THESIS

INTERPLAY BETWEEN SELENIUM HYPERACCUMULATOR PLANTS AND THEIR MICROBIOME

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ABSTRACT

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The plant microbiome includes all microorganisms that occur on the plant root (rhizosphere) and shoot (phyllosphere) or inside plants (endosphere). Many of these microbes benefit their host by promoting growth, helping acquire nutrients or by alleviating biotic or abiotic stress. In addition to its intellectual merit, better knowledge of plant-microbiome interactions is important for agriculture and medicine. Microbiome studies are gaining popularity in multiple research areas, particularly due to advances in next generation sequencing, which has advantages over cultivable methods by revealing the complete microbial community. Still relatively little is known about the microbiomes of plants with extreme properties, including plants that hyperaccumulate (HA) toxic elements such as selenium (Se). Selenium HAs may contain up to 1.5% of their dry weight in Se, which can cause toxicity to herbivores and pathogens as well as neighboring plants. Many advances are yet to be made with regard to the interaction of Se and the plant microbiome: does plant Se affect microbial diversity and composition, and do plant-associated microbes affect plant Se accumulation?

The first chapter of this thesis will discuss aspects of the plant microbiome as well as the discoveries to date with regard to plant-associated microbes and Se, mostly explored through culture-dependent methods. Selenium HA appear to harbor equally diverse endophytic microbial communities as non-hyperaccumulators. Thus, plant Se does not impair associations with microbes. A variety of microbes have been isolated from plants or soil in seleniferous areas,

including some bacteria and fungi with extreme Se tolerance. Inoculation of plants with individual strains or consortia of microbes was able to promote plant growth, Se uptake and/or Se volatilization. Thus, microbes may facilitate their host's fitness in seleniferous areas. Exploiting and optimizing plant-microbe associations may facilitate applications like phytoremediation (bio-based environmental cleanup) or biofortification (nutritionally fortified crops). Plant-derived microbial isolates may also be applicable without their plant host, e.g. for cleanup of wastewaters.

Culture-dependent studies have dominated the plant-microbe interactions research in regards to hyperaccumulators thus far, painting an elaborate but incomplete picture. In the second chapter of this thesis, we use a mix of culture based and culture-independent methods to investigate the bacterial rhizobiome of selenium Se HAs. Using 16S rRNA Illumina sequencing, we show that the rhizobiomes of Se HAs are significantly different from non-accumulators from the same naturally seleniferous site, with a higher occurrence of *Pedobacter* and *Deviosa* surrounding HAs. In addition, we found that HAs harbor a higher species richness when compared to non-accumulators on the same seleniferous site. Thus, hyperaccumulation does not appear to negatively affect rhizobiome diversity, and may select for certain bacterial taxa in the rhizobiome.

The bacterial isolates, independent from site or host plant species were in general extremely resistant to toxic concentrations of Se (up to 200mM selenate or selenite) and could reduce selenite to elemental Se. Thus, microbial Se resistance may be widespread and not be under selection by Se HAs., In future studies it will be interesting to further investigate the mechanisms by which Se HA species similarly shape their rhizobiome; this is perhaps due to Se-

related root exudates. Future studies may also focus on elucidating the effects of microbes on plant Se accumulation and tolerance.

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1.1 Introduction to the Plant Microbiome

1.1.1 General overview

The plant microbiome is becoming an increasingly popular area of study in plant sciences, but is generally still poorly understood. Microbiomes include all microorganisms of a particular environment, which include bacteria, fungi, and even some protists. Here, however, we will be discussing only the active bacteria and fungi in the microbiome. Often plant-associated microbes benefit their host via Plant Growth Promoting Properties (PGPP) while their host offers protection and nutrients (particularly organic carbon compounds) to the associated microbe (Compant et al., 2010; Turner et al., 2013b). The species composition of microbiomes have been shown to vary with host plant species, geography, growth conditions and plant developmental stage (Redford et al., 2010; Chaparro et al., 2014; Mahnert et al., 2015). There is even evidence that the plant microbiome was responsible for the ability of early plants to colonize land (Knack et al., 2015).

Studying the composition of plant microbiomes has become increasingly popular with the onset of affordable next generation sequencing, offering a broader perspective on microbial diversity than culture-based methods. Studies have shown that microbial flora *in planta* is much more diverse and abundant than originally thought, with many plant samples containing hundreds more microbial taxa based on 16S rRNA sequence analysis as compared to culture-dependent methods (Kent and Triplett, 2002; Visioli et al., 2015). Even though microbiomes are generally biogeography-specific, there tends to be taxonomic overlap between plant bacterial communities, with most samples containing *Actinobacteria, Proteobacteria,* and *Bacterioides* (Redford et al.,

2010; Chaparro et al 2014; Turner et al., 2013a; Panke-Buisse et al., 2014). There is also evidence that individual plant species have their own core microbiomes, which can act as a personalized signature for that species (Lundberg et al., 2012).

The plant microbiome is more easily studied when broken up into its three components, the rhizosphere (root zone), endosphere (inside tissues), and phyllosphere (shoot surface). Each of these spheres of the plant microbiome is unique; the spheres have intra- and intercommunity interactions, which are dependent on biotic and abiotic conditions (Turner et al., 2013a). The phyllosphere microbes, unlike those from the endosphere and rhizosphere, are exposed directly to the atmosphere and therefore must be resilient to many abiotic factors including high winds, UV, desiccation, and wet conditions (Turner et al., 2013a). Endophytes live most closely together with their host, inside plant tissues (Alford et al., 2010) and can protect the plant from herbivores and pathogens was well as promote plant growth via the production of plant growth hormones like indole acetic acid (IAA), nitrogen fixation, phosphate solubilization, and production of metal chelators like siderophores (Weyens et al., 2009b). The rhizosphere includes the root surface and the area of soil that is influenced by the plant roots (Turner et al., 2013a). The amount of soil that in included in the rhizosphere is dependent on many factors including the length of roots and root hairs and symbiosis with mycorrhizal fungi. The microbes that make up the rhizosphere also include bacteria with PGPP often referred to as PGP Rhizobacteria (PGPR). Like some endophytes, PGPR have been shown to produce or make available to plants compounds that promote plant growth including IAA, nutrients such as phosphates, nitrogen or iron (via iron-carrying siderophores), or compounds that inhibit pathogens or upregulate plant defenses (Jha et al., 2013). This chapter will focus on rhizosphere and endosphere microbes in relation to selenium (Se); the phyllosphere microbes remain to be studied in relation to Se.

1.1.2 Introduction to selenium in the plant-plant microbiome system

Selenium is an essential micronutrient for many animals including humans as well as for many microbes and algae, but is not essential for higher plants. All plants can accumulate Se to some extent by means of sulfur (S) uptake and assimilation pathways. The degree to which plant species accumulate Se often reflects the activity of these pathways. Selenium is mainly taken up into plants as selenate and can leave plants in a volatile form, usually as dimethyl selenide (DMSe) (Terry et al., 2000). Other ways in which Se can be deposited by plants is via litter, root turnover, or root exudation (Galeas et al., 2007; El Mehdawi et al 2012). Depending on the plant species, the plants can also convert inorganic selenate to selenoamino acids, and make this available to microbes (Terry et al., 2000). These selenoamino acids are an attractive food source to microbes since they provide C, N and Se, all of which are essential nutrients for bacteria. Several other forms of Se may be present inside the plant, some of which can be toxic. Se hyperaccumulator (HA) plant species differ from other species in several ways. They typically accumulate two orders of magnitude higher Se levels, and have evolved ways to avoid Se toxicity by converting selenate to methyl-selenocysteine, gamma-glutamyl-methylselenocysteine and selenocystathionine, which they can sequester in the vacuoles of epidermal tissues or transform to volatile dimethyl diselenide (DMDSe) (Pilon-Smits and LeDuc, 2009; Evans and Johnson 1967).

Bacterial Se metabolism shows similarities to that in plants, with assimilation of inorganic Se to organic forms and capacity to form organic volatile forms, DMSe or DMDSe (Frankenberger and Karlson 1994; Zayed and Terry, 1994; Turner et al., 1998; Winkel et al., 2015). Bacteria are also capable of reducing selenite (and sometimes selenate) to elemental Se nanoparticles (Turner et al., 1998; Zayed et al, 1998; Husen and Siddiqi 2014; Staicu et al

2015a,b; Winkel et al., 2015). These processes are illustrated in Figure 1.1. Bacteria in general seem to be very tolerant to Se, some strains surviving and even benefiting from concentrations of selenate and selenite up to 200 mM; this capacity was not dependent on the Se concentration of the site or plant they were isolated from (Sura de Jong et al., 2015; Cochran et al., unpublished). In one study on Se-dependent litter decomposition by Quinn and coworkers it was found that litter from Se hyperaccumulator species harbored more culturable bacteria and decomposed faster than litter from related non-hyperaccumulator species (Quinn et al., 2011). Thus, while most other ecological partners associated with hyperaccumulators are by default sensitive to Se (see review by El Mehdawi and Pilon-Smits, 2012), bacteria appear to be by default Se-resistant and may even benefit from and seek out high-Se plant material to colonize. Fungi, on the other hand, are much more sensitive to Se than bacteria on an individual basis (Wangeline et al., 2011). Thus, not all plant-associated microbes are equally resistant to Se. Furthermore, it has been shown that the addition of Se was able to protect *Brassica juncea* from fungal pathogens *Fusarium sp.* and *Alternaria brassicicola* (Hanson et al., 2003). This effect has not yet been tested on bacterial pathogens.

1.2 Rhizosphere Microbes

1.2.1 Introduction to the rhizosphere

The rhizosphere is a dynamic environment, constantly changing and influenced by multiple biotic and abiotic factors. Rhizosphere processes are a fascinating area of plant-microbe interaction research; the soil, host plants and microbial components of the system affect each other in a complex relationship triangle (Turner et al., 2013a). The rhizosphere has the highest abundance of microbes compared with the rest of the plant microbiome, about 1000 fold higher

in microbial abundance than in surrounding bulk soil (Berg and Smalla, 2009). This is phenomenon often referred to as the rhizosphere effect, where the plant exudes compounds rich in sugars and acids; they may also produce specific secondary plant compounds that can induce bacterial pathways (Morgan et al., 2001; Berg and Smalla, 2009). The plant uses these strategies to build specific microbial communities in the soil to aid its survival and potentially that of its offspring (Lapsansky et al., 2016).

Some rhizosphere microbes, including strains of *Burkholdia*, *Ralstonia* and *Pseudomonas* are opportunistic pathogens, which can take advantage of a weakened immunity in the host (Berg et al., 2005; Mendes et al., 2013). Even though some rhizosphere microbes are pathogens or parasites, the majority of the bacteria found here are traditionally categorized as mutualistic with their hosts (Newton et al., 2010). There is a multitude of bacterial taxa that fall into the PGPR category, some of which can benefit a wide range of host plants and some of which are host-specific (Kloepper, 1996). In order to identify PGPR, experiments showing that the host plant grows better after inoculation with the specific PGPR strain are necessary. More general PGP mechanisms by which bacteria stimulate different hosts include IAA (plant growth hormone) production, phosphate solubilization, siderophore production, NH_3 production, nitrogen fixation, and defense against pathogens (Ahmad et al., 2008).

Rhizobacteria-legume interactions are one example of a widely studied host-specific interaction. These nitrogen (N_2)-fixing PGPR can enter into the roots and establish themselves inside root nodules, which gives the nodulated plant the ability to fix nitrogen as well. There are multiple genera capable of this symbiosis in the bacterial family Rhizobiaceae (Gray and Smith, 2005). Among the most popular of these genera is *Rhizobium*, usually found in symbiosis with the plant family Fabaceae. The molecular cross-talk between the plant roots and the specific

rhizobacteria often starts with plant root exudate signal compounds that induce bacterial signal compounds, which then leads to nodule formation (Gray and Smith, 2005).

An example of a more promiscuous plant-microbe interaction is the large group of fungi called mycorrhizae that live in association with plant roots; in this symbiotic relationship the plant benefits from the fungus through increased water and nutrient uptake and the fungus benefits from the organic carbon compounds released by the plant (Marschner and Dell, 1994). The most common mycorrhizae are the arbuscular mycorrhizae, defined by the colonization of the host root cortex by the fungal symbiont, which then uses its mycelium to reach into the soil to gather water and minerals (Barea et al., 2005; Wang and Qui, 2006). The fungi responsible for these interactions are generally obligate in their symbioses, needing a host plant to colonize in order to survive and reproduce (Barea et al., 2005). Most plant families (92%) and even plant species (80%) are thought to have mycorrhizal partners (Wang and Qui, 2006).

1.2.2 Selenium and the rhizosphere

The plant family Fabaceae includes 25 species that hyperaccumulate Se, e.g. *Astragalus bisulcatus* (Beath et al. 1939). The enhanced nitrogen acquisition capacity of Fabaceae members associated with root nodulation is not only beneficial for plant growth, but also was found to enhance Se accumulation in the form of seleno-aminoacids in hyperaccumulators (HAs) including *A. bisculatus* (Alford et al., 2014). While it could be hypothesized that high plant concentrations of Se would inhibit root nodule formation in symbioses between *A. bisulcatus* and *Rhizobium*, no evidence was found for this (Alford et al., 2012). Increasing Se concentration in *Astragalus* hyperaccumulators was associated with enhanced nodule- formation, and 10-fold higher levels of the N-rich compound gamma-glutamyl-MetSeCys (Alford et al., 2012, 2014).

Thus, rhizobia in root nodules may play a role in Se hyperaccumulation in *A. bisculatus* by providing nitrogen for the selenoaminoacids that these plants accumulate up to 1% of their dry weight (Alford et al., 2012, 2014). Multiple species of *Rhizobium* have been shown to reduce selenite to elemental Se (Se^0), which may influence Se speciation in plants (Basaglia et al., 2007; Hunter et al., 2007; Valdez Barillas et al., 2012). While organic C-Se-C compounds were found to make up close to 100% of Se in the roots of *A. bisulcatus,* it constituted only 75% of Se in root nodules, where the remaining substantial fraction (25%) was Se^0 (Valdez Barillas et al., 2012).

Many fungi have also been shown to reduce selenite to Se^0, despite the generally lower Se tolerance of fungi to high concentrations of Se, as compared to bacteria (Gharieb et al., 1995; Wangeline et al., 2011; Lindblom et al., 2013). In a study by Wangeline and coworkers, hundreds of fungi were isolated from rhizosphere soil collected from seleniferous and non-seleniferous sites, which were identified and characterized for their Se tolerance. The fungi isolated from seleniferous soils were more tolerant to Se than those isolated from non-seleniferous soils, indicating that the fungi living in seleniferous soils have evolved to be more resistent to the high concentrations of Se in the soil (Wangeline et al., 2011).

In addition to reducing selenite to Se^0, rhizosphere microbes have been shown to volatilize Se in the forms of DMSe or DMDSe from selenate or selenite (de Souza et al., 1999a). Since these volatile forms of Se are less toxic and remove Se from the site, Se volatilization likely serves a detoxification function for the microbes, and also has applications in bioremediation (Barkes and Fleming, 1974; Azaizeh et al., 1997, 2003).

There have been multiple studies on the effects of rhizosphere microbes on growth and plant accumulation of Se and other elements. As shown in the summary of these studies in Table 1.1, the presence of rhizosphere microbes can contribute to the growth and Se accumulation of

Se HA species as well as non-HAs. In many instances, bacterial inoculation increased the biomass of the inoculated plant and enhanced Se accumulation (de Souza et al.,1999a&b; di Gregorio et al., 2005; Wenzel et al., 2009; Durán et al., 2013; El Mehdawi et al., 2015; Sura de Jong et al., 2015). In one study, rhizosphere soil slurry of HA *Symphyotrichum ericoides* stimulated growth and Se accumulation in the same species when grown from surface-sterilized seed on autoclaved naturally seleniferous or non-seleniferous soils (El Mehdawi et al., 2015). In another study, inoculation with a single environmental strain enabled wheat to take up more Se and iron (Yasin et al., 2015). Furthermore, Se accumulation and volatilization could be enhanced in *Brassica juncea* and several aquatic species by inoculation with environmental bacteria isolated from a Se-rich sediment (de Souza et al., 1999a,b) or from the rhizosphere of Se hyperaccumulator *A. bisulcatus* (di Gregorio et al., 2005).

Similar trends were observed after inoculating plants with rhizosphere fungi. Some of the fungi from Se hyperaccumulators were shown to increase Se accumulation in the roots of Se HA *Stanleya pinnata* (Lindblom et al., 2013). There have also been several studies on the effects of mycorrhizal fungi on Se accumulation and uptake. Most of these studies found that when a mycorrhizal relationship formed, the concentration of Se increased in the plant compared to a plant growing in seleniferous conditions without mycorrhizal inoculation (Wanek et al., 1999; Larsen et al., 2006; Yu et al, 2011). However, in some cases the opposite was true, where inoculation with mycorrhizal fungi did not result in the increase of the level of Se in plants even after addition of selenate to the soil (Munier-Lamy et al., 2007; Yu et al., 2011). The identity of host plant and Se speciation may affect these interactions (Munier-Lamy et al., 2007; Yu et al., 2011).

1.3 Endosphere Microbes

1.3.1 Introduction to endophytes

Endophytes are bacteria and fungi that live inside plants, colonizing the roots, shoots, and reproductive portions (Jha et al., 2013). These microbes can either be inherited from the parent plant via the seed, introduced via a vector (e.g. an insect), or can colonize the plant during its life through sites of lateral root emergence or open areas in the plant epidermis (Reinhold-Hurek and Hurek, 2011; Lapsansky et al., 2016). As with rhizosphere microbes, endophytes can be pathogenic, parasitic or mutualistic. The PGPP endophytes are generally host-specific, but the mechanism(s) by which bacteria promote plant growth are similar in different host-endophyte pairs and similar to those in PGPR (Long et al., 2008). Endophytes have been shown to produce IAA, fix nitrogen, solubilize phosphate and produce siderophores (Hardoim et al., 2008; Long et al., 2008; Weyens et al., 2009a; Durán et al., 2014; Lins et al., 2014).

The ability of endophytes to escape the host immunity is still poorly understood. It is known, however, that endophytes are able to modulate ethylene levels in plants, which could have some role in the plant immune response (Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011). Some endophytes can induce attack against endophytic pathogens, increasing the host immunity to defend against these pathogens (Nejad and Johnson, 2000; Arnold et al., 2003). It has been shown that some endophytes do this by triggering the host's systemic jasmonic acid- or salicylic acid-mediated responses and can prime the plant's immune response in preparation for future attacks (Van Wees et al., 2008; Reinhold-Hurek and Hurek, 2011). In addition to growth promotion and immune regulation, endophytes are able to alleviate abiotic stresses and increase nutrient availability by regulation of host genes and by increasing levels of abscisic acid (Hesse et al., 2003; Sziderics et al., 2007; Jha et al., 2013).

1.3.2 Selenium and the endosphere

In a study done by Sura-de Jong and colleagues (2015), endophytic bacteria were isolated from Se hyperaccumulators *A. bisulcatus* and *S. pinnata* and tested for physiological properties as well as the ability to enhance growth and Se uptake in plants. When exposed to Se, the isolates were shown to be tolerant to high concentrations (up to 200 mM) of selenate and selenite, and to have the ability to reduce selenite to Se^0 (Sura de Jong et al., 2015; Staicu et al., 2015a,b). A selection of endophytes from Se hyperaccumulators were inoculated to *Brassica juncea* and *Medicago sativa*, resulting in increased dry weight when compared to un-inoculated control plants; Se accumulation was not significantly affected (Sura de Jong et al., 2015).

Several studies have evaluated the potential use of bacterial endophytes in Se biofortification and phytoremediation. Durán and coworkers (2014) found that endophytic bacteria including *Acinetobacter, Bacillus* and *Klebsiella* tolerated high levels of Se and promoted plant growth (Durán et al., 2014). In addition to these properties, these endophytic bacteria were able to protect wheat crops from *Gaeumannomyces graminis*, a soil-borne pathogen that destroys many cereal crops (Durán et al., 2014). Since endophytes live in the plant and are generally host specific, they often possess abilities to degrade certain pollutants that are found in the HA host plant environment in which they live (Doty et al., 2008). Endophytic microbes have been used in a number of studies on other pollutants and have potential uses in cleaning up polluted areas (Doty et al, 2008). For example, it was shown that an endophytic *Pseudomonas* strain isolated from Se hyperaccumulator *Stanleya pinnata* was able to completely remove up to 100 mM of selenite from water by precipitating it as Se^0 (Staicu et al., 2015a).

A study done by Lindblom and coworkers in 2012 showed that chemical Se speciation in HAs may be affected by fungal endophytes that produce Se^0 (Lindblom et al., 2012a). In

particular, a selenophilic fungus known as *Alternaria astragali* which was isolated from the root of *A. bisulcatus* was used for this experiment. In a laboratory setting, elemental Se was only found in root nodules when plants were inoculated with *A. astragali* (Lindblom et al., 2012a). This is consistent with findings of a study done on the seeds of *A. bisulcatus* showing that seeds containing *A. astragali* had a significantly higher fraction of Se^0 (up to 30%) than those without this endophytic fungus (Valdez Barillas et al., 2012). A follow up study showed that *A. astragali* enhanced the growth of some *Astragalus* species but inhibited the growth of others, however growth of all *Astragalus* species tested was not inhibited when plants were both inoculated and supplied with Se (Lindblom et al., 2012b). This indicates that, like bacteria, endophytic fungi may also be capable of enhancing plant growth, changing Se speciation and affecting the Se accumulation of inoculated plants. These studies are also included in Table 1.1.

1.4 Future Directions

There have been many recent advances and discoveries in the area of plant microbiomes and Se. However, there is still much to be discovered and many questions to be addressed. Is there a core microbiome associated with Se hyperaccumulators that may contribute to Se accumulation and that can be used for bioremediation and phytoremediation? Do individual plants select their microbiomes or have plant species and their microbiomes coevolved? The advances in the understanding of plant microbiomes and Se could very well be useful to increase effectiveness of bioremediation, phytoremediation, and biofortification. Overall, the phyllosphere and endosphere deserve more attention, with almost no studies on the phyllosphere and Se to date. It is expected that the implementation of next generation sequencing will give

additional insights into the role of Se in plant-plant microbiome interactions, which will complement the limited insight from studies to date.

The studies reviewed in this chapter began to explore the complex interactions of Se HAs and their microbiomes. Based on these studies, it could be hypothesized that the microbes associated with HAs facilitate hyperaccumulation, and also that the plant HA environment provides a specific niche that shapes the bacterial community in and around it. The aim of my Master's research was to address these hypotheses. In chapter two of this thesis, I look to answer the following questions about Se HAs and their rhizobiome: (1) How does Se HA affect the bacterial rhizobiome? (2) Are bacteria isolated from Se HAs or seleniferous soils more tolerant to Se than those from non-HAs or non-seleniferous soils? and (3) Can the bacteria isolated from Se HAs increase Se accumulation in a non-accumulator? These questions are just the beginning of uncovering the complex relationships between these fascinating plants and their microbiomes.

1.5 Tables and Figures

Table 1.1: Overview of plant inoculation studies that used fungi or bacteria from Se hyperaccumulators and determined their effects on plant Se metabolism. Boxes with stars denote areas for future research.

	Promoted growth	**Affected Se speciation**	**Can tolerate high Se**	**Increased Se accumulation**
Fungi from HA	Lindblom et al., 2012b	Lindblom et al., 2012a,b	Wangeline et al., 2011	Lindblom et al., 2013
Bacteria from HA	Alford et al., 2014 Sura de jong et al., 2015 Yasin et al., 2015	di Gregorio et al., 2005, 2006 Valdez Barilles et al., 2012 Alford et al., 2014 Staicu et al., 2015b	Di Gregorio et al., 2005 Sura de Jong et al., 2015	de Souza et al., 1999a,b di Gregorio et al., 2005 Alford et al., 2014 Yasin et al., 2015
Microbe Consortium from HA	El Mehdawi et al., 2015	***	***	Quinn et al., 2011 El Mehdawi et al., 2015

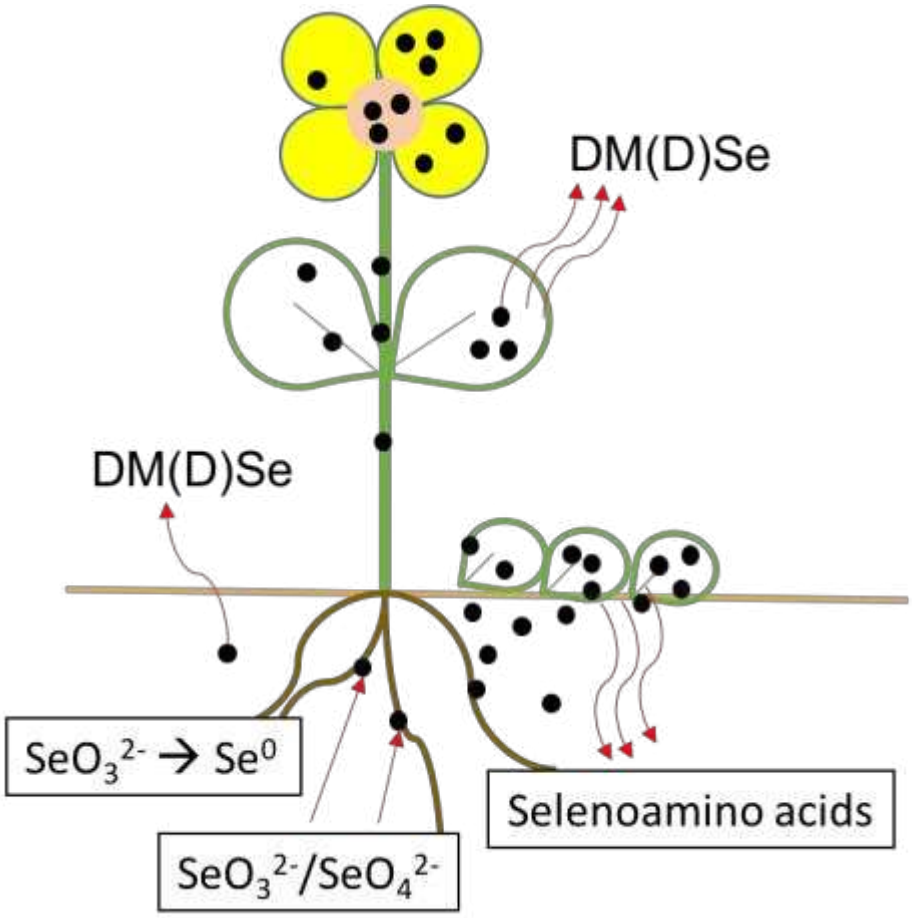

Figure 1.1: Schematic depiction of plant and microbial processes in the plant-rhizosphere-soil system that affect the fate of selenium. Bacteria and fungi occur in rhizosphere, phyllosphere and endosphere of plants. Both plants and microbes can reduce selenate to selenite and produce organic forms of Se, including volatile DMSe/DMDSe. Microbes can also produce elemental Se (Se^0) and increase Se accumulation in plants from selenite and selenate (SeO_3^{2-} and SeO_4^{2-}). Hyperaccumulator plants produce selenoamino acids, which offer an additional source of (organic) Se to microbes when decomposed.

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CHAPTER 2- INTERPLAY BETWEEN SELENIUM HYPERACCUMULATOR PLANTS AND THEIR RHIZOBIOME

2.1 Outside Contributions

Jemma Bauer[1] assisted with Se resistance tests by maintaining cultures and data collection; Jessica L Metcalf[2] assisted with navigation of QIIME and statistical analysis of the rhizobiome data; Petra Novak[3] and Martina Sura de Jong[3] performed the MALDI-TOF; Elizabeth AH Pilon-Smits[1] was the main PI on this project and contributed lab space and resources in addition to assisting with method development, interpretation of results and manuscript development.

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2.2 Introduction

The plant microbiome includes all microorganisms (bacteria, fungi, and protists) that live in association with a particular plant. Here, however, we will be referring to the active microorganisms in this system. The plant microbiome is generally broken up into three components, the rhizosphere, endosphere and phyllosphere (Berg et al., 2014). The phyllosphere includes microbes that occur on the surface of plant shoots, the endosphere contains microbes that live inside of plant tissues, and the rhizosphere is the area underground that is influenced by the roots (Alford et al., 2010; Berg et al., 2014). Many plant-microbe associations are beneficial to the plant: endosphere and rhizosphere microbes have been shown to protect the host plant from pathogens (which are also a part of the microbiome) as well as promote plant growth via

the production of plant growth hormones or by facilitating nutrient acquisition or enhancing abiotic stress resistance (Berg and Hallmann, 2006; Weyens et al., 2009b, Jha et al., 2013).

The rise of next-generation sequencing technologies has made microbiome research an increasingly popular and feasible area of study in plant biology (Berg et al., 2013). These technologies have allowed researchers to study entire plant microbiomes rather than just culture-dependent microbes. This is of particular importance considering that only 1% of microbes on the planet have been estimated to be cultivable, leaving 99% undiscovered and unstudied depending on the type of environment tested (Visoli et al., 2015). The ability to move beyond the cultivable side of the microbiome story has offered a new and more expanded view of the microbial world, which has changed the way researchers look at the microbial communities associated with plants (Berg and Hallmann, 2006; Berg et al., 2013; Visoli et al., 2015).

Microbiome research has also received increasing interest in the area of plant hyperaccumulation, i.e. the capacity of some plant species to accumulate upwards of 100-fold higher levels of a specific toxic element than their non-accumulator counterparts (Baker and Brooks, 1989; Cappa and Pilon-Smits, 2014). Around 500 plant species have been reported to be hyperaccumulators (HAs), and around 10% of HA species have had their rhizospheric interactions studied (Alford et al., 2010; Visoli et al., 2015). Elements reliably reported to be hyperaccumulated by certain plant species include As, Cd, Co, Ni, Pb, Se and Zn; several other elements may also be hyperaccumulated, but this is not as well documented (Cappa and Pilon-Smits, 2014). Thus, one hypothesis could be that the extreme concentration of a toxic element present inside HA may affect the microbiome, and conversely it is possible that the microbiome affects elemental accumulation. It could be hypothesized that the microbial communities of the HA plants have enabled these plants to evolve the ability to hyperaccumulate. The opposite

could also be hypothesized, where HA plants have created a toxic environment that reduces microbial density or diversity. It is also possible that the high levels of these (toxic) elements in and around the plant alter competition between microbial groups, and thus alter the structure of the associated microbial communities or the resistance of the microbiome against the toxic element in question. In order to sufficiently address these broad hypotheses, a combination of culture-dependent and culture-independent methods needs to be applied to this research.

Research to date on microbial communities of HAs has been carried out mostly on rhizosphere microbes with culture-dependent methods. This research has shown that the rhizosphere microbes of Ni and Cd HAs are more tolerant to the metals that their hosts hyperaccumulate than rhizosphere microbes not associated with HAs (Visoli et al., 2015). In addition, inoculation of HA-derived microbes back to their HA hosts in some cases enabled the plants to accumulate more of the element in question (Visoli et al., 2015).

In the current study, the aspect of plant hyperaccumulation of particular interest is the interplay between selenium (Se) HAs and their microbiomes. Selenium is an essential micronutrient for many microbes and animals, including humans, and is considered a beneficial element for higher plants (Terry et al., 2000). Despite not being essential for plants, it is readily taken up by plants and assimilated from selenate or selenite to organic forms via the sulfate assimilation pathway (Terry et al., 2000). Selenium is toxic at high concentrations because it is converted into selenocysteine (SeCys), which disrupts protein function when it is nonspecifically incorporated into proteins in the place of cysteine (Terry et al., 2000). Selenium HAs such as *Stanleya pinnata* (Brassicaceae) and *Astragalus bisulcatus* (Fabaceae) have evolved a way to circumvent this toxicity by converting SeCys into a non-toxic form, methyl-SeCys, which can be sequestered in epidermal vacuoles or further converted to volatile dimethyldiselenide (DMDSe)

(Evans and Johnson 1967; Neuhierl et al., 1999). Many microbes detoxify Se in a similar way, taking up selenate or selenite and converting it to organic forms or volatile forms; they can also reduce selenite or selenate to elemental Se (Se^0) (Frankenberger and Karlson 1994; Zayed and Terry, 1994; Turner et al., 1998; Staicu et al 2015a,b; Winkel et al., 2015).

While high Se concentrations may be hypothesized to negatively affect microbial density or diversity, it has been shown in several cases that high-Se substrates are actually more microbe- rich than corresponding low-Se substrates, including plant litter and ponds (de Souza et al., 2001; Quinn et al., 2011). High-Se substrates may, however, select for taxa that are more Se-resistant: rhizosphere fungi isolated from naturally seleniferous soils were more tolerant to high concentrations of Se than those isolated from a non-seleniferous area (Wangeline et al., 2011). It is not known whether the same is the case for rhizosphere bacteria, because the properties of this group are not well-studied. Bacterial endophytes from HA, however, were able to withstand (and in some cases benefit from) selenate and selenite concentrations up to 200 mM (Sura de Jong et al., 2015); the same may be true for rhizosphere bacteria. When inoculated to plants, both rhizosphere and endosphere microbes from various sources have been shown to promote plant growth, increase Se accumulation and increase pathogen resistance (de Souza et al., 1999a,b; Hanson et al., 2003; di Gregorio et al., 2005; Lindblom et al., 2012b, 2013; Alford et al., 2014; El Mehdawi et al., 2015; Sura de Jong et al., 2015; Yasin et al., 2015). To date, no experiments involving the phyllosphere of Se HAs have been performed.

In this study, a combination of culture-dependent and culture-independent methods was used to study the rhizosphere microbiome (rhizobiome) of plants that hyperaccumulate Se in comparison with non-HA species or unvegetated soil from the same seleniferous area or from a nearby non-seleniferous area. The aim of this study was to answer the following questions: (1)

How does Se HA affect the bacterial rhizobiome? (2) Are bacteria isolated from Se HAs or seleniferous soils more tolerant to Se than those from non-HAs or non-seleniferous soils? and (3) Can the bacteria isolated from Se HAs increase Se accumulation in a non-accumulator?

2.3 Materials and Methods

2.3.1 Sampling

Rhizosphere soil from five plant species were sampled from a seleniferous site, Pine Ridge (PR), in Fort Collins, CO (sandy loam, pH 7.57, SOM 5.8%, 1.7 mg Se kg^{-1}) (described previously by Galeas et al., 2008 and El Mehdawi et al., 2012). The sampled species include three HA species, *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae) and a HA population of *Symphyotrichum ericoides* (Asteraceae) and two non-HA species, *Astragalus tenellus* (Fabaceae) and *Physaria bellii* (Brassicaceae). For comparison, rhizosphere soil was sampled from plants on a nearby non-seleniferous site, Cloudy Pass (CP), in Bellvue, CO (sandy loam, pH 6.57, SOM 4.5%, 0.11 mg Se kg^{-1}) (described by El Mehdawi et al., 2012, 2015). The species sampled here were all non-HA species, including *Astragalus laxmanii* (Fabaceae), *Physaria montana* (Brassicaceae) and a non-Se accumulator population of *S. ericoides* (El Mehdawi et al., 2015). Leaf and rhizosphere soil samples were taken from six individuals of each species sampled on both sites, except for *A. tenellus*, where n=4. Rhizosphere soil was collected by removing the root from the soil, shaking the bulk soil off of the roots, and then collecting remaining soil in association with the roots in a sterile container. Soil samples were then sieved using a 1 mm sieve and stored in sterile 1.5 mL Eppendorf tubes and stored at 4^{o}C. Leaf Se concentrations of plants from PR were evaluated using ICP-OES as described before (Fassel, 1978). Plant species and Se concentrations of the sampled plants are listed in Table 1.1.

2.3.2 Culture-Independent Studies

2.3.2.1 DNA Extraction and 16S rRNA Amplification for Rhizobiome Sequencing

DNA extractions from soil samples and 16S rRNA amplification were completed by the Earth Microbiome Project. DNA extraction was performed using a modified MoBio PowerSoil DNA Isolation 96-well extraction protocol (http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols/dna-extraction-protocol/). 16S rRNA amplification was then performed in triplicate by the Earth Microbiome Project using the barcoded forward primer 515fb and the barcoded reverse primer 806rB as described by Apprill et al (2015). The samples were run on a thermocycler under the following conditions: 94˚C for 3 minutes, 35 cycles consisting of 94˚C for 45 seconds, 50˚C for 60 seconds, 72˚C for 90 seconds and 72˚C for 10 minutes. Resulting amplicons were run on an agarose gel to look for bands at 300-350 bp and quantified with Picogreen. Amplicons from each sample were pooled using MoBio UltraClean PCR Clean-Up Kit #12500. Aliquots were sequenced using the primer constructs designed by the Earth Microbiome Project. (http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols/16s/)

2.3.2.2 Data Processing and Statistics of 16S rRNA Sequences

Data processing and statistics for rhizobiome analysis were done using QIIME. The generated 16S rRNA sequences were filtered for quality and demultiplexed. Chloroplasts were filtered out and the resulting table was rarefied. A closed reference OTU (operational taxonomic unit) picking was then performed against the Greengenes database for 16S taxonomic assignments at 97% OTU picking. A unifrac distance matrix was generated from OTUs detected in each sample and visualized using a PCoA plot. Compare Categories and Group Significance

tests were performed on the unifrac distance matrix using permonova and anosim statistical tests for whole data set as well as within the Pine Ridge set.

2.3.3 Culture Based Studies

2.3.3.1 Rhizosphere bacterial isolation

Rhizosphere soil samples were sieved with a 1 mm screen. A 1:1 (w/v) ratio of soil and autoclaved 50 mM $MgSO_4$ were mixed and diluted to 10^{-6} before plating 200 µL onto Luria Bertani (LB) agar (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) and incubating at 30°C for 3-10 days. Individual colonies were isolated based on morphology and cultured in LB liquid before being stored in a 1:1 (v/v) solution of bacterial culture and 30% glycerol at -80°C. Individual isolates were identified via Matrix Assisted Laser Desportion Ionization- Time of Flight (MALDI-TOF) and MALDI Biotyper as described by Sura de Jong et al. (2015). A list of all bacterial strains and their identification confidence levels used in this study can be found in supplemental table S3.

2.3.3.2 Selenium Resistance

Individual isolates were streaked onto LB plates containing 0, 1, 10, 100 or 200 mM sodium selenate (Na_2SeO_4) or sodium selenite (Na_2SeO_3) and qualitatively scored for Se resistance as well as for the ability to produce red elemental Se. Each isolate that grew on the minus Se plate was then given a selenate and selenite resistance score (0-5) based on the ability to grow on each plate. The scores were assigned based on the following criteria: 0 indicates no growth on any of the Se plates, 1 denotes growth up to 1 mM Se, 2 growth up to 10 mM Se, 3

growth up to 100 mM, 4 growth up to 200 mM and a score of 5 denotes improved growth at 200 mM Se (as compared with the control plate).

Then, each of the selenate and selenite resistance scores were pooled for each individual plant host. The median selenate and selenite resistance scores were calculated for each host plant. Scores from each host plant were then put into the following three groups: (1) Cloudy Pass non-HA and bulk soil; (2) Pine Ridge HA; and (3) Pine Ridge non-HA and bulk soil; statistical comparisons of Se tolerance in these groups were performed using a Kruskal-Wallis test with JMP.

2.3.3.3 Making Consortias for Inoculation

Individual bacterial strains isolated from rhizosphere soil samples from Cloudy Pass (CP), Pine Ridge non-HAs (PRn) and Pine Ridge HAs (PR) were inoculated in LB media and placed on a shaker at 30˚C overnight (see Supplemental Table S4 for consortium info). Strains were selected by bacterial genus and/or morphology from each host to ensure maximum diversity of isolates. The OD_{600} for each culture was measured with a spectrophotometer, spun down, and re-suspended in 1/5 Hoagland's for a final OD_{600} of 1.0. The cultures were then combined into consortia, which were used to inoculate *Brassica juncea* seeds. Portions of *B. juncea* seeds were inoculated overnight at 4°C in the various consortia; the control seeds were mock-inoculated in 1/5 Hoagland's. *B. juncea* was chosen because it grows quickly and is a popular species for Se phytoremediation and biofortification (Banuelos et al., 2005).

2.3.3.4 Post Inoculation Growing and Harvesting

After overnight incubation in inoculation solutions, *B. juncea* seeds were blotted dry and planted in sterilized coarse sand (quartz play sand, washed and pH adjusted to 6.0) and watered with 1/5 Hoagland's. The plants were cultivated in a grow room at 150 μE light intensity, 10/14 L/D photoperiod and 22°C. After one week, the +Se plants were watered with a mixture of 1/5 Hoagland's with 20 μM Na_2SeO_4 while the control groups continued to receive 1/5 Hoagland's. After three weeks, all plants were treated with the pesticide Talstar according to the manufacturer's instructions. The plants were harvested after five weeks of treatment. The roots and shoots were separated, dried, and weighed. The elemental concentrations in the plants were measured using ICP-OES as described previously (Fassel, 1978). Student's t-test, Anova and Tukey-Kramer tests were performed using JMP on dry weight and Se content of the roots, shoots, and total plants.

2.4 Results

2.4.1 Culture-Independent Methods

The program QIIME was used for data visualization and statistics on the rhizobiome data. A taxa summary plot was generated, which indicated that the most prevalent phyla in the data set were Proteobacteria (23.3%), Actinobacteria (17.8%), Bacteroidetes (17.6%), and Acidobacteria (16.4%). This finding is consistent with most microbiome studies conducted on environmental samples (Redford et al., 2010; Chaparro et al 2014; Turner et al., 2013a; Bulgarelli et al., 2013; Panke-Buisse et al., 2014). The distribution of bacterial phyla in each group that was sampled is shown in S1, with the percentages of each bacterial phyla in supplementary material S2. An unweighted unifrac distance matrix was generated from operational taxonomic units (OTUs)

detected in each sample and visualized using a PCoA plot. A compare categories test was performed on the OTUs in the soil samples to see which groups were statistically significant. Permanova and Anosim tests (n=999) were performed on the unifrac distance matrix to compare differences in OTU abundance within site and within HA, non-HA and bulk soil over the whole data set as well as within the Pine Ridge site. Both tests were significant for site ($p < 0.001$ for both tests). Permanova and Anosim were significantly different for HA, non-HA and bulk soil over the whole data set ($p < 0.001$ for both). The CP samples were then filtered out and Anosim and Permanova tests were performed on the PR samples. Both tests found significant differences between HA, non-HA and bulk soil ($p < 0.001$). 2D plots of beta diversity of soil samples over the whole data set as well as within PR are shown in Figures 2.1 and 2.2.

Group significance was also tested to compare OTUs frequencies between groups. The frequency of all bacterial OTUs were significantly different between sites. When looking at Pine Ridge specifically, no significant differences were found in OTU frequency when comparing HA with non-HA and bulk soil. However, some bacterial taxa had a higher mean occurrence than others. This includes *Pedobacter* (HA mean= 56.53; non-HA= 2.56; bulk= 1.16) and *Devosia* (HA mean= 17.5; non-HA= 4.5; bulk= 0.17). Alpha diversity (observed OTUs) was also tested between sites and within PR. Between sites, CP had a significantly higher alpha diversity than PR (ANOVA $p < 0.004$; Figure 2.3), with non-HA from CP being on par with HA from PR. Within PR, however, samples from HA were found to be significantly different than samples taken from non-HAs and bulk soil via Anova ($p < 0.001$; Figure 2.3): rhizosphere samples from hyperaccumulator species had a higher alpha diversity (Figure 2.3). Similar results were obtained for phylogenetic distance (not shown).

Thus, based on the group significance and compare categories tests, the composition of the bacterial microbiome in our sample set was significantly different between the seleniferous (PR) and non-seleniferous (CP) sites as well as between rhizospheres of HA, rhizospheres of non-HA and bulk soil samples.

2.4.2 Culture-based studies

2.4.2.1 Se tolerance of bacterial isolates from seleniferous and non-seleniferous habitats

A non-seleniferous site (Cloudy Pass) and a seleniferous site (Pine Ridge) were sampled in this study. In CP, rhizosphere soil samples were collected from three non-HA plant species and bulk soil. In PR, rhizosphere soil samples were collected from three HA plant species, two non-HA plant species and bulk soil (Table 1). After removing duplicates based on identification and morphology, a total of 142 bacterial isolates were collected from all of the rhizosphere soil samples. 57 of these were isolated from CP samples and 85 were isolated from PR samples. Within CP, 56 isolates were taken from non-HA rhizosphere soil samples and 1 was taken from bulk soil. Within PR, 23 isolates were taken from non-HAs, 58 were taken from HAs, and 4 were taken from bulk soil (Figure 2.4). There is an obvious trend that the rhizosphere samples included more isolates than the bulk soil samples, which is consistent with the rhizosphere effect (Morgan et al., 2001; Berg and Smalla, 2009). Among the plant species, *Astragalus laxmanií* from CP and *Astragalus bisulcatus* from PR appeared to house the greatest richness of cultivable bacteria in their rhizosphere (Figures 2.5 and 2.6). However, it should be noted that the number of bulk soil samples was smaller (6) than the number of rhizosphere samples (12-18) for each site. Also, the samples are not directly comparable, since the amount of soil used from each sample for bacterial isolation was not standardized. The bacterial genus *Bacillus* was found in

rhizosphere soil samples from every host as well as from bulk soil from site CP, but not from bulk soil from site PR. In addition, it should be noted that the medium used to culture bacteria (LB) does not present an ideal environment for soil bacteria and therefore likely excluded many strains present in each soil sample.

The isolates were then streaked onto agar plates spiked with different concentrations of selenite and selenate, to test for Se resistance and for the ability to produce red elemental Se (Se^0). Qualitative resistance scores were assigned to each isolate based on its ability to grow on certain concentrations of Se (S1). Most bacteria have the capacity to convert selenite, but not selenate, to elemental Se, which has a deep red color and is less toxic than selenite (Garbisu et al., 1996). Indeed, red Se was observed for all isolates when grown on selenite. We observed that the bacteria were also able to produce some Se^0 when supplied with very high concentrations of selenate (200 mM), but upon further investigation it was found that the selenate stock included trace concentrations of selenite which was sufficient to result in visible red Se production at high selenate concentrations.

The median tolerance scores for the bacteria isolated from each host at each site are represented in Figures 2.5 and 2.6. A Kruskal-Wallis test was performed on the median selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) tolerance scores of the pooled isolates from each host plant. No significant differences were found between Se tolerance and host plant species, site, host Se concentration, or bacterial genus. All bacteria isolated from rhizosphere samples were tolerant to most concentrations of SeO_4^{2-}, some even grew better in the presence of 200 mM SeO_4^{2-} as compared to control conditions. Most isolates were able to grow on the 10 mM SeO_3^{2-} plates; however, few strains grew on the 200 mM SeO_3^{2-} plates.

2.4.2.2 Inoculation of B. juncea with Rhizobacteria

B. juncea plants were inoculated with bacterial consortia from CP non-HAs, PR non-HAs and PR HAs and given either 20 μM Na_2SeO_4 or no Se and tested for dry weight biomass production as well as Se accumulation. A Student t-test was performed on total dry weight between +Se and –Se treatments. All consortia groups showed a significantly ($p < .05$) smaller biomass for the +Se treatment than the –Se treatment, except for the group inoculated with the Pine Ridge HAs consortium , which was not significantly impaired in growth by the presence of Se (S5). Anova and Tukey-Kramer tests were performed on the total dry weight as well as root and shoot dry weight to look at differences between consortia treatments (Figure 2.7). No significant differences were found with these tests between inoculation treatments. Anova and Tukey-Kramer tests were also performed on Se content between consortia which also showed no significant differences (Figure 2.8).

2.5 Discussion

The questions addressed in this study are (1) How does Se HA affect the bacterial rhizobiome? (2) Are bacteria isolated from Se HAs or seleniferous soils more tolerant to Se than those from non-HAs or non-seleniferous soils? and (3) Can the bacteria isolated from Se HAs increase Se accumulation in a non-accumulator? The results from this study show that there is no difference in Se resistance between bacteria isolated from HA and non-HAs or from seleniferous and non-seleniferous soils, and most bacterial isolates were extremely selenate- and selenite resistant. It was also shown that bacterial communities around Se HAs are significantly different than those from non-HAs and bulk soil from the same site, and that microbial communities differed between seleniferous and non-seleniferous sites. While there is some evidence that

bacterial consortia from the rhizosphere of Se HAs can alleviate Se stress in non-accumulator *B. juncea*, there were no striking effects of inoculation on plant growth or Se accumulation.

While the highest species richness was found at CP, this study revealed a significantly higher species richness in rhizosphere soil samples taken from HAs than those not taken from HAs at Pine Ridge ($p < 0.001$), meaning there was a wider range of bacterial OTUs harbored by HAs at PR (Figure 2.2). This could be because of seleno-amino acids released by Se HAs via litter and roots, providing an extra source of essential C, N and Se to the bacteria (El Mehdawi et al., 2015). The rhizobiome sequencing data presented here indicate that HAs not only have higher bacterial diversity but also select for different bacterial communities to colonize their rhizosphere, since the HA rhizosphere microbe composition was significantly different from the non-HA rhizosphere samples on the same seleniferous site (Fig. 3). This difference in bacterial composition may be Se-related, even if Se resistance was not under selection. Some bacteria may be better able than others to utilize Se and thus benefit from it in terms of fitness. Such microbes may have a higher fitness in the rhizosphere of Se HAs.

The finding that high-Se habitats are rich in microbial diversity is in agreement with earlier studies that showed a large bacterial diversity in Se contaminated areas; for example, in Se contaminated ponds (de Souza et al., 2001), and enhanced numbers of cultivable microbes in Se-rich leaf litter (Quinn et al., 2011). Some studies that have assessed the species richness of microbial communities in soils containing high concentrations of other elements including Cr, Cu and As have shown an opposite effect, where microbial diversity decreases in these conditions (Kong et al., 2006; Sheik et al., 2012;). A potential explanation may be that these other elements are not present in organic forms and/or cannot be utilized by bacteria as essential elements. However, it was found that Ni HA *Thlaspi* (now called *Noccaea*) *caerulescens* also

harbored a rhizosphere rich in microbial diversity with Ni-resistant bacteria (Aboudrar et al., 2007).

The rhizobiome sequencing data revealed that the bacterial communities on the seleniferous and non-seleniferous sites were significantly different (Figure 2.1). This site-dependent difference was an expected result and has been shown before (Berg and Smalla, 2009). It may be attributed to a range of factors that differed between the two sites, including the soil type, pH, or possibly Se level. The most interesting finding in this study is that the bacterial communities were significantly different around HAs as compared to samples from the same site not taken from HAs (Figure 2.2). This is a novel discovery that deserves further investigation. The fact that three HAs from different plant families differ significantly in rhizobiome with two non-HA from the same families suggests Se HAs have a common rhizosphere factor that affects the microbial composition, potentially related to Se. Based on the group significance test performed on the rhizobiome data, the two genera with the highest average occurrence in HAs (when compare with non-HA and bulk soil on PR) are *Pedobacter* and *Devosia*. While this finding was not statically significant, it could be indicative of a core microbiome for Se HAs. More culture-independent studies are needed to follow up on this idea.

While the differences in rhizosphere microbial communities between HA and non-HA may reflect the effects of Se HA, conversely it may be hypothesized that the different, HA-specific rhizobial communities could be one of the factors contributing to the evolution of Se hyperaccumulation, in analogy to what has been speculated before in regards to *Rhizobium* and legumes (Alford et al., 2012). The theory of microbes aiding in evolution of higher plants has been developed by others as well, e.g. allowing plants to colonize land (Knack et al., 2015). The hypothesis that the rhizobiome plays a role in the evolution of Se hyperaccumulation is to some

extent supported by the fact that inoculation of *B. juncea* with rhizosphere bacteria from PR HAs decreased the gap between dry weight production of the -Se and +Se treatments (judged from t-tests). This may indicate that the bacterial consortium alleviated the Se stress on *B. juncea*, a non-hyperaccumulator. However, more studies are needed to confirm this first result.

Bacterial strains associated with plants that HA metals such as Ni, Cd and Zn, are often shown to be more tolerant to the metal being accumulated than similar bacteria taken from non-accumulators (Visoli et al., 2015). This study, however, suggests that rhizobacteria that associate with Se HAs are no more tolerant to Se than rhizobacteria associated with non-accumulators grown on both seleniferous and non-seleniferous soils (Figures 2.5 and 2.6). A similar study which tested rhizosphere fungi taken from HAs and non-HAs on a seleniferous site showed that fungal strains from the rhizosphere of HAs were no more tolerant to 10 mg $Se \cdot L^{-1}$ than strains isolated from non-HAs from the same seleniferous site (Wangeline et al., 2011). However, rhizospheric fungi taken from seleniferous sites were significantly more Se tolerant than those taken from a non-seleniferous site (Wangeline et al., 2011). To date, rhizobacteria tested for Se tolerance have only been tested up to 50 mM (di Gregorio et al., 2004), however this study shows that bacteria isolated from rhizosphere soil can survive, and in some cases show enhanced growth, on concentrations of 200 mM selenate and selenite. A similar trend was noticed in the tolerance of endophytes taken isolated from Se HAs *Stanleya pinnata* and *Astragalus bisulcatus* from the same seleniferous site sampled here (Pine Ridge), where the isolates were also able to withstand concentrations of 200 mM Se (Sura de Jong et al., 2015).

In an earlier study, the Se levels found in soil surrounding these Se HA species were 7-11 times elevated compared to bulk soil, but the soil Se levels were never more than a few hundred mg kg^{-1} (El Mehdawi et al., 2011, 2012), which corresponds with a few mM selenate or selenite

(1 mM = 80 ppm). Most isolates in this study, both from seleniferous and non-seleniferous soil, were not inhibited by a few mM selenate or selenite. This suggests that most bacteria are not sensitive to the concentrations of Se that surround Se HAs, and that the bacterial microbiome in the rhizosphere of HAs is not under selection for Se resistance. This result is quite different from those found for other ecological partners associated with HAs: herbivores, fungi, other plant species, and also pollinators are generally sensitive to the high levels of Se in HAs (Hanson et al., 2003,2004; Freeman et al., 2007, 2009; Galeas et al., 2008; Quinn et al., 2008, 2011b; El Mehdawi et al., 2011a,b, 2012, 2015; Pilon-Smits, 2012a,b, 2015; Valdez Barillas et al., 2012). This has been suggested to lead to a selective effect by Se HAs on their ecological partners, against Se-sensitivity and for Se resistance (El Mehdawi and Pilon-Smits, 2012). Thus, while Se HAs may be hypothesized to significantly affect the Se resistance and perhaps through that, species composition of their associated herbivores, pollinators and vegetation, they likely do not select for enhanced Se resistance in their microbiome.

2.6 Conclusion

This study utilized both culture-dependent and culture-independent approaches to study the rhizobiome of Se HAs. By utilizing both techniques, we were able to uncover multiple facets of Se hyperaccumulation that either approach on its own could not accomplish. Through the use of next-generation sequencing methods we were able to uncover over one thousand bacterial genera living in the samples within this dataset. By culturing isolates directly from the soil, their Se-related properties could be studied and compared, as well as their plant growth promoting properties.

The results from this study indicate that bacteria are in general extremely tolerant to Se, which likely makes them different in their interactions with Se HAs as compared to other ecological partners of these plants. In contrast to animals, fungi or plants, bacteria are generally not sensitive to the increased Se concentrations associated with HA plants. Therefore, HAs may not select their bacterial communities for increased resistance to Se. However, the organic selenocompounds present in HA-associated habitats may be able to shape the microbial species composition, favoring taxa that best utilize the essential element Se. HA rhizobiomes showed increased species richness and significantly different community composition. It will be interesting to study whether there could be a core microbiome for Se HA that plays a role in Se hyperaccumulation by enhancing plant fitness and perhaps by contributing to the Se hyperaccumulation itself. More studies are needed to investigate the nature of the relationships between Se HAs and their specific bacterial microbiome. These findings may help increase the efficiency of bioremediation of Se polluted soils and waterways or Se biofortification of crops. This is of significance, since it could help alleviate Se deficiency in humans and livestock, which affects millions of people worldwide.

2.7 Tables and Figures

Table 2.1: Plant species sampled (n=6 plants per species) for this study, and corresponding leaf Se range determined by ICP-OES. Leaf, root and rhizosphere soil samples were taken from each plant. HA: Se hyperaccumulator species. ND= not determined.

Pine Ridge (seleniferous)	**Cloudy Pass (non-seleniferous)**

• *Astragalus bisulcatus* (HA)
  • 10 - 2,699 mg Se kg^{-1} DW
• *Stanleya pinnata* (HA)
  • 610 - 11,784 mg Se kg^{-1} DW
• *Symphyotrichum ericoides* (HA)
  • 36 – 1,169 mg Se kg^{-1} DW
• *Astragalus tenellus* (n=4; non-HA)
  • ND
• *Physaria bellii* (non-HA)
  • 3 - 24 mg Se kg^{-1} DW
• Unvegetated soil
  • ND
	• *Symphyotrichum ericoides* (non-HA)
	  • ND
	• *Astragalus laxmanii* (non-HA)
	  • 0.07 - 0.11 mg Se kg^{-1} DW
	• *Physaria montana* (non-HA)
	  • 0.02 - 0.35 mg Se kg^{-1} DW
	• Unvegetated soil
	  • ND

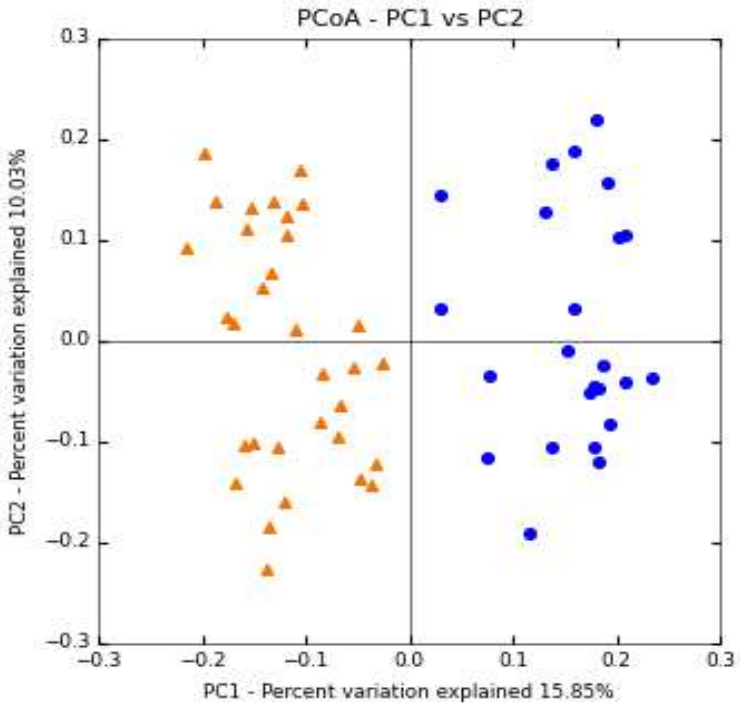

Figure 2.1: 2D PCoA plots of 16S rRNA diversity from rhizosphere soil samples. Orange triangles represent samples from PR from PR and blue circles represent samples from CP. Permanova test suggests microbial communities are significantly different based on site ($p < .0001$).

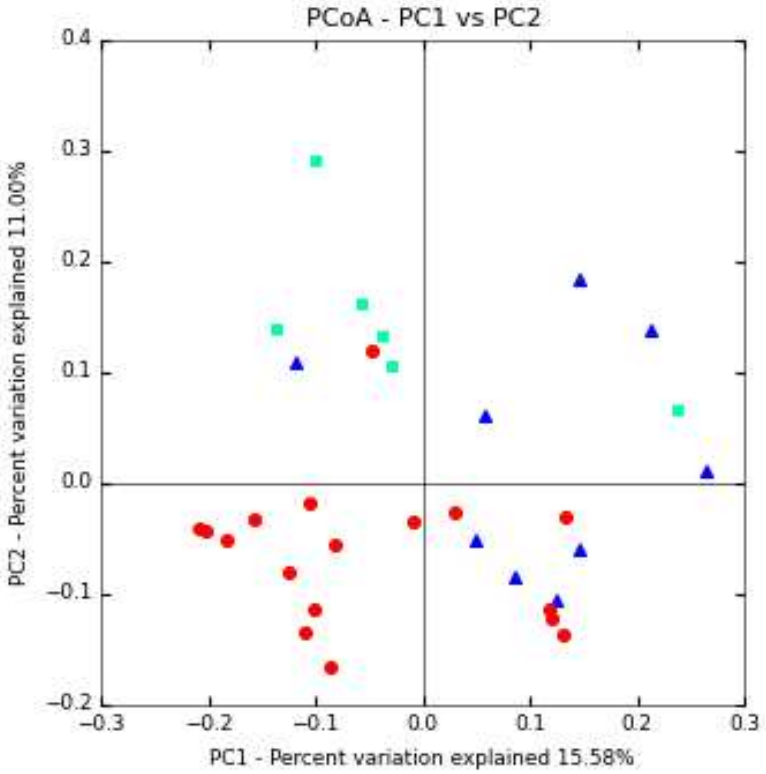

Figure 2.2: 2D PCoA plots of 16S rRNA diversity from rhizosphere soil samples collected from PR. Red circles are samples taken from HA hosts, blue triangles are non-HA hosts and green squares are from bulk soil. Permanova test suggests microbial communities are significantly different based on host ability to HA ($p < .001$).

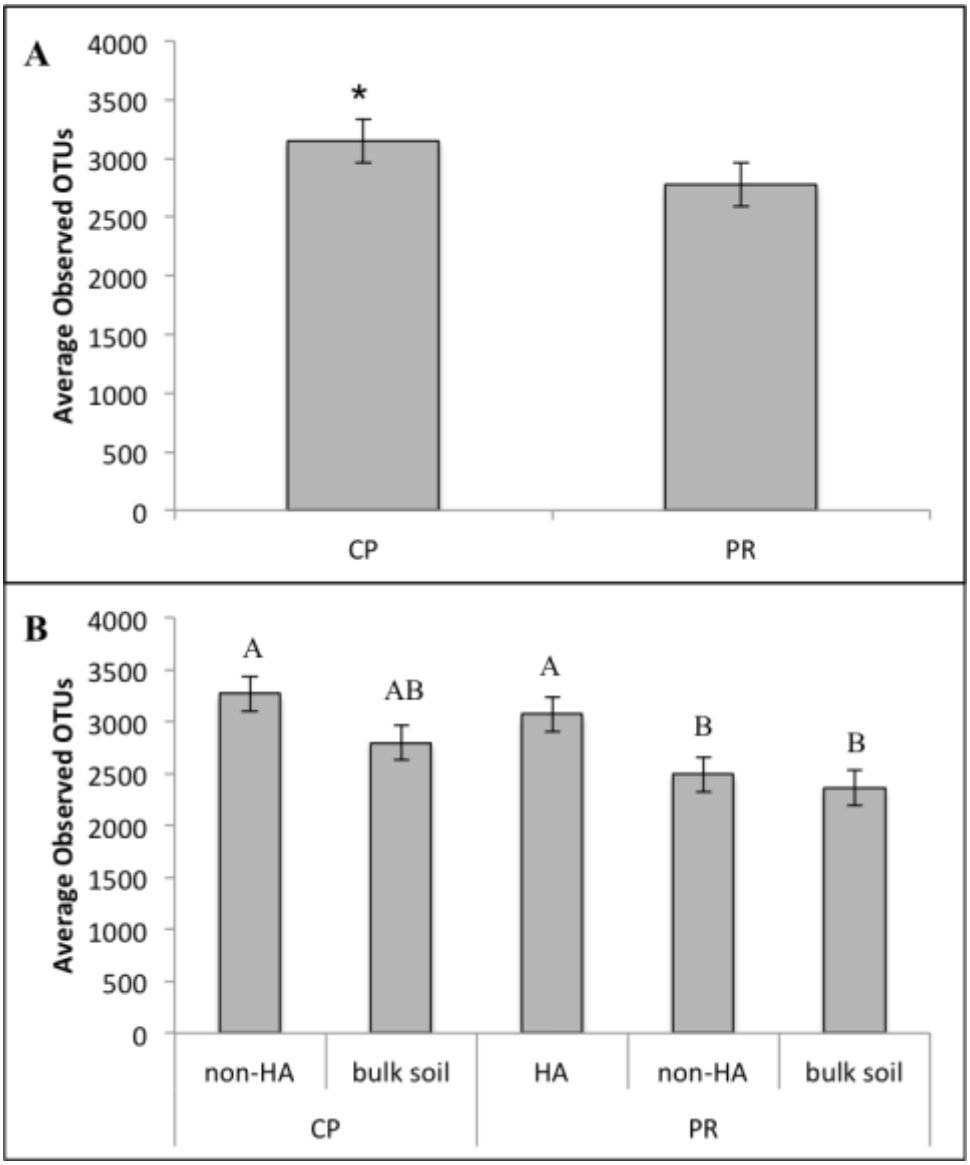

Figure 2.3: Alpha diversity (observed OTUs) of soil samples in **A)** each site and **B)** each group. ANOVA was performed on each data set and both were found to be statistically significant (**A:** $p < .004$; **B:** $p < .0001$). Letters in **B** designated by Tukey HSD indicate significant differences.

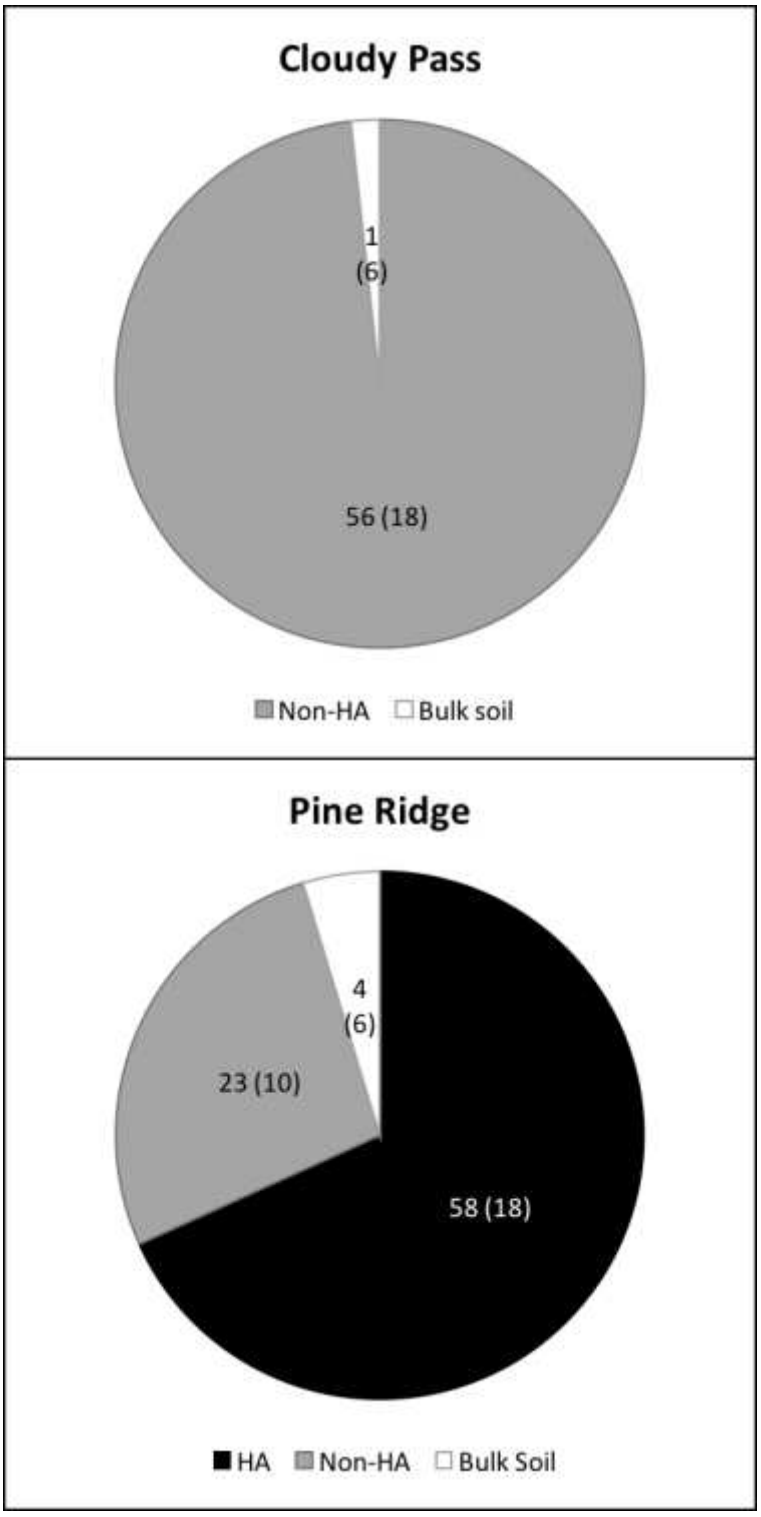

Figure 2.4: Fractions of individual bacterial isolates resulting from hyperaccumulator rhizosphere soil (HA), non-HA rhizosphere soil (non-HA) or bulk soil at Cloudy Pass (top) and Pine Ridge (bottom). Each pie represents all isolates from each site. The total number of isolates cultured from each group are shown in the individual pie slices, with the number of samples per group in parentheses.

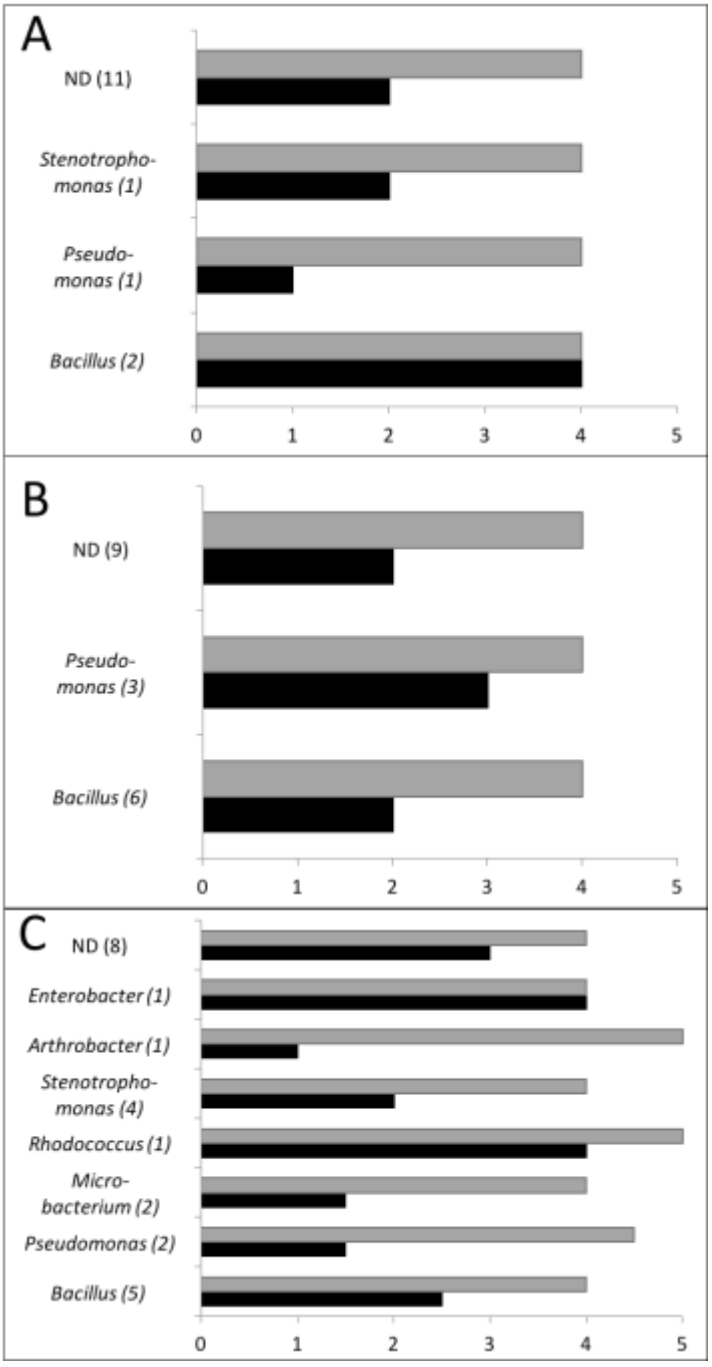

Figure 2.5: Median selenite (black) and selenate (gray) tolerance scores for bacterial genera isolated from rhizosphere soil samples of different non-HA host plants from non-seleniferous area Cloudy Pass. **A:** *Symphyotrichum ericoides;* **B:** *Physaria montana;* **C:** *Astragalus laxmanii.* Unidentified bacteria are pooled into ND. The number of isolates in each genus are in parentheses next to the genus name on the y-axis. Tolerance scores were assigned by the following criteria: 0: no growth $\geq$1 mM Se; 1: no growth $\geq$10 mM Se; 2: no growth $\geq$100 mM Se; 3: no growth $\geq$200mM Se; 4: growth at 200mM Se; 5: enhanced growth on 200 mM Se relative to 0 Se. All isolates grew on 0 Se medium.

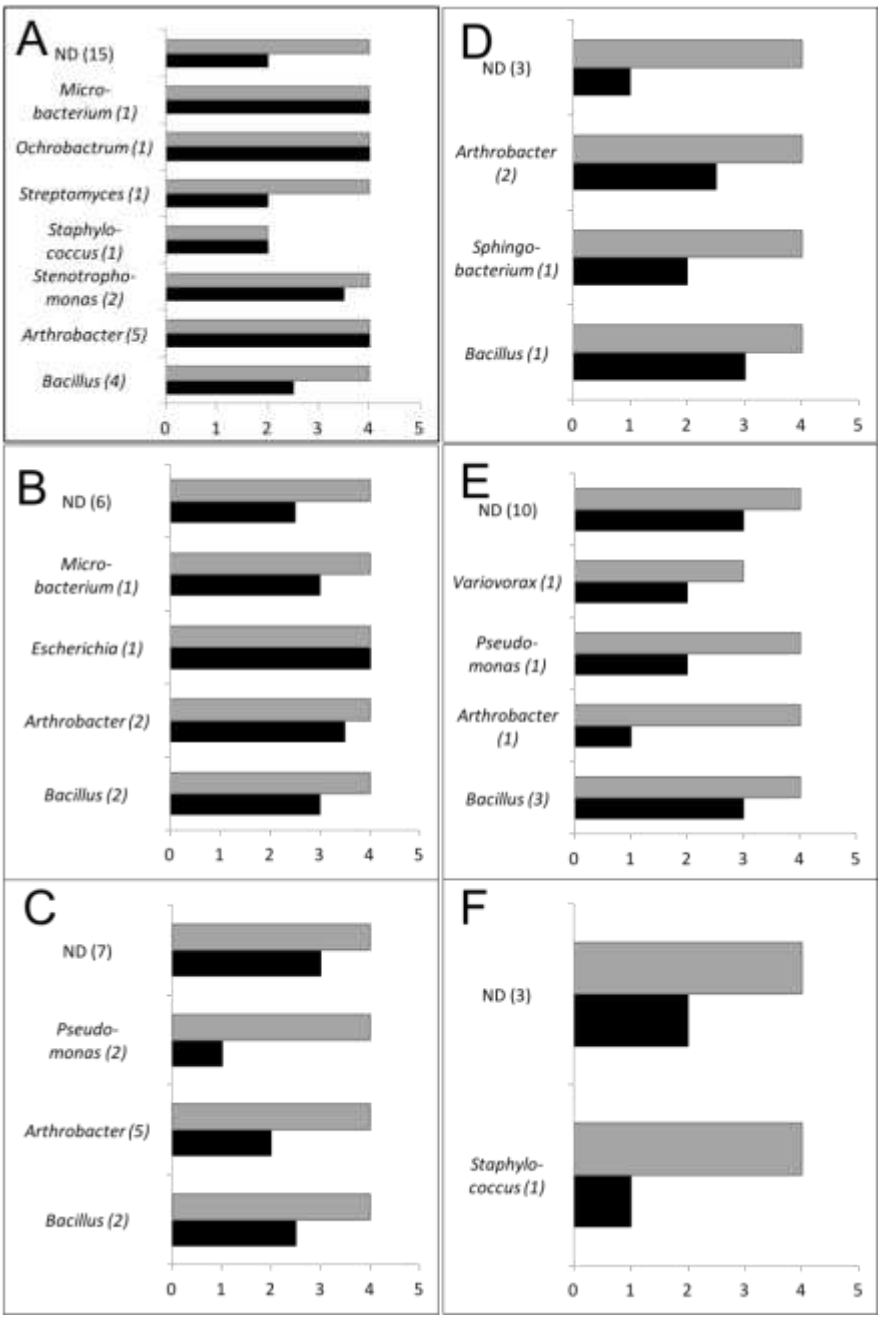

Figure 2.6: Median selenite (black) and selenate (gray) tolerance scores for each bacterial genus isolated from rhizosphere soil samples of different host species (or bulk soil) from seleniferous area Pine Ridge. Isolates from HA hosts are on the left (**A:** *Astragalus bisulcatus*; **B:** *Stanleya pinnata;* **C:** *Symphyotrichum ericoides*). Isolates from non-HA hosts are on the right (**D:** *Astragalus tenellus;* **E:** *Physaria bellii;* **F:** Bulk soil). Unidentified bacteria are pooled into category ND. The number of isolates in each genus are shown in parentheses next to the genus names on the y-axis. Tolerance scores were assigned as described in Figure 6.

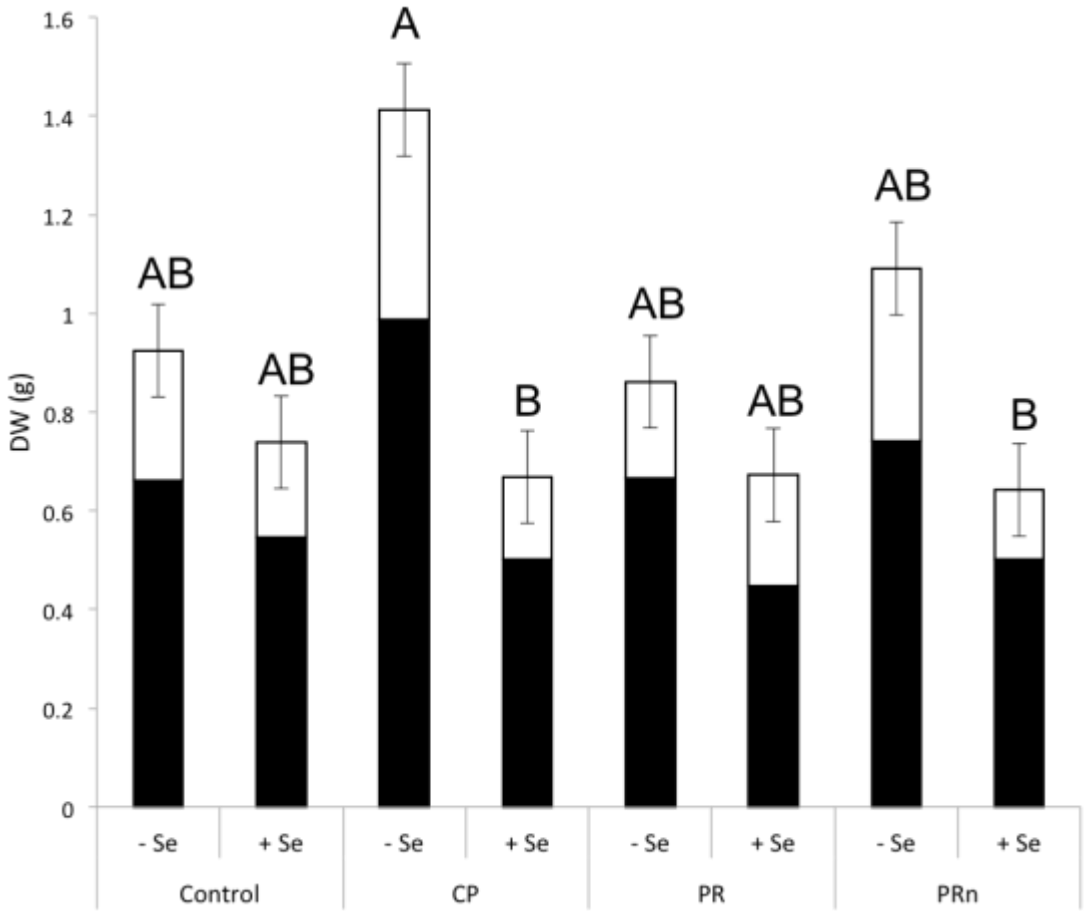

Figure 2.7: Average total dry weight of *B. juncea* split into root average (white) and shoot average (black) after inoculation with rhizosphere bacterial consortia and treatment with or without 20 μM Na_2SeO_4. Differences of total dry weight were tested using Anova ($p < .0062$) and letters above show differences between total dry weight means via Tukey HSD. Control indicates no inoculum and PRn is PR non-HA.

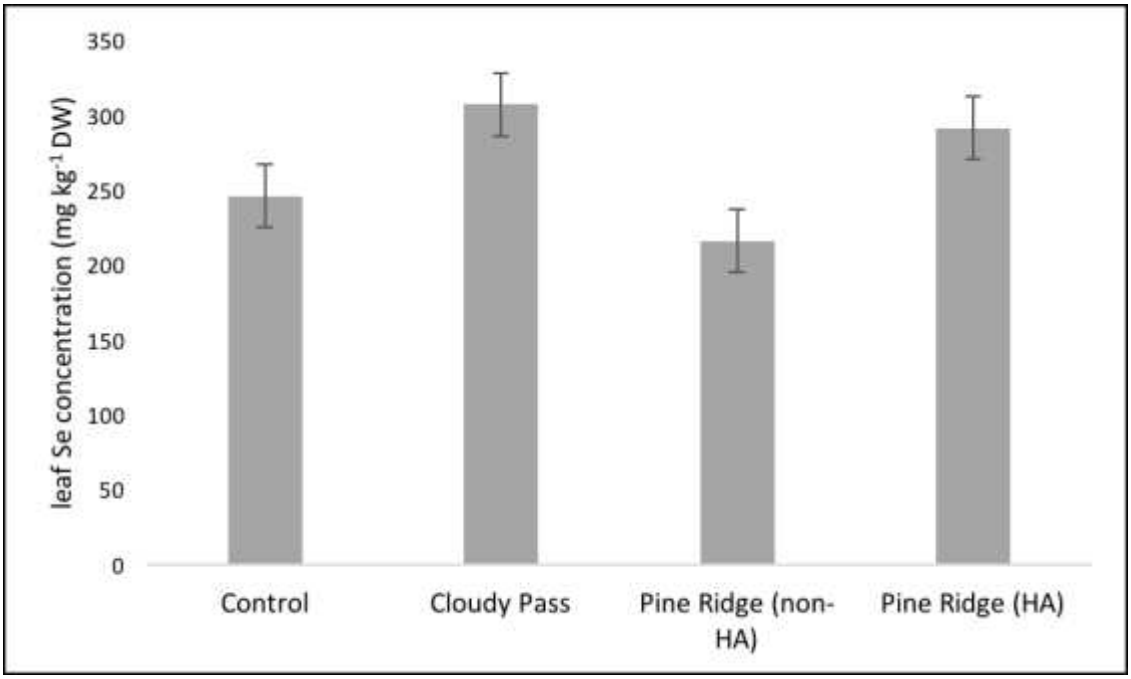

Figure 2.8: Leaf Se concentration of *B. juncea* plants inoculated with different consortia of rhizobacteria (as described in previous figure legends). Se content was measured using ICP-OES on plants treated with 20 µM Na_2SeO_4. There were no significant differences in Se content between bacterial consortium treatments (ANOVA, $p > 0.05$).

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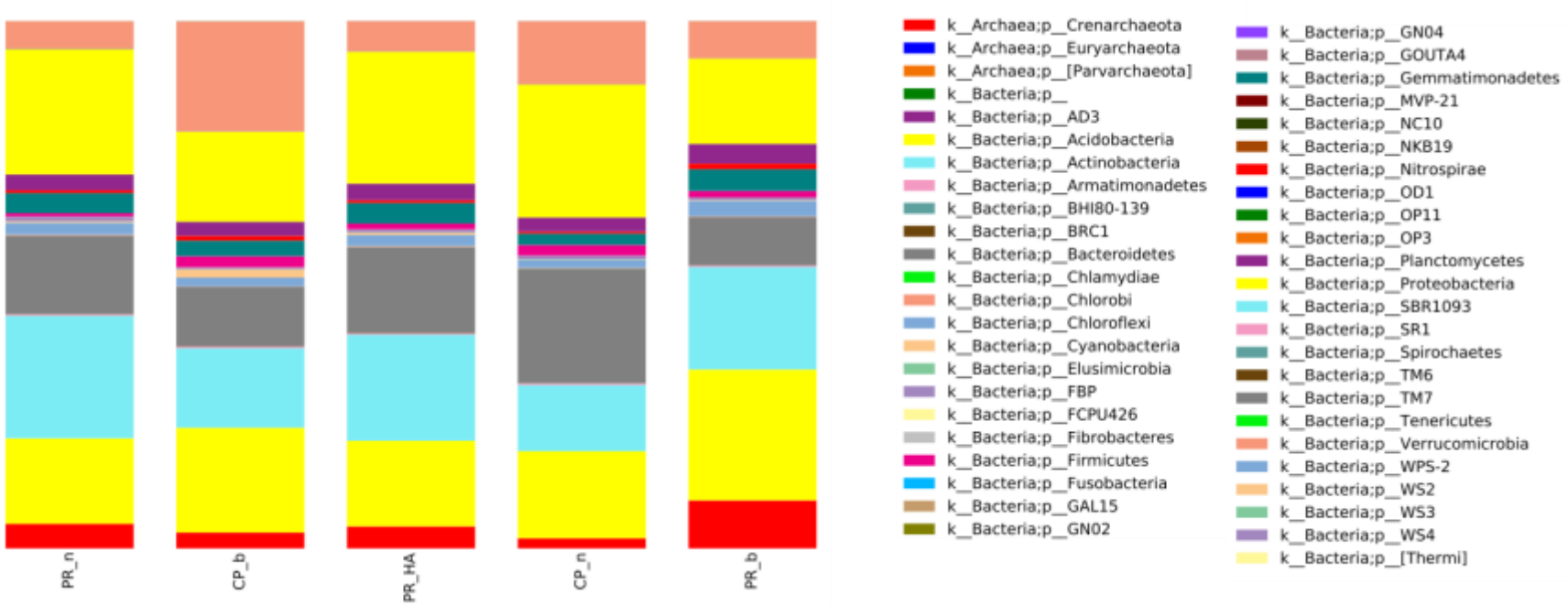


S1: Chart depicting the distribution of bacterial phyla in soil samples taken from Pine Ridge non-HA (Pr_n), Pine Ridge HAs (PR_HA), Pine Ridge bulk soil (PR_b), Cloudy Pass non-HAs (CP_n) and Cloudy Pass bulk soil (CP_b). OTU IDs were assigned with QIIME using closed OTU picking against a Green Genes database. Exact percentages of phyla represented in each sampling category is shown in Supplementary table S2.

S2: Table describing the distribution of bacterial phyla in soil samples taken from Pine Ridge non-HA (Pr_n), Pine Ridge HAs (PR_HA), Pine Ridge bulk soil (PR_b), Cloudy Pass non-HAs (CP_n) and Cloudy Pass bulk soil (CP_b) as depicted in S1. OTU IDs were assigned with QIIME using closed OTU picking against a Green Genes database.

OTU ID	PR_n	CP_b	PR_HA	CP_n	PR_b
k__Archaea;p__Crenarchaeota	4.6939%	3.0528%	4.2001%	1.9392%	9.1308%
k__Archaea;p__Euryarchaeota	0.0005%	0.0004%	0.0013%	0.0007%	0.0000%
k__Archaea;p__[Parvarchaeota]	0.0000%	0.0000%	0.0010%	0.0000%	0.0000%
k__Bacteria;p__	0.0017%	0.0047%	0.0045%	0.0016%	0.0066%
k__Bacteria;p__AD3	0.0000%	0.0000%	0.0000%	0.0006%	0.0000%
k__Bacteria;p__Acidobacteria	16.2038%	19.8448%	16.2223%	16.5260%	24.7920%
k__Bacteria;p__Actinobacteria	23.2367%	15.0842%	20.1218%	12.5330%	19.4222%
k__Bacteria;p__Armatimonadetes	0.2436%	0.3060%	0.2546%	0.3106%	0.2801%
k__Bacteria;p__BHI80-139	0.0012%	0.0047%	0.0021%	0.0038%	0.0022%
k__Bacteria;p__BRC1	0.0860%	0.0390%	0.0840%	0.0330%	0.0240%
k__Bacteria;p__Bacteroidetes	14.8420%	11.2244%	16.2330%	21.7026%	9.1887%
k__Bacteria;p__Chlamydiae	0.0112%	0.0069%	0.0149%	0.0196%	0.0080%
k__Bacteria;p__Chlorobi	0.1656%	0.0991%	0.1763%	0.1175%	0.1129%
k__Bacteria;p__Chloroflexi	2.2210%	1.6638%	2.0657%	1.5241%	2.8874%
k__Bacteria;p__Cyanobacteria	0.2417%	1.5373%	0.3923%	0.0750%	0.1166%
k__Bacteria;p__Elusimicrobia	0.0607%	0.0674%	0.1216%	0.0774%	0.0736%
k__Bacteria;p__FBP	0.8852%	0.2874%	0.5410%	0.5923%	0.4608%
k__Bacteria;p__FCPU426	0.0000%	0.0000%	0.0000%	0.0001%	0.0000%
k__Bacteria;p__Fibrobacteres	0.0357%	0.0747%	0.0482%	0.0625%	0.0193%
k__Bacteria;p__Firmicutes	0.6006%	2.0933%	1.0942%	1.9943%	1.2310%
k__Bacteria;p__Fusobacteria	0.0039%	0.0109%	0.0021%	0.0034%	0.0113%
k__Bacteria;p__GAL15	0.0000%	0.0000%	0.0010%	0.0000%	0.0011%
k__Bacteria;p__GN02	0.0005%	0.0007%	0.0015%	0.0010%	0.0000%
k__Bacteria;p__GN04	0.0002%	0.0004%	0.0001%	0.0000%	0.0004%
k__Bacteria;p__GOUTA4	0.0000%	0.0004%	0.0001%	0.0000%	0.0000%
k__Bacteria;p__Gemmatimonadetes	3.8098%	2.9435%	3.8809%	2.1500%	4.0958%
k__Bacteria;p__MVP-21	0.0002%	0.0000%	0.0006%	0.0002%	0.0007%
k__Bacteria;p__NC10	0.0000%	0.0000%	0.0001%	0.0005%	0.0000%
k__Bacteria;p__NKB19	0.0007%	0.0004%	0.0031%	0.0033%	0.0007%
k__Bacteria;p__Nitrospirae	0.5343%	0.9151%	0.5328%	0.3920%	1.0856%
k__Bacteria;p__OD1	0.0053%	0.0528%	0.0104%	0.0383%	0.0117%
k__Bacteria;p__OP11	0.0007%	0.0011%	0.0026%	0.0017%	0.0004%
k__Bacteria;p__OP3	0.0056%	0.0051%	0.0118%	0.0100%	0.0117%
k__Bacteria;p__Planctomycetes	2.9802%	2.5960%	3.1190%	2.6081%	3.6980%
k__Bacteria;p__Proteobacteria	23.6729%	17.0346%	24.9247%	25.1868%	16.1472%
k__Bacteria;p__SBR1093	0.0000%	0.0000%	0.0001%	0.0000%	0.0000%
k__Bacteria;p__SR1	0.0000%	0.0000%	0.0008%	0.0013%	0.0000%
k__Bacteria;p__Spirochaetes	0.0029%	0.0011%	0.0114%	0.0038%	0.0007%

k__Bacteria;p__TM6	0.0221%	0.0051%	0.0116%	0.0273%	0.0007%
k__Bacteria;p__TM7	0.0153%	0.0219%	0.0216%	0.0301%	0.0077%
k__Bacteria;p__Tenericutes	0.0474%	0.0215%	0.0406%	0.0140%	0.0149%
k__Bacteria;p__Verrucomicrobia	5.2979%	20.9075%	5.7905%	11.9477%	7.0922%
k__Bacteria;p__WPS-2	0.0104%	0.0029%	0.0069%	0.0327%	0.0018%
k__Bacteria;p__WS2	0.0032%	0.0171%	0.0060%	0.0170%	0.0073%
k__Bacteria;p__WS3	0.0058%	0.0648%	0.0197%	0.0109%	0.0295%
k__Bacteria;p__WS4	0.0000%	0.0011%	0.0000%	0.0000%	0.0000%
k__Bacteria;p__[Thermi]	0.0495%	0.0051%	0.0208%	0.0061%	0.0244%

S3: Table of all bacteria used in analyses for Se resistance. Bacteria ID corresponds with the identification number given to each isolate during isolation from soil samples. Identification of isolates was performed using MALDI-TOF, and a MALDI confidence score was given to each isolate to indicate the degree of certainty of the identification. +++; the isolate is confidently described to species level; ++ the isolate is confidently described at genus level; + genus identification is probable; below 1.7 the identification is not successful (Sura de Jong et al., 2015). The host ID corresponds with the individual the isolate was taken from. Site is the site of collection (CP is Cloudy Pass and PR is Pine Ridge). HA? Indicates whether the host plant is able to hyperaccumulate Se. SeO_3 and SeO_4 Resistance Score were determined by the isolate's ability to either not grow on Se (0), grow only on 1 mM (1), grow on plates up to 10 mM (2), grow on plates up to 100 mM (3), grow on plates up to 200 mM (4), or grow better on 200 mM than on the control plate (5).

Bacteria ID	MALDI IDENTIFICATION	MALDI confidence score	Host ID	Host name	Site	HA?	SeO_3 Resistance Score	SeO_4 Resistance Score
ACR 1	*Pseudomonas koreensis*	++	ACAS2	*A. laxmanií*	CP	N	1	4
ACR 2	*Microbacterium* sp.	+	ACAS2	*A. laxmanií*	CP	N	3	4
ACR 3	*Bacillus* sp.	+	ACAS2	*A. laxmanií*	CP	N	3	4
ACR 4			ACAS2	*A. laxmanií*	CP	N	4	4
ACR 5			ACAS2	*A. laxmanií*	CP	N	3	4
ACR11	*Bacillus atrophaeus*	+++	ACAS1	*A. laxmanií*	CP	N	2	4
ACR12	*Bacillus atrophaeus*	+++	ACAS1	*A. laxmanií*	CP	N	4	4
ACR13			ACAS1	*A. laxmanií*	CP	N	3	4
ACR 18			ACAS3	*A. laxmanií*	CP	N	3	4
ACR 19			ACAS3	*A. laxmanií*	CP	N	2	4
ACR 20	*Bacillus atrophaeus*	++	ACAS3	*A. laxmanií*	CP	N	3	4
ACR 22	*Bacillus atrophaeus*	++	ACAS3	*A. laxmanií*	CP	N	2	4
ACR 35	*Rhodococcus fascians*	++	ACAS4	*A. laxmanií*	CP	N	4	5
ACR 36			ACAS4	*A. laxmanií*	CP	N	1	4
ACR 38			ACAS4	*A. laxmanií*	CP	N	1	5
ACR 40			ACAS5	*A. laxmanií*	CP	N	3	4
ACR 42	*Stenotrophomonas rhizophila*	++	ACAS5	*A. laxmanií*	CP	N	4	5
ACR 43	*Arthrobacter* sp.	+	ACAS5	*A. laxmanií*	CP	N	1	5

ACR 44	*Pseudomonas brassicacearum*	++	ACAS5	*A. laxmanií*	CP	N	2	5
ACR 92	*Stenotrophomonas sp*	++	ACAS6	*A. laxmanií*	CP	N	2	4
ACR 93	*Stenotrophomonas sp*	+++	ACAS6	*A. laxmanií*	CP	N	2	4
ACR 95	*Microbacterium luteolum*	++	ACAS6	*A. laxmanií*	CP	N	0	4
ACR 96	*Stenotrophomonas sp*	++	ACAS6	*A. laxmanií*	CP	N	2	4
ACR 97	*Enterobacter cloacae*	+++	ACAS6	*A. laxmanií*	CP	N	4	4
ACR 6	*Bacillus cereus*	++	ACPB4	*P. montana*	CP	N	3	4
ACR 7			ACPB4	*P. montana*	CP	N	2	4
ACR 14	*Pseudomonas koreensis*	++	ACPB3	*P. montana*	CP	N	4	4
ACR 15	*Bacillus* sp.	+	ACPB3	*P. montana*	CP	N	1	4
ACR 16			ACPB3	*P. montana*	CP	N	4	4
ACR 23			ACPB1	*P. montana*	CP	N	2	4
ACR 24	*Bacilllus* sp.	+	ACPB1	*P. montana*	CP	N	1	4
ACR 26	*Bacilllus* sp.	+	ACPB1	*P. montana*	CP	N	4	4
ACR 45	*Pseudomonas koreensis*	++	ACPB9	*P. montana*	CP	N	1	4
ACR 47	*Bacillus magaterium*	++	ACPB9	*P. montana*	CP	N	4	4
ACR 55			ACPB8	*P. montana*	CP	N	2	4
ACR 56			ACPB9	*P. montana*	CP	N	1	4
ACR 57	*Bacillus atrophaeus*	+++	ACPB10	*P. montana*	CP	N	1	4
ACR 66			ACPB10	*P. montana*	CP	N	3	4
ACR 67	*Pseudomonas thivervalensis*	++	ACPB10	*P. montana*	CP	N	3	4
ACR 68			ACPB10	*P. montana*	CP	N	2	4
ACR 69			ACPB10	*P. montana*	CP	N	3	4
ACR 49			ACPB9	*P. montana*	CP	N	0	2
ACR 28			ACSE7	*S. ericoides*	CP	N	3	4
ACR 29	*Stenotrophomonas* sp.	+	ACSE9	*S. ericoides*	CP	N	2	4
ACR 30			ACSE9	*S. ericoides*	CP	N	4	4
ACR 31			ACSE9	*S. ericoides*	CP	N	1	4

ACR 32	*Bacillus simplex*	++	ACSE8	*S. ericoides*	CP	N	4	4
ACR 33			ACSE8	*S. ericoides*	CP	N	3	4
ACR 34			ACSE8	*S. ericoides*	CP	N	1	4
ACR 70	*Bacillus* sp.	+	ACSE13	*S. ericoides*	CP	N	4	4
ACR 71			ACSE13	*S. ericoides*	CP	N	0	2
ACR 74			ACSE13	*S. ericoides*	CP	N	2	4
ACR 75			ACSE12	*S. ericoides*	CP	N	0	2
ACR 76	*Pseudomonas frederiksbergensis*	++	ACSE12	*S. ericoides*	CP	N	1	4
ACR 77			ACSE12	*S. ericoides*	CP	N	3	4
ACR 89			ACSE11	*S. ericoides*	CP	N	2	4
ACR 90			ACSE11	*S. ericoides*	CP	N	0	3
ACR 203	*Arthrobacter globiformis*	++	ACBS6	Bulk Soil	CP	N/A		
ACR 106	*Bacillus* sp.	+	ACAB9	*A. tenellus*	PR	N	3	4
ACR 112			ACAB7	*A. tenellus*	PR	N	1	3
ACR 114	*Sphingobacterium* sp.	+	ACAB7	*A. tenellus*	PR	N	2	4
ACR 191			ACAT2	*A. tenellus*	PR	N	3	4
ACR 192			ACAT2	*A. tenellus*	PR	N	1	4
ACR 193	*Arthrobacter aurescens*	++	ACAT1	*A. tenellus*	PR	N	3	4
ACR 194	*Arthrobacter aurescens*	++	ACAT1	*A. tenellus*	PR	N	2	4
ACR 58	*Variovorax* sp.	+	ACPB11	*P. bellii*	PR	N	2	3
ACR 79			ACPB7	*P. bellii*	PR	N	3	4
ACR 81			ACPB6	*P. bellii*	PR	N	1	4
ACR 125	*Bacillus mycoides*	++	ACPB5	*P. bellii*	PR	N	3	4
ACR 126	*Arthrobacter polychromogenes*	++	ACPB5	*P. bellii*	PR	N	1	4
ACR 127			ACPB5	*P. bellii*	PR	N	4	4
ACR 128			ACPB5	*P. bellii*	PR	N	1	4
ACR 129			ACPB5	*P. bellii*	PR	N	3	4
ACR 130	*Bacillus cereus*	++	ACPB5	*P. bellii*	PR	N	2	4
ACR 133			ACPB6	*P. bellii*	PR	N	4	4

ACR 134			ACPB6	*P. bellii*	PR	N	3	4
ACR 135	*Pseudomonas corrugata*	++	ACPB6	*P. bellii*	PR	N	2	4
ACR 136	*Bacillus mycoides*	++	ACPB6	*P. bellii*	PR	N	3	5
ACR 137			ACPB6	*P. bellii*	PR	N	1	4
ACR 160			ACPB11	*P. bellii*	PR	N	4	4
ACR 167			ACPB13	*P. bellii*	PR	N	2	0
ACR 196			ACBS10	Bulk Soil	PR	N/A	2	4
ACR 199			ACBS10	Bulk Soil	PR	N/A	2	4
ACR 200	*Staphylococcus epidermidis*	++	ACBS9	Bulk Soil	PR	N/A	1	4
ACR 201			ACBS8	Bulk Soil	PR	N/A	3	4
ACR 102	*Arthrobacter aurescens*	++	ACAB8	*A. bisulcatus*	PR	Y	3	4
ACR 103			ACAB8	*A. bisulcatus*	PR	Y	2	4
ACR 105			ACAB8	*A. bisulcatus*	PR	Y	1	4
ACR 107	*Bacillus* sp.	+	ACAB3	*A. bisulcatus*	PR	Y	1	4
ACR 109	*Microbacterium hydrocarbonoxydans*	++	ACAB3	*A. bisulcatus*	PR	Y	4	4
ACR 110			ACAB3	*A. bisulcatus*	PR	Y	0	4
ACR 111	*Arthrobacter aurescens*	+++	ACAB3	*A. bisulcatus*	PR	Y	1	4
ACR 117			ACAB2	*A. bisulcatus*	PR	Y	2	4
ACR 119			ACAB2	*A. bisulcatus*	PR	Y	1	4
ACR 120	*Ochrobactrum grignonense*	++	ACAB2	*A. bisulcatus*	PR	Y	4	4
ACR 121	*Staphylococcus haemolyticus*	++	ACAB2	*A. bisulcatus*	PR	Y	2	2
ACR 123	*Stenotrophomonas sp*	++	ACAB2	*A. bisulcatus*	PR	Y	4	4
ACR 124	*Stenotrophomonas rhizophila*	++	ACAB2	*A. bisulcatus*	PR	Y	3	4
ACR 153	*Bacillus* sp.	+	ACAB1	*A. bisulcatus*	PR	Y	2	4
ACR 154	*Bacillus endophyticus*	++	ACAB1	*A. bisulcatus*	PR	Y	3	4
ACR 155	*Streptomyces* sp.	+	ACAB1	*A. bisulcatus*	PR	Y	2	4

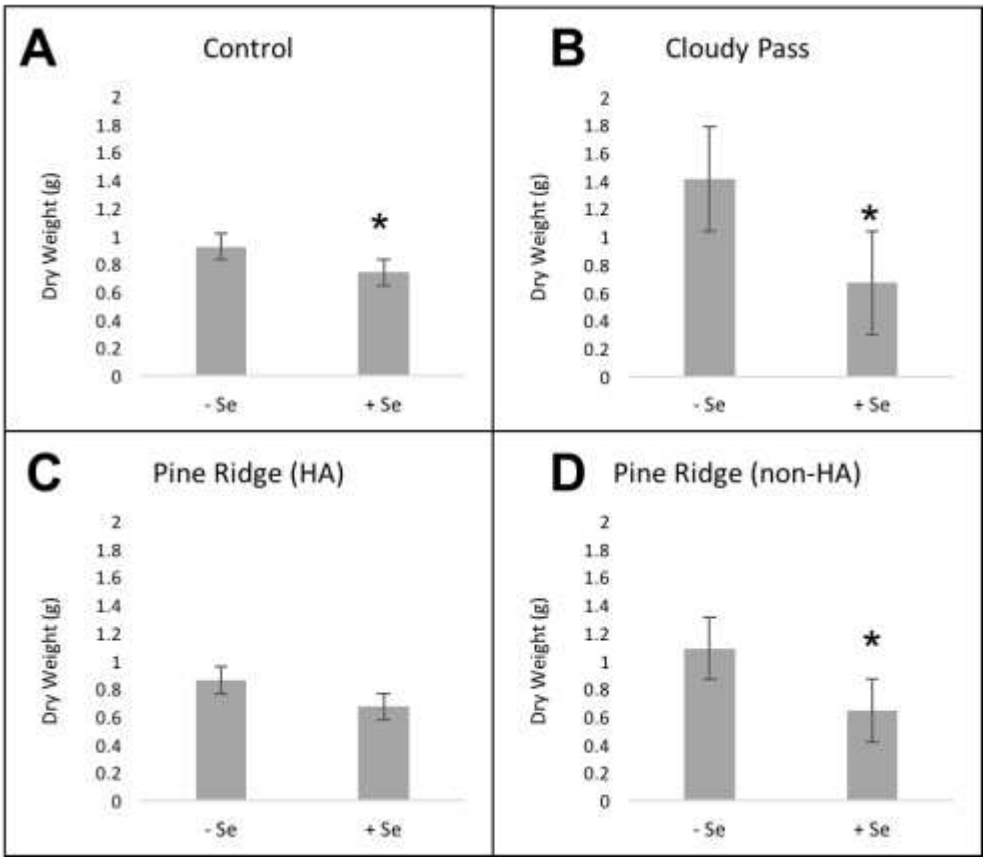
ACR 156			ACAB1	*A. bisulcatus*	PR	Y	1	4
ACR 157	*Bacillus mycoides*	++	ACAB1	*A. bisulcatus*	PR	Y	3	4
ACR 159	*Arthrobacter aurescens*	++	ACAB1	*A. bisulcatus*	PR	Y	4	4
ACR 176	*Arthrobacter aurescens*	++	ACAB11	*A. bisulcatus*	PR	Y	4	4
ACR 177	*Arthrobacter aurescens*	+++	ACAB11	*A. bisulcatus*	PR	Y	4	4
ACR 178			ACAB11	*A. bisulcatus*	PR	Y	4	4
ACR 179			ACAB11	*A. bisulcatus*	PR	Y	2	3
ACR 180			ACAB11	*A. bisulcatus*	PR	Y	2	4
ACR 181			ACAB11	*A. bisulcatus*	PR	Y	4	4
ACR 182			ACAB10	*A. bisulcatus*	PR	Y	2	4
ACR 184			ACAB10	*A. bisulcatus*	PR	Y	2	4
ACR 187			ACAB10	*A. bisulcatus*	PR	Y	4	4
ACR 188			ACAB10	*A. bisulcatus*	PR	Y	3	4
ACR 189			ACAB10	*A. bisulcatus*	PR	Y	3	4
ACR 83			ACSP4	*S. pinnata*	PR	Y	1	4
ACR 84			ACSP4	*S. pinnata*	PR	Y	2	4
ACR 85			ACSP4	*S. pinnata*	PR	Y	2	4
ACR 86	*Bacillus* sp.	+	ACSP6	*S. pinnata*	PR	Y	2	4
ACR 87			ACSP6	*S. pinnata*	PR	Y	4	4
ACR 100			ACSP5	*S. pinnata*	PR	Y	3	4
ACR 101	*Arthrobacter aurescens*	++	ACSP5	*S. pinnata*	PR	Y	3	4
ACR 144	*Arthrobacter* sp.	+	ACSP3	*S. pinnata*	PR	Y	4	4
ACR 161	*Bacillus simplex*	++	ACSP1	*S. pinnata*	PR	Y	4	4
ACR 162	*Escherichia hermanii*	+++	ACSP1	*S. pinnata*	PR	Y	4	4
ACR 163			ACSP1	*S. pinnata*	PR	Y	4	4
ACR 164	*Microbacterium* sp.	+	ACSP1	*S. pinnata*	PR	Y	3	4
ACR 50	*Bacillus simplex*	++	ACSE2	*S. ericoides*	PR	Y	2	4
ACR 51			ACSE2	*S. ericoides*	PR	Y	4	5
ACR 54	*Arthrobacter aurescens*	++	ACSE2	*S. ericoides*	PR	Y	2	4
ACR 59	*Pseudomonas thivervalensis*	++	ACSE1	*S. ericoides*	PR	Y	1	4

ACR 60			ACSE1	*S. ericoides*	PR	Y	3	4
ACR 62	*Arthrobacter aurescens*	++	ACSE1	*S. ericoides*	PR	Y	1	4
ACR 64			ACSE1	*S. ericoides*	PR	Y	3	4
ACR 98			ACSE16	*S. ericoides*	PR	Y	1	3
ACR 149	*Pseudomonas orientalis*	++	ACSE15	*S. ericoides*	PR	Y	1	4
ACR 150	*Arthrobacter aurescens*	++	ACSE15	*S. ericoides*	PR	Y	2	4
ACR 152	*Arthrobacter* sp.	+	ACSE15	*S. ericoides*	PR	Y	3	4
ACR 169	*Bacillus simplex*	++	ACSE14	*S. ericoides*	PR	Y	3	4
ACR 170	*Arthrobacter aurescens*	++	ACSE14	*S. ericoides*	PR	Y	3	4
ACR 172			ACSE14	*S. ericoides*	PR	Y	3	4
ACR 173			ACSE14	*S. ericoides*	PR	Y	1	4
ACR 174			ACSE14	*S. ericoides*	PR	Y	4	4

S4: Isolates used in bacterial consortiums that were used to inoculate *Brassica juncea* in inoculation experiment. Bacterial ID corresponds with the bacterial ID assigned in supplemental table S3. Under MALDI identification, ND signifies that the identification of the isolate was Not Determined.

Consortium	Host name	MALDI Identification	Bacterial ID
Cloudy Pass	*A. laxmanii*	*Bacillus atrophaeus*	12
	A. laxmanii	*Pseudomonas koreensis*	1
	A. laxmanii	*Microbacterium luteolum*	95
	A. laxmanii	*Arthrobacter sp.*	43
	A. laxmanii	*Stenotrophomonas sp*	92
	A. laxmanii	*Rhodococcus fascians*	35
	A. laxmanii	*Enterobacter cloacae*	97
	P. montana	*Bacilllus sp.*	24
	P. montana	*Pseudomonas koreensis*	14
	P. montana	*Bacillus cereus*	6
	P. montana	*Bacillus atrophaeus*	57
	S. ericoides	ND	75
	S. ericoides	*Pseudomonas frederiksbergensis*	76
	S. ericoides	*Stenotrophomonas sp.*	29
	S. ericoides	ND	74
	S. ericoides	ND	33
	S. ericoides	*Bacillus simplex*	32
Pine Ridge (non-HA)	*A. tenellus*	ND	191
	A. tenellus	*Bacillus sp.*	106
	A. tenellus	*Arthrobacter aurescens*	194
	A. tenellus	*Arthrobacter aurescens*	193
	A. tenellus	ND	112
	A. tenellus	*Sphingobacterium sp.*	114
	P. bellii	*Arthrobacter polychromogenes*	126
	P. bellii	*Bacillus mycoides*	125
	P. bellii	ND	129
	P. bellii	ND	127
	P. bellii	*Variovorax sp.*	58

	P. bellii	*Pseudomonas corrugata*	135
	P. bellii	ND	134
	S. ericoides	*Pseudomonas thivervalensis*	59
	S. ericoides	*Arthrobacter aurescens*	62
	S. ericoides	ND	98
	S. ericoides	*Bacillus simplex*	169
Pine Ridge (HA)	*A. bisulcatus*	ND	117
	A. bisulcatus	*Arthrobacter aurescens*	177
	A. bisulcatus	ND	119
	A. bisulcatus	*Stenotrophomonas rhizophila*	124
	A. bisulcatus	*Ochrobactrum grignonense*	120
	A. bisulcatus	*Stenotrophomonas sp*	123
	S. pinnata	*Bacillus sp.*	86
	S. pinnata	ND	87
	S. pinnata	*Arthrobacter sp.*	144
	S. pinnata	ND	100
	S. pinnata	*Arthrobacter aurescens*	101
	S. ericoides	*Pseudomonas orientalis*	149
	S. ericoides	*Bacillus simplex*	50
	S. ericoides	*Arthrobacter aurescens*	54



S5: *Brassica juncea* total (shoot + root) dry weight after treatment with or without 20 μM Na_2SeO_4 and inoculation with rhizobacteria consortia from different sites (CP or PR) and types of host plants (HA or non-HA). Shown values represent means and standard errors. Asterisks denote significant differences between +Se and –Se treatments ($p \leq 0.05$); other results from Anova and Tukey Kramer tests are mentioned in the text. **A**: Control (no inoculation). **B**: Plants treated with a consortium of rhizobacteria from non-hyperaccumulators from Cloudy Pass. **C**: Plants treated with a consortium of rhizobacteria taken from Se hyperaccumulators from Pine Ridge. **D**: Plants treated with a consortium of rhizobacteria taken from non-hyperaccumulators from Pine Ridge.