Ecological and Genetic Investigations of the Nickel Hyperaccumulator *Streptanthus polygaloides* (Brassicaceae)

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 4, 2018

Key words: nickel hyperaccumulation, *Streptanthus polygaloides*, herbivory, root foraging, phylogenetics

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Abstract

Hyperaccumulators are plants that take up, transport, and sequester large amounts of an element in their tissues. By doing this, they often achieve tissue concentrations that are toxic to most organisms. In this dissertation, I combined DNA sequence analyses with ecological studies of allocation strategies and herbivory to broaden our knowledge of the Ni hyperaccumulator *Streptanthus polygaloides* Gray, a species restricted to California serpentine soils. First, I assembled and annotated complete chloroplast genome sequences for all four morphs of *S. polygaloides.* Phylogenetic analyses strongly supported clades aligning with geographic collection localities. Second, I evaluated if seeds of the Ni hyperaccumulator, and a congeneric non-hyperaccumulator species also found on California serpentine soils (*Streptanthus insignis* Jepson), are resistant to predation by a generalist granivore, *Tribolium confusum* Jaquelin du Val. In addition, I investigated Ni toxicity to the granivore using an artificial diet study. I determined that Ni in *S. polygaloides* seeds can, due to its toxicity, act as an elemental defense against seed herbivores even at 300 µg Ni g^{-1}, a level below the 1000 µg Ni g^{-1} hyperaccumulation threshold concentration. In contrast, seeds of the congeneric non-hyperaccumulator species (*S. insignis*) contained little Ni (~5 µg Ni g^{-1}) and their consumption did not affect *T. confusum*. Third, I evaluated if Ni hyperaccumulation is associated with herbivory tolerance by applying levels of artificial herbivory to *S. polygaloides* and *S. insignis* plants grown in either control or Ni-amended soil. Plants of *S. polygaloides* receiving greater damage produced significantly more flowers, and had greater biomass, when grown in Ni-amended soil than plants in unamended soil, but this effect did not occur with the non-hyperaccumulator *S. insignis*. I conclude that *S. polygaloides* better tolerates herbivory when hyperaccumulating Ni, thus increasing plant fitness when herbivory damage is severe. Lastly, I compared root proliferation of *S. polygaloides* and *S. insignis* in control and Ni-amended soil. Root proliferation by *S. polygaloides* was two-fold greater in Ni-amended soil, but *S. insignis* demonstrated no differential response to soil Ni. Directional root growth to Ni also was measured in seedlings of the two species germinated on agar medium. Roots of *S. polygaloides* tended to grow toward a Ni source (the source created a Ni concentration gradient in the agar), produced more lateral roots in the direction of the Ni source, and lateral roots were longer the direction of the Ni source. In contrast, roots of *S. insignis* did not respond to the Ni concentration gradient. I concluded that *S. polygaloides* exhibited several positive root foraging responses to Ni (biomass, directional growth, elongation, and lateral root initiation) that may enhance its Ni uptake ability and suggest the term "nickelophilic root foraging" for this behavior. Overall, my research increases our knowledge of this Ni hyperaccumulator species and expands our understanding of the potential functions of hyperaccumulation in plants.

Acknowledgments

I would like to thank my graduate advisor and committee chair, Dr. Robert S. Boyd for his endless support and encouragement throughout my graduate career. Without his mentorship, this research would not have been possible. I also thank Dr. Roland Dute, Dr. Paul Cobine, and Dr. Les Goertzen for serving on my committee and providing guidance when needed. I thank Dr. Aaron Rashotte for the use of his lab space and materials and Jane Farr for much needed help and guidance in the Auburn University Plant Science Center Greenhouse. I thank Jess Choi for endless hours of work on several chapters of the following dissertation. She has been invaluable in so many ways. I would like to thank my lab mates, undergrads, and other graduate students who have helped with research, provided support and encouraging words, and allowed me to bounce ideas off them. I thank Nicole Agostino and my Yogafly family for their support and for providing a space where I always felt safe to be myself, could let out my emotions, whatever they may be, and could de-stress through yoga (both by teaching and being a student). Last but not least, I sincerely thank my parents, Pam and Dennis Mincey, for their love, support, encouragement, and belief in me, even when I could not see the light at the end of the tunnel.

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CHAPTER I: INTRODUCTION

Serpentine: Ultramafic rocks are made of at least 70% mafic minerals, such as manganese and iron, and are found in the Earth's mantle (Kruckeberg 2002; Brady et al. 2005). Serpentine rock (serpentinite) is formed by the metamorphosis and hydration of ultramafic rock, often peridotite, which is rich in olivine (Walker 1954). Serpentine rocks often have a mottled appearance and range in color from shiny green to bluish/black (Walker 1954; Brady et al. 2005). Serpentine soils are formed by weathering of these rocks. Areas of serpentine soil are widespread but are often patchily distributed and are in many cases defined by the unique plant communities that can be found there (Brady et al. 2005).

Serpentine soils, and the plant species adapted to life in these soils, are growing topics of interest and research. Soil is a major factor determining the relative distribution and abundance of plant species within plant communities and is a major defining characteristic of the plant communities themselves (Brady et al. 2005). Serpentine soils differ from non-serpentine soils due to their chemical composition (Brady et al. 2005), as well as their barren landscapes (Cacho and Strauss 2014), making them unfavorable for plant establishment and growth. They are characterized by low calcium:magnesium ratios, as well as by deficiency in essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K) (Walker 1954; Brooks et al. 1979; Cacho and Strauss 2014). They commonly have elevated levels of heavy metals, such as cobalt (Co), chromium (Cr), iron (Fe), or nickel (Ni), which are toxic to most plants (Walker 1954; Brooks et al. 1979; Cacho and Strauss 2014).

Serpentine soils vary among sites and even within sites; however, serpentine areas are often steep, open landscapes with rocky substrates (Rajakaruna and Boyd 2014). Serpentine outcrops often are characterized by shallow soil resulting in a reduced capacity to retain moisture

(Brady et al. 2005). Steep rocky slopes and sparse plant cover in these areas also encourage continual erosion and water loss (Kruckeberg 1954; Kruckeberg 2002); therefore, plants inhabiting serpentine areas in temperate zones must often tolerate drought in addition to depressed nutrient levels and the harsh soil chemical conditions (Walker 1954).

Although there is variation among sites, Whittaker (1954) identified three traits that characterize the vegetation of most serpentine areas: poor plant productivity, high rates of endemism, and vegetation types distinct from those of neighboring areas. Plant species adapted to serpentine soils often possess morphological features that are distinct from closely related species found on non-serpentine sites (Brady et al. 2005). Features characteristic of serpentine-tolerant species include reduced leaf size, smaller stature when compared to non-serpentine plants, and greater root:shoot ratio when growing on serpentine soils than on neighboring soils (Brady et al. 2005). Serpentine-tolerant species seem to be limited to serpentine soils due to their inability to compete in non-serpentine environments (Kruckeberg 1954; Anacker 2014): thus, these species are often endemic to serpentine regions (Brady et al. 2005). Experiments by Kruckeberg (1951), Anacker and Harrison (2012), and others, indicate that serpentine endemics are typically slow-growing and stress-tolerant species, rather than being fast-growing competitive species. Serpentine soils contribute important selective factors in plant evolution and adaptation, speciation, and plant restriction, all of which are important to the study of serpentine endemism (Anacker 2014).

Serpentine soils are an ideal system for studying plant adaptation, speciation, evolutionary ecology, and conservation (Kruckeberg 1954; Brady et al. 2005; Harrison and Rajakaruna 2011; Anacker 2014). Due to selective pressure from such stressful natural conditions, serpentine soil is a selective factor that promotes speciation and the evolution of serpentine endemism (Anacker 2014; Rajakaruna and Boyd 2014). Adaptations to bare habitats may also enable adaptation to harsh serpentine soils (Cacho and Strauss 2014). Geographic isolation likely plays a role in the speciation of serpentine endemics (Anacker 2014), as serpentine areas often occur as patches in a landscape and may be isolated from the nearest neighboring patch by many kilometers. Isolation may prevent transfer of genes as well as limit the area in which a species may spread. Serpentine endemism has evolved independently many times and has led to both isolation and diversification of many species (Safford et al. 2005).

Hyperaccumulation: Metallophytes can be defined by their ability to survive on soils, such as serpentine soils, that have naturally high levels of heavy metals (van der Ent et al. 2013). They may also be poor competitors on non-metalliferous soils due to their slow-growing nature (Kruckeberg 1954; Anacker and Harrison 2012) or by being relatively vulnerable to herbivores and pathogens when growing on non-metalliferous soils (Martens and Boyd 1994). The term ‘hyperaccumulator’ describes plants that take up, transport, and sequester large amounts of an element in their tissues (Brooks et al. 1979; Pollard and Baker 1996). In doing this, hyperaccumulators can achieve tissue concentrations that are toxic to most organisms (van der Ent et al. 2013). Hyperaccumulation is a trait present in over 500 species of plants (van der Ent et al. 2013). Most hyperaccumulators, approximately 75%, accumulate Ni, and hyperaccumulators have been reported from every continent except Antarctica (Reeves and Baker 2000). The threshold for the definition of hyperaccumulation varies depending on the element (Reeves and Baker 2000). A Ni hyperaccumulator has been defined as a plant with a Ni concentration of at least 1000 µg g^{-1} recorded in the dry matter of leaves when growing in its natural habitat (van der Ent et al. 2013). This follows the general hyperaccumulation threshold criterion that a hyperaccumulating species for a particular element must have a concentration at least two-to-three orders of magnitude greater than foliar concentrations of the same species on non-metalliferous soils, and at least one order of magnitude greater than the usual range in leaves on metalliferous soils (van der Ent et al. 2013).

Identification of the mechanisms by which hyperaccumulators take up elements is important to understanding this trait. Three common features distinguish hyperaccumulators from related non-hyperaccumulating taxa: 1) greater ability to take up elements from the soil; 2) faster and more effective root-to-shoot translocation of elements; and 3) greater ability to detoxify and sequester large amounts of elements in leaf tissue (Rascio and Navari-Izzo 2011). A number of mechanisms or properties of these plants can enhance elemental uptake. These include dense absorption sites on root membranes (Lasat et al. 2000), enhanced internal metal transport (Pence et al. 2000), release of phytometallophores (Hutchinson et al. 2000; Whiting et al. 2000), and localized root proliferation (Schwartz et al. 1999; Whiting et al. 2000; Haines 2002). According to Rascio and Navari-Izzo (2011), hyperaccumulators also rapidly translocate elements to shoots via the xylem, rather than retaining them in root cells like non-hyperaccumulator plants. They rely on enhanced xylem loading by a constitutive overexpression of genes coding for transport systems (Rascio and Navari-Izzo 2011). A class of proteins, P_{1B}-type ATPases (also known as HMAs: Heavy Metal transporting ATPases), operates in heavy metal transport and expression is up-regulated when these plants are exposed to high levels of particular metals (Mils et al. 2003; Papoyan and Kochian 2004). Detoxification and sequestration allow hyperaccumulators to concentrate large amounts of metals in aboveground tissues, particularly leaves, without toxicity. Tolerance mechanisms consist of detoxification by ligands or the removal of heavy metals from metabolically active cytoplasm to inactive compartments of cells, such as cell walls or vacuoles (Rascio and Navari-Izzo 2011).

Essential resources for plant growth are often heterogeneously distributed both above- and belowground, and plants forage for these patchily distributed resources by positioning leaves and roots in areas of greater resource availability (Hutchings and de Kroon 1994). Foraging responses, such as root proliferation in response to soil nutrients and water, have been demonstrated in many plant species and can affect plant performance (Robinson 1994; Hutchinson et al. 2000). The patchy distribution of soil resources can alter resource acquisition and allocation patterns, potentially changing total plant biomass (Hutchinson et al. 2000; Haines 2002).

Metals or other elements found in natural or contaminated soils are typically heterogeneously distributed as well, suggesting that root foraging in substrates with high elemental concentration may benefit hyperaccumulator plants by enhancing accumulation (Haines 2002). Haines (2002) proposed the idea of “zincophilic root foraging,” finding that, through preferential root placement and proliferation in response to Zn-rich patches, Zn uptake was enhanced in a hyperaccumulator species. Localized root proliferation has been observed in several studies using the Zn hyperaccumulator *Thlaspi caerulescens* J. and C. Presl. (Schwartz et al. 1999; Whiting et al. 2000). Positive root growth responses towards patches of Zn-rich soil included increased root branching and length, increased root hair length, and overall increased total root biomass. El Mehdawi et al. (2015) compared populations of the Se hyperaccumulator *Symphyotrichum ericoides* (L.) G.L. Nesom from both seleniferous and non-seleniferous soil to determine root response to Se. They determined that roots proliferated more in Se-amended soil and that the roots and shoots of plants from the seleniferous population had a much larger biomass in Se-amended soil. In terms of seedling growth, the authors reported that roots of the seleniferous population grew towards a central Se source, while roots of the non-seleniferous population showed no growth response to Se.

Herbivore defense: resistance vs. tolerance: Major questions regarding hyperaccumulator plants from serpentine soils are: 1) how did plants evolve hyperaccumulation of heavy metals; and 2) what are the benefits of metal hyperaccumulation? A variety of hypotheses have been proposed to explain the elevated elemental composition of metal hyperaccumulators (Boyd and Martens 1992): metal tolerance/disposal, drought resistance, interference with neighboring plants, inadvertent uptake, and defense against enemies. According to the tolerance/disposal hypothesis, hyperaccumulation allows plants to take heavy metals away from the roots by sequestering them in tolerant leaf tissues that they could eventually lose by shedding. The drought resistance hypothesis suggests that large amounts of heavy metals might increase the ability of hyperaccumulators (perhaps through osmoregulation) to survive in an arid environment. Serpentine soils are often considered to generate drought stress in plants due to their shallow, rocky soil and more open vegetation structure. The interference hypothesis, or "elemental allelopathy," suggests that hyperaccumulator plants interfere with neighboring plants by increasing metal concentration in the surface soil around them. This might avert establishment of less metal-tolerant species. The inadvertent uptake hypothesis suggests that hyperaccumulation might be an incidental consequence of enhanced nutrient uptake capabilities. This hypothesis suggests that hyperaccumulation of metals is not an adaptive feature, but a consequence of a nutrient-scavenging adaptation that enhances uptake of a hyperaccumulated element along with that of a nutrient. The explanation that has received the most attention, known as the "defense hypothesis" by Boyd and Martens (1992), suggests that the elevated concentration of an element in plant tissues is a defense against at least some enemies (herbivores/pathogens).

The most investigated herbivore defense strategy for the hyperaccumulation trait is resistance. The defense hypothesis suggests that hyperaccumulation is a resistance trait that can decrease the amount of damage inflicted on a hyperaccumulator by herbivores and pathogens (Boyd and Martens 1998; Davis et al. 2001). This can be achieved in two ways: 1) by toxic effects to an herbivore after consumption of plant material (Boyd et al. 2002) or, 2) by deterrence, which reduces consumption or prevents it altogether (Pollard and Baker 1996; Boyd 2007). Previous research suggests that hyperaccumulating plants may use toxicity, partial deterrence, or both, as defenses against herbivores (Boyd 2007). The Zn hyperaccumulator, *T. caerulescens*, has been shown to deter herbivores when grown in Zn-amended soil (Pollard and Baker 1996). Other experiments have shown that herbivores prefer to feed on plants with lower elemental concentrations when given a choice between plants with high and low elemental concentrations (e.g. Boyd and Martens 1994; Hanson et al. 2003; Coleman et al. 2005).

A second defense strategy that is much less explored is tolerance of herbivory. Tolerance has been defined as the capacity of plants to reduce the negative effects of damage on fitness and involves a plant's ability to continue to grow and reproduce after herbivory has occurred (Stowe et al. 2000; Palomino et al. 2007). For example, a plant may respond to herbivory damage by compensatory growth: growth that replaces damaged leaves, shoots, or roots and thereby allows a plant to be unaffected by herbivory (Fornoni 2011). To my knowledge, there is only one prior investigation of the role of hyperaccumulation in herbivory tolerance. Palomino et al. (2007) examined the effects of artificial damage and soil Ni concentration on the Ni hyperaccumulator *Noccaea fendleri* (A. Gray) Holub subsp. *glauca* (A. Nelson) Al-Shehbaz and M. Koch (formerly known as *Thlaspi montanum* L. var. *montanum*). Treatments included two levels of soil Ni concentrations (Ni-amended and unamended) and three levels of artificial damage (0, 10, and 50% leaf removal). To quantify the effects of each treatment on plant growth after 7 months, stem diameter at the base of the rosette was measured. Palomino et al. (2007) observed a significant interaction between soil Ni and artificial herbivory. At the most intense levels of damage, the size of plants growing on Ni-amended soil (and therefore hyperaccumulating Ni) was not significantly different from undamaged plants, but this tolerance to damage did not occur when plants were grown on unamended soil. While this study was limited to a measure of plant size, it does suggest that there is a potential for these plants to respond positively to intense herbivory. A direct measurement of overall plant fitness (flower or seed production) would better demonstrate a plant's capability to tolerate herbivory.

Although tolerance and resistance are different plant defense strategies, there are several hypotheses as to how they might have co-evolved. Some evidence suggests that individual plants in a population allocate resources simultaneously to both strategies, therefore exhibiting a mixed pattern of defense (Fineblum and Rausher 1995; Mauricio et al. 1997; Núñez-Farfán et al. 2007). According to Strauss and Agrawal (1999), plant resistance and plant tolerance, as alternative but not mutually exclusive defenses to herbivory, may result in differing dynamics between plant and herbivore populations. Tolerance does not impose selection on herbivores but allows a plant to compensate for damage. However, plant resistance may lead to counter-defenses by herbivores, triggering a "coevolutionary arms race." More research is needed to understand the selective forces and evolutionary processes acting upon the two defensive strategies, particularly in the case of hyperaccumulators.

Hyperaccumulation as a model system for herbivore studies: Hyperaccumulating plants are a model system for experiments studying the interactions between plants and herbivores because the experimenter has relatively direct control of phenotypic expression of defensive chemicals in a plant. In order to create “defended” and “undefended” plants, one may manipulate the concentration of metals in leaves and shoot tissues by controlling metal availability in the growth medium (Pollard 2000). For example, Martens and Boyd (1994) generated high- and low-Ni leaves of *Streptanthus polygaloides* by growing plants on soil amended with high and low concentrations of Ni. This allowed them to use the same species for both treatments of leaves given to insect herbivores during a feeding experiment. Herbivores fed Ni-amended leaves showed poor survival and only those herbivores fed unamended leaves gained weight throughout the experimental period. With the advantages offered by the ability to manipulate defensive chemical concentrations in a plant, such studies may provide insight into interactions between hyperaccumulators and their herbivores. One weakness of this approach, however, lies in the assumption that a change in foliar metal concentration is the only change resulting from soil treatments. Martens and Boyd (1994) analyzed plants used in their experiment to determine if Ni was the only differing parameter. They found other elemental differences in the plants and required another experiment to demonstrate that Ni toxicity was responsible for the lethal response observed.

Another approach to examine herbivore response to Ni is an artificial diet study. For example, Goncalves et al. (2007) conducted no-choice and choice feeding trials with the herbivore *Tribolium castaneum* using synthetic diet disks containing various Ni concentrations. As Ni concentration increased in the no-choice experiments, *T. castaneum* fed significantly less and rejected those disks with the highest concentrations. In the choice trials, the herbivores preferred to eat those disks containing less Ni. Use of artificial diet allows control of other variables that are present when working with whole plants. However, differences between artificial diet and plant material may affect feeding preferences of the herbivores. Although results of an artificial diet study may provide useful evidence, it is important that the work be transferable to studies using whole plants.

Streptanthus: Streptanthus Nutt. is a genus of approximately 35 species in the family Brassicaceae, tribe Thelypodieae (Cacho et al. 2014). Its unique flowers are strongly zygomorphic, with an urceolate calyx, and vary widely in color (Al-Shehbaz 2010), differing from the typical yellow cruciform actinomorphic flowers of other Brassicaceae. *Streptanthus* is often associated with serpentine soil: about 30% of the species are restricted to serpentine and thus are serpentine endemics (Al-Shehbaz 2010; Cacho et al. 2014). A few species, known as serpentine tolerators (Kruckeberg 2006; Cacho et al. 2014), grow on both serpentine and non-serpentine soils. Variation in edaphic adaptations in *Streptanthus* makes it an ideal group in which to study serpentine specialization. Until the recent work of Cacho et al. (2014), there was a lack of phylogenetic resolution within this genus.

Streptanthus polygaloides Gray (Milkwort Jewelflower: Brassicaceae) is an annual Ni hyperaccumulator. While Kruckeberg (1984) stated that almost all *Streptanthus* taxa are endemic to, or tolerant of, serpentine soil, *S. polygaloides* is the only member of the genus that hyperaccumulates Ni (Reeves et al. 1981; Kruckeberg and Reeves 1995). It is also a serpentine endemic, is one of the few hyperaccumulators found growing on the serpentine soils of continental North America (Boyd et al. 2009), and only grows along the western side of the Californian Sierra Nevada (Reeves et al. 1981; Baldwin et al. 2012). *Streptanthus polygaloides* is capable of accumulating Ni in leaves at concentrations as high as 14,800 µg g^{-1} dry mass, but Ni can also be found in flowers, roots, and seeds. Nickel concentrations in all parts of the plant exceed 1000 µg g^{-1} dry mass, the hyperaccumulation threshold for Ni (Reeves et al. 1981; Sánchez-Mata et al. 2013a).

Streptanthus polygaloides consists of four morphs that vary in sepal color, leaf morphology, height, and geographic location (Wall and Boyd 2006; Boyd et al. 2009). Two of the morphs range from northern California to central California (~39°N to ~37.5°N); while the other two have restricted distributions. The most common and widespread is the yellow sepal morph (Y), ranging from serpentine sites in Butte County in the north to Mariposa County in the south. The purple sepal morph (P) is also widespread, from Sierra County to Placer County, and generally grows at higher elevation sites in the northern range of *S. polygaloides*. One geographically isolated morph, found approximately 100 km southeast of all other populations, has pale yellow undulate sepals (U). The other geographically limited morph, the yellow-to-purple morph (Y/P) is unusual in that the flower buds are yellow, but the sepals change to purple during maturation of the inflorescence. Sánchez-Mata et al. (2013b) proposed that the purple morph be recognized as a species, *Streptanthus purpureus*, however this is only based on some morphological characteristics of the plant and pollen grains and lacks data from hybridization or genetic studies.

A study involving a common garden experiment (Boyd et al. 2009) found significant morphological variation (plant size, leaf length and width, degree of lobing) and elemental concentration within the four morphs of *S. polygaloides*. Plants of U and Y/P morphs were the tallest plants with the longest leaves but differed in the lobing of their leaves. Plants of the Y morph were intermediate in both plant height and leaf length, while P morph plants were the shortest of the morphs with the shortest leaves. The P morph differed most greatly in its elemental concentrations, having more Ni and Mg, and less P and Mn, than other morphs. The differences found by Boyd et al. (2009) suggest that these morphs differ physiologically from each other in similar growing conditions. The four morphs may be genetically distinct and deserve taxonomic recognition, as well as conservation management, especially for the Y/P and U morphs due to their restricted distributions.

Pope et al. (2013) investigated the role of both elevation and soil chemistry on the distribution and elemental accumulation of the four morphs of *S. polygaloides*. They found that the Y and P morphs, the most widespread, tend to separate along a climatic gradient. The P morph occurred at colder temperatures with more rainfall than did the Y morph. They did, however, find few differences in the foliar element concentrations of plants collected in the wild. This contrasts with the findings of Boyd et al. (2009) and could be a result of greenhouse-grown plants differing from wild populations due to the variation in soil chemistry within and between population sites.

Streptanthus insignis subsp. *insignis* Jepson (San Benito Jewelflower: Brassicaceae) is an annual herb endemic to California and also grows on open serpentine slopes (Baldwin et al. 2012). It is found growing in the Inner Central Coast Ranges (Baldwin et al. 2012). Flowers have purple urceolate sepals with whitish tips. *Streptanthus insignis,* like all members of the genus except *S. polygaloides,* does not hyperaccumulate Ni (Reeves et al. 1981).

My research, combining DNA sequence analyses with ecological studies of allocation strategies and herbivory, allows for a broad overview of the Ni hyperaccumulator *S. polygaloides.* This research will further knowledge of this species through use of both greenhouse and lab studies as well as high-throughput next-generation sequencing of DNA.

Streptanthus polygaloides, and the comparative non-hyperaccumulator species *S. insignis,* are annual plants, making them unusual because many Ni hyperaccumulators are perennial species (Reeves and Baker 2000). They are also useful for research because the short life span of an annual (and their smaller size at maturity) allows for study of more individuals and experimental replicates in less time. Specifically, the four objectives of this research are:

1) To investigate phylogenetic relationships of the four morphs of *S. polygaloides*;
2) To determine if seeds of *S. polygaloides* and *S. insignis* are resistant to predation by a seed predator (*Tribolium* beetles);
3) To consider the role of tolerance to damage in Ni hyperaccumulators by determining the fitness of *S. polygaloides* when subjected to artificial herbivory; and
4) To compare the foraging responses of roots of *S. polygaloides* and *S. insignis* by quantifying root proliferation and directional growth responses in unamended and Ni-amended media.

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CHAPTER II: INTRASPECIFIC PLASTID GENOME VARIATION AND PHYLOGENETIC STRUCTURE WITHIN THE NICKEL HYPERACCUMULATOR *STREPTANTHUS POLYGALOIDES* (BRASSICACEAE)

Abstract - *Streptanthus polygaloides* (Brassicaceae) is an annual nickel hyperaccumulator endemic to serpentine soils along the western side of the Californian Sierra Nevada. These serpentine areas are often open, steep habitats that are fragmented along the landscape and may be isolated from the nearest patch by many kilometers. Four morphologically diverse morphs of *S. polygaloides* have been described that vary in sepal color, leaf morphology, and height, as well as geographic location. The morphs range from being widespread in the northern and central parts of the Sierra Nevada to being geographically isolated from all other morphs by approximately 100 km. To examine whether the four morphs are genetically different, we examined complete plastid genome sequences that were assembled from high-throughput sequencing data from individuals of eight populations. Plastid genome sequences were assembled in an iterative fashion with contigs assembly confirmed by mapping original reads back at intermediate stages of assembly. All eight full-length plastid genome sequences were annotated and aligned with various outgroup Brassicaceae. Maximum likelihood phylogenetic reconstructions were then performed across individuals utilizing partitioning for substitution rate heterogeneity. Phylogenetic analyses strongly supported clades aligning with geographic collection localities. Numerous morph-specific plastid SNPs were identified that can be tested more widely across the *S. polygaloides* range with additional sampling.

INTRODUCTION

In the past decade, many advances have been made in determining the evolutionary relationships of the main lineages of Brassicaceae (Al-Shehbaz et al. 2006; Cacho et al. 2014). However, even with some of the best-studied model systems and a complete, annotated *Arabidopsis thaliana* (L.) Heynh. genome, phylogenetic relationships within some of the tribes of Brassicaceae remain vague. The Thelypodieae tribe within Brassicaceae, a group exhibiting many unusual floral adaptations, remains among the most difficult tribes to resolve in the context of phylogeny (Warwick et al. 2009).

Streptanthus Nutt. is a genus within Thelypodieae composed of approximately 35 species (Cacho et al. 2014). Its unique flowers are strongly zygomorphic with an urceolate calyx and vary widely in color (Al-Shehbaz 2010) – unlike the typical yellow cruciform actinomorphic flowers of other Brassicaceae. *Streptanthus* is often associated with serpentine soils and about 30% of the species are restricted to serpentine and thus are serpentine endemics (Al-Shehbaz 2010; Cacho et al. 2014). A few species, known as serpentine tolerators (Kruckeberg 2006; Cacho et al. 2014), grow on both serpentine and non-serpentine soils. Additionally, *S. polygaloides* Gray (Milkwort Jewelflower) is an annual nickel (Ni) hyperaccumulator, and while Kruckeberg (1984) stated that almost all *Streptanthus* taxa are endemic to or tolerant of serpentine soil, *S. polygaloides* is the only member of the genus that hyperaccumulates Ni (Reeves et al. 1981; Kruckeberg and Reeves 1995). This serpentine endemic is one of the few hyperaccumulators found growing on the serpentine soils of continental North America (Boyd et al. 2009) and only grows along the western side of the Californian Sierra Nevada (Reeves et al. 1981; Baldwin et al. 2012). It is capable of accumulating Ni in leaves at concentrations as high as 14,800 µg Ni g^{-1} dry mass, but Ni can also be found in flowers, roots, and seeds at concentrations all greater than the hyperaccumulation threshold for Ni (1000 µg Ni g^{-1}) (Reeves et al. 1981; Sánchez-Mata et al. 2013a).

Streptanthus polygaloides consists of four morphs that vary in sepal color, leaf morphology, height, and geographic location (Wall and Boyd 2006; Boyd et al. 2009). The morphs are partitioned based on field observations that primarily focus on sepal color, although the morphs also differ in geographic location (Wall and Boyd 2006; Boyd et al. 2009). The most common and widespread morph is the yellow sepal morph (denoted as Y). The purple sepal morph (P) is also widespread, although its distribution is restricted to relatively higher elevation sites in the northern range of *S. polygaloides*. These two morphs range from northern California to central California (~39°N to ~37.5°N). The other two morphs have more restricted distributions (Boyd et al. 2009). Sepals of the yellow-to-purple morph (Y/P) are unusual in that flower buds are yellow but the sepals change to purple during maturation of the inflorescence. The Y/P morph has been documented in only a few locations scattered throughout the range of *S. polygaloides*. The other geographically isolated morph, approximately 100 km southeast of all other populations, has pale yellow undulate sepals (U). Sánchez-Mata et al. (2013b) have proposed a name for the purple morph, *Streptanthus purpureus*, however this is exclusively based on plant and pollen grain morphology and thus lacks genetic data for phylogenetic support.

There is a complex evolutionary history behind the genus *Streptanthus* and its allied genera (e.g., *Caulanthus*, *Thelypodium*, *Stanleya*, etc.) in the tribe Thelypodieae. Many recognized the tribe to be monophyletic (e.g., Al-Shehbaz et al. 2006; Warwick et al. 2009; Burrell et al. 2011) which led to the naming of the ‘Streptanthoid Complex’ based on affinity with the genus *Streptanthus* and a largely conserved chromosome number of n = 14. However, prior to Cacho et al. (2014) there was a lack of phylogenetic resolution within this genus, thus inhibiting comparative studies in the context of evolution within the genus. Cacho et al. (2014) suggested that *Streptanthus*, *Caulanthus*, and *Thelypodium* are not monophyletic as previously described, but instead are split into two strongly supported clades. They determined an overall correspondence of phylogeny and geography and suggested that both serpentine endemism and tolerance played an important role in shaping the diversity of the clades.

In this study, we reconstructed the complete sequence of the chloroplast genome for each morph collected across localities in California's Sierra Nevada and placed these into a phylogenetic context to address the evolutionary relationships among morphs. Together, our data show a close correspondence between phylogenetic relationship and geographic location, rather than flower morphology, among the collected specimens.

METHODS

Plant upkeep. Plants grown for this analysis were collected as seeds between the summers of 2010 and 2013 from collection locales listed in Table 1. Seeds were germinated in 10 cm square pots in the Auburn University Plant Science Research Center greenhouse in the spring of 2014. Plants were grown in commercial potting soil (Sunshine #8 by Sungro, Agawam, MA) amended with Ni (3.2g $NiCl_2$ added to 7L soil to achieve concentration of ~800 µg Ni g^{-1}) and maintained in the greenhouse under ambient light conditions at an average temperature of 23°C, with a relative humidity of 74%. Plants were watered twice daily and Peter's Peat-lite 20-10-20 fertilizer was added once a week. DNA was extracted from mature leaf tissue of eight plants using a DNeasy Plant mini kit (QIAGEN, Hilden, Germany). Each plant represented a population and at least one population per morph was used (Table 1). Whole genomic DNA was extracted using a modified CTAB protocol (Appendix 1).

Sequencing, assembly, and annotation. Whole genomic extractions were sequenced as 100bp paired-end reads via an Illumina HiSeq platform using a genome skimming approach (Straub et al. 2012). Sequencing was performed by Hudson Alpha Genomics (Huntsville, AL). The FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) was used to assess read quality prior to assembly. Ray v.2.3.1 (Boisvert et al. 2010) was used for *de novo* genome assembly. Ray parameters were default except for k-mer size which were as follows: 31-Bagby (Y), Marshall Rd (Y), Sugar Pine (P), Pine Flat (U); 26-Washington Rd (P); 21-Watts Valley (U), Coulterville (Y/P), SE Coulterville (Y). Chloroplast contigs were identified via BLASTn (Altschul et al. 1990) using *Arabidopsis thaliana* as the query (NC_000932.1). To assemble a primary reference *S. polygaloides* plastid genome, contigs from the Bagby population (Y) data set were assembled manually in SeaView v.4.5.4 (Gouy et al. 2010). To assess quality of assembly, reads were mapped to reference via Bowtie2 v.2.2.9 (Langmead and Salzberg 2012). Bowtie2 settings were as follows: qc-filter, no-unal, no-mixed. The reference sequence was then processed through an iterative mapping, consensus creation tool written in R script by Eric Archer to finalize the plastid genome.

Reads from all other individuals were mapped to the Bagby reference via Bowtie2 with the flags qc-filter, no-unal, and no-mixed, and a consensus of each was made using the Eric Archer R script. SNP-sites v.2.0.3 was used to identify morph- and geography-specific single-nucleotide polymorphisms (SNPs). Outgroups were removed from the nucleotide data matrix and all accessions of *S. polygaloides* were re-aligned using MAFFT. SNP-sites was run using default settings and the output vcf flag, and SNPs were extracted from the vcf file.

Annotation was performed using DOGMA (Wyman et al. 2004) (80% identity cutoff for protein coding genes and RNAa, E-value = $1e^{-5}$). Overlapping annotations with the same identity were consolidated. Short gaps separating tandem identical genes (e.g., 4 copies of *ycf1* separated by less than 50 nucleotides) were interpreted as introns.

Phylogenetics. Plastid genome sequences from members of the Brassicaceae (*Raphanus sativus* L. KJ716483.1, *Capsella bursa-pastoris* (L.) Medik. AP009371.1, *Brassica juncea* (L.) Czern. KT581449.1, *Lepidium virginicum* L. AP009374) were acquired from GenBank (Benson et al. 2005) and used as outgroups. MAFFT v.7.2.5 (Katoh et al. 2002) with default settings was used to align plastid genome sequences, manually trimmed of uninformative sites, and then partitioned using PartitionFinder v.2.0.0 (Lanfear et al. 2012). Maximum likelihood phylogenetic reconstruction was performed using RAxML v.7.0.4 (Stamatakis 2014) using GTR+Γ nucleotide substitution model and 1000 bootstraps.

RESULTS AND DISCUSSION

Basic assembly statistics for each individual plastid genome are available in Table 2. The chloroplast genomes exhibited similar structure and organization. Each was divided into four different regions, a pair of inverted repeated regions (IR_a and IR_b) separated by one large single copy region (LSC) and one small single copy region (SSC) (Figure 1). All individuals displayed the same gene content and order, with 129 genes (four ribosomal RNA genes, 29 tRNA genes and 79 protein-coding genes): 112 of these were unique, with 16 genes duplicated in the IR regions. The eight genomes have 13 genes containing introns (*rps16, atpF, rpoC1, ycf3, trnL-UAA, trnV-UAC, clpP, rp12, ndhB, trnI-GAU, trnA-UGC, ycf1, ndhA*), 10 with one intron, two with two introns, and one with three. For all individuals, the *ycf1* gene starts in the SSC region, but its sequence goes forward through the SSC/IR$_b$ boundary, causing a duplication of the 3’ end of the *ycf1* gene in IR$_a$ and, therefore, producing a *ycf1* pseudogene.

Maximum-likelihood phylogenetic reconstruction was performed on an aligned plastid genome matrix containing 156,889 sites. The yielded phylogeny supports three distinct monophyletic groups clustering according to their most proximal geographic neighbor (Figure 2). Specifically, the three clades include an undulate morph clade (i.e., the southern-most clade), a central clade, and a northern clade. Within their respective clades, sister taxa are best explained by geographic isolation as opposed to flower morphology. For example, the Marshall Road yellow morph specimen was found to be sister to the proximal purple morphs instead of the other, more geographically distant, yellow morphs (i.e., Bagby and SE Coulterville).

Our analyses identified unique SNPs exclusive to each geographic clade, relative to the Bagby specimen which served as a reference for this analysis. We were unable to identify any SNPs unique to yellow or yellow-purple morphs, likely due to their lack of geographic (and thus genetic) separation from other populations of the same morph. In the context of morphology, 25 purple-specific and 401 undulate-specific SNPs were identified. In the context of geography, the northern clade had 124 clade-specific SNPs, the central clade had 86 clade-specific SNPs, and the southern clade had 401 clade-specific SNPs.

Most striking of our reported SNP values per clade, the southern (undulate) group, possessed nearly four times the number of SNPs as the second-highest SNP value. This high value likely indicates geographic isolation has prevented gene flow between the undulate individuals and other morphs, ultimately promoting the accumulation of more mutations within the chloroplast genome relative to the other morphs. Other morphs tended to share SNPs within a geographic area, rather than within the individual morph. This suggests that gene flow has historically been less restricted among the other morphs.

A common garden study of the four morphs of *S. polygaloides* by Boyd et al. (2009) found distinctive morphological variation. The U and Y/P morphs included the tallest plants with the largest leaf size. The P morph plants were the shortest overall with the smallest leaf size, while the Y morph was intermediate in both plant height and leaf size. Given the phylogenetic clustering of a northern, higher-elevation, dominantly purple clade, a central "mixed" clade, and a southern undulate clade, the gradient of small-to-large phenotypes from northern-most to southern-most collection sites, respectively, may represent a gradient of hybridization between any given morph and its most proximal ally. However, experimental evidence on the hybridization potential between morphs, in either laboratory or field conditions, is absent (Boyd et al. 2009). This gradient between morphs is further substantiated by Boyd et al. (2009) whom also performed a principal component analysis which suggested that the morphological differences among the Y morph populations occur along a north/south gradient – a trend compatible with our plastid genome phylogeny. The differences found by Boyd et al. (2009) also suggest that these morphs differ physiologically from each other in similar growing conditions.

Pope et al. (2013) investigated the effect of both elevation and soil chemistry on the geographic distribution and elemental accumulations of the four morphs *in vivo*. Their findings showed large variations in soil and tissue elemental concentrations within each site and they suggest that a climatic gradient may be a driving factor for divergence in floral color among populations of *S. polygaloides.* Future investigation of the genetic isolation between these taxa, using increasingly sensitive molecular methods such as RAD sequencing (Davey and Blaxter 2010), will be invaluable for providing increased phylogenetic resolution among morphs and populations – ultimately informing the potential for taxonomic reconsideration and conservation management within the species *S. polygaloides*.

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TABLES

Table 1. *Streptanthus polygaloides* populations represented in the genome assembly

Population name	County	Latitude/Longitude	Year Collected
Yellow (Y) morph			
Bagby	Mariposa	37°36'48" N/120°08'22" W	2013
Marshall Road	El Dorado	38°50'34" N/120°52'42" W	2011
SE Coulterville	Mariposa	37°38'43" N/120°08'50" W	2013
Purple (P) morph			
Sugar Pine	Sutter	39°07'36" N/120°08'50" W	2011
Washington Road	Nevada	39°20'06" N/120°48'13" W	2011
Yellow/purple (Y/P) morph			
Coulterville	Mariposa	37°44'26" N/120°14'18" W	2010
Undulate (U) morph			
Pine Flat	Fresno	36°52'35" N/119°17'23" W	2010
Watts Valley	Fresno	36°54'56" N/119°25'07" W	2013

Table 2. Comparison of the complete chloroplast genomes among eight individuals of *S. polygaloides*

Population	**# of reads**	**Assembly length (bp)**	**GC (%)**
Bagby	7966556	150425	36.3
Marshall Road	4084529	150423	36.4
SE Coulterville	26247856	150423	36.4
Sugar Pine	21466049	150400	36.4
Washington Road	20073772	150389	36.4
Coulterville	24156382	150389	36.4
Pine Flat	24363727	150412	36.4
Watts Valley	9292084	150411	36.4

Population	**LSC (bp)**	**IR$_a$ (bp)**	**IR$_b$ (bp)**	**SSC (bp)**
Bagby	86196	23579	23631	17019
Marshall Road	86201	23583	23631	17008
SE Coulterville	86197	23579	23631	17016
Sugar Pine	86178	23586	23634	17002
Washington Road	86174	23586	23631	16998
Coulterville	86174	23580	23632	17003
Pine Flat	86195	23580	23628	17009
Watts Valley	86206	23577	23627	17000

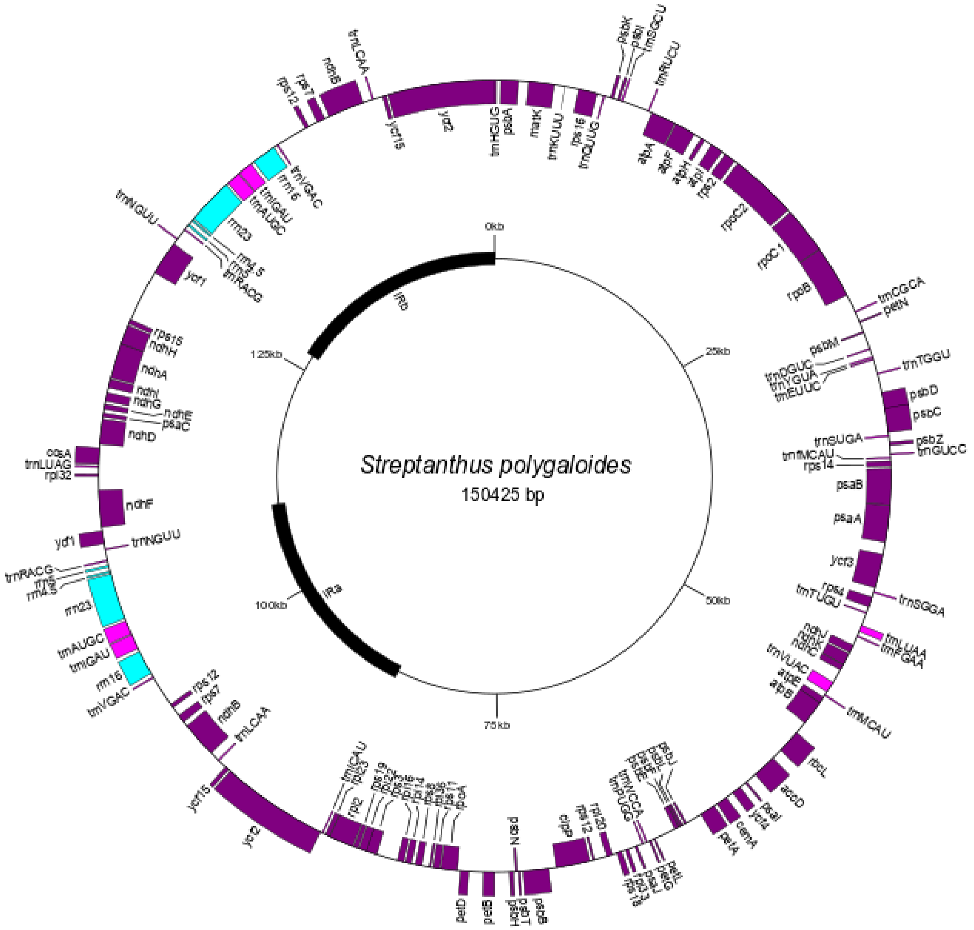

Figure 1. Chloroplast genome map of *S. polygaloides* plastid genome, from the Bagby population individual (as an example). Genes shown inside the circle are transcribed clockwise, and those outside the circle are transcribed counterclockwise. Purple gene diagrams represent protein coding genes, pink represents tRNA, and aqua (light blue) represents rRNA.

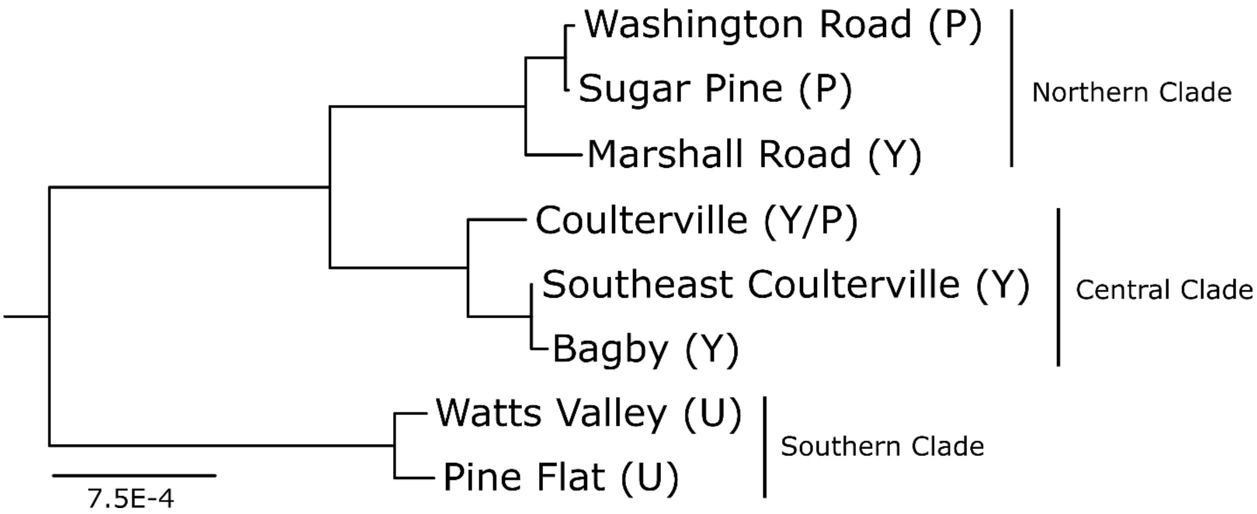

Figure 2. Phylogenetic reconstruction of eight individuals using maximum likelihood analysis based on chloroplast genome sequences. Bootstrap values are 100% at all internal nodes.

APPENDIX 1

Modified CTAB protocol used in extraction of *Streptanthus polygaloides* DNA.

· Fill 2 mL microcentrifuge tube ~1/2 full of leaf material
· Add 600 µL of CTAB
· Heat samples to 65°C in water bath to soften leaf tissue
· Crush using mini-pestle
· Heat at 65°C for ~ 20 minutes
· Centrifuge at 15,000 RPM for 5 minutes
· Transfer new supernatant to new 2 mL tube
· Add 600 µL chloroform and vortex for 15 seconds
· Centrifuge at 15,000 RPM for 10 minutes
· Remove aqueous layer and transfer to new tube
· Add 450 µL of isopropanol and mix
· Centrifuge 15,000 RPM for 15 minutes
· Decant liquid, leaving DNA pellet
· Wash with ~ 1 µL 70% ethanol
· Allow to dry and elute in 100 mL of sterile dH_2O

CHAPTER III: ELEMENTAL DEFENSE OF NICKEL HYPERACCUMULATOR SEEDS AGAINST A GENERALIST INSECT GRANIVORE

Abstract - Hyperaccumulation has been proposed as an elemental defense against herbivores; however, few studies have examined seed defense. This study included two annual *Streptanthus* species (Brassicaceae) from California serpentine soils: a non-hyperaccumulator (*S. insignis*) and three populations (representing Y, P, and U morphs) of a Ni hyperaccumulator (*S. polygaloides*). Adults of the generalist seed herbivore *Tribolium confusum* (Coleoptera: Tenebrionidae) were fed either whole or cut seeds, survival was recorded for seven weeks, and Ni concentrations of both beetles and seeds were determined using ICP-OES. Survival analysis showed significantly more rapid mortality for beetles consuming *S. polygaloides* seeds compared to those consuming *S. insignis*. Mortality of beetles fed whole *S. polygaloides* seeds was more rapid than those fed cut *S. polygaloides* seeds. Seeds of the *S. polygaloides* populations contained approximately 300 µg Ni g^{-1} whereas *S. insignis* contained approximately 5 µg Ni g^{-1}. Beetles fed whole *S. polygaloides* seeds contained more than 2.5-fold greater Ni concentrations than those fed cut seeds (approximately 60 and 25 µg Ni g^{-1}, respectively), whereas beetles fed either cut or whole *S. insignis* seeds contained < 0.3 µg Ni g^{-1}. An artificial diet study, using Ni-amended cornmeal, confirmed that diet Ni concentrations greater than 240 µg Ni g^{-1} were toxic to *T. confusum*. We conclude that Ni in *S. polygaloides* seeds can act as an elemental defense against seed herbivores even at 300 µg Ni g^{-1}, a level below the 1000 µg Ni g^{-1} hyperaccumulation threshold concentration.

INTRODUCTION

Plants respond to herbivory through an intricate and dynamic defense system. These defenses include various morphological, biochemical, and molecular mechanisms to counter and/or offset herbivore attack (War et al. 2012). Herbivores play an important role in driving plant responses and plant defenses limit injury by reducing the preference or performance of herbivores (Strauss and Agrawal 1999). These defenses, collectively referred to as herbivore resistance, can take the form of either toxicity or deterrence (Strauss et al. 2002). Some plants produce or sequester toxic chemicals (e.g. terpenoids, alkaloids, elements) in order to halt or retard the development of herbivores through toxicity (Boyd and Martens 1994; Martens and Boyd 1994; War et al. 2012; Fürstenberg-Hägg et al. 2013). Other plants deter herbivores through physical protection (Massey and Hartley 2009) or by production of unpalatable chemicals to reduce plant tissue damage (Hanley et al. 2007). Deterrence utilizes defenses that are structural (e.g. leaves surrounded by thorns – Gowda 1996), chemical (e.g. production of phenolics that deter herbivores from continued feeding following an initial bite – Hanley and Lamont 2001), or phenological (e.g. rapid turnover of vulnerable parts or avoidance of herbivores through life cycle timing – Saltz and Ward 2000).

Although important for protecting plant integrity, defensive chemicals and structures can be metabolically costly (Strauss et al. 2002). Plants have limited resources and an increase in the amount of resources allocated either to defense or growth must be balanced by a reduction (tradeoff) in resource allocation to the other function (Coley et al. 1985). Plants that invest metabolic resources exclusively towards biomass and reproductive tissues are rapidly affected by herbivory and experience an increased probability of mortality (Kant et al. 2015). Therefore, natural selection favors plant traits that lessen consumption by herbivores (Mortensen 2013).

Hyperaccumulation, a trait found in more than 500 plant taxa, allows plants to take up, transport, and sequester high concentrations of elements in their tissues (van der Ent et al. 2013). Hyperaccumulators generally have elemental concentrations 2-3 orders of magnitude greater than concentrations found in "normal" plant species (Baker et al. 2000). Many aspects of the biology of elemental hyperaccumulation have been studied and several hypotheses have been proposed to explain the evolution of this trait (Boyd and Martens 1992), with most attention focused on the hypothesis that hyperaccumulated metals may act as a defense (Boyd 2014). The "elemental defense hypothesis" formulated by Boyd and Martens (1998) suggests elevated concentrations of a sequestered element protect hyperaccumulating plants against damage by unadapted herbivores (and/or pathogens).

Commonly, elemental defense studies have used feeding-choice trials and attribute successful herbivore deterrence as support for the elemental defense hypothesis. Vesk and Reichman (2009) performed a meta-analysis of 31 feeding-choice trials from ten published studies in which herbivorous invertebrates were provided plant material from hyperaccumulators containing high or low elemental concentrations. These hyperaccumulator studies provided considerable evidence for the elemental defense hypothesis. For example, foliage of *Thlaspi caerulescens* J. and C. Presl. (Brassicaceae), a zinc (Zn) hyperaccumulator, deters herbivores when grown on Zn-amended soil (Pollard and Baker 1997). Other experiments (e.g. Boyd and Martens 1994; Hanson et al. 2003; Coleman et al. 2005) have shown that herbivores prefer to feed on hyperaccumulator plants when they are grown on low-element soils (thus producing low-element tissue concentrations) compared to plants grown on high-element soils. However, because the distribution of herbivores and plants across the studies included by Vesk and Reichman (2009) was limited and uneven, the power of their analysis to accurately estimate the individual effects of herbivore, plant, or element was significantly restricted. Vesk and Reichman (2009) concluded that a greater diversity of variables (e.g. including non-folivore herbivores and/or non-leaf plant structures) needs to be incorporated into elemental defense studies to accurately parse the importance of individual variables to herbivory deterrence.

Studies that use herbivores representative of various feeding modes (chewing, piercing/sucking, etc.) are needed to establish the boundaries of elemental defenses. Plants are consumed by a diverse array of herbivores, and plant–herbivore interactions can be influenced by herbivore feeding mode (Strauss 1991; Karban and Baldwin 1997; Gavloski and Lamb 2000; Jhee et al. 2006). In reviews of herbivory experiments involving hyperaccumulators by both Boyd (2007) and Vesk and Reichman (2009), the authors reported little variation in herbivore feeding mode in experiments, with chewing insects feeding on leaf tissue representing a large proportion of the invertebrates studied. Defense experiments using herbivores of other feeding modes, such as those with piercing/sucking mouthparts (i.e. cellular disruptors, phloem feeders, and xylem feeders) (e.g. Boyd and Martens 1998; Hanson et al. 2003; Jiang et al. 2005; Jhee et al. 2006) are less frequent. In particular, besides Freeman et al. (2012), there has been little exploration of chewing herbivores that consume seeds (granivores) in hyperaccumulator studies and there are no studies of granivory for plants that hyperaccumulate Ni. Since Ni is the most commonly hyperaccumulated element (van der Ent et al. 2013), there is a pressing need for studies that explore granivory and Ni hyperaccumulators. Seed production is central to sexual reproduction; however, the seed stage of a plant is particularly vulnerable to predation (Vanderwall et al. 2005). Many herbivores selectively eat reproductive plant tissues, including seeds, due to their high nutritive value, and often kill the embryo in the process (Janzen 1971; Hulme and Benkman 2002). Maron and Crone (2006) suggest that the impact of granivory on plant populations may be greater than that of other herbivores. Sustained predation by granivores may limit the build-up of a seed bank and potentially lead to a major bottleneck to reproduction (Maron and Crone 2006; Latorre et al. 2013). Hyperaccumulation, however, may provide a competitive advantage for sustaining plant populations over time by protecting seeds from granivory.

Species in the genus *Tribolium* (generalist granivore beetles) have been used as model organisms in feeding experiments in chemical ecology (Alonso-Amelot et al. 1994), fumigation trials for pesticide testing (Hashemi and Safavi 2012; Wu et al. 2015), as well as in hyperaccumulator herbivory studies (Goncalves et al. 2007). While not a granivory study, Goncalves et al. (2007) conducted feeding trials with *Tribolium castaneum* Herbst (Tenebrionidae) using synthetic diet disks containing several Ni concentrations (control, low, intermediate, high). As diet Ni concentration increased, *T. castaneum* fed significantly less and even refused to feed on disks with the highest Ni concentration. Although use of artificial diet provides experimental consistency and controls other variables (e.g. Coleman et al. 2005), differences between artificial diet and seeds will likely affect feeding preferences of granivores. Experiments utilizing seeds of a hyperaccumulator are needed as a next step in examining hyperaccumulation’s effect on granivory. In the current study, we investigate granivory resistance of seeds of the Ni hyperaccumulator *Streptanthus polygaloides* using the granivorous beetle, *Tribolium confusum*. We additionally investigated Ni toxicity to *T. confusum,* using an artificial diet study, to verify that Ni at the concentration found in *S. polygaloides* seeds is toxic to these granivores.

METHODS

Biological materials: The confused flour beetle, *Tribolium confusum* Jaquelin du Val (Coleoptera: Tenebrionidae), is a small insect (adults measure 3-6 mm in length) that commonly infests flour and grain (Haouas et al. 2012). Adults of *T. confusum* used in the experiments were obtained from a population cultured at Auburn University for more than 10 years. Beetles were maintained on cornmeal diet at room temperature in the dark until used for experiments.

Streptanthus polygaloides A. Gray (milkwort jewelflower: Brassicaceae) (abbreviated as SP) is an annual Ni hyperaccumulator endemic to serpentine soils along the western side of the Californian Sierra Nevada (Baldwin et al. 2012). The species consists of four morphs which vary in several morphological characters, including sepal color as well as geographic location (Wall and Boyd 2006; Boyd et al. 2009). The morphs are designated by their sepal characteristics (Boyd et al. 2009): yellow morph (Y), purple morph (P), pale yellow undulate morph (U), and yellow-to-purple morph (YP). The Y and P morphs range from northern California to central California (~39°N to ~37.5°N), whereas the U and YP morphs have more restricted distributions (Pope et al. 2013). Most common and widespread is the Y morph. The P morph is also widespread but mainly grows at higher elevation sites in the northern part of the species' range (Boyd et al. 2009). One morph, geographically isolated approximately 100 km southeast of all other populations, has pale yellow undulate sepals (U), while the sepals of the yellow-to-purple morph (YP) are unusual in that the sepals of the flower buds begin yellow but change to purple during maturation of the flowers (Boyd et al. 2009). Three of the morphs (Y, P, U) were used for this experiment to determine if granivores respond similarly to seeds of different morphs. Seeds were collected in the summer of 2013 from an undulate morph population at Pine Flat (SP-U: 36° 52' N, 119° 17' W, Fresno County), a purple morph population at Washington Road (SP-P:

39° 21' N, 120° 48' W, Nevada County), and a yellow morph population at Marshall Road (SP-Y: 38° 50' N, 120° 52' W, El Dorado County) in California, USA.

Streptanthus insignis subsp. *insignis* Jepson (San Benito jewelflower: Brassicaceae) (abbreviated as SI and hereafter referred to as *S. insignis*) is an annual non-hyperaccumulator species also endemic to serpentine soils of California (Baldwin et al. 2012). Seeds of *S. insignis* were collected in the summer of 2013 from a serpentine site in the Diablo Mountain Range (36° 39' N, 121° 06' W, San Benito County), California, USA. *Streptanthus polygaloides* and *S. insignis* represent two closely related species within sister clades (ASHTB clade: *S. polygaloides* and GH clade: *S. insignis*) within the Streptanthus Clade I (Cacho et al. 2014).

Granivory experiment: Seeds of *S. polygaloides* morphs (SP-Y, SP-P, SP-U) and *S. insignis* (SI) were added to 1.5 mL centrifuge tubes (~0.1 g per tube). A no food treatment was included to provide information about mortality due to starvation in case beetles refused to feed upon seeds. *Tribolium* beetles are unable to attack undamaged grain (Walter 1990). For this reason, a cutting treatment was also included in which seeds of each type (SP-Y, SP-P, SP-U, SI) were cut in half. Adult beetles were starved for 48 h prior to experiments, and were then placed two per tube, with 20 replicates (tubes) of each treatment (four seed types, and seeds either cut or uncut), for a total of 40 beetles per treatment. Beetles were randomly assigned to each of the nine treatments (no food plus two cutting treatments for each of four seed types) and were kept at room temperature in the dark (similar to colony growth conditions). Mortality was recorded daily for 48 days. At the end of the experiment, vials also were checked for visible damage to seeds and for presence of fecal material to confirm beetle feeding.

Whole seeds were analyzed to determine their Ni concentrations. Whole seeds were ground with a mortar and pestle before drying for ease of sample acid digestion. All samples were oven-dried for 48 h, digested with 100 µl concentrated nitric acid (HNO_3), and heated for 1 h at 102°C to dissolve samples completely. Purified milli-q water (200 µl) was added to dilute the sample and then the sample was analyzed via inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, Inc., Weltham, MA). Blanks (containing no sample) were included to determine background Ni values and, in addition, a standard (Sigma-Aldrich, Reference material no. 679 cabbage powder, European Commission, Bureau of Reference) with known Ni concentration was used to verify the accuracy of the analysis. The measured Ni concentration in the standard reference material for this experiment was 27.4 ± 2.6 µg Ni g^{-1} (mean ± SEM, N=14) (reference value of 26.2–27.8 µg Ni g^{-1}).

Additional samples of whole seeds (SP-Y, SP-P, SP-U, SI) were separated into seed coat and embryo portions to quantify their respective Ni concentrations in an attempt to locate tissues of heightened Ni sequestration. Seeds were hydrated on a wetted paper towel for 3 h and then split using a razor blade while viewing with a dissection microscope. The seed coat was separated from the embryo and the two portions were placed in separate microcentrifuge tubes. To obtain sufficient biomass to test Ni concentration via ICP-OES, 30 seeds of each seed type were split and separated to create a pair of samples of embryo and seed coat for analysis. Five seed coat and five embryo samples were prepared for each seed type. Samples were then dried, weighed, digested, and Ni concentrations determined using ICP-OES (see protocol above). Beetles also were analyzed to determine their Ni concentrations using the same protocol used for seeds as described above (drying, wet ashing, etc.). We additionally evaluated the potential for particulate matter adhering to beetles to skew Ni concentration measurements of beetle samples.

This was done by a separate experiment using beetles fed an artificial diet of the highest available Ni concentration (3200 µg Ni g^{-1} – see Artificial Diet Experiment below) for 10 days which ended in 100% mortality. We compared Ni concentrations of unwashed beetles and beetles washed with deionized water to determine if significant surface contamination from the diet occurred. Ten replicates of five beetles each were assigned randomly to treatments. Samples of beetles were washed by placing them in a fine mesh bag (80 mesh count/cm) to keep them intact and swirling them in a petri dish of deionized water for four minutes. Samples were then oven dried for 48 h and analyzed for Ni concentrations using ICP-OES. A significant difference (T-test: t = 2.8, *df* = 1, *P* = 0.038) was found in Ni concentrations between washed (mean = 350 µg Ni g^{-1}, SEM = 49) and unwashed beetles (mean = 510 µg Ni g^{-1}, SEM = 17), suggesting that surface contamination might distort measurements of whole-body Ni concentrations when beetles are fed high-Ni foods. Therefore, all beetles from both the granivory and artificial diet experiments (see below) were washed in deionized water to remove particulate matter (seed fragments or artificial diet) from their bodies before analysis of Ni concentrations.

Statistical analyses were implemented with the open-source statistical platform R, version 3.3.1 (R Core Team 2016). All datasets exhibited normal distribution and equal variance prior to analysis, and treatment significance was determined using a log rank test with $P \leq 0.05$ for all statistical tests. Beetle mortality data were analyzed by survival analysis (R, version 3.3.1, package: survival) to determine if mortality was significantly affected by the treatments. Once an overall significant treatment effect was determined, the dataset was then subdivided to compare specific seed types and cutting treatments, using additional survival analyses to isolate the effects of these factors.

Whole seed Ni concentration data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons tests to compare concentrations among seed types (SP-Y, SP-P, SP-U, SI). We also compared Ni concentrations of seed coats and embryos for each seed type using paired-sample T-tests. Beetles from the granivory experiment were analyzed to determine if experimental factors affected their whole-body Ni concentrations, using 2-way ANOVA, with seed type and cutting treatment as factors.

Artificial diet experiment: An artificial diet study was used to verify that Ni at the concentration found in *S. polygaloides* seeds is toxic to *Tribolium*. Similar to Boyd et al. (2002), yellow cornmeal was amended with powdered $NiCl_2$ (Fisher Scientific, Suwanee, GA) to produce artificial diets containing 140, 240, 440, 830, 1600, and 3200 µg Ni g^{-1} dry mass (as verified by analysis via ICP-OES; methods described below). Two additional diets served as control treatments: an un-amended cornmeal diet and cornmeal supplemented with 300 µg $CaCl_2$ g^{-1}, providing approximately the Cl concentration of the highest Ni (3200 µg Ni g^{-1}) treatment. The latter treatment served as a partial control, as use of $NiCl_2$ (rather than pure elemental Ni) introduces a potential experimental factor of chloride toxicity. To address this factor, if the $CaCl_2$ amended diet (at the highest-available Cl concentration) did not cause significant beetle mortality, then $NiCl_2$-associated mortality could be attributed to Ni, independent of Cl.

Adult beetles were assigned randomly to 1.5 ml microcentrifuge tubes (one beetle per tube) with 15 replicates for each of the eight diet treatments. Beetles were fed 0.5 g of artificial diet at the beginning of the experiment and survival was recorded daily for two weeks. Beetles and artificial diets were dried, digested, and analyzed for Ni concentrations via elemental analysis in the same manner as the granivory experiment (see above).

Data were analyzed to determine if mortality was significantly affected by diet treatments using R, version 3.3.1, package: survival (R Core Team 2016). Treatment significance was determined using a log rank test with $P \leq 0.05$. Analysis of all treatment data revealed a highly significant treatment effect; therefore, the dataset was subdivided to make pairwise comparisons between selected treatments. Specifically, mortality of beetles fed unamended diet was compared to that of beetles fed 220 and 440 µg Ni g^{-1} diets to determine the lowest Ni concentration that caused significantly increased beetle mortality. Beetles were washed and analyzed for whole-body Ni concentration. Only 1 beetle sample per treatment was analyzed because all 10 beetles had to be combined to reach the minimum mass needed for analysis by ICP-OES. Beetle whole-body Ni concentrations were compared to diet Ni concentrations using linear regression (R Core Team 2016).

RESULTS

Granivory experiment: Beetle mortality varied greatly depending on treatment. Survival analysis of the entire dataset showed that treatments significantly affected beetle mortality (log rank test, Chi-square = 370, $df = 8$, $P < 0.0001$). Since an overall significant treatment effect was found, the dataset was subdivided to compare the effects of individual seed types and cutting treatments. Beetles given no food suffered 100% mortality after 18 days, significantly faster than all other treatments (Chi-square = 270, $df = 1$, $P < 0.001$) (Fig. 1), indicating beetles in other treatments were consuming at least some portion of the seeds rather than dying of starvation. All treatments were also checked for visible damage to seeds and for fecal material, by which feeding was confirmed.

Overall, beetles that consumed seeds of any *S. polygaloides* morph suffered greater mortality than beetles that consumed seeds of the non-hyperaccumulator, *S. insignis* (Chi-square = 76, *df* = 1, $P < 0.001$) (Fig. 1). Mortality was similar for beetles fed seeds of the three morphs of *S. polygaloides* (Chi-square = 5.6, *df* = 2, $P = 0.06$), but cutting treatment significantly affected beetle mortality. Cutting treatments (whole versus cut seeds) for each of the three morphs of *S. polygaloides* were compared and significant results were found in each case (SP-Y: Chi-square = 8.1, *df* = 1, $P = 0.004$; SP-P: Chi-square = 4.7, *df* = 1, $P = 0.03$; SP-U: Chi-square = 11.1, *df* = 1, $P < 0.001$). Beetles fed whole hyperaccumulator seeds had greater and faster rates of mortality than those fed cut hyperaccumulator seeds (Fig. 1). At the end of the experiment, mean mortality for beetles fed whole seeds from any of the three hyperaccumulator morphs was 88%, whereas mean mortality for beetles fed cut hyperaccumulator morph seeds was only 56%. In contrast, mortality of beetles fed *S. insignis* (non-hyperaccumulator) seeds was not affected by cutting treatment (Chi-square = 0.01, *df* = 1, $P = 0.96$), and was very low (ca. 10%) at the end of the experiment for beetles fed either cut or uncut seeds (Fig. 1).

Seed types differed significantly in Ni concentrations (ANOVA: $F_{3,16} = 72$, $P < 0.0001$). Whole seeds of *S. polygaloides* contained approximately 300 µg Ni g^{-1} (SP-Y mean = 320 µg Ni g^{-1}, SEM = 24 µg Ni g^{-1}; SP-P mean = 300 µg Ni g^{-1}, SEM = 4.9 µg Ni g^{-1}; SP-U mean = 300 µg Ni g^{-1}, SEM = 26 µg Ni g^{-1}; N = 5 in all cases) while *S. insignis* seeds contained very little Ni (SI mean = 5.2 µg Ni g^{-1}, SEM = 1.4 µg Ni g^{-1}, N = 5). Tukey's post-hoc tests showed that whole seed Ni concentrations of *S. polygaloides* morphs did not differ significantly from one another, but all were significantly greater than *S. insignis*.

Nickel concentrations of coats and embryos were compared for all four seed types using paired sample T-tests. For *S. insignis,* Ni concentrations were low in both portions of the seeds (seed coat mean = 5 µg Ni g^{-1}, SEM = 0.7, embryo mean = 8 µg Ni g^{-1}, SEM = 0.2; Fig. 2) and were not significantly different (T = -1.6, *df* = 9, *P* = 0.14). In contrast, significant differences were found for each hyperaccumulator morph (SP-Y: T = -21, *df* = 9, *P* < 0.001; SP-P: T = -9, *df* = 9, *P* < 0.001; SP-U: T = -7.7, *df* = 9, *P* < 0.001). Embryos of hyperaccumulator seeds had at least 2-fold greater Ni concentrations than seed coats in all cases (Fig. 2).

Two-way ANOVA revealed that beetles fed seeds also differed significantly in Ni concentrations, depending on seed type as well as cutting treatment. A significant seed type effect was present ($F_{4,40} = 29$, $P < 0.001$). Beetles fed *S. polygaloides* seeds had greater Ni concentrations in their bodies than beetles fed seeds of *S. insignis* (Fig. 3). A significant cutting treatment effect was also found ($F_{1,40} = 22$, $P < 0.001$). The greatest difference in beetle Ni concentrations was found for beetles feeding on *S. polygaloides* morphs (mean Ni concentrations: whole seeds: 53 µg Ni g^{-1}; cut seeds: 25 µg Ni g^{-1}) (Fig. 3). The 2-way ANOVA also showed a significant interaction between seed type and cutting treatment ($F_{4,40} = 8.4$, $P < 0.001$). Beetles fed whole seeds of SP-Y and SP-P contained on average more than 2.5-fold greater Ni concentrations than beetles fed cut SP-Y and SP-P seeds (Fig. 3). In contrast, beetles fed either cut or whole seeds of SP-U had similar Ni concentrations (Fig. 3). Beetles fed cut or whole seeds of the non-hyperaccumulator (SI) contained comparable Ni concentrations and contained on average less than 0.3 µg Ni g^{-1} (Fig. 3), much less than beetles fed seeds of either cutting treatment of SP seeds.

Artificial diet experiment: Diet treatments significantly affected beetle mortality (log rank test, Chi-square = 65, *df* = 7, *P* < 0.0001). Mortality was lowest for beetles consuming either unamended diet or diet containing 140 µg Ni g^{-1} and generally increased as Ni diet concentration increased (Fig. 4). There was no significant difference between mortality of beetles fed unamended diet and diet amended with $CaCl_2$ (Chi-square = 0.4, *df* = 1, *P* = 0.53), suggesting there was no chloride effect at the highest level of Cl concentration used in the Ni treatments. Mortality of beetles fed unamended diet did not differ from that of beetles fed 240 µg Ni g^{-1} (Chi-square = 2.3, *df* = 1, *P* = 0.13), but mortality of beetles fed unamended diet was significantly reduced compared to that of beetles fed 440 µg Ni g^{-1} diet (Chi-square = 16, *df* = 1, $P \leq 0.0001$), indicating a toxicity threshold for *T. confusum* adults between diets containing 240 and 440 µg Ni g^{-1}.

Beetles from the artificial diet experiment were also analyzed for whole-body Ni concentrations. Linear regression (Fig. 5) showed a close relationship between beetle body and diet Ni concentrations (R^2 = 0.96). We also noted consistency in beetle Ni concentrations between the granivory and artificial diet experiments. Beetles fed whole hyperaccumulator seeds contained about 60 µg Ni g^{-1} (Fig. 3) while beetles fed diets containing 240 and 440 µg Ni g^{-1} contained 45 and 68 µg Ni g^{-1}, respectively (Fig. 5).

DISCUSSION

Our study suggests that Ni concentrations well below the hyperaccumulation threshold of 1000 µg Ni g^{-1} (van der Ent et al. 2013) are toxic to *T. confusum.* Beetles fed seeds of *S. polygaloides* (seeds contained approximately 300 µg Ni g^{-1} regardless of morph) suffered higher mortality (Fig. 1) than those fed non-hyperaccumulator (*S. insignis*) seeds (which contained 5 µg Ni g^{-1}). These results support the hypothesis that seeds of *S. polygaloides* are defended against granivory by *T. confusum,* a generalist granivore unadapted to high-Ni foods. The cutting treatments were found to have a significant effect on the mortality of the beetles. Beetles consuming whole, intact seeds suffered greater mortality than beetles consuming damaged, cut seeds (Fig. 1). This may be explained by the greater Ni accumulation in the bodies of the beetles fed whole seeds (Fig. 3). However, when seeds were dissected and analyzed for Ni concentration, the seed coat contained less Ni than the embryo. Since beetles fed cut seeds had easier access to the embryo (which contained more Ni overall), we speculate that Ni may be asymmetrically distributed within the embryo. This might allow beetles fed cut seeds to concentrate their feeding on low-Ni embryonic tissues. Further research is needed to learn more about Ni localization in seeds of *S. polygaloides* to potentially explain these results. Particularly interesting would be comparison of Ni localization patterns in embryos of U morph plants with those in Y and P morphs. As mentioned earlier, U morph populations are disjunct from other *S. polygaloides* populations by about 100 km (Boyd et al. 2009) and beetles fed U morph seeds did not differ in Ni concentration due to the cutting treatment (Fig. 3). We hypothesize that the pattern of Ni localization in U morph seeds may differ from that of other morphs, and suggest further research be directed toward this question.

Nickel hyperaccumulators have been noted to have lower Ni concentrations in seeds and fruits than in other tissues (Bhatia et al. 2003) but this varies between species. For *S. polygaloides,* Reeves et al. (1981) reported the range of Ni concentrations in leaves as 3000-14,000 µg Ni g^{-1} while the value for one specimen's seeds was 1150 µg Ni g^{-1}. Lower concentrations (300 µg Ni g^{-1}) were found in the seeds of our study, possibly due to variability in Ni concentrations occurring between years or among *S. polygaloides* populations. Additionally, Ni was found in both the seed coat and embryo of the seeds of *S. polygaloides,* with 2-fold greater Ni concentrations in the embryo (Fig. 2). Several studies have found similar Ni placement in the seeds of other Ni hyperaccumulators (higher in the embryo, lower in the seed coat) (Bhatia et al. 2003; Kachenko et al. 2009; Barillas et al. 2012). Concentration of Ni inside the seed may enhance the reproductive success of a hyperaccumulator plant, providing protection from herbivory for both seed and newly germinating seedling (Freeman et al. 2012). However, this does not explain our finding lower mortality for beetles with access to the embryo, for which further research is needed. Psaras and Manetas (2001) found Ni in the seed coat of *Thlaspi pindicum* Hausskn. (Brassicaceae) to be localized in the micropylar area – providing protection to the embryo at the point of easiest access for seed predators. It is possible that this localization of Ni could be similar in *S. polygaloides* as an explanation for the mortality results of beetles fed whole seeds in the current study.

Our artificial diet study confirmed that Ni was toxic to *T. confusum* at concentrations approximating those of naturally occurring *S. polygaloides* seeds. Mortality of beetles increased (although not significantly) when fed diet containing 240 µg Ni g^{-1}, but mortality significantly increased when fed artificial diet containing 440 µg Ni g^{-1} or more (Fig. 4). Seeds and artificial diets with similar Ni concentrations (300 µg Ni g^{-1} and 440 µg Ni g^{-1}, respectively, notably below the conventional hyperaccumulation threshold) caused significantly increased mortality for the granivorous beetles. Beetle whole-body Ni concentrations also were elevated when they were fed either *S. polygaloides* seeds (Fig. 3) or high Ni artificial diet (Fig. 4). These data suggest that beetles were obtaining Ni from their food and the ingested Ni was responsible for the increased mortality. We conclude that Ni in *S. polygaloides* seeds acts as a defense against this unadapted granivore, consistent with the elemental defense hypothesis (Boyd and Martens 1998).

Our results are consistent with similar studies showing lethal effects of Ni on unadapted herbivores at levels below the conventional hyperaccumulation threshold concentration. Boyd et al. (2002) documented significantly decreased survival of the herbivore *Helix aspersa* Müller (Mollusca: Pulmonidae) using an artificial diet containing 840 µg Ni g^{-1}. Cheruiyot et al. (2013) reported that Ni caused significant mortality of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) at concentrations as low as 230 µg Ni g^{-1}. Coleman et al. (2005) reported reduced survival of *Plutella xylostella* Hübner (Lepidoptera: Plutellidae) at 20 µg Ni g^{-1}. Differences in these effective Ni concentrations may be due to differing sensitivities to Ni of these particular herbivore species. Nevertheless, results from our experiments, compounded with available evidence from other studies, suggest that plants accumulating less than the 1000 µg Ni g^{-1} used to define hyperaccumulation (van der Ent et al. 2013) may still experience a defensive benefit. Beneficial effects yielded by relatively low concentrations of elements indicate that the threshold for effective elemental defense may extend below hyperaccumulation levels (Coleman et al. 2005; Boyd 2007; Cheruiyot et al. 2013).

Lethal effects often occur at higher metal concentrations than sublethal effects, and their defensive value may be greater because they can severely impact herbivore populations (Boyd 2012). However, sublethal effects of elemental sequestration may be evolutionarily effective in some cases (Price et al. 1980; Boyd and Moar 1999; Wise et al. 2006). Determination of both lethal and sublethal levels of elements may provide information helpful to studies of elemental plant defense. Although our study focused on beetle mortality as a defensive effect, consideration of other variables, such as measuring beetle body mass throughout the experiment or measuring fecundity, would provide insight into potential sublethal effects of Ni as well. These sublethal effects may deter herbivores from feeding and thus extend the defensive benefit of Ni to even lower concentrations. Defensive benefits of low Ni concentrations are part of the “defensive enhancement hypothesis” as a potential explanation for the evolution of elemental hyperaccumulation (Boyd 2012). In this scenario, low concentrations of metals, through sublethal and/or lethal effects, can provide an initial defensive benefit to a plant. Natural selection may then drive evolution of even higher concentrations in plant tissues to enhance defense against herbivory.

While toxicity is a useful chemical defense against herbivores (Strauss et al. 2002), deterrence – prevention or halting herbivory prior to significant plant damage (War et al. 2012) – is comparably effective for plant defense. Similar to the present study, Goncalves et al. (2007) conducted feeding trials with the herbivore *Tribolium castaneum* using artificial diet disks containing varying dry weight Ni concentrations (0, 5000, 13000, 25000 µg Ni g^{-1}). As Ni concentration increased, food consumption was significantly reduced, with no feeding on disks of the highest concentration (25000 µg Ni g^{-1}). Goncalves et al. (2007) also found significantly decreased beetle mass with increasing Ni concentrations but not significantly greater mortality due to the experiment only lasting 96 h. To improve upon this, our artificial diet experiment included a more narrow range of Ni concentrations to more appropriately resolve the lethal dose, aiming to determine an approximate Ni toxicity threshold. Treatments including no food and $CaCl_2$ were added to determine that beetles were feeding and to differentiate between Ni and Cl effects. Including even more incremental Ni treatments between 240 and 440 µg Ni g^{-1} would help pinpoint the threshold level for lethal effects on *T. confusum*. In addition to an artificial diet study, our experiment used seeds from both hyperaccumulating and non-hyperaccumulating species. Further strengthening the current study's experimental design, three morphs of *S. polygaloides* (hyperaccumulator) were used, as well as one congeneric non-accumulating species to help isolate the Ni effect.

Our study is the first granivory study involving Ni hyperaccumulation and one of the few examining seed herbivory of any hyperaccumulator. As previously addressed, most hyperaccumulator herbivory defense studies have involved folivores (Boyd 2007; Vesk and Reichman 2009). Studies that use herbivores with a variety of feeding modes are needed to establish the boundaries of elemental defenses. To date, the only other study of hyperaccumulator seed herbivory involves Se hyperaccumulation. Freeman et al. (2012) investigated resistance to Se in chalcid wasps and beetles found feeding on the seeds of two Se hyperaccumulator species, *Astragalus bisulcatus* (Hook) A. Gray and *Stanleya pinnata* (Pursh) Britton (Brassicaceae). Their study suggests that Se-resistant seed herbivores exclude Se from their tissues, allowing them to consume high-Se seeds without suffering toxicity: thus, they are adapted granivores. Future investigations of *S. polygaloides* should examine seed defense using a natural granivore (or one from a closely related non-hyperaccumulating *Streptanthus* species). Unfortunately, to our knowledge, no information is available about granivores that feed on *Streptanthus* seeds in nature. In general, there is a lack of information on the natural herbivores (both adapted and unadapted) of hyperaccumulator species (Boyd 2014). As new hyperaccumulating plant species and their associated herbivores are discovered and characterized (e.g. van der Ent et al. 2015a), research is needed to elucidate ecological interactions between plant and herbivore. There is a pressing need for more ecological information on hyperaccumulator plants, their naturally-occurring herbivores, and the herbivores’ trophic niches so that broad conclusions can be made on the effectiveness and prevalence of hyperaccumulator-associated defenses (van der Ent et al. 2015b).

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FIGURES

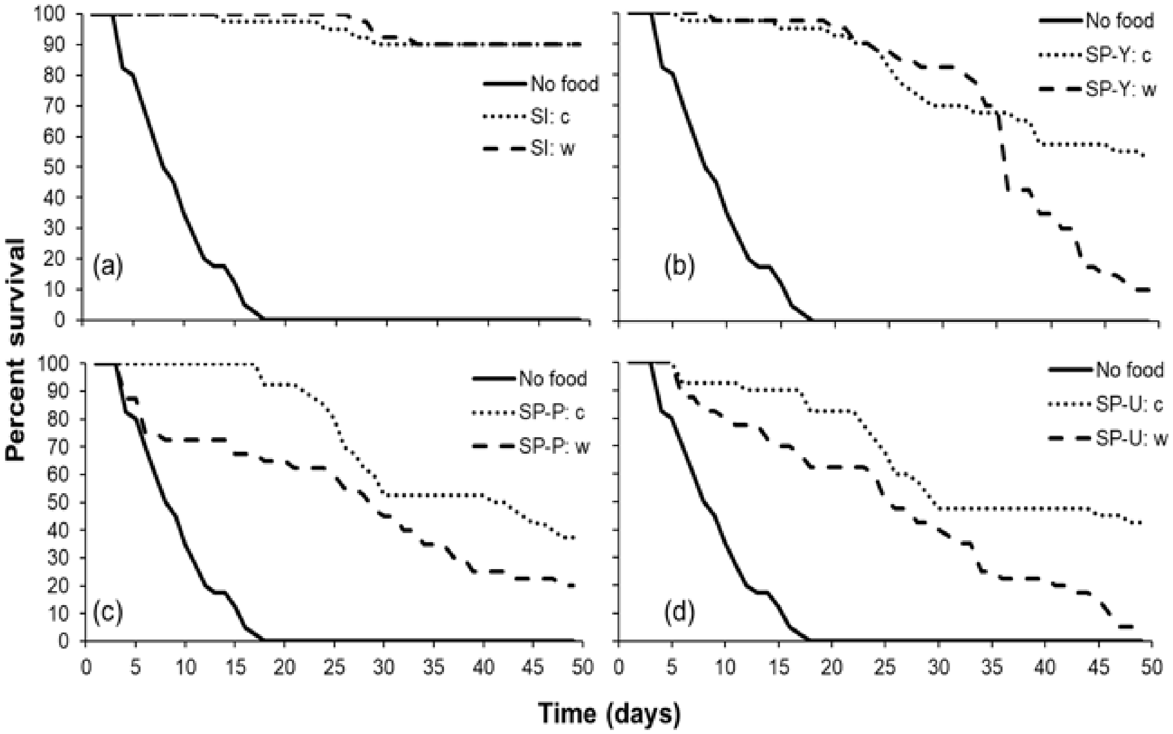

Figure 1. Survival curves (mean percent survival) for *Tribolium confusum* adults in the granivory experiment. Seeds from four sources were used as food: SI – *Streptanthus insignis*, SP-Y – *Streptanthus polygaloides* Marshall Road population (yellow morph), SP-P – *S. polygaloides* Washington Road population (purple morph), SP-U – *S. polygaloides* Pine Flat population (undulate morph). Treatments include cut seeds (c) and whole seeds (w). Panel (a) compares survival of beetles fed no food and those fed SI seed treatments, panel (b) – no food and SP-Y treatments, panel (c) – no food and SP-P treatments, panel (d) – no food and SP-U treatments.

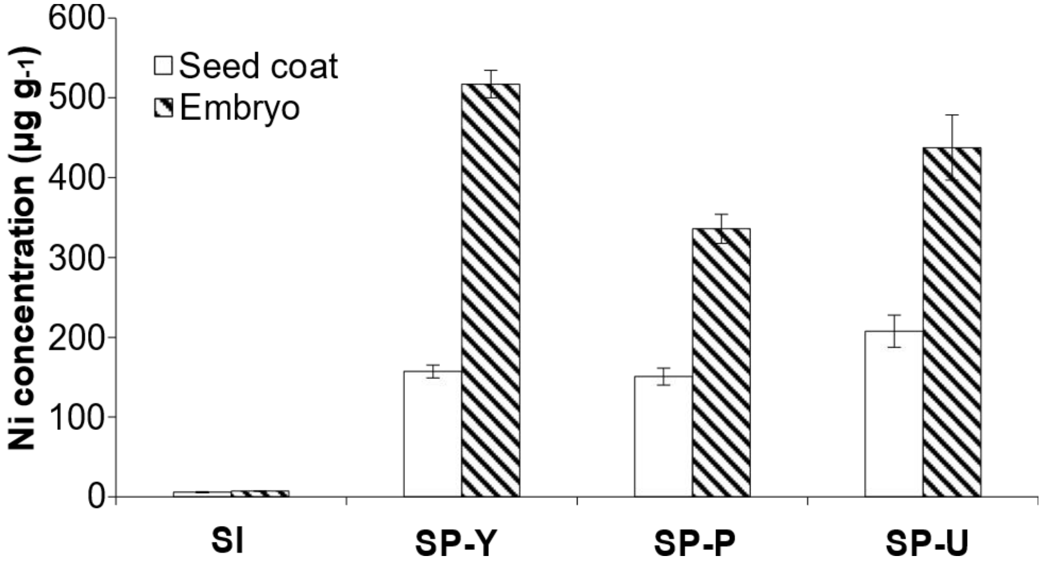

Figure 2. Mean Ni concentrations (error bars = standard error of the mean (SEM)) of seed coats and embryos of the seeds used in the feeding experiment (SI – *Streptanthus insignis,* SP-Y – *Streptanthus polygaloides* (yellow morph), SP-P – *S. polygaloides* (purple morph), SP-U – *S. polygaloides* (undulate morph)). Statistically significant differences (paired T-tests, $P < 0.05$) were found between Ni concentrations of seed coats and embryos for all seed types.

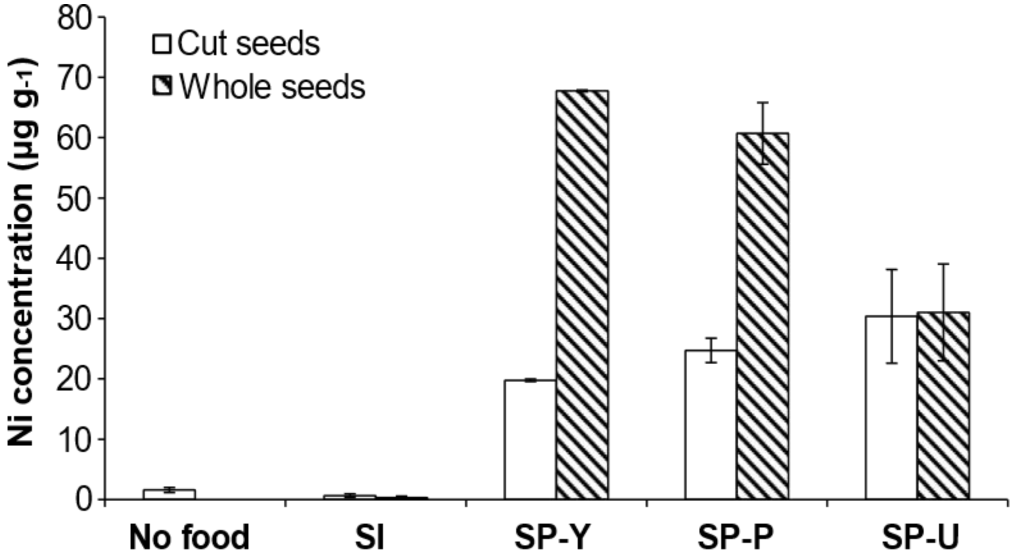

Figure 3. Mean whole-body Ni concentrations (error bars = standard error of the mean (SEM)) of beetles used in the granivory experiment. (SI – beetles fed *Streptanthus insignis*, SP-Y – beetles fed *Streptanthus polygaloides* (yellow morph), SP-P – beetles fed *S. polygaloides* (purple morph), SP-U – beetles fed *S. polygaloides* (undulate morph)).

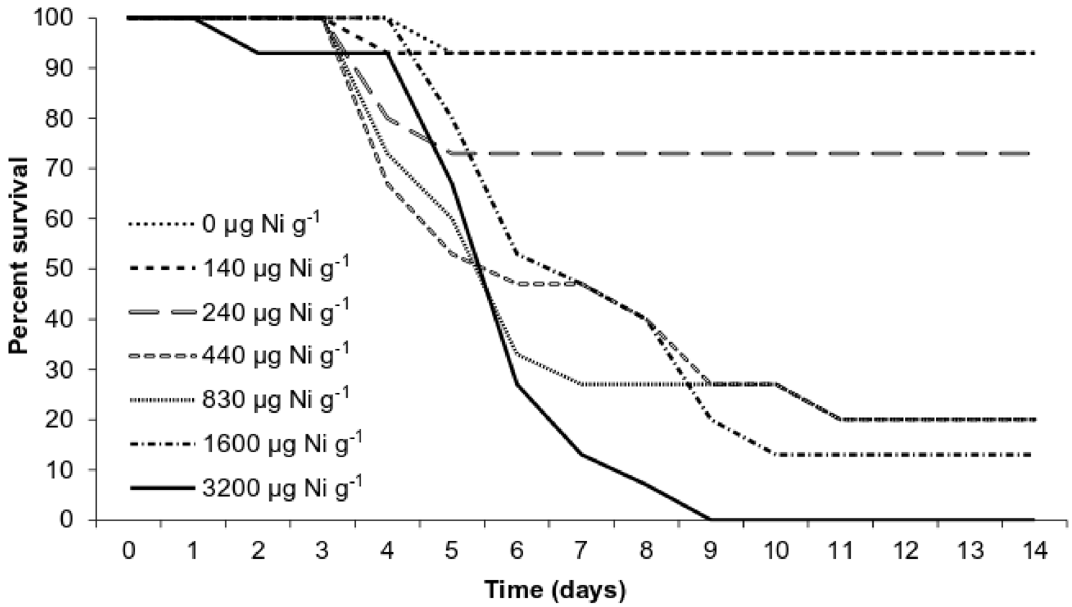

Figure 4. Survival curves (mean percent survival) of *Tribolium confusum* adults fed Ni-amended cornmeal diets. Diet Ni concentrations are measured in µg Ni g^{-1} dry mass.

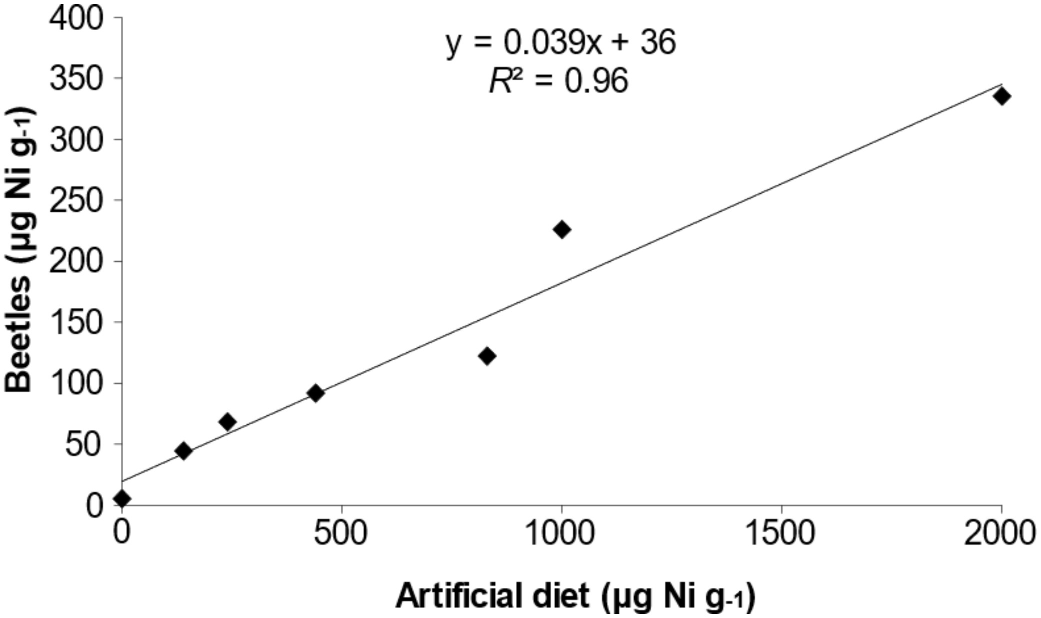

Figure 5. Linear regression of whole-body beetle Ni concentrations and the Ni concentrations of the artificial diet upon which they were feeding.

CHAPTER IV: NICKEL HYPERACCUMULATION BY *STREPTANTHUS POLYGALOIDES* IS ASSOCIATED WITH HERBIVORY TOLERANCE

Abstract- Metal hyperaccumulation can increase plant resistance to herbivory, but tolerance as an herbivore defense has been little investigated. This study explored the interaction between Ni hyperaccumulation and herbivory tolerance using *Streptanthus polygaloides*. Plants were grown in one of two potting soil Ni treatments: Ni-amended (800 µg g^{-1} added Ni) or unamended (0 µg g^{-1} added Ni). One-month-old plants were arbitrarily assigned one of four levels of artificial herbivory damage applied to the leaves. Response variables included aboveground dry biomass and Ni concentration, total leaf number, final plant height, and total number of flowers, and were analyzed by two-way ANOVA. We found no consistent soil Ni effect, artificial herbivory effect, or interaction between soil Ni and artificial herbivory treatments for total leaf number or final plant height. However, there was a soil Ni effect for biomass, flower production, and Ni concentrations: all were greater for plants grown in Ni-amended soil when compared to plants grown in unamended soil. There was also a significant interaction between treatments for number of flowers produced (reflecting plant fitness) as well as for aboveground biomass. Plants of *S. polygaloides* receiving greater damage produced significantly more flowers, and had greater biomass, when grown in Ni-amended soil than plants in unamended soil. We conclude that Ni hyperaccumulation is associated with herbivory tolerance of *S. polygaloides*, increasing plant fitness when herbivory damage is severe. Herbivory tolerance provides an additional dimension to elemental defenses that can supplement the defense trait of herbivory resistance.

INTRODUCTION

Hyperaccumulation is the ability of a plant species to accumulate a high concentration of an element in its tissues (van der Ent et al. 2013). The hyperaccumulation concept has been applied to many elements, including both metals and metalloids. Van der Ent et al. (2013) reports that more than 500 hyperaccumulator plants have been described and hyperaccumulated elements include As, Cd, Co, Cu, Ni, Se, and Zn. However, the vast majority (with estimations as high as 78% of all hyperaccumulator species) accumulate Ni (van der Ent et al. 2013). This phenomenon has many important ecological implications, including trophic transfer of elements (Rascio and Navari-Izzo 2011), alteration of soil chemistry (Fones and Preston 2013), and plant elemental defense (Boyd 2004). A number of hypotheses have been proposed regarding the function of the elevated elemental composition of hyperaccumulators, including the hypothesis that hyperaccumulation evolved as a protective mechanism (Boyd and Martens 1992). This "defense hypothesis" (Boyd and Martens 1994) suggests that elevated concentrations of an element in plant tissues are a defense against unadapted herbivores (and/or pathogens). Elemental defense has been supported by studies showing that unadapted herbivores prefer to feed on plants with low concentrations of hyperaccumulated elements (Pollard and Baker 1996; Boyd et al. 2002; Hanson et al. 2003; Jhee et al. 2005) and that herbivore fitness is reduced when unadapted herbivores consume plants with high elemental concentrations (Boyd and Martens 1994; Boyd and Moar 1999; Boyd et al. 2002; Coleman et al. 2005; Cheruiyot et al. 2013).

Plant defense against natural enemies, such as herbivores and pathogens, has been a major driver of plant evolution (Núñez-Farfán et al. 2007; War et al. 2012). Ehrlich and Raven (1964) attributed many plant traits as defenses against herbivores and, since then, the topic of plant/herbivore interactions has received considerable attention. Herbivory has promoted the evolution of two main defensive strategies in plants, resistance and tolerance, which have different effects on the fitness of both plant and herbivore (Núñez-Farfán et al. 2007). Resistance includes constitutive or induced responses of plants to avoid or reduce herbivory damage (Núñez-Farfán et al. 2007). Tolerance is the degree to which a plant can withstand or repair damage caused by an herbivore, without compromising the plant's growth and reproduction (Stowe et al. 2000; Fornoni 2011). Both strategies are adaptive and the evolution of both defensive strategies should be affected by the relative fitness costs and benefits involved in each strategy (Núñez-Farfán et al. 2007).

Typically, herbivory negatively affects plant fitness and thus can provide a selective incentive for increased levels of plant defense. Plant resistance traits reduce herbivore preference or performance relative to plants that lack the traits (Strauss and Agrawal 1999). There are two main mechanisms of herbivore resistance. First, a trait may exert toxic effects upon an herbivore after it consumes plant material, potentially resulting in herbivore mortality (Núñez-Farfán et al. 2007). Another mechanism is deterrence of herbivory, which reduces the amount of tissue consumed or may even prevent consumption altogether (War et al. 2012). Herbivore resistance has often been the target of plant defense studies and is the more well-investigated topic of herbivore defense, both in general studies (Steppuhn and Baldwin 2007; Karban 2011; War et al. 2012) and in studies of hyperaccumulation (Boyd et al. 2002; Hanson et al. 2003; Jhee et al. 2006).

Most investigations of the elemental defense hypothesis have focused on resistance, demonstrating reduced herbivore damage (e.g., Boyd and Martens 1998; Davis et al. 2001; Hanson et al. 2003; Freeman et al. 2007; Quinn et al. 2010). Across elements and defense studies, resistance often has been demonstrated using plants grown on high- and low-element soils, thus producing defended and undefended plants (e.g., Jhee et al. 1999; Hanson et al. 2003; Boyd and Jhee 2005; Freeman et al. 2007). There have been several recent reviews of research testing the defense hypothesis (Boyd 2007; Vesk and Reichman 2009; El Mehdawi and Pilon-Smits 2012). A Bayesian meta-analysis by Vesk and Reichman (2009) concluded that elemental hyperaccumulation provides a protective effect against herbivores. A review by Boyd (2007) listed 72 tests in 34 separate studies, including studies of defense against both herbivores and pathogens. Of these studies, 44 supported the hypothesis by showing that herbivores prefer to feed on plants with lower elemental concentrations, whereas only four showed no effect. It has also been observed that weight gain of herbivores consuming plant material with high element concentrations is lower than that of those feeding on plant material with low concentrations of the element (Boyd et al. 2002; Hanson et al. 2003; Noret et al. 2005). El Mehdawi and Pilon-Smits (2012) reviewed the literature for Se hyperaccumulation and the defense hypothesis, concluding that there is ample evidence that Se accumulation can defend plants against herbivores. However, as with any plant defense, over time plant resistance to damage may be overcome by herbivores (Núñez-Farfán et al. 2007; War et al. 2012).

Plant tolerance traits can reduce negative effects of damage on fitness by allowing a plant to continue to grow and reproduce after herbivory (Rosenthal and Kotanen 1994; Simms and Triplett 1994; Fornoni and Núñez-Farfán 2000; Stowe et al. 2000). Under certain environmental conditions, tolerance can allow a plant to compensate for damage by replacing damaged leaves, shoots, or roots (Strauss and Agrawal 1999; Mauricio 2000). Plant traits involved in tolerance to herbivory include increased photosynthetic activity and activation of dormant meristems (Tiffin 2000). Several studies have shown that partial leaf defoliation can cause an increased photosynthetic rate in remaining leaf tissue (Rosenthal and Kotanen 1994; Strauss and Agrawal

1999). Compensatory regrowth following partial defoliation may occur by the activation of dormant meristems (e.g. Bergelson et al. 1996; Mabry and Wayne 1997). Identifying the mechanisms of tolerance to herbivore damage facilitates our understanding of the role of tolerance in evolutionary and ecological dynamics of plants and herbivores (Tiffin 2000).

In contrast to the numerous papers on resistance, Boyd (2014) reported that the connection between hyperaccumulation and herbivory tolerance has only been explored once. Palomino et al. (2007) proposed that Ni hyperaccumulation may benefit hyperaccumulator plants by increasing their tolerance to herbivory. They examined effects of artificial damage and soil Ni concentration on the perennial Ni hyperaccumulator *Noccaea fendleri* A. Gray (Brassicaceae) (formerly known as *Thlaspi montanum* L. var. *montanum*) (Holmgren 1971). The authors found that plant growth decreased with damage, yet the magnitude of the negative impacts varied with soil Ni. In low-Ni soil, low and high levels of damage significantly reduced plant growth below that of undamaged plants. However, in high-Ni soil, plants with high levels of damage showed no difference in plant size when compared to plants grown in low-Ni soil with the same amount of damage. They concluded that Ni enhances herbivory tolerance of *N. fendleri* at intense levels of damage. Palomino et al. (2007) suggested that plant fitness response to herbivory should be evaluated to better understand how elemental accumulation and herbivory affect the evolutionary ecology of hyperaccumulating species and that future studies should include a more direct measure of fitness than plant size.

In the current study, we examine the role of herbivory tolerance using another Ni hyperaccumulator, *Streptanthus polygaloides* A. Gray (milkwort jewelflower: Brassicaceae). This species has been the focus of previous research on the resistance effects of hyperaccumulated Ni (Martens and Boyd 1994; Boyd and Moar 1998; Boyd and Jhee 2005; Jhee et al. 2006). Boyd and Moar (1998) found decreased larval weight and survival of herbivores when fed high-Ni leaves of *S. polygaloides.* Boyd and Jhee (2005) concluded that hyperaccumulated Ni can defend *S. polygaloides* from slug herbivory, reporting mortality of all slugs fed high-Ni leaves. Both Martens and Boyd (1994) and Jhee et al. (2006) demonstrated that hyperaccumulated Ni reduced herbivore damage, while Jhee et al. (2006) suggested that toxicity to herbivore larvae and decreased oviposition of adult herbivores positively impacted *Streptanthus polygaloides*. These studies used *S. polygaloides* as the subject for herbivory resistance, however, herbivory tolerance has not been explored. Aside from being well-studied for the role of Ni in herbivory resistance, *S. polygaloides* is an ideal experimental subject for examining fitness due to its lifetime production of flowers in a single growing season. Our study investigates the fitness of *S. polygaloides* as a measure of tolerance to herbivory using artificial leaf removal (damage). We measured total leaf number produced, plant height, aboveground biomass and its Ni concentration, and flower production. Tolerance would be revealed by a significant statistical interaction between soil Ni and artificial herbivory (e.g. a negative effect of herbivory on plant fitness without added soil Ni but a positive effect of herbivory on plant fitness with added soil Ni).

METHODS

Streptanthus polygaloides is an annual Ni hyperaccumulator and the only member of the genus that hyperaccumulates Ni (Reeves et al. 1981; Kruckeberg and Reeves 1995). This serpentine endemic is also one of the few hyperaccumulators found on the serpentine soils of continental North America (Boyd et al. 2009), growing along the western side of the Californian Sierra Nevada (Reeves et al. 1981; Baldwin et al. 2012). As an annual, *S. polygaloides* germinates after fall/winter rains, forms a basal rosette of leaves, and then bolts to flower in late spring/early summer. The species consists of four morphs that vary in floral traits (sepal color and shape), several other morphological characters (including leaf shape/size and plant height), and geographic location, but all hyperaccumulate Ni (Wall and Boyd 2006; Boyd et al. 2009). They are designated (Boyd et al. 2009) as yellow morph, purple morph, pale yellow undulate morph, and yellow-to-purple morph (in which sepals begin yellow but change to purple during flower maturation). Seeds of *S. polygaloides* were collected from an undulate morph population (36°52' N, 119°17' W, Fresno County) in the summer of 2011. The undulate morph was selected due to its relatively entire leaves (Boyd et al. 2009) which made it easier to consistently apply artificial herbivory damage treatments to experimental plants.

Three experimental trials, with each trial taking place during a different year (2013, 2014, 2015), were conducted to strengthen the experimental conclusions and test consistency of the results. All three trials included two soil treatments (unamended and Ni-amended), using commercial potting soil (Sunshine #8 by Sungro, Agawam, MA). A Ni-amended soil was created by adding powdered $NiCl_2$ (Fisher Scientific, Fair Lawn, NJ) to achieve an approximate concentration of 800 µg Ni g^{-1} dry mass. Square pots (10 by 10 cm) were filled with either unamended or Ni-amended soil (2013: 36 of each, 2014 and 2015: 40 of each). To aid in seedling establishment, a layer of perlite was added to cover the potting soil, and seeds were sown onto the perlite layer. Seeds were germinated in a greenhouse at the Auburn University Plant Science Research Center, and plants were maintained under ambient light conditions at an average temperature of 24°C and 74% relative humidity. They were watered twice daily and Peter's Peat-lite 20-10-20 fertilizer (Everris NA Inc., Dublin, OH) was supplied weekly. Plants were thinned to one plant per pot 10 days after germination and grown for approximately five months (from February to June each year). This is similar to the time of year that *S. polygaloides* grows in the field (Wall and Boyd 2002) and was chosen to mimic natural daylength conditions.

Plants were approximately one month old at the start of each trial. Pots were sorted into blocks of eight (to combine four herbivory treatments and two soil treatments) and grouped by similarities in plant size at the beginning of the experiment. This experimental design was used so that initial plant sizes were as similar as possible within blocks at the start of the experiment, thus minimizing the impact of variation in initial plant size on results. In the 2013 trial, four levels of artificial herbivory damage (0, 10, 25, and 50%) were applied. Each treatment had 9 replicates, for a total of 72 plants. In 2014 and 2015, the four levels of artificial herbivory were increased to 0, 25, 50, and 75% because no significant artificial herbivory effect was found for the first experiment. For 2014 and 2015, each treatment was replicated 10 times, to include a total of 80 plants. Artificial herbivory allowed for application of precise amounts of damage to plants. Cutting treatments were arbitrarily assigned to plants in each block and, for cutting treatments, all leaves present at the beginning of the experiment were cut. Distal portions of leaves were removed using scissors to cut across each leaf's midvein and inflict the appropriate amount of damage. Cutting treatments were applied weekly to all new leaves produced by a plant until plants bolted to flower. Every week pots were rotated within each block on the greenhouse bench to even out any positional effects on plant growth. These annual plants were grown for five months, allowing them to reach maturity, flower, and die.

Several variables were measured to quantify treatment effects. To represent plant size, we counted the number of leaves produced by each plant and measured final height of each plant from soil surface to the tallest stem tip. Plant size was also represented by aboveground dry biomass: each plant was harvested, dried for 48 h at 40°C, and weighed. We used total flower production as an estimate of fitness of this annual species. Although seed production would be a preferable measurement of fitness (Younginger et al. 2017), greenhouse-grown plants of *S. polygaloides* are unable to produce seeds without pollination (Boyd et al. 2009).

Elemental analysis was performed to determine Ni concentrations of all samples. Plant samples were ground to a powder using a TissueLyser II (QIAGEN group, Hilden, Germany) at 30 Hz for 15 minutes. Ground samples were analyzed for Ni concentration via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, PerkinElmer, Inc, Weltham, MA). Samples first were digested using concentrated nitric acid (HNO_3), adding 100 µl of nitric acid for every 5 mg of plant mass, and heated for four h at 102°C. Purified milli-q water was added to dilute the samples to a 2:1 ratio of water:nitric acid. Blanks (containing no sample) were prepared with concentrated nitric acid and milli-q water to determine background Ni values. In addition, samples of reference material (Sigma-Aldrich, Reference Material No. 679 Cabbage Powder, European Commission, Bureau of Reference) with known Ni concentration were used to verify analysis accuracy. The Ni concentration in the standard reference material was determined by our procedure to be 27.4 ± 2.6 µg Ni g^{-1} (N=14) (reference value of 26.2–27.84 µg Ni g^{-1}).

Data were analyzed using R, version 3.3.1 (R Core Team 2016), to conduct two-way analyses of variance (ANOVA), using soil Ni and artificial herbivory as the two factors. Effects were considered significant at $P \leq 0.05$. Separate ANOVAs were conducted for each year's experiment because the artificial herbivory treatments differed between 2013 and 2014/2015 and so the experimental protocols were not identical. However, consistency of experimental results between years was useful to demonstrate the generality of treatment effects. We were particularly interested in the interaction term, as a significant interaction would indicate that plant response to artificial herbivory was dependent on soil Ni level and thus whether plants were hyperaccumulating Ni or not.

Wise and Carr (2008) point out that the choice of scales used to measure herbivory damage levels and plant response in studies of herbivory tolerance is critical. Herbivory tolerance studies often use a multiplicative scale for herbivory damage (i.e., proportion of a plant’s leaves that are damaged) and an additive scale for plant fitness (i.e., decrease in number of seeds, flowers, or fruits produced) (e.g. Simms and Triplett 1994; Mauricio et al. 1997; Stowe 1998; Agrawal et al. 1999; Tiffin and Rausher 1999), as was done in our study. However, Wise and Carr (2008) show that it is important for damage and response scales to match, because mixing scales can lead to misleading inferences about a plant’s tolerance to herbivory. It is especially important in our study, as we focus on the interaction between factors (soil Ni and artificial herbivory) to determine herbivory tolerance. To avoid this problem in our study, all response variables were log-transformed before analysis so that both they and the damage levels we used were expressed on matching (multiplicative) scales.

RESULTS

Two-way ANOVA revealed that flower production by *S. polygaloides* was significantly affected by treatments. A significant soil Ni effect was detected for each of the three years (Table 1). Plants on average produced more flowers (between 17-25% more) in Ni-amended soil than in unamended soil (Fig. 1). However, there was no consistent artificial herbivory effect among the three years of experiments (Table 1). Plants grown in unamended soil either had similar flower production across the herbivory treatments, or production decreased with more intense damage (Fig. 1). Plants grown in Ni-amended soil either had similar flower production or increased production as damage increased (Fig. 1). The interaction between soil Ni and artificial herbivory was significant every year (Table 1). Figure 1 shows that flower production was significantly greater with greater amounts of artificial herbivory and the addition of Ni to the soil. Plants with lesser levels of damage (< 50%) had similar flower production between soil treatments, but flower production by plants assigned greater levels of damage (50 and 75%) was from 1.3- to 1.5-fold greater for plants in Ni-amended soil compared to unamended soil (Fig. 1).

Aboveground biomass also varied based on treatment. Table 1 shows that a significant soil Ni effect was found for all three experiments. Plants in Ni-amended soil had greater biomass (approximately 10% more) than those grown in unamended soil (Fig. 1). Similar to flower production, however, biomass was not significantly affected by artificial herbivory (Table 1). Plants grown in unamended soils had relatively similar biomass across herbivory treatments, while plants grown in Ni-amended soil had increasingly greater biomass with more intense damage (Fig. 1). Finally, also similar to flower production, there was a significant interaction between soil Ni and artificial herbivory for biomass (Table 1). More biomass (11%) was produced by plants grown in Ni-amended soil and treated with greater levels of artificial herbivory than similar damage levels of plants in unamended soil (Fig. 1).

The other two variables, total leaf number and height, produced no consistent significant effects for soil Ni or artificial herbivory over the three years (Table 1). Figure 2 shows that over the course of the three trials, plants of all soil treatments and artificial damage treatments produced relatively similar numbers of leaves and grew to similar heights. Furthermore, there also were no consistent significant interactions between soil Ni treatment and artificial herbivory treatment for either total leaf number or final plant height (Table 1). Leaf number and plant height (Fig. 2) were not greater in *S. polygaloides* with the addition of Ni to the soil and high amounts of artificial herbivory to leaves. Those years that did produce significant effects for soil Ni, as well as for the interaction between soil Ni and artificial herbivory (Table 1), had trends consistent with data on biomass and flower production. We suggest that plant height and leaf number were more variable indicators of plant size, whereas biomass was a more consistent indicator of plant size for both soil Ni and interaction effects.

The amount of Ni measured in the aboveground plant material varied based on the soil treatment. Table 1 shows that a significant soil Ni effect was found for all three years. Plants grown in Ni-amended soil were all determined to be at or above hyperaccumulation status (1000 µg Ni g^{-1}), while those grown in unamended soil contained small quantities of Ni (< 50 µg Ni g^{-1}) (Fig. 3). There was no significant variation in Ni concentration due to the artificial herbivory treatment or for the interaction term (Table 1).

DISCUSSION

In our study, the most consistent significant effect was the interaction between soil Ni and artificial damage treatment, found for both biomass and flower production. This interaction suggests that Ni hyperaccumulation may increase the fitness of *S. polygaloides* when plants are damaged by herbivores. Plants with the highest levels of damage applied, and with soil Ni added, increased in size and reproductive output relative to plants grown in unamended soil with similar damage, demonstrating compensation for the additional damage. These results also suggest that soil Ni enhances tolerance of *S. polygaloides* at intense levels of herbivory damage. While the conclusions of our study are limited to greenhouse conditions and artificial herbivory, our data suggest that hyperaccumulators may be tolerant of herbivory in the field under natural conditions.

Tolerance to herbivore damage may play an important role in the ecological and evolutionary dynamics between plants and herbivores. Plant tolerance traits include altering physiological processes such as photosynthetic activity and growth (Strauss and Agrawal 1999; Stowe et al. 2000; Tiffin 2000). In some plant species, partial herbivory may lead to an increased photosynthetic rate in the remaining plant tissues (Strauss and Agrawal 1999; Retuerto et al. 2004), suggesting that compensatory photosynthesis can be a physiological response to tissue damage (Tiffin 2000). Additional experiments, including measurement of photosynthetic rates of plant tissues after herbivory, should be done to further investigate the mechanisms underlying tolerance of herbivory in *S. polygaloides*. Plant tolerance to herbivory could also be produced by changes in phenology (Tiffin 2000). Delayed growth, flower, and fruit production following herbivore damage could promote herbivory tolerance by postponing plant development until the threat of attack has passed (Tiffin 2000; Mitchell et al. 2016).

Elemental defense has become one of the best-supported hypotheses for the function of hyperaccumulation (for review, see Boyd 2007), however, other evolutionary drivers, including a direct benefit of a hyperaccumulated element, may act in concert with elemental defense. Statwick et al. (2016) examined plant performance variables of the Se hyperaccumulator *Astragalus bisulcatus* (Hook.) A. Gray (Fabaceae) when plants were subjected to herbivory by a spidermite, *Tetranychus urticae* Koch (Acarina: Tetranychidae). They found that *A. bisulcatus* responded positively to Se dosage both with and without herbivory. They observed less herbivory on *A. bisulcatus* when dosed with Se, however, plants without herbivory and with addition of Se grew significantly better as measured by every metric. They proposed a new hypothesis of “elemental stimulation” which suggests that elements can enhance the growth of hyperaccumulators even when herbivory is not a significant factor. Meindl and Ashman (2017) suggest that Ni may limit the reproductive ability of non-hyperaccumulator serpentine endemic species, while Ni may increase the reproductive potential for Ni hyperaccumulators. In addition, Ghasemi et al. (2014) reported a stimulatory effect of Ni for the hyperaccumulator *Alyssum inflatum* Nyár. (Brassicaceae), documenting an initial fitness advantage for Ni-treated plants of greater flower production. The current study found similar results, with a consistent soil Ni effect over the three trials for both biomass and flower production (Table 1). Plant growth (measured as aboveground biomass: Fig. 1) was greater in high-Ni soil, indicating Ni stimulation. Flower production was also stimulated (Fig. 1) in high-Ni soil. Our results support the "elemental stimulation" hypothesis of Statwick et al. (2016) since soil Ni enhanced both plant growth and reproductive success in *S. polygaloides*.

Previous herbivory studies on *S. polygaloides* have focused on resistance and suggest that hyperaccumulation reduces herbivory via that mechanism (e.g., Martens and Boyd 1994; Jhee et al. 2006). To date there is only one study (Palomino et al. 2007) focusing on the role that hyperaccumulation may play in the tolerance to herbivory damage. Palomino et al. (2007) used a perennial plant but were unable to directly measure fitness as their experiment did not extend throughout the plant's lifespan. The current study improved upon their experimental design by using an annual, allowing us to measure lifetime fitness. A fundamental life history trait is an organism's reproductive strategy, including when to reproduce, so that survival and success of offspring are maximized (Adler et al. 2013). There are two main plant reproductive strategies: annuals that reproduce once and die and polycarpic perennials that reproduce repeatedly and cycle through vegetative and reproductive phases (Friedman and Rubin 2015). By using an annual plant, we obtained a more comprehensive estimation of fitness because in annual plants energy reserves are directed into reproduction rather than an additional vegetative cycle, as would be the case in a perennial plant.

Inducible plant defenses require that plant tissues be damaged before they are stimulated or manufactured (Karban and Baldwin 1997). Davis and Boyd (2000) reported that Ni-based defenses in *S. polygaloides* are not inducible due to the lack of response of Ni concentrations to both artificial and lepidopteran herbivory. In the current study, we also found no evidence of inducibility of Ni concentrations. *Streptanthus polygaloides* plants grown in Ni-amended soil had similar Ni concentrations (mean: 2400 µg Ni g^{-1}) regardless of artificial herbivory treatment (Table 1, Fig. 3).

Plants in the current study were damaged using scissors to simulate the effects of herbivore attack yet allowing precise control of the degree of damage. A review of the use of artificial damage in plant-herbivore studies (Lehtila and Boalt 2004) reported that similar effects for artificial and natural damage were shown more often for studies of plant tolerance than plant resistance to herbivory. Artificial damage, however, may not adequately mimic natural damage. For example, plants under attack from actual herbivores may be affected by herbivore saliva, eliciting an additional defense response that is not triggered by artificial damage techniques (Agrawal 1998; Shinya et al. 2016). There is a need for natural history studies of hyperaccumulators to identify natural herbivores (Boyd 2009): this would allow researchers to determine if herbivory by natural herbivores has effects similar to the artificial herbivory used in our study.

Tolerance and resistance are different plant defense strategies against herbivores. Metal hyperaccumulation has been largely investigated as an herbivory resistance trait (Boyd and Martens 1992), however, our study suggests hyperaccumulation simultaneously promotes herbivory tolerance. According to Strauss and Agrawal (1999), plant resistance and plant tolerance, as alternative but not mutually exclusive herbivory defenses, may result in differing dynamics between plant and herbivore populations. Plant resistance may lead to counter-defenses by herbivores, triggering a "coevolutionary arms race." In contrast to resistance, herbivory tolerance does not reduce individual herbivore fitness but can decrease attack at the plant population level (Espinosa and Fornoni 2006; Miller et al. 2006; Fornoni 2011). Evidence from natural plant populations reveals that plants often allocate resources simultaneously to both strategies (Núñez-Farfán et al. 2007).

Plants interact with assemblages of herbivores (War et al. 2012) that include both those unadapted and those adapted to particular plant defense mechanisms. As mentioned above, adapted herbivores have evolved counter-defenses that reduce the effectiveness of resistance traits, yet a plant may withstand damage from these adapted herbivores through tolerance. It has been hypothesized that tolerance is favored when resistance is no longer effective against natural enemies (Agrawal and Fishbein 2008; Garrido et al. 2012). While resistance traits are ineffective against adapted herbivores, resistance traits may still be useful to defend against unadapted generalist herbivores. Thus, a plant with both mechanisms of defense will have higher fitness than either strategy alone would provide.

Streptanthus polygaloides has been the focus of numerous herbivory resistance studies (Martens and Boyd 1994; Boyd and Moar 1999; Boyd and Jhee 2005; Jhee et al. 2005; Jhee et al. 2006). Observations from these studies include decreased weight and/or survival for unadapted herbivores (i.e. *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae), *Limax maximus* Linnaeus (Gastropoda: Pulmonata), *Melanoplus femurrubrum* DeGeer (Orthoptera: Acrididae), *Pieris rapae* Linnaeus (Lepidoptera: Pieridae), *Tetranychus urticae*, etc.). However, some generalist herbivores are not affected by Ni-based resistance in *S. polygaloides*. For example, Boyd and Martens (1999) found that the elevated Ni concentration in *S. polygaloides* grown on high-Ni soil was not effective as an elemental defense against *Acyrthosiphon pisum* Harris (Homoptera: Aphidae). Additionally, Jhee et al. (2005) found that the phloem-feeding insects *Lipaphis erysimi* Kaltenbach (Hemiptera: Aphididae) and *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) were unaffected by the Ni concentrations found in *S. polygaloides*. In addition, some herbivores of hyperaccumulators have apparently evolved counter-defenses that render hyperaccumulated elements ineffective in defense. A number of these adapted herbivores have been discovered, including those termed high-Ni insects. Boyd (2009) identified high-Ni insects as those having a whole-body Ni concentration of 500 µg Ni g^{-1} or greater, and most of those identified to date are host-specific to a particular Ni hyperaccumulating plant species. For *S. polygaloides,* a survey of arthropods associated with it revealed a new species, *Melanotrichus boydi* Schwartz and Wall (Hemiptera: Miridae), which has been shown to be monophagous on it and therefore is presumably adapted to feeding upon it (Wall and Boyd 2006). While Ni hyperaccumulation is a valuable resistance trait against some generalist herbivores, tolerance provides a mechanism to cope with other herbivores (both adapted and unadapted) that are not affected by the high Ni concentration in the plant. More research is needed on other hyperaccumulators to determine how both resistance and tolerance defensive strategies influence adapted and unadapted herbivores of those hyperaccumulator species.

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TABLES

Table 1. Statistical results from 2-way analysis of variance (ANOVA) of data from artificial herbivory experiments, showing *P*-values from each trial (listed in order as 2013, 2014, 2015). Bold numbers represent significant effects ($P < 0.05$). The Ni conc. variable is the Ni concentrations of the dried plant material.

Variable	Soil Ni	Artificial herbivory	Interaction
Flowers	< **0.01**, < **0.01**, < **0.01**	0.58, 0.06, **0.01**	**0.01**, **0.02**, **0.01**
Biomass	< **0.01**, < **0.01**, **0.01**	0.22, 0.87, 0.66	**0.01**, **0.02**, < **0.01**
Leaf number	0.68, **0.01**, 0.06	0.07, **0.04**, **0.03**	0.40, 0.51, **0.02**
Height	0.74, 0.07, < **0.01**	0.30, 0.20, **0.01**	0.78, 0.12, **0.01**
Ni conc.	**< 0.01, < 0.01, < 0.01**	0.17, 0.12, 0.16	0.18, 0.12, 0.15

FIGURES

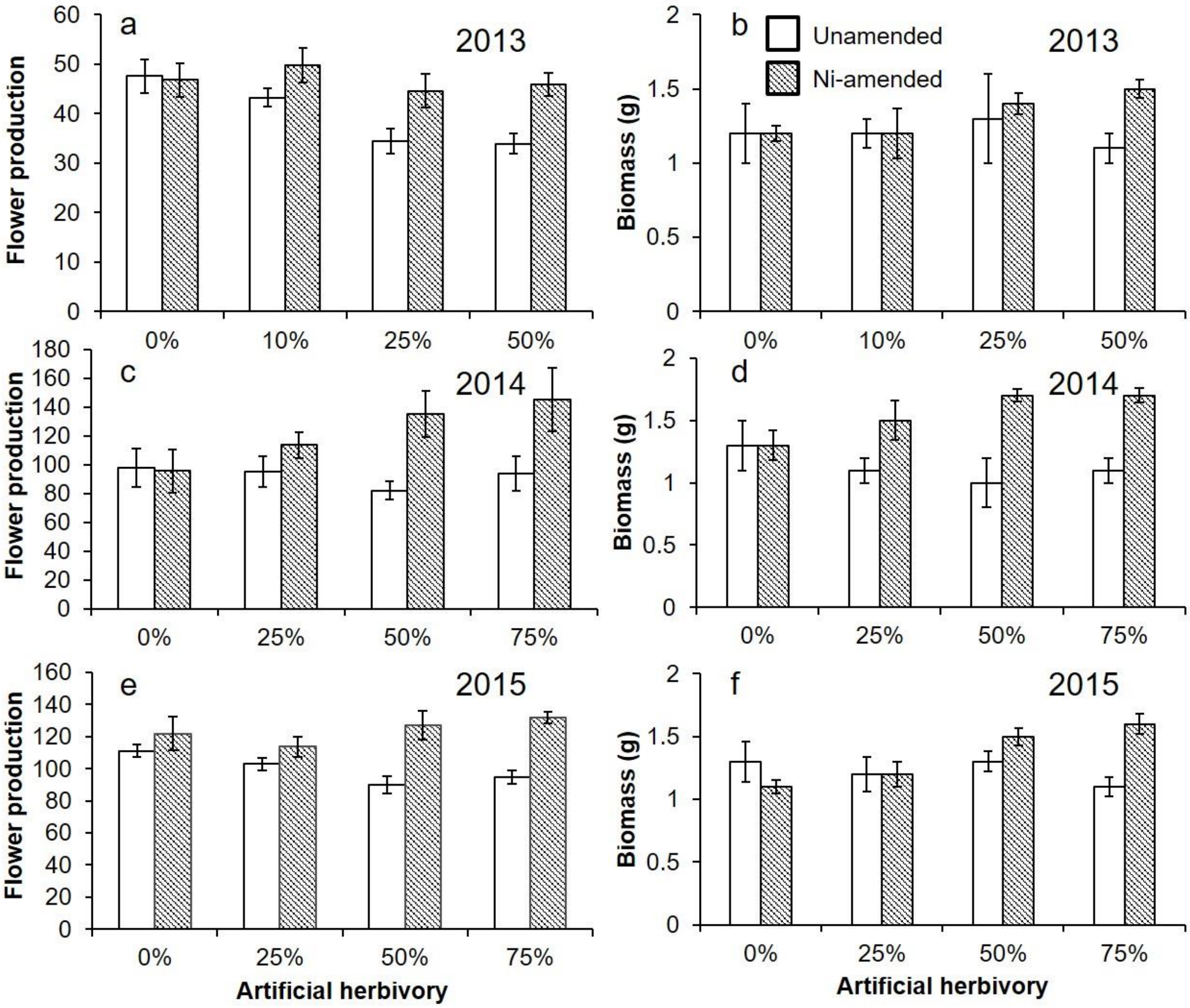

Figure 1. Mean flower production and biomass of *Streptanthus polygaloides* at two soil Ni concentrations (unamended: unfilled bars, Ni-amended: crosshatched bars) and four levels of artificial herbivory damage (2013 (a & b): 0, 10, 25, 50%; 2014 (c & d) and 2015 (e & f): 0, 25, 50, 75%). Error bars represent standard error of the mean.

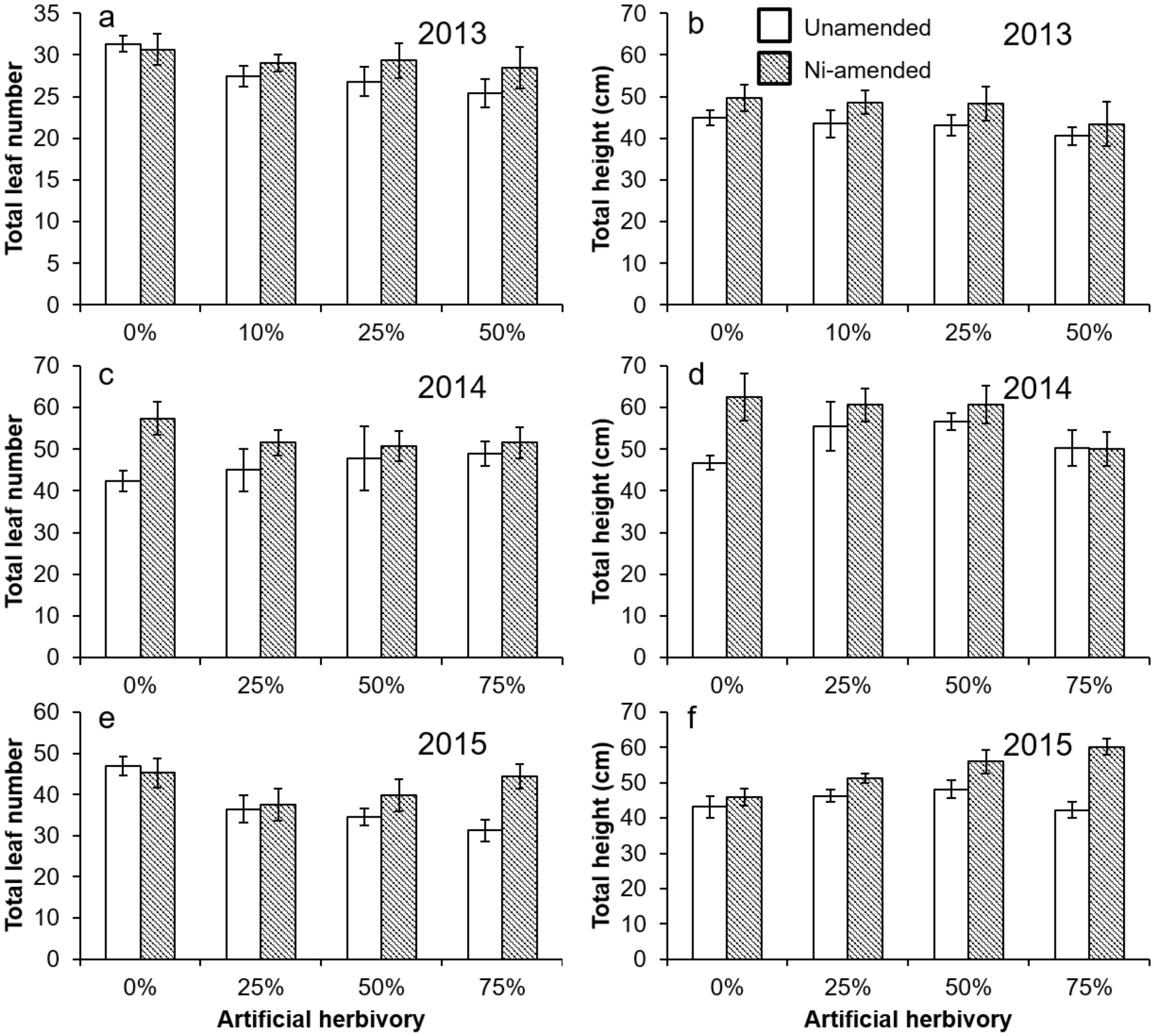

Figure 2. Mean total leaf number and total height of *Streptanthus polygaloides* at two soil Ni concentrations (unamended: unfilled bars; Ni-amended: crosshatched bars) and four levels of artificial herbivory damage (2013 (a & b): 0, 10, 25, 50%; 2014 (c & d) and 2015 (e & f): 0, 25, 50, 75%). Error bars represent standard error of the mean.

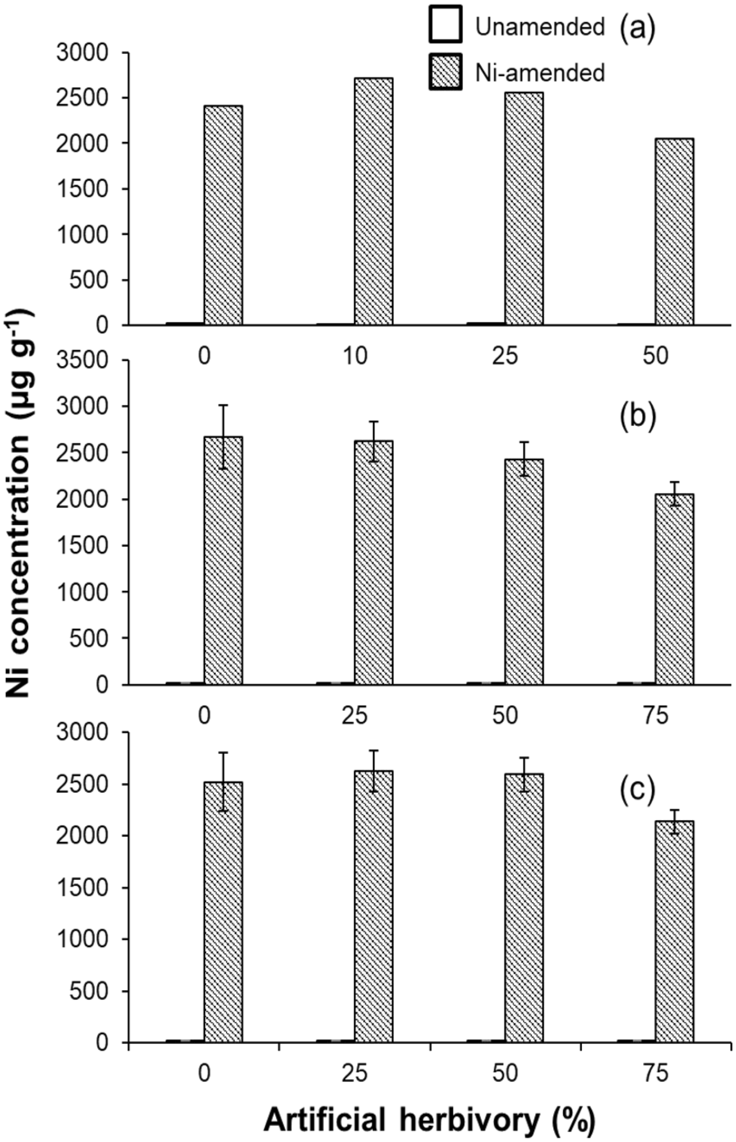

Figure 3. Mean Ni concentrations of aboveground biomass of *Streptanthus polygaloides* at two soil Ni concentrations (unamended: unfilled bars; Ni-amended: crosshatched bars) and four levels of artificial herbivory damage (2013 (a): 0, 10, 25, 50%; 2014 (b) and 2015 (c): 0, 25, 50, 75%). Error bars represent standard error of the mean. Error bars for plants growing on unamended soil are too small to appear on the figure (standard errors of the mean for all unamended soil values were < 42 µg Ni g^{-1}).

CHAPTER V: NICKELOPHILIC FORAGING BY ROOTS OF THE NICKEL HYPERACCUMULATOR, *STREPTANTHUS POLYGALOIDES* (BRASSICACEAE)

Abstract – Root foraging may allow hyperaccumulator plants to enhance element accumulation. This study compared root proliferation in Ni-amended and unamended potting soils by two annual *Streptanthus* species endemic to serpentine soils of California. *Streptanthus polygaloides* is one of the few Ni hyperaccumulators known from continental North America and *Streptanthus insignis* is a non-hyperaccumulator. Seeds were planted in pots divided into halves by a sealed partition, with Ni-amended potting soil (800 g Ni kg^{-1} dry mass) in one half and unamended soil in the other. Seeds were germinated directly over the partition, allowing roots to explore both soils as plants grew. After five months, roots from each side of each pot were washed, dried, and weighed. *Streptanthus polygaloides* root biomass was significantly (2-fold) greater in Ni-amended soil whereas *S. insignis* root biomass was similar in the two soils. Seedlings of both species also were germinated in vertical agar-filled petri dishes to determine if Ni affected seedling root growth. Seedlings were placed on either side of a central piece of filter paper soaked in either a $NiCl_2$ solution or deionized water. Growth direction of the primary root (towards, away, neutral) in regard to the filter paper was recorded. Lateral root numbers (towards, away) and their lengths were also quantified. Primary root direction, lateral root number, and lateral root lengths were significantly increased toward Ni-soaked filter paper for *S. polygaloides* but not for *S. insignis*. We conclude that *S. polygaloides* exhibited positive root foraging responses that may enhance Ni uptake and suggest that the term “nickelophilic root foraging” be applied to this behavior.

INTRODUCTION

Plants have a large amount of phenotypic plasticity which aids individuals in acquiring essential resources (Tilman 1987). Plants generally require both light (an aboveground resource) and various belowground resources, including soil nutrients and water. To obtain light for photosynthesis, a plant must allocate energy to production of stems and leaves, whereas to obtain nutrients/water it must allocate energy to root growth. Phenotypic plasticity allows a plant to adjust the rate at which it acquires each resource. Resources essential for plant growth (i.e. light, water, nutrients) are often heterogeneously distributed within the environment (Hodge 2004; Hodge 2006; Bao et al. 2014). Plants forage for these patchily distributed resources by strategically positioning leaves, stems, and roots in areas of greater resource availability to optimize resource acquisition (Hutchings and de Kroon 1994; Bao et al. 2014). Foraging responses, such as root proliferation in response to soil nutrients and water, have been demonstrated in many plant species (McNickle et al. 2009). Greater growth can be achieved in habitats with patchy nutrient distribution when compared to habitats with the same quality of resources but with homogenous distribution (Robinson 1994; Hutchinson et al. 2000). Patchy distribution of soil resources can positively affect plant performance and can alter resource acquisition and allocation patterns, potentially changing total plant biomass (Hutchinson et al. 2000; Haines 2002).

Serpentine soil is an edaphically stressful, low productivity soil type that hosts stunted vegetation along with a great degree of plant endemism (Brady et al. 2005; Rajakaruna and Boyd 2014; Echevarria et al. 2018). While there is considerable variation in the features of serpentine habitats, they are often characterized by shallow soil resulting in a reduced capacity to retain moisture (Kruckeberg 2002; Brady et al. 2005). Steep rocky slopes and sparse plant cover in these areas also encourage continual erosion and water loss (Kruckeberg 1954; Kruckeberg 2002); therefore, plants inhabiting serpentine areas in temperate zones must often tolerate drought in addition to depressed nutrient levels and the harsh soil chemical conditions (Walker 1954; Latham 1993; Rajakaruna et al. 2009). Serpentine soil differs from non-serpentine soil in chemical composition (Brady et al. 2005), typically having low Ca/Mg ratios, deficiencies of essential nutrients (N, P, and K), and containing high concentrations of elements, such as nickel (Ni), cobalt (Co), and chromium (Cr), that are toxic to most organisms, making them unfavorable for plant establishment and growth (Brooks 1987; Brady et al. 2005).

Serpentine soil hosts plant species that have the ability to hyperaccumulate one or more elements. Hyperaccumulators actively take up large amounts of one (or more) elements from the soil with their roots and translocate them to the shoots and leaves, accumulating them to concentrations 100- to 1000-fold higher than those of non-hyperaccumulating plants (Baker et al. 2000; Rascio and Navari-Izzo 2011; van der Ent et al. 2013). The hyperaccumulation trait occurs in over 500 plant taxa, with a great majority of the reports being of Ni hyperaccumulation and most of these occurring on serpentine soil (van der Ent et al. 2013). The distribution and bioavailability of Ni in serpentine soil differs from one site to another (Hseu 2006), which may be due to several factors including soil pH, type of vegetation, and local climatic conditions. In general, elements found in natural or contaminated soils are typically heterogeneously distributed (Price et al. 1996; Ramsey 1997), suggesting that foraging traits in substrates with high elemental concentrations may be beneficial to plants by enhancing accumulation in hyperaccumulator species (Haines 2002).

Physiological studies have paved the way for a basic understanding of elemental hyperaccumulation mechanisms: these include enhanced elemental uptake by roots, increased xylem loading and transport, phloem translocation, and element sequestration and compartmentation in leaf tissue (Verbruggen et al. 2009; Rascio and Navari-Izzo 2011; Deng et al. 2018). In addition, other properties of these plants' root systems can enhance elemental uptake, including dense absorption sites on root membranes as well as localized root proliferation (Verbruggen et al. 2009). Progress has been made into understanding the physiological mechanisms behind hyperaccumulation, but most research has focused on elemental transport at the cellular level.

A potential role of root proliferation in hyperaccumulation has been suggested in previous studies of hyperaccumulators of zinc (Zn), selenium (Se) and Ni. Localized root proliferation has been observed in several studies using the Zn hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. (Brassicaceae) (Schwartz et al. 1999; Whiting et al. 2000; Haines 2002). For example, Haines (2002) reported positive root growth responses towards patches of Zn-rich soil including increased root branching and length, increased root hair length, and increased root biomass overall, proposing the idea of "zincophilic root foraging." El Mehdawi et al. (2015) compared populations of the Se hyperaccumulator *Symphyotrichum ericoides* (L.) G.L. Nesom (Asteraceae) from seleniferous and non-seleniferous soil to determine root response to Se. They reported that roots of mature plants from the seleniferous population proliferated more in seleniferous soil, and that roots of seedlings from the seleniferous population grew predominantly towards Se. In contrast, roots of mature plants, and roots from seedlings from the non-seleniferous population, showed no response to Se. There are only two studies dealing with Ni hyperaccumulators. A preliminary study (Boyd et al. 2000) on seedling root elongation of the Ni hyperaccumulator *Streptanthus polygaloides* A. Gray (Brassicaceae) reported greater root elongation in serpentine soil than in non-serpentine soil, suggesting that *S. polygaloides* may require elevated soil Ni for optimum root growth. Moradi et al. (2009) reported that roots of the Ni hyperaccumulator *Berkheya coddii* Rossler (Asteraceae) did not grow towards Ni but, in the presence of Ni in soil, did change root morphology, producing thicker roots with less branching. Overall, there is little research to date on root foraging responses of hyperaccumulators and their potential importance to hyperaccumulation.

Nickel was the first element designated as being hyperaccumulated by plants (Brooks et al. 1977; Reeves 1992; van der Ent et al. 2013). Nickel hyperaccumulators contain at least 1000 μg Ni g^{-1} in their leaves when found in their natural habitat (van der Ent et al. 2013). A common approach to investigations of hyperaccumulation mechanisms has been to compare a hyperaccumulator with a closely related non-accumulator (e.g., Davis and Boyd 2000; Boyd and Jhee 2005; El Mehdawi et al. 2015). *Streptanthus polygaloides* is a Ni hyperaccumulator endemic to serpentine soil on the western side of the Sierra Nevada range in California (Reeves et al. 1981; Baldwin et al. 2012). It is the only species of the genus that hyperaccumulates Ni (Reeves et al. 1981; Kruckeberg and Reeves 1995). It is also the most-investigated North American Ni hyperaccumulator, as it has been the subject of studies of morphology, ecology, genetics, herbivore interactions, and elemental concentration (e.g., Boyd et al. 2000; Davis et al. 2001; Jhee et al. 2006; Boyd et al. 2009; Pope et al. 2013; Mincey and Boyd 2018; Mincey et al. 2018). *Streptanthus insignis* Jeps. (Brassicaceae) is also a serpentine endemic species in California (Baldwin et al. 2012) but does not hyperaccumulate Ni (Kruckeberg and Reeves 1995). We compared these two species in two experiments to address whether roots respond to Ni. A soil experiment using mature plants was designed to determine root biomass response, and seedlings were used in an artificial medium experiment to explore multiple measures of individual root responses (including primary root direction as well as lateral root initiation and growth in response to Ni).

METHODS

Biological material: Streptanthus polygaloides (milkwort jewelflower: Brassicaceae) is an annual Ni hyperaccumulator. This serpentine endemic grows along the western side of the Californian Sierra Nevada (Baldwin et al. 2012; Reeves et al. 1981). Seeds of *S. polygaloides* were collected from the Pine Flat serpentine site (36°52'35" N, 119°17'23" W, Fresno County) in 2011. *Streptanthus insignis* (plumed jewelflower: Brassicaceae) is an annual herb also endemic to California. It grows in grassland and chaparral habitats in the Inner South Coast Ranges, generally on serpentine soils (Baldwin et al. 2012). Seeds of *S. insignis* were collected from a serpentine site (36°39'38.3" N, 121°06'43" W, San Benito County) for use in this experiment in 2011.

Root proliferation pot experiment: This study compared root proliferation in the two *Streptanthus* species when plants were provided with soil of two Ni concentrations (unamended and Ni-amended) in each pot. Pots (10 cm square and 10 cm tall) contained a 6.5 cm tall sealed partition extending diagonally across the bottom from corner to corner. The partition was made using a fitted piece of plastic sheeting which was held in place to the bottom and sides of the pot with 100% silicone sealant (General Electric, Huntersville, NC). The soil used for this experiment was a mix of equal parts commercial potting soil (Sunshine #8 by Sungro, Agawam, MA) and sand: this coarser soil made separation of roots from soil easier at the end of the experiment. Unamended soil was placed on one side of the partition and Ni-amended soil was placed on the other. The Ni-amended soil was made by adding powdered $NiCl_2$ (Fisher Scientific, Suwanee, GA) to achieve a Ni concentration of 800 g Ni kg^{-1} dry mass. After each side of the pot was filled with its respective soil to the top of the partition, an additional 3 cm deep layer of unamended soil was placed over the partition and a single layer of perlite was added to aid in seedling germination and establishment. Seeds (~10) were placed on top of the perlite in a line down the pot center directly over the partition. To keep seeds in place over the partition during germination, temporary plastic barriers approximately 5 cm high were placed on either side of the row of seeds 1.5 cm from the pot center and anchored into the soil.

Sequential pot experiments were conducted at the Auburn University Plant Science Research Center greenhouse: one experiment for each *Streptanthus* species. *Streptanthus polygaloides* was planted in February of 2014, while *S. insignis* was planted in February of 2018. Seeds were germinated during spring because spring in Alabama has a daylength regime similar to the locations in California in which these plants grow (seed germination in California occurs during winter rains, and flowering occurs with the onset of summer). Seedlings were thinned (to ~3-5 plants) in each pot one week after germination. Plants were maintained in the greenhouse under ambient light conditions at an average temperature of 23°C, with a relative humidity of 74%. Plants were watered twice daily, with Peter's Peat-lite 20-10-20 fertilizer added once a week.

These experiments included 40 replicates (pots) each of *S. polygaloides* and *S. insignis.* Pots in each experiment were arbitrarily arranged on a greenhouse bench and rotated on the bench once a week to minimize positional effects. Plants were grown for approximately five months, from February to June, until a sufficient amount of root biomass was achieved for measurements (based on root emergence from pot bottom). Roots were harvested from each pot half separately by thorough washing and removal of soil. Roots were dried for five days in an oven at 60°C and weighed to determine root biomass in each half of each pot. Root biomass data from each species were analyzed using paired T-tests in R, version 3.3.1 (R Core Team 2016) to test if root biomass of each species was equal in the two halves of each pot.

Seedling root responses to Ni (agar plates): To test if direction of seedling root growth was influenced by Ni, square petri dishes (10 cm length x 10 cm width x 1.5 cm height square dishes with grid, Fisher Scientific, Suwanee, GA) were filled with a 0.8% Murashige and Skoog (MS) basal salts agar medium (Murashige and Skoog 1962). After the agar solidified, a piece of Whatman 1001-185 Grade 1 filter paper (3 cm wide x 10 cm long) was placed onto the agar in the center of the petri dish to create a central source of experimental material (Ni or a water control). The filter paper either was saturated with a 100 µM Ni solution (using $NiCl_2$ dissolved in deionized water) or with only deionized water (control). Seeds were sterilized via the following lab protocol. Seeds were washed with deionized water for 30 min, soaked in 70% EtOH for 15 min, and soaked in 20% bleach + tween for 15 min. They were then rinsed with sterile water, and soaked in sterile water for 30 min, before plating for germination onto MS medium petri dishes. Seeds were placed in a growth chamber (16 hr light: 22°C/8 hr dark: 18°C) for 3 days for seed germination, and seedlings were then transferred to experimental petri dishes.

A grid system was present on one side of the petri dishes, with grid lines spaced 1.5 cm apart. A seedling of each species was placed in each of 120 petri dishes (60 control and 60 Ni-amended): seedlings were randomly placed (either ca. 1.5 cm left or ca. 1.5 cm right of the edge of the center filter paper) at the intersection of a vertical and a horizontal grid line. The petri dishes were sealed and incubated vertically for 12 days in a growth chamber to allow roots to grow downward. Each petri dish was photographed after 12 days.

Photographs were used for root measurements by two evaluators in a double-blind study to reduce bias (evaluators were unaware of species or treatment conditions in each photograph). Results were consolidated and determined to be identical. The direction of primary roots in relation to the central filter paper location were scored either as displaying positive, negative, or neutral growth. Grid lines on the petri dish were useful to the evaluators in judging the general direction of primary root growth. Primary root direction data (towards/neutral/away) were analyzed using separate Chi-square tests for each species in R, version 3.3.1 (R Core Team 2016). In addition, the number of lateral roots on each side of each seedling (and therefore either growing towards or away from the central filter paper location) were counted and the length of each lateral root was measured. Measurements were done using photographs and adjusted for scale using the known lengths of the gridlines. Mean lateral root length in each direction (towards or away from the central filter paper location) for each seedling was calculated by dividing the sum of the lateral root lengths in that direction by the number of the lateral roots in that direction. Both count data and mean lengths of lateral roots were analyzed using 2-way analysis of variance (ANOVA) in R (R Core Team 2016). The two factors used in the ANOVA included species (*S. polygaloides* and *S. insignis*) as well as a treatment/direction factor. The treatment/direction factor combined filter paper source and direction of root growth to produce four categories: Ni/positive root direction, Ni/negative root direction, control/positive root direction, control/negative root direction). In the two-way ANOVA, we were particularly interested in the interaction of the two factors, as a significant interaction would indicate that the hyperaccumulator and non-hyperaccumulator species responded differently to Ni. Count data and lateral root length data were arc-sin transformed prior to analysis to achieve normality and meet the assumptions of the statistical tests.

Agar medium from the seedling experiment was also analyzed for Ni concentration to document the Ni gradient created in the Ni-treated petri dishes. Agar in each dish was cut into five sections: one interior portion which was underneath the filter paper (and thus contained the Ni source), two middle sections containing agar between the filter paper (source location) and the locations of the seedlings on either side of the source, and two outer sections (each containing agar from a seedling location to the edge of the petri dish). Nickel concentrations were determined via acid digestion and inductively coupled plasma optical emission spectroscopy (ICP-OES, PerkinElmer, Inc., Weltham, MA). Samples were dried for 48 h, digested with 100 µl concentrated nitric acid (HNO_3) and heated for an hour at 102°C. Afterwards, 200 µl of purified milli-q water was added to dilute each sample and Ni concentrations were determined via ICP-OES. Blanks (containing no sample) were included to determine background Ni values and, in addition, a standard (Sigma-Aldrich, Reference material no. 679 cabbage powder, European Commission, Bureau of Reference) with known Ni concentration was used to verify the accuracy of the analysis. The measured Ni concentration in the standard reference material was 27.4 ± 2.6 µg Ni g^{-1} (mean ± SEM, N=14; the reference value for this material was 26.2–27.84 µg Ni g^{-1}). Nickel concentration data from the three locations (center, middle, outer) in each Ni-treated petri dish were analyzed using one-way ANOVA followed by Tukey’s test for pairwise significance comparisons in R (R Core Team 2016).

RESULTS

Root proliferation pot experiment: Comparisons of root masses from each side of the divided pots showed that *S. polygaloides* responded positively to Ni while *S. insignis* exhibited no response. For *S. polygaloides*, root biomass in the Ni-amended soil (Figure 1a) was 2-fold greater than in the unamended soil (paired T-test: $t = 9.2$, $df = 39$, $P < 0.01$). In contrast, *S. insignis* root biomass (Figure 1b) did not differ between the two sides (paired T-test: $t = 0.72$, $df = 39$, $P = 0.64$).

Seedling root responses to Ni (agar plates): Primary roots of the two species responded differently to Ni. *Streptanthus polygaloides* primary roots showed directionality depending on the treatment of the central filter paper (Chi-square test: $x^2 = 33.4$, $df = 2$, $P < 0.01$) (Figure 2a). Most *S. polygaloides* primary roots (78%) grew towards the Ni-treated filter paper (Figure 3a), but there was no directional response (Figure 3b) when Ni was absent (control filter paper). In contrast, most primary roots from seedlings of *S. insignis* grew downward (Figure 2b), showing no directional response to either the central Ni source (Figure 3a) or the control paper (Figure 3b) (Chi-square test: $x^2 = 4.5$, $df = 2$, $P = 0.11$).

For directional lateral root initiation, the 2-way ANOVA showed a significant effect of species ($F_{1,\,250} = 20.6$, $P < 0.01$), no effect of the treatment/direction factor ($F_{1,\,250} = 1.7$, $P = 0.15$), and a significant interaction between the two factors ($F_{1,\,250} = 5.0$, $P < 0.01$). For the species factor, *S. polygaloides* (Figure 2c) produced about 60% more lateral roots than *S. insignis* (Figure 2d). The significant interaction between the species and treatment/direction factors was due to the strong positive response to Ni by *S. polygaloides* seedlings. As shown in Figures 2c & 3c, 90% more lateral roots were initiated towards the central Ni treatment than away. In contrast, similar numbers of lateral roots were produced in each direction by *S. polygaloides* seedlings in the control treatment (Figure 2c). In contrast to *S. polygaloides*, *S. insignis* seedlings produced similar numbers of roots in each direction in both Ni and control treatments (Figures 2d & 3d).

Mean lateral root length data displayed results similar to lateral root count data. The 2-way ANOVA showed a significant species effect ($F_{1,\,250} = 33.4$, $P < 0.01$), no effect of the treatment/direction factor ($F_{1,\,250} = 3.7$, $P = 0.18$), and a significant interaction between the two factors ($F_{1,\,250} = 8.2$, $P < 0.01$). For the species factor, *S. polygaloides* (Figure 2e) produced about 90% longer lateral roots than *S. insignis* (Figure 2f). The significant interaction between the species and treatment/direction factors was due to the greater lengths (ca. 58%) of *S. polygaloides* roots produced towards the central Ni treatment compared to away from the Ni treatment (Figure 2e). In contrast, similar lengths of lateral roots were produced in each direction by *S. polygaloides* seedlings next to the central control treatment (Figure 2e). In contrast to *S. polygaloides*, *S. insignis* seedlings produced similar lengths of lateral roots in each direction in both Ni and control treatments, however, lateral roots growing next to a central control treatment were 65% greater in length than lateral roots growing next to a central Ni source (Figure 2f).

The Ni-amended filter paper treatment successfully created a gradient in agar Ni concentrations across Ni-treated petri dishes. Agar sections differed significantly in Ni concentration (ANOVA: $F_{2,\,177} = 34.5$, $P < 0.01$), with the highest Ni concentration of 1.6 ± 0.32 μg Ni g^{-1} (mean ± SEM; ~88 µM Ni) in the center section (underneath the filter paper Ni source). The middle section contained 0.92 ± 0.14 μg Ni g^{-1} (mean ± SEM; ~46 µM Ni), and the outer portion of the petri dish contained 0.52 ± 0.12 μg Ni g^{-1} (mean ± SEM; ~23 µM Ni). All pairwise comparisons of these Ni values were significantly different (Tukey's test, $P < 0.05$).

DISCUSSION

Hyperaccumulator plants have evolved efficient physiological mechanisms to take up and store particular elements. These mechanisms, recently reviewed by Deng et al. (2018), include stimulated absorption by roots, increased xylem loading, and enhanced ability to transport these elements from roots to storage sites in shoot and leaf tissues. Once hyperaccumulated elements reach leaves, a plant may have specific detoxification mechanisms and/or an ability to store the element in a compartment that is not metabolically active (Rascio and Navari-Izzo 2011). Progress has been made in understanding the roles of these processes in Zn and Cd hyperaccumulation in Brassicaceae (e.g., Krämer 2010), however less is known about the physiology of Ni hyperaccumulator plants (Deng et al. 2018). Most of the research exploring hyperaccumulation mechanisms focuses on the cellular mechanics of Ni uptake and transport within a plant (Deng et al. 2018).

Root growth responses may be an additional important mechanism of hyperaccumulation but seldom have been studied (e.g., Boyd et al. 2000; Whiting et al. 2000; Haines 2002; Moradi et al. 2009; El Mehdawi et al. 2015). Despite the rarity of these studies, all but one of these investigations have demonstrated positive root responses of hyperaccumulators to an element. For the Zn hyperaccumulator, *T. caerulescens*, Haines (2002) demonstrated positive root proliferation in high-Zn soil patches, while Whiting et al. (2000) reported both increased lateral root branching and length, as well as increased root hair length. El Mehdawi et al. (2015) reported that the Se hyperaccumulator, *Symphyotrichum ericoides*, showed directional growth toward Se as well as produced longer and a greater number of lateral roots. The current study showed that the Ni hyperaccumulator species, *Streptanthus polygaloides*, responded positively to Ni by directional growth of the primary root as well as increased lateral root initiation and elongation. These positive root responses, found in multiple species, suggest that root traits may be important to hyperaccumulation ability and as an aid in elemental foraging.

Phenotypic plasticity in root growth, architecture, and spatial placement might be a response to soil's heterogeneous nature (Weiser et al. 2016). Numerous studies have illustrated root proliferation patterns in response to gradients of water and nutrients (e.g. Drew 1975; Hodge et al. 1999; Bao et al. 2014). However, the spatial scale at which this stimulus influences root architecture is not well understood. Bao et al. (2014) determined that differences in the availability of water, across a gradient as small as the diameter of a root, create spatial cues that help determine the position of lateral roots. Similarly, a gradient in Ni concentration that occurs across the diameter of a *S. polygaloides* root may stimulate root directionality and lateral root initiation and elongation. DTPA-extractable (bioavailable) Ni in serpentine soil varies greatly by site but has been reported as ranging from 21 to 280 µg Ni g^{-1} (Bani et al. 2013). In our agar plate study, *S. polygaloides* seedlings responded to very low Ni concentrations created in the gradient (ranging from 0.5–1.6 µg Ni g^{-1}) suggesting a sensitive mechanism exists for detecting variation in Ni concentration. More research is needed to understand the Ni transporters that are involved during root uptake processes (Deng et al. 2018), however, perception, signaling, and response to Ni may be involved in preferentially placing roots and encouraging directional growth in Ni hyperaccumulators.

Statwick et al. (2016) suggested that elements such as Se, Ni, and Cd can benefit hyperaccumulators of these elements and enhance their growth despite the apparent cost of uptake. They proposed the term "elemental stimulation" for this phenomenon. Several other studies (Ghasemi et al. 2014; Meindl and Ashman 2017; Mincey et al. 2018) suggest a hyperaccumulated element improves the growth or reproductive potential of a hyperaccumulator.

The current study's results are in accord with the "elemental stimulation" hypothesis, as Ni enhanced root growth and stimulated lateral root production in *S. polygaloides*. The only other test of *S. polygaloides* roots (Boyd et al. 2000) also reported that the species showed increased root length in the presence of Ni and suggested that *S. polygaloides* may require elevated soil Ni for optimum root growth.

Boyd and Martens (1992) reported several adaptive explanations for the trait of hyperaccumulation, including a number of hypotheses suggesting ecological benefits. The most investigated hypothesis suggests that high elemental concentrations in a hyperaccumulator function as a defense against herbivores (Boyd and Martens 1992). A positive root response toward Ni could be an important mechanism for acquiring Ni when it is distributed in the soil heterogeneously, improving uptake and supporting an adaptive function of Ni. However, one of the hypotheses, the "inadvertent uptake" hypothesis (Boyd and Martens 1992), attributes no selective value to hyperaccumulation: proposing hyperaccumulation as an incidental consequence of enhanced nutrient uptake abilities. The "inadvertent uptake" hypothesis suggests that hyperaccumulation of an element may have evolved from physiological mechanisms that target uptake and sequestration of another soil ion. The results of the current study suggest that root elongation and lateral root initiation towards Ni might be preferential for the plant as an adaptive trait, not supporting the non-adaptive "inadvertent uptake" hypothesis.

The extreme elemental accumulation ability of hyperaccumulator plants has led to the concept of phytoextraction for remediating contaminated soils (Chaney 1983), which has attracted much research effort (e.g., Chaney et al. 1997; Salt et al. 1998; Pilon-Smits 2005; van der Ent et al. 2013). Phytoextraction employs a hyperaccumulating species to transport high quantities of elements from soil into harvestable parts shoots. Optimization of phytoextraction technologies requires in-depth knowledge of metal uptake and transport mechanisms in hyperaccumulator plants. Root foraging strategies, along with uptake and sequestration traits, may enhance the usefulness of hyperaccumulator plants for phytoextraction. Root proliferation by *S. polygaloides* in response to Ni is similar to that exhibited by *T. caerulescens* in response to Zn, for which Haines (2002) suggested the term "zincophilic root foraging." We suggest the responses of *S. polygaloides* roots to Ni display "nickelophilic root foraging" and encourage exploration of root growth responses of other hyperaccumulator species to more fully understand their role in elemental hyperaccumulation.

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FIGURES

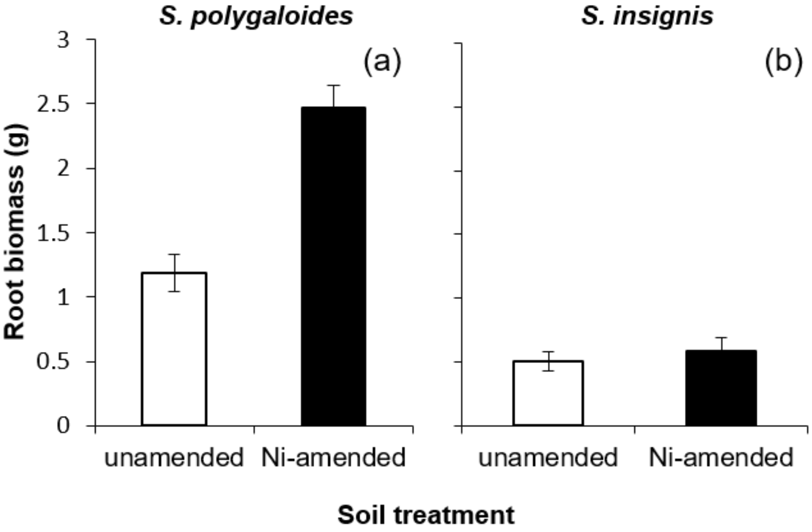

Figure 1. Mean dry root biomass for mature plants from pot experiment, (a) *S. polygaloides* (b) *S. insignis*. Error bars represent standard error of the mean (SEM).

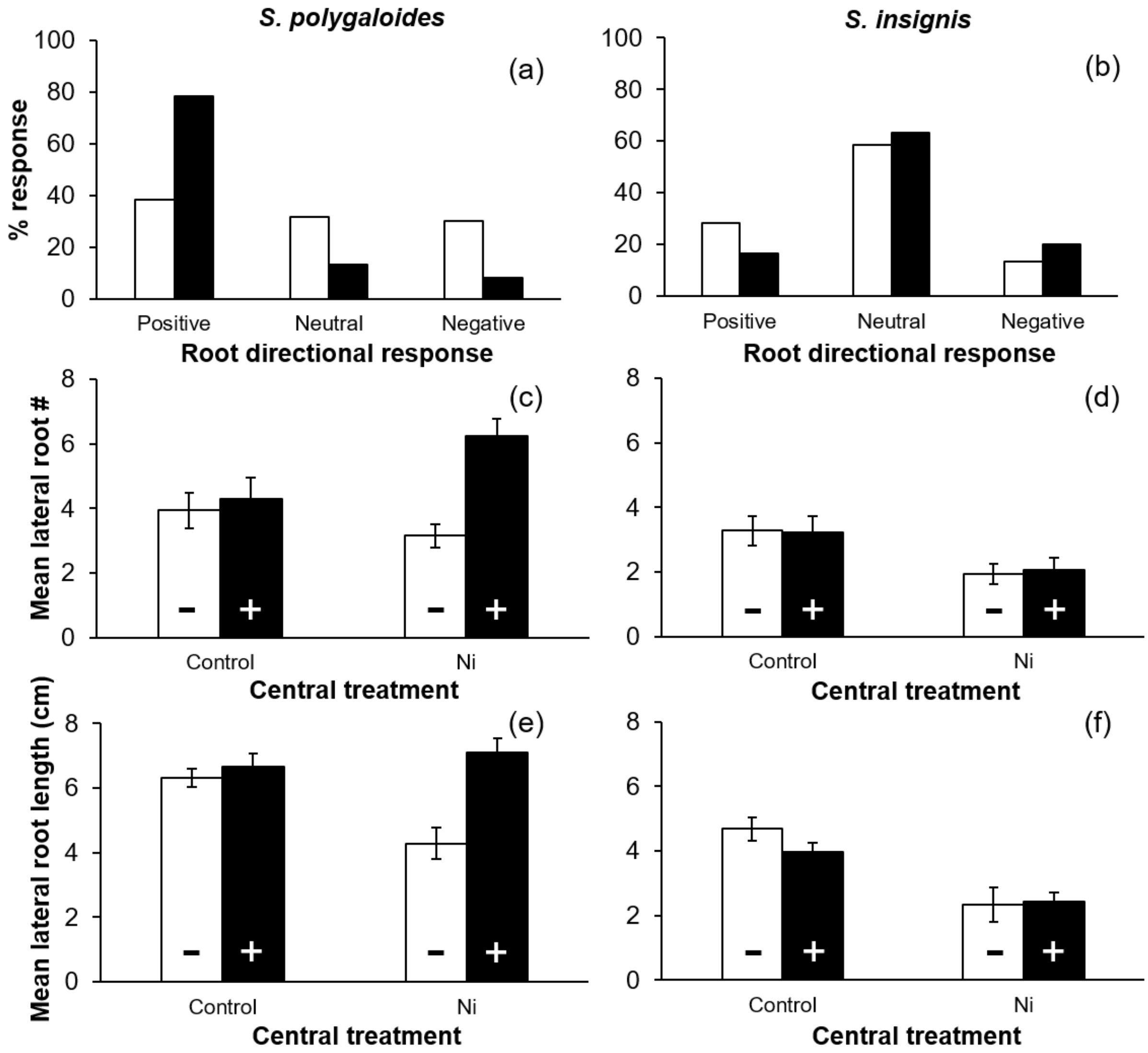

Figure 2. Root responses for petri dish experiment (left column: *S. polygaloides*, right column: *S. insignis*). Panels (a) and (b) compare root directional response percentage in relation to the central treatment (control: open, Ni-amended: solid). Panels (c) and (d) compare mean lateral root number. Panels (e) and (f) compare mean lateral root length in centimeters. For panels (c), (d), (e), and (f), open bars labeled (-) represents root response away from the central treatment whereas (+) on solid bars represents root response towards the central treatment. Error bars represent standard error of the mean (SEM).

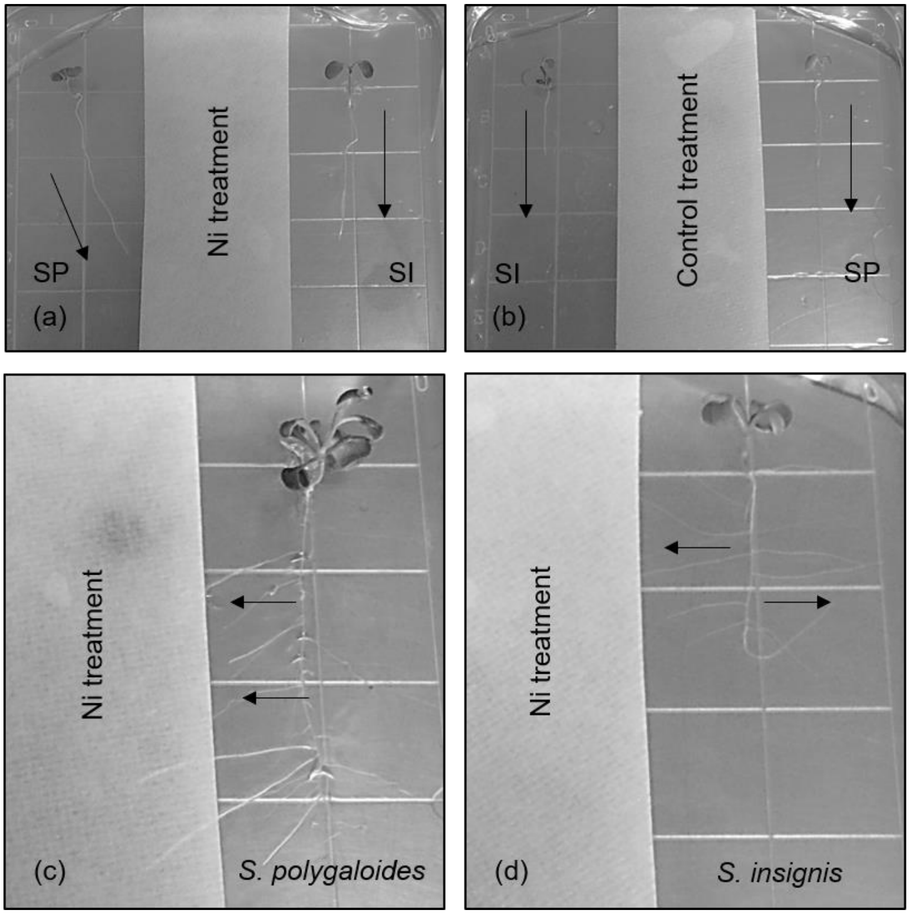

Figure 3. Photographs (a, b) show gridlines of petri dishes and examples of growth of primary roots in relation to central treatment (SP = *S. polygaloides*, SI = *S. insignis*). (a) shows a positive directional response to Ni for *S. polygaloides* and a neutral response for *S. insignis*. (b) shows a neutral response to the central control treatment for both species. Black arrows label the predominant direction of primary root growth in each photograph. Photographs (c, d) show gridlines of petri dishes and examples of growth of lateral roots relative to the central Ni treatment for both species. (c) shows a majority of *S. polygaloides* lateral roots growing towards the central Ni treatment. (d) shows *S. insignis* lateral roots growing both towards and away from the central Ni treatment. Black arrows in (c) and (d) label the predominant direction of lateral root growth in each photograph.