Serpentine populations of *Arabidopsis lyrata* ssp. *lyrata* show evidence for local adaptation in response to nickel exposure at germination and during juvenile growth

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Abstract Evolutionary divergence among disjunct populations that encounter different stressful conditions may be rapid due to limited gene flow. *Arabidopsis lyrata* ssp. *lyrata* (L.) O’Kane & Al-Shehbaz grows throughout the mid-Atlantic region in a variety of fragmented and stressful environments, including serpentine soils. The purpose of this study was to investigate the differential responses to heavy metals (particularly nickel and chromium, naturally occurring in serpentine soils) among three serpentine populations compared to two non-serpentine populations of *A. l.* ssp. *lyrata*, as evidence of local adaptation. The five populations were exposed to a nickel, chromium or control treatment during germination and juvenile growth. None of the populations had a negative growth response to chromium. All five populations had reductions in

growth when exposed to nickel. The greatest reductions were found in the two non-serpentine populations, which had reductions of 97-99% compared to the control. In contrast, the three serpentine populations had reductions of 23-83%, with most reduction in one of the serpentine populations (Robert E. Lee). Compared to the non-serpentine populations and the least tolerant serpentine population, the two most tolerant serpentine populations (Pilot and Soldiers Delight) had early lateral root formation, accumulated less nickel in their shoots and roots, and had high shoot phenolics. All three serpentine populations had increased serine levels in response to nickel and chromium. The tolerance mechanisms we found in the most tolerant serpentine populations serve as evidence for local adaptation to heavy metal stress in the non-hyperaccumulator *A. l* ssp. *lyrata*.

1. Introduction

Serpentine soils are stressful environments that are subject to drought, low calcium to magnesium ratios, and low availability of essential plant nutrients, such as calcium (Ca), phosphorous (P), and potassium (K) (Going et al., 2009; Murren et al., 2006), and high levels of heavy metals such as nickel (Ni) and chromium (Cr) (Bradshaw, 2005; Brady et al., 2005; Powell and Knight, 2009; Proctor, 1999). In addition, serpentine soils are home to a high number of endemics (Asemaneh et al. 2007; O'Dell et al., 2006), but there are species, known as bodenvags, that can tolerate serpentine soils, and are found growing on both serpentine and non-serpentine soils (Marrs and Proctor, 1976; Murren et al., 2006; Rajakaruna et al., 2003). The ability to grow under such a diverse array of conditions may be due to evolution of specialized ecotypes or a more generalist strategy (Bradshaw, 1965; Pigliucci, 2005; Schlichting,

1986). Specialization in the form of local adaptation may result in trade-offs such that specialists do worse in non-native environments (Lortie and Aarssen, 1996; Pigliucci, 2005), thus limiting gene flow and leading to higher degrees of differentiation.

Plants exposed to heavy metals have developed a variety of mechanisms to successfully grow under these stressful conditions. Toxicity prevention mechanisms may include exclusion or compartmentalization of heavy metals in either the roots or shoots to keep them from vital cellular components such as chloroplasts (Cobbett, 2000). Plants may also detoxify heavy metals utilizing phenolics, phytochelatins, metallothioneins, amino acids, among other secondary compounds (Cobbett, 2000; He et al., 2013; Kerkeb and Krämer, 2003; Manara, 2012; Mejáre and Bülow, 2001). Phenolics exert protective effects through chelation of heavy metals, scavenging of free radicals, and /or stabilizing membranes (Davis et al., 2001; Lavid et al., 2001; Michalak, 2006). Phytochelatins and metallothioneins are critical in chelation of heavy metals (Cobbett, 2000; Manara, 2012; Mejáre and Bülow, 2001). Amino acids are also critical in detoxification of a variety of heavy metals. Nickel is able to form relatively stable complexes with amino acids (Sharma and Dietz, 2006). Histidine, particularly free histidine in the xylem, act as ligands for Ni in the Ni hyperaccumulator *Alysum lesbiacum* and improves Ni tolerance in non-tolerant species such as *A. montanum* (Ingle et al., 2005; Krämer et al., 1996). Other amino acids that have been implicated in heavy metal tolerance are proline, glutamine, cysteine, serine, methionine, asparagine, and alanine (Sharma and Dietz, 2006). Maintaining a high internal Ca concentration has also been associated with reduced Ni toxicity in some serpentine species (Chaney et al., 2008). Therefore it seems that there is no universal approach to dealing with heavy metal toxicity.

Arabidopsis lyrata ssp. *lyrata* (L.). O’Kane & Al-Shehbaz (Brassicaceae) has a widespread and fragmented distribution (Clauss et al., 2006; Mable et al., 2005; Mitchell-Olds, 2001; O’Kane and Al-Shehbaz, 1997; Riihimaki and Savolainen, 2004). Throughout its range in the North Eastern and Mid-Atlantic region of the United States *A. l. ssp. lyrata* grows in a range of substrates such as rock, shale, calcareous sand, serpentine soil and alluvial flood plains. A study of microsatellite markers revealed genetic differentiation between serpentine and calcareous or shale populations of *A. l. ssp. lyrata* (Lloyd et al., 2011). Another study by Turner et al. (2010) showed strong differentiation between serpentine and granitic populations at genes that encode for putative heavy metal detoxification metallothioneins. Previous research in our lab showed evidence of cross-tolerance in calcareous, flood plain and serpentine populations to both Ca and Mg deficiency (Veatch-Blohm et al., 2013).

The purpose of this study was twofold. First we wanted to determine if there was local adaptation to heavy metals (particularly Ni and Cr) among serpentine and non-serpentine populations of *A. l. ssp. lyrata*. We also wanted to investigate possible heavy metal tolerance mechanisms employed by serpentine populations of *A. l. ssp. lyrata*.

2. Materials and Methods

2.1 Source material.

Five populations of *A. l. ssp. lyrata* were examined in this study. Three populations were collected from the State Line Serpentine Barrens of Maryland and were collected at the following locations: Soldiers Delight Natural Environment Area, Robert E. Lee Memorial Park, and Pilot Serpentine Barren Nature Preserve. The southernmost population was collected from Jug Bay Wetlands Sanctuary, which is in an alluvial flood

plain. The northernmost population was collected from the Roger Perry Memorial Preserve, which is limestone sand. Seeds were collected by removing one inflorescence per plant, with a distance of at least 1 meter between plants. Seeds from a single maternal plant were considered a half-sib family. Seeds from all populations were collected from 2008-2011, except for Soldiers Delight, where the most recent collection date was 2009. In addition to seed collection, soil samples were collected from each site by removing the first 10 cm of topsoil within a meter of a plant.

2.2 Soil analysis.

Soil samples from each location were analyzed for extractable cation concentration (Ca, Mg, K, Ni, Cr, Zn). Calcium, Mg, and K were extracted from the soil using ammonium acetate according to Bert et al. (2000). Nickel, Cr and Zn were extracted from the soil with 0.1 N HCl according to Jung et al. (2005). Elemental concentration in the extracts was analyzed using flame atomic absorption spectroscopy (FAAS) (Olsen and Ulicny, 2001). Soil pH was measured according to Bert et al. (2000).

2.3 Germination and seedling growth.

For the germination test seeds were taken from 15 half-sib families. All seeds were sorted and surface sterilized in a 50% ethanol: 0.1% Tween mixture for three minutes. The seeds were dried using 95% ethanol. The seeds were then placed in a 0.1% agar: Tween mixture at 4 °C in the dark for 4 days. The germination experiment took place in a growth chamber under 14/10 h day/night cycle at 22 °C and germination recorded for 18 days. Seeds were transferred to 2% agar plates that had modified Hoagland's solution (Epstein and Bloom, 2005) and one of three treatments: control, Ni or Cr. In the Ni containing solutions, Ni was present at $8 \mu\text{g}\cdot\text{g}^{-1}$ and in the Cr treatment Cr was present at

5 $\mu\text{g}\cdot\text{g}^{-1}$ (Shaw, 1991). All seeds were randomized within the plate, with four seeds from each population, and plates were arranged in the growth chamber in a completely randomized design with eight replications. Initially we tested germination and early root growth for the Pilot, Jug Bay and Perry Preserve populations, which was repeated three times. In addition to percent germination we examined uniformity and speed of germination (germination rate). Each day the number of seeds that germinated within a population that day were counted. Germination rate was calculated as $\sum_{i=1}^f S_i / D_i$ where S_i is the number of seeds that germinated on day i , D_i is the number of days since the first day of germination, and f is the final day of germination. (Maguire, 1962). A large number indicates that seeds germinated early and uniformly, while low numbers indicate either late germination or non-uniform germination or both. Root growth was measured for 5 days following germination. Each day a picture was taken of the root using a SPOT Insight digital camera (Diagnostics Instruments Inc., Sterling Heights, MI). Root length was then measured using Image J (Rasband, 1997-2005). During the third run of the germination test, seeds were followed for 11 days, and the day that the first lateral root was formed (root branching) recorded. A germination test as described above was also conducted for the three serpentine populations together (Pilot, Soldiers Delight, and Robert E. Lee) two different times.

2.4 Shoot and root analysis of juvenile plants.

Seeds from 15 half-sib families from each population were sown on pre-wet rockwool cubes and stratified at 4°C for four days. The seeds were then moved to a growth chamber under 14/10 h day/night cycle at 22 °C for 24 days. After 24 days the plants were irrigated with one of three irrigation treatments: control, Ni, and Cr, with all

solutions containing a modified Hoagland's solution as described for the germination experiments (Epstein and Bloom, 2005). The experiment was conducted three times between September 2012 and April 2014. Each run of the experiment was set up as a randomized complete block with six blocks. The plants were grown for 28 days. On a weekly basis, the number of leaves within the rosette was counted, and two perpendicular measurements of rosette diameter were taken.

As two of the populations responded poorly to the Ni treatments, plants were harvested after four weeks of growth to ensure the harvest of live plants in each population. Both the shoots and roots were harvested and washed with deionized water. It was impossible to separate roots completely from the rockwool substrate, but we removed as much of the root as possible. All plant parts were dried at 80 °C and dry weight recorded. A portion of each shoot and root sample was wet digested with nitric acid and prepped according to Pomper and Grusak (2004) and cation (Ca, Mg, Ni, Cr) concentration analyzed via FAAS. A second subset of each sample was subjected to methanol extraction as described by Cai et al. (2004). The methanol extracts were analyzed for antioxidant capacity using the FRAP assay (Benzie and Strain, 1996) and phenolic concentration using the Folin-Ciocalteu method (Ozsoy et al., 2008).

A sample of leaf tissue from the third run was freeze-dried and analyzed for the following amino acids: aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, and methionine. Amino acids were extracted from 1-50 mg of crushed freeze-dried samples by adding 3.0 mL of deionized water (18 Ohm) and holding in a bath at 80°C for 45 minutes (Ingle et al. 2005). The samples were

then centrifuged and the extracted supernatant samples containing the amino acids were frozen for later analysis. Aliquots of 100.0 μ L of the extract were pipetted into centrifuge tubes and dried under a vacuum. Following drying, samples were either immediately derivatized or stored for later derivatization. Derivatization was done using Edman's reagent following the example protocol from ThermoFisher. After derivatization and drying, the derivatized samples were frozen and stored until analysis via HPLC. Standard amino acid samples were prepared following the same procedure using a range of 0-40 μ L of a stock standard solution (ThermoFisher 20088) to generate a calibration curve. An internal standard of hydroxyproline was added to half the samples and standards to monitor retention times and amino acid derivatization. Detection of amino acid standards and samples was done using reverse phase HPLC (Agilent 1100) on a C18 column (15 cm, 4.6 mm, 5 μ m) using a gradient elution with detection at 254 nm using appropriate modification of the methods from González-Castro et al. (1997). Derivatized samples and standards were dissolved in the mobile phase buffer prior to injection into the HPLC.

2.5. Statistical analysis

The data was analyzed with the general linear model using the fit model platform of JMP 10 (SAS Institute, 1998-2008), except root branching, which was analyzed using ordinal logistic regression. The slope of root growth regressed on day was used to compare the differential success of early seedlings from all populations on each of the growth substrates. All germination and seedling characteristics had run, population, nutrient treatment, population x nutrient treatment, and run x nutrient treatment as factors in the model in JMP 8, with plate nested within nutrient treatment (SAS Institute, 1998-

2008). Data associated with juvenile plants had initial leaf number as a covariate, with run, block, population, nutrient treatment, and population x nutrient treatment as factors in the model. Phenolic and antioxidant concentrations, cation concentrations, and amino acid concentrations were natural log transformed to meet the assumption of normality for data analysis. Multiple comparisons were done using Tukey's HSD. A *P*-value of ≤ 0.05 was considered significant throughout.

3. Results

3.1 Soil Analysis.

All populations had a soil pH ≥ 7 , except Jug Bay, which had a pH around 5 (Table 1). Extractable Ni was almost 1000 times greater in the serpentine populations than in the limestone population (Table 1). Extractable Cr concentration was negligible in both Perry Preserve and Jug Bay. Chromium concentration was also low in the serpentine soils, but the soils from Robert E. Lee had 10 times more Cr than the other two serpentine locations (Table 1).

3.2 Germination and Seedling Growth.

Population but not treatment significantly affected germination percent and germination rate and there was no population by treatment interaction (Table 2). Germination percentage and rate was highest in the Perry Preserve population, and reduced in the other four populations (Table 3). Although the Soldiers Delight population had the oldest seeds, the germination success was comparable to the other populations.

Root growth rate was significantly affected by population and nutrient treatment, and there was a significant nutrient treatment by population interaction

(Table 2). Nickel exposure significantly reduced root growth rate in all five populations, with the greatest reductions in Perry Preserve, Jug Bay and Robert E. Lee (Figure 1), which were 14%, 35% and 40% of the control rate, respectively. On the other hand seedlings from Pilot and Soldiers Delight had root growth approximately 60% and 70% the rate of the control rate, respectively.

Lateral root formation (branching) was significantly affected by population and nutrient treatment, and there was a significant population by nutrient treatment interaction (Table 4). By five days of root growth Jug Bay and Pilot had root branching in 27.6% and 36% of the seedlings exposed to Ni compared to only 3.33% in the seedlings from Perry Preserve (Table 4). By 11 days after germination the percent of seedlings in the Jug Bay and Pilot populations in all treatments had root branching in a minimum of 79-96% seeds, while root branching in the Perry Preserve population was approximately 35% lower in the Ni treatment compared to the control and Cr treatments (Table 4). When the three serpentine populations were compared to each other, there were significant differences among populations, but not treatments (Table 4). Lateral root formation was not different among the serpentine populations until 8 days after germination, where percent branching in seedlings from Pilot was the highest (grand mean 65%), from Soldiers Delight moderate (grand mean 53%), and from Robert E. Lee the lowest (grand mean 30%) (Table 4).

3.3 Juvenile Rosette Growth.

Growth, as measured by changes in rosette diameter and leaf number, was significantly affected by population and nutrient treatment, and there was a

significant population by nutrient treatment interaction (Table 5). Change in leaf number and rosette diameter was highly correlated ($R^2 = 0.78$, $p < 0.0001$); therefore, we focused on changes in leaf number.

All five populations had high growth rates in both the control and Cr treatments, with the Jug Bay population generally having the highest growth rate and Perry Preserve the lowest growth rate (Figure 2). In the Ni treatment growth was reduced in all five populations, but there were differences in how the populations responded (Figure 2c). The Pilot population had the most growth under nickel exposure, which was comparable to Soldiers Delight, but significantly higher than Robert E. Lee. All three serpentine populations grew significantly better than Jug Bay or Perry Preserve under Ni exposure, where new leaves stopped being produced somewhere between 14 and 21 days of treatment (Figure 2c). Leaf necrosis in the Jug Bay and Perry Preserve populations started at the youngest leaves and moved progressively to the older leaves (data not shown).

Shoot biomass production (rosette dry weight) was significantly affected by nutrient treatment and population, and there was a significant population by nutrient treatment interaction (Table 5). Jug Bay accumulated significantly more biomass in the control and Cr treatments than any of the other populations (Table 6). All 5 populations experienced significant reductions in biomass when exposed to Ni (Table 6). The reductions under Ni exposure were most dramatic in the Jug Bay and Perry Preserve populations, which experienced reductions of 97-99% compared to the control and chromium treatments. Of the three serpentine populations, Robert E. Lee experienced the greatest biomass reduction under Ni

treatment (83% mean reduction), while Pilot experienced the least (23% mean reduction) (Table 6). We were unable to measure root biomass accurately due to the difficulty of removing roots from the rockwool growth media; however, based on visual estimates during root collection, the control and Cr treatments produced more root biomass than the plants in the Ni treatment.

3.4 Cation concentration of shoots and roots.

Population and nutrient treatment significantly affected shoot and root Ni concentration and there was a significant population by nutrient treatment interaction for both (Table 5). Shoot Ni concentration under Ni treatment was highest in the Jug Bay, Perry Preserve, and Robert E. Lee populations (Table 6). The shoot Ni concentration of the Pilot population under Ni treatment was 43-57% less than the other populations. Root Ni concentration under the Ni treatment in the three serpentine populations was $\leq 50\%$ of the Ni concentration in the Jug Bay and Perry Preserve populations (Table 6). Chromium concentration of the shoot (overall mean $34.70 \mu\text{g}\cdot\text{g}^{-1}$) and roots (overall mean $192.42 \mu\text{g}\cdot\text{g}^{-1}$) were not significantly affected by population or nutrient treatment. Shoot Ca to Mg ratio was significantly affected by nutrient treatment only (Table 5), with the highest Ca to Mg ratio observed in the plants exposed to Ni regardless of population (Table 6). In contrast there was no effect of population or treatment on root Ca to Mg ratio (Table 5), which had an overall mean of 0.37.

3.5 Antioxidant, phenolic, and amino acid production of juvenile plants.

Shoot antioxidant concentration was significantly affected by population and nutrient treatment, and there was a significant population by nutrient treatment

interaction (Table 5). In both the Perry Preserve and Jug Bay populations the shoot antioxidant concentration significantly increased in response to Ni treatment (Table 7). The shoot antioxidant concentration of the three serpentine populations did not significantly differ based on nutrient treatment (Table 7). In contrast, root antioxidant concentration was not affected by population or treatment (Table 5). Shoot Phenolic concentration was affected by population and there was a significant population by treatment interaction (Table 5). Shoot phenolic concentration was highest in the Pilot and Soldiers Delight serpentine populations and was lowest in the Jug Bay population (Table 7). In the Robert E. Lee population phenolics were significantly increased under Ni treatment. The Soldiers Delight population showed a trend of increasing shoot phenolic concentration under Ni treatment; however, this trend was not statistically significant. Root phenolic concentration was significantly affected by nutrient treatment (Table 5), with the highest phenolic concentration on average in response to Ni (Table 7). The Perry Preserve, Jug Bay and Robert E. Lee populations had their highest root phenolic concentration under Ni treatment, while this was not the case for the Pilot or Soldiers Delight populations; despite this difference, there was not a significant population by treatment interaction (Table 7).

Due to limited plant material in the non-serpentine populations under Ni exposure, we were able to analyze the overall change in amino acids for all treatments only in the three serpentine populations. There were no significant population or treatment effects for any amino acids tested, except for serine (Table 5). The mean overall amino acid concentration averaged across treatments ranged

from 2.19 $\mu\text{mol} \cdot \text{g}^{-1}$ of tyrosine to 16.17 $\mu\text{mol} \cdot \text{g}^{-1}$ of serine (Figure 3a). Serine levels in general were highest while tyrosine and alanine were the lowest (Figure 3a). Serine levels were significantly higher in both the Cr and Ni treatments compared to the control (Figure 3b).

4. Discussion

4.1 Response of seedlings and juveniles to Cr

The bodenvag species, *A. l. ssp. lyrata* exhibited limited to no differences in response to Cr, despite the importance ascribed to it in the serpentine barrens (Brady et al., 2005; Briscoe et al., 2009; Pope et al., 2010). The only indication that there may be a Cr specific response was the increased serine concentration after Cr exposure (Fig. 3b). While the Cr levels we tested may not have been sufficiently high to induce stress, they were orders of magnitude higher than the Cr concentration we measured in serpentine soils (Table 1).

4.2 Seedling tolerance to Ni

On the other hand *A. l. ssp. lyrata* exhibited marked population differences in response to Ni. While all soils contained some Ni, only the three serpentine soils contained Ni in large concentrations comparable to or greater than the concentrations used in the experiments (Table 1). Suppression of root growth rate by Ni was apparent for seedlings from all populations, regardless of whether they were from serpentine or non-serpentine habitats (Figure 1). However, reductions in early root growth were most pronounced in the Perry Preserve (85% reduction), Jug Bay (67% reduction) and Robert E. Lee populations (60% reduction). Seedlings

in the Soldiers Delight population faired best under Ni exposure with only a 30% reduction in root growth rate compared to the control.

In addition, in the 2009 and 2010 experiments, where we compared one serpentine population to two non-serpentine populations, we initially noticed that Pilot (serpentine) seedlings appeared to put on lateral roots sooner when exposed to Ni compared to one of the non-serpentine populations, Perry Preserve (Table 4), which encounters almost no Ni in its natural habitat. Changes in root architecture are common responses to heavy metals and often function in stress avoidance (Khare et al., 2016; Moradi et al., 2009). By 11 days of growth, the root branching was consistently high across all populations, except for the Perry Preserve population in the Ni treatment. A slightly different trend was observed in the 2012 and 2013 experiments with the three serpentine populations, where there did not seem to be a differential branching response to Ni among the populations. However, the Pilot population still exhibited earlier root branching and at a higher frequency than either Soldiers Delight and Robert E. Lee populations (Table 4). In addition the Robert E. Lee population, which had the lowest overall growth under Ni of the serpentine populations, also had slower root branching than the more tolerant serpentine populations. This may be indicative of lower overall fitness of this very small, inbred population. To further elucidate the relative importance of root branching in early seedling growth to Ni tolerance future work should focus on the number of lateral roots formed in each population.

4.2 Juvenile rosette tolerance mechanisms

Although we observed early root branching with exposure to Ni in the Jug Bay population, these plants responded very poorly to Ni exposure during seedling growth (Figure 1) and juvenile rosette growth (Figure 2). The plants from the Jug Bay and Perry Preserve populations accumulated more Ni in their shoots than two of the three serpentine populations: Pilot and Soldiers Delight (Table 6). The poor growth experienced by rosettes of the Perry Preserve and Jug Bay populations in response to Ni (Figure 2) may be due to simple Ni toxicity. Shoot antioxidant activity was particularly high in these two populations, which may be indicative of reduced efficacy of detoxification mechanisms leading to increased production of reactive oxygen species (Manara, 2012; Michalak, 2006).

The three serpentine populations all expressed tolerance to Ni stress, but to varying degrees. While all three populations responded to Ni stress with significantly reduced biomass production compared to the control (Table 6), the response of Robert E. Lee plants to Ni was more severe and often resembled the responses of non-serpentine plants. Although all three serpentine populations can tolerate Ni in the substrate, by virtue of sustaining populations in serpentine barrens, they may employ different mechanisms of varying efficacy to accomplish tolerance, in addition to some mechanisms in common.

Shoot Ca to Mg ratio plays an important role in heavy metal tolerance (Chaney et al., 2008), while cell Ca plays an important role in activating cell defenses (Manara, 2012). In a previous study we observed that the Pilot serpentine population had a constitutively higher shoot Ca to Mg ratio than the Perry Preserve and Jug Bay populations regardless of the Ca to Mg ratio of the experimentally

imposed substrate (Veatch-Blohm et al., 2013). In this current study the shoot Ca to Mg ratio increased under Ni exposure in all populations (Table 6). In *Alyssum murale* increasing Ca in the nutrient solution reduced Ni uptake by the plant and improved plant biomass production (Chaney et al., 2008). Increasing shoot Ca to Mg ratio may be one of many tolerance mechanisms utilized by the serpentine populations, and perhaps the increase in shoot Ca to Mg ratio allowed the non-serpentine populations to survive as long as they did under Ni stress.

All three serpentine populations accumulated less Ni in their roots than the non-serpentine populations (Table 6). The plants in the Pilot population, which demonstrated the least biomass reduction under Ni exposure, also accumulated the least Ni within the leaves. Given that the Robert E. Lee had shoot Ni accumulation on par with non-serpentine plants, it appears that only the Pilot and Soldiers Delight populations have mechanisms to somewhat restrict Ni uptake in both shoots and roots, which may reduce their exposure to Ni toxicity. Included among candidate genes with polymorphisms that differed greatly between serpentine and granitic populations of *A. l. ssp. lyrata* are a tonoplast Ni transporter, a cation efflux/metal tolerance protein, and a membrane protein involved in zinc sequestration and homeostasis (Turner et al., 2010). The soils of both Pilot and Soldiers Delight have a fairly high Zn concentration, while the soils of Robert E. Lee do not (Table 1). There is evidence that Ni uptake is facilitated by Zn transporters (Manara, 2012), and that plants exposed to Zn in their native soils demonstrate tolerance to Ni (Baker et al., 1994; Schat and Vooijs, 1997; Von Frenckell-Insam and Hutchinson, 1993), indicating that cross-tolerance to Zn could be driving Ni tolerance in Pilot and

Soldiers Delight. Zinc has been accumulating in the soils of Jug Bay over the last couple of decades (Khan and Brush, 1994), which may apply additional pressure to the Jug Bay population and lead to changes in metal tolerance, or may be responsible for the root branching behavior we observed in the seedlings. Stress-induced morphological responses in the form of changes in architecture in roots exposed to Zn are known to occur in *A. l. ssp. lyrata*'s close relative, *Arabidopsis thaliana* (Remans et al., 2012), and insights from molecular work there may elucidate more about mechanisms in *A. l. ssp. lyrata*'s detoxification strategies.

Another difference between the serpentine populations and the non-serpentine populations were their phenolic concentrations, particularly during Ni stress (Table 7). Phenolics such as flavonoids and tannins may act as antioxidants or detoxify Ni through chelation (Michalak, 2006; Roitto et al., 2005). In scots pine there was an increase in condensed tannins in response to increased Ni, as well as changes in carbohydrate metabolism (Roitto, et al., 2005). Nickel exposure may be responsible for some of the reduced growth in the serpentine populations as assimilated carbon is shuttled to phenolic production. In some Ni hyperaccumulating species it has been observed that phenolics, particularly tannins, are expressed at a constitutively high level (Davis et al., 2001). We may not have observed any effect of Cr on growth because phenolics, particularly tannins, have been shown to effectively chelate Cr (Lavid et al., 2001), and the phenolic concentration present in all populations may have been sufficient to deal with the relatively small amount of Cr they were exposed to.

Due to the tolerance demonstrated by the serpentine populations we expected to see increased concentration of some of the most common amino acids (histidine and proline) involved in heavy metal response due to their role in detoxification of Ni (Sharma and Dietz, 2006). However, not only were these amino acids not elevated in response to nickel, but their average was quite low compared to glutamic acid, glycine, arginine, threonine and serine. Serine was the only amino acid that was significantly enriched during Ni exposure in *A. l. ssp. lyrata* (Figure 3). Serine is a component of and precursor for phytochelatins and glutathione, which are important in Ni detoxification and hyperaccumulation (Sharma and Dietz, 2006). Therefore it is not surprising that serine concentration is elevated in the serpentine populations under both Ni and Cr exposure. Our data on phenolics, antioxidants and amino acids act as a starting point to further elucidate specific compounds involved in tolerance, such as the concentration of important downstream products of serine.

A. l. ssp. lyrata has a disjunct distribution across a wide variety of growing substrates, making it an ideal model organism for studying local adaptation. Serpentine barrens impose very strong selection pressures on plants, which may be due in part to their high concentrations of heavy metals, and thus are considered a model ecosystem (Harrison and Rajakaruna, 2011). The three serpentine populations (Robert E. Lee, Soldiers Delight and Pilot) demonstrate tolerance to Ni exposure that is lacking in the two non-serpentine populations (Perry Preserve and Jug Bay). The Pilot and Soldiers Delight populations, which demonstrated the most tolerance (i.e., the least reduction in biomass), had the widest variety of putative tolerance mechanisms in place. Compared to non-serpentine populations, these

plants had earlier emergence of lateral roots as seedlings, lower accumulation of Ni by both the shoots and roots, and high shoot phenolic concentration in addition to having increased serine production in response to Ni exposure. Therefore our data provide physiological evidence for tolerance mechanisms involved in the local adaptation of these populations of a model organism *A. l. ssp. lyrata*, in a model ecosystem, serpentine barrens.

Figure 1. Mean root growth rate $\pm$ se for five days following germination of 5 disjunct populations of *A. lyrata* ssp. *lyrata* grown under three different nutrient treatments (control, Ni, Cr). Within each treatment each population had the following 'n': Perry Preserve (PP) and Jug Bay (JB) n = 96, for Pilot (P) n = 160, and for Robert E. Lee (REL) and Soldiers Delight (SD) n = 64. Populations with the same letter in the Ni treatment are not significantly different according to Tukey's HSD ($p < 0.05$).

Figure 2. Mean leaf number $\pm$ se measured every seven days for 28 days of irrigation with three different nutrient treatments (control, Ni, Cr) of 5 disjunct populations of *A. lyrata* ssp. *lyrata*. Perry Preserve (PP) and Jug Bay (JB) are non-serpentine populations, while Pilot (P), Robert E. Lee (REL) and Soldiers Delight (SD) are serpentine populations. For each population in each treatment n = 18.

Figure 3. a) Mean shoot amino acid concentration $\pm$ se of amino acids and **b)** Mean shoot serine concentration $\pm$ se after 28 days of irrigation with three different nutrient treatments (control, Ni, Cr) of three disjunct serpentine populations of *A. lyrata* ssp. *lyrata*. Means with the same lower case letter are not significantly different for nutrient treatment according to Tukey's HSD ($p < 0.05$). The treatments had the following 'n': control (26), Cr (31), Ni (9).

474 **Table 1.** Soil characteristics of five disjunct populations of *A. lyrata* ssp. *lyrata*
 475 collected within 1 meter of the plant (n=3).

	Perry Preserve	Jug Bay	Pilot	Robert E. Lee	Soldiers Delight
Substrate	Limestone Sand	Sandy Loam	Serpentine	Serpentine	Serpentine
pH	7.13	4.97	7.7	7.34	7.38
	Soil Extractable Cation Concentration ($\mu\text{g g}^{-1}$)				
Ca	206	110.01	0.5	42.78	3.076
Mg	275.48	203.53	345.48	1563.14	294.1
K	628.53	778.72	643.48	659.11	630.73
Cr	<0.001	<0.001	0.007	0.07	0.007
Ni	0.02	0.58	14.4	14.73	19.84
Zn	<0.001	0.75	5.59	0.02	6.43

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Table 2. *P*-values for population (P), nutrient treatment (T) and population by treatment interaction (P X T) based on the general linear model for germination percent, germination rate and root growth rate.

	Population	Treatment	P X T
Germination (%)	<0.0001	0.4246	0.1324
Germination Rate	<0.0001	0.2652	0.2248
Root Growth Rate	<0.0001	<0.0001	<0.0001

482 **Table 3.** Germination percent (%) and germination rate of 5 disjunct populations of
 483 *A. lyrata* ssp. *lyrata*. Means with the same lower case letter are not significantly
 484 different for populations according to Tukey's HSD ($p < 0.05$).

	Perry Preserve	Jug Bay	Pilot	Robert E. Lee	Soldiers Delight
Substrate	Limestone Sand	Sandy Loam	Serpentine	Serpentine	Serpentine
%	88.5 a	74.5 b	75.9 b	80.7 b	74.0 b
Rate	1.03 a	0.77 b	0.70 c	0.82 b	0.78 b

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Table 4. Mean formation of lateral roots (branching) expressed as a % of population at 5, 8 and 11 days after germination in 5 disjunct populations of *A. lyrata* ssp. *lyrata* under 3 different nutrient treatments (control, Ni, Cr). Presence or absence of root branching was analyzed using ordinal logistic regression. *P*-values from chi-square are listed below the results of each germination test.

Population	Treatment	Days after germination 2009/2010			
		<i>n</i>	5	8	11
Perry Preserve	Control	87	0.00	34.38	93.75
	Cr	86	7.14	32.14	100.00
	Ni	76	3.33	30.00	66.67
	<i>Mean</i>		3.49	32.17	86.81
Jug Bay	Control	69	7.69	46.15	96.15
	Cr	74	3.45	44.83	96.55
	Ni	75	27.59	72.41	89.66
	<i>Mean</i>		12.91	54.46	94.12
Pilot	Control	59	25.00	75.00	87.50
	Cr	64	15.00	70.00	85.00
	Ni	64	36.00	80.00	84.00
	<i>Mean</i>		25.33	75.00	85.5
			Population	Treatment	P X T
Branching by 5 days			0.0080	0.0010	0.0033
Branching by 8 days			0.0291	0.0003	0.1301
Branching by 11 days			<0.0001	<0.0001	0.0011
Soldiers Delight		2013/2014			
	Control	48	0.00	45.83	82.98
	Cr	47	0.00	46.81	80.43
	Ni	43	2.33	69.77	88.37
	<i>Mean</i>		0.78	54.14	83.93
Robert E. Lee					
	Control	47	0.00	29.79	78.72
	Cr	48	0.00	27.59	79.17
	Ni	52	0.00	34.62	74.51
	<i>Mean</i>		0.00	30.67	77.47

Pilot

Control	58	0.00	65.52	86.21
Cr	51	0.00	64.71	94.12
Ni	56	1.79	67.86	92.86
<i>Mean</i>		<i>0.60</i>	<i>66.03</i>	<i>91.06</i>

	Population	Treatment	P X T
Branching by 5 days	1.0000	1.0000	0.7873
Branching by 8 days	<0.0001	0.1520	0.8279
Branching by 11 days	<0.0001	0.1792	0.8507

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Table 5. Juvenile rosette *p*-values for population (P), nutrient treatment (T) and population by treatment interaction (P X T) based on the general linear model.

	P	T	P X T
Rosette Growth	<0.0001	<0.0001	<0.0001
Leaf Number	<0.0001	<0.0001	<0.0001
Rosette Dry Weight	<0.0001	<0.0001	<0.0001
Shoot Ni	0.0032	<0.0001	0.0004
Root Ni	0.0002	<0.0001	<0.0001
Shoot Ca:Mg	0.0653	0.0032	0.0969
Root Ca:Mg	0.7544	0.3994	0.7858
Shoot Antioxidants	0.3042	<0.0001	0.0117
Root Antioxidants	0.0635	0.4377	0.7105
Shoot Phenolics	<0.0001	0.1706	0.0356
Root Phenolics	0.1646	0.0176	0.3835
Shoot Serine	0.6799	0.0094	NA

Table 6. Mean biomass, nickel concentration, and shoot Ca:Mg ratio of five disjunct populations of *A. lyrata* ssp. *lyrata* after 28 days of irrigation with three different nutrient treatments (control, Ni, Cr). Means with the same lower case letter are not significantly different for population by treatment interaction according to Tukey's HSD ($p < 0.05$). Means with the same upper case letter indicate no significant difference in either population or treatment according to Tukey's HSD ($p < 0.05$). The 'n' for each population within a treatment is indicated within the parentheses.

	Perry Preserve	Jug Bay	Pilot	Robert E. Lee	Soldiers Delight	
Treatment	Limestone Sand	Sandy Loam	Serpentine	Serpentine	Serpentine	
Shoot Dry Weight (g)						<i>Mean</i>
Control	0.45 b (17)	0.98 a (18)	0.43 b (18)	0.48 b (14)	0.32 bc (17)	0.54 A
Chromium	0.43 b (16)	0.83 a (18)	0.36 b (18)	0.43 cd (15)	0.36 bc (17)	0.48 A
Nickel	0.01 f (13)	0.01 f (11)	0.33 cd (17)	0.08 e (12)	0.19 de (14)	0.14 B
Mean	0.32 C	0.69 A	0.37 B	0.35 BC	0.30 BC	
Shoot Nickel Concentration ($\mu\text{g}\cdot\text{g}^{-1}$)						
Control	19.66 d (12)	16.44 d (12)	15.79 d (12)	15.57 d (12)	15.59 d (12)	16.60 B
Chromium	23.59 d (12)	13.91 d (12)	22.26 d (12)	15.97 d (12)	20.49 d (12)	19.24 B
Nickel	850.15 a (8)	774.23 a (6)	370.16 c (12)	726.31 ab (7)	487.58 bc (11)	598.54 A
Mean	228.76 A	167 A	136.07 C	176.21 AB	165.61 BC	
Root nickel Concentration ($\mu\text{g}\cdot\text{g}^{-1}$)						
Control	83.68 cd (8)	89.58 cd (10)	63.05 cd (9)	90.06 cd (8)	252.69 c (11)	122.45 B
Chromium	139.94 c (8)	59.05 d (10)	82.43 cd (10)	112.64 cd (12)	58.21 cd (11)	88.75 B
Nickel	1291.39 a (3)	1037.10 a (4)	403.34 b (9)	556.31 b (7)	370.24 b (7)	604.61 A
Mean	298.06 A	234.78 AB	179.35 C	220.98 BC	207.29 C	
Shoot Ca:Mg Ratio						
Control	0.47 (12)	0.47 (12)	1.10 (12)	0.75 (12)	0.87 (12)	0.73 B
Chromium	1.24 (12)	0.32 (12)	1.86 (12)	1.07 (12)	0.86 (12)	1.07 B
Nickel	5.06 (8)	1.36 (6)	2.24 (12)	1.58 (7)	2.40 (11)	2.59 A
Mean	1.9	0.59	1.73	1.04	1.35	

Table 7. Mean shoot and root antioxidant and phenolic concentration of five disjunct populations of *A. lyrata* ssp. *lyrata* after 28 days of irrigation with three different nutrient treatments (control, Ni, Cr). Means with the same lower case letter are not significantly different for population by treatment interaction according to Tukey's HSD ($p < 0.05$). Means with the same upper case letter indicate no significant difference in either population or treatment according to Tukey's HSD ($p < 0.05$). The 'n' for each population within a treatment is indicated within parentheses.

(n)	Perry Preserve	Jug Bay	Pilot	Robert E. Lee	Soldiers Delight	
Treatment	Limestone Sand	Sandy Loam	Serpentine	Serpentine	Serpentine	
Shoot Antioxidant Concentration ($\text{TE} \cdot \text{g}^{-1} \text{ dw}$)						<i>Mean</i>
Control	1245 ef (10)	1150 f (12)	1677 b-f (10)	1258 c-f (8)	1879 b-e (8)	1419 B
Chromium	1236 ef (9)	1255 ef (12)	2434 bcd (10)	1290 def (8)	2080 b (10)	1666 B
Nickel	4141 a (3)	2165 ab (3)	1828 bc (9)	1743 b-e (3)	1840 b-e (6)	2152 A
<i>Mean</i>	1636	1309	1985	1348	1953	
Root Antioxidant Concentration ($\text{TE} \cdot \text{g}^{-1} \text{ dw}$)						
Control	14239 (16)	14010 (18)	18534 (16)	17973 (14)	22213 (14)	17290
Chromium	19318 (15)	12325 (18)	21483 (16)	15013 (14)	18777 (15)	17276
Nickel	19063 (5)	26863 (3)	20045 (14)	32542 (8)	17046 (10)	31708
<i>Mean</i>	16767	14023	20018	19822	19864	
Shoot Phenolic Concentration ($\text{GAE} \cdot \text{g}^{-1} \text{ dw}$)						
Control	0.39 de (10)	0.28 e (12)	0.42 cde (10)	0.31 de (8)	0.53 a-d (8)	0.37
Chromium	0.42 cde (9)	0.29 de (12)	0.75 a (10)	0.29 de (8)	0.46 b-e (10)	0.44
Nickel	0.30 de (3)	0.19 e (3)	0.70 abc (9)	0.80 ab (3)	0.72 abc (6)	0.60
<i>Mean</i>	0.39 BC	0.27 C	0.62 A	0.38 AB	0.55 A	
Root Phenolic Concentration ($\text{GAE} \cdot \text{g}^{-1} \text{ dw}$)						
Control	3.36 (16)	1.76 (18)	4.97 (16)	3.26 (14)	7.75 (14)	4.14 B
Chromium	2.59 (15)	2.06 (18)	6.43 (16)	3.39 (14)	3.44 (15)	3.59 B
Nickel	8.62 (5)	6.32 (3)	3.71 (14)	5.06 (8)	3.83 (10)	4.47 A
<i>Mean</i>	3.25	2.18	5.19	3.67	5.18	

Figure 1. Mean root growth rate $\pm$ se for five days following germination of 5
disjunct populations of *A. lyrata* ssp. *lyrata* grown under three different nutrient
treatments (control, Ni, Cr). Within each treatment each population had the
following 'n': Perry Preserve (PP) and Jug Bay (JB) n = 96, for Pilot (P) n = 160, and
for Robert E. Lee (REL) and Soldiers Delight (SD) n = 64. Populations with the same
letter in the Ni treatment are not significantly different according to Tukey's HSD
($p < 0.05$).

Figure 2. Mean leaf number $\pm$ se measured every seven days for 28 days of irrigation with three different nutrient treatments (control, Ni, Cr) of 5 disjunct populations of *A. lyrata* ssp. *lyrata*. Perry Preserve (PP) and Jug Bay (JB) are non-serpentine populations, while Pilot (P), Robert E. Lee (REL) and Soldiers Delight (SD) are serpentine populations. For each population in each treatment n = 18.

Figure 3. a) Mean shoot amino acid concentration $\pm$ se of amino acids and, **b)** Mean shoot serine concentration $\pm$ se after 28 days of irrigation with three different nutrient treatments (control, Ni, Cr) of three disjunct serpentine populations of *A. lyrata* ssp. *lyrata*. Means with the same lower case letter are not significantly different for nutrient treatment according to Tukey's HSD ($p < 0.05$). The treatments had the following 'n': Control n = 26, Cr n = 31, Ni n = 9.

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