Exploring candidate genes and rhizosphere microbiome in relation to iron cycling in Andean potatoes

Hua Xiao

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Horticulture

Mark A. Williams, Co-Chair

Richard E. Veilleux, Co-Chair

Bingyu Zhao

Boris A. Vinatzer

Chenming Zhang

April 10th, 2017

Blacksburg, VA

Keywords: *Solanum tuberosum*, Andean potato, Iron deficiency, Fe fortification, Micronutrients, Candidate genes, Iron cycling, Rhizosphere, Microbes.

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Abstract (academic)

Fe biofortification of potato is a promising strategy to prevent Fe deficiency worldwide either through traditional breeding or biotechnological approaches. These approaches require the identification of candidate genes to uptake, transport and store Fe in potato tubers. We employed multiple approaches including SNP genotyping, QTL analysis, identifying genes orthologous to Arabidopsis ferrome, yeast complementation assay and genetic transformation to avoid the limitation from a single approach. We revealed several candidate genes potentially associated with potato plant Fe acquisition, *PGSC0003DMG400024976* (metal transporter), *PGSC0003DMG400013297* (oligopeptide transporter), *PGSC0003DMG400021155* (*IRT1*) and *IRTunannotated* (an ortholog to the *IRT* gene that is not annotated in the potato genome). The microorganisms in the rhizosphere react intensely with the various metabolites released by plant roots in a variety of ways such as positive, negative, and neutral. These interactions can influence the uptake and transport of micronutrients in the plant roots. Therefore, the contribution of soil microorganisms in the rhizosphere to improve Fe supply of plants may play a key role in Fe biofortification, especially under real world field-based soil scenarios. We thus investigated rhizosphere microbial community diversity in Andean potato landraces to understand the role of plant-microbial interaction in potato Fe nutrient cycling. From the analysis of the high-throughput Illumina sequences of 16S and ITS region of ribosomal

RNA gene, we found that both potato landraces with low and high Fe content in tubers and a landrace on which low or high Fe content fertilizer was applied to the leaf surface had large impacts on the rhizosphere fungal community composition. Indicator species analysis (ISA) indicated that Operational Taxonomic Units (OTUs) contributing most to these impacts were closely related to *Eurotiomycetes* and *Leotiomycetes* in the phylum *Ascomycota*, *Glomeromycetes* in the phylum *Glomeromycota* and *Microbotryomycetes* in the phylum *Basidiomycota*. Lots of species from these groups have been shown to regulate plant mineral nutrient cycling. Our research revealed potential candidate genes and fungal taxa involved in the potato plant Fe nutrient dynamics, which provides new insights into crop management and breeding strategies for sustainable Fe fortification in agricultural production.

Exploring candidate genes and rhizosphere microbiome in relation to iron cycling in Andean potatoes

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Abstract (public)

Sustainably enriching Fe nutrition and its bioavailability in the potato is a promising strategy to prevent Fe deficiency worldwide either through traditional breeding or biotechnological approaches. All of these approaches require the identification of candidate genes to uptake, transport and store Fe in potato tubers. In this study, we coupled plant molecular methods with analysis of soil microbial community in the rhizosphere (the region of soil within immediate vicinity of plant roots, and a hotspot of this plant-microbial interplay) to uncover relationships among Fe nutritional status in potato, potato genotype and soil microbes. We identified a number of genes that likely control the amount of Fe content in potato using multiple approaches. After functional analysis in yeasts and potato plants, we revealed several elite candidate genes potentially associated with potato plant Fe acquisition, *PGSC0003DMG400024976* (Metal Transporter), *PGSC0003DMG400013297* (Oligopeptide Transporter), *PGSC0003DMG400021155* (Iron-Regulated Transporter 1, IRT1) and *IRTunannotated* (an ortholog to the *IRT* gene that is not annotated in the potato genome). The microorganisms in the rhizosphere react intensely with the various metabolites released by plant roots in a variety of ways such as positive, negative, and neutral. These interactions can influence the uptake and transport of micronutrients in the plant roots.

Therefore, the contribution of soil microorganisms in the rhizosphere to improve Fe supply of plants may play a key role in enriching Fe nutrition, especially under real world field-based scenarios, e.g., high-pH and calcareous soils that occupy one third of agriculture lands limit the Fe bioavailability to crops. We investigated rhizosphere microbial community diversity in Andean potato landraces to understand the role of plant-microbial interaction in potato Fe nutrient cycling using high-throughput Illumina sequencing method. We found that both potato landraces with low and high Fe content in tubers and a landrace on which low or high Fe content fertilizer was applied to the leaf surface had large impacts on the rhizosphere fungal community composition. These impacts were closely related to *Eurotiomycetes* and *Leotiomycetes* in the phylum *Ascomycota*, *Glomeromycetes* in the phylum *Glomeromycota* and *Microbotryomycetes* in the phylum *Basidiomycota*. Lots of species from these groups have been shown to regulate plant mineral nutrient cycling. Our research revealed potential candidate genes and fungal taxa involved in the potato plant Fe nutrient dynamics, which provides new insights into crop management and breeding strategies for sustainable Fe improvement in agricultural production.

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Chapter 1 General introduction and research objectives

1.1 Fe micronutrient required by humans

Micronutrient malnutrition is a serious problem for humans. It is estimated that more than three billion people experience micronutrient deficiency, and iron (Fe) deficiency is one of the most prevalent nutrient deficiencies worldwide, affecting about two billion people and causing almost a million deaths a year. Over 30% of the world's population are anemic that is caused by several factors, but Fe deficiency is the most common and widespread factor (Bouis 1995; WHO 2017). Pregnant women and young children are most commonly and severely affected because of the high Fe demands of pregnancy and infant growth. For instance, in developing countries 50% of pregnant women and 40% of children below the age of five are anemic (WHO 2017). In the USA, 9-11 % of adolescent girls and women of childbearing age and 9% of toddlers were found to be Fe deficient (Gupta et al. 2016; Looker et al. 1997). The Fe deficiency in the early stage of human growth irreversibly affects development of brain structure and function, which cause impaired cognitive development in children, a weakened immune system, and increased risk of morbidity (Black 2003; Beard 2008).

Therefore, the big picture of this dissertation is to explore Fe fortification to ameliorate problems associated with Fe deficiency through plant and microbial approaches. Potato (*Solanum tuberosum* L.) was used as the plant material. The major objective of the study is to determine the genetic factors in potato plant contributing to genetic engineering approach, and to understand plant-microbial interaction underlying Fe cycling in potato plants contributing to microbial management approach.

1.2 Strategies for Fe fortification

1.2.1 Food Fortification for humans

Micronutrient malnutrition in human is closely related to the dietary intake. More than half of Fe-deficiency anemia could be avoided by increasing the amount of Fe in the diet. Thus, Fe supplementation and food fortification with Fe strategies have been proposed to alleviate mineral deficiencies firstly since they are most cost-effective strategies compare with others due to political and technical constraints (Horton 2006). Food Fortification Initiative (http://www.ffinetwork.org/) has created a program involved in Fe fortification of wheat flour in several developing countries to combat deficiencies of Fe and certain B vitamins. It resulted in increased usage from 18% in 2004 to 27% in 2007 and 540 million people avoided Fe deficiency (CDCP 2008; FFI 2013). Barkley et al. (2015) recently provided good evidence that anemia prevalence can be reduced in countries fortifying wheat flour with Fe. However, this strategy is still challenging because Fe fortification of foods is technically difficult. For example, exogenous Fe fortification added during food processing can change the taste and color of food; the antinutrients such as phytic acid, fiber or other constituents that are rich in the plant food are potent inhibitor of dietary Fe absorption by chelating Fe (Mendoza 2002; Hunt 2002). A preferred Fe compound because of its higher bioavailability, ferric sodium EDTA (NaFeEDTA), can overcome the inhibition of antinutrients on Fe absorption, but it is the most expensive solution that is also fraught with losses of Fe due to leaching (Murgia et al. 2012; Frossard et al. 2000).

1.2.2 Fe biofortification of crops

Alternatively, the human diet, especially in the developing countries, mainly relies on

agricultural plant production such as rice, wheat, potato and soybean, as meat, even though representing the most concentrated source of some vitamins and minerals, is limited and not as healthful as plant products. However, some of these crops contain low levels of Fe and high levels of antinutritional compounds such as phytate (Gómez-Galera et al. 2010). Thus, improving the content and bioavailability of Fe in commonly consumed plants is a rational approach to resolving Fe deficiency in humans.

Among all essential micronutrients involving plant growth, Fe is a key nutrient for biomass production and plant product quality as well (Marschner 2012). It functions in various important cellular processes, including electron transport in photosynthesis and cellular respiration, chlorophyll biosynthesis and chloroplast development (Briat et al. 2015; Kobayashi and Nishizawa 2012; Marschner 2012). Fe is one of the four most abundant soil elements with an average concentration ranging from 20 to 40 g/kg (Cornell and Schwertmann 2003), but its bioavailability to plants is often limited especially in high-pH and calcareous soils that occupy one third of agriculture lands. In cultivated soils that possess a pH range 5.0 to 8.5 suitable for most plant growth, Fe is mainly present in insoluble complexes in the forms of hydroxides, oxyhydroxides and oxides so that the soluble Fe remains low (Marschner 2012; Colombo et al. 2014). It has been estimated that the total concentration of soluble Fe in such soil solution is about $10^7 \sim 10^{-10}$ M which is far less than requirements for optimal growth of plants (Kraemer et al. 2006). Therefore, as an alternative sustainable and economical agricultural approach, Fe biofortification, the process of enriching Fe nutrition and its bioavailability in the edible parts of crops, has been of scientific interest for many years.

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Agronomic practice

Various strategies of Fe biofortification based on management of Fe uptake of crops and Fe bioavailability in soil have been reported widely, including agronomic practices, plant breeding and genetic engineering. The application of Fe fertilizer of inorganic Fe compounds, synthetic Fe-chelates, and natural Fe-complexes, and intercropping of grass with non-grass plants are the most commonly used agronomic practices to correct plant Fe deficiency (Saini et al. 2016; Zuo et al. 2000; Zuo and Zhang 2009; Inal et al. 2007). Nevertheless, Fe^{2+} in the inorganic Fe fertilizer such as $FeSO_4$, can easily bind to soil particles and be converted to Fe^{3+} that has low mobility and is unavailable to plants. The Fe-complexes fertilizer is expensive and can increase the cost of food since it needs to be applied regularly. Additionally, because of the limited mobility of Fe in the phloem (Marschner 2012), fertilization does not always adequately increase Fe concentrations in edible parts of the plant (Inal et al. 2007; White and Broadley 2009). Thus Fe fortification through agronomic management has value under certain situations, but may not be the best way of increasing plant Fe for maximum yield and human dietary needs.

Plant breeding

Plant breeding and genetic engineering tend to be more economically and environmentally sustainable since they offer economically and environmentally sustainable alternatives to develop crop cultivars with higher mineral content (Tester and Langridge 2010; Mannar and Sankar 2004; Genc et al. 2005). Both methods seek to modify or insert genes that favor efficient accumulation of bioavailable Fe in crops, across many types of plants including wild, landraces, and cultivated species. For example, the level of Fe in edible tissues ranges from 6 to 24 mg.kg^{-1} in rice (*Oryza*

sativa), 10 to 160 mg.kg^{-1} in maize (*Zea mays*), 15 to 94 mg.kg^{-1} in wheat (*Triticum spp.*), 9 to 176 mg.kg^{-1} in yam (*Dioscorea alata*), 34 to 90 mg.kg^{-1} in common beans (*Phaseolus vulgaris*), 4 to 49 mg.kg^{-1} in cassava roots (*Manihot esculenta*) (White and Broadley 2005; Kumar et al. 2015). This broad range of Fe concentration has been associated with genetic variation. Such genetic diversity, as a prerequisite genomic resource, plays the most important role in these two Fe biofortification strategies to effectively generate crop lines with enriched Fe nutrition. Thus, to characterize and exploit genetic variation within natural plant populations and breeding lines, marker-assisted selection (MAS) employing molecular markers has been extensively utilized to detect several quantitative trait loci (QTLs) for Fe concentration predominantly in wheat, rice, maize, and pearl millet (Lung'aho et al. 2011; Anuradha et al. 2012; Blair and Izquierdo 2012; Xu et al. 2012; Tako et al. 2013). The mapped QTLs followed by MAS technique can be used to select the lines with higher Fe content over many generations from crossing of existing accessions with best performance, and thus pave a way for Fe fortification by plant breeding and engineering (Velu et al. 2014).

Genetic engineering

Genetic engineering must first be supported by an understanding of the genes underlying the natural genetic variation in plant Fe content. Resolving QTLs to single-gene level for identifying candidate genes involved in the establishment and control of Fe homeostasis in plants is critical for achieving this goal. Currently, the investigation of the response to Fe deficiency in plants at the molecular level has been dramatically advanced in some model plant species such as Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) using mutation approaches (Schmidt and Buckhout 2011; Anuradha et al. 2012;

Trijatmiko et al. 2016). To cope with Fe limitation, plants have evolved at least two active mechanisms, strategy I and strategy II, for Fe acquisition in the rhizosphere (Kobayashi and Nishizawa 2012). Strategy I, present in the case of non-graminaceous plants, uses a reduction-based Fe uptake mechanism, and strategy II presenting in graminaceous plants, uses a chelation-based Fe uptake mechanism. These broad mechanisms can thus be used to help pinpoint more specific gene-based approaches for increasing plant Fe.

For strategy I plants under Fe shortage, three processes are involved: 1) **Fe solubilization.** Fe deficiency induces the expression of the H^+-ATPase (*HA*) genes that extrude protons resulting in acidification of the rhizosphere to increase the solubility of Fe^{3+} compounds, or the excretion of organic acid anions that complex Fe^{3+} and hold it in a soluble form that can diffuse to the root surface (Sussman 1994; Eide et al. 1996); 2) **reduction of Fe^{3+} to Fe^{2+}.** The *FRO2* gene (Ferric Reduction Oxidase 2) encodes ferric chelate reductase enzyme that induce trans-plasma membrane electron transfer to reduce Fe^{3+} hydrolysis species to a more soluble form Fe^{2+} at the root surface (Robinson et al. 1999); Moreover, a number of diverse roles of FRO family have been documented and characterized as involving both iron and copper metabolism in plants (Jain et al. 2014); 3) **Fe^{2+} import** into the cell. The iron-regulated transporter gene *IRT1* (Iron-Regulated Transporter 1) encodes the ferrous Fe transporter that absorbs the generated Fe^{2+} across the root plasma membrane into root epidermis cells (Eide et al. 1996; Marschner 2012; Ivanov et al. 2012; Kobayashi and Nishizawa 2012; Mai et al. 2016).

As for strategy II plants under Fe shortage, Fe uptake is based on chelation of Fe^{3+} to strong ligands called phytosiderophores (PS), which are non-proteinogenic amino acid

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derivatives such as mugineic acids (MAs) (Marschner et al. 2011). *TOM1* (Transporter of Mugineic acid family phytosiderophores 1) encode PS efflux transporters. The chelated Fe-phytosiderophore complex can be subsequently taken up into the root cells via yellow stripe (YS)/yellow stripe-like (YSL) family transporters (Curie et al. 2009; Nozoye et al. 2011; Kobayashi and Nishizawa 2012). Particularly, rice has been demonstrated to use a combined strategy, which has all features of a strategy II plant (Inoue et al. 2009; Lee et al. 2009) and some features of a strategy I plant (Ricachenevsky and Sperotto 2014).

Additionally, except for the Fe acquisition from soil, Fe transport from roots to edible parts and the regulation of Fe distribution in various plant organs can be manipulated to produce plant foods with higher Fe content as well. Briefly, the FRD3 (Ferric Chelate Reductase Defective 3), a transporter of the Multidrug and Toxic compound Extrusion (MATE) family and PEZ1 (Phenolics Efflux Zero 1) facilitate efflux of Fe-chelating molecules into the xylem. ZIP (Zinc-regulated transporter, Iron-regulated transporter–like protein) and NRAMP (Natural Resistance-Associated Macrophage Protein) families were discovered as transporters of Fe and other similar divalent metals. Moreover, a number of genes involved in regulation of Fe homeostasis in response to Fe deficiency have been determined by transcriptomic and proteomic studies (Schmidt and Buckhout 2011; Schuler et al. 2011; Yang et al. 2010). The major transcriptional regulators of Fe-deficiency responses are the bHLH transcription factors including two main regulation networks, FIT network and the POPEYE network (Ivanov et al. 2012; Hindt and Guerinot 2012; Long et al. 2010). Many previously undiscovered genes involved in these regulation networks have recently been described (Mai et al. 2016; Palmer et al. 2013; Brumbarova et al. 2015).

In the past decades, the homologs of some of these Fe homeostasis related genes have been isolated and characterized from various non-model plant species, such as *PsFRO1* in pea (*Pisum sativum*) (Waters et al. 2002), *LeIRT1*, *LeIRT2*, *LeNRAMP1*, *LeNRAMP3* and *LeFRO1* in tomato (*Solanum lycopersicum*) (Li et al. 2004; Bereczky et al. 2003; Eckhardt et al. 2001), *CsHA1*, *CsHA2*, *CsFRO1* and *CsIRT1* in cucumber (*Cucumis sativus* L.) (Santi et al. 2005; De Nisi et al. 2012), *MxHA7* in apple (*Malus xiaojinensis*) (Zha et al. 2014). In recent years, with lowering costs and increased access to technologies such as next-generation sequencing (Mardis 2013) and genome editing (Gaj et al. 2013), comparative genomics and transcriptomic studies were performed to uncover key genes involved in Fe metabolism in non-model plant species. For instance, Mamidi et al. (2011) identified 15 candidate genes involved in iron metabolism in soybean (*Glycine max*) using genome-wide association analysis. Later on, RNA-Seq studies have been conducted to identify candidate genes involved in Fe deficiency signaling in soybean (Moran Lauter et al. 2014).

1.2.3 Plant-microbial interaction on micronutrient uptake

Even though mechanisms exist to increase plant absorption capacity for Fe under Fe-limited conditions, they are not always sufficient to prevent plants from suffering Fe deficiency or increase Fe content in the plant food, especially in soils with low bioavailable Fe. Indeed, microbes as mediators of plant Fe may also play a role in the above-described mechanisms currently attributed primarily to plant physiological pathways. The plant-microbial interaction in the rhizosphere ranging from mutualism to competition has been studied widely. The rhizosphere, the region of soil within immediate vicinity of plant roots, is a hotspot of this plant-microbial interplay. It differs

from surrounding bulk soil in most chemical and physical properties and as well in microbial population and community structure (Phillips et al. 2003). The plant-microbial interaction and plant root/rhizosphere-colonizing microbes that have a positive effect on plant growth have been studied in detail. These microbes are collectively known as plant growth-promoting microorganisms (PGPMs). The mechanisms for this plant growth promotion are various, such as inhibition of growth and activity of pathogens, eliciting induced systemic resistance (ISR) and mediating the quality and availability of essential nutrients to plants (Marschner et al. 2011). These mechanisms also influence Fe dynamics in the rhizosphere and Fe uptake by plants and microorganisms (Pii et al. 2016a; Rasouli-Sadaghiani et al. 2014; Mimmo et al. 2014; Jin et al. 2014). Therefore, the contribution of soil microorganisms in the rhizosphere to improve Fe supply of plants may play a key role in Fe biofortification, especially under real world field-based soil scenarios.

The soil microorganisms in the rhizosphere are predominantly affected by rhizodeposits released from plant roots, such as plants root exudates, sloughed border cells, and mucilage, which provide the carbon source supporting higher microbial density and activity (Philippot et al. 2013; Belnap et al. 2003; Bardgett et al. 1998; Haichar et al. 2008). Many rhizospheric microbes also secrete growth-stimulating or -inhibiting substances influencing plant growth and development, such as phytohormones, siderophores, strigolactones involved in microbial processes, such as nitrogen fixation, phosphate solubilization, potassium mobilization, micronutrient mobilization and signaling (Ryu et al. 2005; Govindasamy et al. 2009; Paszkowski 2006; Akiyama and Hayashi 2006). There is no debate that microorganisms are key players in nutrient cycles

and can deter or enhance plant nutrient acquisition (Bulgarelli et al. 2013; Mishra et al. 2012). This knowledge thus supports the development of technologies which integrate beneficial plant-microbial interactions to support nutrient cycling and plant nutrition.

Microbial contribution on plant N and P nutrient cycling

One of the best-known plant-microbial interactions that supports plant nutrient supply is based on the association of N_2-fixing bacteria associated with the roots of plants. In this association, microorganisms convert atmospheric N_2 into ammonium, which is then made available to the plant. This bacterial-driven process of biological nitrogen fixation is underpinned by the protein nitrogenase (Falkowski 1997). In exchange for nitrogen, plants provide carbon to support bacterial activity and growth. Plants have co-evolved with these microorganisms and in many cases are highly dependent upon their support for survival and growth. Some examples of bacteria that fix nitrogen in association with plants include genera *Rhizobium*, *Azotobacter* and *Azospirillum*. In the case of leguminous plants, there is an elegant integration of the plant-bacterial system. Indeed, bacteria that reside in the nodules of legumes change their metabolism and physiology to become bacteroids. The process of the interaction between the plant and bacteria begins with secretion of phenolic molecules, predominantly flavonoids and isoflavonoids, into the rhizosphere. *Rhizobium*, in turn, respond to these signals which ultimately stimulate the formation of root nodules in which atmospheric nitrogen is fixed (Franche et al. 2009; van Rhijn and Vanderleyden 1995). A broadly similar, though mechanistically different mutualism occurs in actinorhizal plants which form nodules to support bacterial from the genera *Frankia*. *Azotobacter, Azospirillum* and other bacteria also are known to "fix" atmospheric nitrogen in non-leguminous crops, especially grasses (Dobbelaere et al. 2002;

Steenhoudt and Vanderleyden 2000). All of these N_2-fixing bacteria enhance plant growth and yields across a range of crops, including maize, tomato, wheat (*Triticum aestivum* L.), common bean (*Phaseolus vulgaris*), mung bean (*Vigna radiate*) and soybean (Faruq et al. 2015; Marini et al. 2015; Patra et al. 2012; Rajpoot and Panwar 2013; Soleimanzadeh and Gooshchi 2013; Trabelsi et al. 2011). Though it is not expected that the same mechanism occurs in support of plant Fe, it is an elegant model system that provides a proof of concept underlying the co-evolution of land plants with soil microorganisms over many tens to hundreds of millions of years. Plant-microbial interactions in soil are thus expected to be a key component of many types of nutrient exchanges.

Similar to Fe, the element phosphorus is abundant in soils but mostly in insoluble complexes such as immobilized inorganic and organic P. These forms of P cannot be directly utilized by plants, and must be solubilized and then mineralized by phosphatase enzymes prior to plant uptake (Giles and Cade-Menun 2014). Bacterial and fungal species have been reported for their phosphate-solubilizing abilities (Bünemann et al. 2012; Oberson et al. 2001; Oberson and Joner 2005). The main mechanism is that microorganisms produce organic anions and acids to solubilize the insoluble P complexes in the soil, and they also produce phosphatase enzymes to mineralize the solubilized organic P to finally release plant-available inorganic orthophosphate in the rhizosphere (Clarholm et al. 2015). The bacteria *Bacillus, Pseudomonas* and *Burkholderia* and fungi *Aspergillus* and *Penicillium* are common species shown to solubilize inorganic and organic P in the liquid media and soil (Clarholm et al. 2015; Giles et al. 2012; Patel et al. 2010). In addition, the arbuscular mycorrhizal fungi (AMF) are well recognized to

support plant survival and growth, including crop plants, by mobilizing soil phosphorus and transport to the host plant. The hyphae act as an extension of the root system to forage a greater surface area of soil. Phosphorus is stored in the form of polyphosphate in vesicles and can be converted into soluble P transported to the plant under phosphate-limited conditions. Because of the much greater surface area explored and the ability to forage in small soil pores, mycorrhizal hyphae more efficiently incorporate phosphorus compared with the plant root system (Smith and Read 2010; Smith and Smith 2011). Inoculation of soil with these microbial species has improved the growth of plants with gains in yield under both greenhouse and field experiments (Karamanos et al. 2010; Yin et al. 2015). For example, Walpola and Yoon (2013) reported that co-inoculation of tomato plants with both *P. agglomerans* and *Burkholderia anthina* showed P solubilization under lab conditions and increased plant growth in the field. Phosphorus shares many of the same chemical properties of Fe in soil, and indeed iron phosphates are common insoluble forms of both nutrients. The knowledge gained regarding microbial support of plant phosphorus may provide a good model system to explore ways microbes can support plant Fe content. It is notable, compared to phosphorus, little research has been conducted on plant-microbial interactions related to the cycling of Fe.

Microbial contribution on plant Fe nutrient cycling

Fe is an essential nutrient for life. Rhizosphere microorganisms are thus similarly in need of Fe since Fe is present in Fe-containing proteins (e.g., Fe-S clusters and heme), involving different metabolic activities (Andrews et al. 2003). It is notable that microorganisms contribute to the mineral weathering in the soil. Bacteria, such as *Thiobacillium* and *Metallogenium* sp. can dissolve Fe-containing primary minerals

(Lowenstam 1981; Lower et al. 2001). Primarily, soil microorganisms acquire Fe under Fe deficient conditions relying on the synthesis and release of siderophores or organic acid anions. Siderophores are relatively low-molecular-weight Fe-chelating molecules (Lemanceau et al. 2009). Siderophores mainly scavenge Fe from the surrounding environment and make them available to microbial cells (Schalk et al. 2011; Saha et al. 2016). Moreover, by depriving pathogens of Fe, siderophores can stimulate the synthesis of antimicrobial compounds and suppress the growth of pathogenic organisms (Shobha and Kumudini 2012).

With respect to the microbial contribution to plant Fe nutrient cycling, indirect and direct mechanisms have been reported by literature. Indirectly, the nitrogen-fixing symbiosis has a great requirement for Fe because many symbiotic proteins incorporate Fe (Tang et al. 1990). For instance, Fe is an essential component of nitrogenases, leghemoglobin and ferredoxin (Dixon and Kahn 2004; Brear et al. 2013). Fe deficiency can affect initiation and development of the nodule (O'HARA et al. 1988; Slatni et al. 2011; Soerensen et al. 1988). The nitrifying bacteria convert ammonia and ammonium to nitrate via nitrification accompanied by release of protons, therefore can be expected to contribute to soil acidification and then increase Fe solubility (Pester et al. 2012). With respect to P, since phosphates generally combines with elements in the soil such as calcium (Ca), magnesium (Mg), aluminum (Al), and Fe, and forms solid compounds, releasing bioavailable P may increase the Fe availability to meet crop needs (Borggaard et al. 1990; Hinsinger 2001; Tomasi et al. 2008). Similarly, some phosphate-solubilizing bacteria also excrete protons. Therefore, the rhizosphere microorganisms influencing plants N and P acquisition may play a critical role indirectly in plants Fe nutrient cycling. Supporting

these speculations, wheat inoculated with the N_2-fixing bacteria *Azospirillum* and mycorrhizae were found to increase uptake of iron, manganese, zinc, and copper (Ardakani et al. 2011). Inoculations with *Bacillus cereus* (N_2-fixing), *Brevibacillus reuszeri* (P-solubilizing), and *Rhizobium rubi* (both N_2-fixing and P-solubilizing) significantly increased Fe content of broccoli in comparison with plants fertilized with manure (control) and mineral fertilizer under field conditions (Yildirim et al. 2011). Clearly, microbes in soil are important determinants of plant Fe. However, details about the plant-Fe-microbe interaction need further investigation.

Directly, the existence of interactions between plant roots and microorganisms in relation to Fe uptake has been reported increasingly. For several decades, it has been known that some microbial siderophores (MS) can promote the growth of various plant species and increase their yield by enhancing Fe uptake to plants. The pyoverdines, yellow-green fluorescent siderophores having a high affinity for Fe^{3+}, are produced by fluorescent *Pseudomonas* spp. Vansuyt et al. (2007) showed that Fe-pyoverdine improved Fe nutrition and growth in Arabidopsis. And they proposed that a putative transporter different than IRT1 in strategy I plants (dicots) can uptake the Fe^{3+}-MS complex. Other microbial siderophores, such as ferrioxamine, rhodothorulic acid, rhizoferrin and fusarinines have been also studied and shown to be relatively effective for delivery of Fe to other plants, such as wheat, rice and tomato (Siebner-Freibach et al. 2003; Crowley 2006; Rungin et al. 2012; Radzki et al. 2013; Rasouli-Sadaghiani et al. 2014; Trapet et al. 2016). Siderophore-producing bacteria, or siderophores extracted from bacteria have been suggested as biofertilizers to supply Fe to plants (Zuo and Zhang 2011; Fernàndez et al. 2005; Radzki et al. 2013). In addition to secreting protons, microorganisms can

increase plant acquisition of Fe via production of hormonal compounds. These hormonal compounds play a signaling role in activating Fe stress response in plants (Hindt and Guerinot 2012; Ivanov et al. 2012). For example, Zhang et al. (2009) reported that a growth-promoting bacterium, *Bacillus subtilis* GB03, facilitated Fe mobility through acidification of the Arabidopsis rhizosphere and induced the expression of ferric reductase *FRO2* and the Fe transporter *IRT1* by transcriptional up-regulation of *AtFIT1*, and finally increased Fe assimilation in Arabidopsis. The effects induced by inoculation of rhizobacteria naturally colonized agronomically important crops on Fe-starved plants have been evaluated. It has been demonstrated that inoculation with *Azospirillum brasilense* and novel bacterial strains isolated from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (*Solanum lycopersicon* L.) plants were able to activate Fe deficiency mechanism in cucumber plants *Cucumis sativus* L., which is supported by an increased expression of the genes encoding for Fe-chelate reductase (CsFRO) and PM H^+-ATPase (CsHA1) and higher Fe uptake rate (Pii et al. 2016b; Pii et al. 2015; Scagliola et al. 2016).

Impact of plant iron nutrition on rhizospheric microorganisms
Within the rhizosphere, the effects of inoculation with soil microorganisms on plant Fe acquisition have not been elucidated and evaluated in the field for developing bioinoculants for agronomic practices, because those mechanisms are even more complex due to the presence of plants. In the rhizosphere, plant rhizodeposits promote the growth and activity of soil microorganisms. These microorganisms and plant roots uptake Fe in the rhizosphere, thus the available Fe content may be even lower in this region. In this case, the growth of microorganisms with a less efficient Fe uptake mechanism through Fe

deficiency is expected to be reduced, and microorganisms in the rhizosphere are less susceptible to Fe depletion compared with bulk soil (Robin et al. 2007; Robin et al. 2006a; Robin et al. 2006b; Alegria Terrazas et al. 2016). Therefore, the influence of plants on rhizosphere microorganisms has been reported widely.

It has been revealed that plant type plays an important role in determining microbial community structure in the rhizosphere, such as plant species, plant growth stage, genotype, phenotypic characters, and abiotic and biotic stress, the nutrient status of the plant, and physiological abnormalities due to pathogen infection (Marques et al. 2014; Van Overbeek and Van Elsas 2008; Poli et al. 2016; Yang and Crowley 2000; Smith et al. 1999; Yang et al. 2001; Turner et al. 2013). Those effects have been mostly reported at the microbial community level but not individual level because physically removing specific microbes from rhizosphere soil and culturing them are both extremely difficult and more innovative approaches have been developed.

The influence of plant type on the microbial community is explained mainly as plant root activity such as root exudation and nutrient uptake (Haichar et al. 2008). For instance, (Robin et al. 2007; Robin et al. 2006b; Robin et al. 2006a) compared microbial communities in the rhizosphere of a tobacco wild type (WT) and a transgenic line that overexpressed ferritin (P6), a protein that stores bioavailable Fe. They found that the structure of the rhizosphere bacterial community was significantly different between WT and P6 because of differences in tobacco Fe acquisition. The bacterial community associated with the P6 transgenic line had greater density cultivating in a soil with low Fe content compared with that associated with the tobacco WT line (Robin et al. 2006b; Robin et al. 2006a). Isolates of fluorescent pseudomonads taken from tobacco WT lines

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and P6 transgenic lines were observed to differ in genetic background, ability to produce specific siderophores and tolerance to Fe starvation (Robin et al. 2007). Jin et al. (2006) also observed variation in the rhizosphere microbial community with regard to the plant's Fe nutritional status. The variation showed that the majority of microbes, which thrived in the medium containing phenolic compounds exuded from red clover roots under Fe-deficient conditions, could secrete siderophores. It was suggested that through specific root exudates plants under Fe deficiency induce siderophore-secreting microbes to help Fe solublization and improve Fe availability for plants (Jin et al. 2014; Jin et al. 2010; Jin et al. 2006). Pii et al. (2016a) demonstrated that tomato (*Solanum lycopersicum* L. cv. Marmande) and barley (*Hordeum vulgare* L. cv. Europa), representative of Strategy I and Strategy II plants, respectively, both promote the development of a particular rhizosphere microbial community composition when they grew in the RHIZOtest system with different concentrations of Fe nutrition solution. However, the major mechanisms of shift of the microbial community composition and the molecular interplay with plants have not been elucidated experimentally.

1.3 Research Objective

Potato, as the fourth most important stable food worldwide after rice, wheat and maize, with a production in 2005 of >323 million tons (FAO 2015), contains abundant vitamin C (ascorbic acid), vitamin B6, vitamin B3, protein and minerals (CIP 2011). It is also a modest contributor of Fe in the human diet. A small potato (100 g serving) provides 6% Daily Value (DV) of Fe (Brown et al. 2010). Moreover, vitamin C is a potent enhancer of Fe uptake in humans via reducing of Fe^{3+} to Fe^{2+} that is more absorbable by cells. Accordingly, biofortification of potato with Fe has tremendous potential to solve the

problem related to Fe deficiency economically and sustainably. In addition, potato is a crop native to the central Andean area of South America with large genetic variations and inheritance patterns observed in the germplasm. Fe concentration in potato tubers ranges from 2.8 to 158 mg kg^{-1} (Lefèvre et al. 2012; Haynes et al. 2012), thus breeding for higher levels of Fe is feasible.

As a valuable source for Fe biofortification, however, potato has been virtually ignored by the projects aiming to enhance micronutrients in plants. Little attention has been paid to uncover key genes for Fe accumulation in potato crop (Legay et al. 2012). Studies of soil microorganisms associated with potato are also relative rare (Cesaro et al. 2008; Senés-Guerrero and Schüßler 2015; Senés-Guerrero et al. 2014). The interaction between potato plants and rhizosphere microbial community associated with Fe acquisition is still unclear. The key players of microbes in nutrient cycles and aid in Fe acquisition have never been studied.

The major objective of this study is to understand the genetic factors and plant-microbial interactions underlying Fe cycling in *S. tuberosum*. To accomplish that, the variation of Fe concentration in tubers of Andean potato landraces was utilized to study the candidate QTL and genes involved in potato Fe metabolism (Chapter 2). Then two Andean potato landraces with relative high and low Fe concentrations in tubers were used to complete a rhizosphere microbial community analysis to identify significant microbial groups associated with potato Fe accumulation (Chapter 3). In chapter 4, an Andean potato landrace with relatively low Fe concentration in tubers growing in Fe deficient soil was treated with different Fe fertilizer foliarly to examine the impact of plant Fe nutritional status on the rhizosphere microbial communities and to characterize the potato

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microbiome contributing to potato Fe uptake. Finally, in chapter 5, the important findings

from the previous chapters were summarized. The information generated by this study

will be useful for establishing strategies for Fe efficiency in potato, which play an

important role in improving the Fe nutritional quality in the native potato cultivars for

enhancing the nutrition of human beings.

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Chapter 2 Identification and characterization of candidate genes for phytonutrient acquisition in potato

Tatiana Boluarte[1], Merideth Bonierbale[2], Elisa Mihovilovich, Mark Williams[1], Hua Xiao[1], Richard E. Veilleux[1]

Author Contributions

TB, MB, MW, HX and REV designed research; MB provided germplasm as well as Fe and Zn data from field studies in Peru; EM provided QTL analysis data; TB, EM and HX performed research; TB, EM, HX and REV analyzed datasets; and TB, HX and REV wrote the paper. All authors approved the manuscript.

Abstract

Fe and Zn biofortification of potato either through traditional breeding or biotechnological approaches will require the identification of candidate genes to uptake, transport and store Fe in potato tubers. The complex of such genes has been described as the ferrome. We employed a small association mapping population, cloning and sequencing of alleles of candidate genes from high and low Andean potato cultivars with respect to Fe and Zn content in tubers, yeast complementation assays and genetic transformation to associate phenotypic variation with potential genetic determinants. Genes in potato orthologous to those described in the Arabidopsis ferrome were also identified. A PhenoGram of potato ferrome, highly significant SNPs from association mapping using the Infinium 8303 potato SNP array, and QTL regions from a mapping

[1] Department of Horticulture, Virginia Tech, Blacksburg, Virginia, USA

[2] The International Potato Center (CIP), Lima, Peru

study of mineral content in a potato dihaploid population revealed several regions of the genome enriched for genetic elements expected to attribute to mineral nutrition. These approaches led to the nomination of several candidate genes with support from more than a single approach, including: *PGSC0003DMG400024976* (metal transporter), *PGSC0003DMG400013297* (oligopeptide transporter), *PGSC0003DMG400021155* (*IRT1*) and *IRTunannotated* (an ortholog to the *IRT* gene that is not annotated in the potato genome).

Introduction

Many nutrients are essential for human health. However, the typical modern diet lacks several important nutrients, and micronutrient malnutrition becomes a serious problem for humans. In many developing countries, where the diet of the low-income populations is based mostly on staple foods, mineral deficiencies are common (Gomez-Galera et al. 2010). It is estimated that more than three billion people experience micronutrient deficiency, and Fe (Fe) deficiency anemia is one of the most common forms of micronutrient malnutrition worldwide, affecting about two billion people and causing almost a million deaths a year (WHO 2017). In the USA, 9-11% of adolescent girls and women of childbearing age and 9% of toddlers were found to be Fe deficient (Gupta et al. 2016; Looker et al. 1997). The Fe deficiency in the early stage of human growth irreversibly affects development of brain structure and function, which cause impaired cognitive development in children, a weakened immune system, and increased risk of morbidity (Black 2003; Beard 2008).

Zinc (Zn) is another essential micronutrient, second only to Fe in its concentration in the body. It is an integral component of hundreds of enzymes and thus obligatory for

metabolism involving cell division, cell growth, wound healing, and the breakdown of carbohydrates (Vallee and Falchuk 1993). Most children suffering from Zn deficiency have stunted growth, which contributes significantly to morbidity and mortality in young children (Alloway 2008). It has been estimated that around 17.3% of the world's human population has diets deficient in Zn (Wessells and Brown 2012). Plants are the primary source of dietary Fe and Zn worldwide; therefore, there is a need for staple crops enriched with bioavailable Fe and Zn to improve human nutrition (Jeong and Guerinot 2009).

Potato (*Solanum tuberosum* L.), is a predominant staple food in many countries with a production in 2015 of >323 million tons (FAO 2015), where its consumption provides the bulk of dietary intake of micronutrients. There are remarkable differences in the nutrient value of potato cultivars (Burlingame et al. 2009). Although nutritional quality of potato is often touted, particularly with regard to Vitamin C, Fe and Zn content, the claim is not true for all varieties, especially with regard to the more than 4,000 native Andean potato varieties, a mixture of which accounts for the dietary staple for much of the South American rural population. Fe concentration in potato tubers ranges from 2.8 to 158 mg/kg of dry weight (DW) (Lefèvre et al. 2012; Haynes et al. 2012; Andre et al. 2007). This variation provides a good resource to identify candidate genes that control Fe acquisition, movement and storage, the complex of which has been described as the ferrome in Arabidopsis (Schmidt and Buckhout 2011).

Strategies to improve micronutrient intake in the human diet include dietary diversification, mineral supplementation and postharvest food fortification. However, these strategies depend on continued investment and infrastructure, and current levels of

postharvest fortification of Fe are often inadequate (Gomez-Galera et al. 2010; Hurrell et al. 2010; White and Broadley 2009). Biofortification is an alternative long-term approach for improving the micronutrient content of crops by increasing mineral levels in the edible parts of staple crops (Gomez-Galera et al. 2010). Improving crop varieties by either conventional breeding or genetic engineering has the advantage that, once the initial research and development are complete, the benefits from nutritionally-enhanced crops will be sustainable with little further investment (Borrill et al. 2014).

Plants capable of hyperaccumulation of minerals have increased activity of metal transporters, which act to extrude toxic metal ions from the cytoplasm and to load them into the xylem and vacuole for transport and storage, respectively (Fones and Preston 2013). The uptake of Fe and Zn from the soil occurs via two processes depending on the plant strategy. In Strategy I plants, Fe^{2+} is absorbed from the soil by metal transmembrane transporters (IRT1 and IRT2, Iron-Regulated Transporter 1 and 2) and Zn^{2+} by ZRT-, whereas in Strategy II plants, mineral absorption occurs via secretion of phytosiderophores (PSs) which chelate Fe^{3+} that are subsequently taken up by yellow stripe like (YSL) transporters (Brumbarova et al. 2015; Briat et al. 2015; Kobayashi and Nishizawa 2012).

Non-graminaceous plants employ a reduction-based Strategy I, which consists of three coordinately induced processes: (1) proton extrusion by H^+-ATPases that acidify the rhizosphere and increase Fe^{3+} solubility (e.g., AtAHA2, Arabidopsis H^+-pump ATPase); (2) Fe^{3+} reduction by membrane-bound ferric reductases (e.g., AtFRO2, PsFRO1, LeFRO1 Ferric Reductase Oxidase); and (3) Fe^{2+} absorption into root epidermal cells by transmembrane Fe transporters (e.g., AtIRT1, Iron-Regulated Transporter 1). H^+-ATPase

activity also allows the establishment of a negative membrane potential that drives cation uptake (Palmgren 2001; Santi and Schmidt 2009; Robinson et al. 1999; Eide et al. 1996; Li et al. 2004). Lucena et al. (2006) proposed a model to explain the regulation of Fe-acquisition genes in Strategy I plants. According to that model, ethylene acts as an activator of SlFER (or AtFIT) expression, and consequently of ferric reductase oxidase (FRO) and iron regulated transporter (IRT), while Fe (probably phloem Fe) acts as an inhibitor of their expression (Lucena et al. 2006). Since that model was proposed, new results have extended the role of ethylene and nitric oxide on the activation and up-regulation of Fe related genes (Amiri et al. 2010; Garcia et al. 2010; Graziano and Lamattina 2007; Chen et al. 2010; Wu et al. 2011).

Major genes have been reported to be involved in Fe and Zn acquisition, transport, accumulation and tolerance (Schmidt and Buckhout 2011; Ling et al. 2002; Robinson et al. 1999; Li et al. 2004; Colangelo and Guerinot 2004; Jakoby et al. 2004; Yuan et al. 2005; Ling et al. 1999; Eide et al. 1996; Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2002; Curie et al. 2000; Thomine et al. 2000b; Amiri et al. 2010; Lin et al. 2009; Ricachenevsky et al. 2013; Blindauer and Schmid 2010; Feng et al. 2006; Wu et al. 2005). In addition to studies of particular genes, Schmidt and Buckhout (2011) described the Arabidopsis "ferrome," a complex of 92 genes involved in Fe acquisition, transport and accumulation processes, most of which were shown to be differentially regulated under Fe deficiency.

New genomic tools associated with the initial publication (The Potato Genome Sequencing Consortium, 2011) and revision (Sharma et al. 2013) of the potato genome along with the potato genome browser have facilitated translational genomics from model

plants to crop plants. The potato genome browser has expanded the possibility to understand gene function and genetic manipulation through plant transformation for the improvement of this important crop. We can now approach the potato ferrome by seeking highly expressed orthologous genes to those in Arabidopsis, another Strategy I plant with regard to metal acquisition.

Another recent potato genomic tool is the abundance of single nucleotide polymorphisms (SNPs) including 8,303 high confidence SNPs included in the SolCAP Infinium 8303 SNP array and genome-wide SNPs that differentiate either allele of heterozygous RH from DM (the RH SNP track). The latter presents some 3.67 million SNPs identified from aligning RH Illumina reads to the PGSC v2.1.11 DM pseudomolecules. Large scale SNP discovery in plants and subsequent genetic applications, such as linkage mapping, population structure analysis, association studies, map-based cloning, marker-assisted plant breeding and functional genomics are enabled by access to large collections of SNPs. The array facilitated a high throughput mapping scheme with wide genome coverage, demonstrating the possibilities for quantitative trait locus (QTL) analysis, genome wide association studies and map-based gene cloning (Felcher et al. 2012).

The objective of this study was to identify genes associated with Fe and Zn acquisition, transport and storage in potato using genomic approaches. These approaches included: candidate gene haplotype analysis and association mapping in a native Andean potato population; identification of ferrome orthologs in potato (for genes reported to be involved in Fe and Zn acquisition, transport and accumulation in the Arabidopsis "ferrome"); and gene identification within QTLs associated with Fe and Zn content in tubers in a study of a segregating population of dihaploids extracted from 'Alca Tarma', a

tetraploid Andean potato cultivar. These three approaches were complemented with transcript expression analysis from quatitative RT-PCR data under Fe deficient conditions, yeast complementation analysis and *Agrobacterium*-mediated plant genetic transformation to verify the function of some candidate genes.

Materials and Methods

Plant materials

A population (n=36) of native Andean potato cultivars (Table A2.1 ID 1-36) previously characterized for Fe and Zn content from field samples of tubers harvested at two locations (Burgos et al. 2007) was used for association mapping and candidate gene amplification. In addition, three Andean cultivars, China Runtush (CIP 703825), Senora Warmi and CIP703580 were used for evaluation of expression and function of selected candidate genes (Table A2.1 ID 11, 37, 38).

Candidate gene approaches and association mapping with Andean cultivars

Selecting from potato genome annotation data

Nine putative candidate genes were selected based on their involvement in metal accumulation in plants (Table 2.1) including genes reported to be instrumental in phytonutrient acquisition (Fe and Zn binding, transport and accumulation). Amino acid sequences of candidate genes were BLAST-searched (blastn function) against version 4.03 of the potato genome (The Potato Genome Sequencing Consortium 2011) to identify orthologous genes. All of the selected candidate genes were proposed for inclusion in the design of the Illumina 8303 potato SNP array; however, none of the SNPs within them fulfilled the restrictive requirements for selection (Hamilton et al. 2011).

Potato gene candidates were also selected based on gene annotation and with high expression levels in roots, stolons and tubers, using either the DM or RH tracks for RNAseq coverage according to the RNA expression profiles within the Potato Genome Browser. The selected genes (Table 2.1) were further analyzed using the "Smart Domain" program (Schultz et al. 1998; Schultz et al. 2012) to identify protein domains likely to be involved in Fe and/or Zn acquisition, transport and accumulation. From the population of 36 primitive cultivars characterized for Fe and Zn content, two high (CIP 703597 and CIP 701243) and two low (CIP 703596 and CIP 704393) genotypes were selected for both Fe and Zn content in tubers for cloning and sequencing of amplicons from candidate genes (Table A2.1 ID 23, 11, 22 and 30). PCR primers were designed to amplify and sequence amplicons within domains of interest.

Primers were designed using DNASTAR® Lasergene 9 to cover exonic regions from the domains of interest. PCR amplification volume was 25 µl containing 10 mM Tris-HCl, pH 8.43, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.64 µM of each primer, 0.5 units of Takara TaqTM and 100-200 ng of genomic DNA template. Standard cycling conditions were 7 min of initial denaturation at 94 °C, 0.5-1 min at 94 °C, 0.5-1 min of annealing at the appropriate Tm and 0.5-1 min of extension time for 30-40 cycles ending with 5 min incubation at 72 °C. DNA fragments (PCR products) were gel purified (Qiagen Gel Extraction Kit, Hilden, Germany), ligated into pGEM®-T Easy Vector (Promega, Madison, WI) following the supplier's instructions and transformed into *E. coli* competent cells by the heat shock method. Transformed *E. coli* were plated in LB/Ampicillin/Xgal solid media and incubated at 37°C overnight (O/N). The following day, single colonies were isolated in the same media and allowed to grow O/N. At least

six white colonies were cultured O/N in 5 ml liquid LB-Amp media, and DNA was extracted and submitted for sequencing at the Biocomplexity Institute of Virginia Tech. Forward and reverse sequences were analyzed to build consensus sequences. Consensus sequences for at least six clones for each gene domain were aligned (DNA Star/Seqman) to identify allelic differences among fragments in each of the four native cultivars.

SNP genotyping

DNA was extracted from 36 Andean potato cultivars and subjected to genotyping using the Illumina 8303 Potato Array. The SNP genotyping facility at Michigan State University processed the samples on an Illumina iScan Reader utilizing the Infinium® HD Assay Ultra (Illumina, Inc., San Diego, CA) and the Infinium 8303 Potato Array. The data from the 8,303 SNPs present on the SNP chip were filtered and used for contingency table analysis and ANOVA based on Fe and Zn concentration in tubers. SNPs that were monomorphic for all individuals, and SNPs with missing values were eliminated from the initial data set.

Arabidopsis Ferrome orthologs

The genes comprising the Arabidopsis ferrome (Schmidt and Buckhout 2011) were BLAST-searched against the potato genome to identify orthologs using blastp. Only genes with hits containing highest similarity of domains were considered orthologous and within those, only genes with good expression depicted by RNAseq data on the Potato eFP Browser (http://bar.utoronto.ca/efp_potato/cgi-bin/efpWeb.cgi) were retained for the "Potato ferrome."

QTL regions of Alca Tarma

A population consisting of 173 sibling dihaploids obtained by gynogenic haploid

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extraction from Andean tetraploid cv. Alca Tarma (LOP868) was characterized for Fe and Zn content in tubers. QTL analysis for Fe and Zn accumulation in the tubers of this population has been conducted (Mihovilovich unpublished). Briefly, a DArT-based linkage groups of this population was used to map the concentrations of Fe and Zn of potato tubers (http://potato.plantbiology.msu.edu/index.shtml). Data from two contrasting environments (Huancayo and La Molina) in Peru were used to locate and determine gene action of QTL, and search for potential candidate genes underlying QTL that were consistent across environments.

PhenoGram

PhenoGram (http://visualization.ritchielab.psu.edu/phenograms/document) was used to plot SNPs that have been highly significantly associated with Fe and Zn content of tubers, potato ferrome and significant QTL regions along the genome. The genome is displayed and divided into separate chromosomes (Figure 2.1).

Evaluation of expression and function of candidate genes

Micropropagation under Fe treatments

Two Andean cultivars with relatively low (CIP703580) and high (CIP701243) Fe concentration in tubers (Table A2.1) were used to investigate the level of ability to uptake Fe under different Fe conditions and the expression of selected candidate gene responses to limited Fe availability. A potato micropropagation and microtuberization system was used for this evaluation to accelerate process and strictly control the environmental condition.

Micropropagation medium was prepared based on MS medium (Murashige and Skoog 1962) with three different concentrations of Fe: 13.9 mg/L (low Fe medium: LIM), 27.8 mg/L (normal medium: NM), and 41.7 mg/L (high Fe medium: HIM) of FeNaEDTA. All media were supplemented with 30 g/L sucrose, 148 mg/L NaH_2PO_4 and 100 mg/L inositol. The pH value was adjusted to 5.9. 30 mL micropropagation medium were distributed to each Delong culture flasks and autoclaved before use. A two-node section and a 1.5 cm long plantlet apex of 3 weeks old *in vitro* potato plants subcultured in the MS basal medium from each genotype were placed into the medium in each flask. All cuttings were cultured at room temperature with 16 h photoperiod. After 3 weeks, roots of each treatment were sampled from three flasks as three replicates for RNA extraction. Shoots were sampled for Fe and Zn concentration analysis. For microtuberization system, the propagation medium was replaced with autoclaved microtuberization media that contain one of three different concentrations of Fe described above, and 80 g/L sucrose. The pH value was adjusted to 5.8. After 3 more weeks, microtubers of each treatment were sampled from three flasks as three replicates for RNA extraction.

Fe and Zn concentration measurement

The dried sample tissues (0.3-0.5 g) 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. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to determine the Fe and Zn concentrations in the digests in the Soil Testing Laboratory at Virginia Tech.

Semi-quantitative RT-PCR analysis

The total RNA was extracted from roots and tubers using Qiagen RNA Isolation Kit (Qiagen) with Trizol (Life Technologies). Briefly, after incubation and vortexing of the homogenized frozen samples in 1 mL of Trizol reagent and 0.2 mL chloroform, the samples were centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was then mixed with Qiagen RLT buffer and 500 µl 100% ethanol, and was transferred into Qiagen MinElute spin columns to centrifuge at 10,000 rpm for 15 s. Then the MinElute column was washed using 500 µl RPE. The RNA was precipitated with 750 µl of 80% ethanol twice. After centrifugation at 10,000 rpm for 15 s at 4°C, the RNA was dissolved in 20 µl of ddH$_2$O, treated with diethyl pyrocarbonate (DEPC). Complementary DNA (cDNA) was synthesized from the total RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

The semi-qPCR amplifications were performed using same amount of cDNA from each sample to preliminarily detect the expression level of selected candidate genes. Elongation factor 1-α (EF1-α) was used as the endogenous reference gene for the PCR (Nicot et al. 2005). Primers (Table A2.2) for the semi-qPCR were designed using DNASTAR® Lasergene 9 to target two exonic regions to avoid DNA amplification. Each 25 µl PCR reaction contained: 12.5 µl 2X ImmoMix master mix, 1 µl each primer (the original conc. is 10 mM), 1 µl cDNA template, and 9.5 µl molecular-grade water. PCR conditions for all genes consisted of an initial denaturation at 95°C for 10 min, followed by 22-28 cycles of 95°C for 30 s and annealing temperature at 55°C for 30 s, and extension at 72°C for 30 s. And a final incubation was implemented at 72°C for 5 min. The ImmoMix master mix (Bioline) for PCR reactions contained dNTPs, Taq polymerase,

MgCl$_2$, and a buffer. In order to obtain semi-quantitative results, we adjusted the number of cycles in the PCR reactions for each gene to obtain barely visible bands in the 1.2% agarose gels with ethidium bromide.

Quantitative real time PCR analysis

Microtuberization and quantitative RT-PCR were conducted to further narrow down the number of candidate genes. Two Andean cultivars with relatively low (CIP703580) and high (CIP703825) Fe concentration in tubers were used (Table A2.1). Plantlets were firstly grown in the micropropagation media for 2 weeks with 27.8 mg/L FeNaEDTA, then 1 week with either of two FeNaEDTA treatments: 0 mg/L (No Fe medium: N); 27.8 mg/L (Control medium with full Fe of MS medium: C). Roots were sampled from three flasks as three replicates for RNA extraction. The detailed procedure, the total RNA isolation and the cDNA synthesis were performed according to the methods described above.

Quantitative RT-PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems) and 7500 System software version 1.3.1. Primers for candidate genes were designed to have a melting temperature of 55°C and a product ranging between 100 and 150 bp (Table A2.3). Elongation factor 1-α (EF1-α) was used as the endogenous reference gene for the PCR (Nicot et al. 2005). For each sample, two replicates were performed in a final 20 µl reaction containing 100 ng of the cDNA, 4 µl of each specific primer (the original conc. is 1 µM) and 10 µl of SYBR Green PCR master mix (Life Technologies) according to the manufacturer's instructions. qRT-PCR amplification was performed as follows: 95°C for 3 min; 95°C for 15 s; 55°C for 30 s; 60°C for 40 s for 40 cycles. PCR efficiencies for the target and reference genes were

determined by generating standard curves. Melting curve analysis was performed to exclude the occurrence of primer dimers and unspecific PCR products. At the end of the PCR, the Ct value was obtained for each sample from the ABI 7500 System software. The amount of the transcripts of each gene normalized to the reference gene EF1-α was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Validation of the role of candidate genes

Yeast complementation assay

The function of six metal transporter candidate genes was investigated by expression in two specific *Saccharomyces cerevisiae* mutant strains, the Fe uptake deficient yeast double mutant *fet3fet4* DEY1453 (*MATα /MATα ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3- 2::HIS3/fet4-1::LEU2/fet4-1::LEU2*), and the Zn uptake deficient yeast strain *zrt1zrt2* ZHY3 (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*) (kindly provided by Dr. David Eide, University of Minnesota School of Medicine, USA). The complete coding sequence of each gene was amplified from RNA isolated from roots of Andean cv. Señora Warmi (CIP 701243), which has comparatively greater Fe and Zn content than other Andean cultivars, by reverse transcription polymerase chain reaction (RT-PCR) with iProof™ High-Fidelity DNA polymerase (Bio-Rad) and the primers in Table A2.4. The PCR product of cDNA was then subcloned into the yeast expression vector pDR196 (kindly provided by Dr. Guillaume Pilot, Virginia Tech, USA) and sequences were confirmed by Sanger sequencing. *AtIRT1*, an Fe transporter having been well characterized from the model plant *Arabidopsis thaliana*, was cloned as a positive control (Eide et al. 1996). These constructs were transformed individually into two yeast mutant backgrounds along with

the empty pDR196 vector as the negative control using the LiAc/SS-DNA/PEG method (Gietz and Schiestl 1995). The yeast wild-type DY1457 (*MATα ade6 can1 his3 leu2 trp1 ura3*) was transformed with the empty pDR196 vector as the positive control. The yeast cells were then used for growth complementation assays on the solid synthetic defined (SD) media without uracil. For selection on metal-limited media, transformants were serially 10-fold diluted and spotted onto SD medium supplemented with 50 µM Fe chelator EDTA and with the pH5.5, 7 and 8, respectively. The spotted yeast cells were photographed after incubating plates for 2 days at 30°C.

Agrobacterium-mediated potato transformation and PCR screening
The method of cloning of candidate genes was described in the yeast complementation assay. Briefly, the coding sequences of Fe candidate genes were amplified from cDNA of Señora Warmi (CIP 701243) (Table A2.5), and verified by Sanger sequencing. Then, the full coding sequence of each gene was cloned into pGEM-T vector (Promega, USA). The genes of interest were subcloned from pGEM-T vectors into *Agrobacterium* binary vector to create the final constructs for transformation.

The plasmid pCAMBIA1305.1 was used as binary vector in this study. It has *hpt*II encoding resistance to hygromycin as selectable marker, and *GUSPlus* reporter gene. Both genes are driven by the CaMV35S promoter. In the present study, the *GUSPlus* gene was replaced with candidate genes. Then the pCAMBIA1305.1 construct carrying each gene of interest was transformed into the competent strain of *Agrobacterium tumefaciens* LBA4404. Andean cultivars CIP703580, CIP703852, CNV7 (a homozygous diploid), DM (double monoploid) and *S. tuberosum* cv. Atlantic (a tetraploid) were used for plant transformation employing a modified protocol based on Beaujean et al. (1998).

Prior to transformation, all cultivar were subcultured as *in vitro* plantlets for 3 weeks on MS basal medium with vitamins, 3% sucrose, 0.7% agar, pH 5.8. The primary transformants were screened on 20 mg/L hygromycin antibiotics in the shoot induction medium, and then regenerants were saved as potential transgenic plantlets for PCR-based validation.

Regenerated shoots from transformation were transferred into the soil and grown in the growth chamber for 1 week before DNA extraction to avoid the *Agrobacterium* DNA contamination. Genomic DNA was isolated from a single expanded leaf of regenerated shoots using a modified CTAB/chloroform-isoamyl alcohol DNA extraction protocol (Doyle 1987). The genotype of putative transgenic plants was confirmed in multiplex PCR, which amplified the hygromycin resistance gene (forward primer HygroF: 5'TATATGCTCAACACATGAGCG3'; reverse primer HygroR: 5'CAAACTGTGATGGACGACACCG3') from the T-DNA region of the binary vector, and a reference gene EF1-α (forward primer StEF-1αF: 5'TGATTGAGAGGTCTACCAACCTTGA3'; reverse primer StEF-1αR: 5'GTTCCTTACCTGAACGCCTGTC3'). Each 25 µl PCR reaction contained: 12.5 µl 2X ImmoMix master mix, 0.5 µl each primer (the original conc. is 10 mM), 1 µl DNA template, and 9.5 µl molecular-grade water. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, 32 cycles at 94°C for 30s, annealing temperature at 55°C for 30s, and extension at 72°C for 1 min, and a final incubation at 72°C for 5 min. Seedlings from which both hygromycin resistant gene and the elongation factor 1-α gene are amplified were identified as putative positive transgenic potato plant.

Genotyping and phenotyping analysis

Micropropagation and quantitative RT-PCR were used for further analysis of positive transgenic potato plants resulted from the PCR screening. The putative transgenic plants were grown in the microtuberization system with six different levels of Fe and Zn nutrient treatments: 27.8 mg/L FeNaEDTA +3.5 mg/L $ZnSO_4$ (NFeLZn: Normal con. of Fe and Low con. of Zn), 27.8 mg/L FeNaEDTA +5.3 mg/L $ZnSO_4$ (NFeMZn: Normal con. of Fe and Moderate con. of Zn), 27.8 mg/L FeNaEDTA +10.6 mg/L $ZnSO_4$ (NFeNZn: Normal con. of Fe and Zn), 9.3 mg/L FeNaEDTA +10.6 mg/L $ZnSO_4$ (LFeNZn), 13.9 mg/L FeNaEDTA +10.6mg/L $ZnSO_4$ (MFeNZn), 13.9 mg/L FeNaEDTA +5.3 mg/L $ZnSO_4$ (MFeMZn). The leaves and roots of each treatment were sampled from three flasks as three replicates for RNA extraction and Fe and Zn concentration measurement. The detail procedure of RNA isolation, cDNA synthesis, qRT-PCR and ICP-AES were performed according to the methods described previously.

Statistic analysis

The data were analyzed by Analysis of variance (ANOVA) to assess differences across treatments. ANOVAs were accomplished using JMP statistical software (SAS Institute Inc., Cary, North Carolina). Means were compared by *t*-test at $p<0.05$ in all cases.

Results

Candidate gene analysis

Cloning and sequencing of amplicons from functional domains of eight candidate genes amplified from four Andean potato cultivars, two with low and two with high Fe and Zn content, revealed the presence of 84 SNPs, 33 synonymous and 41 nonsynonymous, in

exonic regions (Table 2.2). Frequency of SNPs in this limited range of the genome was ten per 1,000 bp for exonic regions and 42 per 1,000 bp for intronic regions, with 66% transitions, 31% transversions and 3% indels (Table 2.2). ANOVA for Fe and Zn association with particular alleles at the nonsynonymous SNP sites resulted in the identification of a single significant SNP associated with Zn content in exon 2 of a zinc ion binding protein (ZIB18122). The SNP resulted in an Arg359Gly substitution within the protein binding domain (HMMPfam). Protein modeling of this substitution confirmed a conformation change that could be associated with an alteration in orientation of Cys residues responsible for ion binding. Individuals with the Arg residue at this position had significantly more Zn (mean = 21.9 mg/g FW) in tubers than those with the Gly residue (mean = 16.7 mg/g FW).

Association mapping

The Infinium 8303 Potato Array was used on a population of 36 Andean potato cultivars. An initial cluster analysis of the population revealed that there were four pairs of duplicate genotypes among the 36 cultivars (Figure A2.1). One of each pair was removed from the population, selecting for retention those with the fewest missing values, leaving a population of 32 for analysis. Of the 8,303 SNPs on the array, filters were applied to remove those of dubious value (1,059 SNPs) according to Felcher et al. (2012) and those with missing values for any genotype (2,683 SNPs). Then SNPs with no polymorphism (211 SNPs) with 31:1 segregation (193 SNPs) and 30:2 segregation (252 SNPs) were removed, leaving 4,524 SNPs for analysis. Each polymorphic SNP was then used as a source of variation in ANOVAs to determine if a particular allelic state was associated with Fe or Zn content of the tubers. Of these, 40 and 37 SNPs (Supplementary Table 1)

were identified as highly significantly ($p<0.001$ for Fe; $p<0.01$ for Zn; $R^2>0.2$) associated with Fe and Zn content in tubers, respectively. The positions of these SNPs on version 4.03 of the potato genome were used to construct ideograms in PhenoGram. Highly significant SNPs for both Fe and Zn were found to be distributed across the genome but formed clusters on particular regions of a linkage group (Figure 2.1); e.g, for Fe, 14 of the 40 (35%) significant SNPs occurred between 35.6 and 51.3 Mb on chr08; for Zn 13 of the 37 (35%) fell between 32.0 and 58.4 Mb on chr03 (Figure 2.1).

The potato ferrome

The 92 genes comprising the Arabidopsis ferrome identified by transcriptional response to Fe deficiency (Schmidt and Buckhout 2011) were BLASTP searched against version 3.4 of the PGSC *S. tuberosum* Group Phureja DM1-3 Proteins to identify candidate orthologs comprising a potato ferrome. In some cases, there was more than a single gene with equivalent homology. In other cases, there were two different Arabidopsis ferrome gene entries that yielded the same ortholog in potato. The expression of candidate potato ferrome genes was examined on the Potato EFP browser and genes with little or no support for expression in roots, stolons or tubers were eliminated. This left a set of 114 candidate genes in potato with equivalency to the Arabidopsis ferrome (Supplementary Table 2). The location of the ferrome genes on draft 4.03 of the potato genome is illustrated in Figure 2.1. Although there were candidate genes across all of the linkage groups, chr01, chr02, chr03 and chr07 jointly accounted for 56% of the ferrome with 21, 14, 13, and 15 candidate genes, respectively.

If we define coincidence of markers with genes as those occurring within 2 Mb, then 31 of 40 (78%) of our highly significant SNPs associated with Fe levels in our population of

32 Andean potato cultivars coincided with 37 of our robust potato ferrome genes (Figure 2.1) on nine linkage groups. A similar analysis of coincidence of highly significant SNPs associated with Zn content in the Andean population and potato ferrome genes yields 18 of 37 (49%) SNPs coinciding with 16 ferrome genes (Figure 2.1) distributed on eight linkage groups. Five of the coincidental ferrome genes for Fe were likewise coincidental for Zn, which may indicate their involvement in a common pathway for metal accumulation. These five genes were distributed on three linkage groups (chr04, chr08 and chr10).

QTL analysis

The QTL positions identified by Mihovilovich et al. (2014) for Fe content in tubers of the Alca Tarma dihaploid population grown at two locations (Huancayo and La Molina) in Peru have been included in the PhenoGram in Figure 2.1. Thirteen significant QTL for mineral concentrations were identified and explained from 7 to 20% of the phenotypic variation for mineral concentration. Coincidence of highly significant SNPs, ferrome gene candidates and QTL regions occurred at four positions in the genome: both arms of chr02, the distal region of chr04 and the proximal region of chr09. These regions encompassed DMG400014936, a Zn/Fe transporter, surrounded by one Zn and two Fe SNPs on the proximal region of chr02; three ferrome genes [DMG400012658 (anthocyanidin 3-0-glucosyltransferase 5DMG); DMG400024972 (conserved gene of unknown function), and DMG00024976 (metal transporter)] coincident with a Zn SNP on the distal region of chr02; DMG400007961 (S-RNase binding protein) and DMG400003342 (cytochrome P450) coinciding with both an Fe and two Zn SNPs on

chr04; and DMG400029195 (ferritin) adjacent to an Fe SNP and on the opposite side of the centromere from a Zn SNP on chr09.

Evaluation of expression and function of candidate genes

Micropropagation under Fe treatments

In a pilot study, the Andean cultivar CIP_703580 with low Fe concentration in tubers showed stunted growth in the micropropagation medium with low Fe (6.9 mg/L) compared with CIP_701243, a relatively high Fe cultivar grown under the same conditions, which suggests the low Fe accumulating cultivar may be more sensitive to Fe deficiency (Figure 2.2). The difference in growth between these two cultivars was still obvious in the higher Fe medium (13.9 mg/L) (Figure A2.2). Since microtuber production was inconsistent among treatments, only the shoots were sent for ICP-AES analysis. The high Fe treatment was eliminated because it was not the condition considered in this study. The result indicated that the Fe concentration in the shoots across treatment is significantly different (p=0.0003) (Figure 2.3). For both cultivars, the Fe concentration was greater in the plants grown in the normal Fe medium (27.8 mg/L), where the high Fe cultivar (CIP701243) accumulated more Fe than the low Fe cultivar (CIP703580). In the low Fe treatment (13.9 mg/L), there was no significant difference between two cultivars.

Identification and confirmation of candidate genes by semi-quantitative RT-PCR
In order to validate the candidate genes detected using SNP genotyping, QTL analysis and Arabidopsis ferrome homologs and to quantitatively evaluate the relative abundance

of the transcripts with possible roles in adaptation to Fe stress, we performed semi-quantitative RT-PCR on 20 candidate genes (Table A2.2). Of the 20 candidates, *PDR13112*, *PMA33034*, *OPT13297*, *CCL28929* and *MTK25861* showed different expression level between low and normal Fe medium in each cultivar in the root, and *SAT18943* showed different expression pattern in the root between two cultivars (Figure A2.3), whereas there is no obvious expression pattern occurred in the microtuber between treatments (Figure A2.4). These six genes with different expression patterns among four treatments were selected for the following quantitative real time PCR analysis (Table A2.3).

Further expression analysis by qRT-PCR

The expression level of nine potential candidate genes was analyzed further by qRT-PCR to validate the results obtained by above-mentioned analyses. The result revealed higher transcript levels of three genes after Fe starvation: metal transporter gene *MT24976*, oligopeptide transporter gene *OPT13297* and germin gene *GER18276* (Table A2.3). The expression of these candidate genes appears to be strongly induced under Fe stress for the low Fe cultivar CIP703580 (Figure 2.4), supporting the above-mentioned result that the low Fe cultivar was more sensitive to Fe starvation. In the present study, there was no significant difference in the transcript levels of the endogenous reference gene, which indicates that the experimental conditions and qRT-PCR analyses were valid. It suggests that *MT24976*, *OPT13297* and *GER18276* could be candidate genes for limited Fe nutrient response.

Validation of the role of candidate genes

Yeast complementation assay

The function of six metal transporter candidate genes was investigated using a yeast complementation assay. As positive controls, DY1457 harboring pDR196 (WT+pDR196) and yeast mutants *fet3fet4* and *zrt1zrt2* containing *AtIRT1*, could grow well on the high pH medium or on the Fe or Zn-limited medium supplemented with 50 µM EDTA (Figure 2.5 A and B, top two rows). In contrast, yeast mutant *fet3fet4* and *zrt1zrt2* transformed with empty vector pDR196 used as a negative control, were not able to grow well on the same medium (Figure 2.5 A and B, third row). Of six metal transporter candidate genes, *IRT21155*, *IRTunannotated* and *MT24976* complemented the growth defect of Fe uptake deficient yeast mutant, while *MTP32189* improved *fet3fet4* growth somewhat on a medium without Fe chelator EDTA under pH5.5. Only *IRTunannotated* restored the ability of *fet3fet4* to grow on minimal medium supplemented with 50 µM EDTA as efficiently as *AtIRT1*, whereas as pH increased, it was not able to rescue Fe uptake mutant yeast. Likewise, *IRT21155* and *IRTunannotated* also restored Zn uptake in the yeast mutant *zrt1zrt2* on medium at pH=5.5 and pH=7 but not at pH=8 and 50 µM EDTA conditions. Expression of different alleles of *MT24976* and *MTP32189* genes did not show differences in growth of transformed yeast mutants. These results suggest that *IRT21155* and *IRTunannotated* are able to transport both Fe and Zn, whereas *MT24976* is able to transport Fe in the yeast.

Plant transformation

The contribution of each candidate gene needs to be clarified for the involvement in Fe homeostasis. Genetic transformation was used as the ultimate way to validate the role of

a candidate gene in the present study. Seven constructs were built for seven candidate genes for *Agrobacterium*-mediated transformation in potato using primers in Table A2.5. The construct for gene *MT24976* and *OPT13297* could not be transformed into *Agrobacterium tumefaciens* successfully since no colony survived on the selection medium when both electro-transformation and heat shock procedure were tried. A construct (empty vector) having no *GusPlus* gene was used as negative control for Fe gene transformation. Firstly, Andean cultivars with low (CIP_703580) and high (CIP_703852) Fe content in tubers were used as plant material, but few shoots regenerated because of *Agrobacterium* overgrowth on the leaf explants. Increasing the time of wash-off and the amount of antibiotics did not solve the problem. Therefore, CNV7 was used as a replacement genotype because it is a homozygous diploid with low Fe content and has a rapid life cycle, which is a good resource for plant transformation. DM, the double monoploid used for potato genome sequencing was also used. However, the procedure failed due to the severity of browning or necrosis of *Agrobacterium*-infected plant leaves. Adding lipoic acid, a unique plant transformation enhancer, did not improve the transformation efficiency. Finally, the tetraploid cv. Atlantic, grown commercially throughout North America, was also used for transformation.

A laborious transformation protocol was conducted on 117 plates of transformation involving thousands of primary leaf explants. Hundreds of shoots were regenerated surviving on the selection shoot induction medium (Table 2.3). However, all positive transgenic plants regenerated from cultivars CIP_703852, CNV7 and DM only contained the empty vector with the exception of one transgenic plant containing the Fe gene construct. Lots of regenerated shoots from Fe gene construct died after subculture on MS

basal medium. The regeneration rate of cultivars CIP703580, CIP703852, CNV7 and DM was low. The tetraploid cultivar 'Atlantic' regenerated more than 200 shoots but most of them died when subcultured on MS basal medium with 20 mg/L hygromycin. The multiplex PCR confirmed that the transgenic plant (s342) containing the Fe construct carries *ZIP18122* (Figure 2.6). The transgenic plant and the wild type including transgene donor CIP701243 and background CIP703852 were exposed to hygromycin antibiotics to evaluate growth responses. The WT plants could not survive on the selection medium but the transgenic plant did not exhibit any signs of damage (Figure 2.7).

The transgenic positive line harboring the ZIB18122 construct was further validated by regular PCR using a set of primers that cross both regions of the binary vector and transgene. The transgene was amplified in the transgenic line (Figure 2.8). The bands generated from primer (forward 20ZIBF: 5'CTCCAATGAAGCTGTAGATGCC3', reverse p1305.1R: 5'TATGATAATCATCGCAAGACCG3') and primer (HygroF and HygroR) were extracted from the gel and sent for sequencing; the sequence matched that of the construct pCambia1305.1 containing *ZIB18122*.

Genotyping and phenotyping analysis

Six different levels of Fe and Zn nutrient treatments in the microtuberization system were set up to investigate the effect of overexpression of Fe candidate gene *ZIB18122* on the Fe and Zn concentration of the transgenic line. Three of six treatments with limited Fe nutrient in the medium impaired the growth of plants, which could not provide enough data for statistical analysis. Another three treatments with full Fe nutrient in the medium were included for sampling. ICP-AES analysis of leaf and root showed no significant difference on the Fe and Zn concentration between wild type and transgenic plants in

each treatment (Table A2.6). The relative expression levels of selected candidate genes and the transgene were analyzed in the transgenic plant and the wild type. No significant transcript level differences were detected in the leaf and root for all tested genes except the gene *MT24976* in the root in the treatment with full Fe and moderate Zn in the medium. The transcript level of transgene *ZIB18122* was higher in the transgenic line but not statistically significant.

Discussion

Our nine initial candidate genes selected for cloning and sequencing in high and low Fe/Zn selections from the Andean cultivars had various levels of support from the literature. The nicotianamine synthase (*NAS*) gene plays a central role in Fe uptake in plants, chelating Fe^{2+} and involved in Fe transport (Ling et al. 1999). Expression of *NAS* is influenced by Fe deficiency although in a study of gene regulation in potato on media supplemented with various levels of Fe, Legay et al. (2012) found that expression of *NAS* was only elevated in stems of plants grown on low Fe medium and remained unchanged in roots and leaves. Our bias, therefore, was that a more efficient *NAS* in stolons, as modified stems, would result in improved transport to and hence enhanced content of Fe in tubers. Only a single gene *NAS02800* annotated as NAS is found in the DM genome and *NAS02800* is the only hit when the *OsNAS2* amino acid sequence is BLAST searched against the DM genome. *OsNAS2* has been used in conjunction with *SferH-1* (ferritin) to boost Fe and Zn concentration in rice, a Strategy II plant (Trijatmiko et al. 2016). Although we identified 13 nonsynonymous SNPs in exons of *NAS02800* in our Andean potato cultivars, we had no support for the association between allelic state in nonsynonymous SNPs and Fe content in tubers. Benke et al. (2015) likewise found no

association between polymorphisms in *NAS* genes of maize with phenotypic response to Fe deficiency.

Of our candidate genes, e.g., zinc ion binding proteins, were more conjectural, as there are dozens of genes with this annotation in the potato genome and they are involved in many different cellular functions. Although the expression of several zinc ion binding proteins has been associated with abnormal Zn uptake in mutant Arabidopsis seedlings resistant to kanamycin (Mentewab et al. 2014), the potato orthologs to these Arabidopsis genes were not among the four zinc ion binding genes that we examined. Ironically, it was one of the zinc ion binding genes *ZIB18122* that yielded a nonsynonymous SNP that was associated with Zn accumulation in our Andean cultivars. In addition, the transcript expression level of this gene in both low and high Fe cultivar was not affected by Fe deficiency in the micropropagation system. The constitutive expression of *ZIB18122* did not have any significant influence on the concentration of Fe and Zn in potato plant leaf and root.

The lack of solid phenotypic data for a sufficient number of accessions severely limited the association analysis. In addition, relationships among the different Andean cultivars (Figure A 2.1) may also have compromised the analysis. However, despite these limitations, the highly significant SNPs for Fe and Zn frequently coincided with ferrome orthologs and QTL regions from the Alca Tarma population (Figure 2.1). Another factor in relating QTL regions with SNPs lies in the genetic differences between the Andean population and the more limited Alca Tarma dihaploids. As the Alca Tarma dihaploids were restricted in polymorphism to whatever was present in the tetraploid cultivar, the

association mapping population was more diverse and may have had SNPs present in chromosomal regions that were homogeneous in the dihaploids.

Many of the genes that have been included in the Arabidopsis ferrome have been verified as differentially regulated in a microarray analysis of Fe-deprived tomato seedlings (Zamboni et al. 2012). In Arabidopsis, two major transcription factors, *FIT* (*FER-like iron-deficiency-induced bHLH transcription factor*) and *PYE* (encoding *bHLH transcription factor*), along with 30 co-expressed genes included in the *FIT-* and *PYE-* modules, have been implicated in the Fe stress response (Li et al. 2015). The potato ortholog of *FIT* (PGSC0003DMG400019017) occurs on the distal arm of chr06, towards the centromere, with no QTL or significant SNP support. The potato ortholog (PGSC0003DMG400000599) most similar to *PYE* (At3g47640) occurs on the distal end of chr03 between three Fe and three Zn SNPs as well as ten other ferrome genes (Figure 2.1). The maize ortholog of the At3g47640 was likewise differentially expressed under Fe limitation (Urbany et al. 2013), suggesting the importance of this transcription factor for Fe metabolism in Strategy II as well as Strategy I plants. Genes that are differentially expressed in response to Fe deficiency are certainly implicated in Fe metabolism in plants; however, whether or not they significantly contribute to Fe accumulation in potato tubers under non-stress conditions is unknown.

Three differentially expressed transcripts by comparing roots of Fe-deficient and Fe-sufficient potato plants confirmed the involvement of the well-known transporter genes in Fe accumulation. The qPCR result was in good agreement with profiles shown in the PhenoGram. The metal transporter gene *MT24976* and the oligopeptide transporter gene *OPT13297* were observed to be up-regulated under Fe shortage. *MT24976* is the ortholog

sharing 88% of homology with At5g67330 (*Atnramp4*) and AT2G23150 (*Atnramp3*) in Arabidopsis and locates in the Zn QTL region and between two Fe and one Zn SNPs (Figure 2.4). It belongs to NRAMP transporters (natural resistance-associated macrophage protein), a broad family of membrane proteins, which were reported for transporting manganese (Mn), Fe and cadmium (Cd) (Ishimaru et al. 2012a; Ishimaru et al. 2012b). It was reported that *Atnramp1*, *Atnramp3*, and *Atnnramp4* are expressed at greater levels upon low Fe supply in plants (Thomine et al. 2000a; Curie et al. 2000). The tomato ortholog of *Atnramp3* was induced by Fe deficiency in roots and is expressed in roots and leaves (Bereczky et al. 2003). *OPT13297* shares 91.7% similarity with AT4G16370 (*AtOPT3*) and locates on chr011. Fe-NA complexes could be transported both in tomato and Arabidopsis plants through OPTs under Fe limitation. AtOPT3 in Fe-deficient Arabidopsis roots (Yang et al. 2010; Buckhout et al. 2009). Zhai et al. (2014) reported that AtOPT3 is a plasma membrane transporter loading Fe into the phloem. It could regulate both shoot-to-root Fe signaling and Fe redistribution from mature to developing tissues. Germin gene *GER18276* shares 80% homology with AT1G09560 and locates on chr01 close to one Zn SNP. Germin has been shown to associate with the defense system (Berna and Bernier 1999; Donaldson et al. 2001), hormonal stimuli (Berna and Bernier 1997) and abiotic stresses, including drought, cold and salt (Bray 2004; Davidson et al. 2010), but so far no detailed information about the relationship between germin and Fe related activities has been reported. However, these three candidate genes may play roles in potato responding to Fe limitation.

The *IRT1* region of the potato genome is incompletely annotated. BLAST searching the *SlIRT1* nucleotide sequence against the potato genome resulted in 21 hits within the

region of chr02 between 28,265,610 and 28,574,280. The annotation within this region encompasses three putative *IRT1* and two *IRT2* genes; however, 14 of the BLAST hits fell in regions without gene annotation and the remaining seven coincide with four of the five annotated genes. One of the *IRT1* annotated gene sequences did not align with sequence of *SLIRT1*. Because highly significant e-values indicated that several of our hits occurred in unannotated regions, we developed *IRT1unannotated* (chr02:28274200..28275700) as a gene model for testing in our yeast bioassays. The ability of *IRT1unannotated* to complement yeast growth on both Fe and Zn deficient media verifies its functionality and elevates it to one of two functional IRT paralogs in the genome (along with *IRT21155*).

Manipulating gene expression through transgenic transformation was used as an ultimate way to determine the role of candidate gene. Constitutive over-expression of five Fe candidate genes on five types of potato cultivars only resulted in one positive transgenic plant that contained the construct carrying the *ZIB18122* gene clones from xxx. However, constitutive expression of *ZIB18122* did not elevate the transcript level of *ZIB18122* and other putative Fe candidate genes. It also did not increase Fe and Zn content in leaves or roots. The reason of this situation can be various. Firstly, *ZIB18122* was not found in the Fe or Zn QTL regions, nor in the potato ferrome, even though a single significant SNP resulting in an Arg residue in the protein coincided with significantly more Zn in tubers. Secondly, when we did gene cloning the PCR-induced recombination for two alleles from *ZIB18122* was observed since the sequences from 40 colonies containing *ZIB18122* gene showed more than two patterns. Thus the sequence that we selected for plant transformation might be not a real coding sequence of *ZIB18122* gene so the function of

this gene might be impacted.

Lots of regenerated shoots produced from putative Fe transporter gene construct transformation survived on the shoot induction medium with 20 mg/L hygromycin but could not root and then died after transfer to the MS basal medium containing same amount of hygromycin. The most likely reason is the metal toxicity because the CaMV35S promoter used for constitutive driving Fe transporter gene expression might lead to too much metal absorbed or translocated into potato plant tissues (Gustin et al. 2009). Especially, these Fe genes selected for our plant tranformation are orthologs of metal transporter genes (IRT1, NRAMP, MTP, OPT) that have been confirmed in various species to directly transport various metals, such as Fe, Zn, Mn and Cd (Tan et al. 2015), but not like the regulator gene leading to adaptation of gene expression state to the changed environment. Therefore, the tissue specific and inducible promoters could be a better choice for this study. Furthermore, it is worthy to consider the sink and source strategy, which means also increasing the expression of metal storage protein gene such as *Ferritin* while overexpressing the metal transporter genes since then it can store the extra Fe in the plant tissues (Trijatmiko et al. 2016; Xiong et al. 2014; Koetle et al. 2015).

All five cultivars were used in this study for plant transformation exhibited low transformation frequencies. Because potato transformation is genotype-dependent, even well-developed transformation protocols are not adaptable to all genotypes (Dale and Hampson 1995). Especially, the present study indicated that most of the cultivars having *andigena* genetic background were inefficient for transformation. A different protocol with greater amount of zeatin could be attempted (Trujillo et al. 2001). The tetraploid cultivar 'Atlantic' was a good resource for plant transformation in a previous study

(Johnson and Veilleux 2003). It produced lots of regenerated shoots compared with other cultivars in the present study; however, no transgenic plants were observed. Han et al. (2015) recently reported that a long period of *in vitro* culture is a critical factor that may result in low transformation efficiency for *Solanum tuberosum* L. cv. Atlantic. The explants from *in vitro* plants that are subcultured every 3 to 4 weeks for longer than 6 months produced significant fewer transgenic plants compared with the explants from plants induced from microtubers.

In conclusion, metal acquisition, transport and accumulation in tubers of potato plants represent complex processes likely to involve many genes that are influenced by various environmental factors, as evidenced by the different QTL regions identified in the Alca Tarma population grown at two different locations. The probability that single amino acid substitutions in implicated genes would have a significant influence on the process is low. Multiple approaches produced some candidate genes occurred in the same regions on the PhenoGram and thus revealed that the potato orthologs of transporter genes (*IRT*, *MT*, *OPT*) in this study might be the elite Fe candidate genes. Yet incremental improvements in the efficiency of several candidate genes identified in this study and their deployment into heavily utilized Andean potato cultivars may lead to the development of biofortified potato with respect to mineral nutrition and concomitant nutritional benefit to those who subsist on them.

Table 2.1 Selected candidate genes for micronutrient acquisition in roots and tubers of Andean potato cultivars used for cloning and sequencing amplicons from two high and two low Andean cultivars with respect to Fe and Zn content in tubers.

Gene Identifier	Gene Name	Annotation	Citation
PGSC0003DMG400027073	ZIB27073	Zinc ion binding protein	(Mentewab et al. 2014)
PGSC0003DMG400025880	ZIB25880	Protein binding /zinc ion binding	
PGSC0003DMG400018122	ZIB18122	Zinc ion binding protein	(Mentewab et al. 2014)
PGSC0003DMG400034304	ZIB34304	Zinc Ion Binding	(Santos et al. 2013)
PGSC0003DMG400022524	IIB22524	Iron ion binding /oxidoreductase	(Farrow and Facchini 2014)
PGSC0003DMG400004697	ISB04697	Ferredoxin, Iron-sulfur binding protein	(Huang et al. 1992)
PGSC0003DMG400003662	IIB03662	Iron ion binding/oxidoreductase	(Farrow and Facchini 2014)
PGSC0003DMG400000002	VIT00002	Vacuolar iron family transporter	(Gollhofer et al. 2014)
PGSC0003DMG400002800	NAS02800	Nicotianamine synthase	(Legay et al. 2011; Ling et al. 1999; Masuda et al. 2012)

Table 2.2 SNP discovery in amplicons of eight candidate metal associated genes from cloning and sequencing selected domains within the genes in four different Andean primitive potato cultivars.

Gene Identifer	Amplicon size (bp)	Exon	Intron	SNPs in exon (S/N)	SNPs in intron	Transitions	Transversions	Indel
PGSC0003DMG400027073	362	285	77	0/4	0	0	4	0
PGSC0003DMG400025880	315	315	0	2/4	na	6	0	0
PGSC0003DMG400018122	575	493	82	5/3	2	7	2	1
PGSC0003DMG400022524	623	340	283	1/2	5	5	1	1
PGSC0003DMG400004697	456	235	221	6/5	17	16	12	1
PGSC0003DMG400003662	390	309	81	6/3	7	14	2	0
PGSC0003DMG400000002	348	348	0	3/7	na	6	4	0
PGSC0003DMG400002800	943	943	0	10/13	ns	15	8	0
Total	4012	3268	744	33/41	31	69	33	3

Table 2.3 Summary for validation of transgenic plants from regenerated shoots in *Agrobacterium*-mediated transformation of potato

Cultivar		Total	Empty vector	Fe gene construct
CIP703580	# of plates	7	3	4
	# of shoots	7	0	7
	positive	0	0	0
	negative	0	0	7
CIP703852	# of plates	42	5	37
	# of shoots	82	10	72
	positive	7	6	**1**
	negative	75	4	71
CNV7	# of plates	25	4	21
	# of shoots	31	8	23
	positive	6	6	0
	negative	25	2	23
DM	# of plates	16	4	12
	# of shoots	8	8	0
	positive	5	5	0
	negative	3	3	0
Atlantic	# of plates	12	0	12
	# of shoots	60	0	60
	positive	0	0	0
	negative	60	0	60

Table A2.1 Primitive potato cultivars used in association studies and candidate gene analysis with their mean Fe and Zn content.

ID	CIP Number	Breeder code or cultivar name	Ploidy	Fe (mg/kg,DW)	Zn (mg/kg,DW)
1	CIP 391058.175	none	4x	17.55	10.53
2	CIP 392025.7	LR-93.221	4x	15.39	26.23
3	CIP 393083.2	none	4x	18.30	18.02
4	CIP 393536.13	95.103	4x	20.91	31.18
5	CIP 393614.3	TXY.3	4x	13.46	15.98
6	CIP 393617.1	TXY.11	4x	14.14	16.54
7	CIP 394613.139	none	4x	15.32	12.59
8	CIP 395446.1	1342	4x	20.74	30.43
9	CIP 399078.11	B1C5033.11	4x	25.00	23.00
10	CIP 399079.22	B1C5035.22	4x	27.00	14.00
11	CIP 701243	Señora Warmi	2x	28.50	26.50
12	CIP 701997	Sullu	4x	22.67	15.16
13	CIP 702588	Wila Ajawiri	2x	27.50	17.50
14	CIP 703265	Yuraq Sole	4x	24.44	18.61
15	CIP 703274	Ch'apiña (same as 705153)	2x	30.82	15.04
16	CIP 703282	Ishkupuru	2x	22.07	20.10
17	CIP 703293	Conga (same as 703594)	2x	22.83	25.10
18	CIP 703294	Unknown	2x	17.84	23.85
19	CIP 703312	Morada Turuna	2x	26.00	24.50
20	CIP 703324	CPY-143-3	2x	18.50	26.89
21	CIP 703594	Unknown (same as 713293)	2x	24.92	22.26
22	CIP 703596	Mambera Morada o Mortena	2x	16.17	13.70
23	CIP 703597	Unknown	2x	27.50	22.00
24	CIP 703693	Unknown (same as 704068)	2x	25.00	23.00
25	CIP 703777	Shumaqperqa	2x	21.00	16.50
26	CIP 704068	Alcca Coillu (same as 703693)	2x	24.50	25.00
27	CIP 704120	Puka Chaucha	2x	21.54	21.13
28	CIP 704203	Chaucha Naranjilla Rosada (same as 704205)	2x	17.25	20.66
29	CIP 704205	Chaucha Tomate (same as 704203)	2x	19.70	22.36
30	CIP 704393	María Cruz	2x	16.50	13.50
31	CIP 704797	Laram Pitu Huayaca	2x	19.50	28.50
32	CIP 705153	Unknown (same as 703274)	2x	18.27	23.52
33	CIP 705607	Urpicha	2x	18.50	32.48
34	CIP 705806	Unknown	2x	23.79	11.77
35	CIP 706822	Cuica	2x	22.15	21.50
36	CIP 395112.32	Unknown	2x	18.09	16.13
37	CIP 703825	China Runtush	2x	26.00	18.00
38	CIP 703580	Unknown	2x	19.85	16.41
		Max		30.82	32.48
		Min		13.46	10.53
		Mean		21.30	20.53

Table A2.2 Candidate genes selected for semi-quantitative RT-PCR

Gene Identifier	Putative Function	Primer Name	sequence 5'--3'
PGSC0003DMG401013112	Pleiotropic drug resistance protein 1	13112F	CACAGCTTTGATGGGTGTTAGT
		13122R	TGAATGGTACAGACGACAGTTCTT
PGSC0003DMG400033034	Plasma membrane H+-ATPase	33034F	ATGCAGCAGCTGCTCTCATG
		33034R	TTGTCACTACAAAGCACGTCCAT
PGSC0003DMG400024754	Respiratory burst oxidase homolog protein B	24754F	ATGGAGCACCAGCACAGGAT
		24754R	CCTTCTTCATAAACGCTGGTGC
PGSC0003DMG400013297	Oligopeptide transporter 3	13297F	GTCATTCAAGCGACTACCAATCA
		13297R	TGTTCCAGCATCCAACGCA
PGSC0003DMG400028929	4-coumarate--CoA ligase 2	28929F	TCACAAGTGTGGCACAACAAGTT
		28929R	AAATGCCAAGCACATAGCCAA
PGSC0003DMG400024976	Metal transporter/NRAMP3	24976F	GGCTATCGCTGGGTATTCTTT
		24976R	TGTCCAGCATAAGTCCCAGTAAT
PGSC0003DMG400025316	ATP binding protein	25316F	GGCAAAGAACTTGCTGTGAAGAT
		25316R	CAAGTAACCGAAGGTTCCAGCTA
PGSC0003DMG400018943	Sulfate adenylyltransferase	18943F	CAGACGCTGTGTTTGCTTTTCA
		18943R	GCATGCCATTGGACTTCTGTT
PGSC0003DMG400020116	Wound-inducible carboxypeptidase	20116F	TCGATGGCTTTGTCTATGAGCAT
		20116R	AGTTGGCACGTATATTCCAGCAT
PGSC0003DMG401016246	Latex cyanogenic beta glucosidase	16246F	ACTGAGAATGGTATGGATGATGAAG
		16246R	GACCGAAGCGTTTTGTATAACCA
PGSC0003DMG400000184	Ferric-chelate reductase	184F	GCAATGGCATCCTTTTACAATCA
		184R	GAGATGAAAGGGGTAATTCCACTT
PGSC0003DMG400025861	Methylthioribose kinase	25861F	TGTGGAAACGTCGAGTTGTGTAG
		25861R	TGAGCCACTCCAACTATTCTCCTT
PGSC0003DMG400006816	ATP binding protein	6816F	CCAGAGGAAGCTTAGAAGAGAAT

			CT
		6816R	GTACTCAGGAGCCAAGTAGCCAA
PGSC0003DMG400031816	Pectinesterase	31816F	ACCATTATTACGGGCAATCATAGC
		31816R	TCGCATTCTCTGTAAAATTGCCTT
PGSC0003DMG400026549	Amino acid transporter	26549F	CACAATTGTTGGTGCAGGAATCA
		26549R	CCAGAGAGCACGTCACCAATTATA
PGSC0003DMG400006866	DNA-damage-inducible protein	6866F	GCAGTCCTTCTTTCTTTGGCTATG
		6866R	CCTGTCCTGCTACAGCTAATCCAT
PGSC0003DMG402003297	Protein zinc induced facilitator-like 1	3297F	CTGATCGATATGGACGAAAACCA
		3297R	CTGAGCAAAGAAGCCTCCTATAGC
PGSC0003DMG400020364	Receptor protein kinase	20364F	TGCTTTGGCTTGATGAAGAACAG
		20364R	CATGGCTTGGAGGAATATGCTTT
PGSC0003DMG400018122	Zinc ion binding protein	ZIB18122F	TGTATGGGTGCTTATCCAGGTC
		ZIB18122R	GATGCTTCCGTTGTTCTTCTTC
PGSC0003DMG400032189	Metal tolerance protein	32189F	CTGCATCTACATTGGATTCGTT
		32189R	ACAAAGTACAAGACACCAAATGTG

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Table A2.3 Candidate genes selected for quantitative RT-PCR

Gene Identifier	Gene Name	Putative Function	Primer Name	sequence 5'--3'
PGSC0003DMG400018276	GER18276	Germin	qPCR18276F	TCCAGACATGCTTCAAGATG
			qPCR18276R	TGCAAAGATAACCGTTTACTG
PGSC0003DMG400024976	MT24976	Metal transporter/NRAMP3	qPCR24976F	TGCGTGGATCTTTGGAGAGA
			qPCR24976R	CACTGCCTGCTTTATTGTCTT
PGSC0003DMG401013112	PDR13112	Pleiotropic drug resistance protein 1	qPCR13112F	TTCATTGCACTTGTCTTTGG
			qPCR13112R	GAGAGTTGCAGCATACATAG
PGSC0003DMG400033034	PMA33034	Plasma membrane H+-ATPase	qPCR33034F	CAAGTTCTTAATCAGATATGC
			qPCR33034R	CATTGAAGCTCACGTTGT
PGSC0003DMG400013297	OPT13297	Oligopeptide transporter 3	qPCR13297F	TCATTCAAGCGACTACCAAT
			qPCR13297R	CCTGGGAGGATATAACCAAT
PGSC0003DMG400028929	CCL28929	4-coumarate--CoA ligase 2	qPCR28929F	TCAAGTGGCTCCTGCTGAA
			qPCR28929R	CACTGGAACTTCTCCTGCTT
PGSC0003DMG400018943	SAT18943	Sulfate adenylyltransferase	qPCR18943F	GCAGATGATGTTCCACTTGA
			qPCR18943R	GCATAGTGCATAGGAGATGG
PGSC0003DMG400025861	MTK25861	Methylthioribose kinase	qPCR25861F	TTTGAGGACACCCTTGGATT
			qPCR25861R	AGCTCGTTTGGCAACATCCT
PGSC0003DMG400026549	AAT26549	Amino acid transporter	qPCR26549F	TGCTGTTTGCATTGGGTTCA
			qPCR26549R	GATCTTGTCCTTCTTTGTTG

| PGSC0003DMG400018122 | ZIB18122 | Zinc ion binding protein | qPCR2018122F | AGGTGTTCGCTGTGATGGTT |
| | | | qPCR2018122R | TGCTGCAGAGATCATAGTTC |

Table A2.4 Candidate genes selected for functional analysis by yeast complementation assay

Gene Identifier	Gene Name	Putative Function	Primer Name	Sequence 5'--3'
PGSC0003DMG400024976	MT24976/(MT-1, MT-2)	Metal transporter/NRAMP3	XmaI24976F2	tccCCCGGGATGCCTCTACACGATGAAGAA
			XhoI24976R2	cccCTCGAGTCAATTATCTATGCTTGAGCTACT
PGSC0003DMG400010373	IRT10373	Iron-regulated transporter 2	XmaI10373F2	tccCCCGGGATGGCGTTTTTCTTCGCAGTAAC
			XhoI10373R2	cccCTCGAGCTAGGCCCAAAT**T**GCCATGACT
PGSC0003DMG400010369	IRT10369	Iron transporter protein IRT1	XmaI10373F2	tccCCCGGGATGGCGTTTTTCTTCGCAGTAAC
			XhoI10373R2	cccCTCGAGCTAGGCCCAAAT**T**GCCATGACT
PGSC0003DMG400021155	IRT21155	Iron transporter protein IRT1	XmaI21155F2	tccCCCGGGATGGCTTCTTCTAATTCCAAGATTATC
			XhoI21155R2	cccCTCGAGTCAAGCCCATTTGGCCATTAG
chr02:28274200..28275700	IRTunannotated	Iron transporter protein homolog	PstIBlankF2	gggCTGCAGATGGCTAATTATAATATCAAGTACATCG
			SalIBlankR2	gagGTCGACTTAAGCCCAAATTGCCATGAC
PGSC0003DMG400032189	MTP32189/(MTP-1, MTP-2)	Metal tolerance protein	XmaI32189F	tccCCCGGGATGGAGGGAGAAGAAATCAAGA
			SalI32189R	gagGTCGACCTAAGGTTCAGTGTTTGGCA
AT4G19690.2	AtIRT1	Iron transporter protein IRT1	XmaIAtIRT1F	tccCCCGGGATGGCTTCAAATTCAGCACTTC
			XholAtIRT1R	cccCTCGAGTTAAGCCCATTTGGCGATAATC

Table A2.5 Candidate genes selected for *Agrobacterium*-mediated transformation

Gene Identifier	Gene name	Putative Function	Primer Name	Sequence 5'--3'
AT4G19690.2	AtIRT1	Iron transporter protein IRT1	BglIIAtIRT1F	ggtAGATCTGATGGCTTCAAATTCAGCACTTC
			BstEIIAtIRT1R	gctGGTNACCTTAAGCCCATTTGGCGATAATC
PGSC0003DMG400018122	ZIB18122	Zinc ion binding protein	NcoI18122F	ccATGGCTATGGAGTCATCTATTGTGAT
			BstEII18122R	GGTNACCCTACTGCTCTCCAGCAATAAG
PGSC0003DMG400032189	MTP32189/(MTP-1, MTP-2)	Metal tolerance protein	NcoI32189F	ccATGGAGGGAGAAGAAATCAAGA
			BstEII32189R	GGTNACCCTAAGGTTCAGTGTTTGGCA
PGSC0003DMG400021155	IRT21155	Iron transporter protein IRT1	BglII21155F	AGATCTGATGGCTTCTTCTAATTCCAAGATTATC
			BstEII21155R	GGTNACCTCAAGCCCATTTGGCCATTAG
chr02:28274200..28275700	IRTunannotated	Iron transporter protein homolog	BglIIBlankF	AGATCTGATGGCTAATTATAATATCAAGTACATCG
			BstEIIBlankR	GGTNACCTTAAGCCCAAATTGCCATGAC
PGSC0003DMG400024976	MT24976	Metal transporter/NRAMP3	NcoI24976F2	tgaccatggtaATGCCTCTACACGATGAAGAA
			BstEII24976R2	gctGGTNACCTCAATTATCTATGCTTGAGCTACT
PGSC0003DMG400013297	OPT13297	Oligopeptide transporter 3	BglII13297F	ggtAGATCTGATGTCCTTAAAAAACTCCGTCC
			BstEI13297R	gctGGTNACCCTATTTGAAAACAGGACATCCTT

Table A2.6 ANOVAs for ICP-AES analysis of leaf and root of transgenic plant and wild type from three treatments in the micropropagation system. Three replications were placed in a RCB design.

Treatment*	Tissue		Fe con. mg/kg	Zn con. mg/kg
NFeLZn	leaf	mean (WT)	901.75	1174.68
		mean(transgenic)	706.87	1364.53
		p-value	0.38	0.65
	root	mean (WT)	677.52	1472.94
		mean(transgenic)	808.01	2312.38
		p-value	0.75	0.42
NFeMZn	leaf	mean (WT)	549.22	869.47
		mean(transgenic)	665.24	1077.96
		p-value	0.33	0.56
	root	mean (WT)	1902.65	2088.39
		mean(transgenic)	1136.09	2192.48
		p-value	0.33	0.89
NFeNZn	leaf	mean (WT)	564.86	989.12
		mean(transgenic)	465.76	1334.82
		p-value	0.64	0.42
	root	mean (WT)	1534.64	3804.70
		mean(transgenic)	1282.86	5626.39
		p-value	0.57	0.23

* NFeLZn: 27.8mg/L FeNaEDTA +3.5mg/L ZnSO4; NFeMZn: 27.8mg/L FeNaEDTA +5.3mg/L ZnSO4; NFeNZn: 27.8mg/L FeNaEDTA +10.6mg/L ZnSO4.
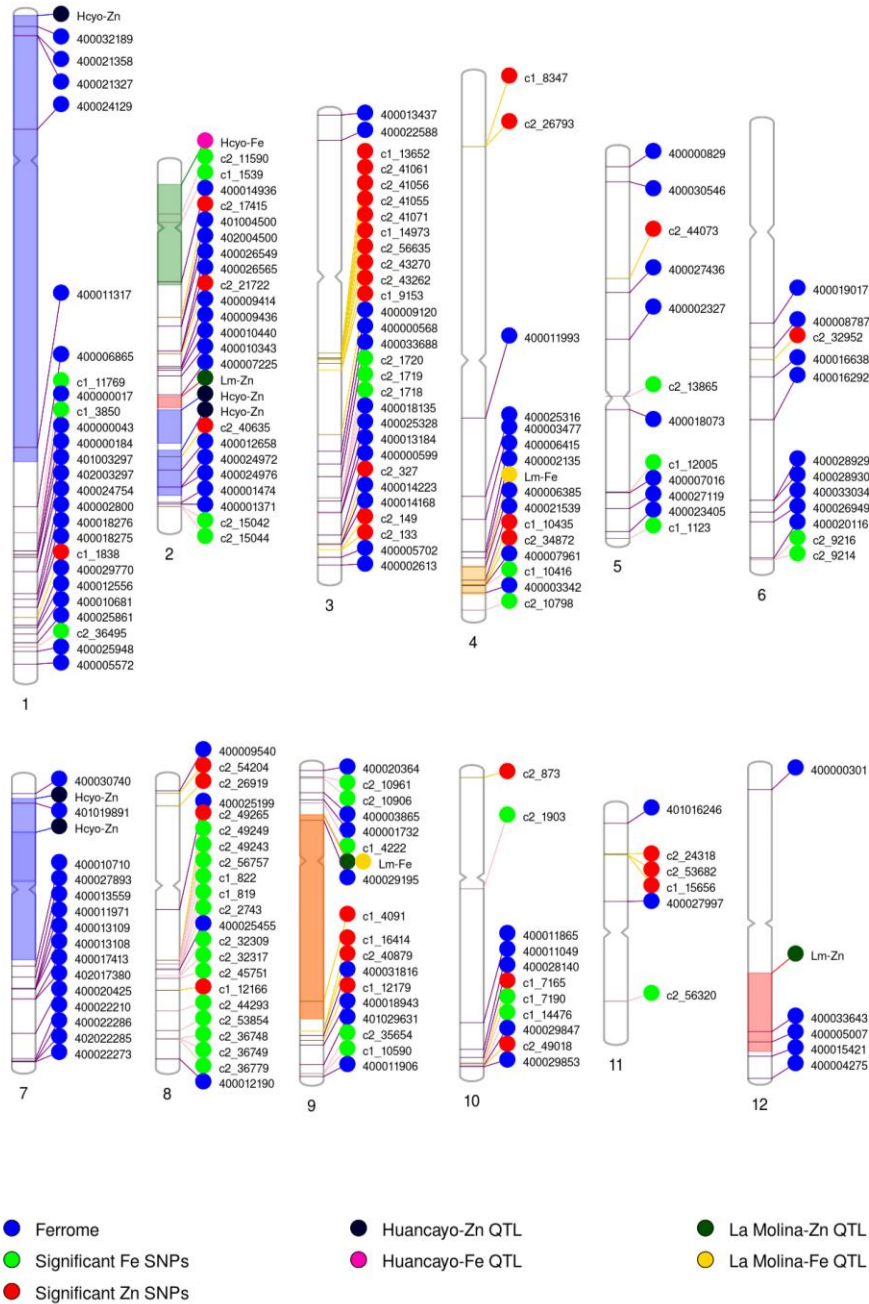
Figure 2.1 PhenoGram of the potato genome with significant QTL regions from a study of Fe and Zn variation in 173 dihaploids of Andean cv. Alca Tarma at two locations (Huancayo and La Molina) in Peru, 40 and 37 highly significant SNPs from association mapping of Fe and Zn variation of 32 Andean potato cultivars grown at two locations and submitted to the Illumina 8303 Potato microarray, and the 114 potato orthologs corresponding to the Arabidopsis ferrome.
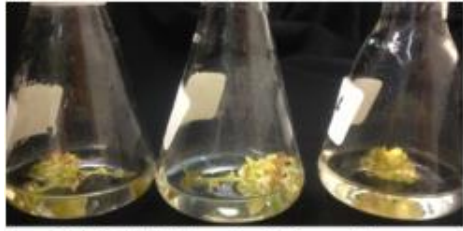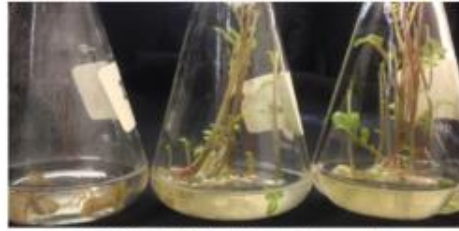
Q47417(low);6.9mg/L   Q47420(high);6.9mg/L

Figure 2.2 3 weeks of growth of Andean potato cultivars with low Fe content (CIP703580/Q47417) and high Fe content (CIP701243/Q47420) in the micropropagation medium with 6.9 mg/L Fe.
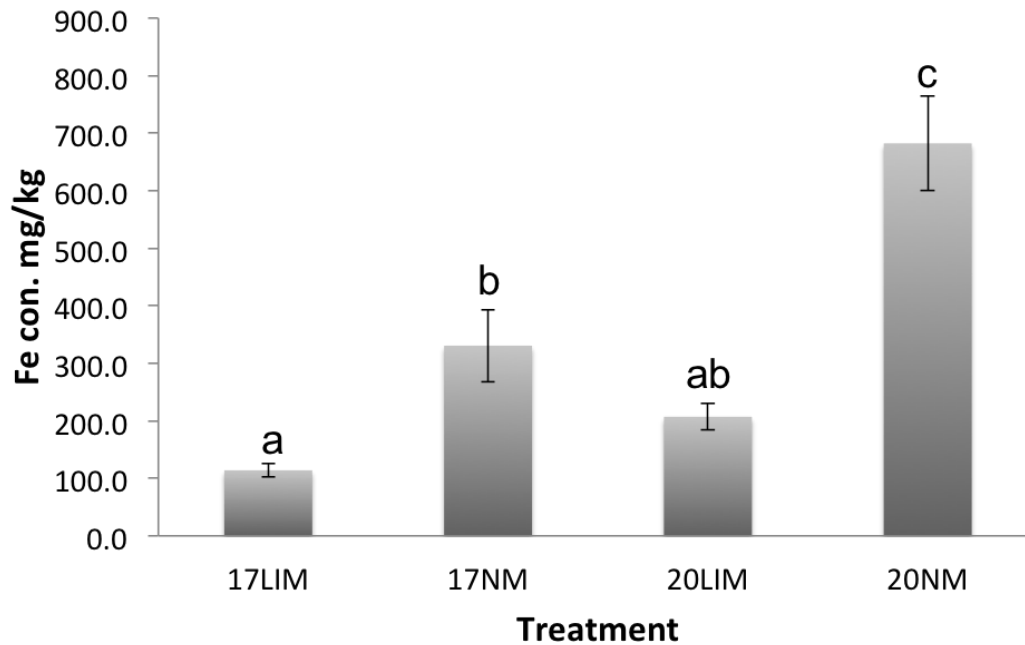
Figure 2.3 Comparison of Fe concentrations in shoots harvested from two Andean potato cultivars. 17: CIP703580; 20: CIP701243 after x weeks on Fe limited or Fe sufficient medium. LIM: low Fe medium with 13.9 mg/L FeNaEDTA; NM: Normal Fe medium with 27.8 mg/L FeNaEDTA. Letters a, b and c indicate statistically significant differences by *t*-test: $P < 0.05$.
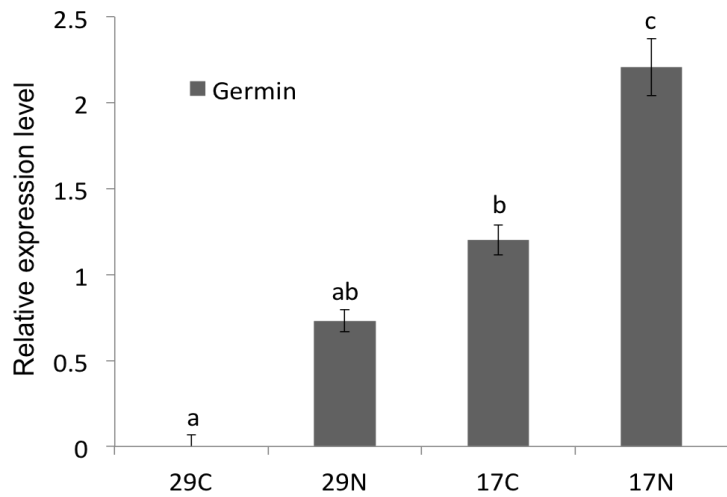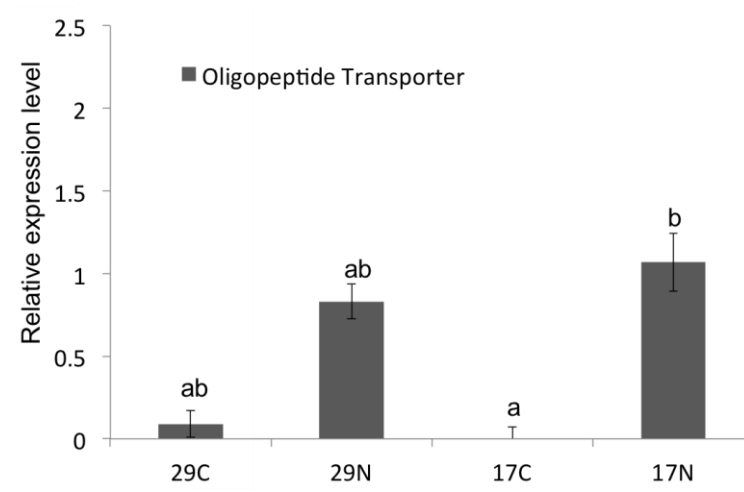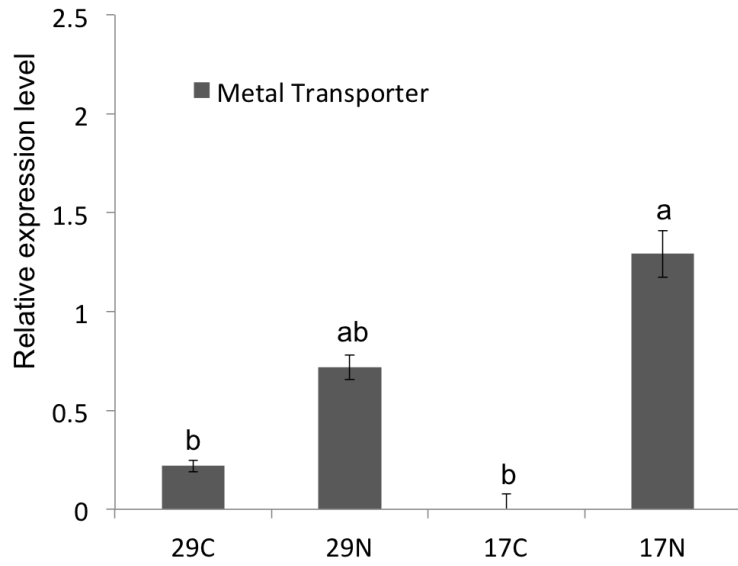
Figure 2.4 Expression levels of *MT24976*, *OPT13297* and *GER18276* in the roots of two Andean cultivars. 29: high Fe cultivar (CIP703825); 17: low Fe cultivar (CIP703580). C: the control medium with normal Fe concentration; N: the medium without adding Fe. Error bars indicate the standard deviation. Letters a, b and c indicate statistically significant differences by *t*-test: $P < 0.05$.

A
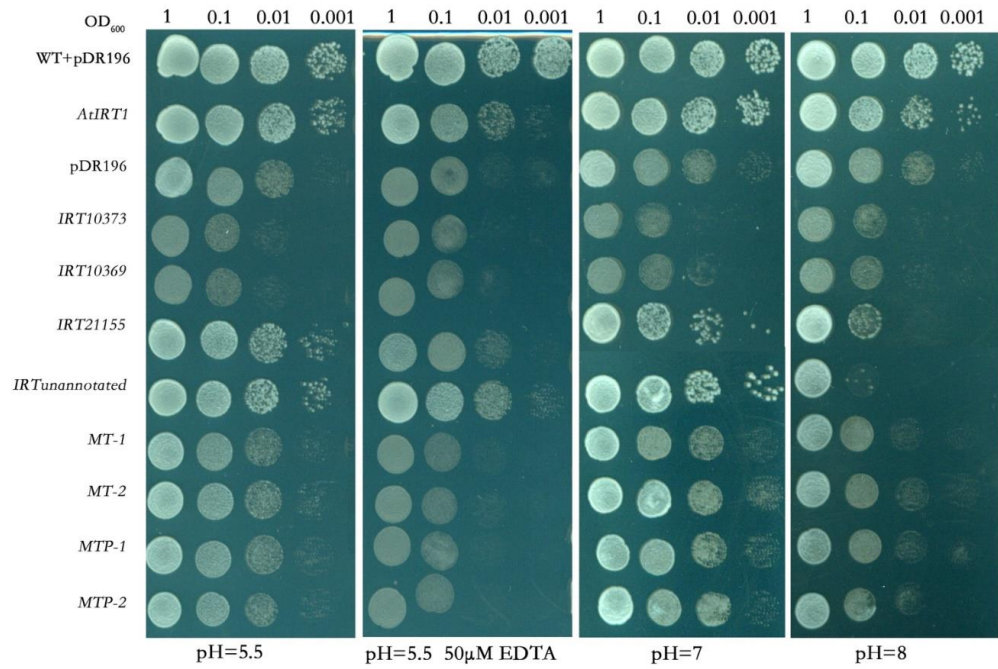
B
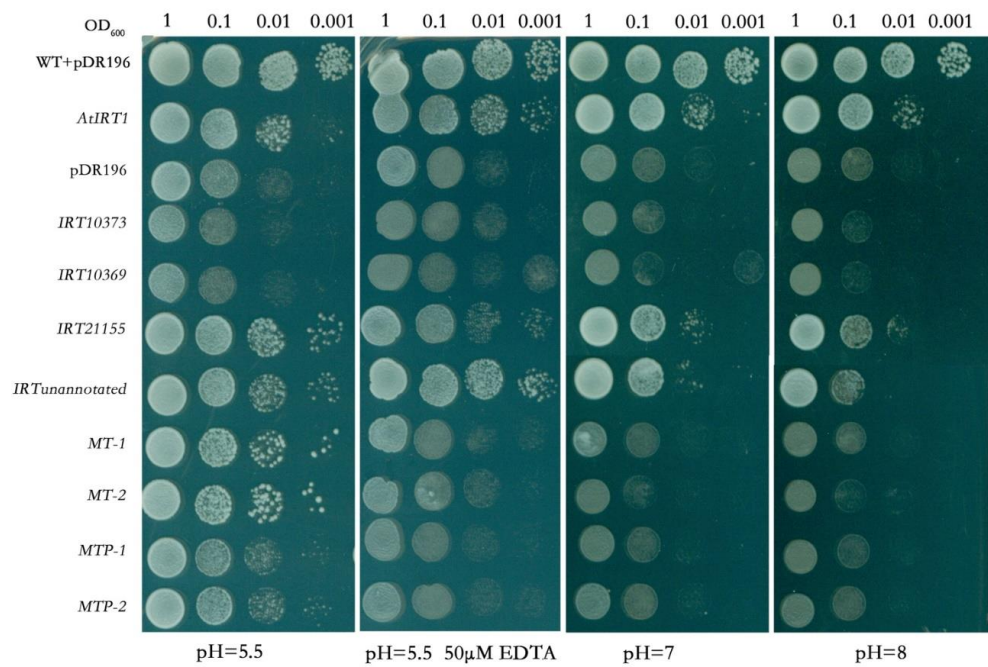
Figure 2.5 Yeast growth on the plate with different pH, Fe and Zn supply conditions. The wild type, the *fet3fet4* mutant (A) and *zrt1zrt2* mutant (B) were transformed with the empty vector (WT+pDR196 and pDR196, respectively), and with potato *IRT* genes,

IRTunannotated, two alleles of *MT*, two alleles of *MTP* genes and Arabidopsis *IRT* gene. Dilution series are indicated on top.
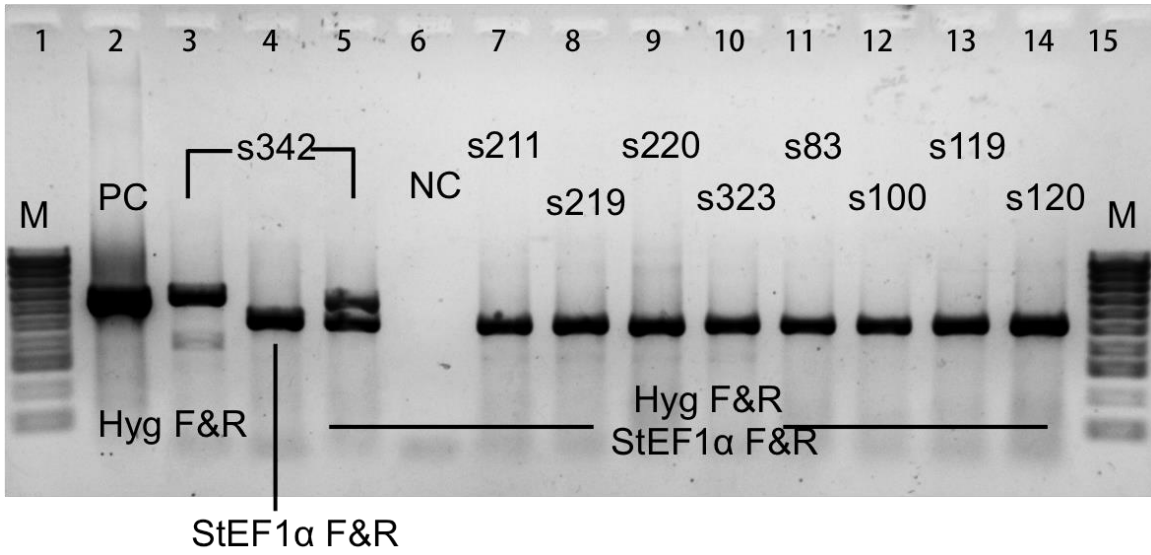
Figure 2.6 PCR analysis of DNA extracted from part of regenerated shoots. Labels above bands are for DNA templates (PC is the positive control, NC is the negative control). Labels below bands are for primers used (Hyg F&R amplify hygrimycin resistant gene, StEF1α F&R amplify the reference gene EF1-α). s342 is the transgenic plant transformed with ZIB18122 gene.
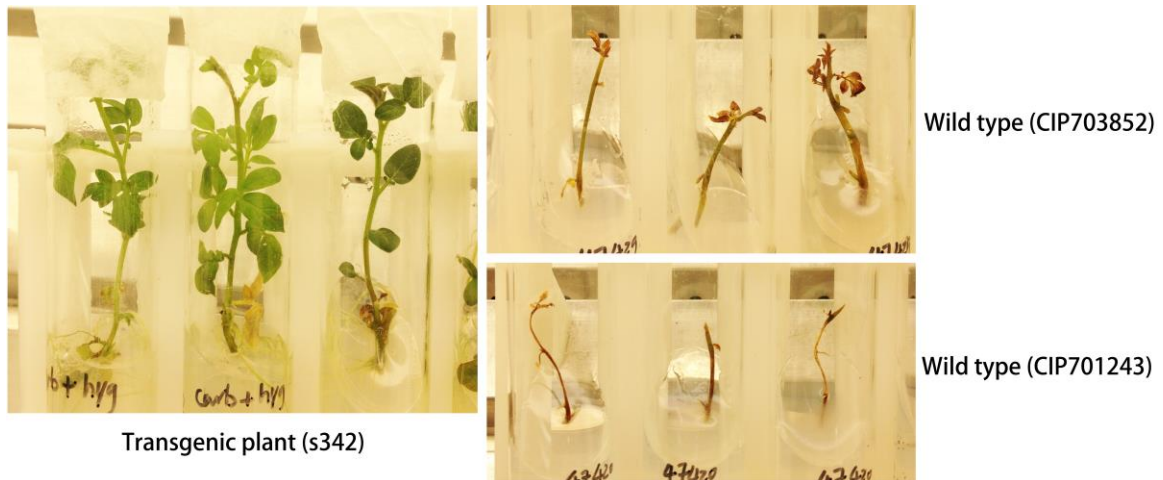
Figure 2.7 The comparison of growth of transgenic plant and wild type in the MS basal medium with 20mg/L of hygromycin antibiotics. s342: transgenic shoot containing *ZIB18122* transgene; CIP703852: wild type that is the background of s342; CIP701243: wild type from which the ZIB18122 transgene was cloned.
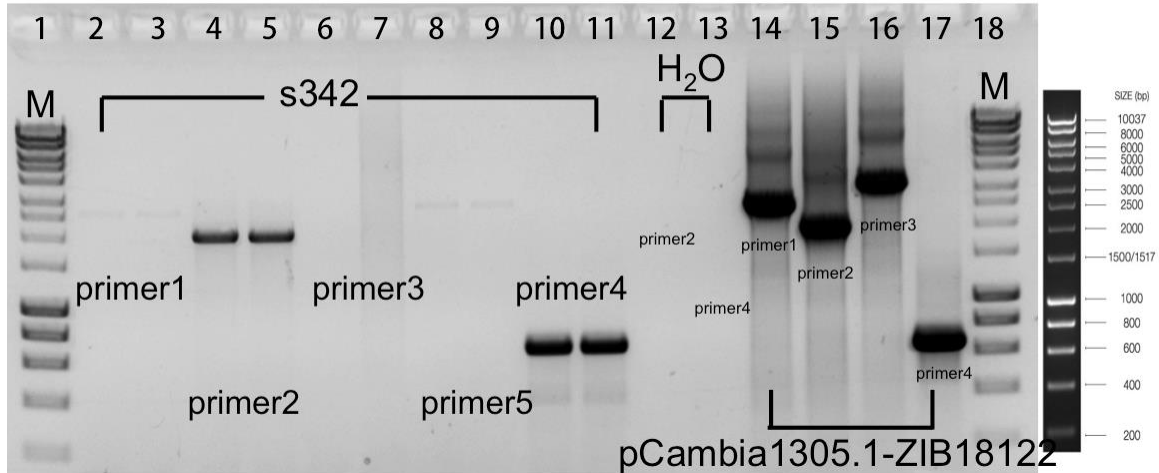
Figure 2.8 PCR analysis of DNA extracted from the transgenic plant s342 transformed with *ZIB18122* gene. pCambia1305.1-ZIB18122 was used as the positive control, H₂O was used as the negative control. Primer1 (p1305.1F2-20ZIBR) resulted in the expected 2543bp fragment. Primer2(20ZIBF-p1305.1R) resulted in the expected 1989bp fragment. Primer3 (p1305.1F2- p1305.1R) resulted in the expected 3152bp fragment. Primer4 (HygroF-HygroR) resulted in the expected 660bp fragment. Primer5 (18122F-p1305.1R) resulted in the expected 2681bp fragment.
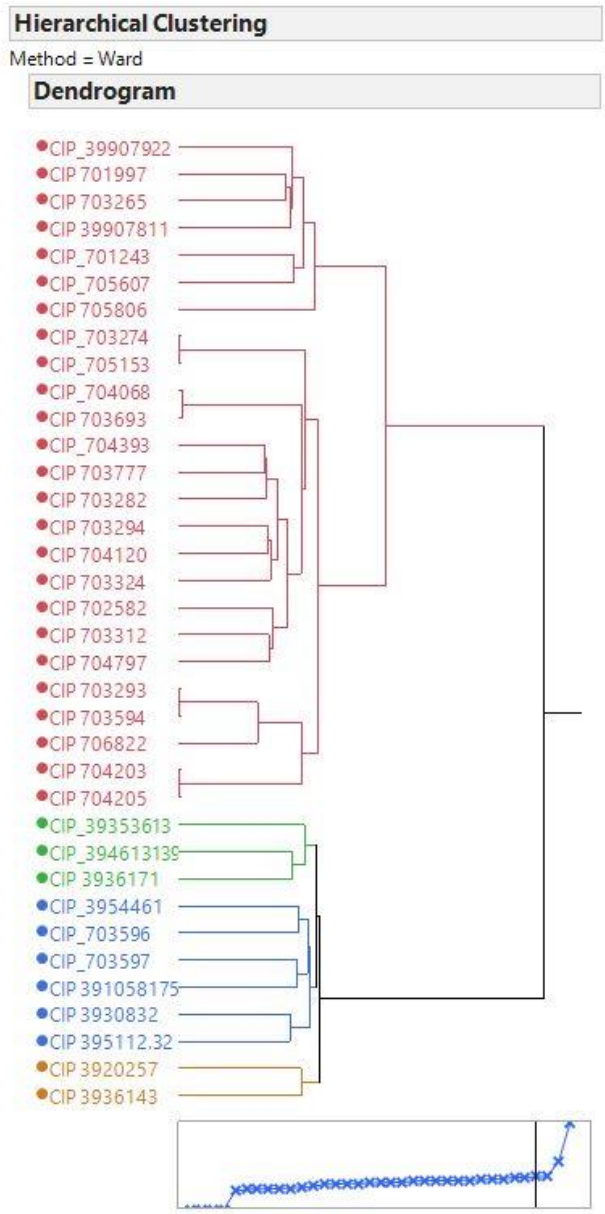
Figure A2.1 Hierarchical cluster of a population of 36 Andean potato cultivars
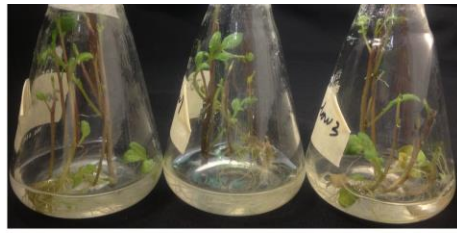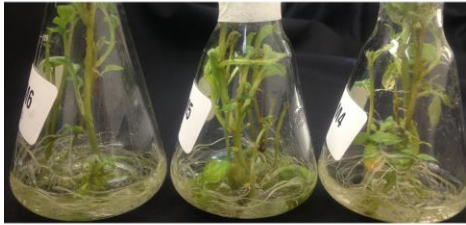
Figure A2.2 The growth of Andean potato cultivars in the micropropagation system. with low Fe content (CIP703580/Q47417) and high Fe content (CIP701243/Q47420) in the micropropagation medium with four different Fe concentrations. A-C, CIP703580; D-F, CIP701243; A and D, the Fe concentration is 13.9mg/L (low Fe medium: LIM); B and E, the Fe concentration is 27.8mg/L (ormal medium: NM); C and F, the Fe concentration is 41.7mg/L (high Fe medium: HIM).
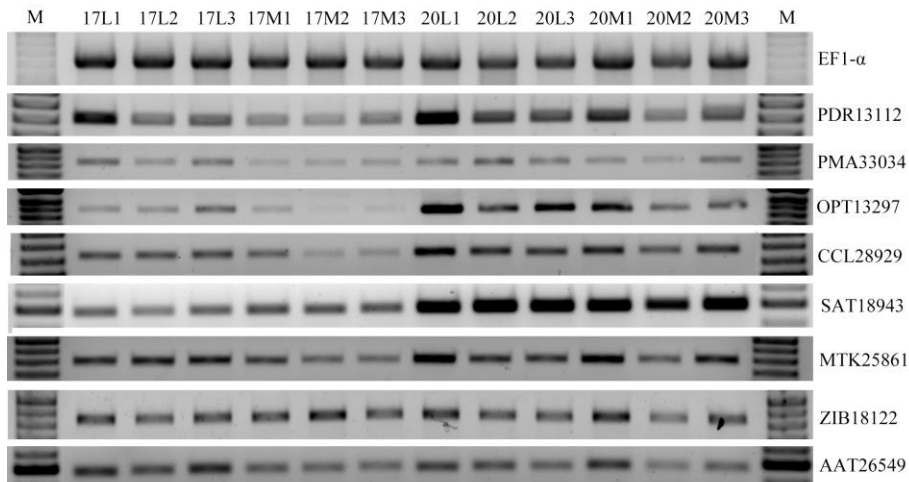
Figure A2.3 Semi-quantitative RT-PCR analysis of candidate genes in roots of two Andean potato cultivars in micropropagation system. 17: CIP703850; 20: CIP701243. L: low Fe medium with 13.9 mg/L FeNaEDTA; M: moderate Fe medium with 27.8 mg/L FeNaEDTA.
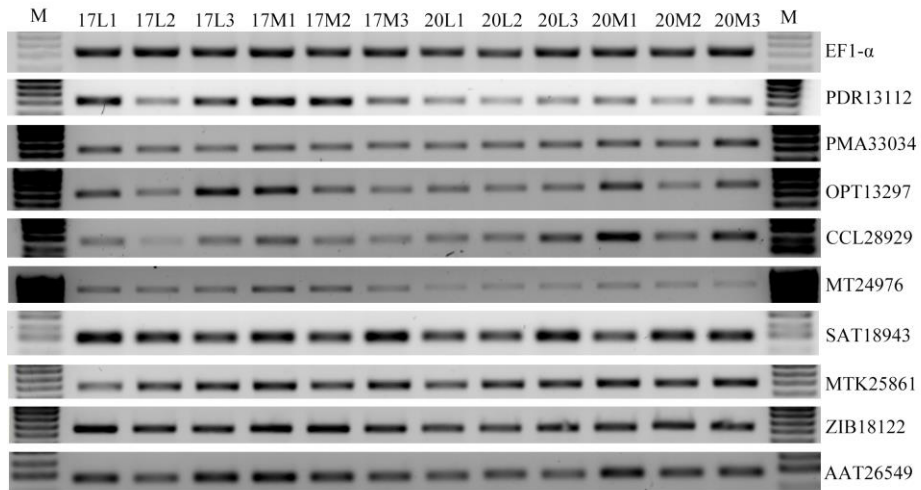
Figure A2.4 Semi-quantitative RT-PCR analysis of candidate genes in microtubers of two Andean potato cultivars in microtuberization system. 17: CIP703850; 20: CIP701243. L: low Fe medium with 13.9 mg/L FeNaEDTA; M: moderate Fe medium with 27.8 mg/L FeNaEDTA.

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Chapter 3 Rhizosphere microbial communities of two Andean potato landraces show consistent differences across two soil types

Hua Xiao[1], Merideth Bonierbale[2] Mark Williams[1], Richard E. Veilleux[1]

Abstract

Plant-microbial interactions are often mediated by or, in turn, mediate nutrient quality and availability to plants. The structural and functional diversity of soil microbial communities are shaped by root exudates, which often differ between plants; in turn rhizosphere microbes can also determine plant health and yield. Andean potato landraces, as ancestral varieties, provide a good resource to understand the structure and the evolution of the microbiota bef[3]ore modern cultivation. In this study, two different Andean potato landraces, namely Llikapa Rurun and Allqa Putis, with high and low tuber iron and zinc, respectively, were grown in two types of field soil in the greenhouse. At the flowering stage, total DNA was extracted from the rhizosphere soil and then sequencing using Illumina Miseq. Quantitative Insights Into Microbial Ecology (QIIME) pipeline was applied in the anlysis of V4 region of the 16S rRNA gene and the spacer ITS1 region of the rRNA gene to describe rhizosphere bacterial and fungal communities, respectively. Both potato landrace and soil type were observed to have similarly large effects on the rhizosphere fungal community composition (Adonis, MRPP, ANOSIM). Indicator species analysis (ISA) revealed that Operational Taxonomic Units (OTUs) of the two landraces were most closely related to Ascomycota, from classes of *Eurotiomycetes* and *Leotiomycetes*. These fungi contain a large variety of dark septate

[1] Department of Horticulture, Virginia Tech, Blacksburg, Virginia, USA

[2] The International Potato Center (CIP), Lima, Peru

endophytic (DSE) which play important roles affecting plant nutrient uptake. There were no significant differences in bacterial communities between the two landraces, but in agreement with previous descriptions of the potato rhizosphere, *Proteobacteria*, *Firmicutes* and *Actinobacteria* were dominant phyla. The present study demonstrated for the first time that plant roots from two different Peruvian landraces of potato play a strong role, across soil types, in selecting the rhizosphere fungal community structure. Further studies are needed to determine the functional implications of fungal community shifts on potato growth and nutrient quantity.

Introduction

The plant-microbial interaction has been studied widely and can take the form of mutualism, commensalism, amensalism, competition and antagonism (e.g. parasitism). These interactions are often mediated by or, in turn, mediate nutrient supply to plants (Marschner et al. 2011). Plant-microbial mutualisms often depend on a quid pro quo whereby soil microbes rely on plant derived carbon resources in trade for nutrients or growth promoting hormones (Beerling and Berner 2005; Demoling et al. 2007). The rhizosphere, the region of soil that surrounds plant roots, is a hotspot of this type of interplay. Plants root exudates, such as low-molecular weight peptides, amino acids, organic acids, sugars, and phenolics are often the carbon source supporting higher microbial density and activity of mutualistic microbes (Philippot et al. 2013). Therefore, the microbial community in the rhizosphere plays a crucial role in promoting root health and maintaining an adequate supply of nutrients for crop growth, which is key for increasing crop yields and productivity. The rhizosphere microbial community structure can be shaped both directly and indirectly by plant root exudates. In the former case, the

molecular signal of exudates support the growth of specific types of microbes, and in the latter can change soil nutrient availability or modulate soil pH to affect plant nutrient uptake (Haichar et al. 2008; Pii et al. 2016; Cassman et al. 2016). While it is known that rhizosphere microbes can have large impacts on plant health and yield, details about the specific changes associated with plants are less well described.

The structural and functional diversity of soil microbial community are shaped by root exudate composition, which in turn can also be impacted by a number of abiotic and biotic factors, including site properties (soil type, climatic conditions and agricultural management regimes) and plant species (Pii et al. 2016; Ladygina and Hedlund 2010; Berg and Smalla 2009). While numerous studies have assessed plant species and soil type effects, there is less known about how plant genotype influence rhizosphere communities.

Plant genotype with different root activities also has the potential to impact the soil microbial community composition. Soil fungal community change related to plant genotypes has been linked to resistance of to diseases, perhaps a result plant-microbial co-evolution (Yu et al. 2016; Yao and Wu 2010; Poli et al. 2016). As an example, Ellouze et al. (2013) reported that the diversity of soil bacteria and fungi in the rhizosphere were influenced by the genotype of chickpea, which had effects on the soil microbial community of the subsequent wheat crop. Thus, in addition to direct and indirect effects associated with plant-microbial interactions, the changing communities can have legacies that have important impacts on downstream cropping systems.

In contrast to the above noted effects, 27 genetically different maize lines were shown to have only a weak association with bacterial community richness (β-diversity) and community structure (β-diversity) when grown across multiple environments (Peiffer et

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al. 2013). These results indicate that genetic change in plant species do not always translate into impacts on rhizosphere communities. Indeed, the many different means of breeding plant species for crop production may have selected for or against growth supportive root-zone microbial communites. Related to potato, cultivar effects on rhizosphere microbial communities have been observed, and found to be associated with tuber starch content (Hannula et al. 2010; İnceoğlu et al. 2012; İnceoğlu et al. 2011; İnceoğlu et al. 2010; Dias et al. 2013). Similar results have been shown with sweet potato (Marques et al. 2014). Yet, it is still difficult to pinpoint what specific factors influence the change in rhizosphere communities across cultivars. Data that link plant traits, such as nutrient content, may help to explain changes in rhizosphere microbial communities. Plant lines with different content of nutritionally important nutrients of Zn and Fe, in particular, are absent from the literature.

Potato (*Solanum tuberosum*), with a worldwide production of >385 million tons in 2014, is currently the world's fourth most important stable food crop, following rice, wheat and maize (FAO 2015). For many populations around the world, it is a major source of antioxidant phytochemicals and mineral nutrients. In the South American Andes, for example, potato constitutes the main staple crop and includes approximately 4,300 native Andean potato varieties. Most family farms grow 10-12 cultivars to reduce their vulnerability to the typically diverse environmental conditions of the region (Brush et al. 1995). Andean potato landraces are highly diverse, containing wide genetic diversity in nutritional contents, specifically for Fe and Zn, for example, which vary over a two-fold range in edible tubers (Burgos et al. 2007; Andre et al. 2007). Plant genotype appears to play an important role in shaping root-associated microbial community composition

(Berg and Smalla 2009), which in turn can feedback to alter plant nutrient acquisition. There is thus opportunity to eventually breed for varieties that attract helper microbes to improve tuber nutrition and yield (Shenton et al. 2016; Pérez-Jaramillo et al. 2016). Andean potato landraces, as ancestral varieties, provide a good resource to understand the structure and the evolution of the microbiota in comparison with modern cultivated potatoes.

To date most studies deciphering the structure of the soil microbial communities have relied on molecular fingerprints, such as Denaturing Gel Gradient Electrophoresis (DGGE), terminal restriction analysis (T-RFLP) and the sequencing of 16S rRNA libraries (Borruso et al. 2014). Those approaches typically identify only a few or no microbes, rather, they are useful for comparing microbial fingerprints across varieties. Few studies applying higher resolution molecular technique such as PhyloChip and 454 pyrosequencing has provided more refined description of microbial community composition including the abundance of rare operational taxonomic units (OTUs) in the complex samples (İnceoğlu et al. 2011; Buée et al. 2009; Dumbrell et al. 2011). To date, however, no studies have described the ITS based fungal community structure associated with the potato rhizosphere, a major focus of the work in this study.

In this study, the composition, evenness and diversity of rhizosphere microbial communities associated with the rhizosphre of two Andean potato landraces growing in two different soil types were described. These two landraces were selected because of their known genetic variation, especially associated with tuber Fe concentration. Our objective was to understand if these two unique varieties were associated with distinct microbial communities, and how soil type might influence these associations. It was

hypothesized that each landrace selects its own fungal and bacterial communities from the pool of microorganisms present in the soil. To our knowledge, this is the first study to apply high-throughput ribosomal RNA gene Illumina sequencing techniques to describe the diverse soil microbial community associated with two potato landraces that differ in iron nutrient acquisition.

Methods

Plant materials and experimental design

In vitro plants of two Andean diploid potato landraces Llikapa Rurun (CIP 705575, high iron and zinc) and Allqa Putis (CIP 705479, low iron and zinc) were transplanted to separate beds, with contrasting types of soil in a greenhouse at CIP station in Huancayo (3300 m a.s.l.) in September 2013. Two types of soil were collected from Huancani that is rich in microbial biomass (hereafter referred to as Huancani) and Santa Ana with low microbial biomass (hereafter referred to as Huancayo), respectively. Ten *in vitro* plants of each potato landrace were planted in each soil type using three replications and three plants per replicate according to completely randomized design (Figure A3.1).

The micronutrient concentration of each variety was determined in the previous field experiments at two locations, Ayacucho and Huancayo (Table A3.1). The analysis of mineral concentrations was carried out at CIP's Quality and Nutrition Laboratory using X-ray fluorescence analysis (XRF) that has previously been used in quantitative elemental analysis of a wide range of organic and inorganic samples (West et al. 2010; Beckhoff et al. 2007). Mineral content values were estimated by XRF based on previous

calibration with selected potato tissue. Nutrient analysis of each soil type was performed by Laboratorio de Quimica Agricola at Valle Grande (Table A3.2).

Sampling and analysis

The soil microbiomes were profiled at 70 dat (days after transplanting) using rhizosphere soil collected from each landrace grown in both Huancani and Huancayo soils. Procedures followed the protocol with some modifications according to Sessitsch et al. (2002). The primary modification was storage of rhizosphere samples in a solution with 15% glycerol and 0.85% NaCl at -70ºC (Figure A3.2). Tubers were sampled, and analysis of variance (ANOVA) was done for fresh weight of tuber. A set of prepared tuber samples were freeze dried and milled for mineral analysis in Adelaide University. Fe and Zn concentration of tubers were determined.

DNA extraction and PCR amplification

Before DNA isolation, rhizosphere soil samples were first treated to get community microbial cell pellets following Lundberg et al. (2012) with small modifications. Briefly, ~20 g of wet rhizosphere soil was suspended in a sterile 50-ml centrifuge tube containing 35 ml phosphate buffer (6.33 g/L $NaH_2PO_4.H_2O$, 16.5 g/L $Na_2HPO_4.7H_2O$, 200µl/L Silwet L-77) by vortexing at maximum speed for 15 sec. The soil solution was then filtered through a 100-um nylon mesh cell strainer to remove large sediments and root pieces. Then the solution was centrifuged at 3,200 g for 15 min to pellet microbial biomass and fine soil particles. The majority of the supernatant was removed and the loose pellets were used to extract microbial community DNA.

For each sample, community DNA was extracted from the soil pellet using PowerSoil

DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacture's protocol. Extracted DNA was quantified with NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Quant-iT PicoGreen dsDNA assay kit (Invitrogen Corp, Carlsbad, CA, USA). DNA quality was checked on a 0.8% (w/v) agarose gel. DNA concentration was determined by fluorometric quantification using the Qubit 2.0 platform with Qubit dsDNA HS Assay Kit (Life Technologies). DNA was diluted to 50 ng/µL and stored in a -20°C freezer.

Bacterial and fungal communities were targeted for Illumina high-throughput sequencing using a PCR approach described previously (Caporaso et al. 2012). PCR primers contain sequencer adapter regions, and the reverse amplification primer contained a twelve base barcode sequence. For bacterial community DNA amplification, the V4 region of the 16S rRNA gene was amplified using 515F/806R primer set in triplicates using a T100™ thermal cycler (Bio-Rad Laboratories Inc., Singapore). Each 25 µl reaction contained: 12.5 µl KAPA HiFi HotStart ReadyMix PCR buffer (KAPA Biosystems, Inc., MA, USA), 0.625 µl each primer (10 mM), 1.25 µl DNA template (50 ng/µl), and 10.0 µl molecular-grade water. Thermocycling consisted of an initial denaturation at 95ºC for 15 min, followed by 35 cycles of 95ºC for 30 sec and annealing temperature at 60ºC for 30 sec, and extension temperature at 72ºC for 30 sec. And a final incubation time was implemented at 72ºC for 5min.

For fungal community DNA amplification, the spacer ITS1 region of the rRNA gene was amplified using ITS1FI2/ ITS2 primer set in triplicates 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 with dNTPs kit buffer (KAPA

Biosystems, Inc., MA, USA), 0.625 µl each primer (10 mM), 2.5 µl DNA template (50ng/ µl), and 8.75 µl molecular-grade water. Thermocycling consisted of an initial denaturation at 95ºC for 15 min, followed by 35 cycles of 98ºC for 15 sec and annealing temperature at 65ºC for 15 sec, and 72ºC for 60 sec. And a final extension time was implemented at 72ºC for 5 min.

The specificity of the PCR products was supported by running on a 1.2% (w/v) agarose gel. The PCR products were gel purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The concentration of purified amplicons was measured by Fluorometric Quantitation (Qubit 2.0 Life Technologies) at Virginia Bioinfomatics Institute (VBI), and then submitted for bar-coded paired-end sequencing (150 bp*2 for 16S and 250 bp*2 for ITS) Illumina MiSeq sequencing.

Sequence data analyses

Quality filtering and de-multiplexing by sample was performed to remove any sequence with a mismatch to the barcode or primer sequence for bacterial and fungal community sequence datasets. The paired end reads with quality scores averaging above 30 were stitched using Pandaseq (Masella et al. 2012). All sequence data were analyzed using QIIME (version 1.7.0) (Caporaso et al. 2010). Operational taxonomic units (OTUs) were delineated at 97% sequence similarity level using *uclust* and *usearch61* (Edgar 2010), yielding the OTU table containing a representative sequence from each OTU. Representative sequences of each OTU for both bacteria and fungi were classified, respectively, using *uclust* against the Greengenes reference database (DeSantis et al. 2006; McDonald et al. 2012) and Ribosomal Database Project (RDP) *classifier* against the UNITE reference (Abarenkov et al. 2010; Wang et al. 2007).

Variations in bacterial and fungal community compositions among treatments were assessed using Python scripts in the QIIME platform. Briefly, to describe the biodiversity and taxonomic summary of the 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 landrace and soil type. The beta diversity was calculated to compare the composition of different communities using weighted and unweighted Unifrac (for bacteria) (Lozupone and Knight 2005), and Bray-Curtis (for fungi) (Beals 1984) distance metrics as input. Multivariate data analysis methods of Multi-Response Permutation Procedures (MRPP), Adonis and Analysis of Similarity (ANOSIM) were conducted to statistically compare differences in the beta diversity between treatments. The ordination patterns and clustering analyses were visualized in 3D-plots in EMPeror (Vázquez-Baeza et al. 2013) using principal coordinates analysis (PCoA) and the significantly correlated OTU were visualized in the plot using nonmetric multidimensional scaling analysis (NMS). Indicator species analysis (ISA) was used to identify OTUs that were significantly (indicator value > 70 and p value < 0.05) correlated with potato variety based on four treatment and when blocked by soil type. A seed of 16 and 18 with 5000 runs was used for the bacteria and fungi, respectively. NMS and ISA were performed using the PC-ORD software version 6.0 (MjM Software, Gleneden Beach, OR, USA).

Results

Plant performance

There were significant differences in fresh weight of tubers (TFW) between landraces (F=3.50, $p<0.05$) and for landrace by soil type interaction (F=4.27, $p<0.05$), but the most significant difference was found between soil types (F=29.24, $p<0.001$). No differences in TFW were found between landraces when grown in the Huancayo soil containing low microbial biomass, whereas the TFW was variable between landraces when grown in Huancani soil rich in microbial biomass. Allqa Putis containing low Fe content in tubers showed greater TFW than Llikapa Rurun with relative high Fe content. This negative correlation between mineral concentration and yield has been observed in previous field trails at CIP. The estimation of Fe concentration in tubers of these two landraces grown in two soil types in the greenhouse showed similar patterns as previous evaluation under field conditions (Table A3.1 and 3.3). The Fe concentration was greater in both landraces under the soil with higher microbial biomass compare to the low microbial biomass soil.

Fungal community analysis

Following removal of low-quality sequences, a total of 73,797 high quality reads of the internal transcribed spacer (ITS) region sequence of rRNA gene were obtained from Illumina MiSeq sequencing. 1087 OTUs were identified across all groups after sequence assembly, clean-up, and clustering in QIIME. The mean and median counts per sample were 6,150 and 4,854 respectively. Since the sample size variation can affect the diversity metrics, a consistent sampling depth threshold was utilized for further analyses by taking a random subsample of 790.

Chao1, observed species, Shannon, and Simpson metrics of the original OTU table were used for analysis of alpha diversity of fungal community. A non-parametric test with the default 999 Monte Carlo permutations with FDR correction indicated that the OTU-based richness and evenness of fungal community was significantly different ($p<0.05$) between soil types across Chao1, observed species and Shannon metrics (Table 3.1). The rarefaction curves also showed trends that soil type was the strongest driver of diversity differences for fungal richness (Figure A3.3). The alpha diversity was higher in the Huancani soil compared to the Huancayo soil. The fungal richness did not vary significantly between landraces, as well as the soil type-landrace interaction, which mean no shifts in fungal OTU richness in response to landrace difference.

Taxonomic summaries showed that the phyla *Ascomycota* (~42.5% in soil Huancani, ~17.5% in soil Huancayo) and *Basidiomycota* (~5.6% in soil Huancani, ~18.0% in soil Huancayo) were the most dominant fungal phyla. A major proportion of taxa could not be assigned (~45.1% in soil Huancani , ~64.1% in soil Huancayo) to known taxa for the fungal data amplification products obtained from the spacer ITS1 region of the rRNA gene (Table 3.2). At finer taxonomic scales, the most abundant fungal classes included the *Eurotiomycetes*, *Sordariomycetes* and *Dothideomycetes* in the phylum *Ascomycota*, and *Tremellomycetes* in the phylum *Basidiomycota*.

When fungal communities were examined based on the fungal phylogenetic composition of relative abundance, community composition was compared by multivariate data analyses using Adonis, ANOSIM, and MRPP based on Bray-Curtis distances. The results showed statistically significant differences ($p<0.01$) in beta diversity due to soil type, landrace, and the interaction of soil type and landrace (Table 3.3). It means that fungal

community composition shifted in response to landraces with different iron concentrations and soil type.

In three-dimensional principal coordinates analysis (PCoA) based on a Bray-Curtis dissimilarity metric, samples within each group clustered together and a clear separation between landrace and soil type samples was observed, in support of expectations based upon statistical tests (Figure 3.2). Soil type and landrace, interestingly, explained similar amounts of variation (PC1=39%, PC2=34%). Fungal OTU composition was also visualized by nonmetric multidimensional scaling (NMS) ordination generated using Bray-Curtis dissimilarity. Consistent with PCoA, the NMS bi-plot showed that both landrace and soil type were associated with fungal community structure shifts, with soil type and landrace clustering separately from one another in the two-dimensional figure (Figure 3.2). Results thus show the strength of the effect of landrace on fungal communities even against the background of a strong soil effect.

To identify the known fungal genera that were most associated with the effect of landrace, we conducted a comparison of the relative abundances of approximately genus-level OTUs in rhizosphere among treatments, using Pearson and Kendall correlation values derived from NMS, and ISA. From the NMS analysis, OTUs that were significantly correlated with ordination axes are showed in Table 3.4. A threshold level of indicator value of 70%, $r^2>0.4$, and significance at $p < 0.05$) were used for identifying indicator species. ISA identified indicator species and the main variables responsible for treatments. It resulted in OTUs and genera having a significant effect on landrace type (Table 3.5; Table 3.6). The results were combined using these criteria and identified 19 OTUs that describe differences in the fungal communities in the rhizosphere of the

landraces (Table 3.7). Seventeen of the OTU within the Ascomycota were associated with differences between the high and low iron landraces. One OTU (#981) was blasted against the NCBI Database and found to be closely matched with an uncultured clone. OTU312 and OTU313 are sequences blasted from potato, which haven't been considered in this study.

Bacterial communities

16S rRNA gene-based Illumina MiSeq sequencing analysis of the V4 region was performed to characterize the bacterial community composition in the rhizosphere. A total of 2,212,983 high-quality sequences (counts) was obtained after sequence assembly, clean-up, and clustering in QIIME. From these sequences, 210,007 OTUs (observations) were identified across all groups. The mean and median counts per sample were 184,415 and 184,386 respectively. The sampling depth threshold was utilized for further analyses by taking a random subsample of 64,000.

Chao1, observed species, PD_whole_tree metrics of the original OTU table were used for analysis of alpha diversity of the bacterial community. The statistical analysis indicated that the OTU-based richness and evenness of the bacterial community were significantly different ($p<0.05$) between soil types across Chao1, observed species, and PD whole tree tested (Table 3.8). The rarefaction curves also showed trends that showed strong differences of bacterial richness in relation to soil type (Figure A3.4). The alpha diversity was higher in the Huancayo compared to the Huancani soil. The bacterial richness did not vary significantly between landraces; however, there was soil by landrace interaction for the observed species and PD whole tree estimates of alpha diversity.

The bacterial community was dominated by phyla from Proteobacteria (~86% in soil Huancani, ~72% in soil Huancayo), followed by the Actinobacteria (~8% in soil Huancani, ~19% in soil Huancayo). These results are consistent with the dominance of these groups in rhizosphere compared to bulk soils (Barnett et al. 2015). At the family level, greater relative sequence abundance were from *Pseudomonadaceae* (Phylum: *Proteobacteria*), *Micrococcaceae* (Phylum: *Actinobacteria*) and *Enterobacteriaceae* (Phylum: *Proteobacteria*) (Figure 3.3).

When bacterial communities were examined based on the phylogenetic composition in relative abundance, community composition was compared by multivariate data analyses using Adonis, ANOSIM, and MRPP on weighted and unweighted Unifrac distances. The results showed statistically significant differences ($p<0.01$) in the beta diversity of the soil type, but not of landrace, and the interaction of soil type and landrace (Table 3.9). Bacterial community composition shifted only in response to soil type.

In three-dimensional principal coordinates analysis (PCoA) based on weighted and unweighted Unifrac matrices, samples within the same soil type clustered together and no clear separation between landrace samples was observed (Figure 3.4). In the weighted Unifrac matrix, the soil type explained the maximum variation (PC1=57%). Overall, these results indicate the effect of soil type but not landrace, on bacterial community structure. In order to better explore the effects of genotype on bacterial communities in potato rhizosphere, separated principal component analysis based on individual soil were conducted and the results showed that the bacterial community structure was still not influenced by potato landrace (Figure A3.5).

Discussion

We examined the effect that two different Andean potato landraces (*Solanum tuberosum* L.) had on the rhizosphere fungal and bacterial communities in two different soil types. These landraces produced many fine roots that made sampling of the rhizosphere straightforward, and showed furthermore, that landrace effects on fungal communities, over a growing season could be as large as soil effects. While soil properties have for a relatively long time been known to play a large role in determining microbial and especially bacterial community structure (Pii et al. 2016; Berg and Smalla 2009; Robin et al. 2006; Marschner et al. 2004; Girvan et al. 2003), plant type (e.g. species) has sometimes, but not always been shown to impact rhizosphere community structure (Manter et al. 2010). A few studies have also shown that plant genotype differences can strongly impact (Philippot et al. 2013; Shenton et al. 2016; Poli et al. 2016; Corneo et al. 2016; Marques et al. 2014; Hannula et al. 2010; Van Overbeek and Van Elsas 2008) microbial community structure in the rhizosphere. These results are consistent with the findings of the current study; however, two other points are relatively unique and important to consider. First, genotype effects on rhizosphere fungal communties were simultaneously observed in two different soils, and the genotype effect was similar in size to that of the soil effect. Second, though each soil seemed to shape rhizosphere community structure in unique ways, landrace also showed elements of consistence across the soil influence. Microbes directly surrounding roots are more likely to be associated with plant-microbial feedbacks and interactions, deliberate and meticulous sampling of the root-zone likely helped better describe of plant-associated microbes against the background of a highly diverse soil microbiome. These results point to the strong role that plant genotype can have on structuring soil communities.

Only a few previous studies have shown a large plant species or genotype effect, relative to soil, predominantly based on sensitive fingerprinting such as PLFA (Aira et al. 2010) and PCR-DGGE (Costa et al. 2006). Though many studies have shown plant effects, generally they are much smaller than those of soil (Poli et al. 2016; İnceoğlu et al. 2012). Furthermore, landraces have been less well studied than those of wild and bred plants (Pérez-Jaramillo et al. 2016; Turner et al. 2013). Andean potato landraces have been shown to have significant genetic diversity, for example, in their concentrations of tuber Fe and Zn (Burgos et al. 2007) as well as other nutrients (Andre et al. 2007). This broad genetic diversity among native potato landraces may help to support the variation in landrace rhizosphere microbial communities.

It is revealing that the fungal but not the bacterial communities in the rhizosphere differed between the two Andean potato landraces. Studies have investigated the composition of the soil bacterial community in and near potato roots under diverse conditions (Lukow et al. 2000; Michelsen et al. 2014; Liu et al. 2014). Unno et al. (2015) reported that macronutrient deficiency influenced the community structure of root-associated bacteria in potato cultivar 'Matilda' cultivation. The abundance of *Rhizobium* spp., for example, increased dramatically under the macronutrient deficiency. İnceoğlu et al. (2011) revealed that potato cultivar effects on bacterial community structures only were found in the young plant stage but were insignificant in the flowering and senescence stages. Soil type also exerted the most profound influence on shaping the fingerprints of the bacterial communities of the cultivars (İnceoğlu et al. 2012). Kobayashi et al. (2015) analyzed the root- and tuber-associated bacteria in eight potato genotypes with different resistance levels against common scab and found that the bacterial community structures were

highly similar in roots. The present study tends to support the findings of these and other researchers whereby root-associated bacterial communities showed little change across cultivars. Much less research has been conducted on the fungal communities of potato, and when done often utilize fingerprinting type methods (Hannula et al. 2012; Hannula et al. 2010; Senés-Guerrero et al. 2014). Based on the results of the current study, fungal community shifts need further study to further verify and understand the functional implications of these changes.

To date, studies of potato-fungal interactions are relative rare. Cesaro et al. (2008) observed a potentially specific interaction between one AM fungal species, *Glomus intraradices*, and potato plants. Senés-Guerrero et al. (2014) and Senés-Guerrero and Schüßler (2015) reported that potato plants were colonized by diverse species from 8 of the 11 *Glomeromycota* families. *Glomeromycota* was an uncommon member of the fungal communities in our study, and no clear-cut differences were found across cultivars. The lack of this phylum may be the result of primer that may tend to favor the description of *Ascomycota* over that of *Glomeromycota*, or possibly the result of soil type and the legacy of communities found in the soil. It was clear, nevertheless, that landrace can have strong effects on fungal community structure.

Dark septate endophytic (DSE) fungi have increasingly been studied and shown to play important roles affecting plant nutrient uptake, generally considered mutualists but also known to be antagonists (Wang et al. 2016; Likar and Regvar 2013). It is interesting to note that the largest differences between the two landraces are related to their membership in *Ascomycota*, which include the phylum of DSE (Berthelot et al. 2016). Indeed the classes of *Eurotiomycetes* and *Leotiomycetes*, contain many DSE, and can

account for >30% of fungal taxa across both landraces. There were also differences in these groups between landraces. The genera *Cryptococcus* from *Basidiomycota* also appeared to be common and can be associated with both disease and plant growth promotion. Overall, the results highlight the strong role that landrace and interactions between landrace and soil type could play in fungal community composition of two contrasting potato landraces, indicating that potato-fungal interactions might be landrace-dependent.

The status of Fe and P might be factors involved in the development of rhizosphere fungal communities (Hinsinger et al. 2015; Treseder 2013). In this regard, DSE are known for their support of plant acquisition of phosphorus and iron (Wang et al. 2016; Mandyam and Jumpponen 2014). These fungi may act in ways similar to those of *Glomeromycota*, by extending hyphae into the surrounding soil and thus dramatically increasing the soil volume explored (Turrini et al. 2016; Smith and Smith 2012), however, because DSE have very fine hyphae and mycelial networks, their mode of assimilation and foraging behavior for nutrients may differ. It is also notable that these two landraces have been and continue to be studied for their differences in iron concentrations (Burgos et al. 2007; Andre et al. 2007). Because Fe and P are highly insoluble in aerated soils, and in the latter case often bound to iron or aluminum sesquioxides (Borggaard et al. 1990; Hinsinger 2001; Tomasi et al. 2008), high demand for phosphorus (Hopkins et al. 2014) and variation in iron content between landraces, may support the development of fungal communities to offset the negative effects of nutrient limitation. There is no direct evidence that there is a link between specific variety traits and their association with particular root-zone communities; however, the results

point to the need for further descriptions of this plant-fungal linkage to better understand its role in potato growth.

In addition to the effects of potential AMF and DSE fungi on the nutrient cycling, saprophytic fungal community also play key roles in ecosystems and mediate many ecological processes that are crucial to ecosystem functioning such as decomposition and soil carbon cycling (Gudiño Gomezjurado et al. 2015). A large proportion of photosynthetically fixed C is directed belowground to roots and associated microorganisms. Hence, fungi found in the root-zone but not directly infecting plant roots may also be important regulators of carbon and nutrient dynamics in this system. Many of the fungi described have been shown (e.g. *Penicillium, Mortierella*) have often been shown to grow in association or near roots and play broad saprotrophic roles in soils (Whitelaw 1999; Barroso and Nahas 2007; Morales et al. 2007).

Though there were no differences in bacterial communities across the two landraces, previous studies have documented the occurrence potato growth-promoting taxa closely related to *Pseudomonadaceae*, *Enterobacteriaceae*, *Xanthomonadaceae*, *Sphingomonadaceae* and *Rhizobiaceae* (Turnbull et al. 2012; Weinert et al. 2011). The results of the study reported herein also observed the above taxa but cannot confirm their potential for growth promotion. The occurrence of *Micrococcaceae* and *Hyphomicrobiaceae* was also observed, and previously shown to play, in some cases, plant growth-promoting roles (Barnett et al. 2015; Manter et al. 2010; Reiter et al. 2002). Our results, furthermore, are consistent with previous studies indicating that *Proteobacteria*, *Firmicutes* and *Actinobacteria* are dominant phyla in potato root endophytes and rhizosphere soil inhabitants (Kobayashi et al. 2015; İnceoğlu et al. 2011).

While the results are consistent with the suppositions of previous studies regarding the possible role of bacteria in plant growth promotion, the lack of differences suggest that bacteria-potato interactions are not influenced by cultivar. However, because plant community succession and plant growth stage can strongly influence the bacterial community (Marques et al. 2014; Sugiyama et al. 2014b; Sugiyama et al. 2014a), further research will be needed to discern more about the nature of potato-bacterial interactions.

The effects of domestication and plant breeding have likely had a strong impact on the microhabitats inhabited by rhizosphere microbes, likely altering traits influencing rhizosphere microbial communities lost during selection of modern genotypes in place of wild ancestors. Bulgarelli et al. (2015) studied root-associated microbial communities of wild and domesticated accessions of barley, finding a small but significant effect of host genotype on the root-associated bacterial communities. In maize, the difference of root system architecture and rhizosphere processes between progenitor and domesticated varieties revealed a small influence of plant genotype on the rhizosphere (Szoboszlay et al. 2015). Therefore, Quiza et al. (2015) has theorized that optimization of microbial activities in the soil without high input cultivation would be a direction for crop improvement. For instance, Shenton et al. (2016) found that wild rice rhizosphere bacterial communities differed in complexity (species richness) and composition compared with cultivated rice rhizosphere, while wild rice had more counts of certain methanotrophs in their rhizosphere. Because methane emission from rice paddies makes a significant contribution to the greenhouse effect, they proposed that breeding rice to optimize the community of methanotrophs in and around rice roots could reduce the damage caused by emissions resulting from food production. Rangjaroen et al. (2015)

proved that inoculation with potentially useful isolates from different highland rice landraces promoted the growth of a commercial rice cultivar. In the present study, the potato landraces have a broader genetic base compare with cultivated potatoes. Quantifying microbes of these lines based on metagenomics and next generation sequencing might reveal some beneficial microbes that are more or less common as a result of domestication. Then introgression of genetic material from these landraces into cultivated varieties may present opportunities to support microbe mediated sustainable agricultural management of agroecosystems.

In conclusion, we used Illumina high-throughput sequencing of ribosomal RNA genes of rhizosphere microbes in two native potato landraces to better investigate the dynamics of rhizosphere microbial communities under the influence of phenotypically diverse potato landraces with regard to Fe content in tubers. During this greenhouse experiment both soil type and of potato landrace had clear impacts on the structure of fungal communities. In particular, some species in the group Ascomycota, which differed between the two potato landraces, may be a useful microbial indicator for evaluating the production properties of different potato landraces, and then provide information for improving microbe mediated sustainable agricultural practices. A deeper study will be needed to investigate the correlation between rhizosphere fungal community and the desired crop function (e.g., micronutrient content) and to provide better insight into preferential rhizosphere fungi colonized in potato landraces.

Table 3.1 Alpha diversity metrics of the rhizosphere fungal community for variety, soil type, and variety × soil type.

	Average ± SE			
	Chao 1	**Observed Species**	**Shannon**	**Simpson**
Soil type				
Huancani	534±42	390±37	5.1±0.2	0.8±0.02
Huancayo	412±51	275±51	4.0±0.5	0.8±0.09
p-value	**0.011**	**0.009**	**0.046**	0.191
Landrace				
Llikapa Rurun	469±57	330±54	4.6±0.5	0.8±0.07
Allqa Putis	477±51	334±50	4.6±0.5	0.8±0.07
p-value	0.696	0.778	0.991	0.786
Soil type x Landrace				
Huancani-Llikapa Rurun	500±77	357±69	5.0±0.4	0.85±0.04
Huancani-Allqa Putis	568±42	423±33	5.1±0.1	0.84±0.01
Huancayo-Llikapa Rurun	438±96	303±95	4.1±0.8	0.78±0.14
Huancayo-Allqa Putis	385±56	246±58	4.0±0.9	0.74±0.15
p-value	0.358	0.355	0.462	0.857

Calculations were based on an equal size of 790 from random sub-sampling on each sample. Bolded values indicate significant (α<0.05) effects.

Table 3.2 The relative abundance of dominant fungal phyla. Numbers describe the % relative abundance of each phyla.

	Huancani	Huancayo	Llikapa Rurun	Allqa Putis	Huancani-Llikapa Rurun	Huancani-Allqa Putis	Huancayo-Llikapa Rurun	Huancayo-Allqa Putis
Ascomycota	42.5	17.5	23.8	36.1	42.9	42.2	14.6	25.8
Basidiomycota	5.6	18.0	12.0	13.4	5.7	5.5	15.1	26.7

Table 3.3 Statistical tests describing differences in fungal community composition (beta diversity).

	Treatment	F.Model	R^2	p-value
Adonis	Soil type x Landrace	5.7901	0.68467	**0.001**
	Landrace	5.0337	0.33483	0.005
	Soil type	5.9987	0.37495	**0.002**

		R statistic	p-value	Number of permutations
ANOSIM	Soil type x Landrace	0.8148	**0.001**	999
	Landrace	0.6037	**0.007**	999
	Soil type	0.6852	**0.007**	999

		A value	p-value	Number of permutations
MRPP	Soil type x Landrace	0.3484	**0.001**	999
	Landrace	0.1485	**0.01**	999
	Soil type	0.1729	**0.005**	999

Bolded values indicate significant (α<0.05) effects.

Table 3.4 *r*-value of Pearson and Kendall correlations between the ordination scores of
the NMS axes of Figure 3.2 based on the OUT abundance table

Axis:	1			2		
	r	r-sq	tau	r	r-sq	tau
OTU312	0.165	0.027	0.121	0.826	**0.683**	0.697
OTU313	-0.496	0.246	-0.455	-0.719	**0.517**	-0.364
OTU607	-0.313	0.098	-0.18	0.717	**0.514**	0.539
OTU768	-0.565	0.319	-0.396	0.697	**0.485**	0.534
OTU981	-0.377	0.142	-0.202	0.685	**0.469**	0.569
OTU646	0.531	0.282	0.495	-0.683	0.467	-0.459
OTU336	0.17	0.029	0.422	-0.68	**0.462**	-0.532
OTU230	-0.581	0.337	-0.357	0.661	**0.437**	0.481
OTU433	-0.457	0.209	-0.359	0.654	**0.428**	0.439
OTU994	0.254	0.064	0.349	-0.649	**0.421**	-0.606
OTU849	-0.605	0.366	-0.461	0.645	**0.417**	0.592
OTU907	-0.083	0.007	-0.095	0.646	0.417	0.445
OTU515	-0.239	0.057	-0.135	0.643	**0.414**	0.584
OTU50	0.072	0.005	0.225	-0.641	0.411	-0.629
OTU573	0.072	0.005	0.225	-0.641	0.411	-0.629

Bolded values indicate significant (α<0.05) effects.

Table 3.5 Monte Carlo test of significance of observed maximum indicator value for variable based on the OTU abundance table

OTU#	(IV)	Mean	S.Dev	*p*
OTU312	99.2	67.8	11.24	**0.0038**
OTU313	92.5	63.2	9.01	**0.0038**
OTU994	74.1	35.9	15.02	**0.0186**
OTU768	72.9	36.1	13.53	**0.0212**
OTU515	100	34.1	18.41	**0.0218**
OTU607	100	31.8	18.23	**0.0218**
OTU981	83.3	36.4	15.71	**0.0218**
OTU849	68.9	38.1	13.1	**0.0218**
OTU230	63.7	39.7	10.44	**0.0218**
OTU336	80.2	38.3	17.09	**0.0334**
OTU433	82.2	33.5	15.78	**0.0416**
OTU646	70.7	35.1	13.45	0.053
OTU907	52.3	38.2	11.26	0.1052
OTU50	53.5	32.8	17.48	0.1728
OTU573	53.5	32.8	17.48	0.1728

Bolded values indicate significant ($\alpha<0.05$) effects.

Table 3.6 Monte Carlo test of significance of observed maximum indicator value for variable based on taxonomic composition at the genus level

Genus#	(IV)	Mean	S.Dev	p
fg101	84.9	48.4	11.67	**0.0128**
fg59	100	31.8	18.57	**0.0166**
fg70	100	32.2	17.39	**0.0166**
fg101	59.1	38	10.86	**0.0284**
fg36	61.4	40.7	9.66	**0.0344**
fg107	78	36.3	15.8	**0.0346**
fg131	66.3	34.4	13.4	**0.0362**
fg50	63.5	38.2	13.18	0.05

Bolded values indicate significant ($\alpha<0.05$) effects.

Table 3.7 Fungal genera with different relative abundance of taxa associated with landrace. Results based on NMS (r^2>0.4) and Indicator Species Analysis (IV > 70 and *p*-value < 0.05).

Maxgrp*		Phylum	Class	Order	Family	Genus	Obs IV	*p*-value	LR(%)	AP(%)
AP	fg101	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	84.9	0.0128	0.02%	0.06%
Huancayo_AP	fg107	Ascomycota	Sordariomycetes	Hypocreales	unidentified	unidentified	78	0.0346	0.01%	0.03%
Huancani_LR	fg131	Ascomycota	Sordariomycetes	Sordariales	unidentified	unidentified	66.3	0.0362	0.02%	0.01%
Huancani-AP	fg36	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Cladophialophora	61.4	0.0344	1.00%	1.44%
Huancani-AP	fg50	Ascomycota	Eurotiomycetes	Incertae_sedis	Incertae_sedis	Calyptrozyma	63.5	0.05	0.55%	0.72%
Huancani-AP	fg59	Ascomycota	Leotiomycetes	Other	Other	Other	100	0.0166	0.00%	0.02%
Huancani-AP	fg70	Ascomycota	Leotiomycetes	Helotiales	Vibrisseaceae	Acephala	100	0.0166	0.00%	0.01%
Huancayo_AP	fg124	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	70.7	0.053	0.39%	0.58%
Huancani_LR	OTU768	Ascomycota	Sordariomycetes				72.9	0.0212		
Huancani_LR	fg46	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	100	0.0218	9.72%	10.93%
Huancayo-AP	OTU994	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium	74.1	0.0186		
Huancani_LR	fg92	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Pochonia	82.2	0.0416	0.27%	0.24%
Huancayo_AP	fg149	Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	Cryptococcus	80.2	0.0334	5.69%	8.70%
Huancani_LR	fg158	Zygomycota	Incertae_sedis	Mortierellales	Mortierellaceae	Mortierella	100	0.0218	3.12%	3.09%

* LR: Llikapa Rurun, potato landrace with high iron and zinc; AP: Allqa Putis, potato landrace with low iron and zinc

Table 3.8 Alpha diversity metrics for variety, soil type, and variety × soil type in rhizosphere bacterial community. Calculations were based on an equal size of 64,000 from random sub-sampling on each sample.

	Average ± SE Chao 1	Observed Species	PD_whole_tree
Soil type			
Huancani	8120±457	3056±147	174±8
Huancayo	9284±253	4219±99	232±5
p-value	**0.041**	**0.001**	**0.001**
Landrace			
Llikapa Rurun	8690±535	3671±335	206±17
Allqa Putis	8714±309	3604±233	199±12
p-value	0.970	0.874	0.750
Soil Type x Landrace			
Huancani-Llikapa Rurun	7948±779	3003±321	174±18
Huancani-Allqa Putis	8292±524	3110±50	175±2
Huancayo-Llikapa Rurun	9432±526	4339±104	239±5
Huancayo-Allqa Putis	9137±151	4099±155	224±8
p-value	0.254	**0.0018**	**0.0033**

Bolded values indicate significant (α<0.05) effects.

Table 3.9 Statistical test for differences in bacterial community composition (beta diversity).

	Treatment	F.Model	R^2		*p*-value
Adonis	Soil type × Landrace	2.0959	0.44008		0.08
	Landrace	0.15285	0.01505		0.98
	Soil type	6.8851	0.40776		**0.003**
		R statistic	***p*-value**		**Number of permutations**
ANOSIM	Soil type × Landrace	0.2531	0.082		999
	Landrace	-0.1389	0.926		999
	Soil type	0.5815	**0.004**		999
		A value	***p*-value**		**Number of permutations**
MRPP	Soil type × Landrace	0.125	0.095		999
	Landrace	-0.0475	0.953		999
	Soil type	0.1944	**0.003**		999

Bolded values indicate significant (α<0.05) effects.

Table A3.1 Diploid landraces with high and low micronutrient concentrations as determined in previous field experiments in two locations (Ayacucho and Huancayo).

Level Fe/Zn	CIP#	Variety name	Ayacucho			Huancayo		
			Fe ppm	Zn ppm	DM %	Fe ppm	Zn ppm	DM %
High Fe	705575	Llikapa Rurun	24.0	14.0	31.1	25.4	20.9	29.2
Low Fe	705479	Allqa Putis	13.8	8.2	32.6	16.4	16.4	29.6

Table A3.2 Soil mineral properties of two soil types.

Parameter	Huancani soil (ppm)	Huancayo soil (ppm)
available Copper (Cu)	1.08	2.40
available Zinc (Zn)	1.32	0.84
available Manganeso (Mn)	41.6	37.60
available Iron (Fe)	93	162.00
pH	3.97	5.13

Table A3.3 Iron concentration measured by XRF in two landraces grown in two soil types.

Level Fe/Zn	CIP#	Variety name	Huancani soil (ppm)	Huancayo soil (ppm)
High Fe	705575	Llikapa Rurun	28.2	13.8±1.6
Low Fe	705479	Allqa Putis	15.7±1.3	12.3±2.4

Table A3.4 *r*-value of Pearson and Kendall correlations between the ordination scores of
the NMS axis based on the OTU abundance table by soil type

Axis:	1			2		
	r	r-sq	tau	r	r-sq	tau
OTU249	0.925	0.856	0.776	-0.43	0.185	-0.293
OTU559	0.918	0.842	0.741	-0.438	0.191	-0.327
OTU57	0.917	0.841	0.667	-0.379	0.143	-0.333
OTU139	0.913	0.833	0.741	-0.402	0.162	-0.327
OTU166	0.909	0.826	0.855	-0.346	0.12	-0.197
OTU183	0.897	0.804	0.667	-0.477	0.228	-0.333
OTU319	0.875	0.765	0.667	-0.381	0.145	-0.273
OTU357	0.874	0.764	0.845	-0.083	0.007	-0.017
OTU413	0.872	0.76	0.789	-0.11	0.012	-0.092
OTU785	0.866	0.75	0.592	-0.44	0.193	-0.428
OTU135	0.864	0.746	0.606	-0.216	0.047	-0.202
OTU749	0.855	0.731	0.707	-0.405	0.164	-0.293
OTU101	0.853	0.728	0.81	-0.273	0.074	-0.259
OTU328	0.853	0.727	0.716	-0.246	0.06	-0.165
OTU60	0.845	0.715	0.691	-0.096	0.009	-0.066
OTU409	-0.842	0.71	-0.672	0.37	0.137	0.259
OTU11	0.84	0.705	0.658	-0.211	0.044	-0.164
OTU270	0.839	0.704	0.699	-0.402	0.161	-0.191
OTU173	0.837	0.7	0.559	-0.512	0.262	-0.362
OTU692	0.836	0.7	0.741	-0.429	0.184	-0.327
OTU789	-0.836	0.699	-0.707	0.352	0.124	0.155
OTU272	0.831	0.691	0.545	-0.506	0.256	-0.394
OTU22	-0.828	0.686	-0.81	0.255	0.065	0.19
OTU500	-0.823	0.678	-0.638	0.392	0.153	0.293
OTU538	-0.821	0.674	-0.776	0.247	0.061	0.224
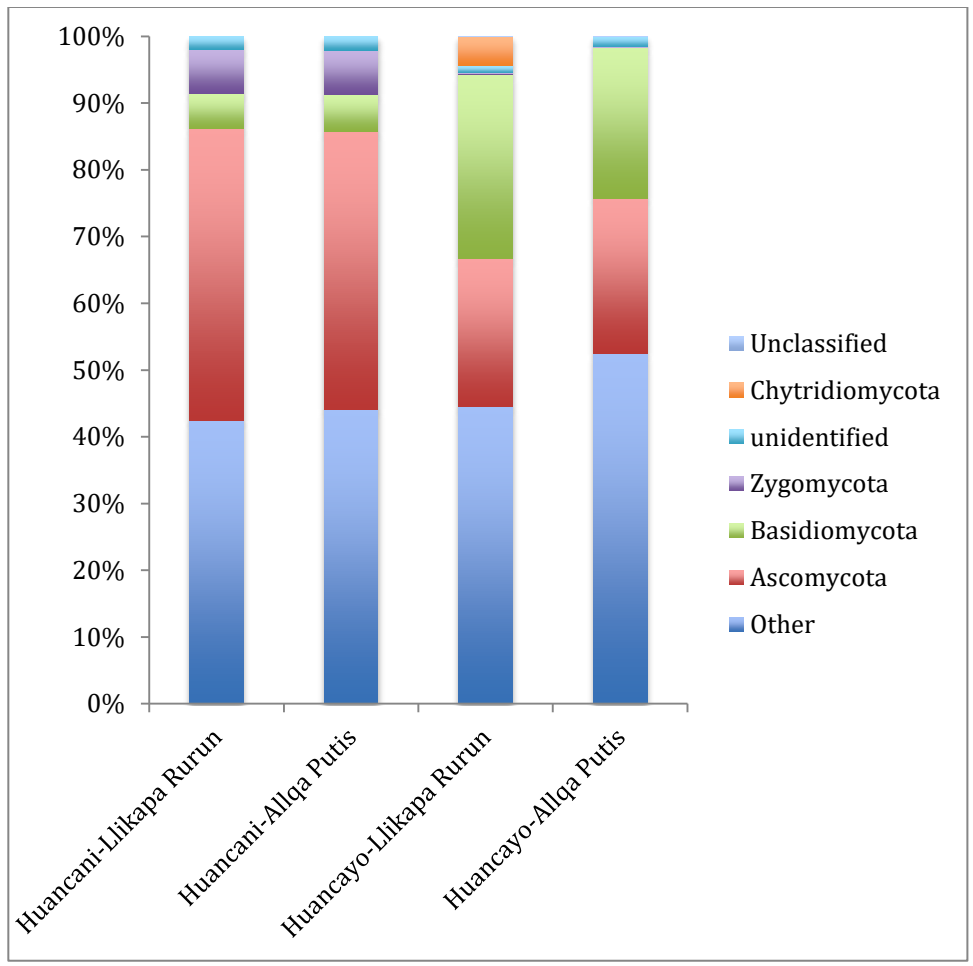
Figure 3.1 Taxonomic summary of the relative abundance of different fungal phyla and classes associated with landrace and soil type.
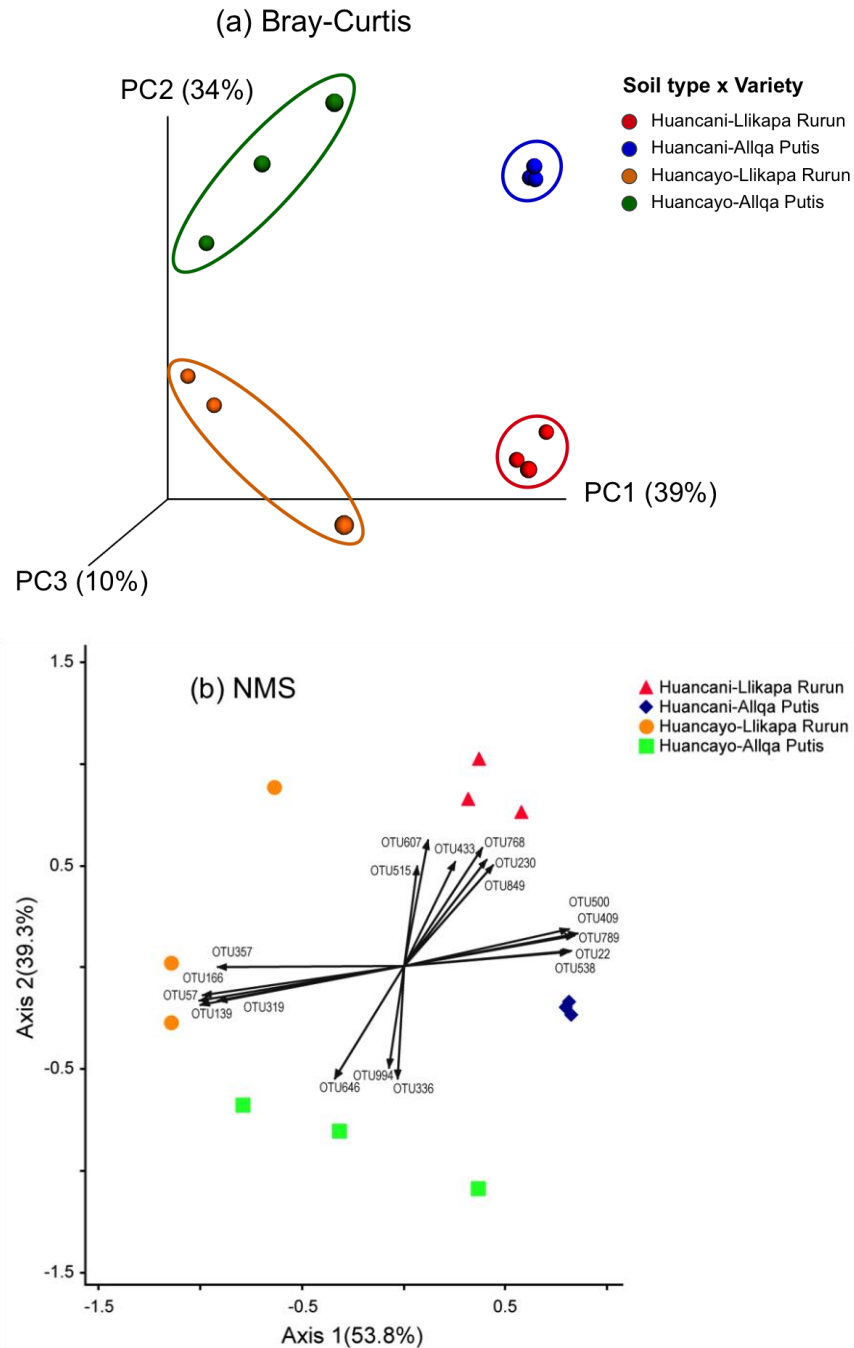
Figure 3.2 (a) PCoA plot (Bray_Curtis) and (b) NMS ordination showing the relationship between landrace and soil type and fungal community composition. Percentages on each axis denote the amount of variability associated with each axis. NMS bi-plot: the final stress for 2-d NMS was 6. The most correlated OTUs were presented as vectors.
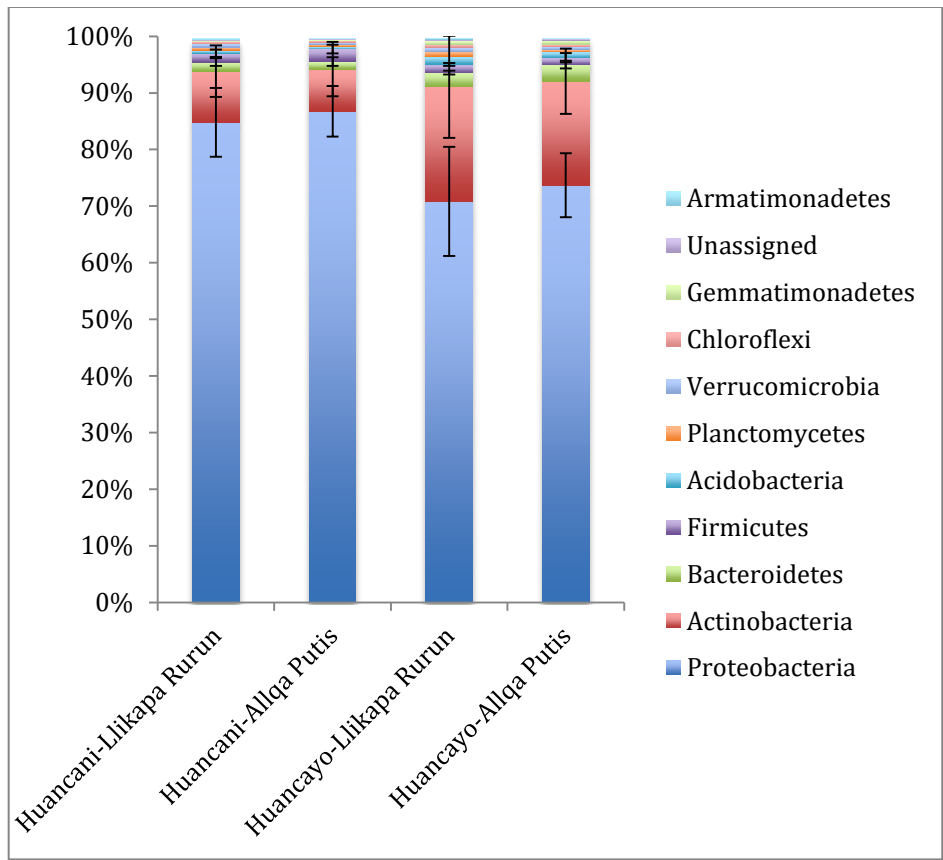
Figure 3.3. Taxonomic summary of the relative abundance of bacterial phyla
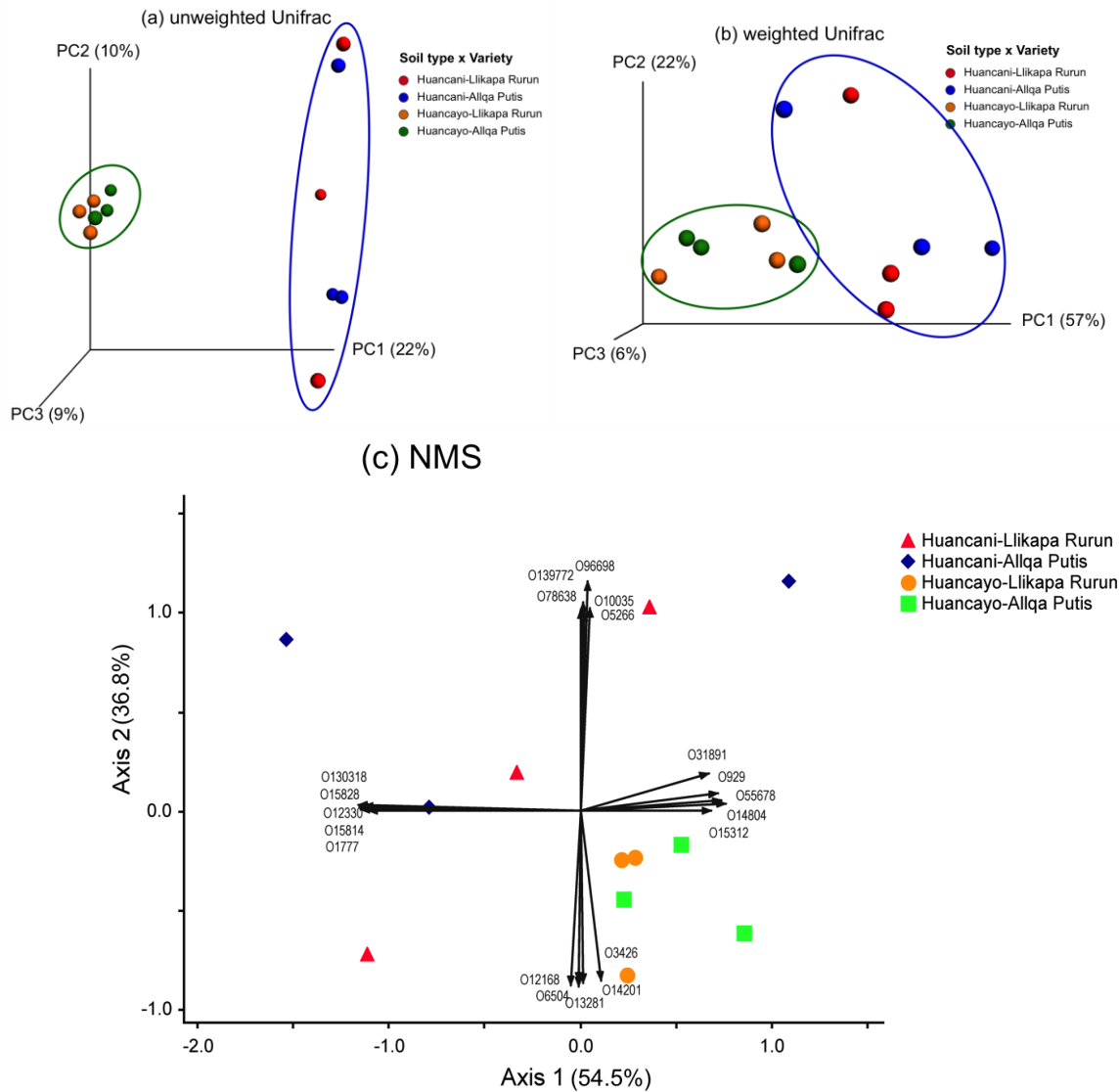
Figure 3.4 PCoA plot describing (a) unweighted and (b) weighted Unifrac and (c) NMS ordination showing the relationship between landrace and soil type and bacterial community composition. The most correlated OTUs were presented as vectors. Percentages on each axis denote the amount of variability associated with each axis.

Figure A3.1 The distribution of treatments in the beds in the greenhouse at transplanting (left) and plants at 45 dat (right).
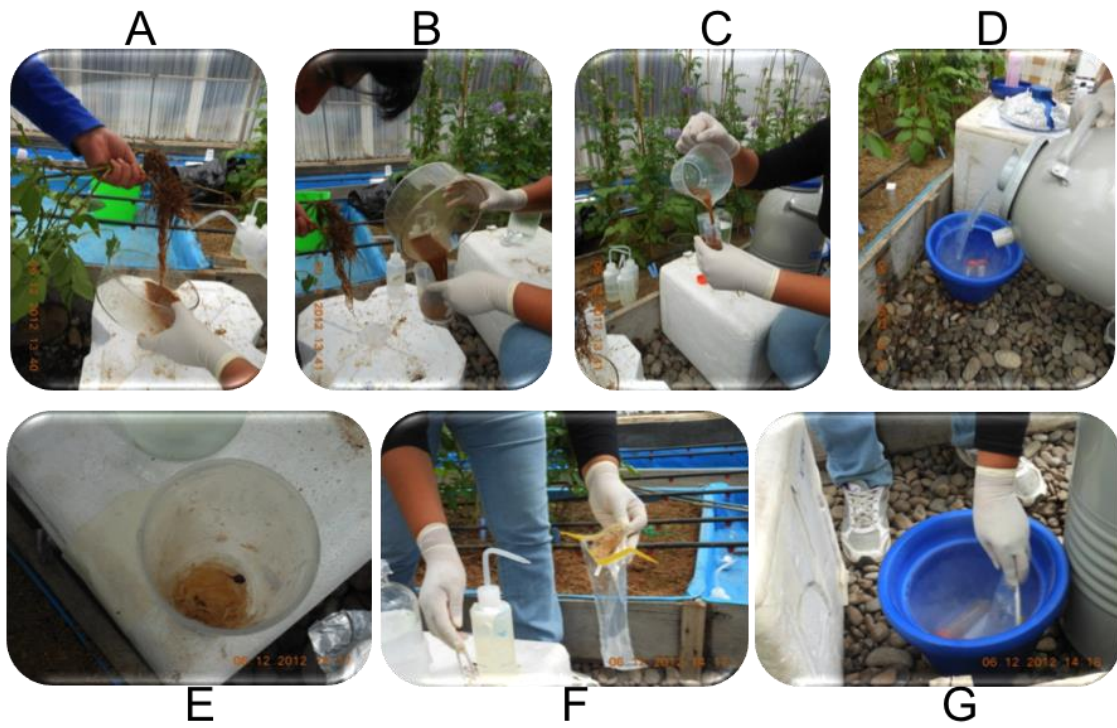
Figure A3.2   (A-D) Rhizosphere extraction from roots and freezing; (E–G) roots
washing and freezing.
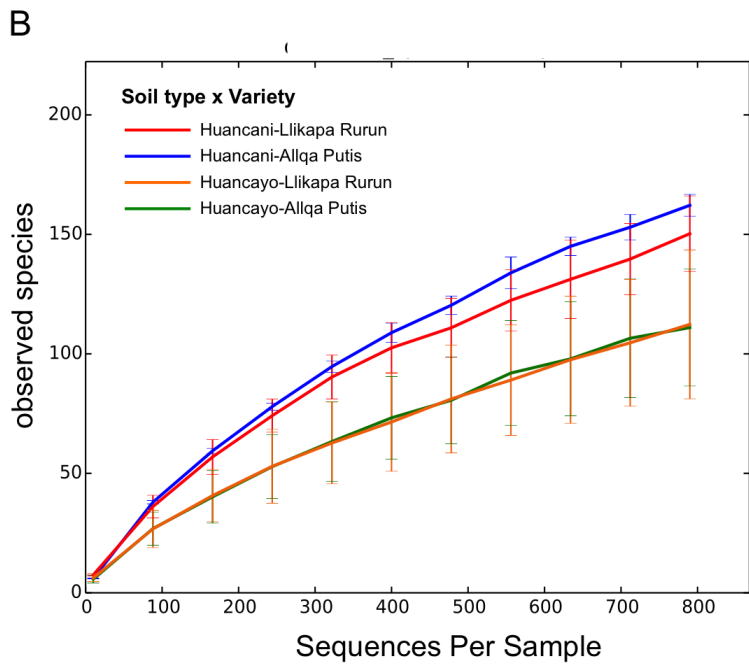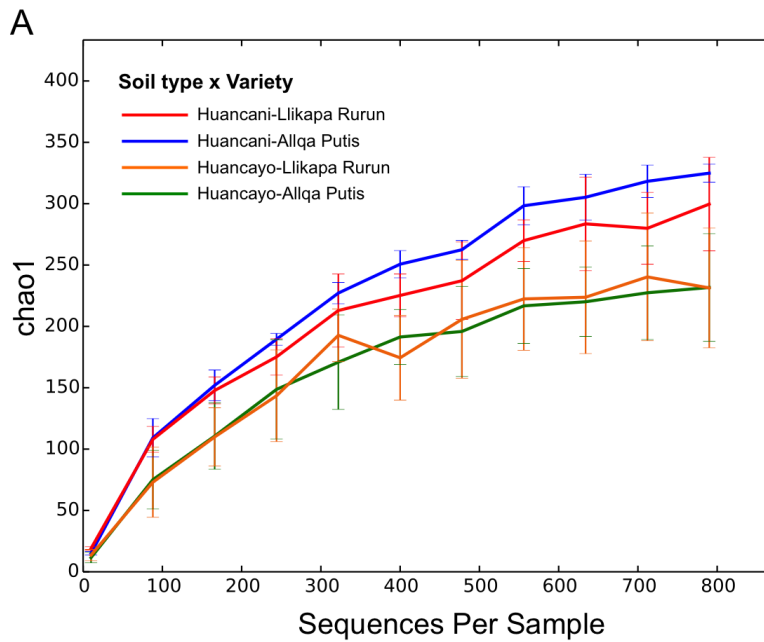
Figure A3.3 Rarefaction plots of fungal alpha diversity for Huancani-Llikapa Rurun, Huancani-Allqa Putis, Huancayo-Llikapa Rurun and HuancayoAllqa Putis samples using chao1 (A), observed species (B).
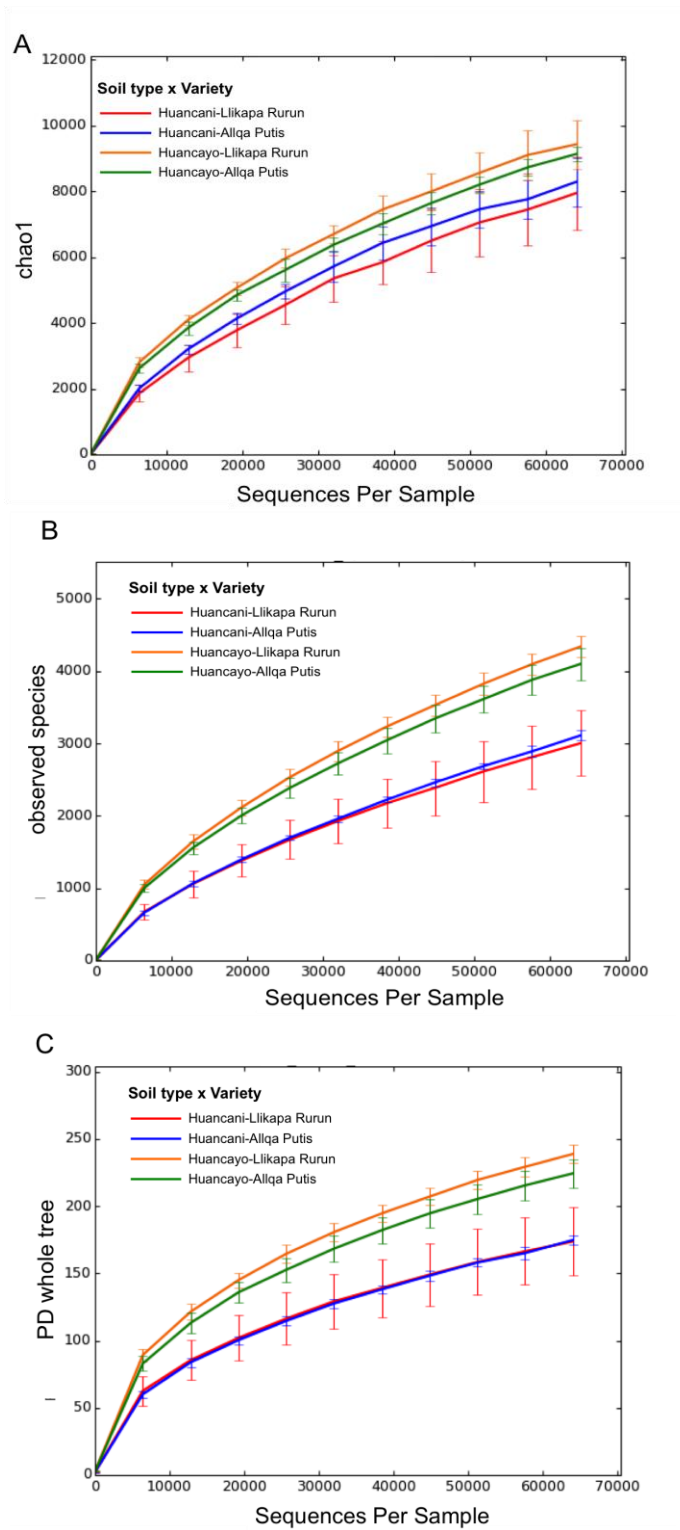
Figure A3.4 Rarefaction plots of bacterial alpha diversity for Huancani-Llikapa Rurun, Huancani-Allqa Putis, Huancayo-Llikapa Rurun and HuancayoAllqa Putis samples using chao1 (A), observed species (B), and PD whole tree (C).

(a) Huancani Soil

PC2 (25%)

● Llikapa Rurun
● Allqa Putis

PC1 (66%)

PC3 (5%)

(b) Huancayo Soil

PC2 (20%)

● Llikapa Rurun
● Allqa Putis

PC1 (59%)

PC3 (13%)
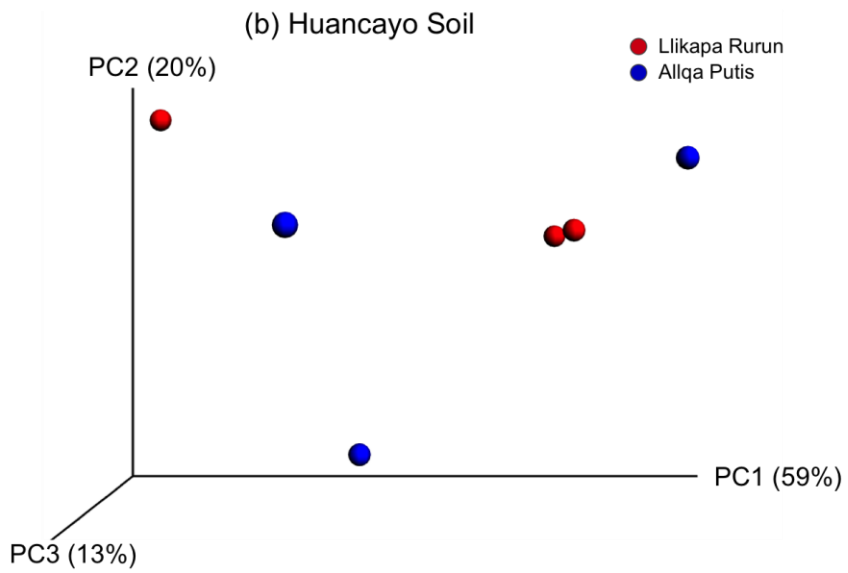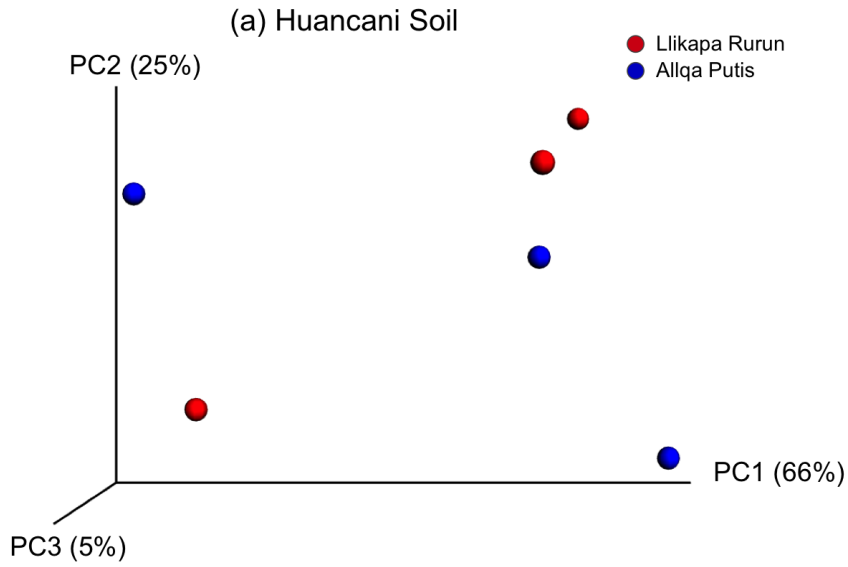
Figure A3.5 PCoA plot showing the relationship between landrace and bacterial community composition in each soil type .

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Chapter 4 Exploiting the linkage between rhizosphere microbiome and iron cycling in Andean potatoes

Hua Xiao[1], Mark Williams[1], Richard Veilleux[1]

Abstract

Iron (Fe) has been considered one of the most important nutrients for plant growth and human health. It is one of the most abundant elements in soil but its low bioavailability can limit plant growth. Fe accumulation by plants can influence the physicochemical conditions in the rhizosphere soil, thus potentially selecting microbial communities that play a role in Fe nutrient cycling and plant production. To assess the effect of plant Fe status on the structure and diversity of microbial communities in the rhizosphere, we grew an Andean native potato landrace in a greenhouse under Fe limitation using three levels of foliar fertilization (FeEDDHA). Application occurred at 45, 60 and 70 days of growth. At the flowering stage (80 days), the rhizosphere soils were sampled. High-throughput sequencing of 16S and ITS region of ribosomal RNA gene amplified from the soil DNA were conducted to examine the bacterial and fungal communities, respectively. Data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME). The taxonomic summary of the relativ[4]e abundance showed that *Ascomycota* (~30.9%) and *Glomeromycota* (~16.3%), and *Proteobacteria* (~26.7%), *Acidobacteria* (~20.0%) and *Actinobacteria* (~10.7%) were the most dominant phyla of fungi and bacteria, respectively. 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. There were no effects on bacterial communities, but PCoA and

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[1] Department of Horticulture, Virginia Tech, Blacksburg, Virginia, USA

multivariate statistical analysis indicated that moderate Fe application levels influenced rhizosphere fungal community structure compared to high and no Fe treatment. In particular, the phylum *Glomeromycota* contributed the greatest variation across Fe application rates. Overall, the results indicate that foliar addition of Fe influences plant Fe that resonates into the root system to affect rhizosphere fungal communities. Potato Fe status thus appears to influence potato root-fungal interactions.

Introduction

Improving biomass yield and plant product quality in a sustainable way is a major challenge for agriculture today (Godfray et al. 2010). Iron (Fe) plays a major role in sustainable agriculture because it can be a limiting factor for biomass and yield production in crops such as tomato (*Solanum lycopersicum*), spinach (*Spinacia oleracea*), and rice (*Oryza sativa*) (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 (WHO 2017; Bouis 1995). 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 that possess a plant physiological pH range (6.0 ~7.0) (Marschner 2012). Because of its importance in the human diet, greater Fe levels in crop production may help support human health, particularly in regions of the world where the availability of animal protein is limited. Therefore, discovering and implementing innovative technology to improve Fe use efficiency play important roles in sustainable agriculture and human diet.

Potato *(Solanum tuberosum* L*.)* ranks fourth as a major crop in the world (FAO 2015). It is a basic food source for humans with abundant vitamin and minerals, and its nutritional

status is important for human health (CIP 2011). For instance, a rich potato source of Fe, a large Russet potato cultivar contains 3.2 mg of Fe and would account for up to 40 and 18 percent of the daily Fe requirements for men and women, respectively (USDA 2014). On average, a small potato (100 g serving) provides 6% Daily Value (DV) of Fe (Brown et al. 2010). Andean potato landraces (*Solanum tuberosum* Group Andigena and others) represent a valuable source covering the largest part of the available genetic diversity of cultivated potato, which are still grown and consumed by Peruvian populations. The previous study showed that the Fe content in the edible portion strongly varies among landraces (Lefèvre et al. 2012), and depends both on genotype as well as the environmental factors (Andre et al. 2007; Burgos et al. 2007). Therefore, increasing micronutrient contents in potato and improving their bioavailability provide sustainable, economic means to solve micronutrient deficiency in the human diet. Andean potato landraces, derived from their habitat of origin, are a fitting resource for this study.

Many research organizations worldwide are investing in the genetic potential of crop plants to improve Fe bioavailability in common staple food crops such as wheat, rice, beans, and oilseeds, through traditional both plant breeding and transgenic approaches (Velu et al. 2014; Trijatmiko et al. 2016). These approaches have their advantages, but because of the importance the soil microbes play in plant nutrient status, integration of their activities into breeding or management are warranted. Recently, inoculation of soil with microbes was shown to increase plant Fe uptake in wheat, white lupin and cucumber plants (de Santiago et al. 2009; Zhao et al. 2014; Pii et al. 2015; Zhang et al. 2009). Plants support abundant and active soil microbial communities by release of rhizodeposits through altering the soil chemistry and acting as selective growth substrates

for soil microorganisms. Microorganisms in turn affect plant growth and nutrient uptake by releasing growth-stimulating or -inhibiting substances that influence root physiology and root system architecture (Govindasamy et al. 2009; Ryu et al. 2005), and thus may naturally regulate Fe acquisition via interaction with roots.

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, the Fe deficiency together with plant rhizodeposits lead to the selection of microbial populations having a more efficient Fe uptake system than those of microorganisms that are more susceptible to Fe starvation. 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. Some bacterial and fungal species have been noted for their phosphate-solubilizing abilities by solubilizing organic P to plant-available inorganic orthophosphate or protons secretion (Bünemann et al. 2012; Oberson et al. 2001; Oberson and Joner 2005). Since the phosphate generally combines with elements in the soil such as calcium (Ca), magnesium (Mg), aluminum (Al), and Fe, and forming solid compounds, releasing bioavailable P may increase the Fe availability to meet crop needs by microorganisms (Borggaard et al. 1990; Hinsinger 2001; Tomasi et al. 2008). In addition, plant Fe nutrition could be directly promoted by siderophores produced by some microbes (Robin et al. 2008; Lemanceau et al. 2009). Arabidopsis, as a strategy I plant, incorporated more complexed Fe when amended with *Pseudomonas fluorescens*-derived siderophore pyoverdine (Vansuyt et al. 2007). More evidence showed that Fe^{3+} complexed with siderophore produced by microorganisms could directly act as Fe donor for plants, restoring the Fe-deficiency condition in hydroponic

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culture (de Santiago et al. 2009; Nagata et al. 2013). Xiong et al. (2013) revealed that Fe^{3+}-DMA complexes could be absorbed directly by strategy I plants by using a transporter codified by a gene belonging to the yellow stripe1-like (YSL) family and located at the root epidermis. It, therefore, appears that some soil microorganisms play an important role in favoring plant Fe uptake. Exploring soil microbial community in the rhizosphere thus provides the potential to improve Fe uptake and Fe use efficiency in plants. However, many soil microorganisms contributing to plant Fe acquisition and the related mechanism remains unknown.

Fe status change in plants affects root exudates, and then can alter the relative abundance of soil microorganisms in the rhizosphere of red clover and maize (Jin et al. 2010; Carvalhais et al. 2011). The plant-microbial interaction involving Fe acquisition has been demonstrated in a few previous studies (Yang and Crowley 2000; Robin et al. 2006; Robin et al. 2007). The changes in microbial community composition in the rhizosphere induced by plant Fe-deficiency should be beneficial for plant Fe uptake because there are more siderophore-producing microorganisms isolated from the plant under Fe shortage (Jin et al. 2008; Jin et al. 2006). Nevertheless, direct evidence for the function of plant-microbial interaction on Fe acquisition is still limited. The mechanisms of microbial activity and community structure in enhancing the Fe uptake of plants still need to be investigated.

As for potato, Fe fertilizer by foliar application during the flowering stage increased the tuber weight and resulted in higher concentrations of Fe in the harvested tubers (Hadi et al. 2014; Al-Jobori and Al-Hadithy 2014). These influences may result from the change of potato root activity since the increased weight and Fe content of tubers need roots to

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take up more nutrients. The change of potato root activity may influence their rhizosphere microbial community. In this study, foliar application of Fe fertilization on potato plants has been conducted in the greenhouse. The objective was, therefore, to examine the impact of plant Fe nutritional status on the rhizosphere bacterial and fungal communities and to potentially screen specific soil microbes that may be impacted by potato Fe acquisition. Discerning whether foliar application of Fe would translate into changes in roots and rhizosphere provides a proof of concept that potato plant Fe status impacts soil microbes.

Methods

Plant materials and experimental design

An Andean native potato landrace (*Solanum tuberosum* L. CIP 703580) was imported from CIP (International Potato Center) in Peru. This potato landrace has relatively low tuber Fe concentration (19.85mg/kg DW Fe) compared with other imported landraces. To make identical plants for replication, we subcultured *in vitro* plants into test tubes (25 x 150mm) 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) and grew them in a growth chamber under a 16h photoperiod with a light intensity of 500 μM 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 (5ppm, pH≈7) in the greenhouse at Virginia Tech in Blacksburg in 2015 spring (Table A4.1). The soil mixture was composed of 30% field soil collected from agricultural land at Kentland Farm, Montgomery County, VA (37.20N, 80.56W), 0-20cm 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 7. Every week, plants were fertilized with half strength Hoagland solution (pH 7) without Fe. Greenhouse conditions were maintained at 22°C and a relative humidity of ~65%. The daily light schedule (intensity 370 μM m^{-2} s^{-1}) consisted of 15 h light and 9 h of darkness.

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 with a pH of 5.5 were prepared, respectively. All foliar applications contained 0.1% (v/v) Tween80 as a surfactant. The soil surface of each pot was covered with plastic wrap during application to remove the potential for direct Fe fertilization into the soil. Misting occurred every other week (3 times) up to flowering stage. Treatments were applied from 6:00 to 7:00 pm to prevent leaf damage. Three treatments with five replicate pots were used to create a complete randomized experimental design.

Sampling and growth parameters

A SPAD chlorophyll meter (SPAD-502, Minolta) was used 2 day prior to each foliar Fe application and 2 days following Fe application from three randomly selected newly formed leaves on each plant. 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 (Figure 4.1) was collected and put in sterile conical tubes and stored at -80ºC. The DNA was then extracted from the soil and further used for 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 60ºC 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 10ml 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.

DNA extraction and PCR amplification

A 0.5g 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 (1µM), 2.5 µL DNA template (5 ng/µL). Thermocycling consisted of an initial

denaturation at 95ºC for 3 min, followed by 30 cycles of 95ºC for 30 sec and annealing temperature at 55ºC for 30 sec, and 72ºC for 30 sec. And a final extension time was implemented at 72ºC for 5min.

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 (1µM), 2.5 µL DNA template (5 ng/µL). Thermocycling consisted of an initial denaturation at 95ºC for 3 min, followed by 30 cycles of 95ºC for 30s and annealing temperature at 60ºC for 30 sec, and 72ºC for 30 sec. And a final extension time was implemented at 72ºC for 5min. 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.

Processing of sequence data and analyses

Quality filtering and de-multiplexing by sample was computationally performed to remove any sequence with a mismatch associated with barcode or primer sequences. 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

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Into Microbial Ecology toolkit-version 1.7.0 (QIIME) (Caporaso et al. 2010). Operational taxonomic units (OTUs) were delineated at 97% sequence similarity level using *uclust* and *usearch61* (Edgar 2010). The most abundant sequence for each OTU was chosen as a representative sequence for that OTU, and was used to create an OTU table. Representative sequence of each OTU in bacterial and fungal community was classified into taxonomy, respectively, using *uclust* against the Greengenes reference database (McDonald et al. 2012; DeSantis et al. 2006) and Ribosomal Database Project (RDP) *classifier* against the UNITE reference database (Abarenkov et al. 2010; Wang et al. 2007).

Variation in bacterial and fungal community compositions among treatments was assessed using Python scripts in the QIIME platform. 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 un-weighted UniFrac distance matrix (for bacteria) (Lozupone and Knight 2005), and Bray-Curtis (for fungi) (Beals 1984) distance matrix. 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. 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).

Results

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 (Figure 4.2). 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 (Figure 4.3). The ratio of shoot length/root dry weight is significant different across three treatments (p=0.0035) with the lowest value in the deionized water treatment (Figure 4.3). These results, importantly, revealed the application of Fe fertilization changed the growth of potato plants as well as the Fe status in the potato tuber, as expected. These results whereby less carbon is allocated to roots relative to shoots is consistent with release from Fe limitation. Fe sufficient plants would be expected to allocate fewer resources to

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roots/rhizosphere relative to shoots whereas Fe limited plants would allocate more carbon to roots to increase Fe uptake potential.

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.

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 (Table 4.1). The rarefaction curves with 94.6% coverage indicated the presence of saturation but also did not show differences for fungal richness (Figure A4.1), and indicated no shifts in fungal OTU richness in response to Fe amendment.

The alignment of normalized sequences dataset with the UNITE reference database identified eight fungal phyla. 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 likely represent as yet unexplored microorganisms that their phylogenetic relationships are poorly understood, or chimeras or other artifacts introduced during the PCR or sequencing process (Tai et al. 2015; Liu et al. 2015; Kröber et al. 2009). 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* (Figure 4.4).

ANOVA was used to compare the difference of each phylum across Fe treatment; however, and no significant effect was detected. Multivariate data analyses in contrast demonstrated clear distinctions of the fungal communities at finer taxonomic levels 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 4.2). There were distinct fungal communities associated with the different amounts of Fe fertilization.

In three-dimensional principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity matrix, samples within the moderate Fe fertilization treatment clustered together and separated well from samples from low and high Fe fertilization treatments along principal coordinate 1 (PC1), PC2 and PC3, explaining 40% of sample variation (Figure 4.5). NMS analysis also separated the moderate Fe fertilization treatment. Overall,

these results indicated that the amount of foliar Fe applied affected the composition of fungal communities in the potato rhizosphere.

For the active fungal genera that were most correlated with effect of Fe fertilization, the community with relative abundance above 0.1% was selected and compared among treatments using the Monte Carlo procedures in indicator species analysis (ISA) based on taxonomic composition at the genus level. ISA identified statistically significant indicator taxa and the main variables responsible for treatments. The genera having a significant effect from Fe application are showed in Table 4.3. Four genera (belonging to *Glomeromycota* and *Basidiomycota*) had significantly different relative abundances (*p*<0.05) among treatments. Three genera in the class *Glomeromycetes* and *Microbotryomycetes* had significantly greater abundance in the high Fe fertilizer treated group. 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 respond to the moderate fertilization application, and *Aspergillus* showed positive response at the low Fe level in soil. Those genera were thought to have potential microorganisms involved with Fe uptake by the plant and associated microbes.

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

Illumina MiSeq sequencing analysis of the V3 and V4 regions of 16S rRNA gene was performed to characterize the bacterial community composition in the rhizosphere. A

total of 1,218,102 high-quality sequences (counts) was 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 three Fe fertilization treatments (Table 4.4). The rarefaction curves did not asymptote, but estimates indicate 92.6% coverage saturation and no strong differences of bacterial richness in relation to treatments (Figure A4.2). Therefore, the richness and evenness of the bacterial community might be similar in the rhizosphere with three different amounts of Fe fertilization.

The alignment of normalized sequences dataset with the Greengenes reference database identified 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*) (Figure 4.6). ANOVA was used to compare the difference of each phylum among treatments and showed significant difference in the phylum *Nitrospirae* ($p=0.012$).

When bacterial communities were examined based on the phylogenetic composition in relative abundance, community composition 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 of different Fe applications (Table 4.5). Bacterial community composition did not shift in response to the Fe status change in the plants. However, in three-dimensional principal coordinates analysis (PCoA) based on weighted Unifrac and unweighted Unifrac matrices, samples within the moderate Fe fertilization treatment clustered together and separated from low and high Fe fertilization treatment visually, whereas no separation between low and high Fe application was observed (Figure 4.7).

Discussion

In this study, we explored the effect of foliar application of Fe amendment on the composition of rhizosphere microbial communities of an Andean potato landrace. 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 alter Fe and perhaps reduce Fe deficiency, which would then have effects on plant-microbial associations in the rhizosphere. The results indicated that Fe fertilization supported greater relative abundance of *Ascomycota*, and some fungi in *Glomeromycota* were relatively more abundant as a result of both low and high Fe treatments. These results support the idea that Fe in some way regulates rhizosphere fungal communities, perhaps through a change in the flow of carbon to roots. Though it is not possible to determine from this study whether the changes in fungal communities and mycorrhizal fungi resulted from a mutualism or competition between

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plant and fungus, it does suggest that further research into the role of plant-microbial interactions in plant Fe acquisition deserves inquiry.

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 the plant Fe nutrition uptake while few fungi, in this regard, have been tested (Zhang et al. 2009; Pii et al. 2015; Nagata et al. 2013; Pii et al. 2016; Bona et al. 2016; Zhou et al. 2016). Therefore, the indicator species analysis was conducted in the present study to discover fungal groups that may be significant contributors in the plant-fungal interaction affecting Fe acquisition. The result showed that some genera in the phyla *Glomermycota*, *Ascomycota* and *Basidiomycota* were relevant groups associated with differences in plant Fe status (Table 4.3).

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 since both elements form various chemical complexes (Borggaard et al. 1990). It is thus straightforward to see that P mobilizing mechanisms may also increase plant Fe availability. It was reported that AMF has significant positive impact on the uptake of immobile nutrients such as Fe, Zn and Cu (Lehmann and Rillig 2015). *Glomus* (order *Glomerales*), *Acaulospora* (order *Diversisporales*) and *Scutellospora* (family *Gigasporaceae*), discovered in this study, were able to produce siderophores and are thought to increase the extent of Fe absorption in *Pennisetum*

glaucum (Pearl millet) and *Sorghum bicolor* (sorghum) growing in Fe^{3+} contaminated soil (Mishra et al. 2016).

In heavy metal contaminated sediments 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) in these sites (Abdel-Azeem et al. 2015), An Fe-enriched fungus was similarly isolated from a natural environment belonging to this class (Zhang et al. 2015). In this study, OTUs in this class had a much greater relative abundance in the high Fe fertilizer application treatment. Thus, it may be that high levels of Fe translated into greater Fe in the rhizosphere, which would 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 phylum *Ascomycota*, *Trichoderma* spp. (order *Hypocreales*) are well-studied filamentous fungi widely used in agriculture as biofertilizers 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 and relatively high microbial activity (de Santiago et al. 2013). The inoculation with *T. asperellum* T6 to the 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 *Eurotiale*s, can excrete organic acids, which can solubilize the unavailable potassium and make available to plant roots (Teotia et al. 2016). Consistent with the role

of *Eurotiale*s, these rhizosphere taxa were sensitive to changes in Fe applied to leaf surfaces

Although the shifts of a microbial community indicate that rhizosphere microorganisms are sensitive to Fe treatment, and therefore may interact with potato roots to aid Fe uptake, the mechanisms involve in this plant-microbial interaction has not been directly demonstrated. However, 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. Several studies have uncovered a linkage between NP nutrient cycling and plant-microbial interaction and found that the plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) can enhance the availability of N and P by production of different enzymes, organic acids and siderophores As for P nutrient cycling, except for AMF as mentioned above, 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 the subsequent availability of P (as phosphate) to plant roots. (Clarholm et al. 2015; Giles et al. 2012; Patel et al. 2010; Richardson and Simpson 2011; Richardson et al. 2011).

A variety of studies have described changes in soil and rhizosphere microbiomes in response to direct fertilization of N and P. Though the approach to fertilization was different, and different nutrients were involved, these experiments can help to understand the potential role that Fe could play in structuring rhizosphere microbial communities in the potato rhizosphere. Nitrogen and P fertilization have been shown to shape rhizosphere microbial community composition in the rhizosphere across soil types, crop species, and

location (Liu et al. 2011; Peyret-Guzzon et al. 2016; Paungfoo-Lonhienne et al. 2015; Chávez-Romero et al. 2016; Cassman 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, respectively. The effects were not restricted to AMF but span a wide range of fungal taxa that 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 the nutrient turnover, which in turn impacted rice productivity. These results do not speak directly to the effects of Fe, in which there is sparse information in the literature, but they do speak to the broader concept of how nutrients play a major role in plant-microbial interactions.

Compared with the macronutrients N and P, there is little information on the plant-microbial interaction associated with Fe nutrient cycling. To the best of my knowledge, the effect of Fe on plant-microbial interaction in agriculture has not been directly tested in an experiment. This is the first study for assessing the impact of Fe availability on the plant-fungal interaction to date. In our previous work, two Andean potato landraces with different Fe concentrations in the tubers showed large effect on the rhizosphere fungal community but the Fe status was not the only factor that differed across those landraces. The present study tried to expand on this finding and thus restricted the treatment to influence plant Fe status. Fe thus appears to play a role in structuring fungal community composition in the rhizosphere. Those changes best fit into the paradigm whereby root exudates change microbial communities. The release of many types of plant root

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exudates such as organic acids improve plant nutrient acquisition and influence the diversity and composition of rhizosphere microbial communities (Haichar et al. 2008; Shi et al. 2013; Huang et al. 2014; Bashir et al. 2016) which inferred roots expansion to acquire more nutrients. The Fe concentration and mass in the tubers and shoots (Figure 4.3) imply that C flow to roots was altered and in turn selectively changed the selection of rhizosphere soil microbes. Further experiments that isolate the role of Fe in soil and its transfer to plants will help to discern mechanisms associated with the plant-microbial interaction.

The present study observed that the rhizosphere bacterial community was not significantly different as a result of Fe fertilization. A similar result has been obtained in a study of Fe fertilization in the ocean using 16S rRNA gene tag pyrosequencing (Thiele et al. 2012). In that study the composition and abundance of the bacterial and archaeal community in the Fe-fertilized water body were remarkably constant. However, 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 (Yu et al. 2015; Michelsen et al. 2014), and/or overshadow impacts of Fe.

In the present study, potato plants might suffer from Fe deficiency stress under low Fe treatment, then the organic acid such as phenolic compounds might be exuded from the root and accumulate in the rhizosphere, which in turn selectively favor microorganisms that can also produce organic acids or siderophores to increase Fe solubility in the soil. Thus, the fungi positively related to the low Fe treatment, would help plant Fe acquisition and might explain the reason why the Fe concentration of roots from three treatments was not significantly distinct. On the other hand, plant-microbe competition might exist in the soil because both microbes and plants have high demands to meet the specific requirements (Colombo et al. 2014; Marschner et al. 2011; Mimmo et al. 2014). Greater relative abundance of fungal groups may have thus competed 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 get enough Fe for growth, then the growth of microorganisms with a less efficient Fe uptake or high Fe demand would increase, which in turn shift the composition of microbial community from the low Fe treatment. The identification of indicator species in this study provides clues to specific groups that may exert beneficial effects on plant productivity and nutrient fortification.

In summary, this is, to our knowledge, the first study to observe rhizosphere fungal community composition changes in response to foliar Fe amendment. Foliar Fe application approach reduced the complexity of effect on the soil microbial community, but directly changed the Fe status of plants such as potato tuber Fe accumulation as well as the plant root growth. 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 *Glomermycota* *Ascomycota* and *Basidiomycota* on potato Fe nutrient cycling are consistent with changes that occur as a result of N and P. Lastly, it was also confirmed that *Ascomycota* and *Glomeromycota* in fungi and *Proteobacteria*, *Acidobacteria* and *Actinobacteria* in bacteria are ubiquitous members of the potato rhizosphere (Senés-Guerrero and Schüßler 2015; Kobayashi et al. 2015; Senés-Guerrero et al. 2014). The next step of this work could determine the relationship between plant root activity, plant Fe status and specific taxa. The relationship between metabolites of roots and fungal taxa could be use to understand the mechanisms by which fungi support potato Fe status.

Table 4.1 Alpha diversity metrics for Fe fertilization in rhizosphere fungal community.

Fe fertilization	Average ± SE			
	Chao 1	Observed Species	Shannon	Simpson
Low	18576±858	9211±931	8.5±0.19	0.986±0.004
Moderate	17865±573	8448±583	8.7±0.04	0.990±0.001
High	19149±1606	9224±1245	8.7±0.04	0.989±0.002
p-value (two-tail)	0.724	0.813	0.478	0.389

Calculations were based on an equal size of 70,500 from random sub-sampling on each sample. Bolded values indicate significant ($\alpha<0.05$) effects.

Table 4.2 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	

Table 4.3 Fungal genera with a greater relative abundance associated with Fe fertilization effect based on Indicator Species Analysis (*p*-value < 0.05) and NMS 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.

Table 4.4 Alpha diversity metrics for Fe fertilization in rhizosphere bacterial community.

Fe fertilization	Average ± SE		
	Chao 1	**Observed Species**	**PD_whole_tree**
Low	18520±110	9235±89	397±6
Moderate	18191±718	9061±234	389±15
High	18181±412	9265±154	400±14
p-value (two-tail)	0.855	0.675	0.777

Calculations were based on an equal size of 37,000 from random sub-sampling on each sample. Bolded values indicate significant ($\alpha<0.05$) effects.

Table 4.5 Multivariate data analysis for differences in bacterial community composition
(beta diversity)

	Treatment	F.Model	R^2		*p*-value
Adonis	Fe fertilization	0.6755	0.0632		0.807

		R statistic	*p*-value	Number of permutations
ANOSIM	Fe fertilization	0.0000	0.454	999

		A value	*p*-value	Number of permutations
MRPP	Fe fertilization	0.0020	0.403	999

Table A4.1 The properties of soil mixture used in the greenhouse experiment

Sample ID	resource	pH	BpH	P ppm	K ppm	Ca ppm	Mg ppm	Zn ppm	Mn ppm	Cu ppm	Fe ppm	B ppm
20515	field soil	6.03	6	21	112	1215	223	2.3	9.5	0.3	**3.4**	0.2
POT04	mixed soil	6.2	6.34	16	43	458	79	1.3	5.3	0.3	**5.1**	0.2
POT25	mixed soil	6.11	6.33	14	48	531	97	1.4	4.9	0.3	**4.5**	0.2
POT44	mixed soil	6.17	6.34	14	50	571	102	1.4	5.4	0.3	**4.7**	0.2

Sample ID	resource	CEC eq /100g	% Acidity	% Base Sat	% Ca Sat	% Mg Sat	% K Sat	P Rating	K Rating	Ca Rating	Mg Rating	SS Rating
20515	field soil	10.6	22.5	77.5	57.4	17.4	2.7	H-	H	VH	VH	
POT04	mixed soil	3.4	10.5	89.5	67.2	19	3.2	M+	M-	M-	H-	
POT25	mixed soil	4	10.4	89.6	66.5	20	3.1	M	M-	M	H+	
POT44	mixed soil	4.2	8.6	91.4	68.3	20	3.1	M	M-	M	H+	

Figure 4.1 Rhizosphere soil for sampling. The scale of length of compact roots is about 15 cm.
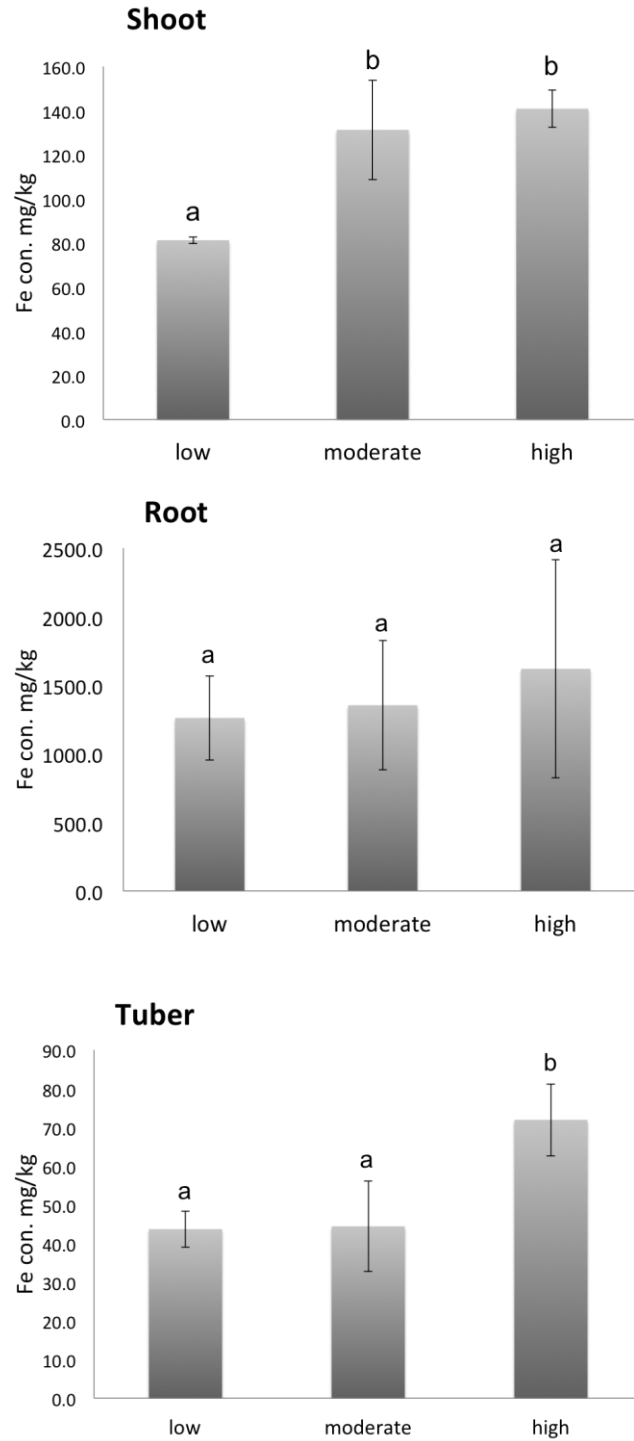
Figure 4.2 Effect of different amount of foliar iron (Fe) application on the Fe concentration of shoots, roots and tubers in an Andean potato landrace (low: deionized water; moderate: 200mg/L FeEDDHA; high: 600mg/L FeEDDHA).
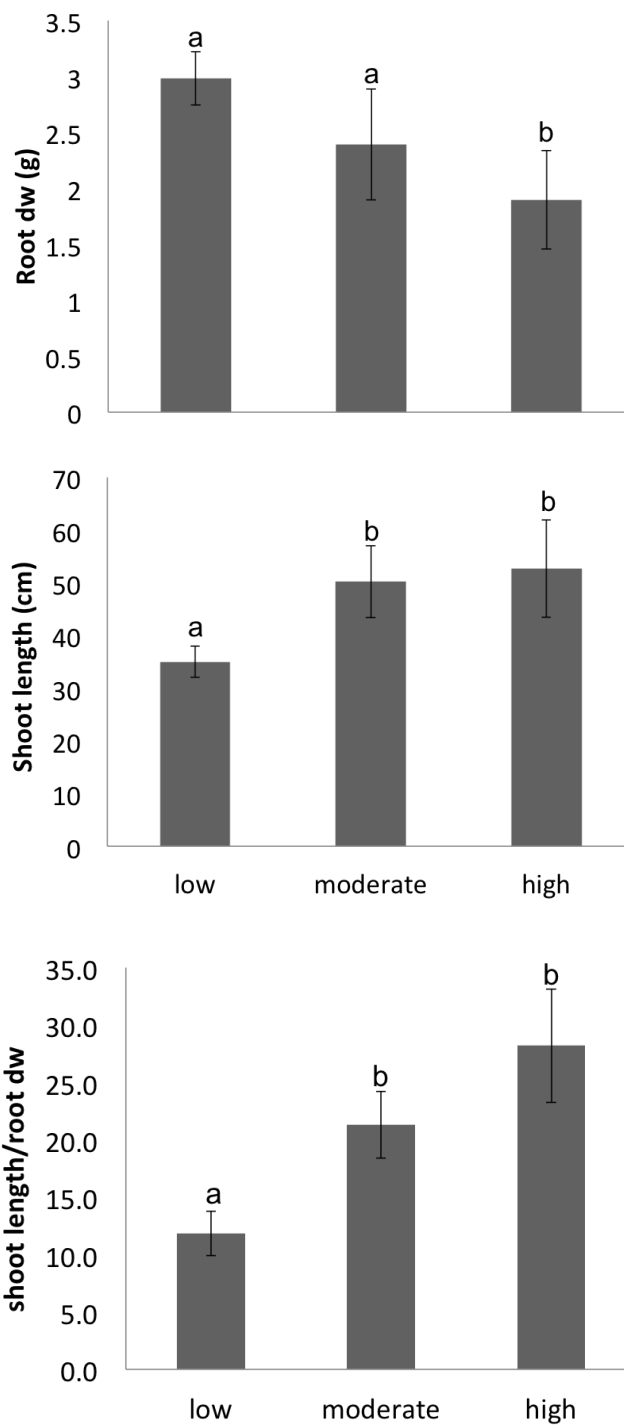
Figure 4.3 Effect of different amounts of foliar iron (Fe) application on the dry weight of roots, shoot length and the ration of shoot length/ root dry weight of an Andean potato landrace (low: deionized water; moderate: 200mg/L FeEDDHA; high: 600mg/L FeEDDHA).
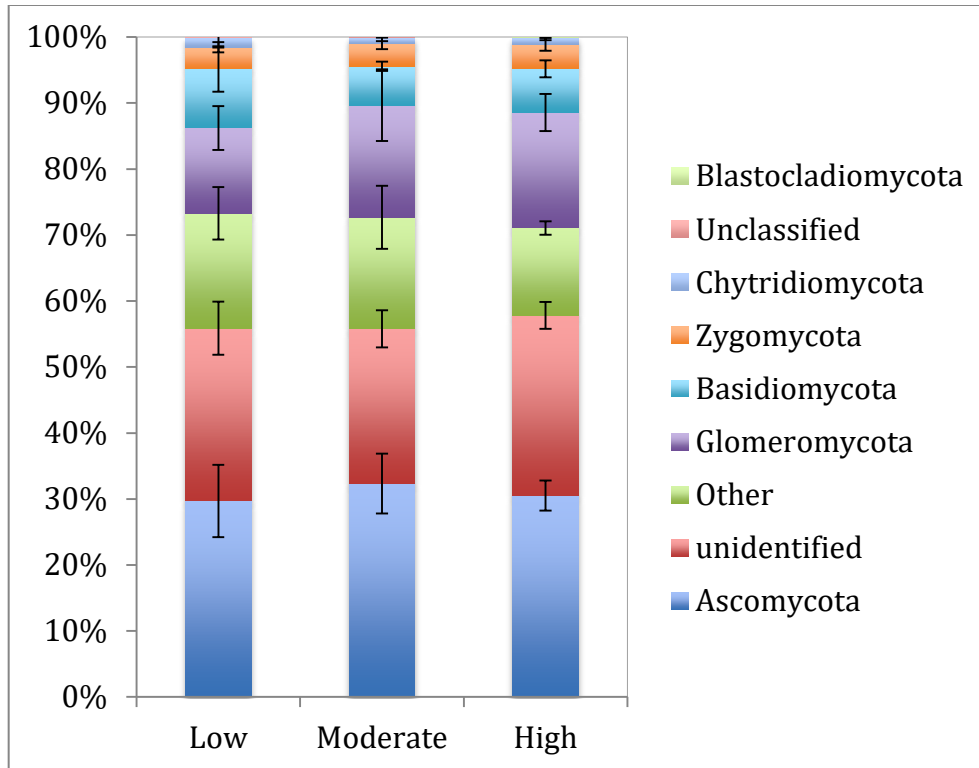
Figure 4.4 Phylum-level taxonomic summaries of fungal communities in Andean potato landrace under different foliar Fe application. 'Unassigned' and less abundant taxa were grouped in 'Other'. Taxa are ordered from bottom to top and sorted as per decreasing abundance.

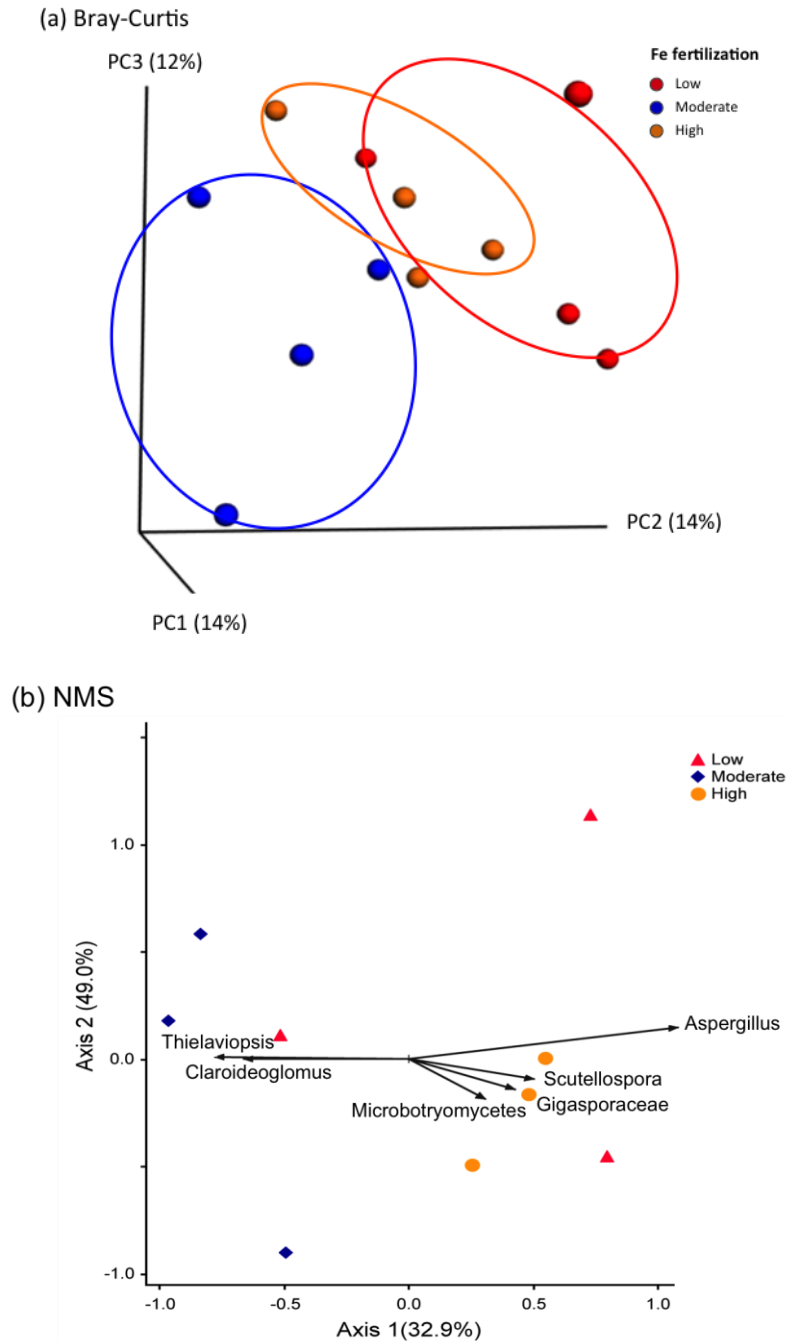Figure 4.5 PCoA plot (Bray_Curtis) (a) and NMS ordination (b) showing the relationship between Fe status and fungal community composition. The most correlated OTUs were presented as vectors. Percentages on each axis denote the amount of variability associated with each axis. The name of taxa for each vector is the cloest match to the lowest level of taxa identified.
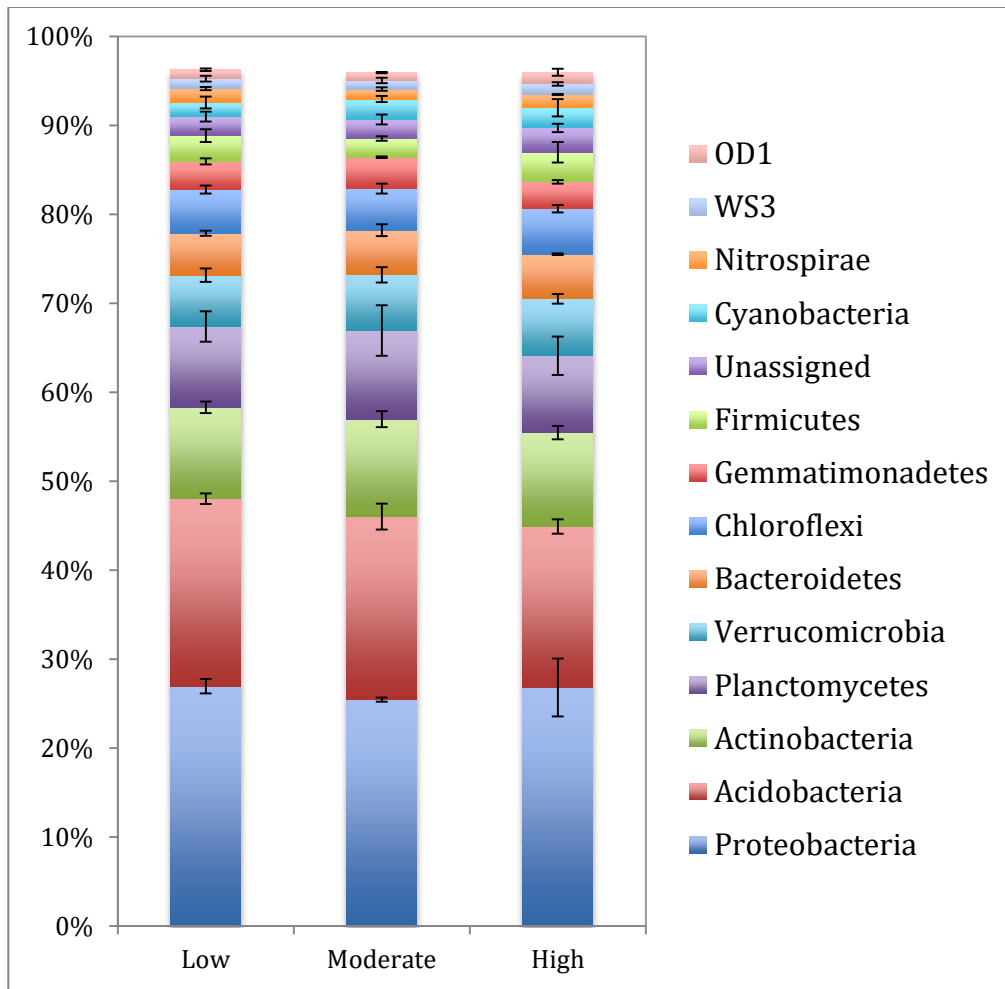
Figure 4.6 Phylum-level taxonomic summaries of bacterial communities in Andean potato landraces under different concentrations of foliar Fe applications. Bars and legend are ordered from most to lowest abundance from bottom to top of figure.
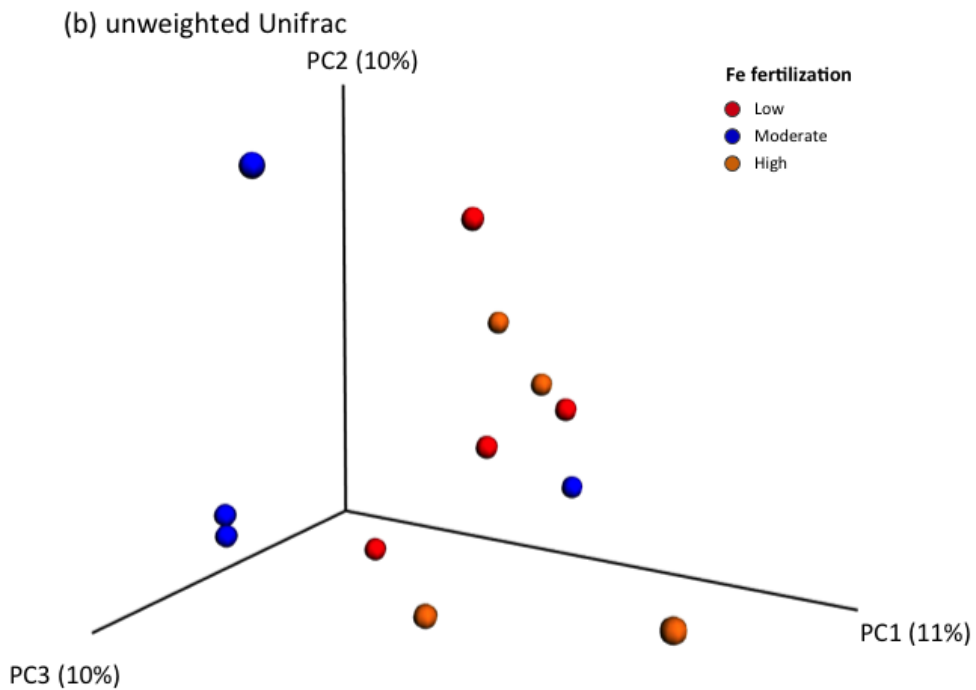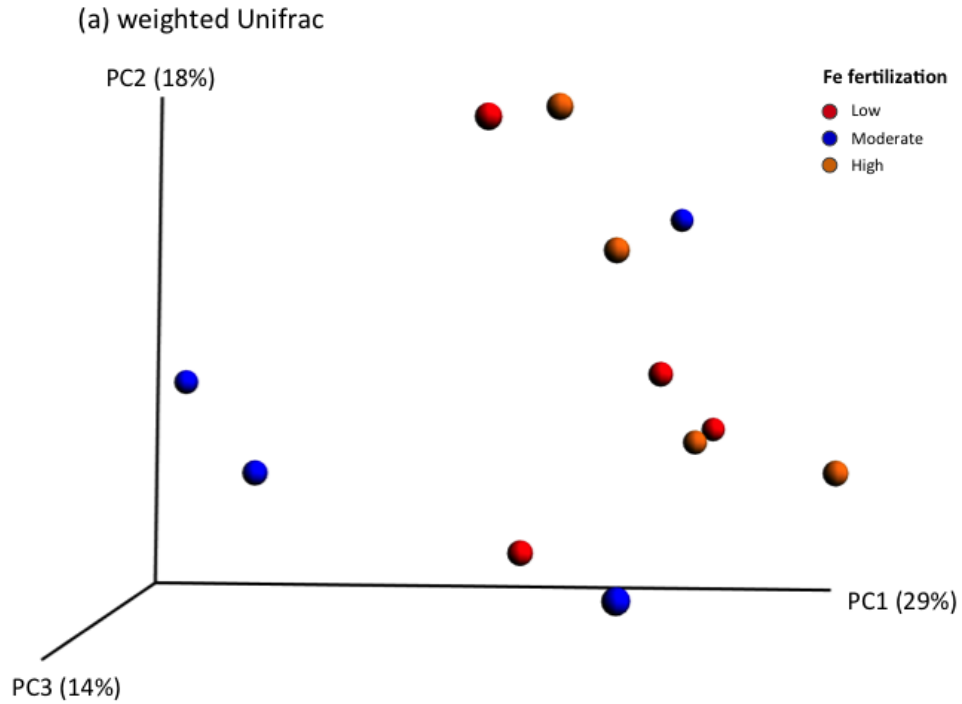
Figure 4.7 PCoA plot describing (a) un-weighted and (b) weighted Unifrac and (c) NMS ordination showing the relationship between Fe status and bacterial community composition. Percentages on each axis denote the amount of variability associated with each axis.
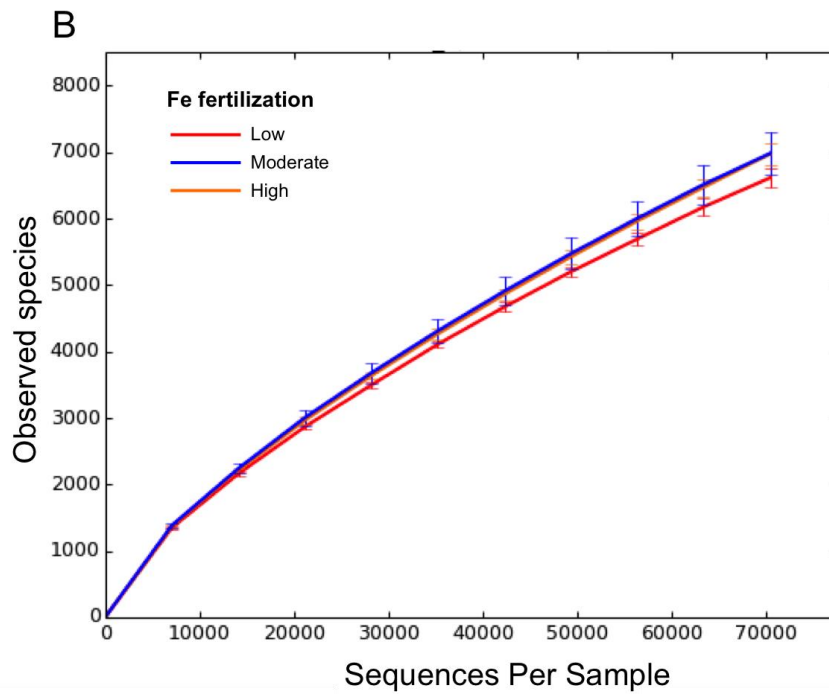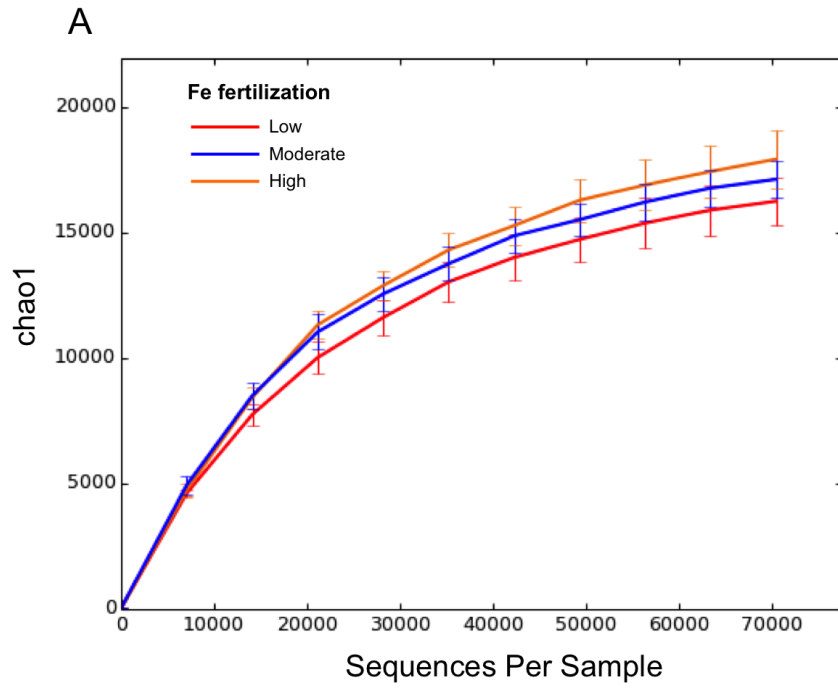
Figure A4.1 Rarefaction plots of fungal alpha diversity for foliar Fe treatment samples using (A) chao1 and (B) observed species.⌞SEP⌟

Figure A4.2 Rarefaction plots of bacterial alpha diversity for foliar Fe treatment samples using (A) chao1, (B) observed species, and (C) PD whole tree.

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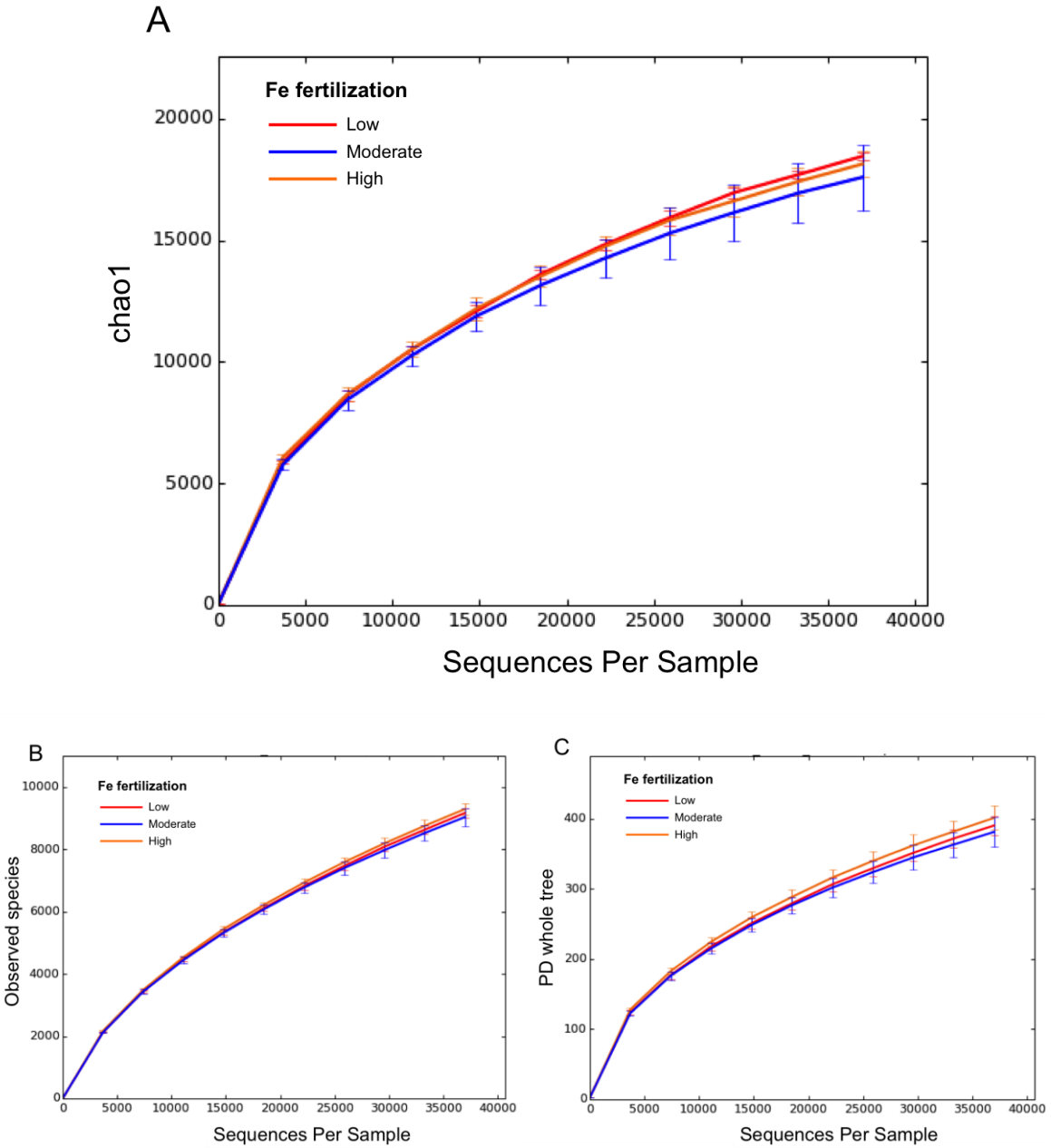Abdel-Azeem A, El-Morsy E, Nour El-Dein M, Rashad H (2015) Occurrence and diversity of mycobiota in heavy metal contaminated sediments of Mediterranean coastal lagoon El-Manzala, Egypt. MYCOSPHERE 6:228-240.

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Chapter 5 Conclusions

Micronutrient malnutrition is a serious problem for humans. Iron-deficiency anemia is one of the most common malnutrition problems and affects over 30 % of the world's population (Bouis 1995; WHO 2017). Fe biofortification of crops, as a promising strategy needed to prevent Fe deficiency worldwide, such as plant breeding and genetic engineering, has been shown to be effective in literature. Plant nutrition is influenced by the rhizosphere microbiome. A unique plant-microbial approach may be another novel strategy to ameliorate problems associated with Fe deficiency. Therefore, it is necessary to undertake appropriate studies to pave the way for these strategies. Potato (*Solanum tuberosum* L.), as the fourth most important stable food worldwide after rice, wheat and maize (FAO 2015), is a modest contributor of Fe in the human diet. For instance, a small potato (100 g serving) provides 6 % Daily Value (DV) of Fe (Brown et al. 2010). Andean potatoes, as dietary mainstay of developing countries, possess large genetic variations and inheritance patterns. They are ideal source for iron biofortification; however, potato has been virtually ignored by the projects aiming to enhance micronutrient in the plants. The interaction between potato plants and rhizosphere microbial community associated with Fe cycling is still unclear.

The primary objective of this dissertation was to identify candidate genes and rhizosphere microbiome involved in the Fe cycling in *S. tuberosum*. We performed association mapping to screen out some genes associated with Fe homeostasis and then demonstrated the potential function of some transporter genes controlling Fe acquisition in yeast and in potato. To explore the role of rhizosphere microbiome in the potato Fe cycling, we first compared the composition, evenness and diversity of microbial communities associated

with the rhizosphere of two Andean potato landraces with different Fe content in tubers growing in two different soil types. We then conducted foliar application of Fe fertilizer on potato plants in the greenhouse to examine the impact of plant Fe nutritional status on the rhizosphere bacterial and fungal communities and to potentially screen out specific soil microbes that may be impacted by potato Fe acquisition.

Major genes have been reported to be involved Fe and Zn acquisition, transport, accumulation and tolerance by many studies in difference species such as Arabisopsis, tomato, pea, cucumber, apple and so forth, but little is known in potato. Multiple approaches were conducted to determine Fe candidate genes in potato, including selecting from potato genome annotation data, association mapping data and Arabidopsis ferrome orthologs. The overlap of potato ferrome, highly significant SNPs from association mapping and QTL regions from a mapping study of mineral content in a potato dihaploid population revealed several regions of the genome enriched for genetic elements associated with Fe cycling. The patterns of expression level of selected candidate genes were investigated from Andean potato cultivars with high and low Fe and Zn content in tubers responding to Fe deficient condition of micropropagation. The quantitative RT-PCR analysis on theses genes revealed greater expression of *PGSC0003DMG400024976* (MT24976, metal transporter) and *PGSC0003DMG400013297* (OPT13297, oligopeptide transporter) in the low Fe cultivar under Fe limitation. The function of transporter candidate genes was verified by the yeast complementation assay and *Agrobacterium*-mediated potato transformation. It turned out that *PGSC0003DMG400021155* (Iron-regulated transporter 1) and an *unannotated IRT* (chr02:28274200..28275700) as well as *MT24976* could rescue the growth of Fe and Zn

uptake deficient yeast mutants. Nevertheless, due to our small mapping population and low plant genetic transformation, future studies would benefit from larger sample sizes to increase power for candidate genes, and tissue-specific promoter that may contribute to successful overexpression to confirm the function of candidate genes.

On the other hand, many researchers have reported the uptake of micronutrients by a variety of crops using various microorganisms. Like that of plants, rhizosphere microbes may also play a role as mediators of potato Fe cycling. Research into the microbiome of potato has revealed the presence of several candidate bacteria and fungi that are strongly linked to the growth of the host potato plant, the tuber yield and nutrients (Rasche et al. 2009; Unno et al. 2015; Barnett et al. 2015). Clues to the plant beneficial effects that bacterial endophytes bring to potato survival and production have received attention (Manter et al. 2010). To investigate if the rhizosphere microbes are related to the potato Fe cycling, we analyzed the microbial community existing in the potato rhizosphere under different conditions.

First, we examined the effect of two different Andean potato landraces with different Fe content on the rhizosphere microbes at the community level using Illumina high-throughput sequencing technique on the ribosomal RNA genes. We found that the potato landrace had clear impacts on the structure of fungal communities despite a large effect of soil type. This finding is unique since much less research has been conducted to identify taxa in the fungal communities of potato and potential relationship between these fungal community and specific traits in potato. The largest differences in the structure of fungal community between the two potato landraces were related to *Ascomycota* and *Basidiomycota*. These two phyla contain dark septate endophytic (DSE) fungi that have

been shown to play important roles affecting plant phosphorus and iron uptake, and *Cryptococcus* that can be associated with both disease and plant growth promotion. With the high demand for phosphorus and the large variation in iron content of Andean potato landraces, we speculated that the status of Fe and P might be factors involved in the difference of relative abundance in *Ascomycota* and *Basidiomycota* groups. There is limited Fe related research on the role played by fungal mutualists of plant, but their role in plant P and N are clear, and thus also support the need to understand their relevance to plant Fe.

There is no direct evidence to pinpoint what specific factors influence change in rhizosphere communities across landraces; a further study is needed to investigate the correlation between rhizosphere fungal community and the specific crop traits (e.g., micronutrient content) and to provide better insight into the role of these groups in potato micronutrient cycling. However, Chapter 3 confirmed the effect of plant genotype on rhizosphere soil microbial community composition. Andean potato landraces have broader genetic diversity compared with modern cultivated potatoes. Quantifying microbes of these lines based on metagenomics and next generation sequencing provide opportunities to reveal some beneficial microbes that are more or less common as a result of plant breeding, which may support microbial mediated sustainable agricultural management of agroecosystems.

In Chapter 4, given the direct evidence is needed to test our assumption that the differing abilities of two landraces to accumulate Fe might be related to the difference in the structure of rhizosphere microbial community between two Andean potato landraces, we first adopted foliar Fe fertilizer application to change the plant Fe status of one Andean

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potato landrace that contain relatively low Fe in tubers and is more sensitive to Fe deficiency (Chapter 2), and then analyzed the influence of Fe status change on the rhizosphere microbial community. Concordant with the finding in Chapter 3, the rhizosphere fungal community structure was distinct across different treatments, while no significant difference in the bacterial community structure was detected. The treatment of foliar Fe fertilization resulted in an increasing trend in Fe concentration in the potato shoot tip and tuber, and in carbon source flow to potato roots. Since Fe application to the leaf surface directly changed the Fe nutritional status of plants it reduced the complexity of other effects on the soil microbial community. We thus concluded that potato plants Fe cycling is related to the structure of rhizosphere fungal communities, perhaps through a change in the flow of carbon to roots (e.g. root exudates).

From the analysis of the active fungal genera that were most correlated with effect of Fe fertilization, Fe application supported greater relative abundance of *Ascomycota* and *Microbotryomycetes*, and some fungi in *Glomeromycota* were relatively more abundant in the low and high Fe treatments. Many studies have reported the role of some fungal species in these taxa in the plant nutrient cycling such as P, N as well as Fe and their ability to produce siderophores. It suggested that the specific taxa screened out from our study are potential candidates interacting with potato plants on Fe acquisition. It has not been directly demonstrated whether the interaction resulted from a mutualism or competition between plant and fungus in this study although it is well established that rhizosphere microbes play an important role in plant productivity by modulating macronutrients (P and N) cycling in the soil through production of different enzymes, organic acids and siderophores. The results suggest that further research into the role of

specific fungal taxa and the profile of plant roots is needed to discern mechanisms associated with the plant-microbial interaction involving plant Fe cycling.

The advent of high-throughput sequencing technologies and decreased sequencing cost make association mapping and metagenomics studies feasible on a genome-wide scale (Korte and Farlow 2013; Schlaeppi and Bulgarelli 2015). In the respect of candidate gene in plants, because the Fe nutrient concentration is a complex nature controlled by hundreds loci, of the many factors limiting the power and accuracy of association mapping, effect size of functional variants plays an important role. Our population size for SNP genotyping is relatively small, and relationships among the different Andean potato cultivars may also limited the analysis. We thus employed more approaches including comparing alleles of candidate genes from high and low Fe cultivars, identifying potato orthologs of Arabidopsis ferrome and yeast complementation assays to improve the efficiency of identification. Our PhenoGram map did show the coincidence of highly significant SNPs for Fe and Zn with ferrome orthologs and QTL regions from the Alca Tarma population. However, false positives may arise in these approaches due to the statistical error and cofounding effect of multiple factors. Such false positives need to be controlled by plant genetic transformation with candidate genes in the future study.

In the respect of candidate soil microbes involved in the plant Fe cycling, the experimental design and sampling are key factors since the soil is a complicated environment. Our soil microbial community study adopted a method of foliar Fe application to reduce the complexity effect on the soil environment, but the treatment with no Fe addition could be better designed to create Fe deficient condition. On the other hand, to better control the condition as much as possible, we can use the transgenic potato

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plant that overexpress or knockout of Fe cycling related genes as material to more directly reveal the plant-microbial interaction. Manipulation of iron-related genes in potato can not only help understand gene function but also determin the interaction between potato and rhizosphere microbes invovled in Fe cycling. It is a good direction for the next step of this work. Association mapping studies that include microbes as part of breeding and genetic engineering of plant hold promise to produce elite, high yielding and nutrient-rich potato varieties.

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