affecting over 30% of the world's population (Bouis, 1995; WHO, 2017). High amounts of Fe are found in soil; however, soluble Fe in its naturally occurring forms of hydroxides, oxyhydroxides and oxides is extremely low in cultivated soils, especially when pH is greater than 6 (Marschner, 2012). Because of its importance in the human diet, greater Fe levels in crop production may help support healthier and more productive plants, but also human health, especially in regions of the world with limited consumption of animal protein.

Many research organizations worldwide are investing in the genetic potential of crop plants to improve Fe bioavailability in common staple food crops through both traditional plant breeding and transgenic approaches (Trijatmiko et al., 2016; Velu et al., 2014). These approaches have their advantages, but because of the importance that soil microbes and microbial-root interactions play in plant nutrient status, integration of their activities into breeding or management are warranted (Govindasamy et al., 2009; Ryu et al., 2005). Recently, inoculation of soil with several types of microbes were shown to differentially increase plant Fe uptake in wheat, white lupin and cucumber plants (de Santiago et al., 2009; Pii et al., 2015; Zhang et al., 2009; Zhao et al., 2014). Hence, microbial type does have an effect on Fe status.

In the rhizosphere, the concentration of bioavailable Fe in solution is decreased due to uptake by roots and microbes (Marschner et al., 2011). As a result, Fe deficiency together with plant rhizodeposits could lead to the selection of microbial populations that aid plant Fe status.

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https://doi.org/10.1016/j.apsoil.2018.08.006
Received 15 May 2018; Received in revised form 14 August 2018; Accepted 15 August 2018
Available online 24 August 2018

These microbial populations may promote plant health by suppressing soil-borne pathogens through siderophore-mediated microbial antagonism and eliciting plant defensive capacity called induced systemic resistance. Microbial driven nutrient availability have probably been most studied via mycorrhizal fungi (Lehmann and Rillig, 2015), but phosphate-solubilizing bacteria (*Pseudomonas* spp.) have been shown to also support plant-available P (Bünemann et al., 2012; Oberson et al., 2001; Oberson and Joner, 2005). Chemically P and Fe and elemental phosphates can combine to form insoluble complexes, and so microbial driven increases in bioavailable plant P may also support increasing Fe availability, and vice-versa (Borggaard et al., 1990; Hinsinger, 2001; Tomasi et al., 2008). There is strong scientific support that soil microorganisms play an important role in nutrient availability, but less is known about iron.

Plant-microbial interaction involving Fe acquisition has been demonstrated in a few previous studies (Robin et al., 2007, 2006; Yang and Crowley, 2000). Red clover and maize Fe content, for example are associated with Fe sequestering microbial siderophores (Carvalhais et al., 2011; Jin et al., 2010). Nevertheless, direct evidence that microbes and their interactions with plants function to support Fe acquisition has not been clearly established.

As for potato, Fe fertilizer by foliar application before and during the flowering stage has been shown to increase tuber weight and result in higher concentrations of Fe in the harvested tubers (Al-Jobori and Al-Hadithy, 2014; Hadi et al., 2014). These influences may result directly from the release of plants from Fe limitation, but if soil microbes are involved in an interaction with plants, the application of iron to leaves and the reduction of iron limitation would likely resonate down the shoots, to the roots, and alter root-microbe interactions and influence the rhizosphere microbial community. In this greenhouse study, foliar application of Fe was conducted to investigate whether plant-microbial communities would be altered by changing plant Fe status. The objective was, therefore, to examine the impact of plant Fe nutritional status on the rhizosphere bacterial and fungal communities and to assess the soil microbes that may be impacted by potato Fe acquisition. It was hypothesized that plant Fe treatment of leaves would translate to roots and affect bacterial and fungal communities in the rhizosphere. Specifically, it was expected that high Fe amendment would alter microbial communities that support Fe acquisition, and in particular the relative abundance of *Glomeromycota* in Fe-limited soil.

2. Methods

2.1. Plant materials and experimental design

An Andean native potato landrace (*Solanum tuberosum* L. CIP 703580) was imported from CIP (International Potato Center). To make identical plants for replication, subcultured *in vitro* plants were grown in test tubes (25 × 150 mm) containing 20 mL of MS basal medium (Murashige and Skoog, 1962) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) with 0.7% agar (Sigma-Aldrich, St. Louis, MO, USA) in a growth chamber under a 16 h photoperiod with a light intensity of 500 $\mu M\, m^{-2}\, s^{-1}$ at 22 °C (day)/16 °C (night) for 3 weeks. Then the subcultured potato plantlets were transplanted to 15 pots (3.8 L) containing a soil mixture with relatively low Fe concentration (5 ppm, pH ≈ 7) in the greenhouse at Virginia Tech in Blacksburg in 2015 spring (Supplementary Table 1). The soil mixture was composed of 30% field soil collected from agricultural land at Kentland Farm, Montgomery County, VA (37.20 N, 80.56 W), 0–20 cm cultivated with soybean plants, 40% sand and 30% perlite. The field soil had been air-dried and passed through a 2-mm sieve. During plant growth, the soil moisture was maintained using an automatic irrigation system delivering tap water as needed. To help ensure Fe deficient conditions, the soil pH was checked weekly to ensure it was maintained above 6. Every week, to ensure adequate nutrient supply, plants were fertilized with half strength Hoagland solution (pH 7) without Fe. Greenhouse conditions were maintained at 28 °C (day), 22 °C night, and a relative humidity of ~65%. The daily light schedule (intensity 370 $\mu M\, m^{-2}\, s^{-1}$) consisted of 15 h light and 9 h of darkness.

Preliminary experiments were conducted to assess level of plant Fe limitation. The potato plants were misted with a foliar spray on both the adaxial and abaxial leaf surface using chelated Fe fertilizer (FeEDDHA, Sprint 138 iron chelate) in deionized water until full wetting. For the low Fe treatment, only deionized water was applied. For moderate and high Fe treatments, 200 mg/L and 600 mg/L of FeEDDHA solution (pH of 5.5) were prepared. Foliar applications contained 0.1% (v/v) Tween80 as a surfactant. Soil of each pot was covered with plastic wrap during application to avoid Fe fertilization of soil. Misting occurred every other week (3 times) up to flowering stage. Fe spraying occurred from 6 to 7 pm to prevent leaf damage. Three treatments with five replicate pots were used to create a complete randomized experimental design.

2.2. Sampling and growth parameters

Rhizosphere soil sampling was done 80 days following transplantation into pots, at flowering. The plant shoots were cut near the soil surface. Roots and rhizosphere soil were sampled by inverting the pots while firmly holding the stems. Non-root associated soil fell from the pots. Roots were further shaken to remove loosely attached soil. Rhizosphere soil adhering firmly on the root surface after gently shaking (Supplementary Fig. 1) was collected and put in sterile conical tubes and stored at −80 °C. The DNA was then extracted from the soil and further used for ITS and 16S rRNA gene-based Illumina sequencing.

Plant length and number of branches were determined. Whole plants and tubers were harvested. The total plant biomass and fresh tuber yield in terms of number of tubers and tuber weight were recorded. For Fe concentration measurement, the shoot tops of each plant were harvested until the third leaf from the apex. Relatively young root tips were also harvested. These shoots and roots were washed of soil using tap water, followed by rinsing with deionized water. Shoots and roots were then placed into paper bags and oven dried at 60 °C for 3 days. The total dry weight of both shoots and roots were similarly measured. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to measure soil and plant Fe concentration (Soil Testing Laboratory at Virginia Tech). For ICP-AES sample preparation, the dried shoots and roots were ground and digested in 10 mL 70% HNO_3 overnight, and then digested in a microwave acid digestion system (MARS 6, CEM corporation, NC, USA) for 30 min and diluted to 50 mL with deionized water.

Analysis of variance (ANOVA) was conducted to assess differences in plant and root properties across treatments. This was accomplished using JMP statistical software (SAS Institute Inc., Cary, North Carolina). Means were compared by *t*-test at $p < 0.05$ in all cases.

2.3. DNA extraction and PCR amplification

A 0.5 g subsample of moist and homogenized rhizosphere soil was used for microbial community DNA extraction using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacture's protocol. DNA quality was checked on a 0.8% (w/v) agarose gel. DNA concentrations were determined by fluorometric quantification using the Qubit1 2.0 platform with Qubit dsDNA HS Assay Kit (Life Technologies). DNA was diluted to 5 ng/μL and stored in the −20 °C freezer during the time preceding amplification of 16S rRNA and ITS gene regions.

Extracted DNA was used for Illumina high-throughput sequencing of the 16S rRNA gene and the ITS region for bacterial and fungal community analyses, respectively. The 16S rRNA gene and the ITS region were targeted using the metagenomic sequencing library preparation protocol described by Illumina (2013) with some modification. Briefly, two stages of PCR were applied for amplifying region of interest and

adding index to each sample. Amplicon primers containing Illumina sequencer adapter regions were used in the 1st stage PCR. Then PCR products were cleaned-up to remove free primers and primer dimer species using AMPure XP beads. The 2nd stage PCR was used to attach the indices and Illumina sequencing adapters. Then the final library was cleaned-up using AMPure XP bead.

For bacterial community DNA amplification, the V3 and V4 region of the 16S rRNA gene was amplified using 16SIlluFor/16SIlluRev primer set. Amplification was conducted using a T100™ thermal cycler (Bio-Rad Laboratories Inc., Singapore) (Klindworth et al., 2013). Each 25 μL reaction contained 12.5 μL KAPA HiFi HotStart ReadyMix PCR buffer (KAPA Biosystems, Inc., MA, USA), 5 μL each primer (1 μM), 2.5 μL DNA template (5 ng/μL). Thermocycling consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s and annealing temperature at 55 °C for 30 s, and 72 °C for 30 s. And a final extension time was implemented at 72 °C for 5 min.

For fungal community DNA amplification, the spacer ITS1 region of the rRNA gene was amplified using ITS1F/ITS2 primer set using a T100™ thermal cycler (Bio-Rad Laboratories Inc., Singapore) (Schmidt et al. 2013). Each 25 μL reaction contained: 12.5 μL KAPA2G Robust DNA Polymerase PCR buffer (KAPA Biosystems, Inc., MA, USA), 5 μL each primer (1 μM), 2.5 μL DNA template (5 ng/μL). Thermocycling consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s and annealing temperature at 60 °C for 30 s, and 72 °C for 30 s. And a final extension time was implemented at 72 °C for 5 min. The specificity of the PCR products from both 16S rRNA gene and ITS region was further evaluated by running on a 1.2% (w/v) agarose gel before the index PCR. The index PCR products were purified and measured by Fluorometric Quantitation (Qubit 2.0 Life Technologies). Lastly, the PCR product of each sample was diluted to 5 ng/μL and 5 μL of each sample were mixed as a sequencing library, and then submitted for bar-coded paired-end (150 bp*2 for 16S and 250 bp*2 for ITS) Illumina Miseq sequencing at the Virginia Biocomplexity Institute at Virginia Tech.

2.4. Processing of sequence data and analyses

Analysis was done as previously described (Rodrigues et al., 2017, 2015). Briefly, samples were demultiplexed and quality filtering was performed to remove low quality sequences (NCBI SRA: PRJNA454291). The paired end reads with quality scores averaging above 30 were stitched using Pandaseq (Masella et al., 2012). Qualified sequences were analyzed using the Quantitative Insights Into Microbial Ecology toolkit-version 1.8.0 (QIIME) (Caporaso et al., 2010). Operational taxonomic units (OTUs) were binned at 97% sequence similarity level using *uclust* and *usearch61* (Edgar, 2010), for bacteria and fungi respectively, using an open reference OTU-picking strategy. Representative sequence of each OTU in bacterial and fungal communities was classified into taxonomy, respectively, using *uclust* against the Greengenes (v13.8) reference database (DeSantis et al., 2006; McDonald et al., 2012) and Ribosomal Database Project (RDP) *classifier* against the UNITE (v12.11) reference database (Abarenkov et al., 2010; Wang et al., 2007).

Variation in microbial community composition among treatments was assessed. Briefly, to describe the biodiversity and taxonomic summary in bacterial and fungal community, we calculated the alpha diversity based on the OTU abundance table, using PD whole tree (for bacteria only), chao1, observed species, and Shannon and Simpson indices for both bacteria and fungi. The chao1 and observed species metrics were used to plot alpha rarefaction curves. The taxonomic summary graphs were produced at different levels to visualize microbial taxonomic summaries of the interaction between Fe application and community composition. To compare the composition of different communities, we assessed beta diversity by using weighted and unweighted UniFrac distance matrices (for bacteria) (Lozupone and Knight, 2005), and Bray-Curtis (for fungi) (Beals, 1984). The non-parametric multivariate statistical analysis methods, Multi-Response Permutation Procedures (MRPP), Adonis and Analysis of Similarity (ANOSIM) were conducted to statistically compare the difference of beta diversity among treatments. The ordination patterns and clustering analyses were visualized in 3D-plots in EMPeror (Vázquez-Baeza et al., 2013) using principal coordinates analysis (PCoA). Indicator species analysis (ISA) was used to identify OTUs that were significantly (indicator value > 70 and p-value < 0.05) correlated with Fe application. Non-metric multidimensional scaling (NMS) and ISA were performed using the PC-ORD software version 6.0 (MjM Software, Gleneden Beach, OR, USA). All statistical differences at the phylum level were performed by ANOVA, and means were separated by the Tukey HSD comparison test at $p \leq 0.05$, by using JMP package (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Foliar Fe fertilization changed the Fe status in potato tuber and plant growth

Foliar Fe fertilization resulted in an increasing trend in Fe concentration in the potato shoot tip and tuber (Fig. 1). There were also significant differences in the shoot length and the dry weight of total roots resulting from Fe fertilization; however, there were no significant differences found in other measured quality parameters including total plant biomass and fresh tuber yield. The dry weight of roots was significantly greater in the low Fe (deionized water) treated plants compared to Fe-treated. Shoot length, in contrast, was greater in high and medium Fe compared to low Fe treated plants (Fig. 2). The ratio of shoot length/root dry weight was significantly different across the three treatments ($p = 0.0035$) with the lowest value in the deionized water treatment. These results, importantly, revealed the application of Fe fertilization changed the growth of potato plants, and as expected, the Fe status in the potato tuber. These results whereby less carbon is allocated to roots relative to shoots are consistent with release from Fe limitation. Fe sufficient plants would be expected to allocate fewer resources to roots/rhizosphere relative to shoots whereas Fe limited plants would allocate more carbon to roots to increase Fe uptake potential. Furthermore, Fe stimulation of growth compared to control treatments support the expect result of Fe deficient soil conditions (Hermans et al., 2006; Trubat et al., 2006).

3.2. The change of plant Fe status affected the composition of rhizosphere fungal community

Following removal of low-quality sequences, a total of 1,258,705 high quality reads of the internal transcribed spacer (ITS) region sequence of rRNA gene was obtained from Illumina MiSeq sequencing. After sequence assembly, clean-up, and clustering in QIIME 40,281 OTUs (observations) were identified across all treatments. The mean and median counts per sample were 104,892 and 104,137, respectively. Since the sample size variation can affect the diversity metrics, the sampling depth threshold was utilized for further analyses by taking a random subsample of 70,500 sequences.

Chao1, observed species, Shannon, and Simpson indices of the original OTU table were used for analysis of alpha diversity and evenness of the fungal community. The Tukey HSD-test result failed to detect significant differences among the fertilization treatments, indicating that the OTU-based richness and evenness of the fungal community were not significantly different among Fe applications across Chao1, observed species Shannon and Simpson indices (Supplementary Table 2). The rarefaction curves with 94.6% coverage indicated the presence of saturation but also did not show differences for fungal richness, and indicated no shifts in fungal OTU richness in response to Fe amendment.

There were different fungal OTUs belonging to eight phyla in all Fe

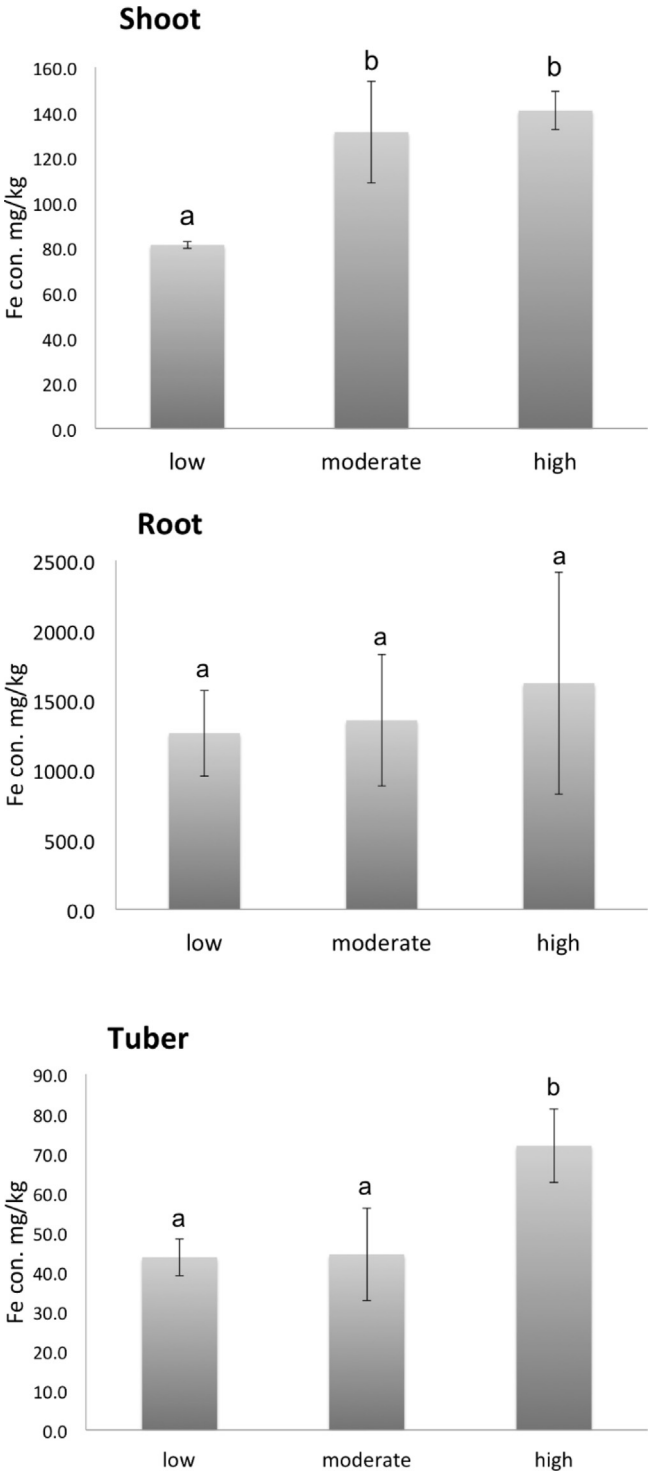

Fig. 1. Effect of different amount of foliar iron (Fe) application on the Fe concentration of shoots, roots and tubers per Kg dry weight in an Andean potato landrace (low: deionized water; moderate: 200 mg/L FeEDDHA; high: 600 mg/L FeEDDHA). Different letters above the bars signify statistically significant differences between plant Fe levels ($p < 0.05$).

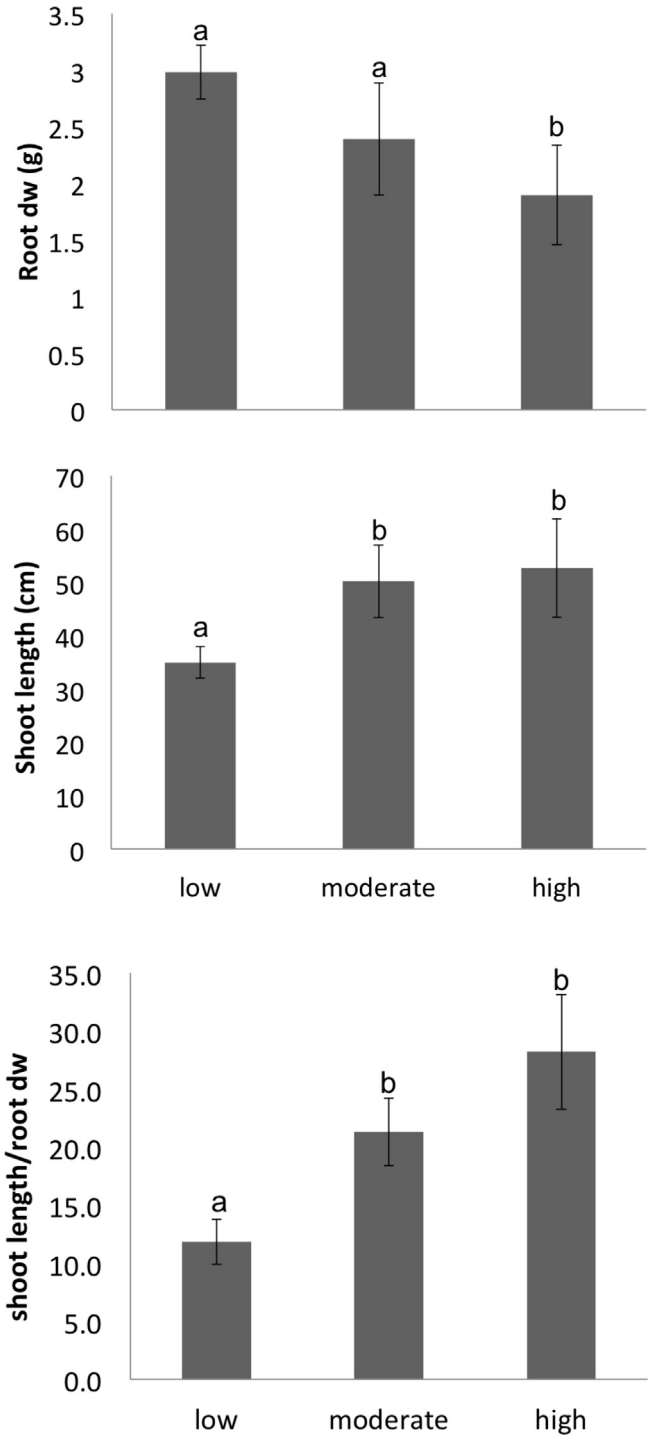

Fig. 2. Effect of different amounts of foliar iron (Fe) application on the dry weight of roots, shoot length and the ratio of shoot length/root dry weight of an Andean potato landrace (low: deionized water; moderate: 200 mg/L FeEDDHA; high: 600 mg/L FeEDDHA). Different letters above the bars signify statistically significant differences between plant Fe levels ($p < 0.05$).

treatments. Taxonomic summaries showed that *Ascomycota* and *Glomeromycota* were the most abundant fungal phyla, amounting to 30.9 and 16.3% of the total number of sequences, respectively. A proportion of sequences could not be assigned (~25.5%) to known taxa for the fungal data. These sequences may represent as yet unexplored microorganisms, or chimeras or other artifacts introduced during the PCR or sequencing process (Kröber et al., 2009; Liu et al., 2015; Tai et al., 2015). At finer taxonomic scales, the most abundant fungal classes included the *Eurotiomycetes*, *Sordariomycetes* and *Dothideomycetes* in the phylum *Ascomycota*, *Glomeromycetes* in the phylum *Glomeromycota*, *Agaricomycetes* and *Tremellomycetes* in the phylum *Basidiomycota* and Incertae sedis in the phylum *Zygomycota* (Fig. 3).

ANOVA was used to compare the difference of each phylum across Fe treatment; however, no significant effect was detected. Multivariate data analyses at finer taxonomic levels in contrast demonstrated clear

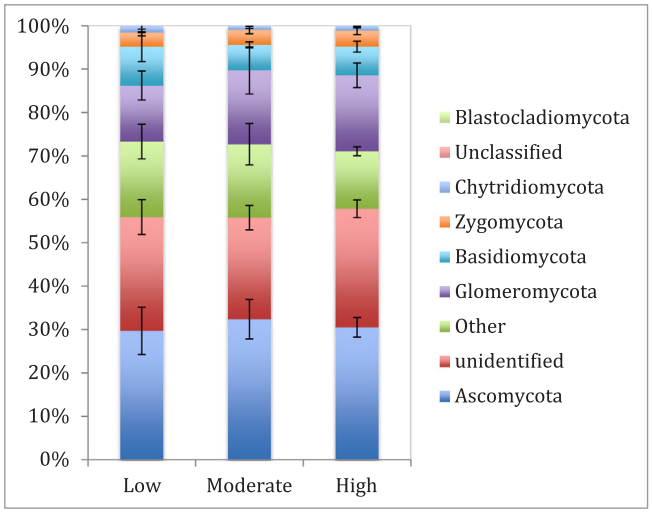

Fig. 3. Phylum-level taxonomic summaries of fungal communities in an Andean potato landrace under different foliar Fe applications. 'Unassigned' and less abundant taxa were grouped in 'Other'. Taxa are ordered from bottom to top and sorted as per decreasing abundance. No statistically significant differences were detected, but not the relatively large contribution from *Glomeromycota*. The larger values in the moderate and high Fe levels were not significantly different, but may help to explain differences at finer taxonomic levels (Table 2).

Table 1
Multivariate data analysis for differences in the composition of rhizosphere fungal community (beta diversity).

	Treatment	F. Model	R^2	*p*-value
Adonis	Fe fertilization	1.0781	0.09731	0.246
		R statistic	*p*-value	Number of permutations
ANOSIM	Fe fertilization	0.2106	0.015*	999
		A value	*p*-value	Number of permutations
MRPP	Fe fertilization	0.02009	0.013*	999

* Significant at the 0.05 probability level.

distinctions of the fungal communities in response to Fe application. The composition of fungal communities was examined in relative abundance at the genus level, and compared using Adonis, ANOSIM, and MRPP on the Bray-Curtis distances. The results of ANOSIM and MRPP showed statistically significant differences ($p < 0.05$) in the beta diversity of different Fe applications (Table 1). There were distinct fungal communities associated with the different amounts of Fe fertilization.

Ordination methods (PCoA and NMS) based on Bray-Curtis dissimilarity matrix, within the moderate Fe fertilization treatment clustered together and separated from samples from low and high Fe fertilization treatments (Fig. 4). Overall, these results indicated that the amount of foliar Fe applied affected the composition of fungal communities in the potato rhizosphere.

For the fungal genera that were most correlated with the effect of Fe fertilization, OTU with relative abundance above 0.1% and taxonomically assigned at the genus level were selected and compared among treatments using the Monte Carlo procedures and ISA (Table 2). Four genera (belonging to *Glomeromycota* and *Basidiomycota*) had significantly different relative abundances ($p < 0.05$) among treatments. Three genera in the classes *Glomeromycetes* and *Microbotryomycetes* had significantly greater abundance in the high Fe fertilizer relative to low and un-treated samples. The genus, *Claroideoglomus*, in the class *Glomeromycetes* had greater abundance in the moderate Fe fertilization. Pearson and Kendall Correlations with Axis 1 from NMS analysis showed two genera in the phylum Ascomycota negatively responded to

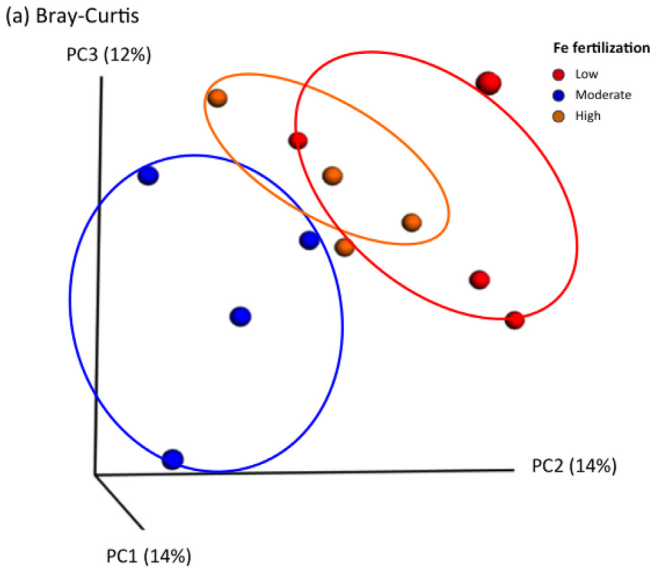

Fig. 4. PCoA plot showing differences between Fe status and fungal community composition. Percentages on each axis denote the amount of variability associated with each axis. Multivariate methods detected significant differences based on level of Fe amendment.

the moderate fertilization application, and *Aspergillus* showed positive response at the low Fe level in soil. Several genera, especially in the high relative to the low Fe treatments were associated with changes in Fe cycling in the potato rhizosphere.

3.3. The effect of plant Fe status on the composition of rhizosphere bacterial community was not significant

Illumina MiSeq sequencing analysis of the 16S rRNA gene was performed to characterize the bacterial communities in the rhizosphere. A total of 1,218,102 high-quality sequences (counts) were obtained after sequence assembly, clean-up, and clustering in QIIME. There were 60,157 OTUs (observations) identified from these sequences across all treatments. The mean and median counts per sample were 101,284 and 101,508 respectively. The sampling depth threshold was utilized for further analyses by taking a random subsample of 37,000.

Chao1, observed species, PD_whole_tree metrics of the original OTU table were used for analysis of alpha diversity of the bacterial community. The Tukey HSD-test indicated that the OTU-based richness and evenness of the bacterial community was not significantly different ($p > 0.05$) among the three Fe fertilization treatments (Supplementary Table 3). Estimates indicate 92.6% coverage of the communities. A higher sequence threshold of 90,000 K showed 99% coverage, but to enable inclusion of all samples and to reduce the impacts of rare and potentially low quality sequences, a respectable coverage of 92.6% was accepted. Therefore, the richness and evenness of the bacterial community might be similar in the rhizosphere with three different amounts of Fe fertilization.

The bacterial communities were dominated by 14 bacterial phyla. The bacterial community was dominated by phyla *Proteobacteria* (~26.7%), followed by the *Acidobacteria* (20.0%) and *Actinobacteria* (~10.7%). The family with greater relative abundance of sequences was the *Sphingomonadaceae* (Phylum: *Proteobacteria*) and *Gaiellaceae* (Phylum: *Actinobacteria*) (Supplementary Fig. 2). ANOVA was used to compare the difference of each phylum among treatments and showed significant difference in the phylum *Nitrospirae* ($p = 0.012$) and thus indicating that alteration in Fe status has the potential to reverberate into the cycling of other nutrients such as nitrogen.

When bacterial communities were examined based on the phylogenetic composition in relative abundance, community composition

Table 2
Fungal genera with a greater relative abundance associated with Fe fertilization effect based on Indicator Species Analysis (p-value < 0.05) and Non-metric Multidimensional Scaling analysis (r^2 > 0.49).

Maxg†	OTU identifier	Phylum	Class	Order	Family	Genus	r	Obs IV	P value	L(%)†	M(%)†	H(%)†
H	OTU343	Glomeromycota	Glomeromycetes	Diversisporales	Gigasporaceae	other	0.547	53.4	0.0166*	2.21%	1.45%	4.19%
M	OTU349	Glomeromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	-0.68	56.2	0.0344*	0.03%	0.07%	0.02%
H	OTU345	Glomeromycota	Glomeromycetes	Diversisporales	Gigasporaceae	Scutellospora	0.592	55.3	0.0394*	0.12%	0.06%	0.22%
H	OTU294	Basidiomycota	Microbotryomycetes	other	other	other	0.461	73.6	0.0394*	0.01%	0.02%	0.10%
M	OTU157	Ascomycota	Sordariomycetes	Hypocreales;	Incertae_sedis	Emericellopsis	-0.718	57	0.0714	0.68%	2.24%	1.00%
M	OTU183	Ascomycota	Sordariomycetes	Microascales	Ceratocystidaceae	Thielaviopsis	-0.737	57.8	0.0722	0.24%	0.75%	0.31%
L	OTU65	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	0.868	52.3	0.1056	0.05%	0.01%	0.04%

† L: low level of Fe fertilization; M: moderate level of Fe fertilization; H: high level of Fe fertilization.

* Significant at the 0.05 probability level.

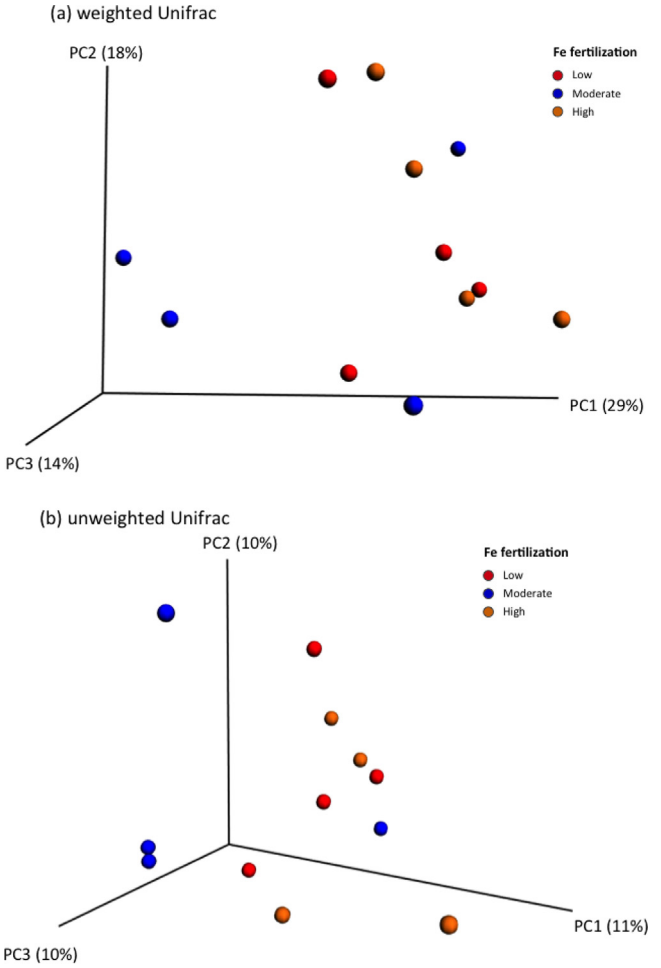

Fig. 5. PCoA plot showing the spread of (a) un-weighted and (b) weighted bacterial community structure using Unifrac. Percentages on each axis denote the amount of variability associated with each axis. There were no significant differences in bacterial communities across levels of fertilization.

was compared by multivariate data analyses using Adonis, ANOSIM, and MRPP based on weighted and unweighted Unifrac distances. The results showed no statistically significant differences (p > 0.05) in the beta diversity resulting from Fe application (Supplementary Table 4). Bacterial community composition, similarly, did not shift in response to the Fe status in the plants in this study (Fig. 5).

4. Discussion

The effect of foliar application of Fe amendment on the composition of rhizosphere microbial communities of an Andean potato landrace were studied. It was reasoned that if Fe was an important mediator of plant-microbial interactions then changing its status in the plant would affect the root-rhizosphere microbial community. Fe application to the leaf surface would reduce plant Fe deficiency, which would then have effects on plant-microbial associations in the rhizosphere, such as those related to the occurrence of mycorrhizal fungi. Though fungal community change was consistent with the hypothesis, there was less evidence that bacterial communities were altered due to Fe application. The results indicated, furthermore that Fe fertilization supported greater (2-3X) relative abundance of the Gigasporaceae, a family widely described as forming arbuscular mycorrhizae with plants (Johnson et al., 2013; Parniske, 2008). *Ascomycota* on the other hand were more abundant as a result of both low and high Fe treatments relative to mid-level treatments. These results support the idea that Fe in some way regulates rhizosphere fungal communities (Lehmann and Rillig, 2015), perhaps through a change in the flow of carbon to roots. These results thus support the findings of other studies, but from a different experimental perspective. Rather than inoculating with mycorrhizal fungi (Ercoli et al., 2017; Nunez et al., 2016), it was opted to change the plant demand and follow those effects to the belowground biota. Still, it is not possible to completely determine from this study whether the changes in fungal communities and mycorrhizal fungi resulted from a change in a positive plant-fungal feedbacks (mutualism). It is possible that foliar application helped reduce Fe limitation that then allowed for greater mycorrhizal infection and thus capture of other limiting nutrients. Release of competition for Fe between plant and fungus, also cannot be ruled out as a regulator of fungal community change (Mimmo et al., 2014). The fact that changes in Gigasporaceae, however, were one of the single largest alterations to fungal communities does support the role of mycorrhizae as some mediator of the interaction. Further research into the role of plant-microbial interactions in plant Fe acquisition, using isotopic methods can help determine if mycorrhzal fungi transport Fe to plant roots.

4.1. Fungal but not bacterial communities impacted by foliar Fe application

The present study showed that the rhizosphere fungal community structure was distinct under different levels of Fe fertilizer application, while no significant difference in the bacterial community structure was detected. To date, more attention has been given to analyzing the effects of specific bacterial inoculants on plant Fe nutrition uptake while few fungi, in this regard, have been tested (Bona et al., 2016; Nagata et al., 2013; Pii et al., 2016, 2015; Zhang et al., 2009; Zhou et al., 2016). It should be noted that there have been numerous studies that have also measured Fe following inoculation with mycorrhizal fungi, and a few that have used radiolabeled Fe to support the idea that at least some plants acquire Fe via mycorrhizal fungi (Lehmann and Rillig, 2015), but most studies have observed changes in micronutrients such as Fe along with other nutrients such as P. Mycorrhizal inoculation

often results in increased values of many nutrients, but it remains a question whether all of the nutrient increases derive directly via fungal hyphae, or through a more indirect route that is the result of greater root and plant growth. The current study does not prove that Fe uptake is supported by mycorrhizal fungi either, but it supports the general consensus that Fe can be a regulator of plant-microbial interactions, such as mycorrhizae.

Indicator Species Analysis was conducted to discover fungal groups that may be significant contributors in the plant-fungal interaction affecting Fe acquisition. The result showed that genera in the phyla *Glomeromycota*, *Ascomycota* and *Basidiomycota* were relevant groups associated with differences in plant Fe status. Arbuscular mycorrhizal fungi (AMF) belong to the phylum *Glomeromycota*, are well known for their involvement in plant P acquisition (Ouahmane et al., 2007). Soil Fe is strongly linked to P availability, primarily related to their tendency to form mineral chemical complexes that are often highly insoluble, iron phosphates (Borggaard et al., 1990). It is thus straightforward to see that P mobilizing mechanisms may be inter-related to plant Fe availability. More research into the linkage between P and Fe and other similar cation and anion nutrient pools could aid in the understanding of the widespread occurrence and diversity of soil mycorrhizae.

In addition to Fe and P, AMF are often associated with mobilization of insoluble nutrients such as, Zn and Cu complexes (Lehmann and Rillig, 2015). In particular, *Glomus* (order *Glomerales*), *Acaulospora* (order *Diversisporales*) and *Scutellospora* (family *Gigasporaceae*), appear to be able to produce siderophores and are thought to increase the extent of Fe absorption in plants such as *Pennisetum glaucum* (Pearl millet) and *Sorghum bicolor* (sorghum) growing in Fe^{3+} contaminated soil (Mishra et al., 2016). In a heavy metal contaminated sediment the class *Microbotryomycetes* in phylum *Basidiomycota* was observed to be resistant to high levels of Fe and other heavy metals (Pb, Mn, Cd, Cu and Zn) (Abdel-Azeem et al., 2015). An Fe-enriched fungus was similarly isolated from a natural environment belonging to this class (Zhang et al., 2015). These results relate well to many of the OTUs in this class which had much greater relative abundance due to high Fe applications to leaf surfaces. Thus, it may be that the higher levels of Fe translated into greater Fe in the rhizosphere, which would also support the growth of fungi such as *Microbotryomycetes*. Though this result is not necessarily consistent with our expectations, it is a relevant possibility that high Fe in the rhizosphere favored certain types of rhizosphere microbes. In the least, the results speak to the importance of Fe in plant-rhizosphere interactions.

In the phylum *Ascomycota*, *Trichoderma* spp. (order *Hypocreales*) are well-studied filamentous fungi sometimes used in agriculture as biofertilizers. This is because they secrete various metabolites such as organic acids, siderophores, and enzymes (Rudresh et al., 2005; Vinale et al., 2008). For example, *T. asperellum* T34 could increase Fe concentration in shoots of cucumber plants grown in a soil under low Fe availability (de Santiago et al., 2013). The inoculation with *T. asperellum* T6 to sterile soil stimulated the activity of root Fe^{3+}-chelate reductase and soluble Fe^{2+} in cucumber tissues, which mobilized insoluble Fe to provide mineral nutrition for plant growth (Zhao et al., 2014). Furthermore, *Aspergillus* spp. and *Penicillium* spp., in the order of *Eurotiales*, can excrete organic acids, which can solubilize solid-phase potassium and into plant available forms taken up by roots (Teotia et al., 2016). Consistent with the reported role of *Eurotiales*, these rhizosphere taxa were sensitive to changes in Fe applied to leaf surfaces in the current study.

Although the shifts of a microbial community indicate that rhizosphere microorganisms are sensitive to Fe treatment, and therefore may interact with potato roots to alter or aid Fe cycling in favor of plant use, the mechanisms involved in this plant-microbial interaction have not been definitively demonstrated. Though, it is well established that rhizosphere microbes play an important role in plant productivity by modulating macronutrients including phosphorus and nitrogen cycling in the soil, the ecological complexity of soils often make it difficult to come to firm conclusions about the role of specific microbes. Linkage between N and P nutrient cycling and plant-microbial interaction in the form of plant growth-promoting rhizobacteria (PGPR) interacting with AMF increase the complexity of understanding mechanisms supporting the availability of nutrients through organic acids and siderophores. As for P nutrient cycling, often attributied to activities of AMF, the bacteria *Bacillus, Pseudomonas* and *Burkolderia* and fungi *Aspergillus* and *Penicillium* have been shown to be species involved in a range of processes that affect P mineralization and solubilization in the soil and thus influence subsequent availability of P to plant roots (Clarholm et al., 2015; Giles et al., 2012; Patel et al., 2010; Richardson et al., 2011; Richardson and Simpson, 2011). Data in the literature support the central role that soil microbes can play in Fe, P, and other nutrient acquisition by many types of plants.

A variety of studies have described changes in soil and rhizosphere microbiomes in response to direct fertilization of highly limiting nutrients such as N (Cassman et al., 2016; Fanin et al., 2015; Jangid et al., 2008; Tian et al., 2015; Zhen et al., 2014). Though the approach to fertilization is different, and a different nutrient involved, the large number of N and P based studies can help to understand the potential role that Fe may play in structuring rhizosphere microbial communities in the rhizosphere. Nitrogen and P fertilization have been shown to shape rhizosphere microbial community composition across soil types, crop species, and location (Cassman et al., 2016; Chávez-Romero et al., 2016; Liu et al., 2011; Paungfoo-Lonhienne et al., 2015; Peyret-Guzzon et al., 2016). For instance, Paungfoo-Lonhienne et al. (2015) and Zhu et al. (2016) reported that different doses of N strongly influence the composition of fungal communities in sugarcane soil and rhizosphere and bacterial communities in the maize rhizosphere. The effects were not restricted to AMF but span a wide range of fungal taxa thought to influence plant health. Phosphorus fertilization (Su et al., 2015) affected both the species evenness of key genera as well as microbial functional structures in rice soil. The authors deduced that the shift in the microbial community might accelerate nutrient turnover, which in turn impacted rice productivity. These results do not speak directly to the effects of Fe, in which there is less information, but they do speak to the broader concept of how nutrients, such as Fe, play a major role in plant-microbial interactions, and thus have great potential for technological development to support more sustainable agricultural practices.

To the best of our knowledge, the effect of Fe on plant-microbial interaction in soils have not been directly tested using a foliar application that was expected to resonate from the aboveground leaves to the root and its interaction with the surrounding rhizosphere community. Though the approach is different than many other studies, it supports previous research on the importance of plant-microbial interactions in the cycling of iron. Fe played a role in structuring fungal community composition in the rhizosphere. Those changes fit into a paradigm whereby root C flow changes fungal, and especially mycorrhizal communities. However, the release of root exudates such as organic acids that can improve plant nutrient acquisition and influence the diversity and composition of rhizosphere microbial communities (Bashir et al., 2016; Haichar et al., 2008; Huang et al., 2014; Shi et al., 2013) could have also responded to changing plant demand and Fe concentrations in the root and rhizosphere. The mass in the roots and shoots (Fig. 2) imply that plant nutrient demand reduced and thus lowered C flow to roots as a result of foliar Fe amendment. Hence, this supports that Fe was a limiting nutrient. Whether this release of Fe as limiting nutrient increased the flow of C to support relatively greater mycorrhizal fungi (Gigasporaceae) and thus acquisition of other nutrients is open to interpretation. They do, however, fit into a hypothesis that Fe directly or indirectly influences plant-fungal feedbacks. Further experiments that isolate the microbial role in Fe transfer to plants will help to discern mechanisms associated with Fe related plant-microbial interactions.

Yang and Crowley (2000) reported that the Fe nutritional status in

barley impacted the bacterial communities in the rhizosphere. And Jin et al. (2010) showed that Fe-stressed red clover secreted higher concentrations of phenolics and altered the composition of siderophore-secreting microbes in the rhizosphere. In addition, considerable work has shown that bacteria and fungal siderophores play an important role in governing plant acquisition of nutrients such as Fe and phosphorus. Because bacterial community composition has been shown to be highly sensitive to environmental factors such as pH they may also interact to play a role in bacterial response (Michelsen et al., 2014; Yu et al., 2015), and/or overshadow impacts of Fe. Results from the current study did not assess changes in microbial function, however, the lack of statistically significant change in bacterial communities in response to foliar Fe amendment should not be inferred as a rebuke to the role of bacteria. This primarily because many of the same multivariate patterns of change across treatments were similar to those of fungal communities.

In the present study, potato plants that suffer from Fe deficiency stress under low Fe treatment, might exude organic and phenolic molecules to the rhizosphere, which in turn selectively favor microorganisms that can also produce organic acids or siderophores to increase Fe solubility in the soil. On the other hand, plant–microbe competition might exist in the soil because both microbes and plants can have high demand to meet specific nutrient requirements (Colombo et al., 2014; Marschner et al., 2011; Mimmo et al., 2014). Greater relative abundance of fungal groups may thus be a side of effect of this competition for Fe with potato plants in the low Fe treatment leading to lower Fe concentration in the tubers. Under Fe fertilized treatments, potato plants might obtain enough Fe for growth, then the growth of microorganisms with high Fe demand would increase, and shift the composition of microbial community from the low Fe treatment. Though relevant possibilities, the result support the hypothesis of overall change and provide identification of indicator species to provide clues to specific groups and possible functions that exert beneficial effects on plant productivity and nutrient fortification. Further verification of these results may provide candidates useful for plant growth promotion under a range of bioavailable iron conditions.

5. Conclusion

To our knowledge, this is the first study to observe rhizosphere fungal community composition changes in response to foliar Fe amendment. The foliar Fe application approach reduced the complexity of effect on the soil microbial community, by directly changing the Fe status of potato plants. These changes significantly affected the rhizosphere fungal community composition suggesting the occurrence of plant-fungal interactions in the rhizosphere related to Fe availability. This finding is important in that it extends the potential role that fungi play in nutrient uptake beyond current foci on N and P. In addition to community-level links, specific changes in the phylum *Glomeromycota* *Ascomycota* and *Basidiomycota* related to Fe were consistent with changes that have been shown to occur as a result of N and P fertilization. The next step of this work would be to determine the movement of isotope labeled Fe from mycorrhizal fungi into the plant root, and attempt to determine the relative importance that roots alone, mycorrhizae and bacteria play in plant Fe status.

Acknowledgements

The authors acknowledge the laboratory and statistical support provided by Dr. Jude Moon. Funding was provided by the Bill and Melinda Gates Foundation, Fralin Life Sciences Institute at Virginia Tech, Institute for Critical Technology and Applied Sciences at Virginia Tech, Fralin Life Sciences Institute at Virginia Tech, and the Department of Horticulture at Virginia Tech.

Data availability

Sequencing data were uploaded as project PRJNA454291. ITS Runs: SRR7084448 - SRR7084459 and 16S Runs: SRR7084474 - SRR7084485.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.apsoil.2018.08.006.

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