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Examining serpentine adaptation in *Aquilegia eximia* (serpentine columbine).

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Arts in Ecology, Evolution, and Marine Biology

by

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Abstract

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The main objective of this study was to identify traits involved in serpentine adaptation in *Aquilegia eximia* and to test whether serpentine tolerance has a simple genetic basis in this species. I assessed seedling survival and growth and measured trichome type frequencies of hybrid crosses between *A. eximia* (serpentine specialist) and *Aquilegia formosa* (soil generalist, and presumed progenitor of *A. eximia*) on field collected serpentine and non-serpentine soil. Seedling growth experiments revealed that *A. eximia* has smaller seeds, smaller seedlings at germination, as well as reduced total seedling biomass after 4 weeks of growth compared to *A. formosa* in nutrient rich non-serpentine soil. Chi^2 tests compared frequencies of seedling survival and growth phenotypes of parent species, F1 and F2 reciprocal cross types on serpentine and non-serpentine soil to test whether any traits follow expected Mendelian ratios for a single locus trait. Serpentine tolerance appears to have a generally dominant inheritance pattern for seedling survival and growth in the F1 and F2 hybrid crosses. Phenotypic frequencies of seedling survival and seedling growth rate on serpentine soil, as well as trichome type appear to segregate in Mendelian ratios in the hybrid mapping populations, suggesting a potentially simple genetic basis for these traits. In contrast, total biomass at harvest of seedlings grown on serpentine soil does not follow

expected Mendelian ratios in the hybrid mapping populations, and therefore appears to be a more complex polygenic trait.

Master's Thesis Chapter I

Overview:

This chapter contains a literature review of plant adaptations to harsh soil types, with a focus on studies detailing the genetic architecture of soil specialization.

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I. Introduction

Different climates, geologic disturbances, and anthropogenic activities across the planet create soils distinct in their physical and chemical characteristics (Rajakaruna 2004; Schimel and Chadwick 2013). Extremely high or low relative concentrations of essential nutrients or high concentrations of phytotoxic metals render soils harsh on the plants and microbes inhabiting them (Schimel and Chadwick 2013; Rajakaruna 2018; Harris 2016). Plant survival is also affected by soil texture and water holding capacity. These combined abiotic stressors interact with biotic factors such as interspecific competition, herbivores, and pathogens to form distinct edaphic boundaries (Rajakaruna 2018, 2004; Harrison and Rajakaruna 2011; Harris 2016).

This literature review focuses on soil specialist ecotype variability and their adaptations to multiple harsh soil types (summarized in Table 1). Harsh edaphic ecosystems are useful models to study how edaphic specialization driven by divergent evolution can contribute to biodiversity (Rajakaruna 2004, 2018; Harris 2016). Soil specialist taxa represent a large proportion of the plant species diversity in the world. For example, although harsh serpentine soil makes up approximately 1% of California's landmass, it supports 12.5% of plant species endemic to the California Floristic Province (Safford, Viers, and Harrison 2005).

Studying the genetic mechanisms underlying edaphic stress tolerance can also help us understand how plants physiologically adapt to survive in these types of environments (Rajakaruna 2018). This information may help scientists cultivate highly stress-resistant

crops (Garner et al. 2016; Cheeseman 2015), or identify harsh soil specialists that may be at risk from impending threats to biodiversity such as climate change, range restriction, or habitat destruction in order to make sure that they are included in conservation plans to protect these plant species from future extinction (Rajakaruna 2018; Harris 2016).

II. Current ecological and genetic approaches to studying edaphic adaptation in plants

Physiological tolerance of harsh soil is a complex response to multiple stressors. This section lists the current research approaches and summarizes the information they provide about the mechanisms behind the observed variation in plant species' adaptations to harsh soils.

1. Phylogenetic comparative studies of soil specialist taxa

Phylogenetic studies have examined large-scale evolutionary trends in edaphic tolerance across multiple taxonomic groups (Rajakaruna 2018), often using the barcoding regions of nuclear ribosomal genes (such as the ITS region) or various chloroplast markers to infer evolutionary relationships. Comparing multiple species in a single lineage can clarify whether edaphic tolerance evolved once in a common ancestor or evolved independently multiple times across the taxonomic group. If a group of closely related plant species, all adapted to the same soil type, form a monophyletic clade, then you can infer that soil tolerance evolved once in the common ancestor for that phylogenetic group. Alternatively, if

soil tolerance has multiple evolutionary origins, tolerant species are classified separately and nested between soil-intolerant species.

Evolutionarily labile traits are easily gained or lost across a phylogenetic group; for example, salt (Na^+) tolerance has evolved at least 59 times at the family level in the angiosperm phylogeny (Saslis-Lagoudakis 2014). Adaptation to serpentine soil has also evolved independently multiple times across many plant lineages (Anacker 2014; Anacker et al. 2011). The same has been found for heavy metal tolerance, although the heterogeneous distribution of metal tolerance across the angiosperm phylogeny suggests that some groups may lack the genetic variation required to evolve this trait (Ernst et al. 2006).

Phylogenetic studies have also explored evolutionary relationships at the genus, species, and population levels. In the plant family Brassicaceae, for example, serpentine tolerance appears to have evolved independently in at least three distinct clades within the "Streptanthoid complex" genus group (*Streptanthus, Caulanthus, Guillenia*), suggesting that serpentine tolerance in this group may be gained or lost through relatively few genetic changes (Pepper and Norwood 2001). Conversely, in a nuclear gene study in the genus *Ceanothus, C. cuneatus* was identified as the likely single progenitor of the widespread *C. cuneatus var. cuneatus* populations endemic to gabbro soil (Burge and Manos 2011).

2. Common garden and reciprocal transplant experiments

Plant species adapted to harsh soils often exhibit a cost to this tolerance, expressed as a decrease in fitness in non-native environments due to the adaptations required to thrive in

the local environment (Rajakaruna 2018). Detecting the costs of tolerance in plant populations can provide evidence that the plant taxon may have undergone local adaptation (although environmental filtering or species sorting may also cause a similar pattern) (Rajakaruna 2018). Alternatively, the plants may exhibit cross-tolerance and thrive equally in native and non-native environments (Rajakaruna et al. 2003; Rajakaruna 2018; Harris 2016).

The extent of local adaptation is commonly tested via reciprocal transplant experiments performed in the field or in a common garden (Hubbs 1941; Clausen, Keck, and Hiesey 1941). Reciprocal transplants growing in the field experience identical environmental conditions acting on the local plant population. Measuring phenotypic traits of field survivors on harsh soils vs. nutrient rich soils can allow investigators to identify traits involved in edaphic tolerance and physiological costs of tolerance, both of which may be driving adaptation to harsh soils. Further, sequencing the genomes of survivors in the field and comparing those genotypes of field survivors on nutrient rich soils can help identify locations in the genome associated with soil tolerance (and potentially genes controlling traits involved in soil adaption) (Anderson, Willis, and Mitchell-Olds 2011). However, field reciprocal transplant experiments are often labor-intensive and time-consuming; thus, many studies also utilize common garden plantings or common gardens combined with soil reciprocal transplant experiments to study potential adaptive traits (de Villemereuil et al. 2016).

In a common garden experiment, plants from many populations are grown on the same plot (usually a greenhouse or growth chamber) to ensure identical environmental conditions. In a soil reciprocal transplant experiment, a soil specialist species and a soil

generalist species are planted in their native and non-native soils. Combining soil reciprocal transplantation with a common garden setup can uncover variation in potential edaphic tolerance traits by eliminating confounding variables such precipitation, climate, and biotic pressures, which may co-occur with geographic variation in soil type (de Villemereuil et al. 2016; Anderson, Willis, and Mitchell-Olds 2011). Further experiments using common gardens coupled with genomic sequencing can help identify the genetic basis of particular traits involved in edaphic tolerance (de Villemereuil et al. 2016).

3. Physiological studies of edaphic tolerance

Once reciprocal transplant experiments have determined the degree of local adaptation, elemental analysis of soil and plant tissues helps scientists understand the physiological mechanisms of tolerance. Measuring the essential nutrient and heavy metal concentrations provides information on the plant's chemical-stress responses and resource allocation in harsh soil.

Plant families growing in serpentine and metal-contaminated mine tailings have evolved diverse strategies of tolerating phytotoxic heavy metals and avoiding cell damage (Harrison and Rajakaruna 2011; Kazakou et al. 2008). Kazakou et al. (2008) reviewed multiple elemental analysis studies (Table 2) in which serpentine-adapted plant taxa were examined to compare the concentrations of metal ions in root/shoot tissues to concentrations of metals in the soil in order to identify different tolerance strategies.

Elemental analysis can also identify preferential uptake of limiting nutrients in depleted soils, such as the preferential uptake of calcium or tolerance of high cellular levels of magnesium in serpentine-endemic plant species (Brady, Kruckeberg, and Bradshaw 2005; Kazakou et al. 2008).

Fertilization experiments can assess how plants respond to available nutrients when growing in nutrient-depleted soils (Kazakou et al. 2008). Adding ions in varying concentrations and combinations can identify limiting nutrients and physiological thresholds of tolerance in soil specialist species compared to soil generalists. Kazakou et al. (2008) describe certain serpentine plants as opportunistic "latent competitors" that grow rapidly when nutrients are added to the system. Alternatively, other serpentine-endemic plants have lost the ability to respond to high levels of available nutrients and have lower growth rates than non-serpentine plants.

Hydroponic experiments involving serpentine-adapted *Mimulus guttatus* and *Lasthenia californica* have been used to assess whether low Ca:Mg ratios, or high Mg or Na concentrations were the primary chemical stressors for each plant species (Palm, Brady, and Van Volkenburgh 2012; Rajakaruna et al. 2003). Palm, Brady, and Van Volkenburgh (2010) found that serpentine-adapted ecotypes of *M. guttatus* tolerate significantly higher Mg tissue concentrations compared to non-adapted ecotypes. Rajakaruna et al. (2003) used hydroponic ion addition on two edaphic races of *Lasthenia* to confirm that the harsh soil specialist Race A can tolerate much higher tissue concentrations of both Na and Mg than the soil generalist

Race C. Studies reviewed by Brady et al. (2005) also pinpoint either a low Ca:Mg ratio or Mg toxicity as the primary driver of serpentine adaptation in multiple plant species.

4. Genetic and genomic studies of soil adaptation

Increasingly cost-effective high throughput sequencing (HTS) techniques and the growing pool of angiosperm reference genomes are revolutionizing genomic research on ecological and edaphic adaptation. Genetic studies can help to determine whether adaptive traits arose from a few large-effect mutations, or from a complex polygenic response to multiple environmental factors.

The main genetic and genomic methods currently used and described in detail below are: (1) genome scan analyses designed to identify highly differentiated/divergent regions of the genome between soil specialist and soil generalist plant populations; (2) scans for selective sweeps to detect regions of the genome that have undergone recent positive selection; (3) QTL analysis of hybrid mapping populations to associate edaphic tolerance with particular locations in the genome; (4) transcriptomic analysis of gene expression to identify differentially expressed genes between edaphically tolerant and intolerant plant taxa.

4.1. Population resequencing/genome scan analyses

Genome scan analyses compare allele frequencies between populations, assuming that locally adapted alleles will occur at a higher frequency than neutral alleles (Strasburg et al. 2012; Anderson, Willis, and Mitchell-Olds 2011). Tests for genetic divergence, such as F_{st}

tests, utilize genome-wide SNP markers to identify highly differentiated regions of the genome between populations. The drawback of this method is the difficulty in detecting outlier loci in species with high baseline levels of genomic diversity (Hoban et al. 2016). The D_N/D_S test is an alternative; it detects the ratio of non-synonymous mutations to synonymous mutations in the protein-coding sequences of genes and can be used to analyze either genomic or transcriptomic datasets (Kryazhimskiy and Plotkin 2008). This test can identify amino-acid-changing mutations in suspected edaphic tolerance genes (Hawkins et al. 2017). The logic behind this test is that genes undergoing positive selection in edaphic tolerance due to changes in protein-coding would have a higher D_N/D_S ratio than genes not involved in edaphic tolerance.

Another genome scan method to detect regions having undergone recent natural selection is the extended haplotype homozygosity test (Sabeti et al. 2002; Arnold et al. 2016). Selection on a new adaptive mutation will carry linked neutral alleles to high frequency in a population (sometimes called genetic hitch-hiking**)** (Kim and Stephan 2002). Over time, this linked neutral variation can become unlinked due to recombination, and its frequency will drop back down. Thus, recently-selected regions can be detected by longer homozygous haplotypes compared to regions that are not part of the selective sweep (Arnold et al. 2016; Sabeti et al. 2002; Kim and Stephan 2002).

4.2. QTL analysis of edaphic tolerance

QTL analysis associates a quantitative phenotypic trait with a particular region of the genome using hybrid mapping populations between soil specialist and soil generalist plant species (Selby and Willis 2018). F1 hybrid plants are either self-pollinated or crossed to create an F2 mapping population. The parent plants and the F2 offspring are grown and phenotyped for soil tolerance traits of interest. F2 individuals are then sequenced across their genomes using HTS. Genetic markers that are linked to a locus influencing adaptive traits will segregate more frequently with the corresponding phenotypes. Statistical tests are then used to correlate the inheritance of specific DNA segments with soil tolerance (Andolfatto et al. 2011; Selby and Willis 2018).

QTL analysis can also identify whether soil tolerance has a simple genetic basis (e.g., few QTL peaks of large/moderate effect) or a complex genetic basis (many QTL peaks of small effect). QTL analysis thus narrows down the list of potential genomic regions involved in edaphic adaptation and their effect on variation seen in adaptive traits such as growth, metal tolerance, flowering time, etc. (Andolfatto et al. 2011; Selby and Willis 2018).

4.3. RNA-sequencing to examine differential gene expression

The previously described genomic analysis methods identify mutations in the DNA sequences but will miss epigenetic influences on phenotypes. RNA-Seq experiments analyze the transcriptomic mRNA present in the cells/tissues at the time of extraction (RNA-Seq methods reviewed by Wolf, 2013), and identify specific genes that are up- or down-regulated

in edaphically tolerant vs. intolerant species. Gene ontology analysis of differentially expressed genes may also identify what types of genes or gene families are involved in harsh soil-tolerance. Arnold et al. (2016) used gene ontology analysis on serpentine-tolerant and intolerant populations of *Mimulus guttatus* to identify several differentially expressed genes coding for cellular ion transport processes. Gene expression experiments are especially useful when coupled with genomic analyses like QTL analysis or genome scans because they can identify differentially expressed genes under QTL peaks or in highly differentiated regions of the genome.

III. Common characteristics of plant taxa adapted to harsh soils

The current literature review of edaphic specialists has identified the following common characteristics: (1) physiological costs of soil-tolerance; (2) specialized water use efficiency, osmoregulation, and ion-homeostasis; (3) speciation caused by edaphically driven reproductive isolation; (4) common patterns of genetic architecture.

1. Physiological costs of adaptation

Soil endemic taxa show a pattern of decreased fitness when grown in "normal" soil environments (Kazakou et al. 2008; Rajakaruna 2018). Numerous studies of serpentine-adapted plant species have shown that in non-serpentine environments, these species tend to have a lower growth rate than non-serpentine taxa. This suggests that soil tolerance has a metabolic or reproductive cost, perhaps due to specialized growth or increased production of

antiherbivory compounds (Harrison and Rajakaruna 2011; Kazakou et al. 2008; Meindl, Bain, and Ashman 2014). In heavy-metal-contaminated soils such as mine tailings, metalliferous plants often have lower growth rates, slower root growth, and lower reproduction than plants growing on non-contaminated soils (Ernst 2006).

2. Specialized water use efficiency, osmoregulation, and ion-homeostasis

The most common physiological adaptations to harsh soil types involve efficient water use and regulation of osmotic potential. Mine tailings, granite outcrops, and serpentine soils all have low water-holding capacities and cause drought stress in many plant species (Table 1). Therefore, drought and high temperatures act as significant selective forces in these coarse-textured, rocky soils (Rajakaruna 2018). Plant species have adapted by growing slower, producing shorter plants with xerophytic leaves, and flowering earlier to escape the summer drought (Brady, Kruckeberg, and Bradshaw 2005; Ferris and Willis 2018; Escudero et al. 2015; Mazer et al. 2010).

Another adaptation to extreme osmotic and chemical stress is specialized ion homeostasis. Many halophyte plants either sequester sodium in their vacuoles or exude it from their tissues (Salis-Lagoudakis et al. 2014; Cheeseman 2015). Plants growing in gabbro, serpentine and other metalliferous soils can exclude heavy metal ions at the root level or translocate them to the shoots and use specialized proteins to tolerate or accumulate them in their cells (Medeiros, Rajakaruna, and Alexander 2015; Harrison and Rajakaruna 2011).

Some edaphic specialist species show cross-resistance to stressful ionic soil conditions by translocating and tolerating extremely high concentrations of multiple different ions (Rajakaruna et al. 2003). A Californian ecotype of *Lasthenia californica*–Race A–shows cross-tolerance to both high levels of Na and Mg, and grows in a variety of harsh soil types including coastal bluffs, alkaline flats, serpentine outcrops, and saline soils (Rajakaruna et al. 2003). Rajakaruna et al. (2003) hypothesized that adaptation to one ionic stressor (either high Na or Mg) may have helped Race A tolerate additional stressor ions in the environment (Harris 2016; Rajakaruna 2018).

In addition to tolerating stressor ions, plants must also tolerate the absence of essential ions (such as N, P, K, etc.). Elemental analysis found that paired endemic and non-endemic species growing in serpentine seeps allocate Ca, Mg, K, and Co differently (DeHart et al. 2014).

Ca:Mg ratios are particularly important in serpentine and gabbro soils where [Ca] is extremely low and [Mg] is toxically high (Medeiros, Rajakaruna, and Alexander 2015; Kazakou et al. 2008). A hydroponic greenhouse study using *M. guttatus* found that high leaf-tissue concentrations of Mg decreased photosynthetic rates and total biomass in serpentine-intolerant ecotypes compared to serpentine-tolerant ecotypes, establishing low Ca:Mg ratios as the primary mechanism of serpentine tolerance in *M. guttatus* (Palm, Brady, and Van Volkenburgh 2012). Similarly, many other serpentine-adapted plant species preferentially uptake Ca and other limiting nutrients while excluding Mg and toxic metals at the root level (Brady, Kruckeberg, and Bradshaw 2005; Harrison and Rajakaruna 2011).

In my previous research project in the Oono lab, we examined how two widespread plant species differ in their foliar fungal endophyte communities and nutrient allocation patterns across the extremely stressful pygmy forest edaphic gradient at the Jughandle state natural reserve. Elemental analysis of *Vaccinium ovatum* leaf tissues (Table 1) revealed preferential uptake and overall higher levels of potassium in plants growing in depleted pygmy forest soil compared to plants growing in non-depleted soil (Oono et al. unpublished). Interestingly, Dehart et al. (2014) found that compared to non-endemics, serpentine-endemic species have higher potassium concentrations within all organ types. They suggested that potassium may be crucial to withstanding biotic and abiotic stressors in the serpentine environment (DeHart et al. 2014). Although serpentine soils and pygmy forest soils have vastly different pH levels, they both are depleted in nutrients and have increased openness, which potentially increases apparency to herbivores or pathogens (Strauss and Cacho 2013). Perhaps these plants are experiencing convergent evolution of K accumulation under extreme edaphic stress.

3. Speciation caused by edaphically driven reproductive isolation

In spite of the homogenizing gene flow between soil ecotypes, strong divergent selection across soil boundaries produces adaptations and reproductively-isolating traits which eventually lead to the divergence and formation of new soil-endemic taxa (Rajakaruna 2004, 2018).

The common pre-zygotic barriers to gene flow–shifts in flowering time and transitions to primarily selfing systems–are presumably adaptive to high temperatures and drought for plant taxa growing on serpentine, metalliferous soils, and granite outcrops (Harrison and Rajakaruna 2011; Ferris and Willis 2018; Mazer et al. 2010). For example, the granite-outcrop-endemic species *M. laciniatus* has earlier flowering, smaller flowers, and higher selfing rates than its primarily-outcrossing progenitor *M. guttatus* (Ferris and Willis 2018; Ferris et al. 2017). Self-compatibility increases the colonization success and reproductive assurance of *M. laciniatus* on granite outcrops (Ferris and Willis 2018; Ferris et al. 2017). The speciation of *M. laciniatus* from *M. guttatus* is an example of peripatric (or budding) speciation, caused by a widespread progenitor's colonizing a harsh marginal habitat (Ferris 2018; Rajakaruna 2018; Harrison and Rajakaruna 2011).

Another pre-zygotic reproductive barrier in certain heavy-metal-accumulating plants is pollinator avoidance of plants with high metal concentrations in their reproductive organs due to the toxic effects of metals on pollinator physiology. High levels of Ni in serpentine soil results in Ni accumulation in leaves, flowers, and pollen of many plant taxa (Meindl, Bain, and Ashman 2014). Plant species which evolved to exclude Ni at the roots had increased pollinator visitation and seed set compared to non-excluder species (Table 2), suggesting that high levels of heavy metals in pollen and other reproductive structures may affect pollinator behavior and cause pollinator avoidance of Ni-accumulating plant species in serpentine ecosystems (Meindl, Bain, and Ashman 2014). Furthermore, heavy metal

pollution from anthropogenic sources may affect pollinator health and behavior and remains an ecologically relevant topic for future studies.

Post-zygotic selection against hybridization in harsh soils reinforces the former pre-zygotic barriers to gene flow. Burge et al. (2011; 2013) examined barriers to gene flow across soil boundaries between the gabbro-endemic shrub, *Ceanothus roderickii*, and its widespread progenitor species, *C. cuneatus.* Burge et al. (2013) genotyped wild seeds and found that while these two species regularly hybridize, hybrid seedlings had lower survival on gabbro soil compared to parent *C. roderickii* seedlings (Table 3). This decrease in hybrid fitness on gabbro soil reproductively isolates *C. roderickii* from *C. cuneatus* when they occur in sympatry.

Overall, these studies show that variation in floral morphology, relationships with pollinators, shifts in phenology, mating system differences (selfing vs. outcrossing), and decreased fitness of hybrid offspring across soil boundaries can all cause reproductive isolation and lead to speciation and formation of new plant taxa endemic to harsh soil types (Rajakaruna 2004, 2018).

4. Simple genetic basis with large-effect mutations

The presence of mutations in a few loci of large effect is a common pattern seen within adaptations to serpentine, granite outcrops, and heavy metals (reviewed in Table 3). The idea that soil tolerance may have a simple genetic basis suggests that this trait may be evolutionarily labile (Rajakaruna 2018; Wright et al. 2013; Ferris et al. 2017). Phylogenetic

studies have confirmed multiple independent origins of salt, metal, and serpentine soil tolerance across several taxonomic groups, supporting the idea that soil adaptation can be easily gained or lost through few genetic changes (Anaker 204; Ernst 2006; Salis-Lagoudakis et al. 2014). It should be noted that many genetic studies of serpentine adaptation and heavy metal tolerance found candidate loci to contain multiple ion transporters either for nutrients, such as K or Ca, or heavy metals (Rajakaruna 2018; Turner et al. 2010; Arnold et al. 2016; Harrison and Rajakaruna 2011). The involvement of ion transport genes suggests that the genetic mechanisms of edaphic tolerance may involve specialized ion homeostasis or osmotic regulation.

Species or populations that are adapted to similar soil types do not always exhibit similar genetic architecture. Recent studies of serpentine-tolerant ecotypes of *Arabidopsis spp.* using population resequencing and genome scans for divergence identified multiple differentiated loci between serpentine tolerant and intolerant ecotypes of both *A. lyrata* and *A. arenosa*, providing evidence that serpentine adaptation appears to be a complex polygenic trait in both species (Arnold et al. 2016; Turner et al. 2010). However, QTL studies of serpentine tolerant ecotypes of *M. guttatus* and local adaptation to granite outcrops in *M. laciniatus* (summarized in Table 2) both identified single large-effect loci controlling edaphic tolerance for each edaphic specialist taxon (although the specific loci in the genome were different between studies), suggesting a simple genetic basis of harsh soil adaptation on both serpentine soil and granite outcrops in these *Mimulus* ecotypes/species (Selby and Willis 2018; Ferris et al. 2017).

Overall, evidence of a simple genetic basis primarily comes from studies using QTL analyses, while whole-genome scans and scans for selection frequently show many differentiated regions between soil ecotypes, suggesting a more complex genetic basis (Hoban et al. 2016; Strasburg et al. 2012). Further research is needed to clarify whether this discrepancy is a product of different evolutionary strategies or the result of method bias.

IV. Discussion

1. Recent breakthroughs in our understanding of natural selection and adaptive evolution

Classic common garden reciprocal transplant experiments provide evidence of a genetic basis to adaptation. However, the most significant breakthroughs in our understanding of adaptive evolution have come from studies using whole-genome sequencing to identify candidate genes and link them to physiological mechanisms of soil tolerance and reproductive isolation between soil endemic and non-endemic species (Clausen, Keck, and Hiesey 1941; Anderson, Willis, and Mitchell-Olds 2011). Identification of genes that confer tolerance to heavy metals, drought stress, and other toxic soil conditions will be important economically and societally as climate change continues to impact our natural resources.

Despite the complex physiological processes involved, harsh soil tolerance appears to result from a few genetic changes with large effects, compared to the classic idea that many genes of small effect contribute to these multifaceted phenotypes (Rajakaruna 2018; Ferris et

al. 2017). Adaptive genes may be pleiotropic or linked through chromosomal inversions, such as the case for *M. guttatus* growing in coastal saline soil (CITATION needed here). This topic will be clarified as more of these chromosomal inversions are identified with whole-genome sequencing (Gould et al. 2017).

2. Strengths and weaknesses of current approaches used to study edaphic specialization in plants

One major strength in the reviewed studies is the combination of field and lab experiments such as reciprocal transplants, elemental analysis of soil and plant tissues, and genetic analyses to identify the physiological and genetic mechanisms of edaphic adaptation. Genomic analyses can identify physical locations on the chromosome that may be involved in edaphic adaptation, but these regions can span thousands of genes. Field or greenhouse studies identify the physiological mechanisms of adaptation and can narrow down the lists of genes identified by QTL analysis or RNA-Seq experiments. For example, in a serpentine-adapted plant species that preferentially uptakes Ca, ion transporter genes contained in loci previously associated with tolerance could be identified as potential candidates for further study.

Whole-genome sequencing is another strong approach due to its high efficiency and cost-effectiveness. It has facilitated the study of the genetic mechanisms involved with edaphic tolerance in the *Phrymaceae* and *Brassicaceae,* which can be expanded to less-studied plant families (Rajakaruna 2018; Harrison and Rajakaruna 2011; Harris 2016). The

rising number of sequenced reference genomes within multiple plant lineages will also simplify genomic studies of adaptation.

A significant weakness in the study of edaphic variation is the binary categorization (e.g., serpentine vs. non-serpentine) of soil classes despite their high chemical and physical variability. A more precise classification of soil properties would help scientists identify the specific selective pressures acting on plants in these soil environments. This information, coupled with the known physiological thresholds of nutrient limitation in plants, could be used to predict plant response and survival to various soil conditions (Palm, Brady, and Van Volkenburgh 2012; DeHart et al. 2014; Rajakaruna et al. 2003).

Another significant weakness in the study of edaphic tolerance in plants is the failure to account for microbial interactions. My work with fungal endophytes across the pygmy forest edaphic gradient shows clear evidence that the nutrient composition of leaves, as well as host plant stress responses, can affect the plant-fungal symbiont abundance and community composition in host plant tissues. Symbiotic mycorrhizal associations at the root level have been shown to facilitate the absorption of otherwise unavailable soil nutrients, such as phosphorus in some metallophyte plants (Rajakaruna 2018; Harrison and Rajakaruna 2011).

Pathogen susceptibility can also be a significant biotic stressor in harsh soils (Rajakaruna 2018). Plants already under chemical and physical stress may also have to allocate resources to make secondary metabolites against pathogen and herbivore attacks (Strauss and Cacho 2013). These plant responses could be a significant contributor to the

costs of tolerance experienced by many soil endemic plant taxa and should not be overlooked.

Including these vital aspects of plant physiological tolerance to soils in future studies will be key to understanding the major drivers of edaphic adaptation. Therefore, to strengthen the study of adaptation within a particular plant ecotype or species, one should consider the physical and chemical properties of the abiotic environment (e.g., soil, climate) as well as the biotic interactions with pollinators, microbial symbionts, pathogens, and herbivores.

3. Avenues of future research

One avenue of future research is the combination of genetic studies (QTL and genome scan analyses), physiological studies in the greenhouse, and community studies of the microbial plant symbionts and pathogens (Strasburg et al. 2012; Rajakaruna 2018). Additionally, testing whether an edaphic specialist is locally adapted to its unique harsh soil environment or is cross-tolerant to multiple harsh soil types can help determine whether tolerance in a particular plant lineage has a simple or complex genetic basis (Rajakaruna 2018; Harris 2016; Rajakaruna et al. 2003).

Plant species that have evolved the ability to accumulate heavy metals are showing promise in soil phytoremediation to keep these pollutants from contaminating our food and water (Ernst 2006). Potential effects of heavy metals on pollinators and insect populations may also be a great cause for concern for pollination services to maintain natural plant populations and global food supplies (Meindl, Bain, and Ashman 2014).

Additionally, human population growth may significantly affect agriculture through rising scarcity of freshwater and prolonged droughts. Therefore, identifying genes that allow plants to tolerate saline soils may help us combat future food insecurity (Meindl, Bain, and Ashman 2014; Cheeseman 2015).

V. Conclusion

Studying plant variation across the vast diversity of soils can provide valuable insight into plant growth and survival in harsh environments. This information can be used to create conservation plans to protect soil endemic plant species in case of future range restriction or land development. Further, candidate genes identified by genetic studies of soil adaptation can be used to engineer crops that can thrive in depleted or polluted agricultural lands.

VI. Tables

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Table 1: Common harsh soil characteristics that act as plant stressors and drive edaphic adaptation.

Soil Type	Soil Description	Chemical Stressors	Physical Stressors	Biotic Stressors	Commonly Described Plant Adaptations	References
Serpentine	Alkaline, ultramafic soils derived from serpentinite rock. Serpentine is often described as coarse-textured rocky soil low in organic matter with high levels of Fe, Mg, and other heavy metals. These outcrops have a disjunct range across the California Floristic Province and other locations worldwide.	-Low Ca, high Mg -Low Ca:Mg ratios -Low macronutrients -High levels of phytotoxic heavy metals such as Cr, Co, Ni, and Cu	-Low water holding capacity -Open environments -High UV light	-Increased herbivory due to the openness of serpentine environments. -Pollinator avoidance due to heavy metal accumulation in the flowers/pollen.	-Slow growth rate -High root:shoot ratios -Xerophytic (sometimes lobed) leaves -Drought adaptation -Early flowering time -High rates of self-fertilization -Regulation of Ca including selective Ca uptake and enhanced root-to-shoot allocation -Mg exclusion or elevated levels of cellular Mg -Heavy metal regulation including root-level exclusion or sequestration. Variable heavy metal uptake and root-to-shoot translocation in accumulator species.	Kruckeberg, 1985; Safford et al., 2005; Brady et al., 2005; Kazakou et al., 2008; Harrison and Rajakaruna, 2011; Strauss and Chacho, 2013; Meindl et al., 2014; Ferris et al., 2015

Table 1: Common harsh soil characteristics that act as plant stressors and drive edaphic adaptation.

Soil Type	Soil Description	Chemical Stressors	Physical Stressors	Biotic Stressors	Commonly Described Plant Adaptations	References
Gabbro	Alkaline, mafic soils derived from plutonic intrusive rock formations. Gabbro soils are chemically similar to basalt and are enriched with iron and Mg. Olivine gabbro often occurs adjacent to serpentine soils in certain areas of CA.	-Low Ca:Mg ratios (not as extreme as in serpentine) -Low K, P in some gabbro soils.	-Low water-holding capacities, depending on the variable amount of organic matter in the soil.	N/A	N/A	Medeiros et al., 2015; Burge et al., 2011; Burge et al., 2013
Metalliferous Soils / Mine Tailings	Soils contaminated by heavy metals as a result of human activities such as mining and/or pollution from agriculture or industrial waste.	-High levels of Ni, Cu, Zn, Cd, or other heavy metals.	-Low water-holding capacity -High UV light and temperature	-Historical or current anthropogenic disturbance	-Drought adaptation. -Evolved physiological tolerance to increased concentrations of toxic metal ions in the soil -Some metal tolerance is said to be mediated by plant associations with mycorrhizal fungi **-Refer to Table 2 for a summary of plant heavy metal allocation strategies.**	Ernst, 2006; Rajakaruna et al., 2018
Granite Outcrops	Exposed, rocky habits creating harsh, marginal habitats for plants. Plants are often found growing in heterogeneously dispersed patches of moss or gravel, which are subject to extreme seasonal drought conditions.	N/A	-High temperatures/UV light -Low water holding capacity	N/A	-Highly lobed leaves -Small statures -Fast development -Early flowering -High rates of self-fertilization	Ferris et al., 2015; Ferris et al., 2017; Ferris and Willis, 2018

Table 1: Common harsh soil characteristics that act as plant stressors and drive edaphic adaptation.

Soil Type	Soil Description	Chemical Stressors	Physical Stressors	Biotic Stressors	Commonly Described Plant Adaptations	References
Saline Soils	Saline soils occur in most biogeographic regions. Soils are high in sodium for multiple reasons including natural inputs such as the ocean, human addition of fertilizer for agriculture, or high salinity due to aridity.	-Sodium ion toxicity -Decreased photosynthetic efficiency -Inhibition of water uptake	-Osmotic stress	N/A	-Reduced osmotic stress through decreasing water loss and increasing overall water use efficiency -Reduced cellular expansion rates -Tight packing of photosynthetic proteins -Compartmentalization of Na in specialized vacuoles or Na secretion via specialized glands.	Salis-Lagoudakis et al., 2014; Cheeseman, 2014
Gypsum Soils	Soils derived from calcium sulfate dihydrate ($CaSO_4 2H_2O$). Gypsum bedrock originates from deposits of sea or lake water evaporation as well as from hot springs. The low solubility of this soil does not significantly increase osmotic stress like typical saline soils and does not create ion-specific toxicity in plants, which is why gypsum is used as a soil amendment on acidic and nutrient-poor soils.	-High sulfate levels -High Ca -High Ca:Mg ratios -Low macronutrients such as N and P	-Drought	-Biocrusts inhabited by cyanobacteria, mosses, and lichens form at soil surface. Plants must be able to physically penetrate these soil crusts with their roots to grow.	-Late flowering -Drought adaptation -Xerophytic leaves -Short stature -Seasonal dimorphism of photosynthetic biomass; the spring shoots having more abundant and productive (longer and thinner) leaves than the summer shoots.	Escudero et al., 2015

Table 1: Common harsh soil characteristics that act as plant stressors and drive edaphic adaptation.

Soil Type	Soil Description	Chemical Stressors	Physical Stressors	Biotic Stressors	Commonly Described Plant Adaptations	References
Pygmy Forest Soils	Pygmy forest soils are a product of continental uplift of 5 marine terraces. Continued weathering results in an edaphic gradient with the 3 older terraces containing depleted acidic soils that house small-statured "pygmy" plants with unique adaptations to environmental stress.	-Extremely acidic -Low macronutrients due to leaching -Limited nutrient exchange capacity	-Iron hardpan limiting rooting depth. -High UV light	-Potentially increased apparency to pathogens and herbivores.	-Small stature -Slower growth and lower specific leaf area, but the same area-based photosynthetic rates and stomatal conductance. -Greater carbon investment in the leaves -Higher tannins and other polyphenols in the leaves	Westman, 1975; Westman, 1978; Cary and Pitterman, 2018

Table 2. Heavy metal tolerance strategies in serpentine and metalliferous soils, characterized by elemental analysis of soil and plant tissues (Kazakou et al. 2008; Ernst et al. 2006)	
Excluders	Plants exclude metals at the root level by limiting the translocation of damaging metals to the shoots. Excluder species have lower concentrations of metal ions in their tissues compared to the soil.
Indicators	Indicator species contain the same concentrations of metal ions as the soil because they cannot exclude metal ions from their tissues.
Accumulators	Accumulator species have evolved the ability to translocate metals into their shoots; therefore, they have higher levels of metal ions in their tissues compared to the soil. They have also evolved mechanisms to tolerate toxic levels of metal ions in their cells.
Hyperaccumulators	Hyperaccumulator species accumulate more than 100x the metal ions in their shoot tissue compared to soil metal concentrations.

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Brassicaceae*	Serpentine vs. non-serpentine ecotypes of *Alyssum serpyllifolium*.	Multiple sites across Portugal and one site in Spain	(1) Are Ni-hyperaccumulating ecotypes of *A. serpyllifolium* genetically distinct species compared to other, non-serpentine A. *serpyllifolium* in this species complex? (2) Are two putative candidate genes involved in metal hyperaccumulation and ion homeostasis in *A. serpyllifoilium?*	- DNA was extracted from approx. 40 individuals from 4 serpentine and 4 non-serpentine populations. - PCR amplification and sanger sequencing of 8 microsatellites; 2 candidate genes involved in metal tolerance in other plant species (NRAMP4 & IREF1), and 1 reference gene not involved in metal tolerance (ASIL1).	- Genetic differentiation between serpentine Ni-hyperaccumulator and Ni-intolerant ecotypes was calculated using F_{st} tests and tests for deviations from HWE.	Ni hyperaccumulation (+/-)	- Genetic differentiation is high in both serpentine and non-serpentine populations. - Candidate genes for metal tolerance & hyperaccumulation NRAMP4 and IREG1 show far higher differentiation between serpentine and non-serpentine ecotypes than ASIL1, suggesting that these genes may be of adaptive significance regarding Ni hyperaccumulation in this species.	-Differentiation between serpentine and non-serpentine sites accounts for very little of the genetic variation seen in *A. serpyllifoilium* populations; however, NRAMP4 and IREG1 may be good candidates for Ni-hyperaccumulation in *A. serpyllifolium.*	Sobczyk et al., 2017

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Serpentine	*Brassicaceae*	Metal-Tolerant vs. metal-intolerant ecotypes of *Arabidopsis halleri*	Multiple sites in Bergamo province of Italy, southern Alps.	(1) What is the genetic basis of Zn-hyperaccumulation in metal-tolerant populations of *A. halleri?*	- Whole-genome sequencing of 4 parents, 2 F1, and 175 F2 hybrids for QTL analysis.	-6 clones from each F2 (1050 total) were grown in the greenhouse in either Zn polluted or non-polluted conditions. -Plants were phenotyped for four biomass and one physiological trait after 0, 2, 4, and 6 weeks of growth.	<u>Zn tolerance via:</u> -Root length -Leaf width -PSII yield -Dry biomass	- A single-effect QTL was associated with PSII yield and explained 27% of the observed phenotypic variation. This QTL signal got stronger with time.	-The identification of only one significant QTL indicates that Zn tolerance in *A. halleri* may have a relatively simple genetic basis. -However, the authors suggest that this result may indicate that Zn tolerance is a complex trait governed by many small-effect QTL not detected by this analysis, or that the traits measured were not appropriate to detect quantitative variation.	Karam et al., 2019

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Brassicaceae*	Serpentine vs. non-serpentine ecotypes of *Arabidopsis lyrata*	*A. lyrata* populations from the USA & *A. lyrata var. petrae* serpentine-tolerant population from Scotland	(1) What are the genetic mechanisms of serpentine tolerance in *A. lyrata*?	-DNA was pooled and sequenced to approximately 30x coverage from 25 individuals from 4 populations (2 serpentine, 2 granitic).	- Examining allele frequency differences and F_{st} tests identified differentiated genomic regions between populations.	Serpentine tolerance (+/-)	-96 identified variants across 82 loci containing soil-type-associated polymorphisms. -A few highly differentiated regions in the US *A. lyrata* populations were also polymorphic in serpentine and non-serpentine populations of distantly related Scottish subspecies *A. lyrata petrae*.	-Differentiated loci were shared across serpentine tolerant US and European *A. lyrata*. -Differentiated regions between soil types were enriched with gene ontology terms involved with metal ion transmembrane transporter activity, potassium transport, and calcium ion binding.	Turner et al., 2010

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Brassicaceae*	Serpentine vs. non-serpentine ecotypes of *Arabidopsis arenosa.*	Populations from Gulsen Mountain in Austria.	(1) What are the genetic mechanisms of serpentine tolerance in *A. arenosa*?	-24 autotetraploid individuals from 3 populations (1 serpentine & 2 non-serpentine) were individually barcoded and sequenced to approximately 21x coverage.	-Measuring nucleotide diversity; absolute divergence (D_{xy}); relative divergence (F_{st}), and tests for selective sweeps across the genome.	-Elemental analysis of soil sites coupled with elemental analysis of plant tissues grown from wild collected seed on fertile soils in the greenhouse.	-Greenhouse plants grown in fertile soil showed that plants from the serpentine population have increased tissue levels and uptake of K, S, Ca:Mg, Cu, Zn, and Cd. -Serpentine plants had much lower nickel than non-serpentine plants suggesting that serpentine population has evolved the ability to exclude Ni from their tissues.	-Multiple loci in serpentine *A. arenosa* appear to be introgressed from *A. lyrata,* suggesting hybridization may play a role in serpentine adaptation. -*A. arenosa* shared 11 highly differentiated genes with *A. lyrata* suggesting convergent evolution of genes encoding Ca and K transporters.	Arnold et al., 2016

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Brassicaceae*	*Caulanthus amplexicaulis var. barbarae* (serpentine tolerant subspecies) vs. *Caulanthus amplexicaulis var. amplexicaulis* (serpentine intolerant subspecies)	-*Caulanthus amplexicaulis var. amplexicaulis* (CAA1) inbred line derived from seed collected on granite outcrops in Los Angeles, CA -*C. amplexicaulis var. barbarae* (CAB1) inbred line derived from a serpentine barren in Santa Barbara County, CA	(1) What non-synonymous mutations in protein coding regions of transcriptomic sequences are under selection in serpentine populations of CAB1?	-Pooling and sequencing RNA from various tissues at various stages of development under differing environmental conditions from inbred lines of *C. amplexicaulis var. barbarae (CAB1)* and *C. amplexicaulis var. amplexicaulis (CAA1).*	-D_n/D_s was measured between CAB and CAA. -High D_n/D_s in this study was used to indicate positive selection (with some caveats). -Orthologous gene pairs with high D_n/D_s ratios underwent gene ontology enrichment analysis.	-Seedling growth on P-depleted media	*C. amplexicaulis* subspecies had a relatively high global mean D_n/D_s ratio compared to comparisons in other study systems (Table 1). -There was both evidence of positive selection and purifying selection in these transcriptomes.	-Authors found multiple enriched gene ontology terms including transcription factors and identified MYB-C transcription factor PHL1 as possible candidate for tolerance to limited phosphate. -Authors further examined PHL1, and confirmed that adaptation to low P environments is a major factor in serpentine tolerance in this species.	Hawkins et al., 2017

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Caryophyllaceae*	Serpentine vs. non-serpentine ecotypes of *Silene Vulgaris*	-Serpentine site is near Davos, Switzerland -Non-serpentine site is near Klosters, Switzerland.	(1) What is the number of QTLs and their magnitudes for Ni tolerance and other traits that differentiate ecotypes of *S. vulgaris?* (2) Are QTLs for different traits associated with serpentine adaptation located in the same genomic region? (3) What is the likelihood that selection for heavy metal tolerance generated the observed phenotypic differences among *S. vulgaris* ecotypes in the field?	-The most Ni-tolerant (serpentine) and most Ni-intolerant (non-serpentine) plants grown in the greenhouse were selected as parents for the cross. -The most Ni-tolerant F1 was selfed to create an F2 mapping population. -263 F2 individuals were phenotyped, then genotyped using AFLP markers.	-300 AFLP markers were used to genotype F2s and create linkage groups.	<u>Morphological Traits:</u> -Plant height -Leaf area -Flower number -Succulence <u>Life History Traits:</u> - # of days to first flower - # of days until germination <u>Physiological Trait:</u> -Nickel tolerance	-15 major-effect and 8 minor-effect QTLs for the 7 investigated traits. - Ni tolerance had 2 major and 2 minor QTLs -Certain QTLs for multiple traits were on the same linkage group segments of the chromosome.	-Nickel tolerance in *S. vulgaris* may have a relatively simple genetic basis. -Overlapping QTLs for multiple serpentine adaptive traits could be evidence of linkage or pleiotropic affects, which is consistent with evidence that serpentine evolution may be the result of few genetic changes of large effect.	Brattler et al., 2006

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine	*Phrymaceae*	Serpentine vs. non-serpentine ecotypes of *Mimulus guttatus*	Reciprocal field transplant experiments were performed at McLaughlin Reserve and Rose Hills BLM Area in Northern California.	(1) What is the genetic architecture of serpentine tolerance in *Mimulus guttatus*?	-DNA of F2 survivors grown on serpentine (n=44) and non-serpentine field sites (n=212) was pooled and sequenced using bulk segregant analysis. -Mean coverage of the serpentine pool = 18x; mean coverage of the non-serpentine pool = 7.5x.	-QTL analysis was performed for seedling survival in the reciprocal transplant and seedling growth. -Putative QTL identified by BSA analysis was confirmed by genotyping 1,216 F2s and parental inbred lines under the QTL peak.	-Height of first leaf at flowering -Flowering date	- Survival on serpentine was associated with a single locus of large effect on chromosome 13. -Linkage disequilibrium/segregation distortion was observed in serpentine survivors under in the QTL region that was consistent across seasons. -Common garden experiments showed that serpentine tolerance is a dominant trait.	-Survivorship of serpentine plants was higher in serpentine sites. On non-serpentine soil, serpentine plants grew larger than plants on serpentine but were smaller than non-serpentine plants, suggesting a cost of serpentine tolerance. -The large effect QTL appears to control a majority of variation in this trait, suggesting serpentine tolerance may have a simple genetic basis in *M. guttatus*.	Selby and Willis, 2018

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Serpentine / Granite Outcrops	*Phrymaceae*	*Mimulus filicifolious* (granite outcrop endemic) *M. laciniatus* (granite outcrop endemic) x *M. guttatus* = lobed leaf cross *M. nudatus* (serpentine endemic) x *M. guttatus* = narrow leaf cross Serpentine ecotype of *M. guttatus* with lobed leaves x *M. guttatus* inbred line	-Species crosses between multiple edaphic specialists of *Mimulus* were used to make F2s, segregating for leaf shape. -Hybrids were grown and phenotyped in the greenhouses at Duke University.	(1) What is the genetic architecture of leaf shape and diversification among three edaphic specialists in the *Mimulus guttatus* species complex?	-*M. laciniatus x M guttatus* inbred lines generated 650 F2s, segregating for leaf lobing. -Pools of the 100 most extreme phenotypes (lobed vs. unlobed) were sequenced using bulk segregant analysis and leaf lobing QTL analysis. -300 *M. laciniatus x M. guttatus* F2s were genotyped at 3 genetic markers under the leaf lobing QTL to confirm BSA.	-108 *M. nudatus* (serpentine endemic) x *M. guttatus* narrow leaf cross F2s and 384 M2L (lobed leaf serpentine *M. guttatus*) x *M. guttatus* (unlobed, non-serpentine) F2s were genotyped at 3 polymorphic markers in the genomic region beneath each *M. laciniatus* QTL.	<u>Leaf Shape:</u> -Leaf narrowness (length x width) -Leaf lobing (using convex hill analysis)	-3 QTLS were confirmed with BSA analysis and single marker genotyping of a random selection of F2s under the QTL peaks. All three loci explain 52% of the difference in mean parental leaf shape. -Divergent leaf shape is quantitative in all three species and a large degree of parallelism exists between the genetic architecture of lobed leaves in *M. laciniatus*, narrow leaves in *M. nudatus* and lobed leaves in the serpentine *M. guttatus*. -Shared genetic architecture of leaf shape variation in the crosses provides evidence of parallel leaf shape evolution at the QTL level.	-Leaf shape may have been derived from segregating variation in ancestral populations of *M. guttatus*. Therefore, *M. laciniatus*, *M. nudatus*, and *M. filicifolious* are all derived peripatric populations of M. guttatus. -Segregating variation in leaf lobing traits may have been repeatedly selected upon when ancestral species encountered dry rocky habitats. Alternatively, leaf lobing may have evolved multiple times through similar genetic mechanisms due t evolutionary constraint due to negative pleiotropy.	Ferris et al., 2015

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Granite Outcrops	*Phrymaceae*	*Mimulus laciniatus* (granite endemic) vs. *Mimulus guttatus* (mesic meadow population).	Sympatric populations of *M. laciniatus* and *M. guttatus* at Shaver Lake, Sierra Nevada Region, CA.	(1) What is the genetic architecture of divergence in flowering time, mating system related traits, and leaf shape between granite outcrop endemic *M. laciniatus* and the adjacent *M. guttatus?*	-1000 F2s, 67 *M. lacinatus* inbred line parents and 133 *M. guttatus* inbred line parents were grown in the greenhouse and phenotyped for morphological and life history traits. -8 *M. laciniatus* + 1 *M. guttatus* parent were sequenced to approx. 31x coverage. 424 F2s were multiplexed and shotgun sequenced to low coverage for genotyping.	-QTL analysis was performed on the genotyping data of the parents and 424 F2s to determine whether these divergent traits between species had a simple genetic basis.	-Leaf shape -Flower size -Node of first flower -Flowering time	-All 5 morphological and life history traits had a relatively simple genetic basis. Each trait was controlled by 4-5 QTL of large-moderate effect, and these loci explained up to 64% of segregating variance in the F2 population. -There was a large-effect pleiotropic QTL on chromosome 8 that controls differences in flowering time, node of first flower, and leaf shape (= LG8b), which explained the largest proportion of variance in the F2 population for all 6 characters. - TCP4is a potential candidate under the largest effect pleiotropic QTL LG8b, which is involved in repression of petal growth, leaf cell differentiation, and the transition to flowering in *A. thaliana*.	-Divergent phenotypes involved with reproductive isolation between sympatric *M. laciniatus* and *M. guttatus* populations appear to have a relatively simple genetic basis, with a large-effect pleiotropic QTL. -Many QTLs colocalize with loci detected in previous genetic mapping experiments of flowering time, flower size, and leaf shape on chromosome 7, 8, and 10, suggesting that variation at these loci was segregating in an ancestral *M. guttatus*-like population.	Ferris et al., 2017

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Soil Type	Plant Family	*Spp.* Comparison	Study Location	Main Study Questions	Expt. Design/ Sample Sizes	Methods	Traits Measured	Results & Evolutionary Patterns Detected	General Conclusions	Refs.
Copper	*Phrymaceae*	Populations of copper-tolerant vs. adjacent copper-intolerant ecotypes of *Mimulus guttatus*	Keystone Mine, Copperopolis, CA	(1) Do copper tolerance and hybrid lethality have the same molecular basis?	-Crossed copper-tolerant and non-tolerant populations to create F1 backcrossed hybrid lines. -Genotyped 4,340 F1 backcrossed individuals at genetic markers along the chromosome to fine-map the copper tolerance locus.	-Collected seed from unique maternal families on the mine (n=108) and 2 off-mine populations located 2 km (n=33) and 9 km (n=29) away. -Measured genetic variation of 8 loci in the Tol1(Cu tolerance fine-mapped region) and 11 Tol1 unlinked loci. -Compared F_{st} of on/off-mine populations at the copper tolerant and unlinked reference loci.	-Cu tolerance(+/-) -Hybrid lethality (scored in multiple grow-outs per line)	-In order to determine if hybrid lethality and copper tolerance were controlled by the same locus, researchers crossed 9 Cu-tolerant and 9 Cu-intolerant lines with a hybrid lethal line which yielded two recombinant plants. -Authors were able to fine-map Cu tolerance and hybrid lethality in this Cu-tolerant population of *M. guttatus* that occurs on a mine tailing in Copperopolis, Ca. - F_{st} was significantly elevated between populations occurring on and off the mine in the Cu-tolerance associated markers compared to the unlinked markers.	-Recombinants between Cu-tolerance and hybrid lethality reveal that this is not a single gene with pleiotropic effects, but two distinct loci that are tightly linked. -Hybrid inviability may have increased in frequency in the Cu-tolerant ecotype due to genetic hitchhiking when the Cu-tolerant alleles rose to fixation in the Copperopolis population due to natural selection.	Wright et al., 2013

Table 3: Studies aiming to discover the genetic architecture and mechanisms of edaphic adaptation in multiple plant taxa.

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Saline	*Phrymaceae*	Perennial coastal vs. annual inland ecotypes of *Mimulus guttatus*	Populations were sampled across California.	(1)What is the genome-wide pattern of allelic differentiation between two geographically widespread plant ecotypes? (2) Do chromosomal inversions have higher levels of differentiation in allele frequencies than colinear regions of the genome? (3) Which genes are most differentiated in allele frequency between coastal and inland ecotypes and thus candidates for adaptation?	-101 individuals from 47 coastal ecotype populations and 92 individuals from 50 inland populations were pooled to form "coastal" and "inland" pools, respectively. -Pools were sequenced using BSA to approx. 262x coverage per pool.	-The F_{st}, G, and the ratio of nucleotide diversity between ecotypes was calculated for each genomic window. -Authors also focused on divergence and differentiation in previously identified adaptive QTL regions containing chromosomal inversions.	-Annual vs. Perennial growth forms -Salt tolerance (+/-)	-Coastal ecotypes of these plants are perennial, have large leaves, wide stems, large flowers, and produce many prostrate vegetative branches and are late-flowering. -Coastal populations are more tolerant to salt spray and soil salinity, and can accumulate Na in their leaves without experiencing necrosis. -Inland populations are annual, produce small leaves, thin stems and small flowers with primarily upright flowering branches and earlier flowering times.	-Only 4 SNPs were completely fixed between coastal and inland population pools; 2 were in the chromosome 8 inversion and only 1 was in a gene region, within an intron. -Authors identified a set of candidate genes for adaptive divergence in development, flowering time, branching architecture, and salt tolerance between the ecotypes.	Gould et al., 2017

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Gabbro	*Rhamnaceae*	*Ceanothus roderickii* (gabbro endemic species) vs. *Ceanothus cuneatus* (soil generalist species)	Pine Hill region, El Dorado County, CA	(1) Does hybridization and introgression affect species reinforcement across edaphic boundaries of gabbro endemic *C. roderickii* and soil generalist *C. cuneatus*?	-288 parent plants from 6 locations (3 populations of *C. roderickii* on gabbro soils and 3 adjacent populations of *C. cuneatus* on non-gabbro soils) and 288 naturally set seeds from select parent plants were genotyped using AFLPs.	-AFLP genome scans were used to examine genetic exchange across edaphic disjunction. -Greenhouse growth trials grew seedlings on/off native soils to test for local adaptation to respective soils types and whether there is selection against hybrid progeny on parental soil types.	-Flowering time -Pollinator guild surveys -Field-based tests of infertility -Seedling germination & growth	-Despite close proximity, there is very little hybridization between the two focal species across soil disjunctions. -Prezygotic barriers to pollination and fertilization were weak. -Greenhouse experiments showed that there was selection against hybrid offspring survival in parent soils	-Selection against hybrids in parent soils in peripatrically occurring species across the edaphic disjunction may maintain reproductive isolation and reinforce speciation of *C. roderickii.*	Burge et al., 2013

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Master's Thesis Chapter II

Overview:

This chapter contains a summary of my research project conducting a QTL experiment measuring multiple serpentine tolerance phenotypes in hybrid crosses of *Aquilegia eximia* and *Aquilegia formosa* on serpentine and non-serpentine soil. The main objective of this study was to test whether serpentine adaptation in *A. eximia* has a simple genetic basis.

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Figure 12. Comparison of seedling growth rate (final/initial seedling biomass) by cross type after 4 weeks of growth on serpentine soil. Average seedling growth rate for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the

number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=1.102, df=3, p=0.35). ANOVA found that the mean seedling growth rate was significantly different between all cross types (F ratio=3.31, df=3, p=0.0215). Connecting letters were generated using Tukey HSD ordered differences report.

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Figure 15. Comparison of seedling growth rate (total biomass at harvest/initial seedling biomass) of F2 hybrids by trichome type on serpentine (S) and non-serpentine soil (NS). Average seedling growth rate for each trichome type is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test for seedlings grown in non-serpentine soil (F ratio=0.004, df=1, p=0.948) and serpentine soil (F ratio=1.80, df=1, p=0.185). ANOVA found that mean seedling biomass was not significantly different between F2s with glandular (E) and non-glandular (F) trichomes when grown in non-serpentine soil (F ratio=0.08, df=1, p=0.784). ANOVA found that mean seedling biomass was significantly different between F2s with glandular and non-glandular trichomes when grown in serpentine soil (F ratio=4.07, df=1, p=0.048).

I. Introduction

Serpentine soil is a well-established model system for the study of plant adaptation to extreme soil and environmental conditions. For many plant taxa, serpentine tolerance involves multiple physiological responses to environmental stressors such as drought, toxic chemicals, and herbivory (Ch I- Table 1). Despite evidence that serpentine specialization has a genetic basis (reviewed in Ch I-Table 3), it is still unclear whether, in general, serpentine adaptation is controlled by single large-effect (potentially pleiotropic) locus, or if serpentine tolerance is more genetically complex, controlled by multiple loci, each having a small effect on the trait. It also completely possible that certain traits associated with serpentine tolerance are simple, while other traits have more complex genetic bases depending on what traits are measured and whether they are involved in serpentine tolerance in the plant species being studied.

Genetic studies in *Arabidopsis spp.* (Turner et al., 2010; Arnold et al., 2016) used population resequencing and whole genome scans to compare serpentine tolerant and intolerant ecotypes in order to identify differentiated regions in the genome that may be involved in serpentine adaptation (summarized in Ch I-Table 3). These studies both identify multiple genetic differences between serpentine tolerant and intolerant ecotype genome sequences, suggesting that serpentine tolerance is potentially a complex polygenic trait in these core Eudicot species.

A genetic study by Bratteler et al. (2006) used QTL analysis to measure 7 traits involved in serpentine tolerance (Ch I- Table 3). They found multiple 15 major and 8 minor

QTL peaks responsible for serpentine tolerance somewhat supporting the idea that serpentine tolerance in *S. vulgaris* is polygenic. However, QTL peaks overlapped for multiple traits, which could alternatively suggest that a simple genetic basis involving genetic linkage or pleiotropic effects. Ni tolerance in *S. vulgaris* only had 2 major QTL, suggesting that the metal tolerance component of serpentine adaptation may have a relatively simple genetic basis (Bratteler, Lexer, & Widmer, 2006). Similarly, a QTL study in *Arabidopsis halleri* also found a single large-effect QTL controlling Zn tolerance in serpentine tolerant F2 populations (Karam et al., 2019). Another recent study by Selby and Willis (2018) used QTL analysis to identify a single locus of large effect responsible for serpentine tolerance in *Mimulus guttatus* F2 hybrid populations, suggesting that survival on serpentine soil may have a simple genetic basis in this species (summarized in ChI- Table 3).

Studies using QTL analysis such as Bratteler et al. 2006, Selby and Willis (2018), and Karam et al. (2019) generally seem to find single large effect loci controlling traits involved in serpentine tolerance, suggesting that serpentine adaptation may have a relatively simple genetic basis. However, there is a caveat that serpentine tolerance may still be a complex trait controlled by small effect loci, but the QTL analysis could not detect those loci because the traits measured as a proxy for serpentine tolerance were not appropriate to detect quantitative variation in the F2s (Karam et al., 2019). Therefore, measuring traits that are actually involved in serpentine tolerance is crucial.

In the current study, I aimed to determine whether serpentine tolerance has a simple genetic basis in two members of the early branching Eudicot family, the *Ranunculaceae*. I

compared the serpentine-endemic species, *Aquilegia eximia*, with its presumed progenitor and sister species, the more-widespread soil generalist, *Aquilegia formosa*. Serpentine adaptation in *A. eximia* may be a complex physiological response to multiple environmental factors, thus, I aimed to measure multiple phenotypic traits in order to detect their potential involvement in serpentine tolerance in this species.

A common trait examined in genetic studies of serpentine adaptation is survival on serpentine soil (+/-). For example, genetic polymorphisms can be associated with survival on serpentine soil by either sequencing survivor pools of parent and hybrid seedlings grown on serpentine and non-serpentine soil in a QTL analysis (Selby & Willis 2018) or by sequencing the genomes of serpentine-adapted and non-adapted plant species or populations (Turner et al., 2010; Arnold et al., 2016). Fewer studies — mostly those examining the heavy metal tolerance component of serpentine tolerance, such as Ni tolerance in *S. vulgaris* and Zn tolerance in *A. halleri* — measure seedling growth, leaf area, or biomass (Bratteler, Lexer, & Widmer, 2006; Karam et al., 2019).

When trying to identify what traits are most adaptive to life as a perennial herb growing on serpentine soil, such as *A. eximia*, it is clear that being able to establish as a seedling is critical to survival until reproductive age. For this reason, to encompass multiple aspects of seedling growth, I measured: seedling survival rates, average seed biomass, initial seedling biomass of newly germinated seedlings at planting, as well as the ability of seedlings to grow on serpentine and non-serpentine soil. Growth measurements on serpentine soil were all quantitative measures of serpentine tolerance. I calculated seedling

root:shoot ratios at the time of harvest because a common adaptation of serpentine plants is a higher allocation of resources to root growth and, therefore, higher root:shoot ratios compared to soil generalist species **(**Harrison & Rajakaruna, 2011**).**

In addition to measuring growth and survival, I also wished to include other phenotypic differences between species, so that I could determine if these traits were involved in serpentine adaptation. However, both species have red and yellow flowers, are hummingbird-pollinated, and can grow in close proximity to one another in nature (especially at serpentine/non-serpentine interfaces), and are therefore morphologically very similar. The only documented species-differentiating traits (aside from *A. eximia's* ability to grow on serpentine soil) are slight differences in flowering time, floral morphology, and most notably: *A. eximia's* viscid leaf and inflorescence surface caused by the presence of glandular trichomes (Dean, 2011). Therefore, in addition to seedling survival and growth, I chose to measure trichome type in the hybrid mapping populations. Even though trichome type is not obviously involved in serpentine tolerance, it is still one of the most taxonomically relevant vegetative traits differentiating *A. eximia* from *A. formosa* (Dean, 2011).

To examine the genetic basis of these species differentiating traits, I produced an F1 and F2 hybrid population from crosses between the soil generalist species, *A. formosa,* and the serpentine specialist species, *A. eximia.* Because previous work has shown strong evidence of inbreeding depression in *Aquilegia* (Yang & Hodges, 2010), I used an outcrossed design to generate the hybrid crosses (Table 1). I also created reciprocal crosses

to test for any possible cyto-nuclear interactions in the F1 and F2 populations (Table 1). To detect whether any traits involved in serpentine tolerance appear to have a simple genetic basis, I compared phenotypic frequencies of cross types (parent species, F1 and F2 reciprocal crosses; Table 1) to expected Mendelian ratios for the F1 and F2 hybrid populations.

The main questions addressed by the experiment described below were: **(1)** What traits in *A. eximia* have differentially evolved from its soil generalist progenitor, *A. formosa,* in order to become a serpentine specialist? **(2)** Is there evidence that serpentine adaptation in *A. eximia* has a simple genetic basis? Do any traits segregate in Mendelian ratios?

II. Methods

1. Field Collections

Soil and seeds from parent populations that generated the experimental crosses were collected from a non-serpentine field site in Nacimiento Fergusson road, Big Sur, CA (35°59'3.95"N, 121°26'4.60"W) and a serpentine seep field site on Prefumo Canyon road, San Luis Obispo, CA (35°15'41.3"N, 120°43'0.3"W) (Figure 1). Soil was dried and sifted through a 2.5in^2 screen to remove large rocks before being used in the seedling growth experiment.

2. Seedling survival rates and growth measurements on serpentine and non-serpentine soil.

To accurately measure the growth dynamics of seedlings, I first recorded average individual seed biomass (mg) by recording the mass of batches of 10 seeds at a time. 650 *A. eximia*, 250 *A. formosa*, 300 EXF1, 210 FOF1, 920 EXF2, and 1120 FOF2 seeds were weighed and plated over the course of this experiment.

Seeds were plated on autoclaved agar plates (8g/L H_2O) in a laminar flow hood and parafilmed to decrease risk of contamination. Plates were then placed under fluorescent lights in order for seeds to germinate (Figure 2). Germination rates were generally low and variable, additionally, some seedlings germinated but were not able to be planted due to contamination by microbial pathogens.

All cross types (Table 1) were planted together in batches and grown for four weeks in a UCSB environmental room, with multiple plantings growing concurrently. Plantings occurred from June 2017 to October 2018. Because germination of seeds was non-synchronous, to control for differences in number days since germination, I plated all seedlings at the same developmental stage: once the cotyledons had expanded but before the presence of a first true leaf (Figure 2). I planted 66 *A. eximia*, 90 *A. formosa,* 84 EXF1, 43 FOF1, 188 EXF2, 226 FOF2 seedlings in either serpentine or non-serpentine soil (Table 2). However, I did not record initial seedling biomass during the first few plantings, so only 44 *A. eximia,* 51 *A. formosa*, 46 EXF1, 13 FOF1, 68 EXF2, 77 FOF2 were weighed before planting.

During planting, I gently removed seedlings from agar plates with clean forceps and measured initial seedling biomass (mg) at the time of planting. I then planted seedlings in either field collected serpentine or non-serpentine soil in 1.5" wide x 5.5" deep cone-tainers (holding approx. 100ml of soil). Seedlings were placed in cone-tainer trays sitting in hydroponic trays filled water, in order to simulate a natural seep or riparian environment. Serpentine and non-serpentine individuals were placed in separate trays so no chemicals leached from the serpentine soil would affect the growth of non-serpentine seedlings and visa-versa. The seedlings grew under fluorescent lights in controlled environmental conditions (18 hours of light/6 hours of dark at approximately 20-22° C) for 4 weeks before harvest (Figure 2).

During harvest, I scored each seedling as either alive or dead after 4 weeks to calculate seedling survival rates. For all surviving seedlings, I completely removed soil from seedling roots in a DI water bath before patting off excess water with clean paper towels. I imaged the seedling, separated root from shoot using a scalpel, placed the root or shoot tissue in 2ml autoclaved centrifuge tubes, measured seedling root and shoot biomass (mg), then flash froze the seedling tissue using liquid nitrogen. Tissue was stored in the -80°C freezer until DNA isolation (Figure 2). On serpentine soil, I harvested 32 *A. eximia,* 0 *A. formosa*, 38 EXF1, 12 FOF1, 77 EXF2, and 91 FOF2 seedlings total (Table 2). On non-serpentine soil, I harvested 21 *A. eximia,* 57 *A. formosa*, 29 EXF1, 16 FOF1, 42 EXF2, and 62 FOF2 seedlings total (Table 2).

3. DNA Isolation Protocol

Frozen tissue was homogenized by steel beads using a Qiagen TissueLyser II. 300mL of DNA isolation buffer [1:1 ratio of 5M potassium acetate: L2 buffer (2M guanadine thiocyanate, 2M NaCl, 25nM tri-sodium citrate)] was added to ground homogenized tissue. Mixture was vortexed until all tissue was dissolved in isolation buffer, then centrifuged for 5 min at max speed. Lysate supernatant was transferred to a sterile 96-well plate containing 20ul of magnetic beads (Qiagen MagAttract Suspension G) per well. DNA was extracted with a Qiagen Biosprint 96 workstation using the plant tissue extraction protocol: (http://www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/biosprint-96-dna.pdf). Because of small amounts of starting tissue for certain individuals, DNA was eluted into 50ul of nanopure DI water.

4. Measuring trichome type frequencies in hybrid mapping populations

A. eximia has a viscid surface covered in glandular trichomes ("sticky" leaves, stems, and inflorescences), while *A. formosa* has long hairs sparsely dispersed among many small capitate trichomes covering to the stems and leaves (both lacking glandular exudates). In order to distinguish between trichome types, I qualitatively examined these differences between parent species using compound light microscopy (Figure 3). I scored trichome type for 127 F1 and 224 F2 seedlings grown to determine the genetic basis of glandular trichomes in *A. eximia* and to infer whether they may be advantageous in serpentine ecosystems. F2 individuals not included in the seedling growth experiment were transplanted into potting

soil and grown in the greenhouse where trichome type was scored by sight to calculate trichome type frequencies.

5. Statistical Analysis of Phenotypic Data

I used contingency table analyses of seedling mortality and trichome type to test whether survival and trichome type frequencies differed significantly across cross types (Table 1). I used Chi^2 tests to see whether these traits segregate in expected Mendelian ratios by comparing the observed versus expected frequencies for seedling survival, seedling growth, and trichome type.

To test for differences in mean total biomass, root:shoot ratio, and seedling growth rate between cross types (Table 1) on each soil type, I first used Levene's test to determine whether the variances between groups were homogeneous. Next, I used ANOVA if the variances were equal and Welsh's ANOVA if the variances were unequal to test for differences in means. I used either Tukey-HSD (equal variances) or Games-Howell HSD (unequal variances) post-hoc ordered difference comparison of all pairs to create the connecting letter reports on seedling growth figures.

III. Results

1 Soil reciprocal transplant of serpentine specialist *A. eximia* and soil generalist *A. formosa.*

I compared survival rates and seedling growth of *A. eximia* and *A. formosa* seedlings grown on their native and non-native field soils to determine whether either species exhibits a reduction in fitness or costs of tolerance when grown in their non-native soil type. If serpentine specialist *A. eximia* is adapted to its native serpentine soil, then it is expected that it will have higher survival and seedling growth rates its native serpentine soil compared to non-serpentine soil.

1.1 Parent Species Seedling Survival

On non-serpentine soil, there was no significant difference in seedling survival rates between *A. eximia* and *A. formosa* (Chi^2= 0.145, p=0.703, Table 3). In contrast, *A. formosa* was completely serpentine intolerant, experiencing 100% seedling mortality on serpentine soil (Table 2). There was no significant difference in *A. eximia's* survival rate between serpentine and non-serpentine soil (Chi^2= 0.865, p=0.352, Table 3).

1.2 Parent Species Seedling Growth on Serpentine and Non-Serpentine Soil

Seedling phenotypes were compared across soil types and between species to identify the ways in which *A. eximia* may be adapted for growth on serpentine soil. *A.*

formosa seedling growth was not measured on serpentine soil because 100% of seedlings died before harvest (Table 2).

I used Welsh's ANOVA tests to compare seedling growth traits between the three surviving parent species groups (*A. eximia* on serpentine and non-serpentine soil and *A. formosa* on non-serpentine soil). The only trait that was significantly different between groups was total seedling biomass after 4 weeks of growth (Figure 4 & Table 4). Although *A. eximia* seedlings grow larger in non-serpentine soil compared to serpentine soil, *A. eximia's* total seedling biomass is significantly lower than that of *A. formosa* seedlings grown in the same non-serpentine soil (Figure 4). Welsh's ANOVA tests detected no significant differences between species in seedling growth rate ($F=1.141$, $p=0.498$, Table 4) or root:shoot ratios ($F=0.501$, $p=0.609$, Table 4). Because there is no evidence that root:shoot ratios play a role in serpentine adaptation between the two species, it was excluded from subsequent analyses of serpentine tolerance traits.

These two species do not differ in their allocation of resources to different organ types nor do they grow at significantly different rates. Yet, there is a clear size difference in seedling total biomass after 4 weeks of growth. To identify the potential cause of this size difference at 4 weeks, I measured average seed individual seed biomass and seedling biomass at the time of planting. *A. eximia* has significantly smaller seeds and initial seedling biomass at germination compared to *A. formosa* at the same developmental stage (Figures 5 & 6).

2. Identifying the factors unrelated to serpentine tolerance that may influence seedling survival and growth

2.1. Maternal Effects on Seedling Survival in Hybrid Crosses

The serpentine tolerance QTL mapping population was generated using an outcrossed design to test for possible cytonuclear interactions or other maternal effects that would affect seedling survival and growth phenotypes in the experimental hybrid crosses. To test for maternal effects on seedling survival unrelated to survival on serpentine, I compared seedling survival rates between the F1 and F2 reciprocal hybrid crosses on non-serpentine soil. Comparisons of survival rates between either the F1 (EXF1 and FOF1) or F2 (EXF2 and FOF2) reciprocal crosses using contingency table analysis found that there were no significant differences between reciprocal crosses on either soil type (Table 5).

2.2 Seed weight and seedling biomass at planting

To identify other factors that may affect total seedling biomass measurements (and therefore may serpentine tolerance measurements), average individual seed biomass and initial seedling biomass at planting were compared across the parent species, F1, and F2 hybrid cross types. All crosses had significantly different average individual seed mass when compared to each other except for *A. formosa* vs. FOF1 seeds (Games Howell HSD p=0.934, Figure 7) and *A. eximia* vs. EXF1 seeds (Games Howell HSD p=1.0, Figure 7).

Although there were significant differences in average individual seed biomass between hybrid crosses, there were no significant differences in seedling biomass at planting between any of the F1 or reciprocal F2 hybrid crosses (EXF1, FOF1, EXF2, FOF2), except

for FOF1 vs. FOF2 (Games Howell HSD p= 0.0266, Figure 8). It should be noted that FOF1 hybrid crosses had the highest average seed biomass and seedling biomass (mg) at planting compared to all other hybrid crosses.

Although reciprocal F1 crosses did not differ significantly with respect to initial seedling biomass or survival rates on non-serpentine soil, FOF1 has higher mean individual seed mass, higher initial seedling biomass, and lower survival rates than EXF1 on serpentine soil compared to *A. eximia* (Table 6, Figures 7 & 8). Therefore, reciprocal F1 cross types will continue to be distinguished in subsequent analyses. Reciprocal F2 hybrid crosses (FOF2 and EXF2) did not differ significantly from each other in survival rates or initial seedling biomass; therefore, F2 reciprocal cross types will be grouped together in subsequent analyses of seedling growth (Table 5, Figure 8).

2.3 Seedling growth on nutrient-rich non-serpentine soil

Seedling total biomass (mg) and seedling growth rate (total/initial biomass) were measured on nutrient-rich non-serpentine soil to identify the growth dynamics of hybrid cross types (Table 1) that may affect seedling growth but are not related to serpentine tolerance. In seedlings grown for four weeks on non-serpentine soil, *A. formosa* and all hybrid seedlings had significantly higher total biomass at harvest compared to *A. eximia*. FOF1 had the highest mean total biomass overall (Figure 9).

A. eximia and *A. formosa* had lower seedling growth rates compared to all hybrid crosses (Figure 10). Although seedling biomass at planting differed between cross types, the

hybrid crosses all had a faster growth rate than the parent species, possibly as a result of hybrid vigor in the F1 and F2 crosses.

3. Examining the genetic basis of serpentine tolerance in hybrid populations.

A crucial question about the genetic basis of serpentine adaptation is whether serpentine tolerance is a dominant or recessive trait. If serpentine tolerance is a dominant trait, then the expectation is that the F1 hybrids will have the same phenotype as *A. eximia* on serpentine soil and that the F2 hybrids will segregate in approximately a 3:1 ratio for each measured trait.

3.1 Seedling Survival on Serpentine Soil

To test whether serpentine tolerance had a dominant inheritance pattern, I first compared survival rates of F1 hybrids to *A. eximia* (Table 6). EXF1 did not have significantly different survivorship from *A. eximia* (Chi^2=0.163, p=0.924), but FOF1 experienced decreased survival on serpentine soil (Chi^2=6.27, p=0.048). Therefore, serpentine tolerance had a dominant inheritance pattern in EXF1, but it did not appear to be completely dominant in FOF1. The differences seen on serpentine soil when comparing F1 survival rates to *A. eximia* were not observed on non-serpentine soil (Table 6).

To further test if serpentine tolerance was segregating in the recombinant F2 generation, reciprocal F2 survival rates were compared to those of *A. eximia* (Table 7). Both F2 reciprocal crosses had significantly lower survival on serpentine soil compared to *A.*

eximia. On non-serpentine, soil survival rates were not significantly different between *A. eximia* and reciprocal F2 crosses.

I calculated the approximate number of seedlings that were serpentine tolerant (alive), seedlings that didn't survive on serpentine due to being serpentine intolerant, and seedlings that didn't survive due to other causes of seedling mortality (transplant shock, fungal infection, etc.). *A. eximia* had a mortality rate of approximately 16% on serpentine soil, while the F2 hybrid population on serpentine soil has a mortality rate of 37%. Therefore, approximately 16% (n=43) of F2 seedlings died on serpentine soil due to factors unrelated to serpentine tolerance, and approximately 21% (n= 56) of F2 seedlings died on serpentine soil because they were serpentine intolerant. In total, 168 seedlings were serpentine tolerant (alive) and 56 were serpentine intolerant (dead), which results in an exact 3:1 ratio of serpentine-tolerant: serpentine-intolerant seedlings in the F2 population on serpentine soil (Chi^2=0, p-value=1).

3.2 Seedling growth on serpentine soil

There were no significant differences in total seedling biomass between cross types on serpentine soil, except that FOF1 had a higher average total biomass compared to all other crosses (Figure 11). *A. eximia* had a significantly higher growth rate than the F2 hybrids when grown on serpentine soil (Figure 12); the opposite trend was seen on non-serpentine soil (Figure 10). The variance of the F2 hybrid population for serpentine tolerance related traits, including total biomass and seedling growth rate was much larger

than that of *A. eximia* (Figures 11 & 12), suggesting possible segregation of phenotypes in the F2 mapping population.

I examined the phenotypic frequency distributions of *A. eximia* compared to the F2 hybrids and identified 28 F2 seedlings that had lower total biomass, and 30 F2 seedlings that had lower growth rates than the minimum measurements in *A. eximia* (Figure 13). For each trait, these individuals were grouped into a serpentine semi-intolerant class of seedlings that survived on serpentine soil, but experienced reduced seedling biomass or growth rates compared to *A. eximia.*

I conducted Chi^2 tests comparing observed with expected phenotypic frequencies of serpentine tolerant and semi-intolerant F2 seedlings to determine whether either trait was segregating in a 3(serpentine tolerant):1(serpentine semi-intolerant) Mendelian ratio. Seedling growth did not significantly differ from Mendelian ratios (Chi^2=0.24, p=0.622, Table 8), while total seedling biomass at harvest phenotypic frequencies did not follow a 3:1 ratio (Chi^2=6.02, p=0.014, Table 8), due to a higher number of seedlings than expected with a high biomass at harvest (in the serpentine-tolerant class). This result was not surprising because previous results have shown that total biomass may be influenced by other factors such as initial seedling biomass at planting and potentially differential seed reserves at germination (Figure 7).

4. Trichome types in hybrid crosses and possible association with serpentine tolerance.

4.1 Trichome type frequencies in the F2 hybrid mapping population on multiple soil types

All F1 hybrids had glandular trichomes (E), suggesting that the presence of glandular trichomes is a dominant trait. Trichome type appeared to segregate in the F2 populations. I used contingency table analysis of seedlings grown on field collected serpentine, field collected non-serpentine soil, and potting soil from the greenhouse to determine that the glandular: non-glandular (E:F) trichome type frequencies of F2 seedlings were not significantly different from each other on any soil type (Chi^2= 0.987, df= 2, p-value= 0.610).

Chi^2 analysis of observed and expected values for the F2 population found that the trichome frequencies did not significantly differ from expected 3(E):1(F) Mendelian ratios (Table 9), suggesting that trichome type may indeed have a simple genetic basis. However, it should be noted that F2's that were grown in nutrient right potting soil in the greenhouse (not included in the seedling growth experiment) had and almost significantly higher number of seedlings with the non-glandular (F) trichomes compared to the expected 3:1 ratio (Chi^2= 3.7, p=0.054, Table 9).

4.2 Trichome type and seedling growth

When examining F2 seedlings grown on non-serpentine soil, there was no significant difference between F2s with glandular (E) vs. non-glandular (F) trichomes when comparing total biomass (F=0.09, p=0.77, Figure 14) or seedling growth rate (F=0.08, p=0.784, Figure 15) after 4 weeks of growth. However, on serpentine soil, F2 seedlings with glandular trichomes had significantly higher total biomass (F=4.93, p=0.03, Figure 14) and seedling

growth rate (F=4.07, p=0.048, Figure 15) compared to F2 seedlings with non-glandular trichomes.

IV. Discussion

This project adds vital information toward the study of serpentine adaptation in plants because, while most prior studies focus on serpentine tolerant ecotypes of annual forbs in core Eudicot families (primarily *Brassicaceae* and *Phrymaceae*), I have examined serpentine tolerance in a perennial herb in the early branching eudicot family the *Ranunculaceae*. Therefore, this study will add phylogenetic breadth to the study of serpentine tolerance in the Eudicot plant lineage.

I measured not only seedling survival on serpentine soil, but I also included multiple facets of seedling growth on field collected serpentine and non-serpentine soil in order to measure serpentine tolerance in *A. eximia.* Examining seedling growth of serpentine specialist species, soil generalist species, and hybrid crosses helped me uncover how *A. eximia* has evolved to specialize for life in serpentine seeps and what aspects of serpentine tolerance may have a simple genetic basis.

1. *A. eximia* seedlings do not experience decreased fitness in their non-native soil and can survive equally well in either soil type.

The first hurdle for any serpentine tolerant plant taxon is establishing and surviving in serpentine's physically harsh and chemically toxic soil environment. Growing parent

species on serpentine and non-serpentine soil collected from their native field sites allowed me to compare serpentine specialist and soil generalist plant species to identify potential trade-offs in survival or growth non-native soil types.

Because *A. formosa* experiences 100% seedling mortality on serpentine soil (Table 2), this species most likely would not able to re-colonize serpentine outcrops and compete for resources with *A. eximia.* On non-serpentine soil, there is no significant difference in survival rates between the two species (Table 3). Therefore, there is no clear fitness cost to *A. eximia* in terms of seedling survival compared to *A. formosa* on non-serpentine soil. I also found no significant difference in *A. eximia* seedling survival between serpentine and non-serpentine soil treatments (Table 3). This suggests that *A. eximia* is not locally adapted for survival on serpentine and can survive equally well on either soil type.

However, even though it appears that *A. eximia* can survive on nutrient rich non-serpentine soil, in nature, this species is only found in serpentine seep habitats and is not found growing on non-serpentine soil types. Perhaps other factors besides the ability of *A. eximia* seedlings to survive on non-serpentine soil, such as differential growth or competition with soil generalist plant species on nutrient rich soil types, that keep *A. eximia*'s range restricted to serpentine seeps.

2. Serpentine specialist *A. eximia* has evolved smaller seeds, smaller seedlings, and lower total biomass at harvest on non-serpentine soil compared to soil generalist *A. formosa*.

Although there did not appear to be significant differences in *A. eximia* seedling survival rates on either soil type compared to *A. formosa* survival rates on non-serpentine soil (Table 3), I wanted to further compare seedling growth of the parent species seedlings that survived in serpentine and non-serpentine soil. Even if a serpentine specialist is able to germinate on non-serpentine soil, it still may not be able to compete with soil generalist species in terms of seedling growth due to possible costs of tolerance. However, in the present study, *A. eximia* exhibited significantly higher mean total seedling biomass on non-serpentine soil compared to serpentine soil (Figure 4), suggesting that *A. eximia* does not appear to be adapted for optimal growth on serpentine soil but actually grows larger in its non-native soil type (likely due to the higher nutrient content of the non-serpentine soil).

However, on non-serpentine soil, *A eximia's* mean seedling biomass at harvest is still significantly lower than that of *A. formosa*, despite the fact that both species have similar growth rates and root:shoot ratios (Table 4). This difference in biomass is most likely due to *A. formosa's* larger average seed biomass (mg) and therefore larger seed reserves, most likely resulting in the observed larger seedling biomass(mg) at germination compared to *A. eximia* (Figures 5 & 6). This suggests that part of serpentine adaptation in *A. eximia* is the evolution of smaller seeds, and therefore seedlings at the time of germination compared to *A. formosa*. This result also suggests that *A. eximia* may be at a competitive disadvantage when trying to compete for resources with *A. formosa* on non-serpentine soil. If *A. formosa*

seedlings are outcompeting *A. eximia* seedlings in nutrient-rich soil types due to their size, the competitive exclusion could potentially restrict *A. eximia's* range to serpentine outcrops.

Despite the apparent competitive disadvantage of smaller seeds and seedlings for *A. eximia* when growing off serpentine, there are many potential reasons why it may have evolved these traits. Reduced plant size has been observed in many serpentine plants as an adaptation to drought to reduce water loss (Ch I-Table 1; Harrison & Rajakaruna, 2011), however, since *A. eximia* grows in serpentine seeps, it is unlikely that seedlings experience significant drought stress during germination and seedling establishment. Alternatively, smaller stature of seedlings may be a way to reduce apparency of seedlings to herbivores in open serpentine outcrops (Strauss & Ivalu Cancho, 2013). Evidence of herbivory is apparent when observing *A. eximia* in the field, and multi-species interactions among herbivores on the sticky glandular surface of *A. eximia* has been previously studied by Lo Presti et al. (2015).

It could also be argued that reduced seed size in *A. eximia* may be due to environmental factors such as to the lack of resources in serpentine soil. However, the lower average seed biomass was consistently observed even when *A. eximia* seeds were harvested from plants grown in nutrient-rich soil in the UCSB greenhouses, suggesting seed size has a genetic basis in this species. Similarly, the reduced seedling biomass observed in *A. eximia* compared to *A. formosa* at planting and subsequent harvest also appears to have a genetic basis because these traits were retained even when seedlings were grown in nutrient-rich soil conditions (Figure 4). These results also highlight the importance of measuring seedling biomass at germination to calculate seedling growth rate (total biomass at harvest/initial

seedling biomass at planting), so that the underlying cause of biomass differences at harvest are not misinterpreted.

3. Measurements on non-serpentine soil helped to identify outside factors influencing seed weight and seedling biomass and germination.

A significant goal of this project was to measure enough traits to accurately quantify the phenotypic variation of serpentine tolerance traits in the F2 hybrid populations. Accurately phenotyping seedling growth is crucial when using growth on serpentine soil as a proxy for serpentine tolerance. Results from seedling growth analyses on non-serpentine soil highlight the importance of measuring not only total seedling biomass at harvest but also other factors that may affect seedling growth, such as initial seedling biomass and average individual seed biomass. Average individual seed biomass across cross types (Figure 7) shows similar trends to initial seedling biomass (Figure 8), and is an obvious potential explanation for the differential initial seedling biomass at germination observed between cross types (Table 1).

Initial seedling biomass helped determine whether seedlings had high total seedling biomass at harvest because they had a faster growth rate or simply because they had larger seedlings at germination and subsequent planting. Seedling growth rates identified how fast seedlings were growing regardless of whether they had high or low total biomass at harvest. Measuring root:shoot ratios can also identify differences in the allocation of resources to shoot or root tissues between species. However, our results found no root:shoot differences

between species or across soil types (Table 4). Therefore, differential allocation of resources to root vs. shoot tissues does not appear to play a role in serpentine adaptation in *A. eximia*.

Using growth phenotypes on non-serpentine soil helped take into account multiple factors that influence seedling growth in general and helped to more accurately measure seedling growth dynamics not specifically involved with growth on serpentine. This information will further help accurately characterize seedling growth on serpentine soil (serpentine tolerance) in parental species and hybrid crosses.

4. Seedling survival on serpentine soil appears to have a simple genetic basis

Comparing the survival rates of F1 hybrid cross types to serpentine specialist *A. eximia* on serpentine soil suggests that serpentine tolerance is a dominant trait in EXF1 seedlings (Table 6). Lower survival rates in FOF1 crosses compared to *A. eximia* on serpentine soil suggest that there may be potential maternal effects on seedling survival on serpentine (Table 6). These maternal effects on the survival of F1 hybrids may indicate that hybrids resulting from *A. eximia* maternal plants pollinated by *A. formosa* pollen could have higher fitness on serpentine soil than hybrids from *A. formosa* pollinated by *A. eximia.* However, the sample sizes of FOF1 individuals were small, and more seedlings should be phenotyped to confirm this result.

Both F2 reciprocal crosses had significantly lower survival than *A. eximia* on serpentine soil but not on non-serpentine soil (Table 7), supporting the expectation that some F2 seedlings were indeed serpentine intolerant and that this trait is segregating in the F2 populations. Further, when accounting for other causes of seedling mortality to calculate the

approximate number of F2 seedlings that were completely serpentine intolerant, seedling survival rate segregates in exactly a 3 (serpentine tolerant=alive):1 (serpentine intolerant=dead) ratio (Table 2), suggesting that seedling survival on serpentine soil may indeed have a simple genetic basis.

5. Seedling growth rate may have a simple genetic basis, but seedling biomass does not

Survival on serpentine is a common trait used to represent serpentine tolerance (+/-) in genetic analysis (Turner et al., 2010; Arnold et al., 2016; Selby & Willis, 2018), while fewer studies actually measure seedling growth on serpentine soil (Bratteler, Lexer, & Widmer, 2006; Karam et al., 2019). By combining both aspects of serpentine tolerance, I was able not only to identify a class of seedlings that were completely serpentine intolerant (i.e., unable to survive on serpentine soil), but also to identify a class of serpentine semi-intolerant seedlings that survived on serpentine but experienced reduced biomass or growth rates.

Comparing the ratios of serpentine tolerant: semi-intolerant F2 seedlings for seedling growth traits showed that seedling growth rate may segregate in a 3:1 Mendelian ratio, but seedling total biomass did not (Table 8). The fact that total seedling biomass at harvest does not have a simple genetic basis is concordant with the observations that average seed biomass and initial seedling biomass–which appear to have a genetic basis of their own–can affect seedling biomass independent of growth on serpentine soil (Figures 7 & 8). The total seedling biomass at harvest on serpentine soil is affected by differential serpentine tolerance of F2 seedlings, but total biomass is also affected by other factors relating to seedling

growth, all of which may be controlled by different genes, suggesting that the genetic basis of this trait is probably more complex than that of seedling growth rate or seedling survival on serpentine.

6. Trichome type appears to have a simple genetic basis and may be associated with seedling growth on serpentine soil

I selected trichome type as an additional trait to measure in the QTL mapping experiment because it is one of the primary traits that differ between these species and may be adaptive on serpentine soil. Additionally, extensive research has been conducted on the function of trichomes in many plant species, and many candidate genes have been identified for glandular trichome production (Hendrick et al., 2016; Kärkkäinen & Agren, 2002), giving a high probability of being able to map and identify the causal gene(s) for this phenotype.

Lo Presti et al. (2015) suggested that the stickiness of *A. eximia*, caused by the presence of exudates from glandular trichomes on the plant surface, may help deter herbivory. The "stickiness" of *A. eximia's* leaves and stems results in plants often being covered in dead insects, which LoPresti et al. (2015) suggest could attract predatory bugs. Increased herbivory is a risk for plants growing in open serpentine outcrops (Strauss & Ivalu Cacho, 2013), so glandular trichomes may function as an adaptation that attracts insect predators that reduce herbivory. Alternatively, this trait may have reached fixation through a neutral process (such as genetic drift or founder effect) when *A. eximia* first colonized serpentine habitats and have no effect on *A. eximia's* survival or growth on serpentine soil.

If glandular trichomes (E) were associated with survival on serpentine and non-glandular trichomes (F) were associated with serpentine intolerance (death on serpentine) in the F2 hybrids, then we would expect less non-glandular trichomes on serpentine soil, skewing the 3:1 ratio. All phenotypic frequencies of trichome type did not significantly differ from 3:1 ratios in the F2 populations grown in serpentine, non-serpentine, and potting soil (Table 9). These results suggest trichome type probably does have a simple genetic basis, but that it is not associated with survival on serpentine soil, and soil type does not affect how trichome type is segregating in the F2 populations.

Although there is no effect of soil type on trichome frequency, on serpentine soil, F2 seedlings with glandular (E) trichomes did tend to have significantly higher total biomass and seedling growth rates compared to F2s with non-glandular (F) trichomes (Figures 14 & 15). This trend is not observed on non-serpentine soil (Figures 14 & 15), suggesting that seedling growth on serpentine soil may be associated with trichome type, although it is not clear how trichome type would affect seedling growth. In this experiment, the difference in total seedling biomass at harvest between F2 hybrids with glandular vs. non-glandular trichomes is not associated with herbivory, as suggested by LoPresti et al. (2015), because seedlings were grown in an environmental chamber in the absence of herbivores.

One possible explanation for this trend is that the gene(s) that code for glandular trichome type may be close in proximity to genes that control seedling growth on serpentine. If these two traits are genetically linked, that could be a possible explanation for increased growth also being associated with glandular trichome type in the F2 seedlings. If QTL peaks for seedling growth and trichome type overlap in a QTL analysis of these seedlings, this

could provide further confirmation of genetic linkage or potentially a single pleiotropic locus controlling both seedling growth and trichome type in *A. eximia.*

7. QTL analysis will confirm whether serpentine tolerance traits in *A. eximia* have a simple or complex genetic basis.

My work forms a solid foundation for future studies aiming to map the genetic variants responsible for serpentine adaptation. Whole-genome high-throughput genotype-by-sequencing (GBS) techniques (Andolfatto et al., 2011) could be used to sequence phenotyped parent species and F2 seedlings. Since there is an assembled genome for *Aquilegia*, genomic sequences can be aligned to this reference genome in order to identify the seedling's genotypes across the genome (Filiault et al., 2018). The groupings of these serpentine tolerant: semi-intolerant seedlings can also be used to aid in potential future QTL analysis. Once these phenotyped F2 seedlings are genotyped using whole genome HTS-genotype-by-sequencing analysis (Andolfatto et al., 2011), each trait such as seedling biomass and seedling growth rate on serpentine soil (Figures 11 & 12) can be associated to physical locations on particular chromosomes.

A complication of this system is that extreme serpentine intolerant phenotypes result in death, and thus I cannot sequence these completely serpentine intolerant individuals' DNA to determine their genotypes. Because both species grow well on non-serpentine soil, the non-serpentine dataset can be used to identify the frequency of genotypes across the genome in the F2 population, as well as serpentine intolerant genotypes that won't be seen in gseedlings grown on serpentine soil due to death. Then, by sequencing the F2 plants that

survive on serpentine soil, it will be possible to identify genomic regions with genotypes at significantly lower frequencies from the non-serpentine dataset, and thus find the regions controlling seedling survival on serpentine soil.

V. Conclusion

This study highlighted key differentiating traits between serpentine specialist and soil generalist *Aquilegia* species. *A. eximia* has evolved smaller seeds and smaller seedlings compared to its soil generalist progenitor *A. formosa*, perhaps an adaptation to become less apparent in open environments as a defense against herbivory (Harrison & Rajakaruna, 2011; Strauss & Ivalu Cacho, 2013). Traits involved in serpentine tolerance in *A. eximia* appear to have a generally dominant inheritance pattern in the F1 and F2 hybrid crosses. Some aspects of serpentine tolerance appear to have a simple genetic basis (seedling survival, seedling growth rate, and trichome type). In contrast, total biomass at harvest of seedlings grown on serpentine soil appears to be a more complex polygenic trait.

The only way to confirm these findings is to perform QTL analysis to associate particular genotypes in the genome to the measured serpentine tolerance traits (Andolfatto et al., 2011). Genotypes that occur in high frequency on serpentine soil compared to non-serpentine soil in the F2 populations can identify regions that control seedling survival on serpentine soil. Using QTL analysis to examine whether each trait has a single QTL of large effect or multiple QTL peaks of small effect will further provide evidence as to what traits

are involved in serpentine tolerance, and whether these traits have evolved a simple or complex genetic architecture in *A. eximia*.

VI. Tables & Figures

Table 1: The outcrossed design used to generate reciprocal cross types for the F1 (EXF1 & FOF1) and F2 (EXF2 & FOF2) hybrid crosses. Notation for each cross is: [maternal parent (carpel)/paternal parent (pollen)].

Generation	Reciprocal Cross Types (Maternal/Paternal)
F0 (Parent Species)	*A. eximia* (**EX**) or *A. formosa* (**FO**)
F1	EX/FO = **EXF1** Or FO/EX = **FOF1**
F2	(EX/FO)/(FO/EX) = **EXF2** or (FO/EX)/(EX/FO) = **FOF2**

Table 2. Counts of seedlings scored alive or dead after 4 weeks of growth and survival rates of each cross type on serpentine and non-serpentine soil.

Cross	Seedlings Alive on Serpentine	Seedlings Dead on Serpentine	Seedling Survival Rates on Serpentine Soil	Seedlings Alive on Non-Serpentine	Seedlings Dead on Non-Serpentine	Seedling Survival Rates on Non-Serpentine Soil
***A. eximia* (EX)**	32	6	0.84	21	7	0.75
***A. formosa* (FO)**	0	18	0	57	23	0.71
EX-F1	38	9	0.81	29	8	0.78
FO-F1	12	10	0.55	16	5	0.76
EX-F2	77	48	0.62	42	21	0.67
FO-F2	91	51	0.64	62	22	0.74

Table 3: Contingency table analysis of survival rates of serpentine and soil generalist species.

Comparison	Chi^2 Value	P-value
A. eximia survival on serpentine vs. non-serpentine soil	0.865	0.352
A. eximia vs. *A. formosa* survival on non-serpentine soil.	0.145	0.703

Table 4. Welsh's ANOVA tests comparing seedling growth traits of *A. eximia* grown on serpentine and non-serpentine soil and *A. formosa* grown on non-serpentine soil. Welsh's ANOVA p-values were corrected for multiple comparisons using the Benjamini-Hochberg correction.

Trait	F	Degrees of Freedom	Benjamini-Hochberg q-value
Total Biomass at 4 weeks (mg)	46.935	2	0.0003
Total/Initial Biomass (Seedling Growth)	1.141	2	0.498
Root:Shoot	0.501	2	0.609

Table 5. Contingency table analyses Chi^2 values for comparisons of survival rates of reciprocal F1 and F2 hybrid crosses on serpentine and non-serpentine soil. Chi^2 p-values were corrected for multiple comparisons using the Benjamini-Hochberg correction.

Comparison	Chi^2 Value	Benjamini-Hochberg q-Value
EXF1 vs. FOF1 on Serpentine Soil	5.197	0.092
EXF1 vs. FOF1 on Non-Serpentine Soil	0.037	0.848
EXF2 vs. FOF2 on Serpentine Soil	0.176	0.848
EXF2 vs. FOF2 on Non-Serpentine Soil	0.888	0.692

Table 6. Contingency table analyses Chi^2 values for comparisons of *A. eximia* survival rates to reciprocal F1 crosses on serpentine and non-serpentine soil. Chi^2 p-values were corrected for multiple comparisons using the Benjamini-Hochberg correction.

Comparison	Chi^2 Value	Benjamini-Hochberg q-Value
EX vs. EXF1 Survivorship on Serpentine Soil	0.163	0.924
EX vs. FOF1 Survivorship on Serpentine Soil	6.27	0.048
EX vs. EXF1 Survivorship on Non-Serpentine Soil	0.102	0.924
EX vs. FOF1 Survivorship on Non-Serpentine Soil	0.0092	0.94

Table 7. Contingency table analyses Chi^2 values for comparisons of *A. eximia* survival rates to reciprocal F2 crosses on serpentine and non-serpentine soil. Chi^2 p-values were corrected for multiple comparisons using the Benjamini-Hochberg correction.

Comparison	Chi^2 Value	Benjamini-Hochberg q-Value
EX vs. EXF2 Survivorship on Serpentine Soil	6.725	0.0356
EX vs. FOF2 Survivorship on Serpentine Soil	5.611	0.0356
EX vs. EXF2 Survivorship on Non-Serpentine Soil	0.632	0.5693
EX vs. FOF2 Survivorship on Non-Serpentine Soil	0.016	0.901

Table 8. Observed and expected phenotypic frequencies of seedling biomass at harvest and seedling growth rate (final/initial biomass) of F2 hybrids. Serpentine semi-intolerant seedlings in the F2s were defined as individuals that had phenotypic values under *A. eximia's* minimum values for each trait.

Trait	Serpentine Tolerant F2s (Observed)	Serpentine Semi-Intolerant F2s (Observed)	Serpentine Tolerant F2s (Expected)	Serpentine Semi-Intolerant F2s (Expected)	Chi^2	P-value
Seedling Biomass at 4 weeks (mg)	139	28	125.25	41.75	6.02	0.014
Seedling Growth (final/initial biomass)	81	30	83.25	27.75	0.24	0.622

Table 9. Observed and expected phenotypic frequencies of glandular trichomes (E) and non-glandular trichomes (F) in the F2 hybrid seedlings grown on field-collected serpentine and non-serpentine soil for the seedling growth experiment, as well additional F2s planted in potting soil and grown in the UCSB greenhouses. Chi^2 tests were performed to test if trichome types were segregating in a 3:1 Mendelian ratio.

Soil Type	Observed (E)	Observed (F)	Expected (E)	Expected (F)	Chi^2	P-value
QTL F2 Seedlings on Serpentine Soil	42	18	45	15	0.8	0.371
QTL F2 Seedlings on Non-Serpentine Soil	26	8	25.5	8.5	0.04	0.843
Greenhouse F2 Seedlings in Potting Soil	88	42	97.5	32.5	3.7	0.054

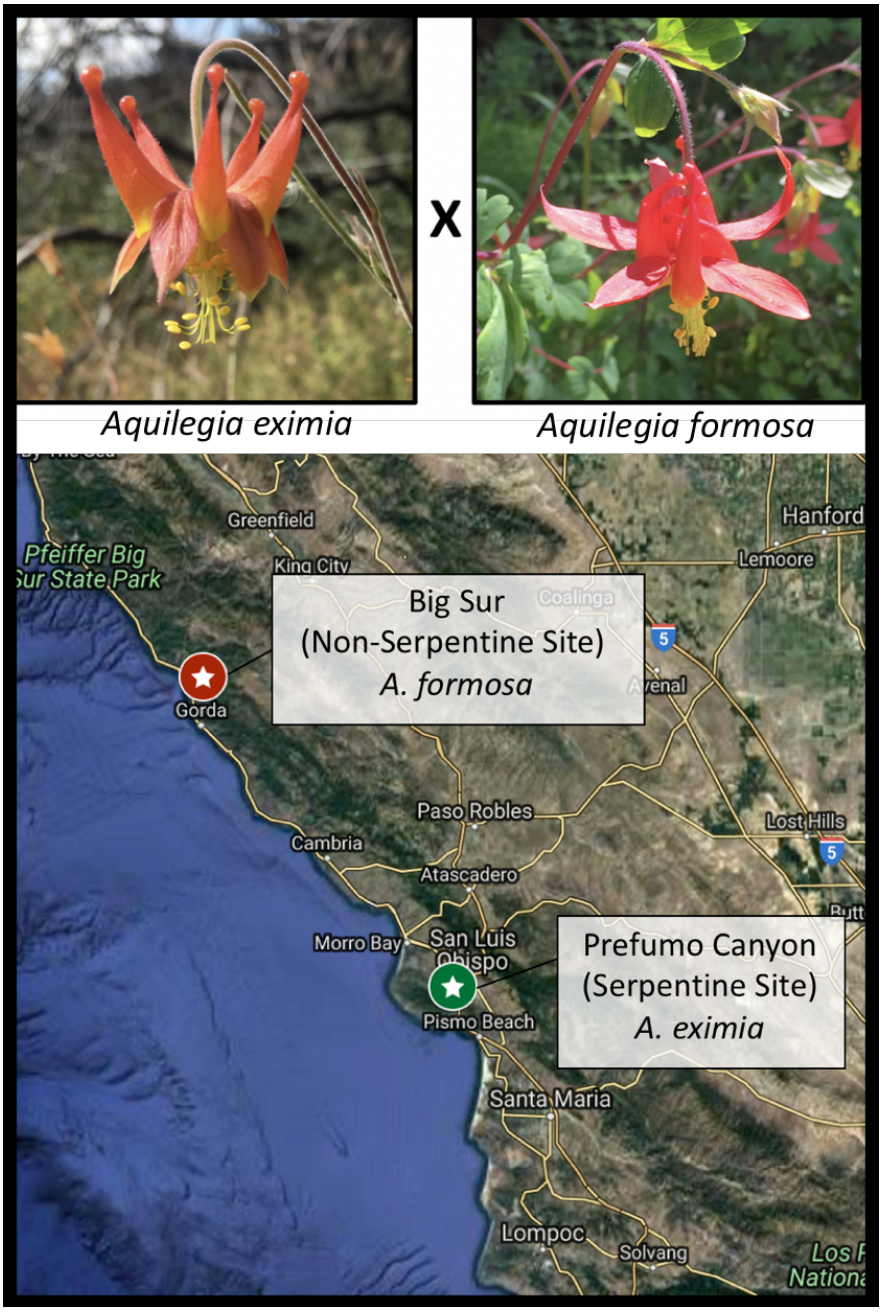

Figure 1. Examples of *A. eximia* and *A. formosa* flowers. Soil and seed for experimental crosses used in the seedling growth experiment were collected from a non-serpentine field site on Nacimiento Fergusson road, Big Sur, CA (35°59'3.95"N, 121°26'4.60"W) and a serpentine seep field site on Prefumo Canyon road, San Luis Obispo San Luis Obispo, CA (35°15'41.3"N, 120°43'0.3"W).

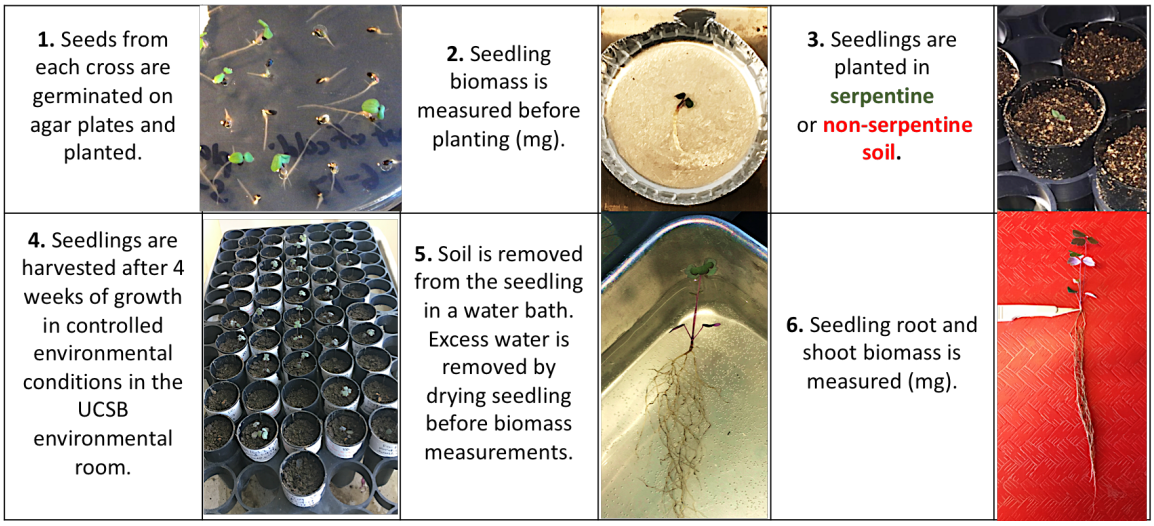

Figure 2. A visual representation of the steps involved from planting to harvest in the seedling growth experiment.

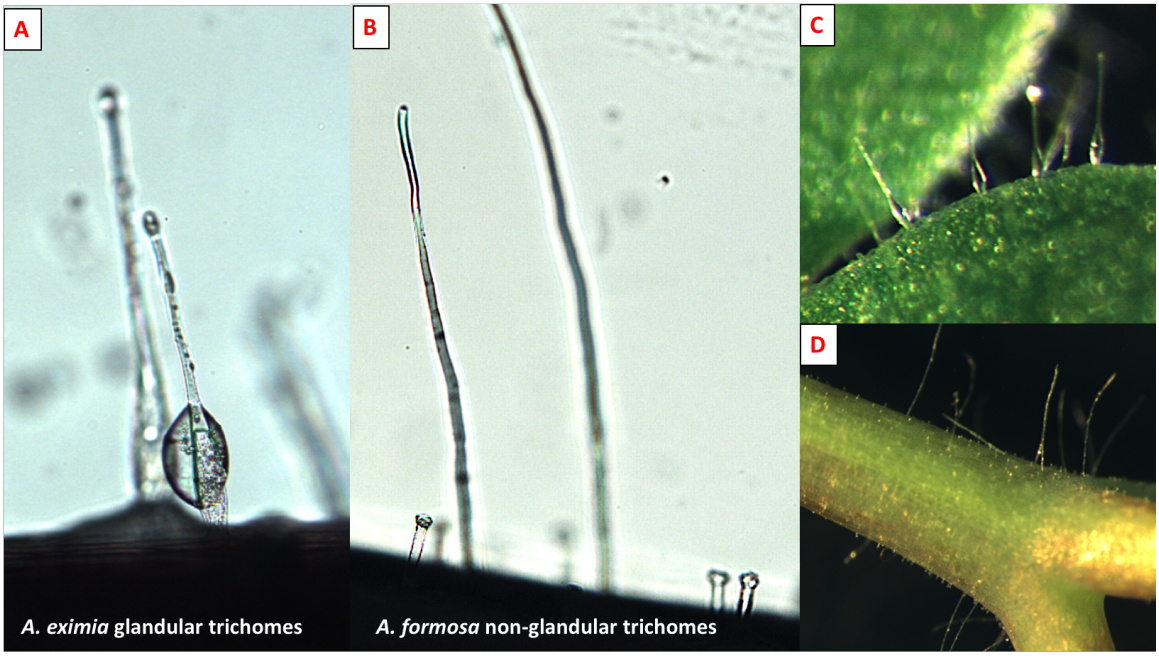

Figure 3. *A. eximia* and *A. formosa* trichome types were differentiated using compound light microscopy. **A:** *A. eximia* has glandular trichomes with a swollen base and drops of glandular exudate on the trichome surface. **B:** *A. formosa* has non-glandular trichomes composed of long sparse hairs along stems and leaf surface and short capitate trichomes along the stem surface, both of which lack glandular exudates. **C:** *A. eximia* glandular trichomes on both adaxial and abaxial leaf surfaces. **D:** Both types of non-glandular trichomes on *A. formosa* leaf petiole. (Images are not to scale).

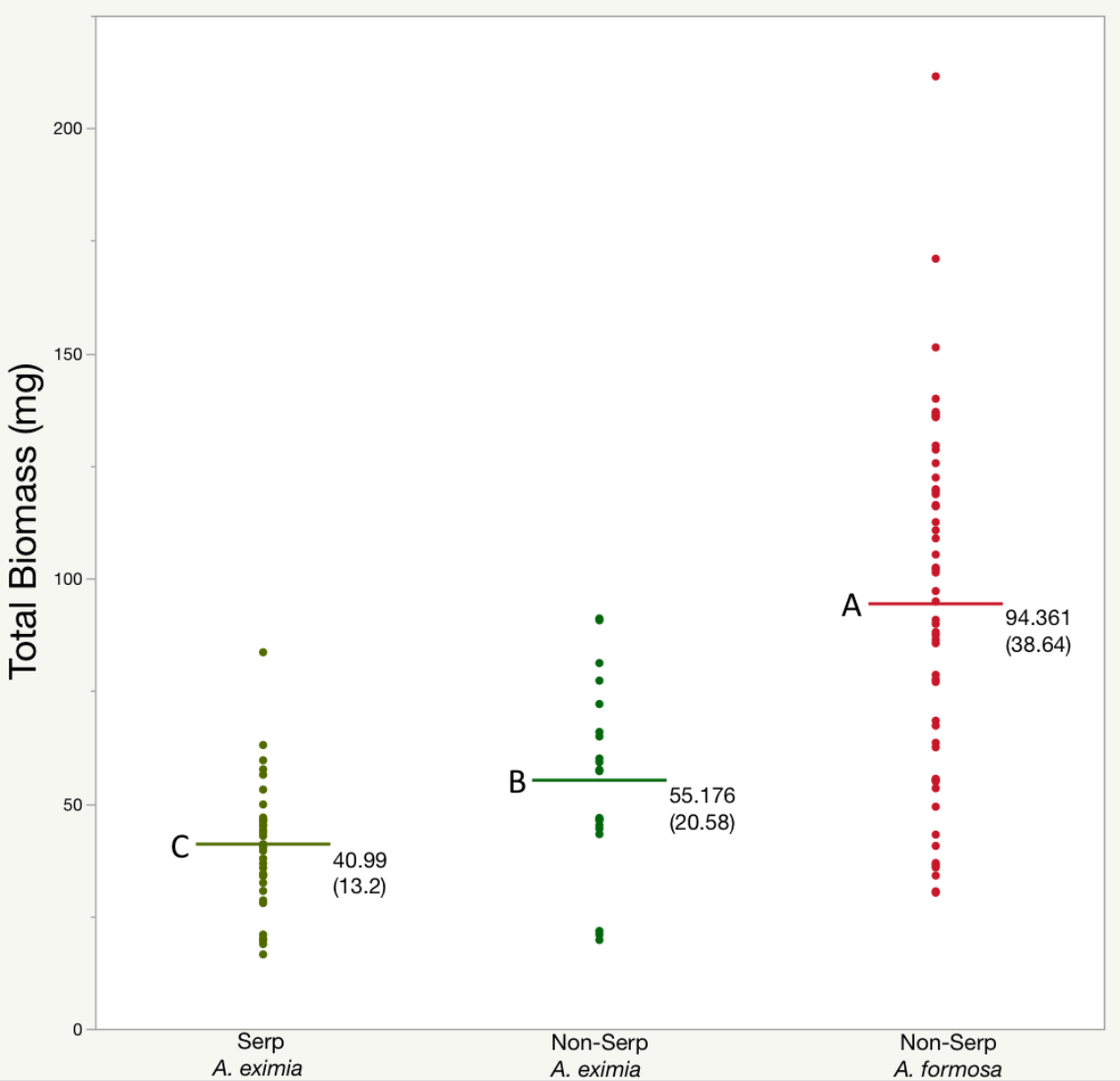

Figure 4. Total seedling biomass (mg) at harvest after four weeks of growth for *A. eximia* on serpentine and non-serpentine soil and *A. formosa* on non-serpentine soil. Average total biomass at harvest is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=19.876, df=2, p= <0.0001). Connecting letters were generated using the Games-Howell HSD ordered differences report.

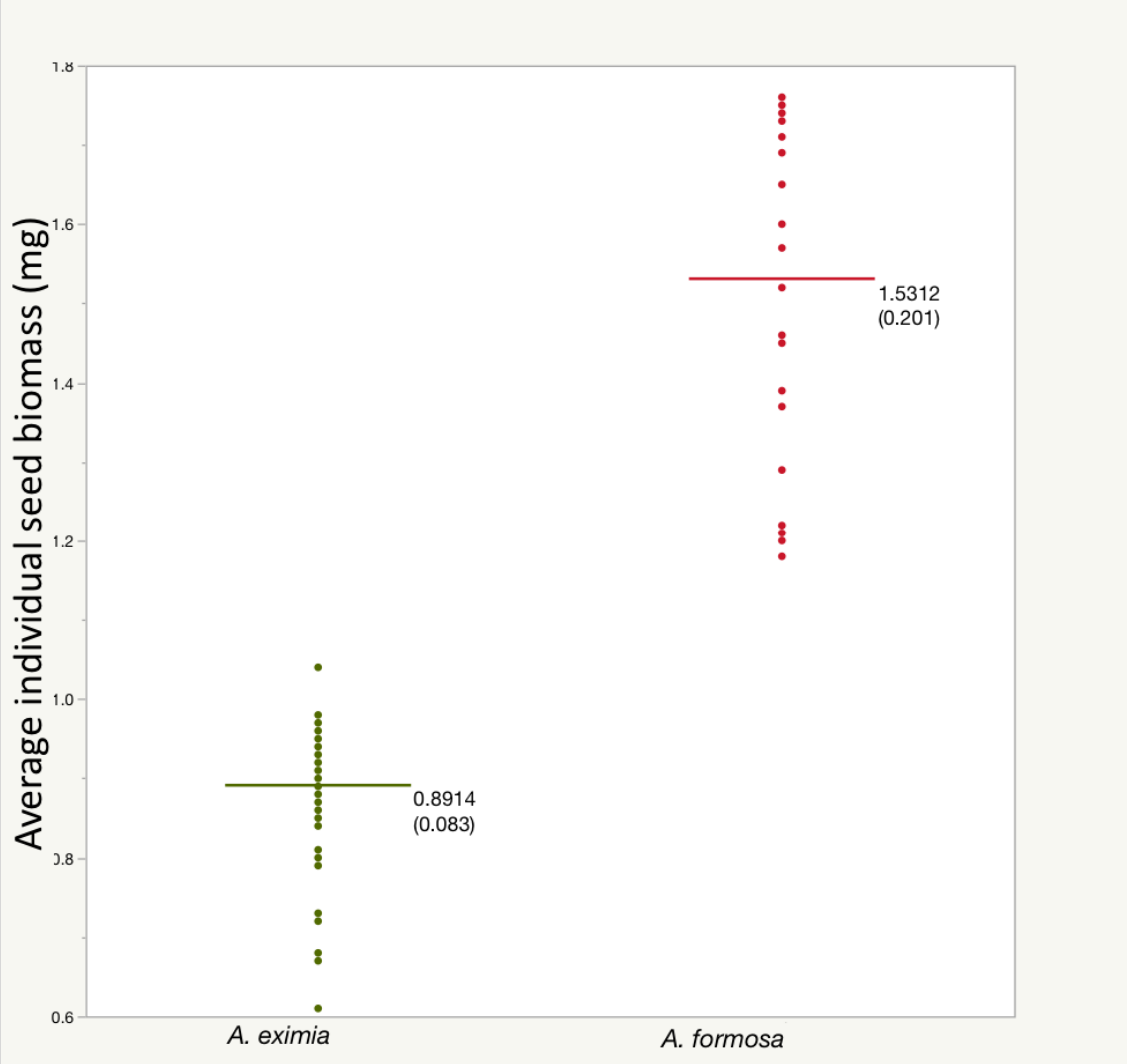

Figure 5. Average individual seed mass (mg) of *A. eximia* and *A. formosa*. Batches of 10 seeds were weighed and then divided by ten to calculate mean individual seed mass= [10 seeds (mg)/10]. Average individual seed biomass for each species is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio= 50.918, df=1, p= <0.0001). Welsh's t-test found that average individual seed mass was significantly different between *A. eximia* and *A. formosa* (F ratio =238.4, df=1, p=<0.0001).

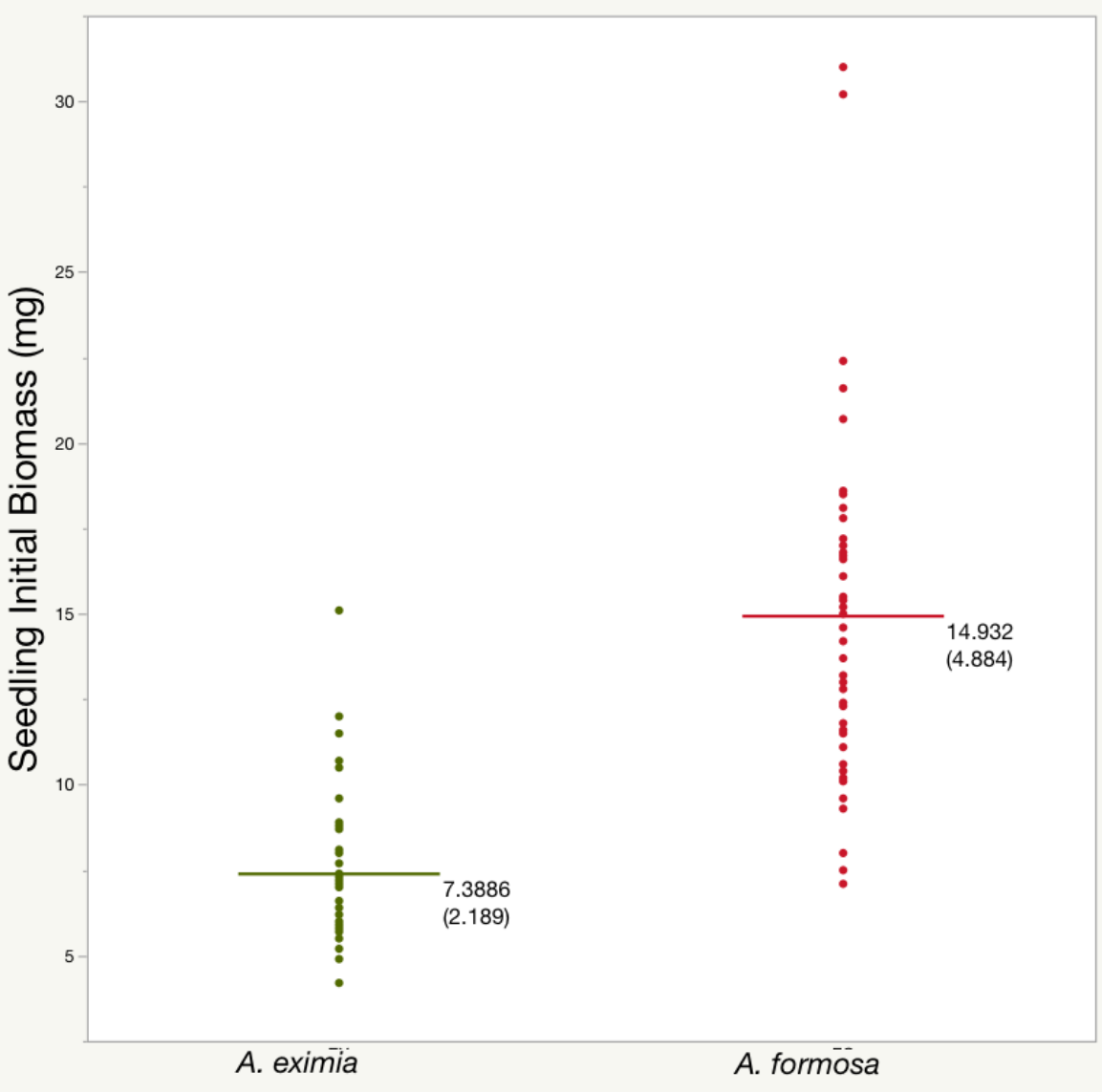

Figure 6. Initial seedling biomass (mg) of newly germinated *A. eximia* and *A. formosa* seedlings on the day of planting in serpentine or non-serpentine soil. Average initial seedling biomass at planting for each species is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=14.094, df=1, p= <0.0001=3). Welsh's t-test found that mean seedling biomass at planting was significantly different between *A. eximia* and *A. formosa* (F ratio = 97.095, df=1, p=<0.0001).

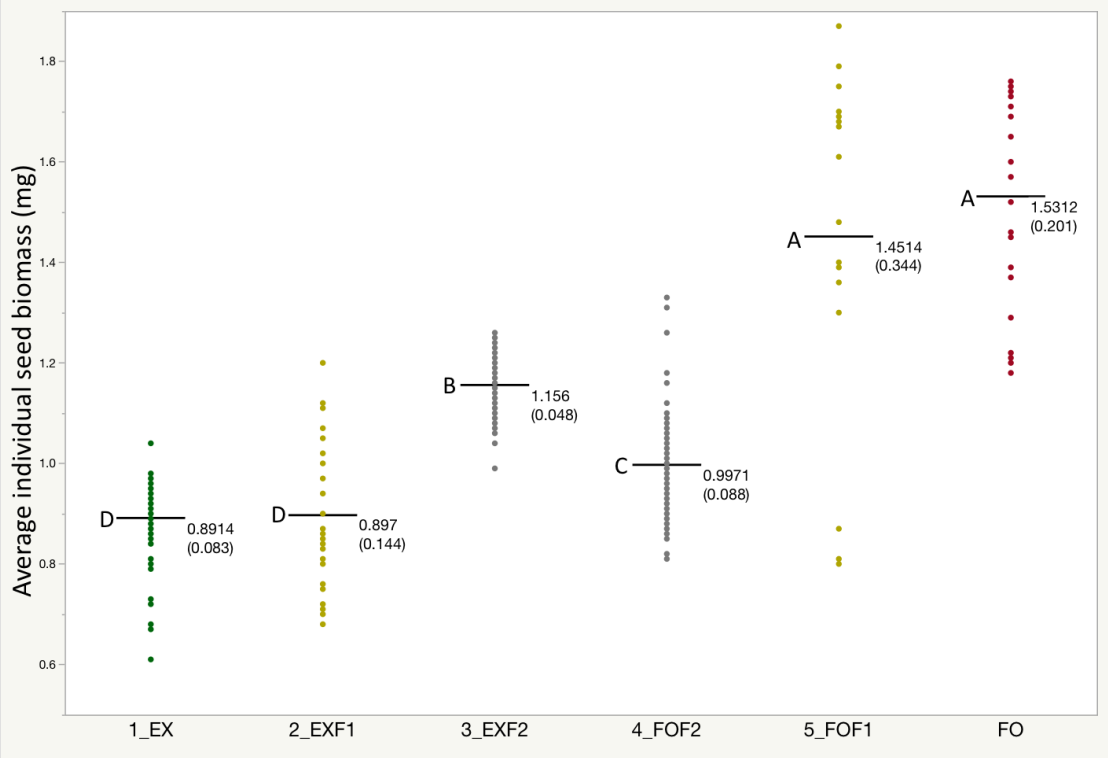

Figure 7. Average individual seed biomass (mg) by cross type. Average individual seed mass is estimated as the total mass of 10 seeds divided by 10. Average individual seed biomass for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance for average individual seed mass was tested using Levene's test (F ratio= 46.739, df=5, p= <0.0001). Welsh's ANOVA found that average individual seed mass was significantly different between all cross types (F ratio = 168.52, df=5, p=<0.0001). Connecting letters were generated using Games-Howell HSD ordered differences report.

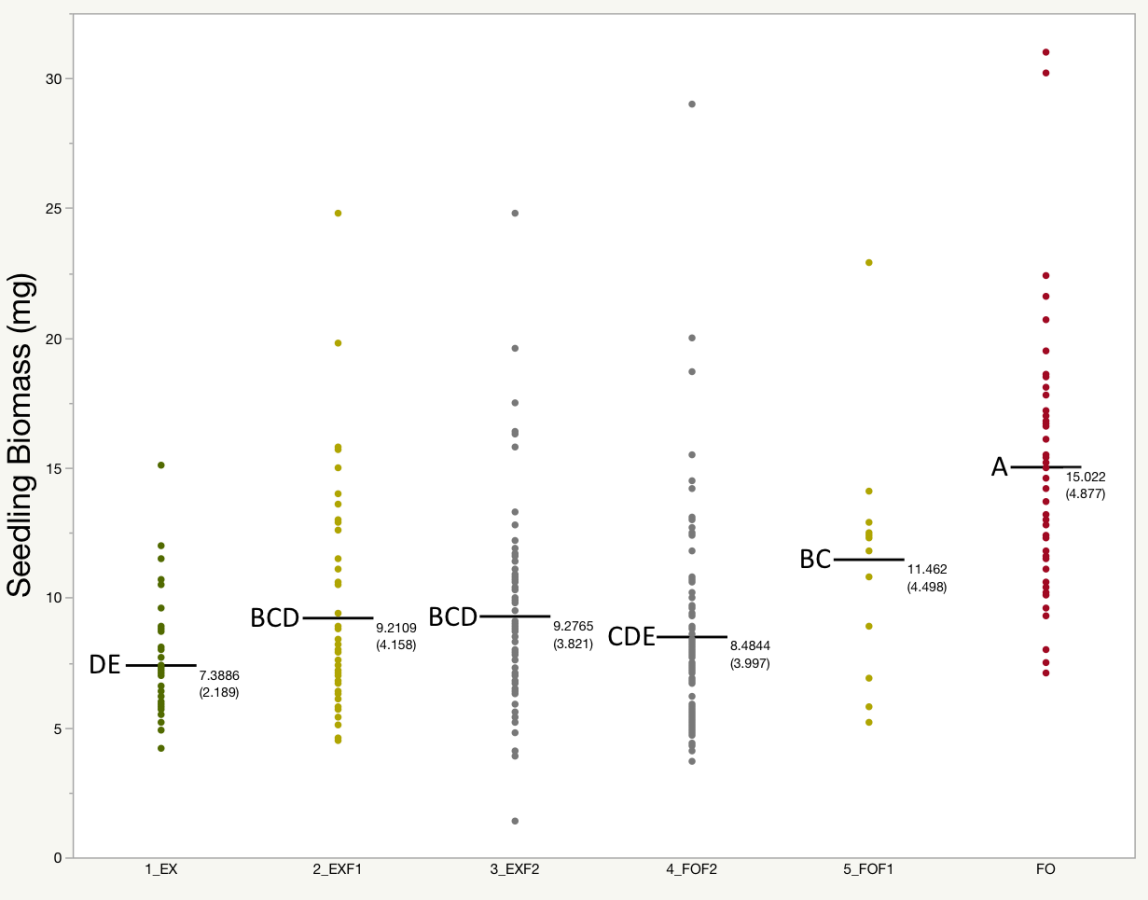

Figure 8. Comparison of initial seedling biomass (mg) at the time of planting by cross type. Average initial seedling biomass at the time of planting for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=2.687, df=5, p=0.0215). Welsh's ANOVA found that mean seedling biomass at planting was significantly different between all cross types (F ratio = 20.772, df=5, p=<0.0001). Connecting letters were generated using Games-Howell HSD ordered differences report.

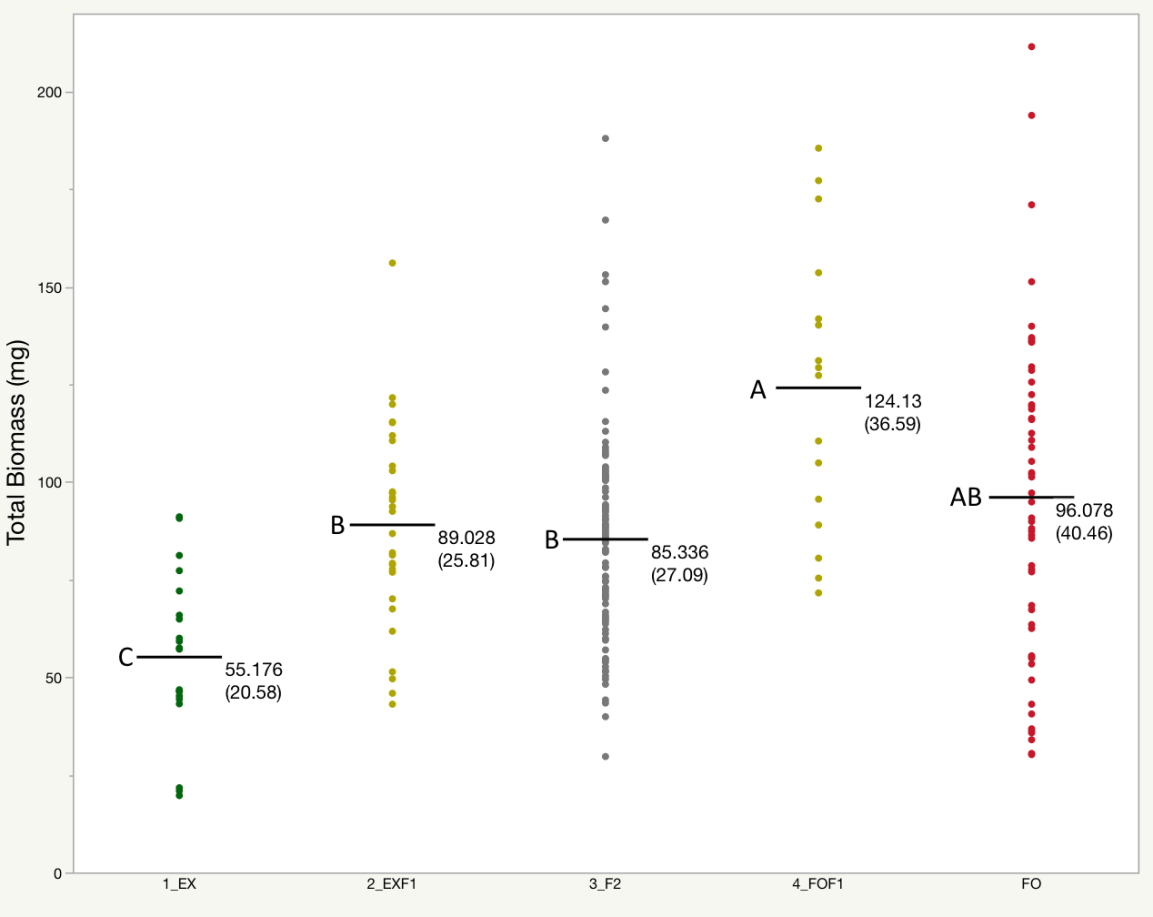

Figure 9. Comparison of total seedling biomass (mg) by cross type after 4 weeks of growth on non-serpentine soil. Average total biomass at harvest for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio= 5.404, df=4, p=0.0004). Welsh's ANOVA found that mean seedling biomass at harvest was significantly different between all cross types (F ratio = 16.175, df=4, p=0.0001). Connecting letters were generated using Games-Howell HSD ordered differences report.

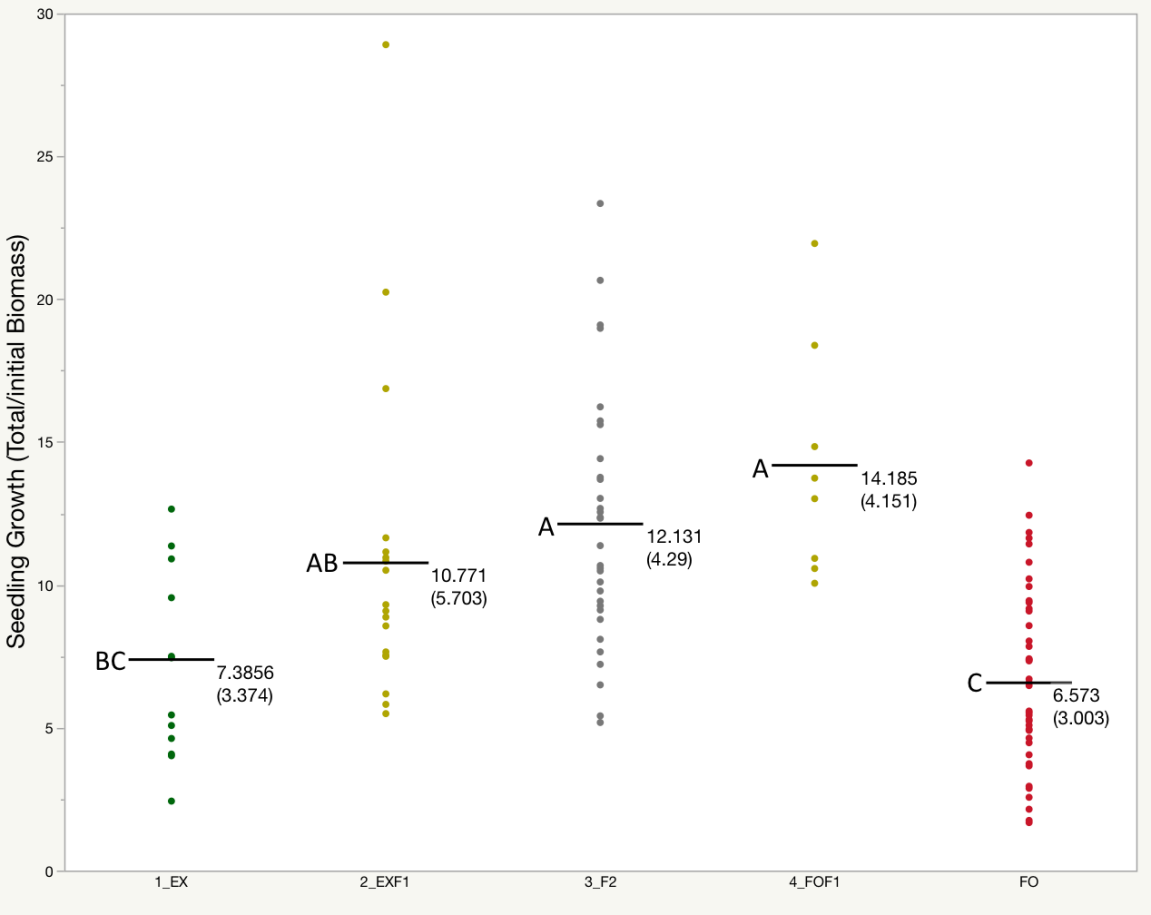

Figure 10. Comparison of seedling growth rate (final/initial seedling biomass) by cross type after 4 weeks of growth on non-serpentine soil. Average seedling growth rate for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio= 1.094, df=4, p=0.363). ANOVA found that the mean seedling growth rate was significantly different between all cross types (F ratio =14.593, df=4, p<0.0001). Connecting letters were generated using Tukey HSD ordered differences report.

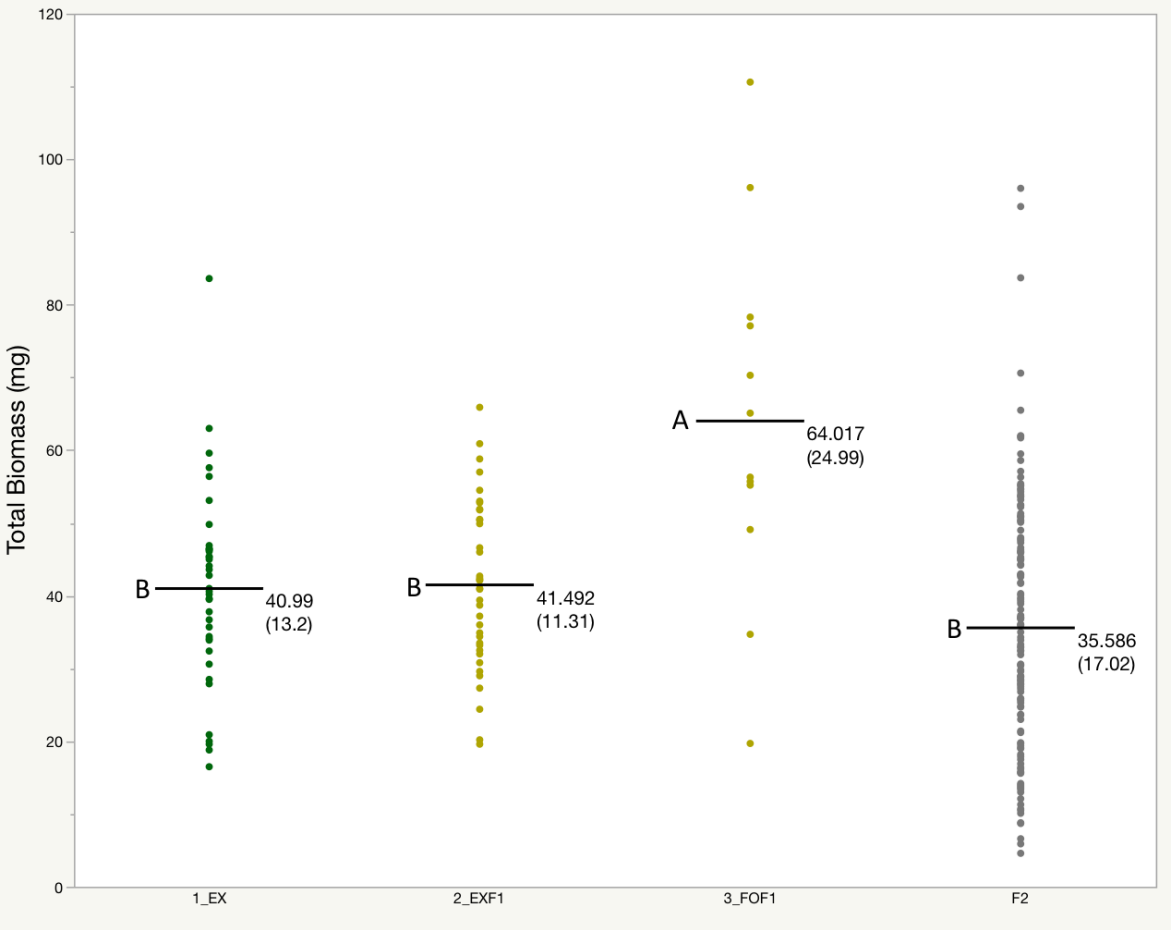

Figure 11. Comparison of total seedling biomass (mg) by cross type after 4 weeks of growth in serpentine soil. Average total seedling biomass at harvest for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=5.236, df=3, p=0.0016). Welsh's ANOVA found that mean seedling biomass at harvest was significantly different between all cross types (F ratio=6.921, df=3, p=0.0007). Connecting letters were generated using Games-Howell HSD ordered differences report.

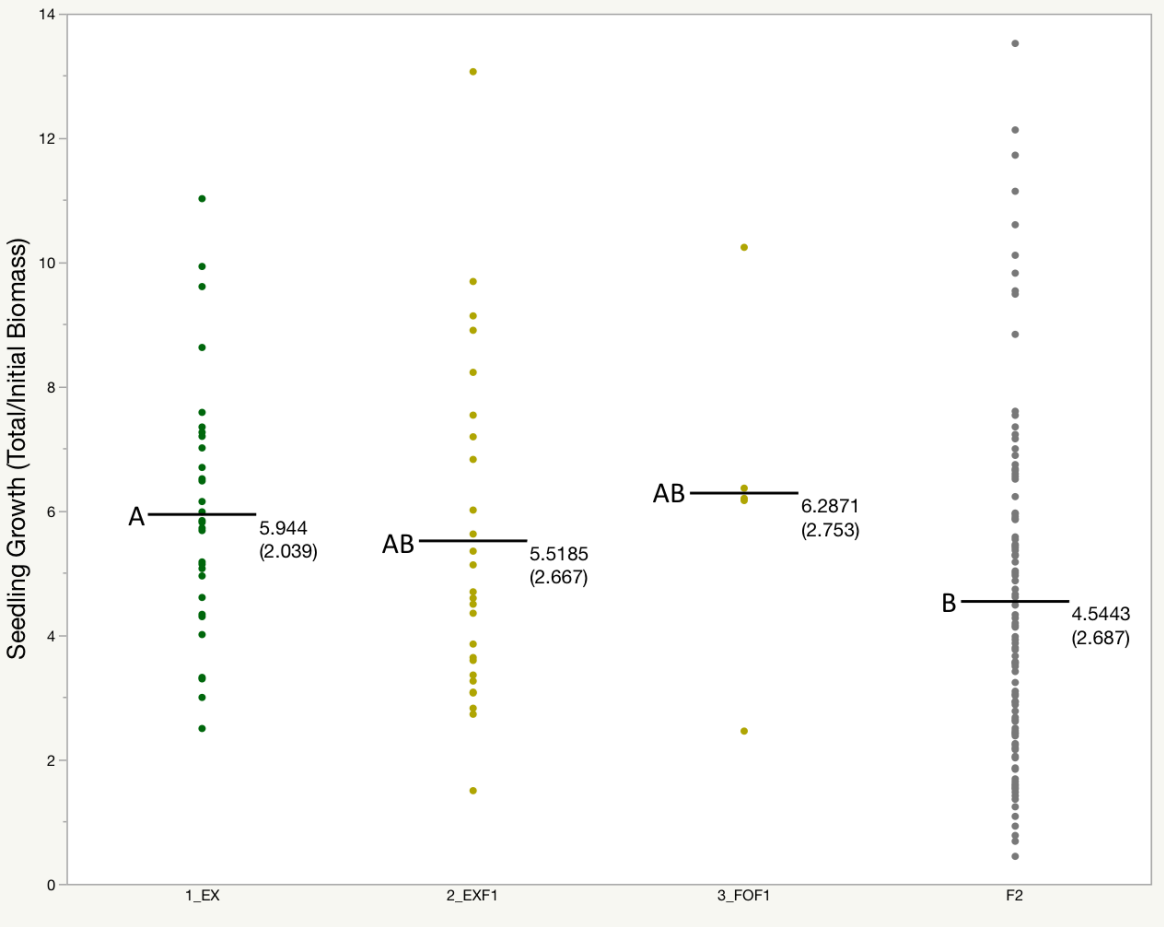

Figure 12. Comparison of seedling growth rate (final/initial seedling biomass) by cross type after 4 weeks of growth on serpentine soil. Average seedling growth rate for each cross is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test (F ratio=1.102, df=3, p=0.35). ANOVA found that the mean seedling growth rate was significantly different between all cross types (F ratio=3.31, df=3, p=0.0215). Connecting letters were generated using Tukey HSD ordered differences report.

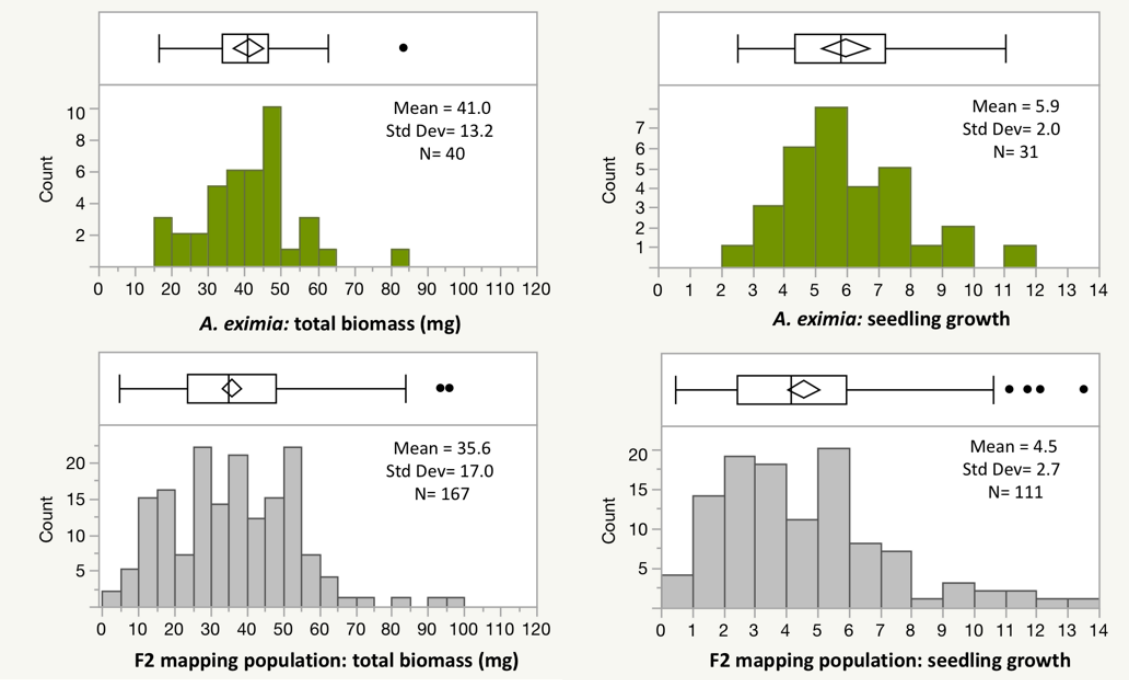

Figure 13. Seedling growth phenotypic frequency distribution histograms for *A. eximia* and F2 hybrid mapping population on serpentine soil. Above each histogram is the associated quantile box plot. These measurements are a proxy for serpentine tolerance relating to seedling growth in serpentine soil.

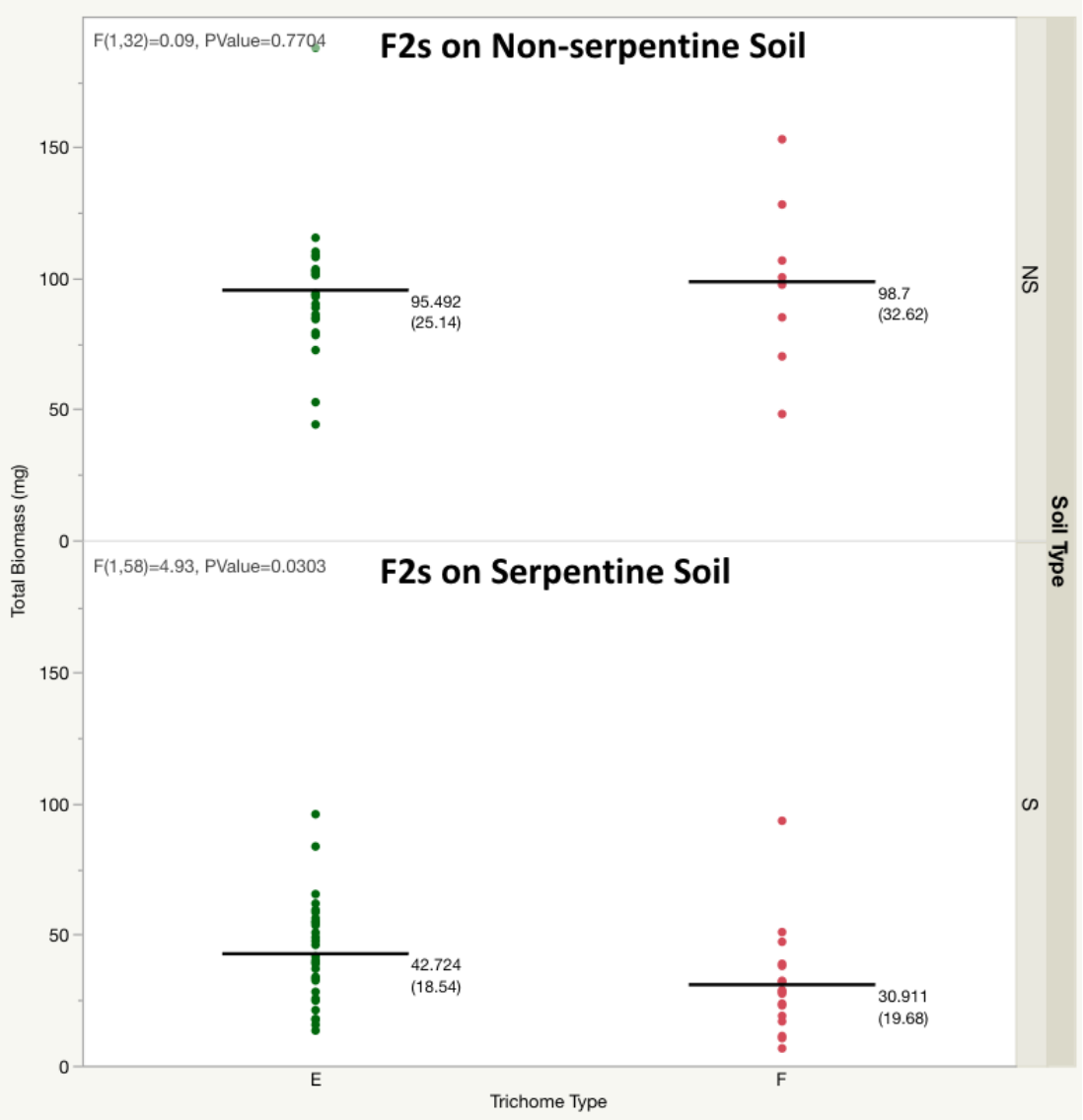

Figure 14. Comparison of total seedling biomass (mg) at harvest of F2 hybrids by trichome type on serpentine (S) and non-serpentine soil (NS). Average total biomass for each trichome type is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test for seedlings grown in non-serpentine soil (F ratio=0.983, df=1, p=0.804) and serpentine soil (F ratio=0.248, df=1, p=0.62). ANOVA found that mean seedling biomass was not significantly different between F2s with glandular (E) and non-glandular (F) trichomes when grown in non-serpentine soil (F ratio=0.09, df=1, p=0.77). ANOVA found that mean seedling biomass was significantly different between F2s with glandular and non-glandular trichomes when grown in serpentine soil (F ratio=4.93, df=1, p=0.03).

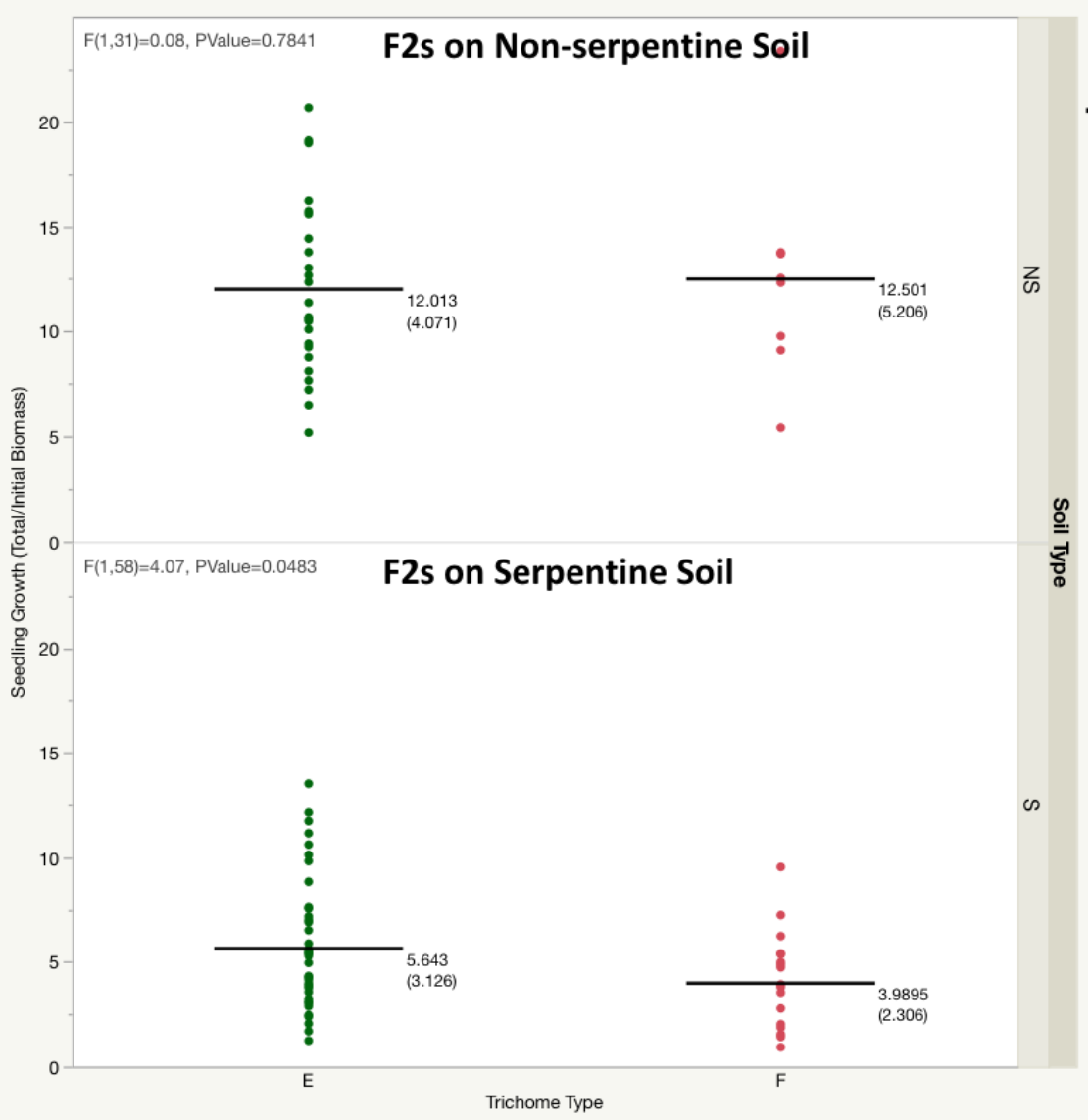

Figure 15. Comparison of seedling growth rate (total biomass at harvest/initial seedling biomass) of F2 hybrids by trichome type on serpentine (S) and non-serpentine soil (NS). Average seedling growth rate for each trichome type is indicated by the horizontal line, the number to the right of the line is the mean value and the number in the parentheses is the standard deviation. Homogeneity of variance was tested using Levene's test for seedlings grown in non-serpentine soil (F ratio=0.004, df=1, p=0.948) and serpentine soil (F ratio=1.80, df=1, p=0.185). ANOVA found that mean seedling biomass was not significantly different between F2s with glandular (E) and non-glandular (F) trichomes when grown in non-serpentine soil (F ratio=0.08, df=1, p=0.784). ANOVA found that mean seedling biomass was significantly different between F2s with glandular and non-glandular trichomes when grown in serpentine soil (F ratio=4.07, df=1, p=0.048)

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