EVOLUTION OF ROOT TRAITS IN *HELIANTHUS*

by

ALAN W. BOWSHER

(Under the Direction of Lisa A. Donovan)

ABSTRACT

It has long been recognized that species distributions are related to their ability to acquire and use resources such as water and nutrients. However, our understanding of root system variation in relation to resource availability is extremely limited, despite the central importance of roots in resource acquisition. Using the genus *Helianthus* as a phylogenetic framework, we conducted a series of controlled environment studies to examine genetically-based differentiation among species in root structure and function. Contrary to expectations, we found little evidence for tradeoffs in fine root morphology, chemistry, and anatomy across populations of 26 *Helianthus* species. However, in comparisons of six *Helianthus* species chosen as phylogenetically-independent contrasts, species native to low nutrient soils consistently produced lower total root length and greater nitrogen uptake rates than species native to high nutrient soils. This suggests that a slow-growing root system, with a high capacity to exploit nutrient pulses in soil, is favored by selection in low fertility soils. We also found that species native to low nutrient soils exhibit constitutively high exudation of primary metabolites, suggesting repeated selection for high root exudation in low nutrient soils. However, all species, regardless of their native soil fertility, responded similarly to low nutrient treatments by drastically increasing exudation of carboxylic acids, which are known to increase mineral

nutrient availability in soils. Taken together, these findings generally fit with the trait syndromes expected to characterize species native to infertile soils. However, we found little evidence for the adaptive value of specific root length, root tissue density, and root nitrogen concentrations in low resource environments, either in fine roots or the whole root system level, despite the general expectation that these traits summarize species' ecological strategies. Therefore, although several traits appear to be under strong differential selection across environmental gradients, there are likely a variety of different trait combinations that are compatible with a given environment, even in closely-related species.

INDEX WORDS: adaptation, fine roots, nutrient uptake, root exudation, plant resource use

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ALAN W. BOWSHER

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ALAN W. BOWSHER

Major Professor:	Lisa A. Donovan
Committee:	Miguel Cabrera Marc van Iersel Russell Malmberg Chung-Jui Tsai

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
May 2015

DEDICATION

To Stephanie, for her endless patience and unwavering support for me in this pursuit.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Understanding functional trait variation and its consequences for species' distributions is a central goal of plant ecology. It has long been recognized that species distributions are governed in part by their ability to acquire and use essential resources such as water and nutrients (*Grime*, 1977; *Chapin*, 1980). Therefore, traits related to resource uptake, use, and loss are of key importance in predicting the success of plant species in a given environment. Over the last several decades, numerous studies have documented patterns of co-variation among resource-use traits (termed plant 'strategies') associated with local environmental conditions (*Grime*, 1977; *Tilman*, 1980; *Chapin*, 1980; *Wright* et al., 2005; *Ordonez* et al., 2009). Specifically, species found in high resource environments typically exhibit fast growth rates, with thin, short-lived tissues capable of rapid resource acquisition, while species found in low resource environments are expected to exhibit the opposite traits in a coordinated strategy better suited for resource conservation (*Grime* 1977; *Chapin*, 1980; *Tilman*, 1982). The best evidence supporting this resource 'acquisition-conservation' tradeoff and its relevance for environmental adaptation has been in global studies of leaf tissues (*Reich* et al., 1997; *Wright* et al., 2004). However, despite the importance of roots in resource acquisition, our understanding of how root form and function relate to species' ecological strategies and distributions is limited (*Lavorel* et al., 2007).

Within plant root systems, the thinnest, most distal branches of the root system (the fine roots), are considered the most important for resource uptake. Similar to the acquisition-conservation tradeoff found in leaves, it is expected that fine root trait variation should range

from species native to resource-rich sites with thin, short-lived roots with high absorption capacity, to species native to resource-poor sites with thick, long-lived roots with lower absorption capacity (*Chapin*, 1980; *Eissenstat* and *Yanai*, 1997; *Mommer* and *Weemstra*, 2012). This focus on fine roots is largely derived from studies of woody plants, in which first- and second-order roots (the most distal branches of the root system) tend to have the thinnest diameter, highest specific root length, and highest N concentrations: traits well-suited for maximizing resource uptake (*Pregitzer* et al., 2002; *Guo* et al., 2008). However, in herbaceous plants which do not produce woody tissues in higher-order roots, different root branching orders may overlap in function (*Comas* et al., 2012). Therefore, whole root system traits, such as morphology and distribution in soil, are also expected to be under differential selection across environments in herbaceous plants. For example, species found in resource-rich sites typically develop large, fast-growing, exploratory root systems capable of rapidly exploiting soil resources, whereas those found in resource-poor sites typically have higher proportional allocation to the root system, with thick, dense roots that can tolerate long periods of nutrient limitation (*Chapin*, 1980; *Fransen* et al., 1998). As described for fine root traits, these opposing strategies at the whole root system level (rapid resource acquisition versus greater resource conservation) are expected to maximize fitness in high-resource and low-resource environments, respectively (*Grime*, 1977; *Chapin*, 1980).

In addition to root structural and nutrient uptake characteristics, root chemical exudation is another important function of plant root systems related to resource acquisition. For example, root exudation of carboxylic acid anions in response to phosphorus deficiency can release phosphorus into soil solution through ligand exchange and dissolution of soil minerals, increasing its availability for plant uptake (*Gerke* et al., 1994; *Kirk* et al., 1999). Root exudates

can also serve as chemo-attractants for beneficial soil microbes and stimulate processes of soil nutrient cycling (*Kuzyakev* and *Cheng*, 2001; *Akiyama* et al., 2005). Therefore, it is widely believed that root exudate composition, and its response to nutrient deficiency, are important adaptations to low fertility soils (*Marschner* et al., 1986; *Dakora* and *Phillips*, 2002; *Denton* et al., 2007). Species native to high fertility soils, however, are expected to maintain constitutively high root exudation rates due to their fast growth rate (*De Deyn* et al., 2008).

Despite these long-standing ecological and evolutionary theories surrounding root trait variation and its adaptive value across environments, there have been few tests in a phylogenetically-informed framework to test their predictions. Given that species are hierarchically related in branched phylogenies, it is now widely recognized that trait variation among species is influenced by their evolutionary relatedness (*Felsenstein*, 1985; *Harvey* and *Purvis*, 1991; *Blomberg* et al., 2003). Thus, accounting for species' relatedness is essential in studies of trait evolution (*Felsenstein*, 1985; *Harvey* and *Purvis*, 1991). In addition to phylogenetic influences, trait variation among species also arises from environmental influences on trait expression. Common garden studies which minimize environmental variation allow for the detection of genetically-based differences among taxa. In a phylogenetic framework, genetic differentiation between taxa native to contrasting environments in multiple lineages would provide strong support for adaptive differentiation among species.

The main objective of this research was to investigate evolutionary patterns in root trait variation and test whether these patterns provide evidence for adaptive differentiation in different environments. We conducted a series of controlled environment studies to assess whether variation in fine root and whole root system form and function is associated with characteristics of species' native environments in genus *Helianthus*. *Helianthus* has emerged as a model system

for evolutionary ecology due to the large phenotypic diversity seen among members of this genus, and the wide variety of habitats they occupy throughout North America (*Heiser* et al., 1969, *Donovan* et al., 2014). In addition, the recent use of a target enrichment approach to infer evolutionary relationships within *Helianthus* using nearly 200 genes (*Stephens* et al. in review) provides a well-supported phylogenetic framework for analyses of trait differentiation across the genus.

Specifically, we asked the following questions: [1] Is genetically-based variation in fine root traits associated with soil and climate variables in species' native environments? [2] Do whole root system morphology and nutrient uptake consistently differ between species native to high versus low fertility soils? [3] Does root exudate composition consistently differ between species native to high versus low fertility soils? In addition, whether methodological aspects of root exudate analysis, such as sampling duration, can influence assessment of treatment effects is still an open question. Therefore, we also asked: [4] Does the duration of root exudate collection period influence analyses of the effects of nutrient supply on root exudate composition? Across all of these studies, consistent associations between genetically-based differentiation for root system traits and species' native site characteristics would indicate repeated evolution of root traits in particular environments, providing strong evidence for their adaptive value.

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CHAPTER 2

FINE ROOT TRAIT DIFFERENTIATION ACROSS ENVIRONMENTS IN 26 *HELIANTHUS* SPECIES[1]

[1] Bowsher, A.W. and Donovan, L.A. To be submitted to *International Journal of Plant Sciences.*

Abstract

Recent work suggests the existence of a fundamental trade-off governing plant form and function, from a rapidly-growing, resource-acquisitive strategy to a resource-conservative, stress-tolerant strategy. While evidence supporting this trade-off has been found for leaves, our knowledge of belowground trait strategies across species and environments is limited. In this study, we investigated root trait evolution across populations of 26 species of North American *Helianthus* in a common garden environment. Although root morphological, chemical, and anatomical traits varied widely across the genus, there was a prevalent lack of phylogenetic signal, suggesting that these traits are highly evolutionarily labile. Among three of the most commonly measured root traits which are expected to summarize species ecological strategies, specific root length and root tissue density were only weakly correlated, and neither trait was associated with root nitrogen. Pairwise correlations between traits measured in the common garden and native site characteristics were generally weak, suggesting that there are a variety of viable root trait combinations both within and across environments, even in genera of closely-related species. In addition, the low phylogenetic signal detected for all root traits examined in this study suggests that root traits evolve rapidly, allowing trait combinations to rapidly re-assemble over evolutionary time. Roots, unlike leaves, must simultaneously balance acquisition of both water and nutrients, along with functions such as anchorage, chemical exudation, and microbial symbioses. Therefore, the weak evidence for correlated evolution of fine roots likely reflects the complexity of plant responses to interacting selection pressures in the belowground environment.

INDEX WORDS*:* specific root length, root tissue density, root nitrogen, adaptation, selection

Introduction

Understanding functional trait variation across species and its relevance to environmental adaptation are two of the main goals of plant physiological ecology. Over the last several decades, both theoretical and experimental work have described a global spectrum of recurring leaf trait combinations, ranging from species that produce thin, short-lived leaves capable of rapid resource acquisition to those with tough, long-lived leaves better suited for resource conservation (*Chapin*, 1980; *Grime*, 1977; *Reich* et al., 1997; *Wright* et al., 2004). Evidence indicates that the "strategies" at the opposite ends of this acquisition-conservation spectrum broadly represent adaptation in resource-rich versus resource-poor environments, respectively (*Cunningham* et al., 1999; *Wright* and *Westoby*, 1999; *Ordonez* et al., 2009; *Reich*, 2014).

Fine roots (the thinnest, most distal branches of the root system) are often considered functionally analogous to leaves in that both tissues are ephemeral and mainly involved in resource uptake. As a result, fine root trait variation has been hypothesized to reflect the tradeoff found in leaves, ranging from species native to resource-rich sites with thin, short-lived roots with high absorptive capacity, to species from resource-poor sites with thick, long-lived roots with lower absorptive capacity (*Chapin*, 1980; *Eissenstat* and *Yanai*, 1997; *Mommer* and *Weemstra*, 2012). Thin roots with high specific root length (SRL; cm g^{-1}), low root tissue density (RTD; g cm^{-3}), and high root nitrogen concentrations (root N; g g^{-1}) are expected to represent the 'acquisition' end of the spectrum, as these traits are expected to be positively associated with root elongation rates (*Eissenstat*, 1991), nutrient uptake rates (*Comas* et al., 2002), and hydraulic conductivity (*Solari* et al., 2006), and negatively correlated with root lifespan (*Tjoelker* et al., 2005). At the putative 'resource conservation' end of the spectrum, thicker, denser roots with low SRL and root N are expected to exhibit lower resource uptake capacity and slower growth

rates, but their production is compensated by greater lifespan (*Eissenstat* et al., 2000; *Mommer* and *Weemstra*, 2012). Although a few studies have supported these expected trait-trait and trait-environment correlations (*Weaver* and *Zink*, 1946; *Reich* et al., 1998; *Comas* et al., 2002; *Espeleta* et al., 2009), a number of exceptions have also been reported (e.g. *Korner* and *Renhardt*, 1987; *Paz*, 2003; *Zangaro* et al., 2008; *Holdaway* et al., 2011; *Comas* et al., 2012). As a result, our understanding of belowground trait strategies across species and environments is still limited (*Chen* et al., 2013; *Kong* et al., 2014).

The general lack of agreement among studies aiming to identify the major axes of root trait variation across species and environments could stem from several different factors. First, roots must simultaneously balance both water and nutrient acquisition, as well as other functions such as root exudation and support of mycorrhizal fungi (*Brundrett*, 2002; *Comas* et al., 2012). Given that a variety of root traits are expected to relate to these functions, many different combinations of root traits may be equally suited for similar environmental conditions (*Comas* et al., 2012). The opposite may also be true: similar combinations of root traits may be equally suited for different environmental conditions (*Ryser*, 2006). For example, thin roots with high SRL may allow efficient exploitation of soil resources, and may therefore be beneficial to highly competitive, fast-growing species in productive sites. Alternatively, thin, high SRL roots may be beneficial for plants from unproductive sites in order to maximize efficiency (root surface area per unit investment), or to aid in resource foraging (*Ryser*, 2006; *Zangaro* et al., 2008). Studies that investigate species distributed across environmental gradients could help establish whether generalizations exist. In addition, exploration of root internal anatomy should shed light on the anatomical traits underlying variation among species in ecologically-important traits such as SRL, RTD, and root N (*Eissenstat* and *Achor*, 1999; *Wahl* and *Ryser*, 2000; *Hummel* et al.,

2007). For example, RTD has been associated with number of xylem vessels, and the proportion of root cross sectional area in xylem and in the stele, suggesting that higher investment in thick secondary cell walls contributes to higher RTD (*Wahl* and *Ryser*, 2000), as well as lower SRL (*Eissenstat* and *Achor*, 1999). Furthermore, root anatomical traits themselves have been associated with species' overall growth strategies (*Wahl* and *Ryser*, 2000) and climate variables such as native site rainfall (*Nicotra* et al., 2002).

Second, plastic responses to environmental variation may have contributed to the lack of correspondence among studies examining root trait co-variation. Most large-scale (> 25 species) studies to date assessing root trait variation across species have been conducted in the field (e.g. *Craine* et al., 2001; *Chen* et al., 2013; *Kong* et al., 2014; but see *Wright* and *Westoby*, 1999); therefore, the trait-trait and trait-environment correlations observed in such studies might have arisen from plastic responses to the environment (*Rausher*, 1992; *Reich* et al., 2003). Such observational studies are undoubtedly crucial for the development of hypotheses regarding the role of selection in shaping trait evolution. However, common garden studies which minimize the influence of environmental gradients on trait variation are essential for identifying genetically-based differences among species, allowing interpretation of how these genetically-based differences might reflect adaptive differentiation across environments (*sensu Wright* and *Westoby*,1999; *Nicotra* et al., 2002).

Third, there is a growing recognition that trait co-variation among species is influenced by their evolutionary relationships (*Felsenstein* et al., 1985; *Harvey* and *Purvis*,1991). Because of their hierarchical relationships, closely-related species often have a tendency to resemble one another more closely in trait values than more distantly-related species; a condition called phylogenetic signal (*Abouheif* et al., 1999; *Blomberg* and *Garland*, 2002). In situations with high

phylogenetic signal, species cannot be viewed as statistically independent data points, violating a main assumption of correlation analyses (*Felsenstein*, 1985; *Harvey* and *Purvis*, 1991). Moreover, environmental variables can also exhibit phylogenetic signal due to niche conservatism, leading to phylogenetic structuring of trait evolution in cases of strong environmental selection (*Garland* et al., 2005). A number of methods for accounting for phylogeny in investigations of trait co-variation exist (*Felsenstein*, 1985; *Grafen*, 1989; *Housworth* et al., 2004). However, the use of such methods in the absence of phylogenetic signal is inappropriate (*Blomberg* et al., 2003; *Rheindt* et al., 2004; *Garland* et al., 2005), and can even produce artifactual results due to violations of the evolutionary models implicit in phylogenetic comparative methods (*Abouheif*, 1999; *Rheindt* et al., 2004). Therefore, assessment of levels of phylogenetic signal is essential in comparative studies examining trait coevolution (*Gittleman* and *Luh*, 1992; *Blomberg* et al., 2003).

In this study, our broad objective was to investigate evolutionary patterns in fine root trait variation and test whether these patterns support adaptive differentiation across environments. In a common garden environment, we examined genetic differentiation for 12 root morphological, chemical, and anatomical traits across populations of 26 *Helianthus* species native to diverse habitats across North America. The genus *Helianthus* is a diverse assemblage of approximately 50 herbaceous dicot species which contains both annuals and perennials. Members of this genus occupy a wide variety of habitats throughout the United States and have adopted a number of different growth forms, making the genus well-suited for investigations of evolutionary ecology (*Heiser* et al., 1969; *Donovan* et al., 2014). First, we tested whether there is support for correlated evolution of root traits. We expected SRL and root N would be positively correlated, and these two traits would be negatively correlated with RTD. We also expected that anatomical

traits reflecting investment in secondary cell walls, such as xylem vessel number and high proportional investment in xylem and stele, would be positively correlated with RTD. Tight correlations among traits would support the existence of a 'spectrum' of root ecological strategies. Second, we tested whether root traits are correlated with the native environment of each species. We expected that species exhibiting high SRL and root N, and low RTD, would be associated with higher native site resource availability, reflecting selection for rapid resource acquisition in such sites. Correlations among root traits and source environment characteristics would indicate repeated evolution of particular traits in particular environments, providing evidence for their adaptive value.

Materials and Methods

Plant material and native habitat characterization

Seeds were collected from two to three populations across the geographic range of each of 26 *Helianthus* species either directly from wild populations or from accessions maintained at the USDA National Genetic Resources Program (see geographic locations and accession numbers in Appendix Table 2.1) in 2011 and 2012. *Phoebanthus tenuifolius* seeds were also collected as an outgroup for *Helianthus* (as in *Stephens* et al., in review). To characterize the native site of each population seed source, soil and climate data were collected for each site. Five soil cores (5 cm diameter, 0-20 cm depth) were collected randomly across each soil site, dried at 60^{o}C, and analyzed for soil fertility characteristics by A & L Laboratories (North Chesterfield, VA, USA). Organic matter (OM) was assessed by loss-on-ignition at 400^{o}C. Available phosphorus (P), and extractable potassium (K), calcium (Ca), and magnesium (Mg) were determined using inductively coupled plasma spectrometry on samples extracted with the Mehlich III extractant (*Mehlich*, 1984), and cation exchange capacity (CEC) was determined by

the sum of exchangeable cations (K, Ca, and Mg). Soil pH was assessed on a 1:1 mixture of soil:deionized water. A subsample of each soil core was ground to fine powder and analyzed for total N concentration and C:N ratio (Micro-Dumas combustion; NA1500, Carlo Erba Strumentazione, Milan, Italy) by the Stable Isotope Laboratory at the University of Georgia. Climate data from each seed collection site were extracted from the WorldClim database (*Hijmans* et al., 2005): mean annual temperature and precipitation (MAT and MAP, respectively), as well as precipitation of the driest month and precipitation of the warmest quarter.

Experimental design and growth conditions

Due to the large number of plants in this study, subsets of the 26 species were grown in two separate growing seasons in the UGA greenhouse facility. Due to seedling mortality in years one and two, replicates of several populations were re-grown in year three (see Appendix Table 2.2 for years that replicates of each population were grown). Three populations of *H. annuus* and one population of *H. longifolius* were re-grown each of the three consecutive summers to evaluate potential variation across years. Each year of the study, seeds were germinated in late May. The blunt end of each seed was scarified and seeds were placed on moist filter paper in petri dishes in darkness for 24 hours at room temperature (20^{o}C). Seed coats were removed with forceps, and petri dishes were moved to a controlled-environment growth chamber (Conviron, Winnipeg, Canada) set to a 12 hour 25/20^{o}C day/night cycle with 70% relative humidity. Seedlings were misted daily with deionized water for five days. Seedlings were then individually transplanted to 7.5 cm deep plugs filled with sand and fertilized daily with a complete nutrient solution (Jack's 20-10-20; JR Peters, Inc., Allentown, PA) to allow establishment. One week later, seedlings were transplanted to 22 cm deep pots filled with a 3:1 mix of sand:Turface

(fritted clay; Profile Products, Buffalo Grove, IL) and moved to the greenhouse in a randomized complete block design, with eight blocks and one replicate plant per population per block. Plants were watered to field capacity daily, and 5 g of Osmocote Plus 15-9-12 slow-release fertilizer with micronutrients (Scotts, Marysville, OH) was added to the soil surface in each pot.

Fine root trait measurements

Each year of the study, root tissue collection took place over four days in late July (eight weeks after germination), with two complete blocks harvested per day. Plants were removed from the pots and soil was gently brushed from the root system. Studies have demonstrated that root structure varies with root order, such that first- and second-order roots (the two most distal branching orders) are expected to be the most involved in resource uptake (*Pregitzer* et al., 2002; *Guo* et al. 2008). To ensure comparison of analogous root tissues across species, a random subsample of first- and - and second-order roots (*sensu Pregitzer* et al., 2002) was collected for each individual plant and placed in plastic bags in a cooler before analysis in the laboratory (*Comas* and *Eissenstat*, 2004).

Fine root samples were rinsed in deionized water and a single 5 mm segment 2.0 cm from the root tip was cut from a random first-order root of each sample for anatomical analysis (*Hummel* et al., 2007). Fine root samples were then separated into two representative subsamples. One subsample, to be used for morphological analysis (SRL and RTD), was stained for three minutes with 0.01% (w/v) Toluidine Blue O (Carolina Biological Supply, Burlington, NC), spread in a thin layer of water in a clear plastic tray to minimize overlap, and imaged with a desktop scanner at a resolution of 400 dpi. Length and volume for each sample were determined using the software winRHIZO (v. 2002c, Regent Instruments, Quebec, Canada). As root diameter distributions exhibited a left-skewed distribution, root volume was calculated by the

sum of the volumes of each diameter class, rather than by mean diameter (*Ryser*, 2006). Scanned root samples were dried in a forced air drying oven at 60^{o}C for 72 hours and weighed. Specific root length was calculated for each sample from total root length and dry mass, and RTD was calculated from dry mass and volume. The second (unstained) fine root subsample was dried at 60^{o}C for 72 hours then ground to a fine powder, and root chemistry (root N and C:N) were determined as with the soil samples.

For anatomical analyses, the 5 mm fine root segments were fixed in 4% glutaraldehye buffered 1:1 (v/v) with potassium phosphate (0.1 M KH_2PO_4 and 0.1 M K_2HPO_4), moved through an ethanol dehydration series, and embedded in LR White acrylic resin (Electron Microscopy Sciences, Hatfield, PA). Samples were sliced to 1.0 µm thick sections with an ultramicrotome, and stained with Toluidine Blue O (0.1% w/v). Sections were photographed under light microscopy and analyzed with ZEN software (Zeiss, Oberkochen, Germany). Root anatomical traits (root cross sectional area (CSA), total xylem CSA, number of xylem vessels, number of large xylem vessels (> 225 µm^2), mean vessel CSA, proportion of root CSA in xylem, proportion of root CSA in stele, and number of vessels per unit root CSA) were measured by tracing the appropriate structures with the cursor.

At the conclusion of the study, the entire dataset, with population replicates pooled across years, included an average of 2.1 populations per species. For each class of traits (morphological, chemical, and anatomical), there were a minimum of three replicates per population, with the following average number of replicate plants (± standard deviation) per population: 9.3 (± 4.8) replicates for root morphological analyses, 7.8 (± 4.1) for chemical analyses, and 7.1 (± 4.0) for anatomical analyses. Due to insufficient root material for chemical and anatomical analyses,

these traits were not measured in several populations, resulting in an average of 1.9 and 2.0 populations per species in these analyses, respectively.

Data analysis

To account for potential year and block effects on trait variation, population least-squares means were calculated for SRL, RTD, root N, and root C:N using ANOVA. Due to logistical constraints, root subsamples assessed for anatomical characteristics were bulked by population; thus, 'block' was not recorded for root anatomy data. However, least-squares means for SRL, RTD, root N, and root C:N both with and without inclusion of block as an explanatory variable were extremely highly correlated ($r^2 > 0.98$; $p < 0.001$), as were population rankings ($r^2 > 0.98$; $p < 0.001$). Therefore, block effects were not considered further, and we assessed least-squares means for all traits using population and year as explanatory variables in ANOVA.

Pairwise correlations among trait least-squares means and environmental characteristics were assessed using JMP Pro v. 11 (SAS Institute Inc., Cary, NC). Correlations were considered significant at $p < 0.05$. One population of *H. neglectus*, and one population of *H. porteri* exhibited several root anatomical traits which were extreme outliers in our regression analyses (approximately three standard deviations from the mean), and several statistically significant trait-environment correlations became non-significant when these two populations were excluded. As such, we present results both including and excluding these populations.

We also calculated phylogenetic signal for each class of traits and habitat variables (root morphology, chemistry, and anatomy, soil, altitude, and climate) using both Pagel's λ (*Pagel*, 1999), and a multivariate extension of Blomberg's K (*Blomberg* et al., 2003) described by (*Adams*, 2014). We used the most recent diploid phylogeny of the genus (*Stephens* et al., in review). The K_{mult} function of the ape package in R (R Core Development Team) was used to

calculate Pagel's λ, as well as the multivariate K statistic, with p-values for the K-statistic based on comparing the observed data with estimates of phylogenetic signal in trees with species randomly reshuffled in 999 permutations (*Adams*, 2014).

Results

Phylogenetic signal

Among classes of variables, both root anatomy and native site soil characteristics exhibited a value of λ significantly different from one (Table 2.1), indicating that branch transformations are needed to approximate Brownian motion trait evolution for these variables (Pagel, 1999). In addition, none of the classes of root traits or environmental characteristics exhibited a significant K-statistic (Table 2.1), indicating that K is no greater than if species were randomized across the tips of the phylogeny (*Blomberg* et al., 2003). Therefore, we were unable to reject the null hypothesis of less phylogenetic signal than expected under Brownian motion, and deemed it inappropriate to use phylogenetic comparative methods to correct for phylogeny (*Gittleman* and *Luh*, 1992; *Blomberg* et al., 2003).

Trait-trait correlations

Across populations of the 26 species, root morphological and chemical traits varied roughly two-fold, with coefficients of variation between 0.14 and 0.24 for SRL, RTD, root N, and root C:N. Root anatomical traits varied from two-fold to eight-fold across species, with coefficients of variation ranging from 0.30 to 0.72 (Table 2.2).

Among morphological and chemical traits, SRL and root RTD were significantly negatively correlated, although neither trait was related to root N or root C:N (Table 2.3). Root N and root C:N were tightly negatively correlated ($r^2 = 0.94$). In terms of the anatomical characteristics underlying these traits, SRL was negatively correlated with root CSA and number

of large vessels, but no anatomical traits were related to RTD (Table 2.3). Root N, on the other hand, was positively correlated with root CSA and negatively correlated with most other anatomical traits (xylem CSA, number of vessels, proportion of root CSA in xylem and stele, and number of vessels per unit root CSA; Table 2.3). Among root anatomical traits, numerous significant correlations were observed. Root CSA was positively correlated with the number of large diameter vessels, while both total xylem CSA and number of large diameter vessels were positively correlated with mean vessel CSA, proportion of root CSA in xylem, and proportion of root CSA in stele. Number of xylem vessels was positively correlated with number of large vessels, proportion of root CSA in xylem, proportion of root CSA in stele, and number of xylem vessels per unit root CSA (Table 2.3).

Trait-environment correlations

In addition to root morphological, chemical, and anatomical traits, there was substantial variation among populations the 26 study species in characteristics of their native environments (Appendix Table 2.2, 2.3). Among soil characteristics, native sites varied from two to four-fold across species in soil pH and C:N, and varied greater than an order of magnitude in soil N, OM, CEC, K, Mg, and Ca (Appendix Table 2.2). Among climate variables, MAT ranged from 7.7 - 22.8^{o}C, and MAP ranged from 63.5 - 1679 cm yr^{-1}, while native site altitude ranged from 5 – 1489 meters above sea level (Appendix Table 2.3).

In general, trait-environment correlations were weak. We found negative correlations between SRL and native site soil CEC, K, Mg, and Ca, while RTD was significantly positively correlated with soil CEC, Mg, Ca, and pH (Table 2.4). Root N was negatively correlated with soil P, but positively with both precipitation of the driest month and precipitation of the warmest quarter (Table 2.4). Among anatomical traits, number of xylem vessels was positively associated

with soil P, K, Mg, Ca, pH, and CEC. Total xylem CSA, number of vessels, number of large vessels, proportion of root CSA in xylem, and number of vessels per root CSA were all negatively correlated with both precipitation of the driest month and precipitation of the warmest quarter. No measured root traits were significantly correlated with native site soil N or MAP (Table 2.4).

Discussion

Trait-trait correlations

Across populations of 26 *Helianthus* species, we detected substantial variation in fine root morphological, chemical, and anatomical traits. In a single genus in a controlled environment, fine root traits varied from two- to eight-fold, and exhibited little phylogenetic signal, suggesting the high evolutionary lability of these traits (*Donovan* et al., 2014). However, despite this wide variation observed in root traits among *Helianthus* populations, we found little evidence for trait co-variation.

Specific root length, RTD, and root N are among the most commonly measured root traits in comparative studies (*Comas* et al., 2002; *Comas* and *Eissenstat*, 2009; *Chen* et al., 2013, *Kong* et al., 2014). As a proxy of root system costs (mass) versus potential benefits (root length for resource uptake), SRL is expected to effectively summarize root system economy (*Ryser*, 2006), while root N is expected to reflect root metabolic activity, such as nutrient uptake (*Comas* et al., 2012). Root tissue density has been considered to reflect plant growth strategies due to its association with root system relative growth rate (*Wahl* and *Ryser*, 2000). Given the overlap in the functional correlates of SRL, root N, and RTD, these three traits are expected to tightly co-vary, with high SRL and root N associated with low RTD (*Mommer* and *Weemstra*, 2012). In agreement with the mixed support found for this expectation in field studies (*Tjoelker* et al.,

2005; *Comas* and *Eissenstat*, 2009; *Kembel* and *Cahill*, 2011; *Chen* et al., 2013; *Kong* et al., 2014), we found a weak (albeit significant) negative relationship between SRL and RTD. Neither trait was related to root N, indicating a lack of correlated evolution between these traits. In conjunction with the inconsistencies in the relationships among these root traits reported in the literature, our study lends further support to the notion that there is no single axis which summarizes variation in root traits across species (*Fort* et al., 2012; *Chen* et al., 2013). Although SRL, RTD, and root N are undoubtedly important components of species growth strategies (*Ryser* 1996; *Wahl* and *Ryser*, 2000), it seems clear that these traits have evolved largely independently of one another as separate components of an integrated plant growth strategy in *Helianthus*.

In terms of the anatomical characteristics associated with SRL and RTD, SRL was negatively correlated with root CSA as expected, given that SRL is a function of root mass, length, and CSA. Specific root length was also negatively correlated with number of large vessels, likely reflecting a diminished transport capacity in thin roots of high SRL. In contrast to SRL, however, variation in RTD could not be explained by variation in any root anatomical characteristics. This is in contrast to previous studies which have shown strong relationships between RTD and number of xylem vessels, proportion of root CSA in xylem, proportion of root CSA in stele, and number of xylem vessels per unit CSA (*Wahl* and *Ryser*, 2000). Both our study and that of *Wahl* and *Ryser* (2000) involved species native to diverse environments grown in a common garden. The lack of correspondence between studies is surprising given that the overall range of RTD observed in that study was less than that observed in the present study. However, the *Wahl* and *Ryser* (2000) study was conducted on grasses, which have been shown to differ from other functional groups in other trait correlations (*Tjoelker* et al., 2005). The lack of

relationships between RTD and root anatomical characteristics in our study likely reflects the fact that that many different combinations of root anatomical traits can produce high (or low) RTD.

In contrast, root N was related to a number of anatomical characteristics. Root N was positively correlated with root CSA, and negatively correlated with most other characteristics associated with xylem investment, including vessel number, total xylem area, and proportional investment in xylem and stele. This finding was not surprising, given that xylem tissues tend to have low nutrient content (*Li* et al., 2010). These correlations provide some evidence for fine root trade-offs in *Helianthus*: ranging from thick roots with high N concentrations, small xylem investment, and little transport capacity, to thin, low N roots with higher xylem investment and transport capacity. However, root CSA, but not root N, was actually positively correlated with number of large (> 225 μm^2) xylem vessels, which suggests exactly the opposite: higher capacity for transport in thicker roots. Given the opposing implications of these two findings, it seems evident that no consistent trade-off exists between root chemistry, root thickness, and root transport capacity.

Trait-environment correlations

Given the resource acquisition and growth rate capacities associated with SRL (*Eissenstat*, 1991; *Comas* et al., 2002), high SRL is often expected to characterize fast-growing, competitive species of high resource sites (*Eissenstat* and *Yanai*, 1997; *Comas* et al., 2002). Contrary to expectations however, SRL was negatively correlated with soil CEC, Mg, K, and Ca. It has been noted in previous studies that SRL increases in response to Mg deficiency (*Garcez* et al., 2011); therefore, the evolutionary correlation between SRL and native soil Mg levels may reflect selection across environments to maximize uptake efficiency of these mineral

macronutrients. Although previous studies have reported strong relationships between SRL and environmental variables, these findings have often been contradictory. For example, across species, SRL has been shown to show both positive and negative correlations with soil nutrient availability (compare *Ryser* and *Eek*, 2000; *Craine* et al., 2001; with *Paz*, 2003; *Tjoelker* et al., 2005; *Holdaway* et al., 2011), and altitude (compare *Korner* and *Renhardt*, 1987; with *Craine* and *Lee*, 2003). While several common garden studies have reported that species native to high rainfall sites consistently have higher SRL than those native to low rainfall sites (*Wright* and *Westoby*, 1999; *Nicotra* et al., 2002), we found no relationship between SRL and MAP. However, our study differs in that the species in our study were not chosen explicitly as phylogenetically-independent contrasts with respect to rainfall, but also differ widely in native soil nutrient availability. The lack of a relationship between SRL and MAP found in our study may reflect interacting and potentially opposing selection pressures between rainfall, nutrient availability, and potentially other unmeasured environmental variables (discussed below; *Ryser*, 2006; *Comas* et al., 2012).

As with SRL, RTD has been cited as a key determinant of the ecological strategies of herbaceous species (*Wahl* and *Ryser*, 2000; *Birouste* et al., 2014). Root tissue density tends to increase under low nutrient supply (*Ryser* and *Lambers*, 1995), and field studies have shown that RTD tends to be higher in species found in the low nutrient end of a soil fertility gradient (*Ostonen* et al., 2007). In addition, in common garden studies of species native to contrasting environments, RTD has been negatively correlated with proxies of native soil nutrient availability, presumably to fit the need for mechanically robust roots that are suited for resource conservation in stressful environments (*Wahl* and *Ryser*, 2000). Contrary to expectations, we detected weak positive correlations between RTD and CEC, soil Ca, Mg, and soil pH, suggesting

repeated evolution of tougher, more resistant fine roots in more fertile environments. In addition, we detected no significant relationships between RTD and climate variables. This result was unexpected, as field studies across latitudinal gradients have reported that RTD was negatively correlated with both MAT and MAP (*Ostonen* et al., 2007).

Among root anatomical traits, xylem vessel number was positively correlated with soil P, CEC, K, Mg, and Ca, likely reflecting the rapid nutrient uptake required for successfully competing in fertile soils. Contrary to expectations, however, number of xylem vessels was negatively correlated with mean annual precipitation, and anatomical traits involving both xylem size and number were negatively correlated with precipitation of the driest month and precipitation of the warmest quarter. These findings conflict with the traditional assumption that large vessels are especially susceptible to water-stress induced cavitation and embolism (*Pockman* and *Sperry*, 2000). However, this may reflect the prevalence of the 'live fast, die young' strategy of rapid resource acquisition in water-limited environments described previously in several *Helianthus* species (*Ludwig* et al., 2004, 2006). Large xylem vessels might allow such species to quickly complete their life cycles by maximizing water transport during early-season episodes of high water availability (*Nicotra* et al., 2002).

Trade-offs in plant structure and function

Our study adds to the growing list of reports which find little support for a single axis of variation describing fine root trait tradeoffs among species. Among three of the most commonly measured root traits which are expected to effectively summarize species ecological strategies (SRL, RTD, and root N), SRL and RTD were only weakly correlated, while neither trait was associated with root N. In addition, pairwise relationships between individual fine root traits and native environment characteristics were generally weak. The lack of phylogenetic signal in fine

root traits, combined with the large variation observed for most of the traits measured in this study, suggests that fine root traits are evolutionarily labile (*Blomberg* et al., 2003, *Rheindt* et al., 2004). Moreover, they suggest that there are a variety of equally viable trait combinations both within and across environments (*Ryser*, 2006; *Comas* et al., 2012).

Ecological and evolutionary theories of root trait variation have largely been based on the tight correlations in leaf structure and physiology observed at a global scale (*Reich* et al., 1997; *Wright* et al., 2004). This pattern of leaf trait correlations, ranging from resource-acquisitive to resource-conservative strategies, is referred to as the leaf economics spectrum, as it summarizes investment and returns in traits related to carbon acquisition (rates of photosynthesis and respiration, leaf N, leaf mass per area, and leaf lifespan; *Reich* et al., 1997; *Wright* et al., 2004). However, roots must function in the acquisition of water and numerous mineral nutrients, which vary in their mobility, spatial distribution, and temporal availability in soils. As a result, the selection pressures on fine roots to simultaneously optimize water and nutrient acquisition, along with processes such as chemical exudation, mycorrhizal symbioses, and hydraulic lift, may even constrain one another (*Ryser*, 2006). For example, it has been suggested that thin, highly-branched roots are the most effective for the acquisition of immobile nutrients such as phosphorus (*Brundrett*, 2002; *Holdaway* et al., 2011). However, higher mycorrhizal colonization rates, which are also linked with higher phosphorus acquisition (*Smith* and *Read*, 2008; *Deguchi* et al., 2012), have been associated with thicker roots (*Kong* et al., 2014). Similarly, there may be selection for higher SRL in low nutrient soils to maximize efficiency of nutrient acquisition (*Paz*, 2003; *Holdaway* et al., 2011), but also for lower SRL in arid environments to increase drought tolerance (*Nicotra* et al. 2002; *Ryser*, 2006), resulting in conflicting selection pressures in low nutrient, low rainfall habitats. Indeed, the lack of strong evolutionary correlations in root traits

detected in our study suggests that different combinations of root traits may equally optimize resource acquisition and conservation (*Comas* et al., 2012). Finally, as opposed to leaves, which are more functionally distinct from the branches supporting them, fine roots are members of a hierarchically-branched root system, with subtending roots that may overlap in function with fine roots (*Comas* et al., 2012). This may be especially true for herbaceous species such as *Helianthus*, which do not develop woody tissues in higher root branching orders (*Comas* et al., 2012). Therefore, in herbaceous species, selection may operate on the root system as a whole, rather than on fine roots as the functional unit in root systems. Clearly, the highly variable selection pressures shaping root form and function differ from those of leaves, and may explain why studies investigating correlations among leaf and root traits have found mixed results (*Withington* et al., 2006; *Zangaro* et al., 2008; *Kembel* and *Cahill*, 2011).

Although the lack of strong correlations between SRL, RTD, root N, and the anatomical components underlying these traits, may be due to the small variation within *Helianthus* relative to global variation in these traits, our findings were comparable to those reported in large-scale (> 25 species) field studies across diverse species and biomes (*Comas* and *Eissenstat*, 2009; *Chen* et al., 2013; *Kong* et al., 2014). Together with these field studies, our common garden study of genetic differentiation across environments found little evidence for the existence of a single spectrum of trait strategies analogous to that of leaves (*Chen* et al., 2013). However, in addition to the traits measured in this study, numerous other characteristics of root systems, such as root lifespan, chemical exudation, and mycorrhizal colonization, may play an important role in adaptation across environmental gradients. Future studies investigating root trait variation across species should target these rarely-measured traits in order to shed further light on potential belowground trade-offs and ecological strategies in plants.

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Table 2.1. Multivariate K-statistics (*Adams*, 2014) and Pagel's λ (*Pagel*, 1999) values for classes of root traits and native site characteristics. Root traits and native site characteristics included within each class of variables are described in the text. Significant p-values ($p < 0.05$) in boldface.

Variable Class	Multivariate K	Multivariate K P-value	Pagel's λ	Pagel's λ P-value
Root morphology	0.651	0.418	1	1
Root chemistry	1.174	0.062	1	1
Root anatomy	0.627	0.536	0.0001	**0.002**
Native site soils	0.675	0.283	0.245	**0.0001**
Native site altitude	0.845	0.103	1	1
Native site climate	0.947	0.150	0.763	0.439

Table 2.2. Summary statistics of fine root traits among 26 *Helianthus* species. Specific root length (SRL); root tissue density (RTD); root nitrogen concentration (root N); root cross sectional area (root CSA); total xylem CSA (xylem CSA); number of vessels (no. vess.); number of large vessels (> 225 μm^2; large vess.); mean xylem vessel CSA (mean vessel CSA); proportion of root CSA in xylem (xylem/root CSA); proportion of root CSA in stele (stele/root CSA); number of vessels per unit root CSA (vess./root CSA).

Root Trait	Units	Minimum	Maximum	Mean	CV
SRL	$m\ g^{-1}$	16.16	42.12	27.60	0.19
RTD	$g\ cm^{-3}$	0.022	0.046	0.034	0.14
Root N	$g\ g^{-1}$	2.20	5.58	3.50	0.21
Root C:N	-	6.20	21.43	12.53	0.24
Root CSA	μm^2	100746.17	395034.94	215797.55	0.32
Xylem CSA	μm^2	309.01	7296.09	1740.00	0.70
No. vess.	count	1.08	8.48	3.99	0.34
Large Vess.	count	1.00	3.80	2.26	0.30
Mean Vessel CSA	μm^2	142.09	1430.75	407.18	0.46
Xylem/Root CSA	-	0.002	0.039	0.009	0.72
Stele/Root CSA	-	0.026	0.102	0.046	0.33
Vess./Root CSA	-	$2.97x10^{-06}$	$4.79x10^{-05}$	$2.19x10^{-05}$	0.43

Table 2.3. Significant ($p < 0.05$) coefficients of determination among root morphological, chemical, and anatomical traits for all species. Not significant (ns). Correlations which became non-significant when the two outlier populations were excluded (*H. neglectus* and *H. petiolaris*) are indicated by (*). Trait abbreviations as in Table 2.2.

	RTD	Root N	Root C:N	Root CSA	Xylem CSA	No. Vess.	Large Vess.	Mean Vessel CSA	Xylem/ Root CSA	Stele/ Root CSA	Vess./ Root CSA
SRL	**-0.18**	ns	ns	**-0.10**	ns	ns	**-0.10**	ns	ns	ns	ns
RTD	-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Root N	-	-	**+0.95**	**+0.10**	**-0.20**	**-0.19**	ns	**-0.08***	**-0.25**	**-0.26**	**-0.27**
Root C:N	-	-	-	**-0.10**	**+0.19**	**+0.20**	ns	**+0.09***	**+0.23**	**+0.25**	**+0.26**
Root CSA	-	-	-	-	-	**-0.08**	**+0.08**	ns	**-0.20**	**-0.13**	**-0.44**
Xylem CSA	-	-	-	-	-	**+0.31**	**+0.67**	**+0.67**	**+0.70**	**+0.61**	**+0.21**
No. vessels	-	-	-	-	-	-	**+0.18**	ns	**+0.31**	**+0.31**	**+0.72**
Large Vessels	-	-	-	-	-	-	-	**+0.49**	**+0.29**	**+0.27**	ns
Mean VesselCSA	-	-	-	-	-	-	-	-	**+0.46**	**+0.35**	ns
Xylem/RootCSA	-	-	-	-	-	-	-	-	-	**+0.76**	**+0.46**
Stele/RootCSA	-	-	-	-	-	-	-	-	-	-	**+0.44**

Table 2.4. Significant ($p < 0.05$) coefficients of determination among root traits and native site characteristics across all species. Correlations which became non-significant when the two outlier populations were excluded (*H. neglectus* and *H. petiolaris*) are indicated by (*). Altitude (Alt.); mean annual temperature (MAT); mean annual precipitation (MAP); precipitation of the driest month (Prec. Driest Month); precipitation of the warmest quarter (Prec. Warm. Qtr.). Trait abbreviations as in Table 2.2.

	Soil Characteristics									**Climate and Altitude**				
Root Trait	OM (%)	N (%)	C:N	P (ppm)	CEC (meq/g)	K (ppm)	Mg (ppm)	Ca (ppm)	pH	Alt. (m)	MAT (^{o}C)	MAP (mm)	Prec. Driest Month	Prec. Warm. Qtr.
SRL	ns	ns	ns	ns	**-0.14**	**-0.08**	**-0.21**	**-0.14**	ns	ns	ns	ns	**+0.08**	ns
RTD	ns	ns	ns	ns	**+0.08**	ns	**+0.09**	**+0.09**	**+0.09**	ns	ns	ns	ns	ns
Root N	ns	ns	ns	**-0.13**	ns	ns	ns	ns	ns	ns	ns	ns	**+0.11**	**+0.15**
Root C:N	**+0.08**	ns	ns	**+0.13**	ns	**+0.10**	ns	ns	ns	ns	ns	ns	**-0.13**	**-0.20**
Root CSA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
XylemCSA	ns	ns	**+0.08***	**+0.08***	ns	ns	ns	ns	ns	ns	ns	ns	**-0.09***	**-0.08***
No. vessels	ns	ns	ns	**+0.16**	**+0.08**	**+0.12**	**+0.10**	**+0.10**	**+0.09**	ns	**-0.15**	ns	**-0.19**	**-0.16**
Large Vessels	ns	ns	ns	ns	**+0.12***	ns	**+0.10**	**+0.11***	ns	ns	ns	ns	**-0.10***	**-0.08***
Mean Vessel CSA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Xylem/Root CSA	ns	ns	ns	ns	ns	ns	ns	ns	ns	**+0.08***	ns	ns	**-0.09***	**-0.11**
Stele/Root CSA	ns	ns	ns	ns	ns	**+0.08**	ns	ns	ns	**+0.14**	**-0.08**	ns	ns	**-0.15**
Vessels/Root CSA	ns	ns	ns	**+0.11**	ns	ns	ns	ns	ns	**+0.08**	**-0.12**	ns	**-0.08**	**-0.11**

Appendix Table 2.1. Population collection sites and USDA National Genetic Resources Program (GRIN) accession numbers. Seeds used in the study were either directly wild-collected (W) or collected from accessions at GRIN (G).

Species	Population	State	Latitude	Longitude	Wild/GRIN	GRIN Accession
H. agrestis	GLA	FL	26.950980	-81.134900	G	Ames 30848
H. agrestis	HEN	FL	26.423050	-81.248400	G	Ames 30847
H. angustifolius	BAS	LA	32.985960	-91.769620	W	Ames 30800
H. angustifolius	CRP	FL	28.954300	-82.648900	W	Ames 31035
H. angustifolius	MAN	GA	33.759500	-84.855500	W	PI 649937
H. annuus	FIR	CA	36.89027778	-120.5027778	G	PI 649859
H. annuus	KON	KS	39.102117	-96.610334	W	Ames 32161
H. annuus	UTA	UT	39.716000	-112.207000	W	Ames 32162
H. argophyllus	DAY	FL	29.25388889	-81.02055556	G	PI 468651
H. argophyllus	FLB	TX	27.659363	-97.313225	W	Ames 32163
H. atrorubens	WAR	AL	33.890556	-86.825833	G	PI 649940
H. carnosus	FCR	FL	29.500656	-81.265022	W	Ames 32166
H. carnosus	POT	FL	29.609651	-81.471558	W	Ames 32167
H. carnosus	SOF	FL	29.320000	-81.310220	W	Ames 32168
H. cusickii	LIT	CA	40.41839	-120.283260	W	PI649966
H. cusickii	RAV	CA	40.67595	-120.285280	W	PI649967
H. debilis ssp. *tardiflorus*	CDK	FL	29.183200	-83.017100	W	Ames 31038
H. floridanus	APL	FL	29.714700	-85.025160	W	Ames 30843
H. floridanus	OCK	FL	29.065185	-81.950769	W	Ames 32740
H. giganteus	BUR	NC	35.811667	-82.197222	G	PI 664710
H. giganteus	LCN	OH	41.591062	-83.765136	W	PI 664647
H. grosseserratus	ATK	IL	41.635620	-89.535740	W	Ames 32174
H. grosseserratus	SAN	IL	41.069070	-87.675540	W	Ames 32176
H. heterophyllus	ANS	FL	30.058846	-85.015507	W	-
H. heterophyllus	RAM	LA	30.531060	-90.149090	W	Ames 30808
H. heterophyllus	SUP	NC	34.068611	-78.293611	G	PI 664732
H. longifolius	ELL	GA	32.425278	-84.374444	W	PI 650000
H. longifolius	FLR	AL	34.757778	-85.696389	W	PI 664680
H. longifolius	FTP	AL	34.431944	-85.675278	W	PI 650001
H. maximiliani	KON	KS	39.110012	-96.562510	W	Ames 32178
H. maximiliani	LAW	IA	42.459705	-96.194167	W	PI 613794
H. microcephalus	MTR	SC	34.947500	-83.089167	W	Ames 32179
H. microcephalus	SUN	SC	34.961111	-82.845000	W	PI 664703
H. mollis	DAR	OH	39.893363	-83.200568	G	Ames 28246
H. mollis	PEM	IL	41.089934	-87.565920	W	Ames 32180

Appendix Table 2.1 (continued)

Species	Population	State	Latitude	Longitude	Wild/GRIN	GRIN Accession
H. neglectus	KER	TX	31.825840	-103.078130	W	Ames 32182
H. neglectus	MON	TX	31.631510	-102.809990	W	Ames 32183
H. occidentalis ssp. *occidentalis*	LCN	OH	41.591337	-83.765347	W	PI 664648
H. occidentalis ssp. *occidentalis*	OQK	IL	41.029348	-90.927048	W	Ames 32185
H. petiolaris ssp. *petiolaris*	GSD	CO	37.767450	-105.515010	W	Ames 32188
H. petiolaris ssp. *petiolaris*	OQK	IL	41.053800	-90.934900	W	Ames 32189
H. porteri	CMR	GA	33.250700	-85.146599	W	Ames 32743
H. porteri	HR	GA	33.539613	-82.251380	W	Ames 32744
H. porteri	PM	GA	33.636276	-84.169514	W	Ames 32745
H. praecox ssp. *runyonii*	FBB	TX	27.649400	-97.308850	W	Ames 32191
H. praecox ssp. *runyonii*	PDI	TX	27.612450	-97.233833	W	-
P. tenuifolius	BSP	FL	30.044100	-85.011900	W	Ames 32195
H. radula	HAR	SC	32.228056	-81.074444	W	PI 664738
H. radula	RAM	LA	30.531090	-90.159410	W	Ames 30809
H. salicifolius	BRO	KS	37.877778	-95.112500	W	PI 664773
H. salicifolius	PAO	KS	38.563333	-94.790556	W	PI 664768
H. silphioides	COL	LA	32.325530	-92.208320	W	Ames 30802
H. silphioides	WEP	MO	36.663334	-91.695555	W	PI 664793
H. niveus ssp. *tephrodes*	GWR	CA	32.726667	-114.906667	G	PI 650021
H. niveus ssp. *tephrodes*	IVS	CA	32.737500	-114.913333	G	PI 650020
H. verticillatus	ALV	AL	34.141780	-85.437214	W	PI 650110
H. verticillatus	TNV	TN	35.484849	-88.711325	W	PI 650109

Appendix Table 2.2. Soil fertility characteristics of population native sites. Nitrogen (N); carbon:nitrogen (C:N); organic matter (OM); available phosphorus (P); cation exchange capacity (CEC); potassium (K); magnesium (Mg); calcium (Ca). Data are the mean of five soil cores collected at random across each population site.

Species	Population	Soil N (%)	Soil C:N	Soil OM (%)	Soil P (ppm)	Soil CEC (meq/100g)	Soil pH	Soil K (ppm)	Soil Mg (ppm)	Soil Ca (ppm)
H. agrestis	GLA	0.34	11.40	3.50	51.4	12.40	5.72	38.4	77.0	1822.6
H. agrestis	HEN	0.22	13.24	3.38	20.0	43.24	7.48	44.2	124.0	8418.4
H. angustifolius	BAS	0.07	14.86	2.14	8.4	6.80	5.00	83.4	137.0	526.4
H. angustifolius	CRP	0.06	24.06	1.92	9.2	4.20	5.38	14.4	56.6	491.6
H. angustifolius	MAN	0.08	15.09	1.94	8.4	3.52	4.62	65.0	60.4	188.6
H. annuus	FIR	0.10	10.39	1.54	153.0	53.58	7.52	832.2	1106.0	8445.6
H. annuus	KON	0.25	11.47	3.88	22.0	31.32	6.92	496.4	313.8	5334.8
H. annuus	UTA	0.07	16.32	1.10	37.4	39.76	8.20	804.0	480.2	6737.6
H. argophyllus	DAY	0.10	21.18	2.00	67.2	61.44	7.96	16.8	129.0	12060.8
H. argophyllus	FLB	0.04	13.10	0.92	11.8	4.92	7.18	80.8	94.6	782.8
H. atrorubens	WAR	0.31	10.73	4.28	10.4	28.14	7.48	191.4	447.8	4779.8
H. carnosus	FCR	0.11	21.56	0.14	20.8	5.64	8.20	32.0	171.2	821.8
H. carnosus	POT	0.06	19.92	2.53	8.3	4.00	4.95	15.0	43.5	377.0
H. carnosus	SOF	0.08	17.61	1.78	8.8	3.24	4.74	11.2	31.2	271.6
H. cusickii	LIT	0.08	12.34	2.14	15.4	5.22	5.08	23.4	81.2	501.6
H. cusickii	RAV	0.04	11.74	0.96	14.8	48.26	7.14	431.6	2030.0	6048.2
H. debilis ssp. *tardiflorus*	CDK	0.07	17.65	2.30	11.0	5.98	6.40	18.2	38.8	1071.8
H. floridanus	APL	0.12	25.08	2.50	24.6	10.80	6.82	24.8	107.4	6501.4
H. floridanus	OCK	0.12	16.77	3.22	24.2	8.42	4.42	21.0	45.2	522.0
H. giganteus	BUR	0.04	16.45	1.46	16.0	2.08	5.16	37.4	38.4	181.8
H. giganteus	LCN	0.15	13.77	3.52	68.4	7.96	6.28	64.4	197.2	1072.2
H. grosseserratus	ATK	0.23	12.47	3.88	86.4	17.44	6.96	202.4	527.0	2477.6
H. grosseserratus	SAN	0.09	16.83	1.84	44.4	7.52	7.16	76.6	219.8	1095.2
H. heterophyllus	ANS	0.09	15.06	1.84	5.4	2.48	4.46	11.8	32.4	141.2
H. heterophyllus	RAM	0.06	16.19	1.38	7.0	2.56	4.42	22.0	33.6	128.4
H. heterophyllus	SUP	0.07	31.39	3.24	9.4	3.00	4.24	11.2	28.8	126.8
H. longifolius	ELL	0.02	20.39	0.74	11.2	1.56	4.84	14.4	25.8	120.6
H. longifolius	FLR	0.29	16.46	4.50	25.0	4.10	5.08	42.0	35.4	437.0
H. longifolius	FTP	0.25	14.75	3.80	16.0	2.82	4.44	57.0	40.4	156.6
H. maximiliani	KON	0.43	15.77	5.94	18.2	42.08	7.34	462.2	176.8	7888.2
H. maximiliani	LAW	0.12	20.04	1.70	43.8	41.34	7.86	418.8	432.6	7335.6
H. microcephalus	MTR	0.11	16.35	2.48	9.0	2.62	4.60	64.8	39.6	128.2

Appendix Table 2.2. (continued)

Species	Population	Soil N (%)	Soil C:N	Soil OM (%)	Soil P (ppm)	Soil CEC (meq/100g)	Soil pH	Soil K (ppm)	Soil Mg (ppm)	Soil Ca (ppm)
H. microcephalus	SUN	0.21	18.41	4.12	14.2	8.54	5.60	167.0	186.6	912.4
H. mollis	DAR	0.17	30.33	2.94	9.4	26.06	7.48	130.8	355.2	4550.8
H. mollis	PEM	0.09	18.16	2.12	44.6	6.62	6.34	37.4	194.2	860.8
H. neglectus	KER	0.10	64.85	1.64	22.4	139.48	7.96	128.0	319.2	27300.4
H. neglectus	MON	0.00	16.19	0.30	10.2	2.60	7.60	40.4	41.4	430.6
H. occidentalis ssp. *occidentalis*	LCN	0.02	15.06	0.86	25.6	2.30	5.30	23.2	29.2	260.4
H. occidentalis ssp. *occidentalis*	OQK	0.04	16.46	1.16	47.4	9.06	7.40	28.8	99.6	1630.2
H. petiolaris ssp. *petiolaris*	GSD	0.01	7.22	0.10	19.6	4.06	7.78	185.8	101.0	549.0
H. petiolaris ssp. *petiolaris*	OQK	0.03	16.75	0.96	58.2	5.10	7.40	41.8	78.6	866.0
H. porteri	CMR	0.62	21.79	7.34	14.2	4.98	4.66	46.4	36.6	423.0
H. porteri	HR	0.45	10.18	7.44	37.8	1.68	4.44	35.6	22.2	75.8
H. porteri	PM	0.65	16.31	8.22	105.8	3.34	4.46	60.0	24.4	172.4
H. praecox ssp. *runyonii*	FBB	0.04	17.84	1.38	1.0	4.58	6.68	65.4	42.0	760.6
H. praecox ssp. *runyonii*	PDI	0.01	28.53	0.36	19.4	19.84	8.44	50.4	441.2	3206.8
P. tenuifolius	BSP	0.04	25.76	1.64	7.8	2.50	4.98	24.0	39.6	216.6
H. radula	HAR	0.07	17.17	0.10	17.4	13.18	9.00	82.4	109.8	2408.0
H. radula	RAM	0.06	13.77	0.10	17.2	13.58	8.90	85.4	129.8	2454.8
H. salicifolius	BRO	0.16	35.07	0.10	14.4	13.52	8.92	80.2	120.6	2459.8
H. salicifolius	PAO	0.27	19.15	1.36	42.0	4.32	6.36	45.0	92.2	577.4
H. silphioides	COL	0.06	16.51	1.36	8.4	6.16	6.42	62.2	67.8	949.0
H. silphioides	WEP	0.08	20.72	2.14	11.0	11.96	7.68	89.8	421.4	1638.8
H. niveus ssp. *tephrodes*	GWR	0.02	17.88	2.28	29.8	9.66	7.46	56.4	272.4	1448.6
H. niveus ssp. *tephrodes*	IVS	0.01	56.35	0.78	15.0	10.10	7.74	42.8	242.6	1591.0
H. verticillatus	ALV	0.08	18.15	2.96	9.6	5.92	5.30	38.4	125.8	533.4
H. verticillatus	TNV	0.07	12.23	1.34	5.2	11.08	4.80	85.8	299.2	635.6

Appendix Table 2.3. Climate characteristics and altitude of population native sites. Mean annual temperature and precipitation (MAT and MAP, respectively); Precipitation of the driest month (Prec. Driest Month); precipitation of the warmest quarter (Prec. Warm. Qtr).

Species	Population	Altitude (m)	MAT (^{o}C)	MAP (mm)	Prec. Driest Month (mm)	Prec. Warm. Qtr. (mm)
H. agrestis	GLA	8	22.7	1188	40	500
H. agrestis	HEN	12	22.9	1321	38	562
H. angustifolius	BAS	47	17.3	1395	79	290
H. angustifolius	CRP	17	21.1	1357	55	578
H. angustifolius	MAN	305	15.5	1359	80	321
H. annuus	FIR	44	16.6	211	0	2
H. annuus	KON	340	12.2	859	20	323
H. annuus	UTA	1580	9.8	306	18	59
H. argophyllus	DAY	1	21.2	1250	61	455
H. argophyllus	FLB	7	22.3	762	27	189
H. atrorubens	WAR	272	15.5	1461	82	325
H. carnosus	FCR	4	22.4	772	62	478
H. carnosus	POT	16	20.9	1286	60	493
H. carnosus	SOF	9	20.8	1301	62	499
H. cusickii	LIT	7	21.1	1303	8	35
H. cusickii	RAV	1619	7.2	309	9	43
H. debilis ssp. *tardiflorus*	CDK	12	20.7	1162	46	484
H. floridanus	APL	4	20.2	1459	70	523
H. floridanus	OCK	27	21	1282	54	518
H. giganteus	BUR	856	11	1391	100	363
H. giganteus	LCN	195	9.6	842	44	261
H. grosseserratus	ATK	201	9.2	908	32	310
H. grosseserratus	SAN	197	9.7	964	38	298
H. heterophyllus	ANS	13	19.8	1557	83	559
H. heterophyllus	RAM	19	19.1	1582	80	431
H. heterophyllus	SUP	17	17	1350	73	471
H. longifolius	ELL	178	17.7	1228	57	333
H. longifolius	FLR	427	14	1501	88	331
H. longifolius	FTP	478	13.9	1507	86	336

Appendix Table 2.3. (continued)

Species	Population	Altitude (m)	MAT (^{o}C)	MAP (mm)	Prec. Driest Month (mm)	Prec. Warm. Qtr. (mm)
H. maximiliani	KON	349	12.2	863	20	324
H. maximiliani	LAW	382	8.9	701	15	277
H. microcephalus	MTR	700	13	1839	135	451
H. microcephalus	SUN	299	15.1	1519	109	382
H. mollis	DAR	279	10.6	959	55	284
H. mollis	PEM	208	9.6	972	39	300
H. neglectus	KER	868	17.8	302	7	117
H. neglectus	MON	834	17.8	320	8	108
H. occidentalis ssp. *occidentalis*	LCN	195	9.6	842	44	261
H. occidentalis ssp. *occidentalis*	OQK	173	10.3	892	29	301
H. petiolaris ssp. *petiolaris*	GSD	2566	5.1	323	11	138
H. petiolaris ssp. *petiolaris*	OQK	171	10.3	891	29	301
H. porteri	CMR	251	16.2	1389	78	324
H. porteri	HR	103	16.8	1189	71	321
H. porteri	PM	212	16.3	1260	78	307
H. praecox ssp. *runyonii*	FBB	7	22.3	761	27	189
H. praecox ssp. *runyonii*	PDI	3	22.3	769	27	189
P. tenuifolius	BSP	15	19.9	1554	83	558
H. radula	HAR	67	22.5	63	53	477
H. radula	RAM	87	22.5	60	80	430
H. salicifolius	BRO	74	22.5	64	35	338
H. salicifolius	PAO	186	9.1	895	32	343
H. silphioides	COL	56	18	1362	77	281
H. silphioides	WEP	280	13	1137	64	267
H. niveus ssp. *tephrodes*	GWR	186	9.1	895	0	15
H. niveus ssp. *tephrodes*	IVS	178	9.9	939	0	16
H. verticillatus	ALV	187	15.6	1388	78	314
H. verticillatus	TNV	116	15.2	1346	77	292

Appendix Table 2.4. Number of replicate seedlings used for analysis of root morphology (morph.), chemistry (chem.), and anatomy (anat.) in each year of the study. (-) indicates no replicates.

		Common Garden 1			Common Garden 2			Common Garden 3			Total Replicates		
Species	**Population**	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.
H. agrestis	GLA	7	5	4	-	-	-	8	8	2	15	13	6
H. agrestis	HEN	-	-	-	-	1	1	4	4	4	4	5	5
H. angustifolius	BAS	5	5	2	-	-	-	4	5	2	9	10	4
H. angustifolius	CRP	4	3	4	-	-	-	-	-	-	4	3	4
H. angustifolius	MAN	6	6	5	8	8	8	1	1	1	7	7	6
H. annuus	FIR	8	6	6	8	6	7	5	5	5	21	19	19
H. annuus	KON	8	6	6	8	7	6	8	8	5	24	20	19
H. annuus	UTA	8	4	6	-	-	-	7	8	8	23	19	20
H. argophyllus	DAY	-	-	-	4	4	2	7	6	4	11	10	6
H. argophyllus	FLB	-	-	-	3	3	3	8	8	6	11	11	9
H. atrorubens	WAR	5	3	4	-	-	-	6	5	4	11	8	8
H. carnosus	FCR	8	4	4	1	-	-	-	-	-	9	4	4
H. carnosus	POT	7	3	5	3	2	4	-	-	-	10	5	9
H. carnosus	SOF	5	-	3	-	-	-	-	-	-	5	-	3
H. cusickii	LIT	-	-	-	8	3	7	6	5	5	14	8	12
H. cusickii	RAV	-	-	-	8	-	4	5	5	5	13	5	4
H. debilis ssp. *tardiflorus*	CDK	-	-	-	4	4	5	7	7	5	11	11	10
H. floridanus	APL	8	6	4	-	-	-	5	5	3	13	11	7
H. floridanus	OCK	7	6	5	-	-	-	-	-	-	7	6	5
H. giganteus	BUR	-	-	-	4	4	4	-	-	-	4	4	4
H. giganteus	LCN	-	-	-	9	8	7	-	-	-	9	8	7
H. grosseserratus	ATK	-	-	-	7	6	7	-	-	-	7	6	7
H. grosseserratus	SAN	-	-	-	8	8	7	-	-	-	8	8	7

Appendix Table 2.4. (continued)

		Common Garden 1			Common Garden 2			Common Garden 3			Total Replicates		
Species	**Population**	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.
H. heterophyllus	ANS	5	-	4	-	-	-	-	-	-	5	-	4
H. heterophyllus	RAM	5	3	-	-	-	-	-	-	-	5	3	-
H. heterophyllus	SUP	5	-	3	-	-	-	-	-	-	5	-	3
H. longifolius	ELL	8	4	5	5	2	5	2	1	2	15	7	12
H. longifolius	FLR	7	4	4	-	2	2	4	4	3	11	10	7
H. longifolius	FTP	7	7	6	8	4	3	5	5	3	20	16	12
H. maximiliani	KON	-	-	-	8	7	8	-	-	-	8	7	8
H. maximiliani	LAW	-	-	-	8	7	6	-	-	-	8	7	6
H. microcephalus	MTR	8	6	2	-	-	-	4	5	4	12	12	10
H. microcephalus	SUN	5	5	4	-	-	-	1	1	1	6	6	5
H. mollis	DAR	-	-	-	4	4	4	-	-	-	4	4	4
H. mollis	PEM	-	-	-	5	4	5	-	-	-	5	4	5
H. neglectus	KER	-	-	-	1	1		5	5	5	6	6	
H. neglectus	MON	-	-	-	4	3	3	8	8	8	12	11	11
H. occidentalis ssp. *occidentalis*	LCN	-	-	-	-	-	-	3	3	-	3	3	-
H. occidentalis ssp. *occidentalis*	OQK	-	-	-	1	1	2	5	5	3	6	6	5
H. petiolaris ssp. *petiolaris*	GSD	-	-	-	7	5	7	-	-	-	7	5	7
H. petiolaris ssp. *petiolaris*	OQK	-	-	-	8	7	5	-	-	-	8	7	5
H. porteri	CMR	8	6	4	-	-	-	6	5	2	14	11	6
H. porteri	HR	-	-	-	-	-	-	5	6	5	5	6	5
H. porteri	PM	8	6	6	-	-	-	-	-	-	8	6	6
H. praecox ssp. *runyonii*	FBB	-	-	-	-	2	2	-	2	2	-	4	4

Appendix Table 2.4. (continued)

		Common Garden 1			Common Garden 2			Common Garden 3			Total Replicates		
Species	**Population**	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.	Morph.	Chem.	Anat.
H. praecox ssp. *runyonii*	PDI	-	-	-	5	4	3	2	2	2	7	6	5
P. tenuifolius	BSP	4	3		-	-	-	-	-	-	4	3	
H. radula	HAR	7	5	4	-	-	-	-	-	-	7	5	4
H. radula	RAM	8	6	8	-	-	-	-	-	-	8	6	8
H. salicifolius	BRO	-	-	-	6	5	2	8	8	6	14	13	8
H. salicifolius	PAO	-	-	-	6	-	5	8	4	8	14	4	13
H. silphioides	COL	-	-	-	3	3	3	1	1	1	4	4	4
H. silphioides	WEP	8	5	3	-	-	-	3	3	-	11	8	3
H. niveus ssp. *tephrodes*	GWR	-	-	-	4	-	-	-	-	-	4	-	-
H. niveus ssp. *tephrodes*	IVS	-	-	-	6	-	3	-	-	-	6	-	3
H. verticillatus	ALV	8	6	6	-	-	-	-	-	-	8	6	6
H. verticillatus	TNV	8	6	8	-	-	-	-	-	-	8	6	8

Appendix Table 2.5. Least-squares means for fine root traits in populations of 26 *Helianthus* species. Least-squares means were calculated by ANOVA, with 'year grown' and 'population' as main effects. Specific root length (SRL); root tissue density (RTD); root nitrogen concentration (root N); root cross sectional area (root CSA); total xylem CSA (xylem CSA); number of vessels (no. vessels); number of large vessels (> 225 μm^2; large vessels); mean xylem vessel CSA (mean vessel CSA); proportion of root CSA in xylem (xylem/root CSA); proportion of root CSA in stele (stele/root CSA); number of vessels per unit root CSA (vessels/root CSA). (-) indicates no data collected.

Species	Population	SRL	RTD	Root N	Root C:N	Root CSA	Xylem CSA	No. vessels	Large Vessels	Mean Vessel CSA	Xylem/ Root CSA	Stele/ Root CSA	Vessels/ Root CSA
H. agrestis	GLA	25.59	0.033	3.43	12.35	236875.77	1144.80	3.09	2.03	313.62	0.005	0.036	1.50E-05
H. agrestis	HEN	19.27	0.043	3.60	12.18	193302.44	1225.22	3.52	2.07	365.11	0.007	0.034	2.10E-05
H. angustifolius	BAS	34.52	0.030	4.15	9.60	209358.35	1214.49	4.26	2.63	282.58	0.006	0.037	2.27E-05
H. angustifolius	CRP	31.18	0.031	4.31	8.13	220250.15	2213.83	3.99	2.35	402.06	0.010	0.038	2.39E-05
H. angustifolius	MAN	31.68	0.031	3.55	11.34	285803.73	1186.94	3.42	2.11	305.26	0.004	0.031	1.29E-05
H. annuus	FIR	23.75	0.033	2.67	16.15	260569.07	2723.25	5.11	2.90	576.52	0.010	0.052	2.09E-05
H. annuus	KON	23.06	0.037	2.71	16.73	176141.51	1832.61	7.48	2.57	350.58	0.010	0.047	3.70E-05
H. annuus	UTA	26.18	0.031	2.81	15.88	395034.94	3482.51	4.88	3.59	622.67	0.015	0.081	2.25E-05
H. argophyllus	DAY	30.14	0.032	3.10	13.96	223642.67	1886.18	5.01	2.69	362.26	0.008	0.049	2.38E-05
H. argophyllus	FLB	26.15	0.032	2.63	16.60	124544.11	1213.01	3.90	1.85	337.31	0.010	0.044	2.92E-05
H. atrorubens	WAR	27.12	0.033	3.71	11.41	217668.06	1996.81	5.26	2.50	331.13	0.010	0.054	2.69E-05
H. carnosus	FCR	29.62	0.032	3.98	9.39	337904.84	1264.60	2.99	2.10	394.80	0.003	0.027	8.02E-06
H. carnosus	POT	27.42	0.034	4.65	8.54	245353.75	1173.58	3.29	2.03	390.12	0.005	0.035	1.46E-05
H. carnosus	SOF	24.15	0.030	-	-	332185.73	1065.60	2.41	2.51	360.35	0.003	0.026	5.67E-06

Appendix Table 2.5. (continued)

Species	Population	SRL	RTD	Root N	Root C:N	Root CSA	Xylem CSA	No. vessels	Large Vessels	Mean Vessel CSA	Xylem/ Root CSA	Stele/ Root CSA	Vessels/ Root CSA
H. cusickii	LIT	19.38	0.034	4.05	11.14	285899.37	2122.47	4.35	3.35	581.57	0.008	0.051	1.65E-05
H. cusickii	RAV	16.16	0.035	4.27	10.15	273273.87	1816.50	4.42	3.11	429.13	0.007	0.048	1.70E-05
H. debilis ssp. *tardiflorus*	CDK	32.19	0.034	3.10	13.28	154919.66	1845.58	4.03	2.18	528.46	0.012	0.052	2.50E-05
H. floridanus	APL	26.16	0.030	3.44	12.21	320232.91	2533.68	3.47	3.23	573.31	0.007	0.031	1.15E-05
H. floridanus	OCK	23.06	0.039	3.78	11.22	378108.58	1114.99	1.94	2.45	436.18	0.002	0.028	2.97E-06
H. giganteus	BUR	23.31	0.036	3.96	11.31	229687.92	732.06	2.98	1.75	306.37	0.003	0.037	1.33E-05
H. giganteus	LCN	22.67	0.036	3.15	13.64	171398.75	1084.26	4.62	1.89	247.10	0.007	0.040	2.86E-05
H. grosseserratus	ATK	20.80	0.036	3.55	12.67	232112.56	2634.99	5.23	3.00	576.25	0.012	0.052	2.24E-05
H. grosseserratus	SAN	21.69	0.036	2.47	16.21	175739.51	2526.95	6.48	3.03	326.84	0.014	0.066	3.54E-05
H. heterophyllus	ANS	28.36	0.027	-	-	357155.05	1653.93	4.24	2.35	336.64	0.008	0.052	1.98E-05
H. heterophyllus	RAM	31.08	0.029	5.42	6.21	-	-	-	-	-	-	-	-
H. heterophyllus	SUP	25.95	0.030	-	-	272715.22	1209.76	1.08	1.85	564.10	0.006	0.041	5.23E-06
H. longifolius	ELL	37.03	0.029	4.01	9.46	160437.33	1075.16	4.47	1.65	265.81	0.007	0.042	3.05E-05
H. longifolius	FLR	36.79	0.031	3.78	10.54	170745.65	766.33	3.92	1.23	202.87	0.005	0.038	2.40E-05
H. longifolius	FTP	30.95	0.032	3.94	10.65	180412.66	882.33	2.60	1.55	329.17	0.006	0.042	1.89E-05
H. maximiliani	KON	23.74	0.036	3.57	12.66	238923.13	796.80	5.10	1.75	184.02	0.004	0.035	2.62E-05
H. maximiliani	LAW	29.71	0.035	3.21	13.16	197699.51	2099.46	5.31	2.75	396.56	0.012	0.052	2.85E-05
H. microcephalus	MTR	27.37	0.036	3.77	11.13	148027.34	2357.73	4.77	2.88	476.52	0.017	0.076	3.42E-05
H. microcephalus	SUN	28.84	0.035	3.38	12.82	207645.37	1526.20	2.35	2.36	522.49	0.009	0.060	1.57E-05
H. mollis	DAR	27.99	0.033	3.84	11.67	143157.49	309.01	3.73	1.00	142.09	0.003	0.029	2.63E-05
H. mollis	PEM	29.33	0.036	2.95	14.07	180941.03	796.34	4.48	1.35	193.02	0.005	0.034	2.76E-05
H. neglectus	KER	26.57	0.031	2.64	16.53	100746.17	7296.09	4.98	3.80	1430.75	0.039	0.088	3.54E-05
H. neglectus	MON	27.34	0.029	2.64	16.39	145580.31	3038.99	4.70	2.95	610.83	0.021	0.068	3.18E-05

Appendix Table 2.5. (continued)

Species	Population	SRL	RTD	Root N	Root C:N	Root CSA	Xylem CSA	No. vessels	Large Vessels	Mean Vessel CSA	Xylem/ Root CSA	Stele/ Root CSA	Vessels/ Root CSA
H. occidentalis ssp. *occidentalis*	LCN	32.51	0.028	3.65	11.90	-	-	-	-	-	-	-	-
H. occidentalis ssp. *occidentalis*	OQK	29.67	0.036	3.81	11.02	164668.78	918.23	3.86	1.54	250.98	0.006	0.038	2.28E-05
H. petiolaris ssp. *petiolaris*	GSD	30.66	0.033	3.15	13.59	156185.48	1243.19	3.33	1.61	431.50	0.009	0.052	2.12E-05
H. petiolaris ssp. *petiolaris*	OQK	28.80	0.030	2.70	15.11	161775.26	6086.36	8.48	2.55	543.83	0.028	0.102	4.79E-05
H. porteri	CMR	40.46	0.031	2.48	16.66	189375.05	2706.12	3.26	2.70	632.01	0.013	0.061	1.92E-05
H. porteri	HR	42.12	0.030	2.95	14.13	115274.05	1242.71	3.78	1.00	320.23	0.009	0.043	2.75E-05
H. porteri	PM	35.45	0.040	2.20	21.43	207849.50	2990.75	5.08	2.51	494.88	0.014	0.059	3.88E-05
H. praecox ssp. *runyonii*	FBB	-	-	3.20	13.76	152321.62	1478.14	3.63	2.08	440.73	0.008	0.041	2.19E-05
H. praecox ssp. *runyonii*	PDI	38.33	0.034	2.75	15.86	149544.43	1420.44	5.00	2.01	312.49	0.010	0.049	3.35E-05
P. tenuifolius	BSP	24.31	0.022	3.89	9.05	-	-	-	-	-	-	-	-
H. radula	HAR	27.92	0.037	4.63	7.86	273475.78	760.10	1.74	1.35	302.51	0.003	0.031	6.22E-06
H. radula	RAM	24.24	0.043	5.58	6.20	229782.02	941.40	1.99	1.97	374.22	0.004	0.037	1.32E-05
H. salicifolius	BRO	22.76	0.040	3.31	13.23	128822.44	980.42	2.83	1.61	336.06	0.007	0.052	2.01E-05
H. salicifolius	PAO	26.83	0.035	3.63	11.72	126531.05	1265.96	3.82	2.31	334.01	0.010	0.055	2.90E-05
H. silphioides	COL	22.01	0.045	4.29	10.78	183878.24	1265.80	3.55	1.91	469.80	0.007	0.036	1.75E-05
H. silphioides	WEP	24.80	0.033	4.20	9.17	289122.21	1237.03	3.41	2.18	264.22	0.005	0.039	1.37E-05
H. niveus ssp. *tephrodes*	GWR	26.84	0.037	-	-	-	-	-	-	-	-	-	-
H. niveus ssp. *tephrodes*	IVS	25.71	0.046	-	-	257091.56	2078.60	5.48	3.75	362.94	0.009	0.043	2.46E-05
H. verticillatus	ALV	26.10	0.041	2.96	13.59	214175.86	631.95	2.24	1.18	266.37	0.003	0.035	1.31E-05
H. verticillatus	TNV	19.65	0.042	2.48	17.28	253202.27	1129.72	2.12	2.10	389.34	0.005	0.037	1.00E-05

CHAPTER 3

NITROGEN UPTAKE AND ROOT SYSTEM MORPHOLOGY IN ECOLOGICALLY-CONTRASTING WILD SPECIES[2]

[2] Bowsher, A.B., Miller, B.J, and Donovan, L.A. To be submitted to *Functional Plant Biology*.

Abstract

Despite the importance of roots in nutrient acquisition, little is known about how root structure and function vary across fertility gradients at ecological and evolutionary scales. As a result, our understanding of how root system variation relates to plant growth strategies and distributions is limited. In this study, we examined three pairs of *Helianthus* species chosen as phylogenetically-independent contrasts with respect to native soil nutrients. Under controlled environmental conditions, we compared these six species for root morphology traits and nitrogen (N) uptake (using a ^{15}N tracer) under both high and low N supply. Species native to low nutrient soils had significantly lower total root length than those native to high nutrient soils, reflecting the inherently slow growth rates of species native to low resource environments. However, contrary to expectations, root mass ratio was consistently lower in species native to low nutrient soils, and species did not consistently differ in specific root length or root tissue density. Species native to low nutrient soils also had higher ^{15}N uptake rates (per root dry mass) than those native to high nutrient soils, but species did not differ in total ^{15}N uptake. Although several traits were influenced by N fertilization, these effects generally did not differ among species, suggesting that root system plasticity is not under differential selection in low nutrient versus high nutrient soils. Overall, the consistent evolutionary divergences detected in this study provide support for adaptive differentiation among species, with repeated evolution of slow-growing root systems suited for tolerating low resource availability in species native to low nutrient soils. However, species native to low nutrient soils maintain a high capacity for nitrate uptake, likely allowing them to maximize nutrient acquisition in times of increased nutrient availability.

INDEX WORDS: *Helianthus*, plant growth strategies, stable isotopes, specific root length, ^{15}N

Introduction

It has long been recognized that species distributions are governed in part by their ability to acquire and use essential resources such as water and nutrients (*Grime*, 1977; *Chapin*, 1980). Over the last several decades, efforts to make generalizations about the extensive variation in plant resource-use traits have culminated in several well-known theories of plant growth "strategies" (*Grime*, 1977; *Tilman*, 1980; *Chapin*, 1980; *Westoby*, 1998). Importantly, large-scale field studies have established linkages between plant growth strategies and the environments in which they are found (e.g. *Wright* et al., 2005; *Ordonez* et al., 2009), providing critical inputs for vegetation models (*Lambers* et al., 2010). While aboveground traits are well-represented in global datasets used for such purposes, root traits are not, despite the fundamental importance of roots in resource acquisition (*Kattge* et al., 2011). In addition, studies which examine root system differentiation across environmental gradients have rarely done so while also assessing its functional consequences, such as nutrient uptake (e.g. *Wahl* and *Ryser*, 2000; *Lavorel* et al., 2007; *Liu* et al., 2010; *Chen* et al., 2013). As a result, our understanding of how root form and function relate to species' ecological strategies and distributions is still extremely limited (*Lavorel* et al., 2007).

Considering the importance of mineral nutrient inputs for crop production, the best-studied factor influencing root trait variation both ecologically and evolutionarily is nutrient availability. Because nutrient uptake in low nutrient soils (LNS) is generally more limited by nutrient supply to the root surface than by root uptake processes, the maximum nutrient uptake capacity of species native to such sites is expected to be low relative to species native to high nutrient soils (HNS) (*Chapin*, 1980; *Eissenstat* and *Yanai*, 1997). However, this may not be the case for highly mobile nutrients, for which maximum uptake capacity is expected to be high in

both species native to LNS and those native to HNS (*Aerts* and *Chapin*, 2000). For the highly mobile nitrate ion, this prediction has been supported in cultivated crops bred in high resource conditions versus their wild relatives native to LNS (*Bloom*, 1985; *Epstein* and *Bloom*, 2005). However, a study of four wild herbaceous species actually found a higher capacity for nitrate uptake species native to LNS versus HNS (*Van de Dijk* et al., 1982). Whether species native to LNS versus HNS consistently differ in nutrient uptake capacity is still an open question.

While nutrient uptake processes are key components of nutrient acquisition, root morphology and distribution in the soil are also crucial elements (*Lynch*, 1995; *Fitter* et al., 2002). Species native to LNS are typically characterized by slow growth rates and low nutrient demand (*Grime* and *Hunt*, 1975; *Grime*, 1977; *Chapin*, 1980; *Poorter* and *Remkes*, 1990; *Lambers* and *Poorter*, 1992). As a result, species native to LNS are expected to adopt a long-lived, slow-growing root system with thick, dense tissues best-suited for resource conservation (*Eissenstat* and *Yanai*, 1997; *Craine* et al., 2001). Conversely, species native to HNS are expected to exhibit the opposite traits, with short-lived, thin, and fast-growing root systems capable of exploring large volumes of soil relatively quickly. Specific root length (root length per unit dry mass) is generally expected to be higher in species native to HNS, as it has been associated with root elongation rate and may therefore confer a greater competitive ability for nutrient acquisition (*Eissenstat*, 1991). As with nutrient uptake capacity, these predictions have received mixed support from the literature (*Wright* and *Westoby*; 1999; *Zangaro* et al., 2008; *Holdaway* et al., 2011; *Comas* et al., 2012). As a result, it is clear that within communities, tremendous diversity exists among species in strategies for nutrient acquisition and use, particularly among functional groups with different growth forms and life histories (*Ryser* and *Lambers*, 1995; *Wright* et al., 2004, 2005; *Lambers* et al., 2010). Along these lines, root system

differentiation among species is not only due to selection pressures in their native environments, but also due to phylogenetic constraints due to species shared evolutionary histories (*Comas* et al., 2012; *Chen* et al., 2013). Therefore, studies assessing cross-species patterns in root system evolution would be best informed using a known phylogenetic framework to account for species' relatedness (*Nicotra* et al., 2002; *Shishkova* et al., 2013).

Plastic responses to nutrient pulses may also be under differential selection in species native to LNS versus those native to HNS. It has been predicted that species native to LNS should exhibit relatively little morphological plasticity of the root system in response to nutrient additions, due to the generally short temporal availability of nutrients in LNS (*Grime* et al., 1986). Rapid root proliferation in response to nutrient pulses in generally LNS may be detrimental in the long-term as those nutrients are depleted, due to the costs of maintaining added root length. As a result, species native to LNS are generally expected to produce slow-growing, long-lived root systems which respond to nutrient additions by physiological rather than morphological plasticity (i.e. enhanced nutrient uptake; *Grime*, 1994; *Hutchings* and *de Kroon*, 1994; *Fransen* et al., 1998). On the other hand, species native to HNS are expected to display high levels of morphological plasticity in response to nutrient additions, leading to the capacity for the fast, competitive growth strategy typical of species native to such sites (*Grime*, 1994; *Fransen* et al., 1998). As a result, when grown under low nutrient supply, all plants are expected to exhibit many of the same characteristics that species native to LNS exhibit regardless of nutrient supply (*Chapin* et al., 1993). In response to high nutrient supply, however, the high morphological plasticity in species native to HNS should result in large differences in root system morphology between species native to LNS versus HNS. Therefore, assessing trait differentiation among species would most informative under more than one level of nutrient

availability, as differences might only be detected under certain nutrient supply regimes (*Chapin* 1980; *Chapin* et al., 1993). In addition, assessing trait differentiation under multiple levels of nutrient availability allows for examination of whether species differ in their plastic responses to nutrient supply, as the responses themselves may be adaptive (*Grime* et al., 1994).

In this study, we investigated evolutionary responses in root form and function to soil N availability using the genus *Helianthus* (sunflowers). *Helianthus* has emerged as a model system for evolutionary ecology due to the large phenotypic diversity within the genus (*Donovan* et al., 2014) as well as the wide variety of habitats explored by its constituent species (*Heiser* et al., 1969). We focused our study on three pairs of *Helianthus* species chosen as phylogenetically-independent contrasts: each pair includes one species native to a relatively HNS and the other native to a LNS. Using a common garden approach to minimize the influence of environmental variation on trait differences among species, we compared the species for root morphology traits and N uptake (using a ^{15}N tracer) under both high and low N supply. Specifically, we asked the following questions: **[1]** Do root morphology and N uptake consistently differ between species native to LNS versus HNS? **[2]** Do plastic responses to N fertilization consistently differ between species native to LNS versus HNS?

We predicted that, under controlled environmental conditions, species native to LNS would consistently exhibit a shorter, thicker, and denser root system with a lower capacity for N uptake than their sister taxa native to HNS. We also predicted that large morphological responses in species of HNS to N fertilization would magnify cross-species differences under high N supply relative to low. Consistent differences within each species pair in root traits (or in the response to N fertilization) would indicate repeated evolutionary responses to soil N, providing evidence for the significance of those traits across environments.

Materials and Methods

Study system and native site characterization

Seeds of 28 *Helianthus* species were collected in 2011 and 2012 and stored at 4^{o}C. Seeds were either wild-collected, or from accessions established from wild-collected seeds at the USDA National Genetic Resources Program. Five soil cores (5 cm diameter, 0 – 20 cm depth) were collected randomly across the site of each population and dried at 60^{o}C for 72 hours. Soils were analyzed for fertility characteristics by A & L Laboratories (North Chesterfield, VA, USA). Available phosphorus (P), and extractable potassium (K), calcium (Ca), and magnesium (Mg) were determined using inductively coupled plasma spectrometry on samples extracted with the Mehlich III extractant (*Mehlich*, 1984). Soil organic matter (OM) was determined by loss-on-ignition at 400^{o}C, while soil pH was measured on a 1:1 mixture of soil:deionized water. A subsample of each soil core was ground to a fine powder and analyzed for root N concentration (Micro-Dumas combustion; NA1500, Carlo Erba Strumentazione, Milan, Italy) by the Stable Isotope Laboratory at the University of Georgia. For this study, three pairs of *Helianthus* species were chosen from different clades in the *Helianthus* phylogeny (*Stephens* et al, in review) as phylogenetically-independent contrasts with respect to soil fertility (Figure 3.1). Within each pair, the native soil of one species was significantly higher in N, P, and K (Figure 3.1), as well as OM, Ca, and Mg (data not shown) than its sister taxon. The only exception to this pattern is in the *H. annuus*-*H. argophyllus* clade, as these two species did not differ in soil P (Figure 3.1). These six focal species were all directly collected from field sites: *H. annuus* (Kansas; N39°06'N 96°36'W), *H. argophyllus* (Texas; 27°38'N 97°13'W), *H. petiolaris* (Illinois; 41°55'N 90°06'W), *H. neglectus* (Texas; 31°37N 102°48'W), *H. grossesseratus* (Iowa; 42°01'N 96°01'W), *H. microcephalus* (South Carolina; 34°15'N 82°39'W). All are erect, branched

herbaceous species: *H. annuus*, *H. argophyllus*, *H. petiolaris*, and *H. neglectus* are annuals, while *H. grossesseratus* and *H. microcephalus* are perennials (*Heiser* et al., 1969).

Growth conditions and nitrogen treatments

In summer 2014, the blunt end of each seed was scarified and seeds were placed on moist filter paper in petri dishes in darkness at room temperature (20^{o}C) for 24 hours. Seed coats were gently removed using forceps, and petri dishes were moved to a controlled-environment growth chamber (Conviron, Winnipeg, Canada) under a 12-hour 25/20^{o}C day/night cycle at 70% relative humidity. Seedlings were misted daily with deionized water. Five days after scarification, 38 seedlings per species (total N = 228) were individually transplanted to 10 cm wide x 22 cm deep pots (Steuwe and Sons Inc, Corvallis, Oregon) filled with river sand. For the first three days following transplant, seedlings received a modified half-strength Hoagland's nutrient solution (Table 3.1; *Epstein* and *Bloom*, 2005) to allow establishment. On the third day, pots were thoroughly flushed with water, and plants were then randomly assigned to either a high N or low N nutrient treatment for growth. Plants of the high N treatment (n = 19 individuals per species) continued to receive the modified half-strength Hoagland's nutrient solution. Plants of the low N treatment (n = 19 individuals per species) received a solution of the same composition as the high, but with a 95% reduction in N, and additions of K_2SO_4, KH_2PO_4, and $CaSO_4$ to achieve ion balance (Table 3.1). Seedlings were watered to field capacity daily, followed by fertilization with 75 mL of the assigned nutrient solution for the first seven days of nutrient treatments, and every second day thereafter. Within each species and N treatment, 8 replicates were used for assessment of root morphology, 8 replicates were watered with a ^{15}N-enriched fertilizer [as $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$)] for assessment of N uptake, and 3 replicates served as controls to correct for background tissue ^{15}N levels (described in detail below; total n = 19 individuals per

species at each N level). Thus, both root morphology and N uptake were assessed on all six species under both high N and low N supply. Although it would be preferable to measure N uptake and root morphology on the same root systems, root systems required a nitrogenous dye to improve contrast in scanned images for assessing root morphology. Depending on the amount of dye each root system takes up, staining roots with a nitrogenous dye may differentially influence ^{15}N levels across root samples, producing an experimental artifact. Therefore, root morphology and N uptake were analyzed on separate root systems of each species (*sensu Comas* et al., 2002).

Nitrogen uptake measurements

As root system traits are known to vary with plant ontogeny (*Araújo* and *Teixeira*, 2000; *Alvarez-Flores* et al., 2014), measurements were conducted on individual plants at the same developmental stage. This procedure was selected to avoid comparing species or N treatments at different developmental stages due to their differing growth rates. Upon the emergence of the third true pair of leaves, N uptake measurements were initiated. A random subset of plants within each species and N treatment (n = 8 within each species and treatment) were watered with a complete fertilizer solution of the same total N content as the low N fertilizer, but containing 10.00 atom % ^{15}N as nitrate ($K^{15}NO_3$), and 10.00 atom % ^{15}N as ammonium [$(^{15}NH_4)_2SO_4$)] (Table 3.1). Stable isotopes were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plants received 75 mL of this solution at 0800 hours (two hours after the growth chamber lights turned on each day) and 1700 hours of the first day, and at 0800 hours of the second day. A subset of plants within each species and N treatment (n = 3 within each species and treatment) received the low N treatment (same total N content as the ^{15}N fertilizer, but lacking the ^{15}N isotope) to serve as controls for background plant ^{15}N levels. At 1400 hours on the second day of ^{15}N treatments

(30 hours after the initial ^{15}N treatment), root systems of both ^{15}N-treated and control plants were removed from the soil and rinsed with deionized water. Root systems were then freeze-dried and ground to a fine powder using a mortar and pestle, and analyzed for ^{15}N content by the Stable Isotope Laboratory at the University of Georgia. For calculation of ^{15}N uptake rates, plant ^{15}N excess was first calculated according to the excess of ^{15}N in ^{15}N-treated versus control plants (*Gioseffi* et al., 2012):

$$[APE(^{15}N) = AP_p - AP_{ptp}]$$

Where $APE(^{15}N)$ = ^{15}N atom % excess in the ^{15}N-treated plant, AP_p = ^{15}N atom % in the ^{15}N-treated plant, AP_{ptp} = mean ^{15}N atom % in control plants. Atom percent excess was then converted to mass percent excess using the following equation:

$$MPE(^{15}N) = \frac{[APE(N)*u(^{15}N)]}{[APE(N)*u(^{15}N)] + [(100-APE(N))*u(^{14}N)]}$$

Where $MPE(^{15}N)$ = ^{15}N mass % excess in ^{15}N-treated plants, u(^{15}N) = ^{15}N atomic mass, and u(^{14}N) = ^{14}N atomic mass. Finally, total excess ^{15}N content in ^{15}N-treated plants was calculated as:

$$^{15}N_{plant} = [DM \times \%N \times MPE(^{15}N)]$$

Where *DM* = whole plant dry mass, *%N* = plant N concentration. Plant ^{15}N uptake rates were then calculated as total excess ^{15}N content in ^{15}N-treated plants per unit root dry mass.

Root morphological and architectural measurements

A third subset of plants within each species and N treatment (n = 8 within each species and treatment) were used to assess root morphology, and were harvested on the second day after emergence of the third true pair of leaves, as with the ^{15}N-treated and control plants described above. Pots (still containing root systems) were sliced horizontally with a razor blade into two

sections (the upper 7 cm and the lower 15 cm) for analysis of root mass distribution. Both root system portions were stained with 0.01% (w/v) Toludine Blue O (Carolina Biological Supply, Burlington, NC) for three minutes, then rinsed in deionized water to remove excess stain. Root systems were placed in a clear plastic tray in a thin layer of deionized water to minimize overlap, and scanned on a desktop scanner at 800 dpi. Images were analyzed using the software WinRHIZO (v. 2002c, Regent Instruments, Quebec, Canada) for calculation of total length as well as diameter distributions plots, which display the total root length allocated to defined diameter classes. Both root system portions were then dried at 60^{o}C for 72 hours before weighing. Specific root length (SRL; root length per unit dry mass) and root tissue density (RTD; dry mass per unit volume) were calculated for each entire root system.

Statistical Analysis

Root traits and nutrient uptake were analyzed using two-way ANOVA within each of the two annual clades (*H. annuus-H. argophyllus*, and *H. petiolaris-H. neglectus*), with species, N supply, and their interaction as explanatory variables. Due to poor germination in *H. microcephalus*, the high N treatment was eliminated for this species: root morphology and ^{15}N uptake data were only collected under low N for *H. microcephalus*. Therefore, root characteristics of the *H. grossesseratus-H. microcephalus* clade were analyzed using t-tests to assess the effects of species (under low N only) and N supply (for *H. grossesseratus* only). Data were transformed as needed (logarithmic or arcsine-square root transformations) to approximate statistical assumptions of normality and homoscedasticity.

For diameter distribution plots, data were log-transformed, and the best-fitting polynomial curve was found for each species at each N treatment using an extra sums of squares test (*Motulsky* and *Christopoulos*, 2003). Next, these best-fit models were compared between

species within each clade, and within each species (across N treatments) using F-tests. This procedure compares models representing two different species (or treatments) by considering the best-fitting model of the pooled data (the combined model) versus representing the species (or treatments) with two separate curves (the alternative model) (*Motulsky* and *Christopoulos*, 2003). Statistical analyses were performed using JMP Pro v. 11 (SAS Institute Inc., Cary, NC).

Results

Across all species, individuals under the low N treatment reached the three-leaf-pair stage in an average of 28.7 (± 3.4) days; significantly longer than those of the high N treatment, which took an average of 21.3 (± 2.5) days (t-test, $p < 0.0001$). This difference was also significant within each species ($p < 0.05$ within each species). Therefore, we concluded that the low N treatment resulted in N-limited conditions compared to the high N treatment for all species examined. In all aspects of dry mass partitioning measured (shoot, root, total plant dry mass, and root: total mass ratio), species native to LNS consistently had lower values than species native to HNS (Figure 3.2a-d). All species produced significantly lower root dry mass (Figure 3.2b), but exhibited no change in shoot dry mass in response to high N availability (Figure 3.2c). As a result, root: total mass ratio (RMR) significantly decreased in response to high N availability in all species (Figure 3.2d). Biomass responses to N supply did not differ among species native to LNS versus those native to HNS, as indicated by the lack of significant species by N interaction terms for these biomass variables (Figure 3.2a-d).

As with RMR, species native to LNS consistently exhibited significantly lower total root length than their sister taxa native to HNS (Figure 3.3a). In addition, total root length significantly decreased in response to high N in all species examined. The magnitude of this response only differed between species in the *H. petiolaris-H. neglectus* clade , as indicated by

the significant species by N treatment interaction term (Figure 3.3a). Although the proportion of total root length in the upper 7 cm of soil significantly increased under high N in all species examined, species did not consistently differ in proportional allocation, regardless of N supply (Figure 3.3b). Neither SRL nor RTD consistently differed between species of LNS and their sister taxa native to HNS. In addition, SRL only responded to N supply in one species (SRL significantly increased under high N in *H. petiolaris*), while RTD was not modified in response to N supply in any species examined (Figure 3.3c-d).

Diameter distribution plots differed significantly between species native to contrasting soil types in both annual clades, but not in the *H. grossesseratus-H microcephalus* clade (Figure 3.4a-c). In both the *H. annuus-H. argophyllus* and the *H. petiolaris-H. neglectus* clades, species native to LNS had significantly lower root length of small diameter classes (< 0.5 mm) than their sister taxa native to HNS (Figure 3.4a-c) This within-clade pattern remained consistent both under low and high N supply. In addition, N fertilization consistently resulted in significantly higher total root length in roots of small diameter classes for all species (Figure 3.4a-c). In contrast to total root length across diameter classes, however, plots of the proportion of total root length in each diameter class did not differ among species (Figure 3.4d-f). Only the proportion of total root length by diameter plot of *H. grossesseratus* significantly differed in response to N, as this species shifted higher proportional allocation to roots greater than 0.4 mm in diameter under low N (Figure 3.4f).

In the N uptake component of the study, ^{15}N atom percent in all plants of the control group was equivalent to the natural abundance of atmospheric ^{15}N (~ 0.37%), while it was more than two-fold greater than natural abundance in all plants receiving the ^{15}N treatment (data not shown), indicating that plants of the treatment group took up the ^{15}N tracer applied. In all three

clades, species native to LNS consistently exhibited a significantly higher rate of ^{15}N uptake (μmol ^{15}N g $root^{-1}$ hr^{-1}) than their sister species native to HNS (Figure 3.5a). This pattern remained consistent regardless of N fertilization. Although there was a consistent trend towards increasing ^{15}N uptake rates in response to N fertilization in all species examined, this difference was only significant in the *H. petiolaris-H. neglectus* clade, with both species exhibiting a significant increase in ^{15}N uptake under the high N treatment (Figure 3.5a). In contrast to the rate of ^{15}N uptake, which significantly differed between species in all three clades, total ^{15}N uptake significantly differed only in the *H. grossesseratus-H. microcephalus* clade under low N supply (Figure 3.5b).

Discussion

Root morphology, ^{15}N uptake, and the influence of N supply

In this study of evolutionary divergences in root system traits using phylogenetically-independent contrasts, species native to LNS consistently produced lower total root length than their sister taxa native to HNS, regardless of N fertilization. Similar results have been seen in species native to high rainfall versus low rainfall sites, with the former producing substantially higher total root length in a common garden study (*Nicotra* et al., 2002). These findings agree with the long-standing notion that species native to HNS should produce large, exploratory root systems, an important prerequisite for successfully competing in nutrient-rich soils (*Grime*, 1977; *Chapin*, 1980; *Campbell* et al., 1991). In terms of mass allocation, all species consistently decreased RMR in response to N fertilization, a typical response to nutrient supply (*Shipley* and *Peters*, 1990; *Lynch* et al., 1991). Contrary to theoretical expectations (*Chapin*, 1980), however, species native to LNS consistently exhibited lower RMR than taxa native to HNS. Although previous studies have reported that there is little empirical evidence to support inherent

differences in mass allocation between species native to soils differing in fertility (*Campbell* et al., 1991), our study actually found significantly higher RMR in species native to HNS than their sister taxa native to LNS, possibly because our study avoided phylogenetic influences by accounting for species evolutionary relatedness.

Specific root length has historically been considered an important root system trait modulated in response to short-term changes in nutrient supply, as well as a long-term evolutionary response to nutrient availability (*Ryser*, 2006). Specific root length is typically expected to increase under multiple nutrient deficiencies, thereby increasing root exploration per unit root volume as well as nutrient uptake efficiency (*Eissenstat*, 1992; *Zobel* et al., 2006). Contrary to expectations, species native to LNS versus HNS did not consistently differ in SRL, and species did not alter SRL in response to N supply. Although mixed results for evolutionary divergences in SRL across species put into question whether generalizations can be made about SRL as an ecologically-relevant trait (*Wright* and *Westoby*, 1999; *Zobel* et al., 2006), it has been argued that high SRL may be adaptive in multiple environments (*Ryser*, 2006; *Holdaway* et al., 2011). For example, high SRL roots might be more beneficial for plants from unproductive sites in order to maximize root surface area per unit investment, or to aid in resource foraging. Alternatively, it is equally reasonable to expect that high SRL may allow efficient exploitation of soil resources, and may therefore be beneficial to highly competitive, fast-growing species in HNS (*Ryser*, 2006; *Holdaway* et al., 2011). This may explain the lack of a clear significant relationship seen between SRL and native soil fertility in the present study.

Root tissue density has also received attention as an important descriptor of species' ecological strategies (*Birouste* et al., 2014). For example, a common garden study of 19 perennial grass species found that RTD was negatively correlated with nutrient indicator values

of the species' native sites (*Wahl* and *Ryser*, 2000). However, contrary to expectations, we found no differences in RTD in species native to LNS versus HNS, potentially reflecting inherent growth differences between grasses and dicots. In addition, although species native to LNS produced significantly less root length in small diameter classes (< 0.5 mm) than species native to HNS, species did not consistently differ in the proportion of total root length across diameter classes. All species, regardless of native soil fertility, produced significantly greater root length in small diameter classes in response to higher N supply. The thinner the root, typically the more efficient it becomes, as thinner roots are able to exploit a greater volume of soil per unit volume of root (*Fitter*, 1987; *White* et al., 2013). As a result, other studies have also noted an increase in the length of small-diameter roots (< 0.5 mm) in response to N-deficiency (*Zobel* et al., 2007) as well as P-deficiency in chicory (*Zobel* et al., 2006).

In the N uptake component of the study, species native to LNS consistently exhibited significantly higher rates of ^{15}N uptake than their sister taxa native to HNS, regardless of N supply. Similar results have been found within both *Plantago* and *Hypochaeris* species native to contrasting soil environments (*Van de Dijk* et al., 1982), agreeing with the theoretical expectation that there should be uniformly strong selection across species for high nutrient uptake capacity of mobile nutrients, regardless of local soil fertility (*Aerts* and *Chapin*, 2000).

Although species native to LNS exhibited significantly higher ^{15}N uptake rates per root mass, they produced significantly less root mass than their sister taxa native to HNS, resulting in similar total ^{15}N uptake across species. In this study, N uptake was assessed using ^{15}N-labelled sources consisting mostly of nitrate, a highly mobile form of N in most soils. For acquisition of highly mobile nutrients such as nitrate, low root densities may be equally as effective as high rooting densities for plants grown in isolation (*Nye* and *Tinker*, 1977; *Fitter*, 1987; *Hodge* et al.,

1999). Thus, intra-root system competition for ^{15}N in the large root systems of HNS species may explain both: 1) why the substantially smaller total root length of species native to LNS still permitted equivalent total ^{15}N uptake to species native to HNS, and 2) why species native to LNS were found to have higher ^{15}N uptake rates than species native to HNS. Thus, for plants native to HNS, there was no additional benefit (in terms of ^{15}N uptake) received from producing higher total root system length than species native to HNS. However, in a competitive scenario, as opposed to individual plants grown in isolation, increased root proliferation results in increased nitrate acquisition relative to competitors (*Hodge* et al., 1999), and has likely selected for high root length densities that may not be as advantageous in situations with no competitors (*Craine*, 2006). This conclusion is supported by previous studies reporting that a relationship exists between nitrate uptake and root proliferation in N-rich soil patches only when species are grown in competition with one another (*Hodge* et al., 1999), but not when species are grown in isolation (*Hodge* et al., 1998). Therefore, although the high root system length produced by species native to HNS did not result in increased nitrate uptake in the present study, production of a large, fast-growing root system likely confers the ability to successfully compete for nutrients in natural environments.

A study of Kentucky bluegrass (*Poa pratensis*) found that total root length was highly positively correlated with nitrate uptake, indicating that root morphology likely plays a key role in preventing nitrate leaching (*Sullivan* et al., 2000, *Zhang* et al., 2013). At least in the short term (30 hours), this does not seem to be the case in *Helianthus*. Despite large differences in total root length, total ^{15}N uptake did not differ across species. These contradictory results may be due to methodological differences, as nutrient uptake in the bluegrass study was conducted in solution culture, preventing the formation of nutrient depletion zones common in soil (*Sullivan* et

al., 2000). Although our study was conducted in sand, likely increasing nutrient mobility and availability relative to natural soils, nitrate is highly mobile in most soil types. Indeed, the value of root system morphology in N-leaching environments has been the subject of both theoretical work (*Lynch*, 2013) and simulation modeling (*Dunbabin* et al., 2003). For example, species native to LNS are expected to produce relatively deep root systems, with little biomass in the upper soil layers, in order to capture leached nitrate at lower soil layers (*Dunbabin* et al., 2003). However, our study did not support this expectation: although all species produced proportionally lower root length in the upper soil layers under low N supply, species native to LNS versus HNS did not differ in root length allocation in the soil column.

Conclusions

Overall, this phylogenetically-informed common garden study found consistent differences in root morphology traits and ^{15}N uptake between sister taxa native to environments contrasting in soil fertility. Consistent with the expectation that selection favors a slow-growing, resource conservative growth strategy in LNS, species native to LNS consistently produced lower total root length than those native to HNS. In addition, species native to LNS had significantly lower RMR, and higher ^{15}N uptake rates, than their sister taxa native to HNS. However, due to significantly higher root masses in species native to HNS relative to species native to LNS, species did not differ in total ^{15}N uptake. The maintenance of a high capacity for N uptake in species native to both LNS and HNS likely reflects the high mobility of nitrate in different soil types, and therefore selection for a high nitrate uptake capacity regardless of native site fertility. Although several root system traits were affected by N fertilization, these affects generally did not differ among species indicating that root system plasticity for the traits we measured is likely not under differential selection in environments with contrasting soil

fertilities. While other root traits such as root lifespan, mycorrhizal status, and root exudation are likely key adaptations with respect to nutrient availability, our study detected repeated evolutionary responses to LNS, providing support for the adaptive significance of root morphological and uptake traits in soils differing in nutrient availability.

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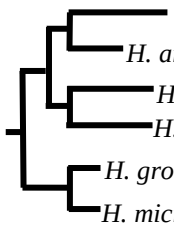

	GPS Coordinates		Native Site Soil Nutrients		
	Latitude	**Longitude**	**%N**	**P (ppm)**	**K (ppm)**
H. annuus	39.102° N	96.610° W	0.246 (0.038)[a]	22.0 (10.1)	496.4 (89.2)[a]
*H. argophyllus**	27.630° N	97.224° W	0.010 (0.001)[b]	20.8 (7.8)	32.0 (4.7)[b]
H. petiolaris	41.921° N	90.113° W	0.083 (0.026)[a]	29.8 (11.9)[a]	56.4 (7.8)[a]
*H. neglectus**	31.632° N	102.81° W	0.003 (0.001)[b]	10.2 (0.8)[b]	40.4 (5.3)[b]
H. grossesseratus	41.636° N	89.536° W	0.293 (0.027)[a]	59.4 (10.9)[a]	596.6 (92.2)[a]
*H. microcephalus**	34.262° N	82.663° W	0.064 (0.020)[b]	8.4 (1.7)[b]	89.6 (21.7)[b]

Figure 3.1. Phylogeny (pruned from that of Stephens et al. in review) and native soil characteristics of the six study species. Data are the mean (SD) of five replicates per species. Different letters indicate significant differences ($P < 0.05$) between the two sister taxa within a given clade. Species native to low nutrient soils (relative to its sister taxon) indicated by an asterisk (*). (N) Nitrogen; (P) phosphorus; (K) potassium.

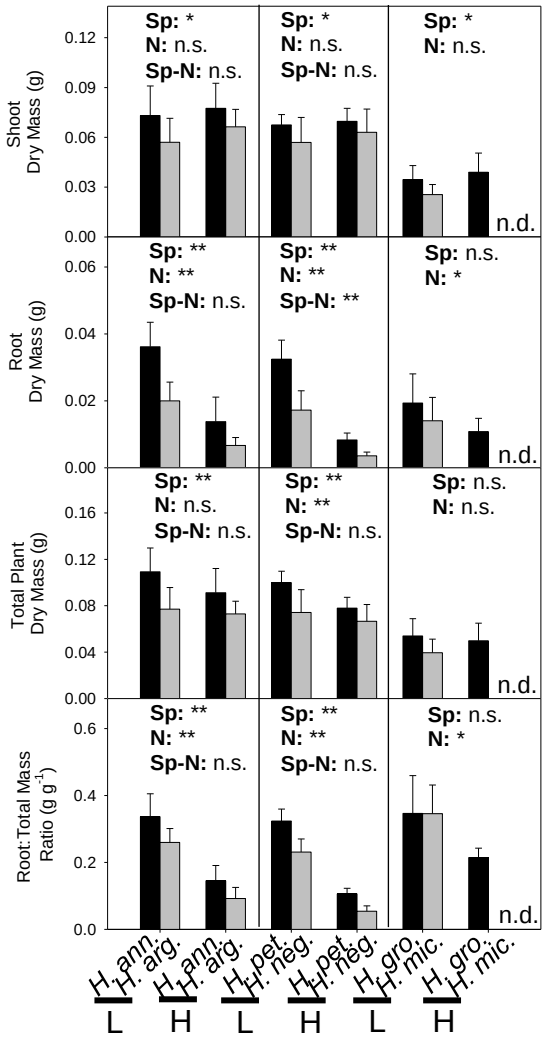

Figure 3.2. Biomass allocation in *H. annuus* (*ann.*), *H. argophyllus* (*arg.*), *H. petiolaris* (*pet.*), *H. neglectus* (*neg.*), *H. grossesseratus* (*gro.*), and *H. microcephalus* (*mic.*) grown under low (L) or high (H) nitrogen (means ± SD, n = 7-8 per species and treatment). No data (n.d.) was collected for *H. mic.* under high nitrogen. Species are arranged by clade next to its sister taxon: species native to relatively high nutrient soils are in black bars; those native to low nutrient soils are in gray bars. (a) shoot dry mass; (b) root dry mass; (c) total dry mass; (d) root:total mass ratio. One-way or two-way ANOVA effects of species (Sp), nitrogen (N), and their interaction are shown: (n.s.) not significant; (*) P<0.05; (**) P<0.01.

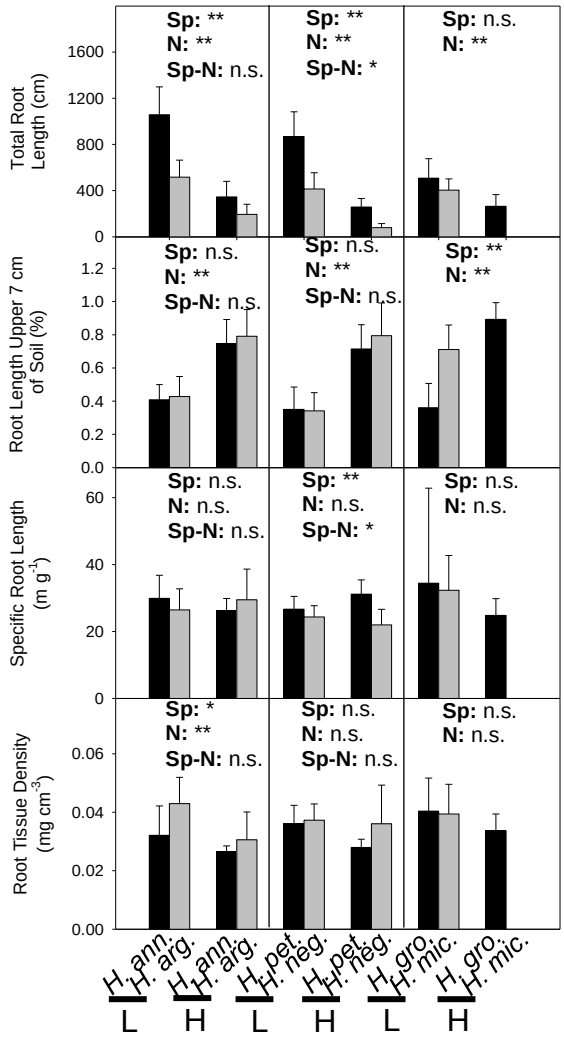

Figure 3.3. Root system morphology and distribution in six Helianthus species grown under either low (L) or high (H) nitrogen, as in Figure 3.2. (a) Total root length; (b) proportion of total root length in the upper 7 cm of soil; (c) specific root length; (d) root tissue density.

Figure 3.4. Diameter distribution plots for six *Helianthus* species grown under low (L) or high (H) nitrogen (means ± SE, n = 7-8 per species and treatment). Species native to relatively high nutrient soils are in triangles; those native to low nutrient soils are in squares. (a-c) Total root length per diameter class; (d-f) proportion of total root length per diameter class. No data was collected for *H. microcephalus* under high nitrogen. Curves separated by a (*) indicate significantly different best-fit polynomial equations ($p < 0.05$).

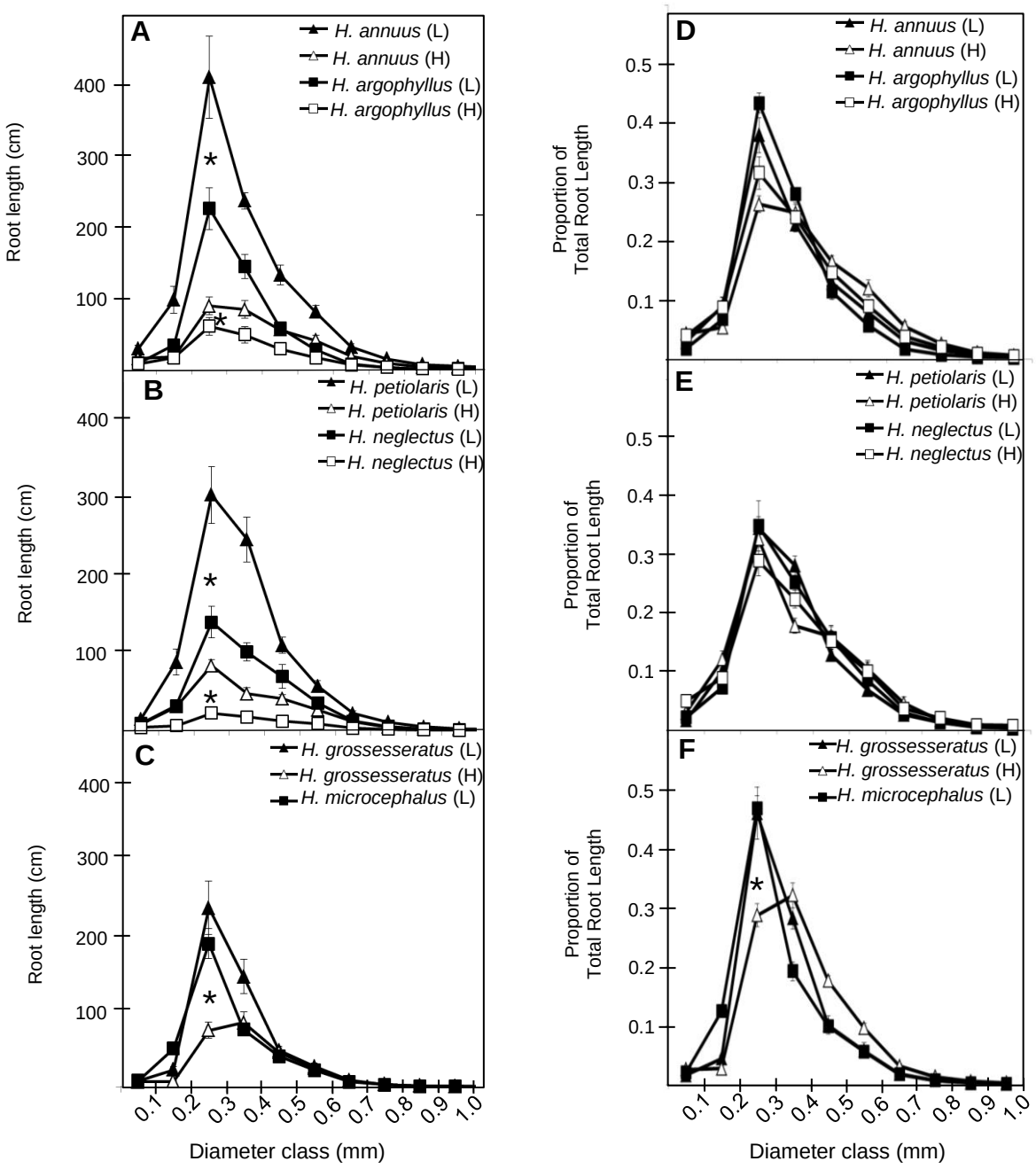
A
H. annuus (L)
H. annuus (H)
H. argophyllus (L)
H. argophyllus (H)
B
H. petiolaris (L)
H. petiolaris (H)
H. neglectus (L)
H. neglectus (H)
C
H. grossesseratus (L)
H. grossesseratus (H)
H. microcephalus (L)
Root length (cm)
Diameter class (mm)
D
E
F
Proportion of Total Root Length

Figure 3.4.

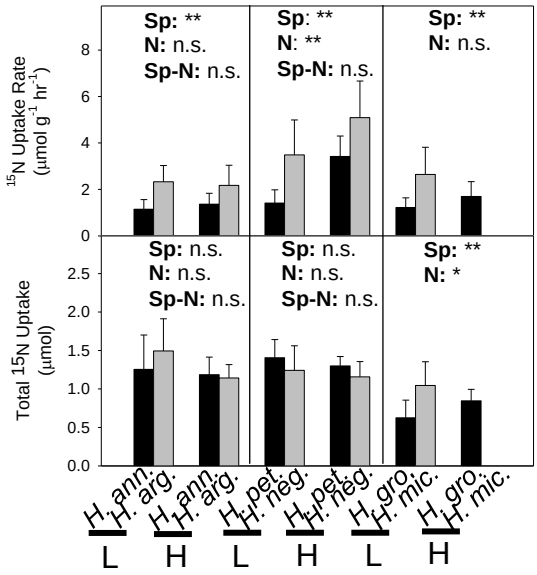

Figure 3.5. Nutrient uptake in six Helianthus species grown under either low (L) or high (H) nitrogen, as in Figure 3.2 (a) ^{15}N uptake rate; (b) total ^{15}N uptake.

Table 3.1. Composition of high nitrogen (high N), low nitrogen (low N), and ^{15}N isotope-labeled nutrient solutions applied to six *Helianthus* species. Replicates of each species received either high N or low N solution until emergence of the third true leaf pair. Seedlings used for the nutrient uptake component of the experiment then received either the ^{15}N isotope solution, or the unlabeled low N solution (to correct for background ^{15}N levels). Nutrient solutions are specified by compound (a) or by element composition (b). All values are in mmol/L.

(a)	Nutrient Solutions		
Compound	High N	Low N	^{15}N Isotope
KNO_3	3	0.35	0.35
$Ca(NO_3)_2$	2	-	-
K_2SO_4	-	0.825	0.825
KH_2PO_4	-	1	1
$NH_4H_2PO_4$	1	0.05	-
$(NH_4)_2SO_4$	-	-	0.025
$MgSO_4$	0.5	0.5	0.5
$CaSO_4$	-	2	2

(b)	Nutrient Solutions		
Element	High N	Low N	^{15}N Isotope
N	8	0.40	0.40
NO_3	7	0.35	0.35
NH_4	1	0.05	0.05
K	3	3	3
Ca	2	2	2
P	1	1.05	1
S	0.5	3.33	3.33
Mg, Cl, B, Mn, Zn, Cu, Mo, Fe	0.5	0.5	0.5

CHAPTER 4

METABOLITE ANALYSIS OF WILD SUNFLOWER (*HELIANTHUS ANNUUS* L.) ROOT EXUDATES[3]

[3] Bowsher, A.B., Ali, R., Harding, S.A., Tsai, C-J., Donovan, L.A. To be submitted to *Journal of Plant Nutrition and Soil Science*.

Abstract

Plant root systems mediate ecological processes in the rhizosphere through the exudation of organic compounds. Although exudate composition is thought to depend strongly on plant nutrient status, little is known about the influence of multi-nutrient stresses. In this study, we examined responses to short-term (three days) nutrient limitation in *Helianthus annuus* (common sunflower). Root exudates were collected for two, four, or six hours by the trap solution method. Root exudates, analyzed by gas chromatography-mass spectrometry, consisted of over 60 sugars, sugar alcohols, amino acids, organic acids, and phosphates. Abundances of over half these metabolites were affected by either nutrient supply or collection duration. Low nutrient treatments resulted in higher exudation of several sugars and organic acids, potentially representing an adaptive response to nutrient limitation in sunflower. However, as sampling interval exerted a strong influence on the apparent effects of nutrient supply, future studies should consider the potential impacts of sampling interval in comparative analyses among different genotypes or treatments.

INDEX WORDS: root exudation, metabolomics, nutrient stress, rhizosphere

Introduction

Plant roots exude a diverse array of sugars, phenolics, organic acids, lipids, and other organic substances into the rhizophere (the zone of soil immediately surrounding plant roots). This process can strongly impact rhizosphere ecology (reviewed extensively by *Bertin* et al., 2003; *Bais* et al., 2006; *Badri* and *Vivanco*, 2009), as up to 10% of photosynthetically-fixed carbon may eventually end up in the soil due to root exudation (*Barber* et al., 1976; *Jones* et al., 2004). For example, root exudates can affect microbial community structure by attracting or deterring specific microbial taxa (*Bowen* and *Rovira*, 1991; *Broeckling* et al., 2008; *Rudrappa* et al., 2008; *Eilers* et al., 2010), and several have been shown to facilitate spore germination (*Tsai* and *Phillips*, 1991; *Bücking* et al., 2008) and hyphal branching of mycorrhizal fungi (*Buée* et al., 2000; *Akiyama* et al., 2005). Root exudates also contribute to ecosystem-level processes such as nutrient cycling through their influence on microbial denitrification rates and decomposition of organic matter by affecting carbon:nitrogen ratios in soils (*Helal* and *Sauerbeck*, 1986; *Botmer* et al., 1988; *van der Krift* et al., 2001; *Michalet* et al., 2013). Metabolites such as organic acid anions can directly improve nutrient bioavailability in soils through ligand exchange, competition for adsorption sites, and changes in soil pH and redox state (*Moghimi* et al., 1978; *Marschner* et al., 1986). Nevertheless, our knowledge of rhizosphere chemistry in diverse species is still extremely limited (*Micallef* et al., 2009), as the majority of root exudate profiling studies to date have been conducted on agricultural crops or genetic model species (e.g. *Kraffczyk* et al., 1984; *Czarnota* et al., 2003; *Badri* et al., 2008; *Suzuki* et al., 2009; *Khorassani* et al., 2011).

In the species examined to date, numerous factors have been shown to influence root exudate composition, including plant genotype (*Neumann* and *Römheld*, 1999; *Fan* et al., 2001;

Badri et al., 2008; *Micallef* et al., 2009), developmental stage (*Aulakh* et al., 2001; *Chaparro* et al., 2013), water status (*Reid* and *Mexal*, 1977), local microbial communities (*Dakora* et al., 1993; *Bais* et al., 2002), and soil structure and composition (*Barber* and *Gunn*, 1974; *Neumann* et al., 2014). In addition, nutrient availability, a key factor limiting plant growth worldwide, is known to alter plant metabolism and resource allocation, which in turn should affect root exudate composition and rhizosphere interactions (*Chapin*, 1980; *Dakora* and *Phillips*, 2002). For example, nitrogen deficiency has been shown to lead to decreased exudation of amino acids, presumably as a means to conserve these nitrogen-rich metabolites (*Carvalhais* et al., 2011). Most studies which have investigated root exudation in response to nutrient limitation have generally explored the response to a single nutrient stress (often nitrogen, phosphorus, or potassium), with all other nutrients assumed to be non-limiting to plant growth (e.g. *Suzuki* et al., 2009; *Carvalhais* et al., 2011; *Khorassani* et al., 2011). However, plants in most natural environments experience co-limitation by several nutrients at some point in their life cycle, impacting their ability to respond to any one nutrient (*Bloom* et al., 1985; *Lynch* and *St. Clair*, 2004). Therefore, assessing exudate responses to multi-nutrient stresses may better represent natural conditions for many species.

In addition to the effects of environmental variables such as nutrient supply, methodological aspects such as sampling duration can also impact analyses of root exudate composition (*Aulakh* et al., 2001). Most studies of root exudation collect exudates over a single time period; typically an interval of six hours or less (*Neumann* et al., 2009). However, the rate of root exudation is not necessarily constant through time, and metabolites may differ in the magnitude and/or direction of these shifts (*Aulakh* et al., 2001). As a result, studies which sample root exudates at a single time interval may reach different conclusions than if a longer or shorter

time period was chosen, particularly when comparing exudates among treatment groups. Furthermore, collection of root exudates over extended periods may lead to underestimation of root exudation due to decomposition or re-uptake of metabolites into the root system (*Aulakh* et al., 2001, *Neumann* et al., 2009, *Carvalhais* et al., 2011). Investigations of the effects of sampling duration on root exudate composition are needed to assess the temporal dynamics of root exudation and inform future studies on the appropriate time intervals for exudate sampling.

In this study, we utilized gas chromatography coupled to mass spectrometry (GC-MS) to investigate the influence of nutrient supply and sampling interval on the root exudate composition of common wild sunflower (*Helianthus annuus* L.). *Helianthus annuus*, the wild progenitor of the domesticated sunflower, is a widely distributed annual species found in diverse habitats across most of the continental United States and southern Canada (*Heiser* et al., 1969). We hypothesized that under low nutrient supply, *H. annuus* would exhibit relatively higher exudation of organic acids and sugars (compounds known to impact soil nutrient bioavailability and plant-microbe associations), as well as lower exudation of amino acids, reflecting conservation of nitrogenous compounds. We also hypothesized that as sampling interval increased, metabolite abundance would initially increase due to accumulation in the rhizosphere, then reach a steady-state due to processes such as degradation and/or re-uptake into the root system.

Materials and Methods

Plant material, growth conditions, and nutrient treatments

Seeds of *H. annuus* were collected from a field site in central Utah, USA (39°45'N 112°18'W) in autumn 2002 and stored at 4^{o}C. On June 1, 2012, seeds were scarified near the hilum, placed on moist filter paper in petri dishes, and kept in darkness for 24 hours. Seed coats

were removed, and petri dishes were moved to a controlled-environment growth chamber (Conviron, Winnipeg, Canada) at the UGA greenhouses, with 12-hour 25/20^{o}C day/night cycle and 70% relative humidity. Seedlings were misted daily with deionized water. Five days after scarification, seedlings were transplanted to a 8.0 cm^3 section of rock wool with the roots emerging through the bottom of the wool, and placed in individual 125 mL Erlenmeyer flasks containing 50% strength modified Hoagland's nutrient solution (*Epstein* and *Bloom*, 2005), consisting of KNO_3 (3 mmol/L), $Ca(NO_3)_2$ (2 mmol/L), $NH_4H_2PO_4$ (1 mmol/L), $MgSO_4$ (0.5 mmol/L), KCl (0.025 mmol/L), H_3BO_3 (0.0125 mmol/L), $MnSO_4$ (0.001 mmol/L), $ZnSO_4$ (0.001 mmol/L), $CuSO_4$ (0.0003 mmol/L), MoO_3 (0.0003 mmol/L), and Fe-EDTA (0.025 mmol/L). The hydroponic culture method was chosen in order to eliminate soil adsorption of root exuded metabolites and to improve efficiency of root exudate collection (*Neumann* et al., 2009). Each flask was aerated by an aquarium pump and covered with aluminium foil to exclude light. Nutrient solutions and rock wool were replaced every second day to minimize changes in nutrient composition due to precipitation and uptake.

After 21 days of hydroponic growth in 50% strength modified Hoagland's nutrient solution, seedlings with two pairs of true leaves were randomly assigned to either the high (H) or low (L) nutrient treatment, consisting of 50% strength or 2.5% strength modified Hoagland's nutrient solution, respectively. Seedling root systems were rinsed for 30 seconds with deionized water to remove surface-adhering salts, then individually placed into foil-wrapped 125 mL Erlenmeyer flasks containing the appropriate nutrient treatment. Nutrient treatment solutions were renewed daily for three days in order to maintain consistent nutrient concentrations prior to exudate collection.

Root exudate collection

Seventy-two hours after the nutrient treatments were initiated, root exudates were collected by the trap-solution method (*Neumann* et al., 2009). Briefly, at 0900 hours (three hours into the photoperiod), root systems were rinsed for 30 seconds with deionized water, and intact seedlings were transferred to 15 mL glass vials covered in aluminium foil and containing 12 mL of deionized water (enough to cover the entire root system of each seedling). Seedlings from each nutrient treatment were randomly assigned to the exudate collection vials for either two, four, or six hours. Thus, our experimental design was a full factorial of nutrient treatments (H vs. L) and sampling intervals (2, 4, or 6 hours), hereafter designated H2, H4, and H6, and L2, L4, and L6, respectively. Each nutrient treatment by sampling interval combination included between five and seven individual replicates arranged in a completely randomized design. The root exudate collection medium was collected from each vial at the appropriate time interval and re-diluted to a total volume of 12 mL to correct for differences in transpirational water loss (and therefore differential concentration of root exudates) across individuals. Aliquots were snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis. Entire shoot and root tissues from each individual plant were dried at 60^{o}C for 72 hours before weighing.

Metabolite analysis

Aliquots (one mL) of the collected root exudate samples were evaporated to dryness in a CentriVap (Labconco), and analyzed by GC-MS as described (*Jeong* et al., 2004; *Frost* et al., 2012). Samples were resuspended in 50 µL of 40% methanol with 400 µM $^{13}C_6$-transcinnamic acid (Sigma-Aldrich, Milwaukee, WI) added as a loading standard. Samples were re-evaporated to dryness, then dissolved in pyridine for methoximation with methoxyamine hydrochloride (Acros, Hanover Park, IL) and derivatization with *N*-methyl-*N*-(trimethylsilyl)-

trifluoroacetamine (MSTFA; Sigma-Aldrich). Following sample randomization, 1 µL was injected in splitless mode onto a DB-5MS column (30 m x 0.25 mm ID, and 0.25 µm film, with deactivated guard column). Gas chromatographic separation was performed in an Agilent 7890A GC oven (Agilent Technologies, Wilmington, DE) with an 80°C hold for 1 minute, 20 °C per minute ramping to 200°C, and then 10°C per minute ramping to 310°C with a 6.5 minute hold at 310 °C. An Agilent 5975C quadrupole mass spectrometer was used for metabolite detection, with source and quadrupole mass filter set at 230^{o}C and 150^{o}C, respectively. Mass spectra were collected in scanning ion mode (m/z 50-500) by Chemstation (Agilent) and deconvoluted with AnalyzerPro (SpectralWorks, Runcom, UK). Peak identities were assigned using NIST08 (*Babushok* et al., 2007), Fiehnlib (*Kind* et al., 2009), and in-house spectral libraries. MetaLab, a custom web-based program (*Xue* et al. 2013), was used for compound matching between samples based on retention times and mass spectra, followed by manual curation. Metabolite peak areas were normalized to that of the loading standard and expressed per unit root dry mass.

Statistical analysis

Two-way ANOVA, with sampling interval, nutrient treatment, and their interaction as explanatory variables, was used to model normalized metabolite peak areas in JMP Pro v. 11 (SAS Institute Inc., Cary, NC). Effects were considered statistically significant at $p < 0.05$. In addition, one of the main goals of our study was to examine the influence of sampling interval on comparisons between nutrient treatments. Therefore, for each metabolite, we also expressed peak areas relative to the L2 mean, allowing for overall assessment of the influence of nutrient treatments and sampling intervals by heat maps. Heat maps displaying fold-changes in metabolite abundance between treatments were generated using conditional formatting in Excel 2010 (Microsoft Corp, Redmond, WA) after log-transforming normalized peak areas.

Results

Sampling time and nutrient supply both affect metabolite abundance

In total, 62 metabolites were detected across all six treatments and were grouped into five descriptive classes: sugars (including one glycoside), sugar alcohols, phosphates, amino acids and their derivatives, and organic acids (Figure 4.1, Appendix Table 4.1). Over one third of the detected metabolites (25/62) were significantly affected by sampling interval (Figure 4.1). These included roughly half of the detected organic acids, sugars, and sugar alcohols, as well as several amino acids and phosphates, all of which exhibited higher abundance in sampling intervals greater than two hours (Figure 4.1). Similarly, 24 of the 62 detected metabolites were significantly affected by nutrient supply, with 20 exhibiting significantly higher abundance in the high nutrient treatment, including half of the detected amino acids, phosphates, and sugar alcohols, as well as several organic acids and one sugar (Figure 4.1). The remaining four metabolites (fumaric acid, quinic acid, glucose, and isoleucine) were all detected in significantly higher abundance in the low nutrient treatment. In addition, several major organic acids (citric, malic, and succinic acid), as well as the two largest sugar peaks besides glucose (sucrose and fructose), all trended towards higher abundance in the low nutrient treatment (Figure 4.1, 4.2).

Sampling interval affects nutrient responses

The majority of metabolites detected in this study tended to exhibit a proportionally larger increase in abundance from the two to four hour intervals compared to that from the four to six hour intervals, as the major metabolites (in terms of abundance) plateaued in abundance from the four to six hour intervals (Figure 4.1, 4.2a-c). As a result, patterns of root exudate composition were very similar at the four and six hour intervals as shown in a heat map depiction (Figure 4.1). However, the magnitude and direction of these shifts varied among metabolites, and

was dependent upon the effects of nutrient supply (Figure 4.1, 4.2a-c). For example, four metabolites (ribitol, glutamine, methionine, and shikimic acid) exhibited a significant sampling interval by nutrient supply interaction due to a larger increase in abundance from the four to six hour intervals under high, relative to low, nutrient supply (Figure 4.1). Additionally, two metabolites exhibited a significant increase in abundance from the two to four hour intervals, followed by a significant decrease from the four to six hour intervals: glutamine (only at high nutrient supply) and fumaric acid (only at low nutrient supply) (Figure 4.2a,b). Three other compounds tended to continually decrease in abundance from the two to four to six hour intervals: malonic acid (only under high nutrient supply), serine, and threonine (only under low nutrient supply), although these trends were not significant (Figure 4.1, 4.2a).

As a result of the variation among metabolites in the size and direction of shifts in abundance over time, the apparent effects of nutrient supply were dependent upon sampling interval. High nutrient supply, relative to low, resulted in trends toward higher abundance of 28 of the 62 detected metabolites (45.2%) at two hours, 44/62 (70.9%) at four hours, and 46/62 (74.2%) at six hours (Figure 4.1), such that many H vs. L comparisons reversed in direction between the two and four hour intervals, but remained largely unchanged between the four and six hour intervals (Figure 4.1). In addition, high nutrient supply resulted in overall significantly higher abundance of half of the detected amino acids (Figure 4.1). However, this H vs. L differential either remained consistent (i.e. aspartic acid) or increased in magnitude (i.e. glutamine, methionine, lysine, serine, and threonine) as sampling interval increased (Figure 4.1, 4.2a). At both the two and four hour intervals, 11 amino acids trended towards higher abundance under high nutrient supply, with only one of these being statistically significant at each time interval. At the six hour interval, however, 14 of the 16 detected amino acids trended towards

higher exudation under high nutrients, with six of these being statistically significant (Figure 4.1). Similar results were seen for the majority of the sugars, organic acids, phosphates, and sugar alcohols, which generally tended to increase under high nutrient supply and with sampling duration (Figure 4.1, Appendix Table 4.1). However, low nutrient-treated plants tended to exhibit higher exudation of the major (most abundant) sugars and organic acids at all time-intervals examined (Figure 4.2b,c).

Discussion

Sampling interval and nutrient supply effects on metabolite abundance

In this controlled hydroponic study, root exudate composition of *H. annuus* was strongly influenced by both nutrient supply (H vs. L nutrient treatment) and sampling interval (2, 4 or 6 hours), with relative abundance of over half of the detected compounds significantly affected by one of the two factors. However, the effects of nutrient supply were strongly dependent upon sampling interval, with many H vs. L comparisons changing in either magnitude and/or direction as sampling interval increased.

Nearly all amino acids were detected in lower abundance under low nutrient supply; a pattern which grew stronger as sampling interval increased. Other studies have reported an overall reduction in amino acid exudation in response to N deprivation (*Bowen*, 1969; *Carvalhais* et al., 2011). Reduced amino acid concentrations in roots is a typical response to N-stress (*von Wirén* et al., 2000), likely resulting in reduced exudation either by passive diffusion or membrane transport. In addition, the observed patterns could reflect re-uptake of amino acids into the root system of low nutrient-treated plants, as amino acid uptake transporters may be up-regulated in response to N-deficiency (*Hirner* et al., 2006). Either scenario results in the reduced

net exudation of amino acids under low nutrient supply, potentially representing plant strategies for N conservation.

Sugars and organic acids comprise a major portion of root exudates in most species examined to date (*Neumann* and *Römheld*, 2001), and tended to accumulate over time in this study. However, nutrient supply also influenced several of these metabolites. Glucose, fumaric, succinic, and quinic acids were all detected in higher abundance under low nutrient supply, and a similar but non-significant trend was observed for sucrose, fructose, malic, citric, and malonic acids at all three time intervals examined. Sugars and organic acids are expected to accumulate in the roots of nutrient-deficient plants due to decreased nutrient assimilation, biosynthesis, and growth (*Marschner*, 1995; *Hernàndez* et al., 2007), and increased exudation of these compounds in response to N, P, and K deficiency has been noted in several studies (*Kraffczyk* et al., 1984; *Lipton* et al., 1987; *Paterson* and *Sim*, 1999; *Carvalhais* et al., 2011). Exudation of organic acids is expected to improve plant P acquisition from soils primarily by ligand exchange and dissolution of phosphate minerals (*Gerke* et al., 1994; *Kirk* et al., 1999). One study showed that calcicole species tend to exude higher levels of organic acids from their roots than calcifuge species, potentially contributing to their ability to colonize P- and Fe-limited calcareous soils (*Tyler* and *Strom*, 1995). In addition, exudation of sugars and organic acids in response to nutrient stress has been hypothesized as a mechanism for stimulating root-microbe relationships (*Carvalhais* et al., 2011). Though little is known about root-microbe associations in wild *Helianthus* (*van Auken* and *Freidrich*, 2006), cultivated *H. annuus* associates in the field with several bacterial species capable of producing siderophores and solubilizing phosphate (*Ambrosini* et al., 2012). Inoculation with several of these microbial species has been shown to increase growth rates and N and P uptake in cultivated sunflower (*Ambrosini* et al., 2012).

Although ruderal species such as wild *H. annuus* are expected to have low dependence on mycorrhizae, studies have shown *H. annuus* to be a facultative mycotroph which forms mycorrhizal relationships in its native soils (*van Auken* and *Freidrich*, 2006). Further studies are needed to examine the nature of these root-microbe relationships in wild *H. annuus*, and to investigate whether sunflower regulates them through exudation of sugars or organic acids.

Sampling interval effects on root exudate composition

In contrast to the observation that most metabolites tended to increase in abundance from the two to four hour intervals, the majority remained at similar abundances (or slightly decreased) from the four to six hour intervals, suggesting a decrease in exudation rates over time. Similar results were seen by *Aulakh* et al. (2001), who observed in rice that exudation rates of both organic acids and total carbohydrates declined over time. This observation may be the result of several factors. First, it may reflect re-uptake of metabolites by the roots, as mentioned above. Plants possess the ability to take up amino acids (*Williams* and *Miller*, 2001), sugars (*Yamada* et al., 2011), sugar alcohols (*Lawlor*, 1970), and organic acids (*Jones* and *Darrah*, 1995) into the root system. Secondly, since our study was not conducted under aseptic conditions, microbial degradation of compounds may have contributed (*Aulakh* et al., 2001). A study which examined exudates of maize under both sterile and non-sterile conditions found that accumulation of several sugars tended to be lower under non-sterile conditions (*Kraffczyk* et al., 1984). However, amino and organic acid abundance was unaffected, even though that study collected root exudates for a much longer time interval than the present study (10 days) (*Kraffczyk* et al., 1984). Third, declining exudation rates could have resulted from collection taking place in deionized water, which potentially induced an initial burst of exudation from roots to re-establish membrane potentials (*Aulakh* et al., 2001; *Vranova* et al., 2013). The resulting reduction in the

concentration gradient from root to rhizosphere due to metabolite accumulation in the collection media could have then led to the apparent drop in exudation rates observed in longer time intervals. However, root concentrations of sugars, amino acids, and organic acids are also expected to be several thousand-fold larger than those in soil solution (*Farrar* et al., 2003), and collecting exudates in deionized water (versus in a salt solution) has been shown to either increase or decrease exudation rates at time intervals as short as two hours, depending on metabolite identity (*Aulakh* et al., 2001). Finally, diurnal or light-induced effects may have contributed. Exudation of organic acids (*Watt* and *Evans*, 1999) and siderophores (*Zhang* et al., 1991) has been shown to peak several hours after the onset of the light period and declines thereafter. Since exudates in the present study were collected three hours after the light period began, similar effects may have resulted in the decrease in exudation rates seen for many metabolites from the four to six hour intervals.

Most likely, the initial increases followed by declines in apparent exudation rates for most of the metabolites detected represents a combination of the above factors. In any case, although exudation rates appeared to decrease as sampling interval increased, most metabolites tended to increase in abundance in intervals longer than two hours. As a result, longer sampling intervals tended to strengthen comparisons between the H and L treatments and produced repeatable patterns, as depicted by the overall similarity between the four and six hour heat maps (Figure 4.1). Consequently, although collecting exudates for periods greater than two hours can impact estimates of exudation rates, they allow for the accumulation of compounds in the collection media and assessment of treatment effects. Studies have collected exudates for intervals ranging from minutes (*Erickson* et al., 2001; *Gransee* and *Whittenmayer*, 2000) to hours (*Fan* et al., 1997; *Carvalhais* et al., 2011) to days (*Kraffczyk* et al., 1984; *Badri* et al., 2008).

Clearly, the relative importance of exudation rate versus raw abundance depends on the objectives of the study at hand.

An important caveat is that our study was conducted in hydroponic culture, which differs from natural soils in mechanical impedance, nutrient gradients, microbial communities, and aeration, among other factors (*Jones*, 1998; *Vranova* et al., 2013). In addition, plants grown in solution culture may differ from those grown in soil in root morphology, and therefore, root exudation (*Vranova* et al., 2013). However, the compounds detected in this study were qualitatively similar to those detected by GC-MS in soil-grown plants, with sugars, amino acids, and organic acids being present in high amounts (*Neumann* et al., 2014). Although root exudate composition may vary qualitatively between hydroponically-grown and soil-grown plants, similar treatment responses, such as high exudation of citrate in phosphorus-deficient conditions, have been detected in both hydroponics (e.g. *Neumann* and *Römheld*, 1999) and in soil (*Dinkelaker* et al., 1999; *Kirk* et al., 1999). As a result, it is widely accepted that stress responses detected in hydroponically-grown plants can be extrapolated to real-world soil conditions (*Neumann* and *Römheld*, 1999; *Broeckling* et al., 2008; *Carvalhais* et al., 2011; *Chaparro* et al., 2013).

Conclusions

In this study, we utilized GC-MS to examine the temporal dynamics of root exudation in the nutrient stress response of wild sunflower. Although the majority of studies to date have focused on limitation by single nutrients, we investigated responses to simultaneous limitation by multiple nutrients, which is more likely to be representative of natural environments. Across treatments, over 60 metabolites were detected, including sugars, sugar alcohols, phosphates, amino acids, and organic acids. While most metabolites were higher in abundance in the high

nutrient treatment, several exudates with putative ecological effects were higher in abundance in the low nutrient treatment, potentially representing an adaptive response to nutrient limitation in sunflower. In addition, the responses detected under multi-nutrient stress are qualitatively similar to those seen in studies of model species under single nutrient stresses, potentially highlighting the evolutionary significance of these responses to nutrient deficiency (*Fan* et al., 2001; *Carvalhais* et al., 2011). For the majority of detected metabolites, however, the effects of nutrient supply were dependent upon the duration of sampling. Although many of the H vs. L comparisons were not statistically significant at the metabolite level, obvious patterns were shared between the four and six hour intervals, suggesting overall differential metabolic allocation in response to nutrient supply. In addition, the trends observed at the four and six hour intervals match both theoretical expectations and empirical results seen in studies examining single-nutrient stress responses. Therefore, we recommend that future studies collect root exudates for a time period of between four and six hours to maximize abundance of exudates and more reliably detect treatment effects than if shorter time periods were chosen.

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Figure 4.1. Relative metabolite abundances in the root exudates of *H. annuus* plants in the low (L) or high (H) nutrient treatment, and collected for either 2, 4, or 6 hours (L2, L4, L6, or H2, H4, H6, respectively). Metabolite peak areas (the mean of three to seven replicate plants within each treatment) were normalized by root dry mass and expressed relative to the L2 group. Darker shades indicate higher values relative to L2, while lighter shades indicate lower values relative to L2. Asterisks indicate significant effects ($p < 0.05$) of sampling interval (I), nutrient supply (N), or their interaction (*/*/*, respectively).

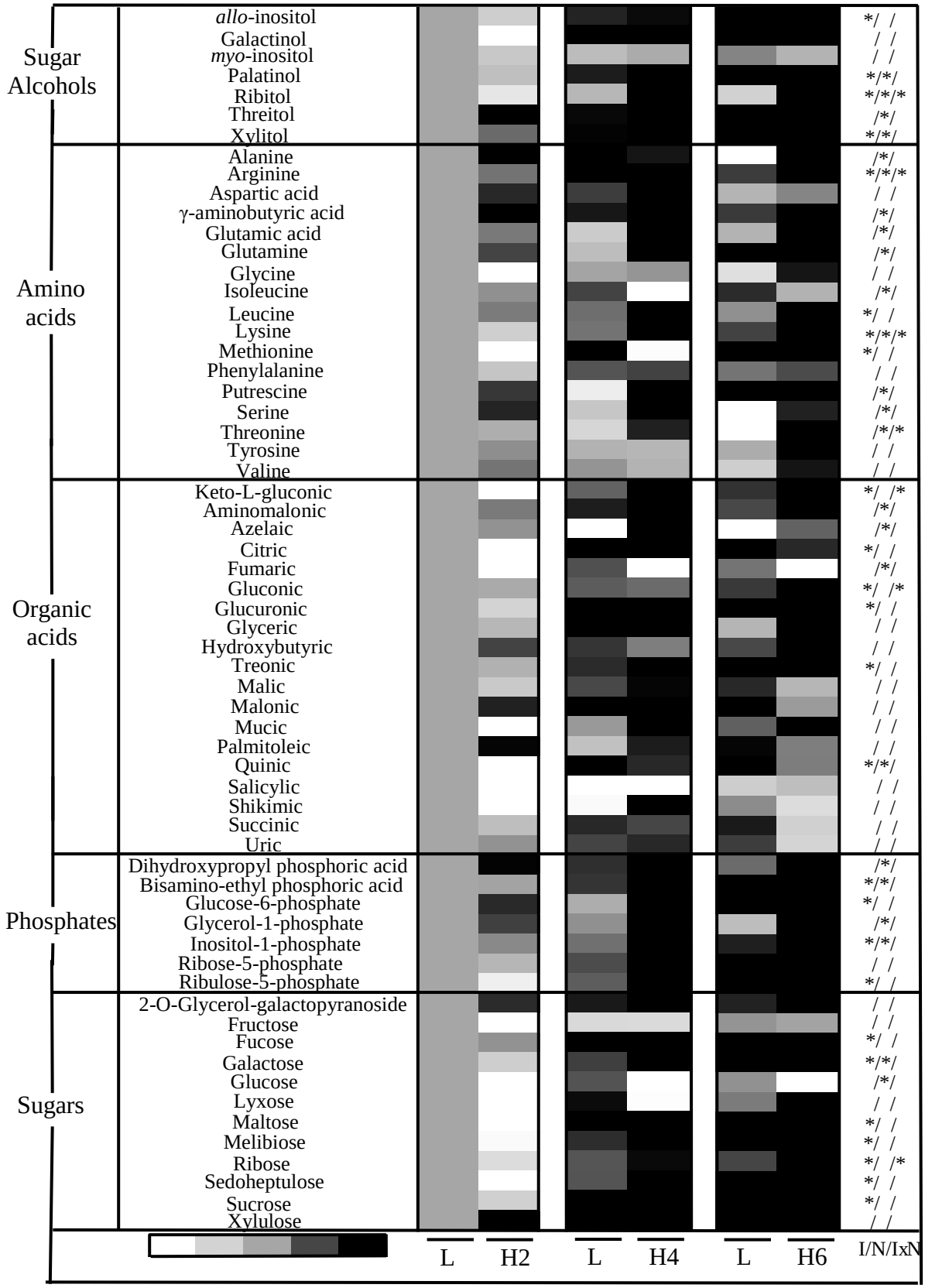
Sugar Alcohols
allo-inositol
Galactinol
myo-inositol
Palatinol
Ribitol
Threitol
Xylitol
Amino acids
Alanine
Arginine
Aspartic acid
γ-aminobutyric acid
Glutamic acid
Glutamine
Glycine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Putrescine
Serine
Threonine
Tyrosine
Valine
Organic acids
Keto-L-gluconic
Aminomalonic
Azelaic
Citric
Fumaric
Gluconic
Glucuronic
Glyceric
Hydroxybutyric
Treonic
Malic
Malonic
Mucic
Palmitoleic
Quinic
Salicylic
Shikimic
Succinic
Uric
Phosphates
Dihydroxypropyl phosphoric acid
Bisamino-ethyl phosphoric acid
Glucose-6-phosphate
Glycerol-1-phosphate
Inositol-1-phosphate
Ribose-5-phosphate
Ribulose-5-phosphate
Sugars
2-O-Glycerol-galactopyranoside
Fructose
Fucose
Galactose
Glucose
Lyxose
Maltose
Melibiose
Ribose
Sedoheptulose
Sucrose
Xylulose
L
H2
L
H4
L
H6
I/N/IxN

Figure 4.1.

Figure 4.2. Normalized peak areas of the major (most abundant) amino acids (A), organic acids (B), and sugars (C) in the root exudates of *H. annuus* plants as detected by GC-MS. Plants were treated with low (L) or high (H) nutrients, and collected for either 2, 4, or 6 hours (L2, L4, L6, or H2, H4, H6, respectively). Bars represent the mean value for between three and seven replicate plants (± S.E.).

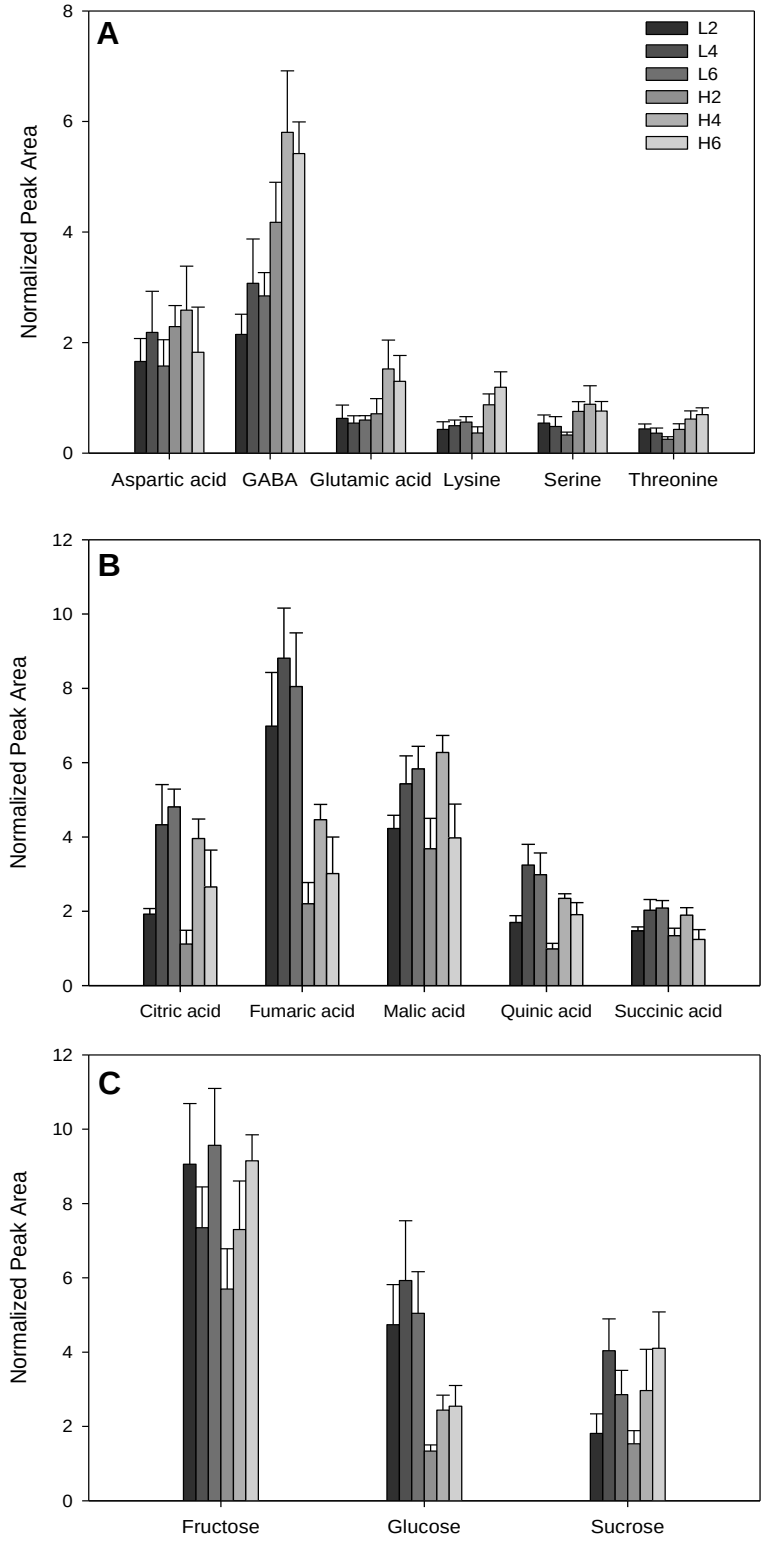
A
L2
L4
L6
H2
H4
H6
Normalized Peak Area
0
2
4
6
8
Aspartic acid
GABA
Glutamic acid
Lysine
Serine
Threonine
B
Normalized Peak Area
0
2
4
6
8
10
12
Citric acid
Fumaric acid
Malic acid
Quinic acid
Succinic acid
C
Normalized Peak Area
0
2
4
6
8
10
12
Fructose
Glucose
Sucrose

Figure 4.2.

Appendix Table 4.1. Normalized peak areas detected by GC-MS in the root exudates of *H. annuus* plants treated with low or high nutrients, and collected for either 2, 4, or 6 hours (L2, L4, L6, or H2, H4, H6, respectively). Metabolite peak areas were normalized by that of the loading standard and expressed per unit dry mass. Data are the mean of 5-7 replicates (±SE).

Compound	L2	L4	L6	H2	H4	H6
allo-inositol	3.94 ± 0.23	5.50 ± 0.81	6.51 ± 0.57	3.37 ± 0.36	5.80 ± 0.62	7.11 ± 1.35
Galactinol	0.03 ± 0.02	0.09 ± 0.01	0.13 ± 0.05	0.01 ± 0.01	0.10 ± 0.07	0.19 ± 0.17
myo-inositol	0.14 ± 0.03	0.13 ± 0.03	0.16 ± 0.04	0.12 ± 0.04	0.14 ± 0.01	0.13 ± 0.03
Palatinitol	0.15 ± 0.04	0.22 ± 0.06	0.26 ± 0.08	0.14 ± 0.03	0.48 ± 0.12	0.41 ± 0.10
Ribitol	0.12 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	0.22 ± 0.04	0.25 ± 0.05
Threitol	0.04 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.11 ± 0.02	0.09 ± 0.03
Xylitol	0.18 ± 0.02	0.26 ± 0.04	0.35 ± 0.03	0.21 ± 0.05	0.39 ± 0.06	0.46 ± 0.06
Alanine	0.10 ± 0.04	0.20 ± 0.12	0.04 ± 0.01	0.36 ± 0.13	0.14 ± 0.03	0.26 ± 0.13
Arginine	0.05 ± 0.01	0.08 ± 0.04	0.06 ± 0.01	0.05 ± 0.00	0.15 ± 0.08	0.31 ± 0.14
Aspartic acid	1.66 ± 0.42	2.18 ± 0.74	1.57 ± 0.48	2.29 ± 0.38	2.59 ± 0.80	1.82 ± 0.82
GABA	2.15 ± 0.37	3.07 ± 0.80	2.85 ± 0.42	4.17 ± 0.73	5.80 ± 1.11	5.42 ± 0.57
Glutamic acid	0.63 ± 0.24	0.54 ± 0.13	0.60 ± 0.08	0.71 ± 0.27	1.52 ± 0.52	1.30 ± 0.47
Glutamine	0.03 ± 0.01	0.03 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.20 ± 0.04	0.09 ± 0.02
Glycine	0.24 ± 0.03	0.25 ± 0.08	0.19 ± 0.03	0.11 ± 0.01	0.26 ± 0.05	0.35 ± 0.21
Isoleucine	0.07 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.05 ± 0.02	0.07 ± 0.01
Leucine	0.15 ± 0.03	0.17 ± 0.04	0.16 ± 0.02	0.17 ± 0.03	0.24 ± 0.04	0.35 ± 0.12
Lysine	0.43 ± 0.14	0.50 ± 0.10	0.56 ± 0.10	0.36 ± 0.11	0.87 ± 0.20	1.19 ± 0.28
Methionine	0.04 ± 0.01	0.09 ± 0.02	0.11 ± 0.02	0.03 ± 0.00	0.03 ± 0.01	0.10 ± 0.05
Phenylalanine	0.35 ± 0.03	0.44 ± 0.10	0.41 ± 0.04	0.31 ± 0.05	0.46 ± 0.07	0.45 ± 0.11
Putrescine	0.06 ± 0.01	0.04 ± 0.01	0.09 ± 0.02	0.08 ± 0.02	0.19 ± 0.06	0.16 ± 0.08
Serine	0.54 ± 0.15	0.48 ± 0.18	0.33 ± 0.05	0.76 ± 0.18	0.88 ± 0.34	0.76 ± 0.17
Threonine	0.44 ± 0.09	0.36 ± 0.09	0.25 ± 0.04	0.43 ± 0.10	0.62 ± 0.15	0.70 ± 0.12
Tyrosine	0.37 ± 0.03	0.35 ± 0.09	0.36 ± 0.08	0.40 ± 0.08	0.35 ± 0.10	0.76 ± 0.18
Valine	0.26 ± 0.04	0.28 ± 0.06	0.22 ± 0.05	0.30 ± 0.04	0.25 ± 0.07	0.38 ± 0.05

Appendix Table 4.1 (continued)

Compound	L2	L4	L6	H2	H4	H6
Keto-gluconic acid	0.16 ± 0.02	0.19 ± 0.04	0.22 ± 0.03	0.09 ± 0.02	0.28 ± 0.05	0.35 ± 0.07
Aminomalonic acid	0.05 ± 0.00	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.00	0.12 ± 0.02	0.09 ± 0.02
Azelaic acid	0.19 ± 0.05	0.12 ± 0.03	0.11 ± 0.05	0.20 ± 0.04	0.29 ± 0.07	0.23 ± 0.10
Citric Acid	1.92 ± 0.15	4.33 ± 1.09	4.80 ± 0.48	1.12 ± 0.37	3.95 ± 0.53	2.65 ± 0.99
Fumaric acid	6.98 ± 1.45	8.81 ± 1.35	8.05 ± 1.44	2.20 ± 0.57	4.47 ± 0.41	3.01 ± 0.98
Gluconic acid	0.29 ± 0.07	0.36 ± 0.07	0.39 ± 0.02	0.29 ± 0.06	0.34 ± 0.08	0.79 ± 0.22
Glucuronic acid	0.34 ± 0.04	0.54 ± 0.07	0.58 ± 0.07	0.28 ± 0.04	0.57 ± 0.11	0.73 ± 0.13
Glyceric acid	0.07 ± 0.01	0.12 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.14 ± 0.04	0.14 ± 0.05
Hydroxybutyric acid	0.24 ± 0.04	0.32 ± 0.07	0.30 ± 0.06	0.31 ± 0.03	0.27 ± 0.07	0.55 ± 0.12
L-Treonic acid	0.07 ± 0.02	0.09 ± 0.03	0.10 ± 0.02	0.06 ± 0.01	0.15 ± 0.01	0.12 ± 0.02
Malic acid	4.22 ± 0.36	5.43 ± 0.75	5.83 ± 0.61	3.68 ± 0.82	6.28 ± 0.46	3.97 ± 0.91
Malonic acid	0.03 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.04	0.05 ± 0.01	0.04 ± 0.00
Mucic Acid	1.65 ± 0.32	1.72 ± 0.48	2.00 ± 0.39	1.03 ± 0.27	3.87 ± 0.89	2.60 ± 1.06
Palmitoleic	0.09 ± 0.02	0.08 ± 0.02	0.13 ± 0.01	0.13 ± 0.04	0.13 ± 0.01	0.10 ± 0.07
Quinic acid	1.70 ± 0.18	3.24 ± 0.56	2.98 ± 0.59	0.99 ± 0.15	2.35 ± 0.12	1.91 ± 0.33
Salicylic acid	0.09 ± 0.07	0.05 ± 0.02	0.08 ± 0.03	0.06 ± 0.05	0.02 ± 0.01	0.08 ± 0.02
Shikimic acid	0.54 ± 0.18	0.37 ± 0.07	0.58 ± 0.07	0.32 ± 0.06	1.16 ± 0.35	0.43 ± 0.06
Succinic acid	1.47 ± 0.11	2.03 ± 0.29	2.09 ± 0.20	1.34 ± 0.20	1.89 ± 0.20	1.24 ± 0.27
Uric acid	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.02
Dihydroxypropyl phosphoric acid	0.07 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.18 ± 0.05	0.11 ± 0.02
Bisamino-ethyl phosphoric acid	0.19 ± 0.04	0.25 ± 0.05	0.32 ± 0.01	0.19 ± 0.03	0.59 ± 0.28	0.76 ± 0.28
Glucose-6-P	0.13 ± 0.08	0.13 ± 0.04	1.03 ± 0.58	0.18 ± 0.02	0.88 ± 0.30	1.14 ± 0.54
Glycerol-1-P	0.58 ± 0.09	0.62 ± 0.13	0.53 ± 0.03	0.76 ± 0.09	1.31 ± 0.26	1.01 ± 0.22
Inositol 1-P	0.06 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.15 ± 0.02	0.16 ± 0.04
Ribose-5-P	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.05 ± 0.01	0.03 ± 0.01

Appendix Table 4.1 (continued)

Compound	L2	L4	L6	H2	H4	H6
Ribulose-5-P	0.04 ± 0.01	0.05 ± 0.01	0.08 ± 0.03	0.03 ± 0.01	0.07 ± 0.02	0.12 ± 0.03
GG	0.20 ± 0.01	0.28 ± 0.04	0.27 ± 0.05	0.27 ± 0.04	0.31 ± 0.11	0.37 ± 0.10
Fructose	9.06 ± 1.63	7.35 ± 1.10	9.57 ± 1.53	5.70 ± 1.08	7.30 ± 1.31	9.15 ± 0.70
Fucose	0.08 ± 0.01	0.15 ± 0.02	0.18 ± 0.03	0.09 ± 0.01	0.17 ± 0.03	0.17 ± 0.03
Galactose	0.16 ± 0.02	0.21 ± 0.03	0.25 ± 0.03	0.14 ± 0.03	0.36 ± 0.09	0.36 ± 0.08
Glucose	4.74 ± 1.08	5.93 ± 1.61	5.04 ± 1.12	1.34 ± 0.16	2.44 ± 0.40	2.54 ± 0.56
Lyxose	0.65 ± 0.09	0.95 ± 0.14	0.73 ± 0.17	0.42 ± 0.08	0.44 ± 0.10	1.31 ± 0.72
Maltose	0.29 ± 0.04	0.71 ± 0.14	1.06 ± 0.19	0.19 ± 0.06	0.58 ± 0.16	1.09 ± 0.28
Melibiose	0.23 ± 0.01	0.31 ± 0.06	0.41 ± 0.03	0.16 ± 0.03	0.36 ± 0.08	0.52 ± 0.08
Ribose	1.57 ± 0.23	1.96 ± 0.48	2.03 ± 0.31	1.25 ± 0.20	2.31 ± 0.13	3.52 ± 0.38
Sedoheptulose	0.55 ± 0.06	0.68 ± 0.12	0.84 ± 0.14	0.32 ± 0.06	0.84 ± 0.13	1.03 ± 0.19
Sucrose	1.81 ± 0.52	4.04 ± 0.86	2.86 ± 0.65	1.53 ± 0.36	2.96 ± 1.11	4.10 ± 0.98
Xylulose	0.27 ± 0.08	0.91 ± 0.55	0.49 ± 0.10	0.65 ± 0.21	1.27 ± 0.58	1.67 ± 0.89

CHAPTER 5

EVOLUTION OF ROOT EXUDATE COMPOSITION IN ECOLOGICALLY-CONTRASTING *HELIANTHUS*[4]

[4] Bowsher, A.B., Ali, R., Harding, S.A., Tsai, C-J., Donovan, L.A. To be submitted to *Frontiers in Plant Science.*

Abstract

Plant roots exude numerous metabolites into the soil which influence nutrient availability. Although exudate composition is considered a key adaptation to low fertility soils, few studies have tested this hypothesis in a phylogenetic framework. In this study, we examined the root exudates of six *Helianthus* species chosen as phylogenetically-independent contrasts with respect to native soil nutrient availability. Plants were grown under controlled conditions and exposed to either high or low nutrient supply for five days. Using gas chromatography-mass spectrometry, we detected 37 metabolites across species. Under high nutrient supply, species native to low nutrient soils exhibited consistently higher overall exudation than species native to high nutrient soils. Under low nutrient supply, however, species native to contrasting soil fertilities did not differ in overall exudate abundance. As expected, all species had higher exudation of carboxylic acids under low nutrient supply than under high nutrient supply. However, contrary to expectations, species native to high nutrient soils also responded to low nutrients with higher exudation of numerous other metabolites. These findings demonstrate that species native to low nutrient soils have constitutively high levels of root exudation, but that species native to high nutrient soils have a greater increase in root exudation in response to low nutrient conditions. These consistent evolutionary divergences among species native to low nutrient versus high nutrient soils provide evidence for the adaptive value of root exudate abundance in contrasting soil types.

INDEX WORDS: rhizosphere, GC-MS, phylogenetically-independent contrasts, plant nutrition

Introduction

Mineral nutrient availability in soils is considered a key factor influencing plant productivity and species distributions (*Coudun* et al., 2006; *John* et al., 2007; *Condit* et al., 2013). Although nutrient limitation is common in terrestrial ecosystems, plants have evolved numerous mechanisms to acquire nutrients from soils via their complex root systems. For example, root system morphology, distribution, and architecture play vital roles in exploring the soil for nutrients (*Lynch*, 1995). In addition to these physical mechanisms for securing nutrient uptake, plants can chemically influence nutrient availability in soils through root exudation (*Marschner* et al., 1986; *Hinsinger*, 2001). Root exudation, the passive or active release of inorganic ions, volatiles, and primary and secondary metabolites from roots, is a ubiquitous phenomenon in higher plants (*Jones* et al., 2009). Root exudates can increase local nutrient availability in the rhizosphere by influencing soil pH and redox state, competing for mineral adsorption sites, chelating mineral nutrients, and dissolving soil minerals (*Moghimi* et al., 1978; *Marschner* et al., 1986; *Gerke* et al., 1994; *Jones* and *Darrah*, 1994; *Veneklaas* et al., 2003). Exudates can also indirectly improve plant nutrient acquisition through interactions with microbes. For example, exudation of flavonoids is a critical component for nodule establishment in N_2-fixing symbioses, and can play an important role in establishment of mycorrhizal relationships (*Akiyama* et al., 2005; *López-Ráez* et al., 2008; *Cesco* et al., 2010). Root exudates also impact rhizosphere community composition, and serve as primers for processes of soil nutrient cycling (*Weisskopf* et al., 2005; *Henry* et al., 2008). Consequently, root exudation is considered to play an important role in plant nutrient acquisition, particularly in low fertility environments (*Marschner* et al., 1986; *Dakora* and *Phillips*, 2002).

Given the potential for root exudation to improve nutrient acquisition by plants, numerous studies have investigated root exudate composition, revealing both qualitative and quantitative variation among species (e.g. *Tyler* and *Strom*, 1995; *Grayston* et al., 1996; *Pearse* et al., 2007). In addition, exudation of sugars and organic acids, which comprise the majority of root exudates, is generally observed to increase in response to low nutrient supply (*Kraffczyk* et al., 1984; *Neumann* and *Römheld*, 2001; *Carvalhais* et al., 2011). However, these plastic responses are species-specific (*Jones*, 1998; *Neumann* and *Römheld*, 1999; *Wouterlood* et al., 2004). For example, cluster-rooted species, such as white lupin and members of the Proteaceae which tolerate extremely infertile soils, exude larger quantities of phosphorus-mobilizing carboxylates under phosphorus-deficient conditions in comparison to other species (*Jones*, 1998; *Neumann* and *Römheld*, 1999; *Lambers* et al., 2013). Given that this response is assumed to be adaptive, we would predict that species native to low fertility soils have evolved a larger response to nutrient deficiency than species native to high fertility soils. However, large increases in carboxylate exudation under nutrient deficiency have also been detected in cultivated species such as oilseed rape (*Hoffland* et al., 1992) and maize (*Jones*, 1995), although these species have presumably been bred under conditions of high nutrient availability. In addition, other studies predict root exudate abundance to be highest in fast-growing species characteristic of fertile soils due to their rapid metabolic activity and high nutrient demand (*De Deyn* et al., 2008; *Orwin* et al., 2010). Higher exudation in such species may contribute to the faster rates of nutrient cycling observed in the rhizosphere of species native to fertile soils in common garden studies (*van der Krift* et al., 2001; *Orwin* et al., 2010). Given these apparent inconsistencies in the literature, the role that root exudation plays in adaptation to soil fertility is still an open question (*Rengel*, 2002).

Of the studies which have examined root exudate composition in relation to native soil characteristics (*Tyler* and *Strom*, 1995; *Strom*, 1997; *Denton* et al., 2007), few have done so in a phylogenetically-informed framework. Consideration of species' relatedness is essential in comparative studies in order to remove the confounding influence of species shared evolutionary histories (*Felsenstein*, 1985; *Harvey* and *Purvis*, 1991). While comparative analyses of trait differentiation across species native to contrasting environments is useful for generating testable hypotheses, studies which investigate trait differentiation in multiple lineages are particularly informative for making adaptive inferences. Consistent shifts in trait values across species in multiple lineages indicate repeated trait evolution in response to the local environment, providing evidence for the adaptive value of that trait (*Nicotra* et al., 2002; *Pineda-García* et al., 2011).

In this controlled environment study, we used gas chromatography-mass spectrometry (GC-MS) to analyze the root exudates of six *Helianthus* species native to habitats differing in soil fertility. Species were chosen as three pairs of phylogenetically-independent contrasts, with each pair including one species native to a relatively low nutrient soil (LNS) and the other native to a relatively high nutrient soil (HNS). Specifically, we asked the following questions: [1] Does root exudate composition differ between species native to LNS versus HNS? [2] Do differences among species depend on nutrient availability? In addition, the *response* to changes in nutrient availability (i.e. the change in root exudate composition between high nutrient-treated and low nutrient-treated plants) may also be adaptive. Therefore, we also asked [3] Do species native to LNS versus HNS differ in their response to low nutrient supply?

We predicted that species native to HNS would exhibit higher root exudation than species native to LNS under high nutrient supply due to their faster growth rates and rapid metabolic activity. We also predicted that species native to LNS would increase exudation to a greater

extent than species native to HNS in response to low nutrient supply. Consistent differences between species native to LNS versus HNS in each lineage would indicate independent, repeated evolution of root exudate composition (or its response to nutrient supply), providing strong evidence for its adaptive value.

Materials and Methods

Study system

Helianthus (sunflowers) is an assemblage of approximately 50 herbaceous species native to North America. Members of the genus have adopted diverse growth forms, and occupy a wide variety of habitats across the continent, making it particularly suited for investigations of evolutionary ecology (*Heiser* et al., 1969). Seeds were collected for 28 diploid *Helianthus* species in 2011 and 2012, either directly from wild populations, or from accessions established at the USDA National Genetic Resources Program. In addition, five soil cores (5 cm diameter, 0-20 cm depth) were also collected randomly across the site of each seed source. Soil samples were dried at 60^{o}C for 72 hours and analyzed for fertility characteristics by A&L Eastern Laboratories (North Chesterfield, VA). Organic matter (OM) was assessed by loss-on-ignition at 400^{o}C. Available phosphorus (P), exchangeable potassium (K), magnesium (Mg), and calcium (Ca) were determined using inductively coupled plasma spectrometry on samples extracted with the Mehlich III extractant (*Mehlich*, 1984). Cation exchange capacity (CEC) was assessed by the sum of exchangeable cations (K, Mg, and Ca), and pH was determined on a 1:1 mixture of soil:deionized water. A subsample of each soil core was ground to fine powder in a ball mill and analyzed for total nitrogen (N) concentration by Micro-Dumas combustion (UGA Stable Isotope/Soil Biology Laboratory).

Based on the native site soil data for each species, we chose three pairs of sister species (six species total) from different clades in the *Helianthus* phylogeny (*Stephens* et al., in review), such that each pair contains one species native to a low nutrient soil (LNS) and the other native to a high nutrient soil (HNS) (Figure 5.1). Within each species pair, the native soil of one species was significantly higher in N, P, and K (Figure 5.1), as well as OM, Ca, and Mg (data not shown) in comparison to its sister taxon. The only exception to this pattern is in the *H. annuus-H. argophyllus* clade: these two species did not differ in soil P (Figure 5.1). All six focal species were directly collected from field sites: *H. annuus* (Kansas; N39°06'N 96°36'W), *H. argophyllus* (Texas; 27°38'N 97°13'W), *H. petiolaris* (Illinois; 41°55'N 90°06'W), *H. praecox* ssp. *runyonii* (Texas; 27°39'N 97°18'W), *H. grossesseratus* (Illinois; 41°38'N 89°32'W), *H. microcephalus* (South Carolina; 34°15'N 82°39'W). All six species are herbaceous dicots: *H. annuus, H. argophyllus, H. petiolaris,* and *H. praecox* are annuals, while *H. grossesseratus* and *H. microcephalus* are perennials (*Heiser* et al., 1969).

Growth conditions and nutrient treatments

Seeds were scarified, placed on moist filter paper in petri dishes, and kept in darkness for 24 hours at room temperature (20^{o}C). After removing the seed coats with forceps, seeds were moved to a controlled-environment growth chamber in a completely randomized design (Conviron, Winnipeg, Canada) programmed for a 12-hour 25/20^{o}C day/night cycle with 70% relative humidity. One week after scarification, seedlings were wrapped with a small section of rock wool so that the roots emerged from the bottom of the wool. Seedlings were then suspended in individual 125 mL Erlenmeyer flasks filled with modified half-strength Hoagland's nutrient solution (*Epstein* and *Bloom*, 2005). The nutrient solution consisted of: KNO_3 (3 mmol/L), $Ca(NO_3)_2$ (2 mmol/L), $NH_4H_2PO_4$ (1 mmol/L), $MgSO_4$ (0.05 mmol/L), KCl (0.025 mmol/L),

H_3BO_3 (0.0125 mmol/L), $MnSO_4$ (0.001 mmol/L), $ZnSO_4$ (0.001 mmol/L), $CuSO_4$ (0.0003 mmol/L), MoO_3 (0.0003 mmol/L), and Fe-EDTA (0.025 mmol/L). Flasks were wrapped in foil and aerated using aquarium pumps. Nutrient solutions and rock wool were replaced every second day.

Due to seedling mortality in *H. microcephalus* and *H. praecox*, these two species were replaced with a second set of germinated seedlings within 4 weeks of the first set. To assess the potential for confounding temporal effects on root exudation, replicate *H. petiolaris* seedlings were germinated in both sets (n = 3 seedlings per set). No significant temporal effects were observed for the abundance of any metabolite detected in *H. petiolaris* (t-test, all $p < 0.05$); therefore, temporal effects were not considered further.

As root exudate composition is known to vary with plant ontogeny (*Aulakh* et al., 2001), nutrient treatments were initiated when plants had reached the same developmental stage to control for the different growth rates among species. At the emergence of the third true leaf pair, seedling root systems were rinsed with deionized water, then placed in individual 125 mL Erlenmeyer flasks containing either half-strength (high) or 1.25% strength (low) modified Hoagland's solution (composition as described above). For each of the six species, between five and seven individual replicates were randomly assigned to each nutrient treatment. Nutrient treatment solutions were replaced every second day to maintain treatment conditions.

Root exudate collection

Five days after initiating the nutrient treatments, root exudates were collected via the trap-solution method (*Neumann* et al., 2009). After rinsing root systems with deionized water, root systems of intact seedlings were then placed in 15 mL glass vials wrapped in foil to exclude light. Vials were then filled with enough deionized water to cover the root system, and these

volumes were recorded. After five hours, the root exudate collection medium was collected from each vial and re-diluted to the original volume to correct for differences in evapotranspirational water loss across individuals, snap-frozen in liquid nitrogen, and stored at -80^{o}C. Root systems for each individual plant were dried at 60^{o}C for 72 hours before weighing.

Exudate analysis

One mL aliquots of the root exudate collection medium were evaporated to dryness in a CentriVap (Labconco) before GC-MS analysis, as described (*Jeong* et al., 2004; *Frost* et al., 2012). Briefly, concentrated samples were dissolved in pyridine, then methoximated with methoxyamine hydrochloride (Acros, Hanover Park, IL) and derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamine (MSTFA; Sigma-Aldrich). Samples (1 µL) were injected onto a DB-5MS column (30 m x 0.25 mm ID, and 0.25 µm film, with deactivated guard column) in splitless mode. Separation was completed in an Agilent 7890A GC oven (Agilent Technologies, Wilmington, DE) with an 80°C hold for 1 minute, ramping of 20 °C per minute to 200°C, and then 10°C per minute ramping to 310°C followed by a 6.5 minute hold. Detection was with an Agilent 5975C quadrupole mass spectrometer, with source set at 230^{o}C and quadrupole mass filter set at 150^{o}C. Mass spectra were collected in scanning ion mode (m/z 50-500) by Chemstation (Agilent) and deconvoluted with AnalyzerPro (SpectralWorks, Runcom, UK).

Peak identities were assigned using NIST08 (*Babushok* et al., 2007), Fiehnlib (*Kind* et al., 2009) as well as in-house spectral libraries. Based on retention times and mass spectra, peaks were aligned across samples using Metalab (*Xue* et al., 2013), followed by manual curation. Peak areas of each sample were normalized according to the volume of deionized water in which the sample was collected, as well as according to root system dry mass. Normalized peak areas were modeled within each *Helianthus* clade using two-way ANOVA, with species, nutrient supply,

and their interaction as explanatory variables. Effects were considered significant at $p < 0.05$. We then conducted a principal component analysis of log-transformed peak areas for visualization of overall differences among species and nutrient treatments. All statistical analyses were completed in JMP Pro v. 11 (SAS Institute Inc., Cary, NC).

Results

In total, 37 metabolites were identified across species and treatments, including organic and amino acids, sugars, and sugar derivatives (Table 5.1). In a principal components analysis of root exudate composition across all individuals, the majority of the variation was accounted for by two principal component axes (Figure 5.2). The first principal component (PC1), which accounted for 64.3% of the variation among samples, revealed a strong influence of root exudate abundance, as all compounds detected were positively loaded with this axis (Table 5.1, Figure 5.2). The second principal component (PC2), which accounted for an additional 9.8% of the variation among samples, primarily revealed the influence of nutrient treatment, as indicated by the clear separation between seedlings in the low nutrient treatment (black symbols) and high nutrient treatment (gray symbols) on this axis (Figure 5.2). The loadings on PC2 demonstrate that the carboxylic acids fumaric acid, citric acid, and malic acid were higher in the low nutrient treatment, while several amino acids, including glutamic acid and alanine, were lower in low nutrient treatment (Table 5.1).

Given the clear influence of nutrient supply on variation among seedlings, we further explored differences between species in the principal components plot by comparing species within each nutrient treatment separately. In the high nutrient treatment, species native to LNS consistently clustered towards significantly higher values than their sister taxa native to HNS on PC1, although this difference was marginally significant in the *H. annuus*-*H. argophyllus* clade

($p = 0.071$; Figure 5.2, 5.3a). This indicated that species native to LNS consistently exhibited higher overall root exudation than species native to HNS, as all compounds detected positively loaded on PC1 (Table 5.1). In contrast, in the low nutrient treatment, differences among species were primarily due to phylogenetic effects, as species tended to tightly cluster tightly with their sister taxa (Figure 5.2). As a result, species native to LNS did not significantly differ from their sister taxa native to HNS in PC1 scores under the low nutrient treatment (Figure 5.3a).

For PC2 scores, species native to LNS did not significantly differ from their sister taxa native to HNS, either in the high or the low nutrient treatment (Figure 5.3b). However, all species exhibited a significantly higher PC2 score under the low nutrient treatment, relative to the high (Figure 5.2, 5.3b). Compared to the high nutrient treatment, all species responded to low nutrient supply with significantly higher exudation of citric and malic acids, and lower exudation of aspartic and glutamic acids, indicated by significant ANOVA effects of nutrient supply in all three clades (Table 5.2, Figure 5.4). In addition, the presence of significant species by nutrient supply interactions for numerous metabolites within each clade indicated that species responded differently to changes in nutrient supply (Table 5.2). However, all of these interaction terms (30/30) were due to relatively larger increases (or smaller decreases in the case of amino acids) in root exudate abundance in species of HNS in response to low nutrient supply (Table 5.2, Figure 5.4, 5.5). No significant species by nutrient supply interactions were shared across all three clades, indicating for any given metabolite, species of HNS did not consistently differ from species of LNS in the response to nutrient supply (Table 5.2, Figure 5.4, 5.5).

Discussion

In this controlled environment study, we detected large differences among six wild *Helianthus* species in root exudation of amino acids, organic acids, sugars, and sugar derivatives.

Principal component analysis revealed a single axis which accounted for the majority of the variation among samples, and was positively associated with the abundance of all detected metabolites. Under high nutrient supply, species native to LNS consistently clustered towards higher values of this axis than their sister taxa native to HNS, providing evidence for repeated evolutionary shifts towards higher exudation in species of LNS. In addition, it is worthy to note that species of LNS did not cluster together separately from species of HNS in this multivariate analysis; rather, consistent differences in root exudation were observed between species of LNS and those of HNS within each clade. This observation highlights the fundamental importance of taking phylogeny into account in comparative analyses.

In terms of individual metabolites, significantly higher levels of threonic acid were detected in the root exudates of species native to LNS relative to their sister taxa native to HNS. Threonic acid, a carboxylated derivative of the sugar threose, is expected to play a role in heavy metal mobilization and accumulation in metal hyperaccumulators (*Luo* et al., 2014). Concentrations of carboxylates have indeed been shown to increase in the rhizosphere of cultivated sunflower in response to cadmium additions, presumably as a mechanism for increasing cadmium solubility for uptake (*Chiang* et al., 2006). However, threonic acid was detected in small quantities relative to other organic acids in the present study, so their relative effects in the rhizosphere may be minimal. Regardless, the consistently higher overall exudation in species native to LNS provides evidence for adaptive differentiation among species. Higher exudation of sugars, amino acids, and organic acids may represent a strategy for initiating beneficial root-microbe associations, or for accessing otherwise immobile nutrients in the native sites of these species (*Lambers* and *Poorter*, 1992; *Moe*, 2013).

In contrast to the interspecific differences detected under high nutrient supply, species did not differ in root exudation according to their native site fertility under low nutrient supply. Principal components analysis indicated that both the *H. annuus-H. argophyllus* and *H. grossesseratus-H. microcephalus* clades clustered together with overall lower exudation than the *H. petiolaris-H. praecox* clade. No shifts in exudate composition from species of LNS to those of HNS were observed in any of the three clades, indicating that there is no evidence for consistent differentiation among species under low nutrient supply.

In addition to differences across species under either high or low nutrient supply, species *responses* to changes in nutrient supply may also be adaptive. For numerous metabolites, species differed strongly in their responses to changes in nutrient supply. All 30 of the metabolites with significant species by nutrient supply interaction terms were a result of larger increases (or smaller decreases) in root exudate abundance in species of HNS in response to low nutrient supply. In contrast to these metabolites, all species examined responded similarly to low nutrient supply with higher exudation of citric and malic acids than under high nutrient supply. Higher exudation of carboxylic acids in response to low nutrient supply has been noted in numerous studies as a mechanism to release mineral nutrients into soil solution (*Gardner* et al., 1983; *Dinkelaker* et al., 1989; *Neumann* and *Römheld*, 1999). However, although all species in the present study increased exudation of carboxylic acids, the magnitude of this response was not related to native soil fertility levels, but was instead phylogenetically-conserved among species. Similar results were seen in a common garden study of widespread and narrowly endemic *Banksia* species: despite differing widely in edaphic distributions, these species did not differ in the response of carboxylate exudation to nutrient supply (*Denton* et al., 2007). These findings highlight the importance of increased carboxylic acid exudation in response to nutrient

deficiency as a phylogenetically conserved mechanism to improve nutrient concentrations in the rhizosphere.

Overall, these findings show that species native to LNS have constitutively high levels of root exudation, but that species native HNS have a greater increase in exudation in response to low nutrient availability. Why should species native to LNS exhibit higher root exudation than species native to HNS under high nutrient supply, but not low nutrient supply? Or, alternatively, why should species native to HNS exhibit a greater response to low nutrient supply than species native to LNS?

Given the unpredictability of nutrient pulses in infertile soils, species of LNS may have evolved to maintain constitutively high exudation rates regardless of nutrient supply in order to capitalize during times of high nutrient availability. Such a strategy may help plants of LNS to take advantage of temporary increases in resource availability to 'prime' the rhizosphere for future situations of low nutrient availability (*Kuzyakov* and *Cheng*, 2001). Viewed from the opposite perspective, species native to chronically HNS may rely on their rapid growth rates to a greater extent than root exudation, in order to exploit nutrients and gain an edge over competitors in high nutrient conditions. Such a strategy is not viable in LNS, even in the presence of high nutrient patches, as rapid production of new roots may be too expensive to maintain as those patches are depleted (*Grime*, 1994; *Hutchings* and *de Kroon*, 1994; *Fransen* et al., 1998). Species native to such infertile soils are expected to grow slowly, but maintain long-lived root systems which can tolerate extended periods of low nutrient availability (*Grime*, 1994; *Hutchings* and *de Kroon*, 1994). In contrast to allocating resources for increased root length production, root exudation may therefore represent a more cost-effective strategy for plants native to LNS to take advantage during brief intervals of high nutrient availability.

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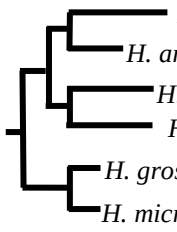

	GPS Coordinates		Native Site Soil Nutrients		
	Latitude	**Longitude**	**%N**	**P (ppm)**	**K (ppm)**
H. annuus	39.102° N	96.610° W	0.246 (0.038)[a]	22.0 (10.1)	496.4 (89.2)[a]
*H. argophyllus**	27.630° N	97.224° W	0.010 (0.001)[b]	20.8 (7.8)	32.0 (4.7)[b]
H. petiolaris	41.921° N	90.113° W	0.083 (0.026)[a]	29.8 (11.9)[a]	56.4 (7.8)[a]
*H. praecox**	27.659 ° N	97.311 ° W	0.020 (0.008)[b]	8.0 (9.1)[b]	38.6(13.8)[b]
H. grossesseratus	41.636 ° N	89.536 ° W	0.228 (0.025)[a]	86.4 (3.0)[a]	202.4 (18.0)[a]
*H. microcephalus**	34.262° N	82.663° W	0.064 (0.020)[b]	8.4 (1.7)[b]	89.6 (21.7)[b]

Figure 5.1. Phylogeny (pruned from that of *Stephens* et al., in review) and native soil characteristics of the six study species. Data are the mean (standard deviation) of five replicates per species. Different letters indicate significant differences ($p < 0.05$) between the two sister taxa within a given clade. Species native to a low nutrient soil (relative to its sister taxa) indicated by (*). (N) Nitrogen; (P) phosphorus; (K) potassium.

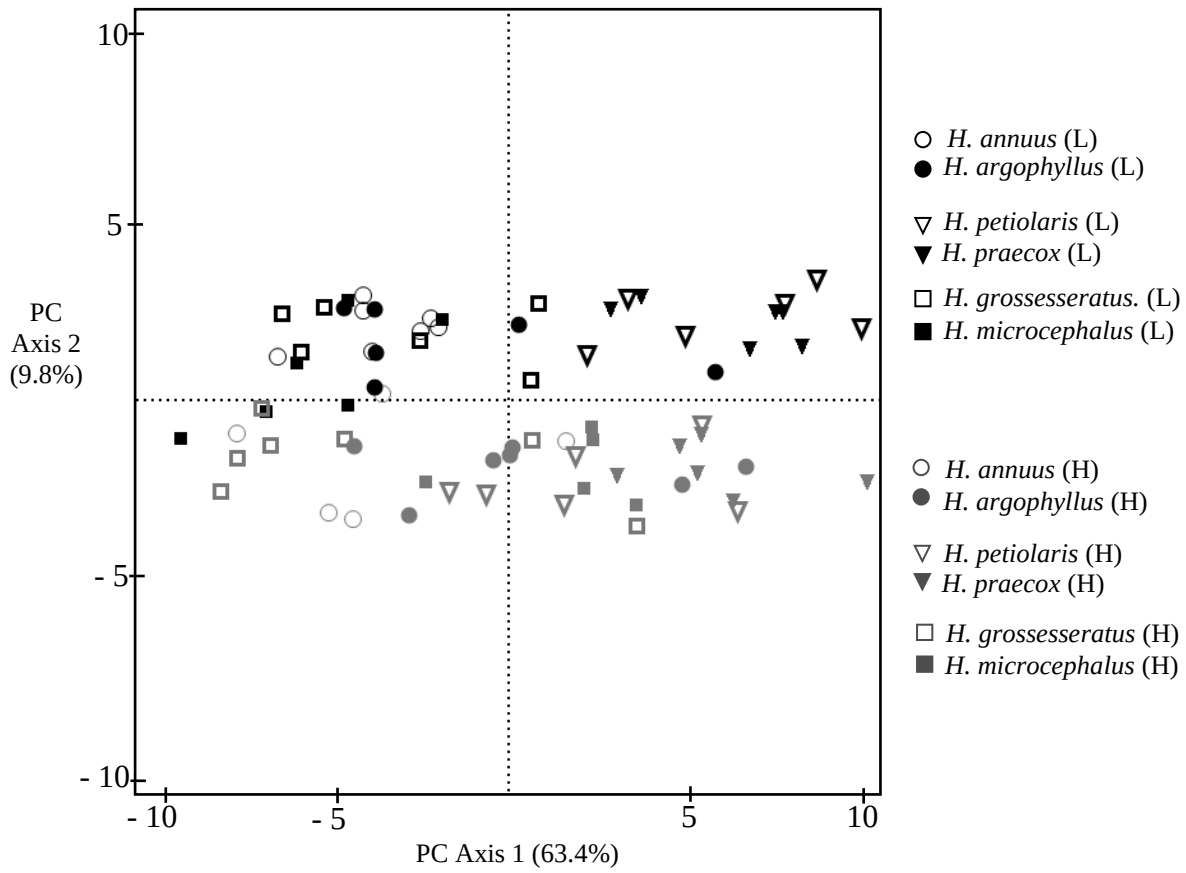

Figure 5.2. Principal components analysis of abundance of 37 metabolites detected in root exudates of six *Helianthus* species. Each point represents an individual seedling. Species native to low nutrient soils are filled symbols, while those native to high nutrient soils are open symbols. Seedlings in the low nutrient treatment (L) are black symbols and seedlings in the high nutrient treatment (H) are gray symbols.

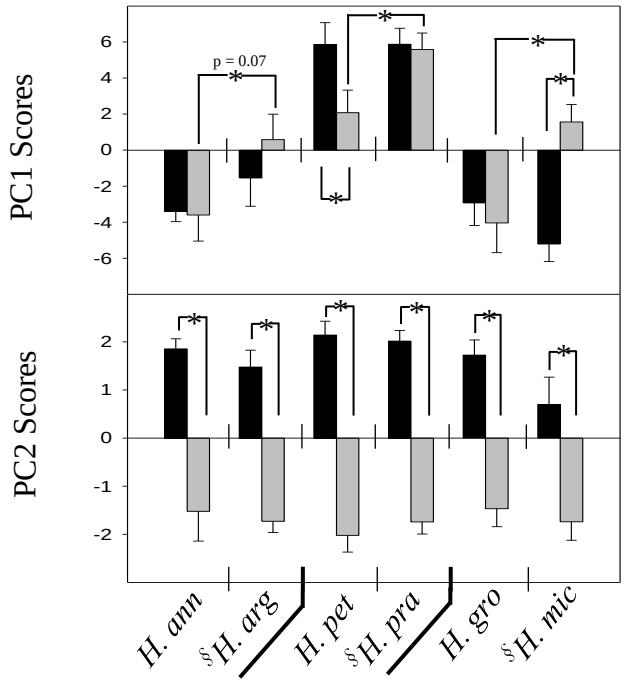

Figure 5.3. Scores of the first (a) and second (b) axes (PC1 and PC2, respectively) of the principal components analysis for root exudate composition of six *Helianthus* species depicted in Figure 5.3: *H. annuus* (*H. ann*), *H. argophyllus* (*H. arg*), *H. petiolaris* (*H. pet*), *H. praecox* (*H. pra*), *H. grossesseratus* (*H. gro*); *H. microcephalus* (*H. mic*). Data are the mean of 5-7replicates (± standard error) for seedlings treated with either low (black bars) or high (gray bars) nutrient supply. Species are arranged by clade, with the taxa native to low nutrient soils (relative to its sister taxa) indicated by (§).Significant differences between species or treatments indicated by (*).

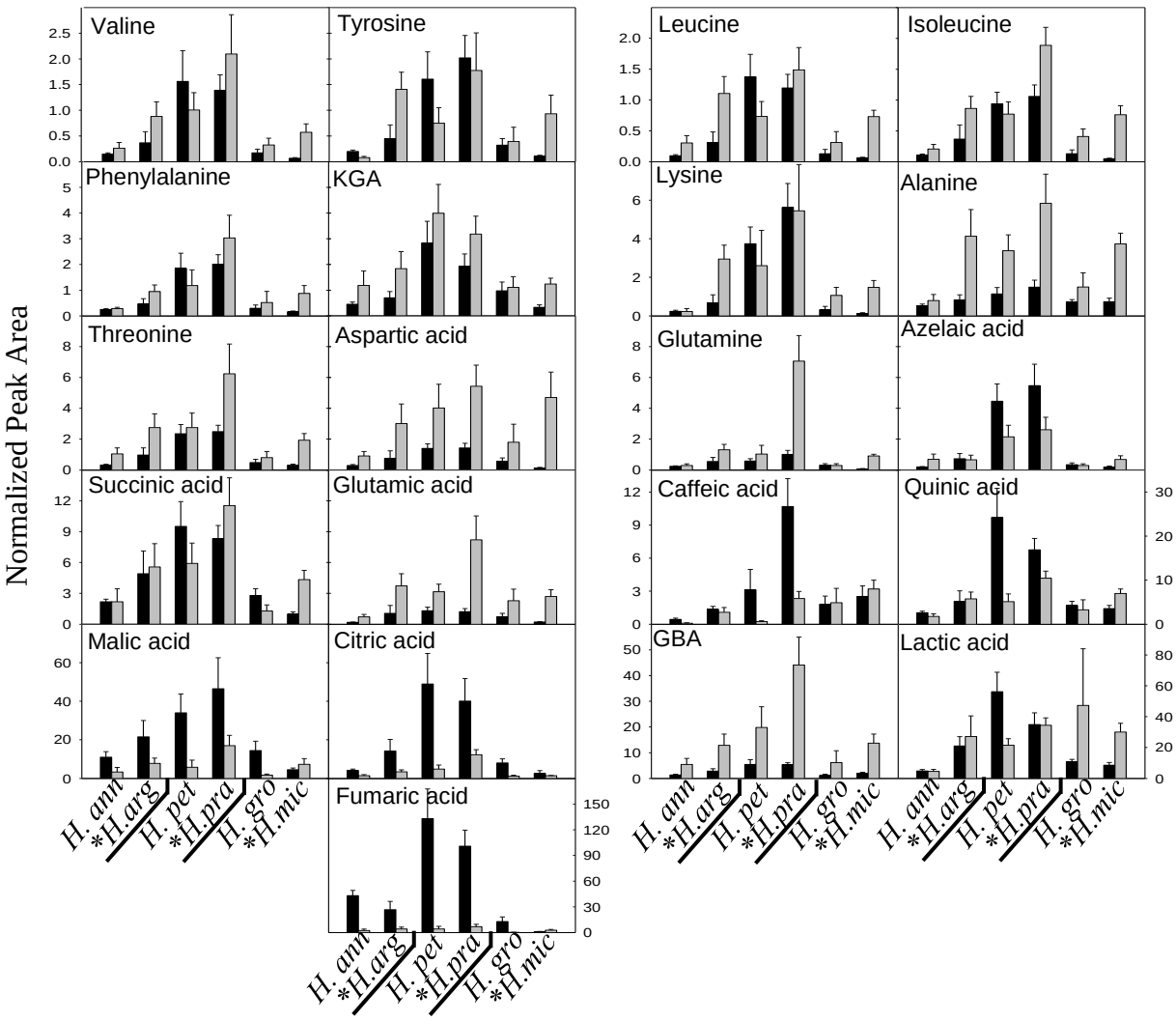

Figure 5.4. Normalized peak areas of the organic acids, amino acids, and derivatives detected under low (black bars) and high (gray bars) nutrient supply in six *Helianthus* species. Data are the mean of 3-7 replicates (± standard error). Species are arranged by clade, with the taxa native to low nutrient soils (relative to its sister taxa) indicated by (*). γ-guanidobutyric acid (GBA); α-keto-glutaric acid (KGA). Species abbreviations are as in Figure 5.3.

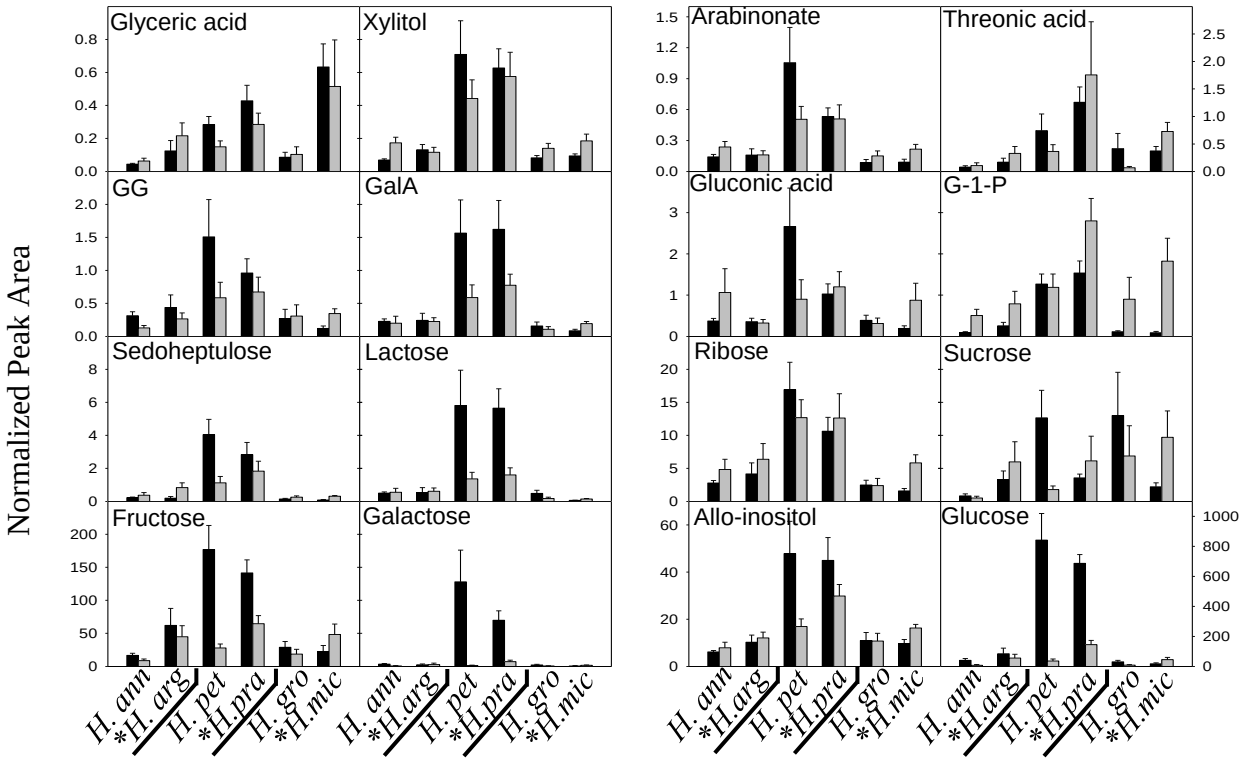

Figure 5.5. Normalized peak areas of the sugars and sugar derivatives detected under low (black bars) and high (gray bars) nutrient supply in six *Helianthus* species. Data are the mean of 3-7 replicates (± standard error). Species are arranged by clade, with the taxa native to low nutrient soils (relative to its sister taxa) indicated by (*).Glycerol-1-phosphate (G-1-P); galacturonic acid (GalA); 2-O-Glycerol-galactopyranoside (GG). Species abbreviations are as in Figure 5.3.

Table 5.1. Loading scores of the detected compounds on the first and second principal components axes (PC1 and PC2, respectively) depicted in Figure 5.2. Glycerol-1-phosphate (G-1-P); γ-guanidobutyric acid (GBA); galacturonic acid (GalA); 2-O-Glycerol-galactopyranoside (GG); keto-glutaric acid (KGA).

Compound	Loadings	
	PC1	PC2
Alanine	0.69	-0.52
Allo-inositol	0.93	0.08
Arabinonate	0.81	-0.03
Aspartic acid	0.77	-0.47
Azelaic acid	0.91	0.11
Caffeic acid	0.47	0.35
Citric acid	0.73	0.55
Fructose	0.83	0.32
Fumaric acid	0.56	0.62
G-1-P	0.84	-0.31
GalA	0.90	0.20
Galactose	0.82	0.43
GBA	0.72	-0.43
GG	0.83	0.25
Gluconic acid	0.76	0.21
Glucose	0.84	0.44
Glutamic acid	0.73	-0.55
Glutamine	0.73	-0.34
Glyceric acid	0.60	-0.04
Isoleucine	0.83	-0.38
KGA	0.82	-0.21
Lactic acid	0.81	0.03
Lactose	0.84	0.25
Leucine	0.85	-0.29
Lysine	0.79	-0.12
Malic acid	0.65	0.59
Phenylalanine	0.88	-0.12
Quinic acid	0.84	0.20
Ribose	0.92	-0.05
Sedoheptulose	0.87	-0.02
Succinic acid	0.88	0.14
Sucrose	0.46	0.09
Threonic acid	0.80	0.08
Threonine	0.90	-0.36
Tyrosine	0.83	-0.08
Valine	0.90	-0.24
Xylitol	0.81	-0.05

Table 5.2. Significant ($p < 0.05$) p-values of the effects of species (Sp), nutrient supply (N), and their interaction as assessed by two-way ANOVA on the abundance of root exudates within three different *Helianthus* clades. Not significant (-); Glycerol-1-phosphate (G-1-P); γ-guanidobutyric acid (GBA); galacturonic acid (GalA); 2-O-Glycerol-galactopyranoside (GG); keto-glutaric acid (KGA). Species abbreviations are as in Figure 5.3.

	H. ann-H. arg			***H. pet-H. pra***			***H. gro-H. mic***		
Compound	**Sp**	**N**	**Sp*N**	**Sp**	**N**	**Sp*N**	**Sp**	**N**	**Sp*N**
Alanine	**0.015**	-	-	-	**0.002**	-	**0.042**	**0.003**	**0.028**
Allo-inositol	-	-	-	-	**0.017**	-	-	-	-
Arabinonate	-	-	-	-	-	-	-	**0.031**	-
Aspartic acid	-	**0.017**	-	-	**<0.001**	-	-	**<0.001**	**0.002**
Azelaic acid	-	-	-	-	**0.008**	-	-	-	-
Caffeic acid	**<0.001**	**0.006**	-	**<0.001**	**<0.001**	-	-	-	-
Citric acid	**0.029**	**0.002**	-	-	**<0.001**	-	-	**0.006**	**0.045**
Fructose	**0.001**	-	-	-	**<0.001**	-	-	-	**0.043**
Fumaric acid	-	**<0.001**	-	-	**<0.001**	-	-	-	**0.007**
G-1-P	-	**<0.001**	-	**0.019**	-	-	-	**0.001**	-
GalA	-	-	-	-	**0.019**	-	-	-	-
Galactose	-	-	-	-	**<0.001**	**0.011**	-	-	**0.042**
GBA	-	**0.023**	-	**0.043**	**<0.001**	-	**0.011**	**0.049**	-
GG	-	**0.035**	-	-	-	-	-	-	-
Gluconic acid	-	-	-	-	-	-	-	-	**0.013**
Glucose	**0.005**	**0.005**	-	-	**<0.001**	-	-	-	**0.012**
Glutamic acid	**0.014**	**0.002**	-	-	**<0.001**	-	-	**0.001**	-
Glutamine	**0.044**	-	-	**<0.001**	**0.005**	**0.015**	-	**0.001**	**<0.001**
Glyceric acid	**0.004**	-	-	**0.045**	**0.047**	-	**<0.001**	-	-
Isoleucine	**0.003**	**0.013**	-	**0.011**	-	**0.036**	-	**<0.001**	-
KGA	-	**0.036**	-	-	-	-	-	**0.022**	-
Lactic acid	**<0.001**	-	-	-	**0.042**	**0.047**	-	-	-
Lactose	-	-	-	-	**<0.001**	-	**0.025**	-	**0.004**
Leucine	**0.003**	**<0.001**	-	-	-	-	-	**0.001**	**0.042**
Lysine	**<0.001**	-	**0.0066**	-	-	-	-	**0.003**	-
Malic acid	**0.046**	**0.002**	-	**0.033**	**<0.001**	-	-	**0.018**	**0.007**
Phenylalanine	-	-	-	**0.029**	-	-	**0.011**	-	**0.034**
Quinic acid	**0.042**	-	-	-	**<0.001**	**0.027**	**0.036**	-	**0.011**
Ribose	-	-	-	-	-	-	-	-	**0.025**
Sedoheptulose	-	**0.036**	-	-	**0.011**	-	-	**0.009**	-
Succinic acid	-	-	-	-	-	-	-	-	**<0.001**
Sucrose	**<0.001**	-	-	-	**0.013**	**0.035**	-	-	**0.049**
Threonic acid	**0.014**	-	-	**0.008**	-	-	**<0.001**	-	**0.005**
Threonine	**0.030**	**0.008**	-	-	-	-	-	**0.009**	**0.035**
Tyrosine	**<0.001**	-	**0.0006**	-	-	-	-	-	**0.026**
Valine	-	-	-	-	-	-	-	**0.007**	**0.043**
Xylitol	-	-	**0.035**	-	-	-	-	**0.021**	-

CHAPTER 6

CONCLUSIONS

The overarching goal of this research was to investigate evolutionary patterns in root trait variation and test whether these patterns support adaptive evolution in different environments. Using the genus *Helianthus* as a model system, we conducted a series of controlled environment studies to assess genetically-based trait differentiation across the genus. Combined with data on the soil and climate characteristics of the native sites these species, we investigated whether genetic differentiation for root system traits was associated with species' native environments.

First, we investigated both inter- and intraspecific differentiation in fine root traits across the diploids of genus *Helianthus*. Contrary to expectations, we found that, across 26 species, specific root length, root tissue density, and root nitrogen concentration, traits which are expected to characterize the resource economy of a root system, were only weakly correlated. In addition, pairwise relationships between individual fine root traits and native soil and climate characteristics were generally weak, providing little evidence for a single axis of growth strategies in fine root traits. Contrary to expectations, total and number of large xylem vessels and xylem cross-sectional area were negatively correlated with mean annual precipitation, possibly reflecting the early-flowering drought escape strategy described for several short-lived desert species in *Helianthus* (*Ludwig* et al., 2004, 2006). However, xylem vessel number was positively correlated with several soil nutrients, presumably as a mechanism for rapid nutrient transport in fertile soils. The absence of consistent tradeoffs in fine root traits suggests that multiple trait combinations may be suited for a given set of environment conditions.

Additionally, the lack of apparent tradeoffs in fine root trait variation could indicate that root system traits are under selection at the whole root system level, rather than the fine root level, in contrasting environments. Therefore, we targeted six species chosen as phylogenetically-independent contrasts with respect to soil fertility to examine whether whole root system morphology and nutrient uptake consistently differ between species native to contrasting soil fertilities. We found that species native to low nutrient soils consistently produced lower total root length than species native to high nutrient soils, consistent with a slow-growing, resource conservative strategy. Although species of low nutrient soils exhibited significantly higher nitrogen uptake rates than species native to high nutrient soils, total nitrogen uptake did not differ across species, likely due to the high mobility of nitrogen in soils. This suggests that, for mobile nutrients, there is consistent selection for high uptake capacities, regardless of species' local soil fertility levels (*Aerts* and *Chapin*, 2000). Overall, this study provides evidence for the adaptive value of a slow-growing root system suited for tolerating resource limitation, as well as high capacity for capitalizing on nutrient pulses with fast nutrient uptake, in low fertility soils.

Given the importance of root exudation for nutrient acquisition, we were also interested in investigating whether root exudate composition is under selection in relation to soil nutrient levels in species' native sites. However, methodological aspects of root exudate analysis such as sampling duration can impact analyses of root exudate composition (*Aulakh* et al., 2001). Therefore, we first used *H. annuus* to assess the temporal dynamics of root exudation and inform on the appropriate time intervals for exudate sampling in *Helianthus*. Using the trap-solution method, we sampled exudates of plants treated with either low or high nutrient supply at two, four, and six hours, and analyzed their composition using gas chromatography-mass

spectrometry. Overall, we found that the duration of root exudate collection strongly influences interpretation of the effects of nutrient availability on root exudate composition. Differences in root exudate composition between low and high nutrient-treated plants were largest in magnitude at the four and six hour time intervals, presumably due to accumulation of metabolites in the collection media. However, it has been suggested that collection intervals greater than six hours may lead to underestimation of root exudation due to processes such as degradation, microbial metabolism, and re-uptake by plant roots (*Neumann* et al., 2009; *Carvalhais* et al., 2011). Therefore, collecting root exudates in the four-to-six hour range may maximize root exudate accumulation, while minimizing losses due to such processes.

Informed by our study of *H. annuus*, we asked whether root exudate composition consistently differs between species native to high versus low fertility soils. We again utilized six species chosen as phylogenetically-independent contrasts with respect to soil fertility. We found that species native to low nutrient soils consistently exhibit higher exudation of primary metabolites under high nutrient supply. All species, regardless of their native soil fertility, responded similarly to low nutrient supply by drastically increasing exudation of carboxylic acids. However, species native to high nutrient soils also exhibited higher exudation of numerous other metabolites in response to low nutrients. These findings show that species native to low nutrient soils have constitutively high levels of root exudation, but species native to high nutrient soils exhibit a greater increase in root exudation in response to nutrient deprivation, likely reflecting a high nutrient demand to support their rapid growth rates.

Taken together, these studies provide evidence that there has been repeated selection for slow-growing root systems in low nutrient soils. This finding supports theoretical expectations for the adaptive value of a resource-conservative strategy in infertile soils (*Chapin*, 1980; *Aerts*

and *Chapin*, 2000). However, our findings indicate that root trait evolution does not strictly reflect a tradeoff between resource acquisition and conservation. For example, we also found evidence for repeated selection for a high capacity for uptake of mobile nutrients and constitutively high root exudation in low nutrient soils. Other traits, such as increased exudation of carboxylic acids in response to nutrient deprivation, were phylogenetically conserved across species, possibly reflecting selection on this response as a fundamentally important response to low nutrient supply. In addition, we found little evidence for the adaptive value of specific root length or root tissue density across environments, either at the fine root or the whole root system level, despite the prevalent belief that these traits summarize species' ecological strategies (*Ryser*, 2006; *Birouste* et al., 2014). Therefore, although several traits appear to be under strong differential selection across environmental gradients, there are likely a variety of different root trait combinations that may be selected for in a given environment, even in closely-related species. Future studies using reciprocal transplants and phenotypic selection analyses could test this prediction, as these methods remain the gold standards for demonstrating the adaptive value of traits (*Lande* and *Arnold*, 1983; *Farris* and *Lechowicz*, 1990; *Ackerly* et al. 2000).

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