Testing the Proximate Mechanisms for the Process of Character Displacement on the Evolution of Root Traits

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Ecology and Evolutionary Biology)
in the University of Michigan
2020

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Dedication

This dissertation dedicated to my loving husband, Andres Ibarra Meade, who always believed in me, and supported me endlessly. To my mother, who encouraged me to continue, and visited me when things got a little heavy. And in memory of my father, who has always guided me to pursue my dreams.

Acknowledgements

I would like to thank Regina Baucom Ph.D. (Gina) for being an incredible advisor. She never gave up on me, and always encouraged me to push to my potential as a scientist. I honestly do not know if I would complete my Ph.D. journey without her careful and consistent guidance. Gina helped me build strong skills in developing hypothesis, experimental design, data analysis and research writing through her expert guidance. She has provided me with a gamma of advice, from professional to work-life balance, and instilled confidence in my research when others doubted it. In short, she was an excellent advisor, mentor and ally. I want to thank Deborah Goldberg Ph.D. for her excellent input on all things plant ecology and plant dynamics—especially her patience and help during the final stages of my dissertation amidst the COVID pandemic. I also thank her for expressing thoughtful and encouraging words throughout my journey. I want to thank Pat Schloss Ph.D. for always being a present and encouraging committee member. His thoughtful feedback on my research ideas, his insights on how to analyze the microbiome, and his friendly disposition to help if I ever got stuck. I am grateful to Mark Hunter Ph.D. for sharing his insights on plant ecology with me and providing very helpful feedback on my thesis work throughout the span of my enrollment as a graduate student. I thank Mark for his constructive criticism on my dissertation proposal, and committee meeting update slides. Together, his input has helped me think more in depth about my work and mature on my thinking and scientific presentation. In summary, I am very thankful for all members of my committee for pushing me to be better while being sincere and constructive. I appreciate their collective effort to help me reach my goals and their open-door attitudes that have helped me feel both welcomed and valued.

I am very lucky to be part of the Baucom Lab, because everyone I have known and worked with there were nothing short of amazing, inspirational and humble individuals. I am forever grateful to Megan Van Etten Ph.D. and her husband Dan York. They have been with me since the very beginning and they helped me immensely in setting up my research both conceptually and in practice. Aside from being an amazing mentor for me, Megan also became a great friend who I value very dearly. Without a doubt, Megan has had a great and positive influence over me as a woman in science.

Other past/present members in the lab including, Malia Santos, Diego Alvarado-Serrano, Sonal Gupta and Nia Johnson, Yoav Jacob, Eliot Jackson and Johanna Nifonsi also went above and beyond in my time of need. They helped me carry out my huge 2017 and 2018 field experiments and did a lot of digging! I literally could not have done this dissertation without each of you helping me out in the field. Further, each of you have also contributed greatly to my work and peace of mind in the office (you too Anah Sobel!) Either by sharing a laugh or proof-reading my work to helping me analyze my data. I appreciate each and one of you greatly.

To all the undergraduate students that helped me with my project, Lourdes A. Abreu Torres, Teresa Dorado, Benjamin Lilleskov, Amirah Bin-Mahfouz, Jazyln Marcos and Donàa Williams, I thank you immensely! The hours each of you put into planting, weeding, digging, harvesting, seed-counting and more, were literally the back-bone of this project. Mentoring each of you was a privilege and a pleasure. I had a lot of fun teaching you about research, and I am grateful you worked so hard with me. It was extremely rewarding to be your mentor.

I thank past/present EEB peers (Jill, Jon, Joe, Michelle, Alex, Jeff, Pascal, Jackie and many more!) for being supportive members of my community and EEB staff for helping me navigate my Ph.D. Especially, Cindy Carl, Gail Kuhnlein and Kati Ellis for their particular attention. I am also extremely grateful to the help of many fellow staff members at the Matthaei Botanical Gardens. Without their help, my research would not be possible. I am especially thankful to, Paul Girard, Michael Palmer and Jeremy Moghtader.

To my close friends, Francheska Alers and Iván Chaar López for helping me get settled when I first moved to Ann Arbor, and for providing me continual love and support. You two mean so much to me, and you have helped me when I needed support the most. I thank you. To my besties in Puerto Rico (Jari, Elvi, Eliza, Tina, Rysa and Francine), I love you and I greatly appreciate the love and support you have shown me all throughout my life. And to my ‘Fuller Park’ community, for keeping me sane with soccer and friendship!

I thank my family. I thank my mother, Gloria Braña, who always wanted me to be happy, and have a great education. Who came to Michigan from Puerto Rico when I was unwell so I can bounce back and continue following my aspirations and goals. She is my rock, and I am forever grateful for her patience and love. To all my six siblings! Gloria, Guiller, Juan, Francisco and Santiago. They provided me a safe space all my life, and throughout graduate school, they each have reminded me of the support and love I can find in them. Especially, Gloria, who always quelled my anxiety, visited me when I have felt unwell and has been endlessly encouraging and dear.

Last and most importantly, to my husband Andres Ibarra. Who has lived with me for the last three years and has been the one to witness me day in and day out throughout this academic journey. He has never doubted me, always encouraged me to finish and always gave me the support and love unconditionally. He has helped me keep my head high and provided me a shoulder to cry and lean on when things got rough. He has helped me mature and gain patience with myself and being okay with making mistakes. Thank you for helping me become a better person, and thus, a better sister, daughter, friend and scientist.

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Abstract

Character displacement, is a process wherein closely resembling species diverge in their resource-linked traits as a response to intense competition. Research evaluating whether character displacement can influence the evolution of a plant's belowground root system remains unreported in the literature, despite the importance of root systems in capturing resources from the soil environment and mediating belowground competition. Thus, my dissertation addresses the overarching question, *Can character displacement influence the evolution of root traits between two closely related species*?

In the first two data chapters of this dissertation I tested for the potential that root traits can evolve *via* character displacement using *Ipomoea purpurea* and *I. hederacea*. In my first data chapter (Chapter 2) I performed a greenhouse common garden experiment to test if root traits were genetically variable and a competition field experiment to test if belowground competition can impose selection on root traits. In my second data chapter (Chapter 3) I expanded on my findings from Chapter 2 and performed a second competition field experiment to test for the main prediction of character displacement.

In addition to the root system, the root-associated microbiome can play a major role in a plant's realized niche and affect how plants access and compete for belowground resources. Moreover, the root-associated microbiome can potentially influence root phenotypes and vice versa. Consequently, plant-microbe interactions can potentially feedback into plant ecology and evolution and alter the outcome of processes such as belowground plant-plant competition. To this end in my third data chapter (Chapter 4). I asked the broad question, Does the rhizosphere microbial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment? I subsampled and analyzed the bacterial microbiome from rhizosphere soil taken from individuals of *I. purpurea* and *I. hederacea* grown in the presence and absence of belowground competition. I tested if root

phenotypes and measures of the rhizosphere microbial community were linked with each other and determined the relative impact of the rhizosphere microbial community on plant fitness in context of belowground competition

In brief, my thesis demonstrates that belowgound competition and root traits represent a viable and overlooked agent and target of selection. Most importantly, it demonstrates that belowground competition may potentially result in character *convergence*, not displacement of root traits. It provides initial evidence for the possibility that the rhizosphere microbiome and root traits can influence each other and effect how plants compete belowground. My work demonstrates the potential for belowground competition to shape plant evolution and diversity and suggests that plant-microbe interactions itself may play an important role in how plants respond and adapt to belowground competition. Collectively, this work represents a novel first step in linking plant ecology and evolution to the ‘hidden’ half.

Chapter 1. Introduction

Background and problem statement

Understanding the relative role of competition in the evolution and distribution of closely related species is a major and unresolved challenge in evolutionary ecology (Losos 2000; Pfennig and Pfennig 2009; Stuart and Losos 2013). Geographic patterns of plant species distributions show that closely related species can co-occur (sympatry) and when they do, they tend to be more phenotypically diverged compared to regions where they do not co-occur (allopatry) (Armbruster 1985; Levin 1985; Whalen 1978; Fishman and Wyatt 1999; Veech and Jenkins 2000; Muchhala 2007). Theory, however, predicts that closely related species are unlikely to coexist in similar habitats because their high similarity in resource-associated traits should result in strong competitive interactions for limiting resources. In turn, strong competition between closely related species should then ultimately result in the exclusion of the weaker congener in regions where they come into contact (Gause 1936). How do closely related plant species coexist? A possible solution to this central issue is that similar species have diverged through the process of character displacement.

Character displacement is a phenomenon whereby closely related species evolve in their resource use and uptake-associated traits as a response to competition for limiting resources (Brown and Wilson 1956; Pfennig and Pfennig 2009). The end result of character displacement is greater trait divergence of competitors in regions of sympatry relative to those in allopatry (Figure 1-1). In general, research on character displacement in plant systems is lacking (Schluter 2000; Dayan and Simberloff 2005, Beans 2014), and the available work has focused on aboveground interactions and traits such as growth form and flower morphology (*reviewed in* Beans 2014). Surprisingly, despite the fundamental role belowground root traits play in resource uptake and mediating competition with other plants, consideration of how readily character displacement can influence the evolution of root systems remains a significant gap in the literature.

While research testing for character displacement as a potential driver in the evolution of belowground plant traits is novel, research on aboveground plant traits, however, has begun to build evidence that the process of character displacement has played an important role in shaping geographical patterns of diversity and morphological adaptations between competing plant species (*e.g.*, character displacement in floral traits, growth morphology, mating system and seed mass; Beans 2014). Because above-ground interactions and traits only tell half the story, in order to fully understand the mechanisms driving patterns of plant species diversity and coexistence we must also begin to ask whether the process of character displacement is important for the evolution of root traits.

I address this gap as the subject of my dissertation, where I assessed the potential for character displacement to influence the evolution of root traits between closely related plant species. To this end, I tested whether the criteria necessary for the process of character displacement to occur--*i.e.*, (1) genetic variation underlying resource associated traits, (2) selection on resource associated traits imposed by belowground competition and (3) a positive relationship between the phenotypic distance of competitors in their resource associated traits and species fitness (McPhail and Schluter 1992; Losos 2000**)--**were satisfied using two sister species of common morning glories, *Ipomoea purpurea* and *I. hederacea*.

Testing the potential for character displacement to drive the evolution of belowground root traits in closely related plant species

The process of character displacement is a specific case of evolution by natural selection, wherein competition for limiting resources is the selective agent and resource associated traits are the targets of selection, *e.g.,* adaptive divergence of flower morphology between plants that compete for pollinators (Brown and Wilson 1956; Pfennig and Pfennig 2009). A critical prediction for the process of character displacement is that the intensity of competition between any two species is inversely proportional to how similar two species are in their resource associated traits (Pfennig and Pfennig 2009). In other words, character displacement may be implicated if selection favors individuals bearing greater dissimilarity in their resource associated traits relative to their competitor. To test whether character displacement may be an important process in the adaptive evolution of resource associated traits, demonstrating a negative relationship between phenotypic similarity and fitness of competing individuals is necessary, however, insufficient. As stated earlier, character displacement is an evolutionary process, therefore evidence that phenotypic variation includes a genetic component must be established because only then can they respond to selection (Anderson et al. 2014). Equally, evidence that competition for limiting resources *per se* is the agent of selection on resource associated traits is key since other ecological factors aside from competition could trigger phenotypic divergence of functionally important traits (*e.g.*, selection from shared predators; Rundle et al. 2003).

Disparate lines of evidence spanning from community ecology, plant breeding and experimental evolution suggest variation in specific root traits meet the criteria for character displacement. First, differences in the root foraging strategies between diverse plant species is considered by some as a critical component for maintaining plant diversity and abundance via resource partitioning (*reviewed in* Silvertown 2004). For example, differences in the root system's spatial arrangement (*e.g.,* rooting depth) between co-occurring plant species has been demonstrated across many plant communities which is thought to contribute to niche-partitioning between species (Callaway and Mahall 1991; Mueller et al. 2013). Furthermore, in a long-term grassland study, Tilman et al. (2001) grew seventeen grass species in different treatments where they varied the number of different functional plant groups according to their nitrogen use strategy. Their study found evidence that more functionally diverse groups of plant species had significantly higher plant productivity. Thus, this study suggests that greater dissimilarity in resource use between plant species can have a positive impact on plant performance and vice versa.

In addition, plant breeding studies have uncovered significant phenotypic and genotypic variation between naturally occurring plant populations in their belowground root traits for multiple model organisms and, they have begun to show that specific root traits can provide competitive advantages in resource limited environments (Dorlodot et al. 2007; Hochholdinger and Tuberosa 2009; Brown and Lynch 2012). From the limited literature in evolutionary ecology, Ferguson et al. (2016), studied natural accessions of *Arabidopsis thaliana* across multiple populations in their native geographic ranges and found significant associations between root phenotypes and soil nutrients, as well as population differences in primary root length, total root length and the number of lateral roots. This suggests that various root trait phenotypes have adapted as a response to different soil environments. In brief, these works indicate that variation in specific root traits can respond to selection from local environmental pressures and result in adaptive phenotypes.

Despite a strong consensus that belowground competition can significantly reduce plant yield/fitness and direct evidence that differences in root traits are important for resource partitioning, research demonstrating root phenotypes as causal adaptations to belowground competition remains a major gap in the literature (Hodge 2009). Because character displacement is essentially adaptive evolution in response to competition, studying the potential for character displacement to influence the evolution of belowground root structures can provide insight on how belowground competition influences plant adaptation and in turn species diversity and distributions. Hence, my research will not only contribute to our understanding of the relative importance of character displacement in driving the adaptive phenotypic evolution of plants but also represents the first work to directly test this process as a potential driver in the evolution of root traits.

To this end, the overarching goal of my dissertation is to address the question, *Can character displacement influence the evolution of root traits*, by examining the criteria for root traits of closely related plant species to undergo the process of character displacement. As a first step, in my first data chapter, I conducted a paired common greenhouse and field experiment to determine whether root traits of *I. purpurea* and *I. hederacea* meet the basic criteria to evolve by natural selection--*i.e.,* (1) evidence for phenotypic and genotypic variation in root traits, and (2) evidence that belowground competition imposes selection on root traits. In my second data chapter I tested for the critical prediction of character displacement, which maintains that phenotypic distance between closely related species is positively and significantly correlated with the fitness of a focal species. Further, I expanded on my findings from my first data chapter to test whether root traits show evidence of genotypic variation and re-assessed evidence for selection from belowground competition to act on root traits, when grown in field conditions.

Aside from the importance of root traits to mediate resource uptake and influence how plants compete belowground, the root-associated microbiome can also play an important role in plant function and fitness, *e.g.*, influencing host plants' realized niches (Shoresh et al. 2010; Friesen et al. 2011; Pieterse et al. 2012). Moreover, the root associated microbiome can influence the phenotypic expression of root traits and vice versa (*discussed in* Friesen et al. 2011). Consequently, root-microbial interactions can feedback into plant ecology and evolution and potentially impact eco-evolutionary processes such as competition for limiting resources, niche-partitioning and character displacement (Bever et al. 2010 Fuente Cantó et al. 2020). Despite the potential for root-microbial interactions to shape plant ecology and evolution, research testing if and how root phenotypes and root associated microbial communities can influence each other and impact plant fitness in context of belowground competition, is lacking. As such, for my third data chapter (**Chapter 4**) I performed a series of exploratory analysis to address the broad question, *does the rhizosphere microbial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment?* Below I provide detailed information on my study system and an overview of my dissertation.

Study system

Ivy leaf morning glory, *Ipomoea hederacea* (L.) Jacquin and the common morning glory, *I. purpurea* (L.) Roth (Convolvulaceae) (Figure 1-2) are self-compatible annual climbing vines that commonly co-occur throughout Eastern United States in ruderal habitat types (e.g. side of train tracks, agricultural fields, road sides and waste areas). The history of coexistence between *I. hederacea* and *I. purpurea* is only partially known. Evidence suggests that *I. purpurea* is native to Central America (Gray 1886; Barkley 1986; Hickman 1993), and it has been present in the eastern United States since at least the early 1700s (Pursh 1814). The history of *I. hederacea* is less clear; it has been in the United States for at least 150 years (Bright 1998) but whether it is native to the United States (Mohr 1901; Stevens 1948) or was introduced from tropical America is uncertain (Shreve et al. 1910; Strausbaugh and Core 1964; Long Lakela 1971; Wunderline 1982; Mahler 1984).

Aboveground, both species have similar growth patterns and produce long stems that branch occasionally, however, *I. purpurea* is slightly larger (up to 10’ long) compared to *I. hederacea* (up to 6’ long). *I. hederacea* and *I. purpurea* are diploid with a mixture of selfing and outcrossing mating systems, where *I. hederacea* is highly selfing with a rate of approximately 93% (Ennos 1981).

Dissertation Overview

In brief, my dissertation encompasses three chapters that address the following: **Chapter 2)** examines whether a few specific root traits meet the core criteria to evolve by natural selection, and that belowground competition can impose selection on root traits in *I. purpurea* and *I. hederacea* and **Chapter 3)** tests for the critical prediction of character displacement in root traits of *I. purpurea* as a focal species and expands my second chapter's tests for maternal line variation and selection from belowground competition on a wider set of root traits. **Chapter 4)** tests for preliminary evidence that root traits and the rhizosphere microbial community can influence each other and potentially influence belowground plant-plant competition using rhizosphere soil samples taken from individuals of *I. purpurea* grown in the presence and absence of competition from *I. hederacea*. Finally, in **Chapter 5)** I synthesize the outcomes of my three data chapters and their general contributions to the field of evolutionary ecology, and discuss how future work should be directed in order to address the great unknowns in the evolution and ecology of 'the hidden half'.

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Figures

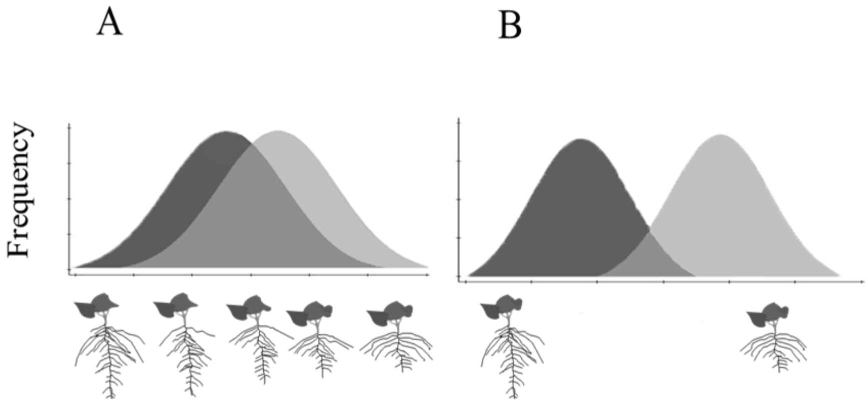

Figure 1-1 Process of character displacement in the belowground root traits of two closely related plant species A-B) Hypothetical representation of character displacement acting on the belowground root traits of two closely related plant species, before and after character displacement. (A) Initially two species come into contact and overlap in their belowground root traits, (B) then strong interspecific competition selects for divergent root traits, and the end result is a significant difference between species in their mean trait value.

Figure 1-2 Pictures of the ivy leaf morning glory (*Ipomoea hederacea*, left photo) and the common morning glory (*I. purpurea*, right photo) taken from our 2017 field experiment. Photo credit to Regina Baucom Ph.D.

Chapter 2. Belowground Competition Influences the Evolution of Root Traits

Abstract

Although root traits play a critical role in mediating plant-plant interactions and resource acquisition from the soil environment, research examining if and how belowground competition can influence the evolution of root traits remains largely unexplored. Here we examine the potential that root traits may evolve as a target of selection from interspecific competition using *Ipomoea purpurea* and *I. hederacea,* two closely related morning glory species that commonly co-occur in the United States as a model system. We show that belowground competitive interactions between the two species can alter the pattern of selection on root traits in each species. Specifically, competition with *I. purpurea* changes the pattern of selection on root angle in *I. hederacea,* and competitive interactions with *I. hederacea* changes the pattern of selection on root size in *I. purpurea*. However, we did not uncover evidence that intraspecific competition altered the pattern of selection on any root traits within *I. hederacea*. Overall, our results suggest that belowground competition between closely related species can influence the phenotypic evolution of root traits in natural populations. Our findings provide a microevolutionary perspective of how competitive belowground interactions may impact plant fitness, potentially leading to patterns of plant community structure.

Keywords: character displacement, root trait evolution, natural selection, *Ipomoea*

Introduction

One of the key reasons plant species are thought to coexist in a given habitat is through niche partitioning (Aarssen 1997; Huston 1997; Tilman et al. 1997, 2001; Loreau 2000). Such niche partitioning is hypothesized to occur following competitive exclusion (competitive exclusion under limiting similarity; Gause 1936; Hutchinson 1957; Hardin 1960; MacArthur and Levins 1967), or from trait divergence stemming from competitive interactions between species (*i.e.*, character displacement; Brown and Wilson 1965; Pfennig and Pfennig 2009). Because of the relevance of these ideas to the formation of plant community structure, there is a large body of literature examining competitive interactions among plants (Faget et al. 2013). Most of this work, however, focuses on above-ground interactions, and as a result, little is known about root-root interactions belowground.

Roots, which provide a vital resource acquisition function for the plant (Fitter, 2002), also serve as a structure through which plants experience competitive interactions with neighboring plants, whether indirectly through alterations of the soil environment—*i.e.*, reduction of water, space and nutrients—or directly by the excretion of signaling and/or allelopathic molecules (*reviewed in* Schenk, 2006 & Callaway, 2002).

The plant root system can be roughly characterized into both size and architectural traits. Root surface area, width, and root length are size proxies, whereas traits describing the spatial organization the root system, such as root angle, lateral root branching pattern, and internode branching distance are root architecture traits (Fitter et al. 1991; Lynch, 1995). These root phenotypes strongly influence how a plant accesses nutrients and water (Fitter et al. 1991; BassiriRad, 2005; Manschadi et al. 2006; *reviewed in* Lynch 2007; Kellermeier and Amtmann, 2013). For example, shallow root architectures are linked to increases in the uptake of immobile resources such as phosphorus (Lynch and Brown, 2001; Fitter et al., 2002; Beebe et al. 2006; Lambers et al. 2006), whereas deep root architectures are linked to an increase in water uptake (Beebe et al. 2006; Ho et al. 2005). Thus, shallow root systems may be more advantageous and lead to higher fitness in nutrient-limited soils, and deeper root systems may provide a fitness advantage in water limited environments. How different root traits may influence fitness in the field is most often studied in crop plants (Lynch 2007), leading to a significant gap in our understanding of the factors that influence root trait evolution in natural plant populations. In light of this, how belowground interactions between competing species can mediate plant resource acquisition patterns and potentially alter selection on root architecture and size traits—especially in wild plant species—is largely unknown.

There are plausible reasons to expect competitive interactions to influence the adaptive evolution of root traits. The intensity of competition between plants is greater when rooting zones overlap (*reviewed in* Casper and Jackson, 1997; Casper et al. 2003; Rubio et al. 2003), indicating that occupying the same belowground niche has a deleterious effect on plant fitness. Although studies hint that differences in root systems between competitors leads to higher fitness (*reviewed in* Silvertown, 2014), most of the research characterizes the root system at a coarse level (*e.g.*, belowground biomass, root length density; Poorter and Ryser, 2015), and has yet to include specific root size and architectural traits. Competition between co-occurring, closely related species can be especially intense due to greater overlap in physical space or niche use (MacArthur and Levins, 1967; Pfennig and Pfennig 2009; Burns and Strauss, 2011). To reduce the effects of this competition, selection would be expected to favor the divergence of root traits (*e.g.*, root angle, root length, and overall root system size) that play important roles in water and nutrient acquisition.

Despite this expectation, there are other explanations for particular root trait shapes or sizes in a species. As above, roots may evolve shallower, deeper, or larger root systems (among other changes) to optimize resource uptake in particular soil environments (Manschadi et al. 2006; Ferguson et al. 2016). Thus, specific root traits may reflect responses to factors in the environment that are distinct from competition. The only way to differentiate competition from other environmental factors that influence root trait evolution is to manipulate the presence of the competitor under otherwise identical conditions and determine if the pattern of selection on the trait is altered as a result (Wade and Kalisz, 1990; Dudley, 1996; Mauricio and Rausher, 1997). While there are many studies assessing how competition influences plant fitness (*reviewed in* Casper and Jackson, 1997 and Faget et al. 2013), there are no studies, to our knowledge, that have examined the potential that competition from a closely related species acts as an agent of selection on root morphology.

The purpose of this work is to determine if belowground competition between two morning glory species—*Ipomoea purpurea* and *I. hederacea*—can influence the phenotypic evolution of root traits in either species. *I. purpurea* and *I. hederacea* are two closely related vines and are common weeds of agriculture in the southeastern and Midwest US. They are most commonly found growing naturally in agricultural fields or in areas of high disturbance (Baucom et al. 2011). In some fields, both species are found to co-occur and intensely compete by vining together above ground; in other fields only one of the species may be present (personal observation, RS Baucom). Previous work has established that competition from one species can alter the pattern of selection on the other. Smith and Rausher (2008) manipulated the presence of *I. purpurea* and experimentally showed that competition between the two species for pollinators can lead to divergence in the floral morphology of *I. hederacea*. Because these species interact in other ways, and share similar morphology as well as resource needs, it is likely that other competitive interactions between the two can lead to trait divergence—namely, root trait divergence following belowground competitive interactions.

Here, we examine the potential that competitive interactions between these two closely related species can drive the evolution of root traits, and we do so by addressing some of the criteria for demonstrating the process of character displacement (*detailed in* Schluter and McPhail, 1992). We first characterize the extent of phenotypic overlap in early growth root traits between *I. purpurea*, and *I. hederacea* to determine if the species overlap in the same below-ground niche and then examine the potential for genetic variation underlying these traits. We likewise investigate the potential that natural selection can drive the evolution of root traits in field conditions. We specifically asked the following questions: How do root traits vary within and between species, and to what extent do the species exhibit phenotypic overlap? Is there evidence for genetic variation underlying root traits of either species, indicating that traits can respond to selection? Does belowground interspecific competition between *I. purpurea* and *I. hederacea* impose selection on root traits, and is there evidence that within-species competition (specifically, *I. hederacea-I. hederacea* competition) similarly acts as an agent of selection? Because the adaptive potential of traits can be obscured by plasticity when in competition, we also examine the potential that the presence of a competitor can directly impact root phenotypes.

To our knowledge, this is the first study to explicitly test the potential that root traits may exhibit evidence of selection as a result of competitive interactions.

Materials and methods

Study system—The common morning glory, *Ipomoea purpurea* (L.) Roth (Convolvulaceae) and ivy leaf morning glory, *I. hederacea* (L.) Jacquin are self-compatible annual climbing vines that commonly co-occur throughout the eastern United States. The two closely related sister species occur in similar habitat types (*e.g.*, side of train tracks, agricultural fields, road sides and waste areas). Both species germinate between the months of May and August and begin to flower about six weeks after germination and continue to flower until they die at first frost. The species have similar above-ground growth patterns and produce long stems that branch occasionally. *I. purpurea* is larger (up to 3 m long) compared to *I. hederacea* (up to 1.82 m long). Belowground, *I. purpurea* and *I. hederacea* have fibrous root systems consisting of a primary root with branched lateral roots, and both species vary greatly in the degree of lateral root branching (*personal observation*; *see* fig. 2-1).

The history of coexistence between *I. purpurea* and *I. hederacea* is only partially known. Evidence suggests that *I. purpurea* is native to Central America (Gray 1886; Barkley 1986; Hickman 1993, Fang et al. 2013*b*), and it has been present in the eastern United States since at least the early 1700s (Pursh 1814). In contrast, *I. hederacea* has been in the United States for at least 150 years (Bright 1998) but whether *I. hederacea* is native to the United States (Mohr 1901; Stevens 1948) or was introduced from tropical America is uncertain (Shreve et al. 1910; Strausbaugh and Core 1964; Wunderlin 1982; Mahler 1984).

Plant material and growth conditions—We performed complementary greenhouse and field studies to investigate the potential that root traits of these two sister *Ipomoea* species could respond to natural selection. To generate experimental seeds for our common garden and field experiments we selfed 25 and 35 maternal lines of *I. purpurea* and *I. hederacea*, respectively, which were previously sampled as seed from five populations located in Pennsylvania and Ohio. Seeds were scarified and planted in a randomized design in the Matthaei Botanical Gardens (Ann

Arbor, MI) greenhouse in November of 2015 and plants were allowed to set seed from selfing for all subsequent experiments.

Greenhouse experiment

We performed a greenhouse experiment to characterize early growth root traits between and within *I. purpurea* and *I. hederacea* in the summer of 2016. We planted replicate once-selfed seeds in custom built rhizotrons containing generic potting soil (fig. 2-1) in greenhouse conditions at the Matthaei Botanical Gardens (Ann Arbor, MI). Rhizotrons consisted of 20.32 cm *x* 25.4 cm frames made out of cut pieces of corrugated plastic and a transparent polystyrene sheet held to the frame by duct tape. Each rhizotron was filled with 20.45 grams of soil and a single seed was placed in the center of the rhizotron approximately one inch below the soil surface against the transparent polystyrene sheet.

We planted three replicates per maternal line per species in the rhizotrons and positioned the rhizotrons in custom-built wooden frames at 30° in a completely randomized design (see fig. 2-A1 in appendix A for root image from rhizotron, and instructions on building rhizotron frames in app. fig. 2-B2; both apps. A and B are available online). We replicated this experiment in its entirety, twice. Thus, we planted 150 individuals of *I. purpurea* (3 biological replicates × 25 maternal lines × 2 experimental replicates) and 210 individuals of *I. hederacea* (3 biological replicates × 35 maternal lines × 2 experimental replicates) for a total of 360 individuals. We watered each individual daily by hand to standardize water availability across all individuals for three weeks.

Greenhouse root phenotyping—Two weeks after germination, we scanned each rhizotron to measure below-ground root traits using a CanoScan LIDE 110® scanner bed. For each image, we traced the roots in Photoshop version CS6 to facilitate automated quantification of root size based on their pixels in ImageJ version 3.0 (Abràmoff and Magalhães 2004).

We focused on root size and root architecture by measuring root system pixels (root size), root system width, primary root length and root angle on the two week old seedlings (fig. 2-1). We elected to focus on these specific traits because they are relatively straightforward to measure across different growing conditions and they also play a vital role in plant resource use and uptake (Wasson et al. 2012; Paez-Garcia, et al. 2015). Prior to data collection, ImageJ was first globally calibrated with the set scale tool in order to obtain measurements in metric units for all of the following procedures. To obtain primary root length, root system width and root angle, first we used the multi-pointer tool and placed a total of four points along the root tips of the root system image in the following order: 1) primary root at the root stem surface, 2) root tip of the left outermost root, 3) primary root tip and 4) root tip of the right outermost root.

We used the statistical programming language R (R Core Team, 2017) to calculate primary root length, root system width and root angle (the script is available in the Dryad Digital Repository https://doi.org/10.5061/dryad.jh9w0vt6s). For primary root length, we calculated the vertical distance between the primary root at the soil surface and the tip of the primary root. We estimated root system width as the euclidean distance between the outermost lateral root tips. To estimate root angle (θ) we used the cosine formula,

$$\theta = \cos^{-1}\left(\frac{b^2+c^2-a^2}{2bc}\right),$$

on a right triangle formed by the primary root (the longest root perpendicular to the soil surface), and each of the outer lateral roots. Here, *b* is the distance between the primary root and the outermost lateral root tip, *c* is a measure of the length of the primary root calculated above, and *a* is the length between the outermost lateral root tip and the primary root tip (fig. 2-1). Root angle was calculated for both the right and left lateral roots, separately averaged, and reported in degrees. We elected to use the angle made between the primary root and the outermost left and right lateral roots because previous research has shown that this trait is indicative of root architecture types (Lynch and Brown, 2001; Uga et al. 2013; Colombi et al. 2015). Finally, to obtain root size, we converted the traced root images into binary images, selected ‘area’ as a measurement output in the ‘measurement’ option, and performed the ‘Analyze Particles’ function in ImageJ. This function quantified the total number of black pixels (all pixels from the root system) and reported the values in centimeters squared. We separated the belowground root system of all plants from aboveground structures at three weeks of growth. We cleaned the roots, dried them for three days at 60C, and then weighed them to obtain belowground biomass.

Field experiment

Field design and planting—We conducted a field experiment in the summer of 2017 to characterize root trait variation in *I. purpurea* and *I. hederacea* grown in field conditions and to determine if natural selection acts on root traits in the context of interspecific competition. We planted replicate selfed seed from maternal lines of *I. purpurea* and *I. hederacea* in two treatments: 'alone' and interspecific competition (fig. 2-2) in a field plot at Matthaei Botanical Gardens (Ann Arbor, MI) on June 2, 2017. We likewise planted replicate selfed seed from *I. hederacea* maternal lines in the presence of intraspecific competition to determine if, at least for this species, within-species competition could influence the evolution of root traits. We used eight maternal lines of *I. purpurea* and *I. hederacea* from a single population from Pennsylvania (PA4). We decided to use maternal lines from this population since preliminary greenhouse data demonstrated high phenotypic overlap between both species for this population (fig. S1-3). The field was plowed two days prior to planting, however, we did not fertilize the field beforehand, nor is it land that has crop rotation. We planted eight replicates of each maternal line per species across four blocks for our alone treatment (8 maternal lines × 2 biological replicates × 4 blocks × 2 species = 128 plants). For our interspecific competition treatment we paired each maternal line of *I. purpurea* with each maternal line of *I. hederacea* for each possible pairwise combination, and planted 2 replicates of each pairing across 4 blocks (64 unique interspecific competition combinations × 2 biological replicates × 4 blocks = 512 individuals × 2 species = 1024 plants). For our intraspecific competition treatment we planted 2 replicates of each unique combination of the 8 *I. hederacea* maternal lines within each block (28 unique interspecific competition combinations × 2 biological replicates × 4 blocks = 224 experimental units × 2 plants = 448 plants). Each pairwise competition pairing was replicated 8 times across the experiment. It is important to note that although we were likewise interested in the potential that *intraspecific* competition in *I. purpurea* could influence root trait evolution in this species, we elected to examine this only in *I. hederacea* due to both field space limitations and the experimental difficulty of phenotyping large numbers of root systems in the field.

Seeds were planted into experimental units (*i.e.,* cell with either a single plant for the absence of competition, or two plants for the competition environment), which were arrayed across the four spatial blocks in a completely random block design. Experimental units were spaced uniformly

1m from each other; individuals in competition treatments were planted 8 cm apart from each other within their experimental unit. One week after planting we scored germination. Due to intense drought < 48% of seeds germinated overall. The average precipitation for June in 2017 in Ann Arbor, MI was 0.10 cm. In comparison, the average precipitation was 0.18 cm and 0.20 cm in 2016 and 2018, respectively based on data collected online from National centers for Environmental Information (NCEI; Menne et al. 2012). We thus planted a second experimental cohort (cohort 2) on June 19, 2017 to increase sample size, and this cohort was planted to conserve the same level of replication across all experimental units. We had 86 % germination success with replanted individuals, and ended with a total 1177 plants of which 670 plants were in interspecific competition, 341 plants were in intraspecific competition, and 166 plants in the alone treatment; of our final sample, 56% came from cohort 1 and 44% came from cohort 2. Throughout the timespan of the field experiment we kept the immediate surroundings of each experimental unit (~15 cm from base of plants) clear of weeds. Three weeks after the first planting date we placed 1m tall bamboo stakes at the base of every experimental plant at a 45° angle, which allowed us to train vines of competing plants away from another, thus removing the potential for above-ground competition.

Field root excavation—To characterize the phenotypic variation of root traits of *I. purpurea* and *I. hederacea* grown in field conditions with and without competition, we adapted the ‘shovelomics’ excavation method described by Colombi et al. (2015). We harvested roots after three weeks of growth on a subset of plants in the field. For root phenotyping, we sampled individuals only from cohort 1 because individuals from cohort 2 were small and not reproductively mature, whereas most individuals of cohort 1 had developed flower buds. We sampled between two to four replicates per maternal line from both competition treatments—specifically, we sampled a total of 165 *I. purpurea* individuals, (N = 23 and N = 142 from the alone and interspecific competition treatments, respectively), and a total of 304 *I. hederacea* individuals (N = 31, N = 132, N = 141 from alone, interspecific and intraspecific competition treatments, respectively). To excavate roots, we cut the stem 5 cm from the soil surface, marked the side of the stem facing the competitor with a permanent marker and then dug the root system with a shovel by placing the shovel head at 45 degree angle, 15 cm from the plant stem. This method unearthed the first 15 cm of the root system. The excavated root core was then shaken gently to remove adhering soil and placed in plastic bags for root imaging. After imaging, we dried the root system at 60C, and weighed them to record belowground biomass.

Field root imaging and phenotyping—Root imaging was carried out indoors with the use of a cubic photo shooting tent (MVPOWER, 40 cm *x* 40 cm *x* 40 cm) in order to standardize imaging between samples and facilitate the use of REST (Colombi et al. 2015), an automated root phenotyping program developed to characterize the root systems of plants grown in the field. To image the root system, roots were hung in the center of the photo shooting box and photographed with a Canon EOS Rebel XSi 12.2 MP (18-55 mm IS Lens). Images were imported into REST (Colombi et al. 2015), and we manually specified where the stem at the soil surface was for each image (shown in fig. 2-3). After user specification of the stem/soil surface, REST draws a rectangular region of interest for pixel analysis to standardize measurements across images. All root measurements were quantified from the pixels lying within this region of interest. REST returned the root angle (right and left root angle), root system width (‘Max width’) and a root system size proxy (‘Area convex hull’), among other morphological and architectural traits. We focused on these three root traits because they are similar to the traits captured in our greenhouse study. Root angle (left and right) are determined by calculating the outermost angle between the top most lateral root and the soil surface plane at the plant stem, and then subtracting this value from a perpendicular line (*i.e.*, 90°) drawn at the plant stem. The root system width captured in REST is the same measurement as taken in the greenhouse rhizotron study as they were both estimated as the Euclidean distance between the root tips of the left and right outermost lateral roots. In contrast, root system size estimated from the greenhouse rhizotron study and REST program were similar, but not identical. Root size from the greenhouse rhizotron study was based on the total area of root derived pixels, and root size in REST was based on the convex hull of all root derived pixels. Preliminary linear regression analysis showed that these two variables predicted variation in belowground biomass, albeit the relationships were moderate (Rhizotron study: $R^2 = 0.30$, p-value < 0.001; Field study: $R^2 = 0.12$, p-value < 0.001). We do not have data for primary root length from plants grown in the field because this trait was destroyed in the process of sampling the roots.

Field plant fitness data—We began to collect mature fruit in the month of September and continued to do so until late October when all plants have senesced. The entire aboveground structure of all remaining plants were collected by first frost and seeds were manually removed, cleaned, and counted with a seed counter to obtain an estimate of total fitness. We sampled 165 individuals of *I. purpurea* (N = 23 and N = 142, from alone and competition treatments, respectively), and 304 individuals of *I. hederacea* (N = 31, N = 132, and N = 141, from alone, interspecific and intraspecific competition treatments, respectively). Ultimately, we sampled seeds from a total of 508 plants; 27% of these individuals came from cohort 1 and 72% from cohort 2.

Data analysis

Greenhouse experiment—All statistical analyses were carried out in R (version 3.3.1). We fit separate linear mixed models using the 'lmer' function of the lme4 package (Bates et al. 2015) for each of the root traits measured to test for the presence of variation in root traits between species, populations, and maternal lines. Each respective linear mixed model consisted of the root trait as the response variable, species, population and experimental replicate (*i.e.,* temporal replicate; 'Experiment') as fixed effects and maternal line as a random effect; *i.e.,* $\text{Root trait} \sim \text{Experiment} + \text{Population} + \text{Species} + (1|\text{Population:Maternal line}) + \varepsilon.$ We treated 'Experiment' and 'Population' as fixed because there were few levels of each (Experiment N = 2, Population N = 4). To ascertain the significance of the predictor variables we used F-statistics for the fixed effects, with Satterthwaite's method to estimate denominator degrees of freedom, and a log likelihood ratio test to estimate chi statistic (χ^2) for the random effect (using the 'anova' and 'ranova' functions from the lmerTest package; Kuznetsova et al. 2017). We ran additional linear mixed models for each species separately to test for evidence of maternal line variation within *I. purpurea* and *I. hederacea*, where root trait was the response variable, population and experimental replicate were fixed effects and maternal line was a random effect. We further examined how roots varied between species in trait space by performing principal component analysis (PCA) using a correlation matrix of all root traits measured in the greenhouse with the 'PCA' function from the FactoMineRPackage (Lê et al, 2008).

Field experiment—To examine how root traits vary between *I. purpurea* and *I. hederacea* grown in field conditions, and to determine if root phenotypes were influenced by competitive interactions, we ran mixed linear models as above. We fit a separate model for each root trait where the trait was the response variable and block, treatment, and block × treatment interaction were fixed effects and maternal line and maternal line × treatment interaction were random effects, *i.e.*, $\text{Root trait} \sim \text{Block} + \text{Treatment} + \text{Block} \times \text{Treatment} + (1|\text{Maternal line}) + \varepsilon$. Preliminary analyses indicated that there were no significant maternal line × treatment interactions for any trait. We thus elected to exclude these effects from our final models. As above, we visualized phenotypic variation in root traits between species when grown in field conditions by performing principal component analysis (PCA) with a correlation matrix on all traits including root system size, root system width and average root angle. In addition, we generated a correlation matrix using the family mean values for all the root traits measured for each species separately to examine relationships between the three traits.

Selection analyses—We used genotypic selection analyses (Lande and Arnold, 1983; Rausher 1992) to estimate selection gradients on each root trait in each competition environment, and ANCOVA to determine if competition and experimental block altered selection on root traits of the two species. We elected to perform a joint selection analysis using maternal line averages of the root traits because it allowed us to examine direct selection acting on each trait while controlling for environmentally induced biases (Rausher 1992). We estimated selection gradients on root system width, root system size, and root angle of both species in each competitive treatment environment (alone and, interspecific and intraspecific competition) by performing multiple regression with the focal root traits included as predictor variables and relative fitness as the dependent variable. Relative fitness was calculated as the total seed number divided by the mean seed number by species, cohort and treatment. For all selection analysis we mean standardized root traits (*i.e.*, subtracted the mean and divide by the standard deviation) and used untransformed values of relative fitness. Preliminary analysis indicated that individuals of both species from the second cohort produced significantly fewer total seeds than individuals from the first cohort (*I. purpurea*: $F_1 = 100.3$, p-value < 0.001; *I. hederacea*: $F_1 = 213.9$, p-value < 0.001), but preliminary analyses also provided no evidence that selection gradients differed between cohorts within either species for any root trait. Thus, we elected to combine cohorts in the genotypic selection analyses (cohort 1 N = 141 and cohort 2 N = 367). Further, while we examined the potential for non-linear selection influencing root traits in preliminary analyses, we did not find evidence of either stabilizing or disruptive selection acting and thus present only the results of linear selection analyses.

We used analysis of covariance (ANCOVA) to determine if the direction and/or intensity of selection varied between the presence and absence of competition (Wade and Kalisz, 1990) separately for each species. For *I. purpurea*, we compared selection gradients between plants grown in interspecific competition or grown alone, and for *I. hederacea*, we compared selection gradients from inter- or intraspecific competition with that of plants grown alone. In each analysis, models included competition treatment, block, the standardized root trait values, and all interactions as predictors of relative fitness. Significant interactions between the competition treatment and standardized root traits indicate that selection gradients differed between treatments. Block and block × treatment interactions were likewise included within the ANCOVAs.

Results

Greenhouse experiment—In our greenhouse rhizotron study assessing early root traits, we found significant variation between species in root system width and average root angle (table 2-1). The root system of *I. hederacea* was wider (8.49 cm, table 2-1) than that of *I. purpurea* (6.92 cm, table 2-1) and the overall root size of *I. hederacea* was on average greater (3.95 cm^2, table 2-1) than *I. purpurea* (2.37 cm^2, table 2-1). *I. purpurea* exhibited lateral roots that were closer to the soil surface (root angle: 37.33 degrees on average, table 2-1) compared to *I. hederacea* (30.44 degrees on average, table 2-1). Although species varied in the above traits (table 2-1), a visualization of the four root traits in a PCA identified considerable overlap of root phenotypes between species (fig. S1-2*A*). The root system width, root angle and primary root length loaded most strongly on the first principal component, which captured 37.3% of the total variation (fig. S1-2*B*), and root size loaded most strongly on the second principal component, which explained 29.1% of the total variation (fig. S1-2*C*). These first two PCA's can thus serve as descriptors of root system architecture (*i.e.*, spatial arrangement of root system) and root size, respectively.

Additionally, we found evidence for both population and maternal line variation in root traits. We found significant population variation for root system width and root system size, and variation among maternal lines for root system width, root angle, and primary root length (table 2-1). Separate mixed models, performed per species, identified significant maternal line variation within *I. purpurea* for root system width ($\chi^2 = 7.46$, *p*-value = 0.01) and root angle ($\chi^2 = 4.05$, *p*-value = 0.04), and marginally significant maternal line variation for root size ($\chi^2 = 3.63$, *p*-value = 0.06). We identified maternal line variation within *I. hederacea* for root angle ($\chi^2 = 8.63$, *p*-value < 0.01), and marginally significant maternal line variation for primary root length ($\chi^2 = 3.10$, *p*-value = 0.08).

Field experiment—A visualization of root system width, size, and root angle in a principal component analysis showed a high overlap between species in root phenotypes (fig. S1-1) in plants grown in the field. We identified maternal line variation in root system width (table 2-B1); a within species examination revealed this result to be driven by *I. purpurea* ($\chi^2 = 4.69$, *p*-value = 0.03). We further found a significant and strong correlation between root size and root width (r = 0.85, *p*-value < 0.001; table 2-B2) in *I. purpurea*, whereas there was evidence for strong and significant positive correlations between all root traits within *I. hederacea* (root width and root angle r = 0.59; root size and root angle r = 0.60; root size and root width r = 0.80; *p*-value < 0.001 for all pairwise traits; table 2-B2).

With the exception of a marginally significant treatment effect on root size ($F_{2,\ 455.74} = 2.33$, *p*-value = 0.10; table 2-B1), we found that interspecific competition in the field did not strongly influence root phenotypes of either species. A closer examination of the linear mixed models within species suggested that this treatment effect likely impacts *I. hederacea* ($F_{2,298} = 2.10$, *p*-value = 0.12) but not *I. purpurea* ($F_{1,12.58} = 0.04$, *p*-value = 0.84). However, there was a strong effect of competition on fitness, with *I. purpurea* experiencing a fitness reduction of 30.31% and *I. hederacea* a reduction of 36.47% when in interspecific competition. *I. hederacea* planted in intraspecific competition likewise experienced a significant fitness reduction (39.67 % lower than plants grown alone). Intraspecific competition between *I. hederacea* plants led to slightly lower fitness than when grown in interspecific competition (*i.e.*, 6.16% reduction in intra- versus interspecific competition), but this difference was not significant (table 2-2).

Selection on root traits in field conditions—From our selection gradient analyses, we identified positive linear selection on root angle in *I. hederacea* in the presence of interspecific competition ($\beta = 0.23$, *p*-value = 0.03; fig. 2-4; table 2-B3) but no evidence of selection when grown alone ($\beta = 0.01$, *p*-value = 0.86; table 2-B3). ANCOVA revealed a significant treatment interaction for root angle in *I. hederacea* ($F_{1,\,70} = 4.37$, *p*-value = 0.04; table 2-B4), supporting the idea that the pattern of selection for root angle differs according to competitive context for this species. Further, we found a marginally significant block × treatment interaction ($F_{3,\,70} = 2.28$, *p*-value = 0.09; fig. 2-4; table 2-B2) for root angle in *I. hederacea* when in interspecific competition, suggesting that environmental differences can influence the strength and/or direction of selection on this trait. In comparison, we found no evidence of selection when *I. hederacea* was grown in intraspecific competition ($\beta = -0.27$, *p*-value = 0.29; fig. 2-4; table 2-B3 and table 2-B4).

For *I. purpurea*, we found marginally significant positive selection for root system size when grown in the absence of interspecific competition ($\beta = 0.56$, *p*-value = 0.08; table 2-B3), but no evidence of selection on root size when in the presence of competition ($\beta = -0.15$, *p*-value = 0.51; table 2-B3). ANCOVA revealed a significant treatment interaction with root size ($F_{1,\,53} = 4.88$, *p*-value = 0.03; table 2-B4) indicating that selection on root size differs according to competitive context in this species. We likewise found a significant treatment × block interaction for root system width within *I. purpurea* ($F_{3,\,53} = 6.29$, *p*-value <0.01; table 2-B4) and a marginally significant treatment × block effect for root angle ($F_{3,\,53} = 2.20$, *p*-value = 0.10; table 2-B4), indicating that the pattern of selection on these two traits are impacted by both competitive context and other unmeasured environmental variables (*i.e.*, block effect).

Discussion

Given the functional importance of root systems, we hypothesized that competition between two closely related species could impose selection on root traits, and that selection could promote divergence in such traits. However, there are few, if any, examinations of the potential for selection on root traits in field conditions. Thus, we characterized the phenotypic variation in root traits of two closely related species, determined if genetic variation in these traits was present both within the greenhouse and in the field, and examined the potential that competition changed the pattern of selection on roots. From our greenhouse experiment, we found early growth root traits to differ significantly between the species and found evidence for genetic variation underlying traits—both between population variation and maternal line variation. Results from our manipulative field experiment showed that in the absence of competition there was a trend for positive linear selection acting on root size in *I. purpurea,* but no evidence for the same pattern of selection in the presence of competition. Interestingly, we found selection acting on different traits in *I. hederacea*: in this species, we found positive selection acting on root angle when in the presence of competition with *I. purpurea*, but no evidence of selection on this trait in the absence of competition. Somewhat surprisingly, we found no evidence of selection on root angle (or any root trait) in *I. hederacea* when in the presence of *intraspecific* competition (i.e., *I. hederacea-I. hederacea* competition). Thus, competition below-ground from *I. purpurea* promotes the evolution of broader root angles (*i.e.*, a more shallow root system) in *I. hederacea*, but the same effect is not seen in *I. hederacea* when in within-species competition.

Because water, ion and minerals are heterogeneously spread in the soil according to their chemical and physical properties, differences in root architecture between plants determine what specific resources are readily available for uptake and in turn how plants compete for such resources (Ho et al. 2005). This provides a likely explanation for the pattern of selection we identified for *I. hederacea* in the presence of interspecific competition; because shallow lateral roots enable the exploitation of nutrients near the soil surface, individuals with shallow roots may be at a fitness advantage when in the presence of a competitor compared to individuals with deeper root systems. In support of this idea, shallow rooting systems have been shown to be advantageous in common bean, maize and rice when grown in environments that are limited by phosphorus and other resources that accumulate in the topsoil (Rubio et al. 2003; Lynch and Brown, 2001; York et al. 2015; Sandhu et al. 2016).

From an ecological standpoint, it is somewhat puzzling that selection favors a larger root system in *I. purpurea* when competition is absent, but not when competition is present. Larger root systems allow for greater exploitation of soil nutrients and water and have been shown to be correlated with increased fitness in other species (Svačina and Chloupek, 2014; Ehdaie and

Waines, 2008). As such, we expected to identify selection for larger root systems regardless of competitive environment. A potential explanation for our findings is that root traits that were not measured here—primary root length, lateral root placement, and/or hair root density—may play an important role in resource uptake in the presence of competition. An investment in greater root foraging precision, as well as selection on traits that optimize resource uptake efficiency could potentially reduce the deleterious effects of competition. Thus, it is possible that root size is not under direct selection when these two species compete because selection is instead acting on traits that increase resource uptake efficiency (Fitter et al. 1991; Hodge et al. 1999; York et al. 2015).

Although we identified selection on only two traits—root size and angle—the strong correlations we uncovered between root width, size, and root angle suggests traits not under direct selection will likely evolve due to indirect selection. We identified strong positive correlations between root width, size, and angle in *I. hederacea*, indicating that width and size may evolve indirectly given selection on root angle. In *I. purpurea*, the strong positive correlation between root width and size, and pattern of positive selection on root size, suggests that root width should likewise experience indirect positive selection. That we found no evidence of correlations between root angle and root width and size in *I. purpurea* suggests root angle may evolve with fewer constraints in this species. It is likewise notable that we uncovered genetic variation underlying only root width in *I. purpurea* in the field experiment; however, this result is not particularly surprising given that genetic variation in field conditions is often obscured by high environmental variation (Conner and Stewart, 2003). Notably, in our greenhouse experiment, we found evidence for both population and maternal line variation on root traits in both species, suggesting these traits have the capacity to evolve either through selective pressures or as a result of genetic drift.

Further, while we identified different patterns of selection across the competitive environments between the two species, we found suggestive, but limited, evidence for plasticity in the root traits of either species as a result of competition. Plant root systems can impact the root growth of other closely neighboring plants either indirectly *via* altering the physical and chemical soil environment and/or directly through the excretion of signaling and/or allelopathic molecules

(Schenk 2006; reviewed in Cahill and McNickle 2011 & Depuydt, 2014). As such, we expected to find a significant treatment effect on root trait phenotypes, and thus evidence of phenotypic plasticity in root architecture and size traits. Other experiments characterizing root phenotypes in a range of species have found mixed results when plants are grown in competition, ranging from genotypic- and species-specific responses in root growth to no response whatsoever (Mahall and Callaway 1991; Falik et al. 2003; Bartelheimer et al. 2006; Dudley and File, 2007; Fang et al. 2013*a*; Belter and Cahill 2015; Litav and Harper 1967; Semchenko et al. 2007). Therefore, the results we report here suggest that these two *Ipomoea* species may lack a mechanism to modify their root growth in competition, that plasticity may be occurring in other, unmeasured traits, including aboveground traits, or simply that the effect sizes on root trait changes due to competition were small, and high variance due to other environmental factors (e.g., potentially the influence of drought in the 2017 field season) reduced our ability to identify significant plasticity in root traits given competition.

Importantly, the belowground plant-plant competition imposed by our experimental design led to reduced fitness of both species—around 35% fewer seeds produced by each species in the presence of competition (whether interspecific and intraspecific)—indicating that although we did not uncover root trait plasticity, there was clearly a cost imposed by the presence of belowground competition between and within species. We note, however, that the strongest trend in reduced root size occurred when *I. hederacea* was planted in *intraspecific* competition relative to interspecific competition. This suggests *I. hederacea* may potentially be decreasing overall plant growth as an adaptive response to reduce intraspecific competition. Such a potential plastic response within *I. hederacea* when in competition with a congener may explain why we did not detect evidence for selection from intraspecific competition on root traits in this species.

Overall, our finding of different patterns of selection acting on root traits in the different competitive treatments indicates that plant-plant competition can act as a selective agent on root traits. That we identified selection on different root traits between species is consistent with the idea of niche partitioning, which predicts greater divergence in resource associated traits between species to reduce competition for limiting resources (MacArthur and Levins 1967). Multiple field studies examining the relationship between different rooting depths of various co-occurring plant species have shown that a decrease in overlap between rooting zones of neighboring plants positively impacts plant yield and biomass (*i.e.*, plant fitness; Fargione and Tilman 2005; Mueller et al. 2013). Our results extend this finding to show that interactions between two closely related, co-occurring species elicits selection for different patterns of root traits. Hence, it is possible that competition between the two *Ipomoea* sister species promotes the divergence in resource-related root traits.

Finally, although our research provides the first experimental evidence that belowground competition can influence the evolution of root traits in these two related species, we are not showing the outcome of such competitive interactions across many natural populations. More specifically, while our study supports the idea that the adaptive process can occur in root traits as a response to belowground competition, we do not explicitly test for broad-scale patterns that would suggest such interactions have led to trait divergence (*i.e*., divergence in root traits where the species co-occur versus similarity in areas where they do not co-occur). Future work testing for patterns of phenotypic evolution in root traits between multiple naturally occurring populations of these two species is thus needed to draw conclusions for, if and how competition belowground has influenced the evolution of root traits in natural populations across the landscape.

Acknowledgements

We thank Corlett Wood for helpful comments on earlier versions of this article. We also thank Tyler Marrs for help with the construction of rhizotrons and wooden frames, and Andres Ibarra, Megan Van Etten, Diego Alvarado-Serreno, Sonal Gupta, Teresa Dorado and Lourdes Abreu Torres for their invaluable assistance with planting, maintenance of the field site and sample collection. We thank Gloria Brana and Gloria Colom for help designing rhizotrons and wooden frames, and employees of Matthaei Botanical Gardens, especially Michael Palmer and Paul Girard for their expertise and loan of field equipment and machinery. This work was made possible with financial support of internal grants at the University of Michigan and Matthaei Botanical Gardens Winifred B. Chase Fellowship.

Data availability statement--The R code and data is available in the Dryad Digital Repository https://doi.org/10.5061/dryad.jh9w0vt6s.

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Figures and Tables

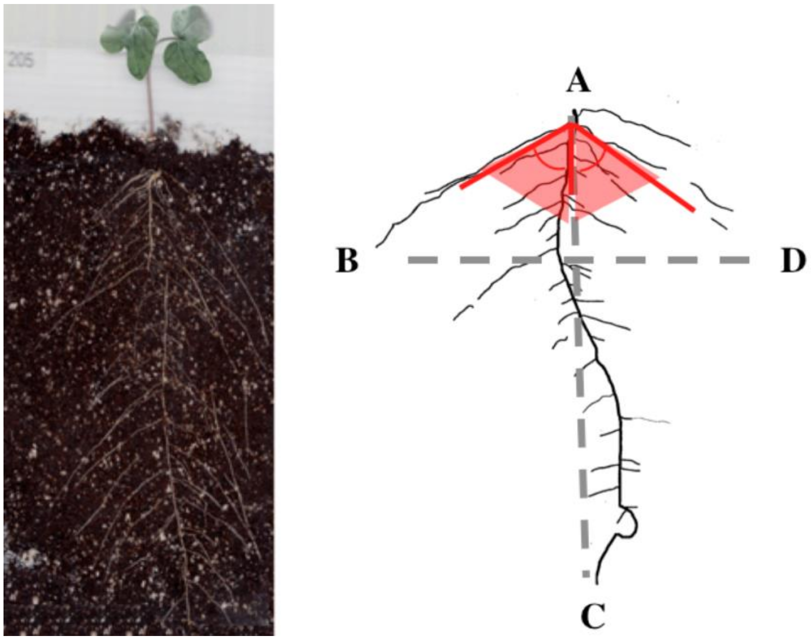

Figure 2-1: Example of an Ipomoea individual growing in rhizotron containing soil (left) and root traced in photoshop (right). Landmarks are placed to estimate root system width (distance between landmark B and D), primary root length (vertical distance between la

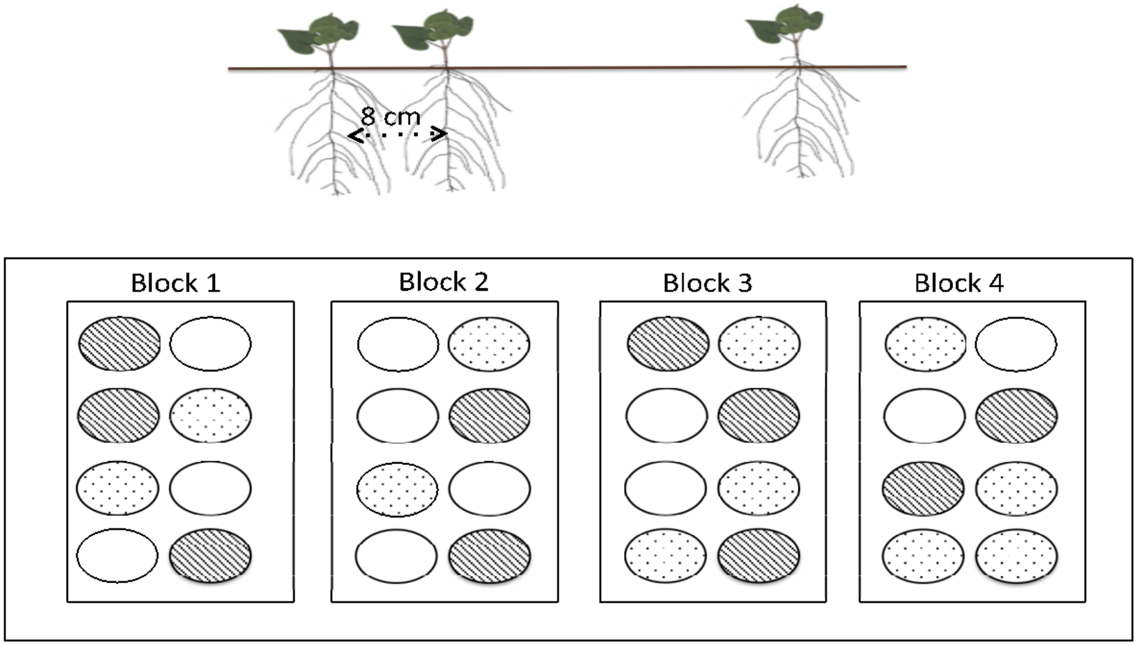

Figure 2-2 Diagram of the field experiment showing Ipomoea plants grown in the presence of competition (inter- or intraspecific) or alone. Inter- or intraspecific competition treatments are indicated by white circles with a black dotted pattern, or grey solid circles with diagonal lines, respectively. The alone treatment is indicated by a white solid circles. Each experimental unit (i.e., each unique competition pairing and alone treatments) was replicated eight times and randomly arrayed with two biological replicates per experimental unit per block.

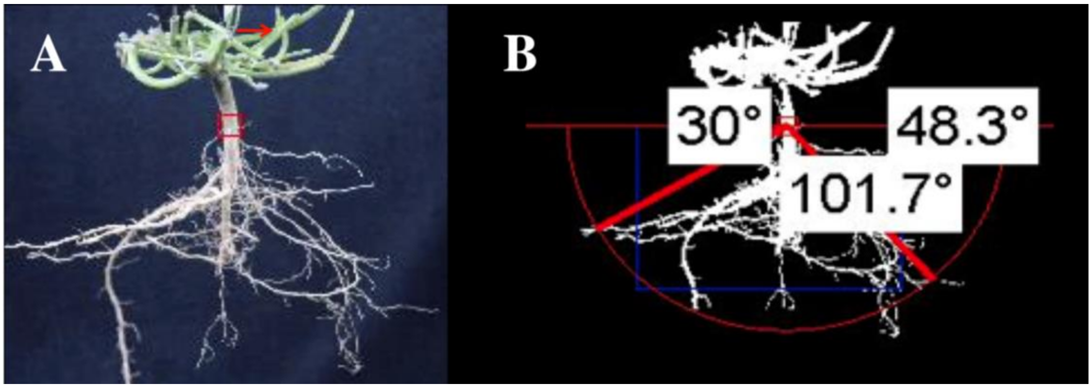

Figure 2-3 REST output showing the original image of an Ipomoea species excavated from the field (A) and its binary form with an arc superimposed by REST to obtain the outer right and left root angles (here 30 degrees on the left and 48.3 degrees on the right) from the horizontal place in red. (B) The blue box shows the region of interest detected automatically by REST program of which total root based pixels were quantified from within to obtain root size ('area convex hull' in REST) and measure root system width ('max root width' in REST) based on the distance between the right and left outermost roots in the box.

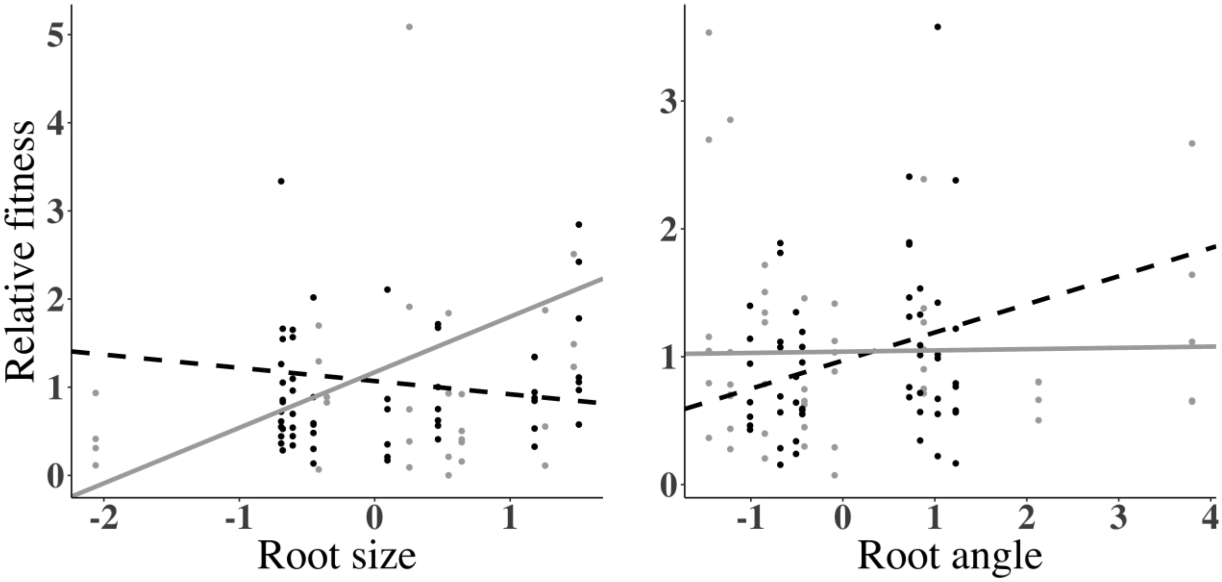

Figure 2-4 Interspecific belowground competition alters the pattern of selection for root size in *I. purpurea* (A), and root angle in *I. hederacea* (B). Solid grey and solid black circles represent the family mean values of standardized root traits (root size in *I. purpurea* (A) and root angle in *I. hederacea* (B)), with family mean values of relative fitness regressed onto each trait when plants were grown in interspecific competition or grown alone, respectively. Solid grey lines and dashed black lines represent the slope (β) for plants grown in interspecific competition, and plants grown alone.) The β for root size in *I. purpurea* grown alone (0.56 ± 0.29) differed significantly ($F_{1,53}$=4.88, *p*-value=0.03; Table B4) from the β of *I. purpurea* grown in interspecific competition (-0.15 ± 0.22). The β for root angle in *I. hederacea* grown alone (0.01 ± 0.08), differed significantly ($F_{1,70}$=4.37, *p*-value=0.04; Table B4) from the β of *I. hederacea* grown in interspecific competition (0.23 ± 0.09).

Table 2-1: SPECIES DIFFERENCES IN I. PURPUREA AND I. HEDERACEA ROOT TRAITS MEASURED FROM

	Species		*F*-statistics			χ^2
Trait	*I. purpurea*	*I. hederacea*	Experiment *df* = 1	Species *df* = 1	Population *df* = 4	Maternal Line *df* = 1
Root system width (cm)	6.90 ± 0.28	8.49 ± 0.22	315.79***	17.18***	6.82***	5.10*
Primary root length (cm)	11.30 ± 0.61	11.40 ± 0.46	22.52***	< 0.01	0.76	5.22*
Root angle (degrees)	37.40 ± 1.33	30.40 ± 1.01	61.42***	15.04***	0.22	12.47***
Root system size (cm^2)	2.36 ± 0.21	3.95 ± 0.16	35.22***	31.41***	7.29***	2.22

NOTE.—Least-squares means ±1 SE for each trait by species and F-statistics and likelihood ratio test statistics (χ^2) values showing the effects of experimental replicate, species, population, and maternal line variation on plant phenotypes. Maternal lines were nested within populations.

* $p< .05$

** $p< .01$

*** $p< .001$

Table 2-2 INFLUENCE OF COMPETITIVE TREATMENT ON *I. PURPUREA* AND *I. HEDERACEA* ROOT TRAITS WHEN GROWN IN THE FIELD

	I. purpurea		*I. hederacea*		
Trait	Alone	Competition	Alone	Competition (inter)	Competition (intra)
Root system width (cm)	7.12 ± 0.37	7.00 ± 0.25	7.43 ± 0.36	7.31 ± 0.25	7.03 ± 0.25
Root system size (cm^2)	77.50 ± 3.94	76.69 ± 2.16	78.08 ± 3.77	77.28 ± 2.22	71.07 ± 2.23
Root angle (degrees)	26.62 ± 2.05	24.43 ±1.14	27.00 ±1.96	24.81 ±1.16	22.63 ±1.17
Seed number	750.01 ±75.69	491.15 ±53.88	709.84 ±68.42	450.98 ±53.48	428.26 ±55.10

NOTE.—Least-squares means ±1 SE for each trait in each treatment.

Chapter 3. Potential for Character Convergence, but Not Displacement, to Influence the Evolution of the Root System of the Common Morning Glory

Abstract

Character displacement describes a type of phenotypic evolution by natural selection where competition between closely related species results in the phenotypic divergence of resource associated traits. Although character displacement can have important repercussions on plant evolution and ecology, research into character displacement as a potential driver of root trait evolution remains untested. Here we investigated the potential for the process of character displacement to result in the evolution of root traits using two closely related morning glory species, *Ipomoea purpurea* and *I. hederacea*. We performed a field experiment where we grew replicate maternal lines of *I. purpurea* in the presence and absence of competition from *I. hederacea* and determined if the phenotypic distance in multivariate measures of 33 root traits between competitors was positively associated with increased fitness, which would indicate that character displacement can lead to root trait evolution. We found maternal line variation in root morphology as a modular trait and in specific root architecture and morphology traits, as well as evidence that belowground competition acts as an agent of selection on these traits. Our test for the prediction of character displacement, however, showed evidence for character *convergenc*e in root architecture (*i.e.,* an increase in relative fitness with a decrease in phenotypic distance between competitors) rather than a pattern of character displacement. This result suggests that plants are either responding plastically to their specific competitive environment and/or indicates the possibility that phenotypic plasticity in root architecture may represent an important mechanism for how plants compete and acquire multiple essential resources belowground.

Introduction

Character displacement, where closely related species diverge in their resource-associated traits as a response to competition, has long been considered an important mechanism that may facilitate species coexistence, result in the evolution of novel phenotypes, and potentially promote the adaptive radiation of species (Brown and Wilson, 1956; Losos 2000; Pfennig et al. 2006; Pfennig and Pfennig 2009). Character displacement is hypothesized to occur due to high phenotypic similarity between related species, which leads to increased competition and a concomitant reduction in fitness (Schluter 2000; Pritchard and Schluter 2001; Day and Young 2004; Dayan and Simberloff 2005). Despite the importance of character displacement in evolutionary processes, the majority of character displacement work has been performed in animal systems (Schluter and Grant 1984; Schluter et al.1985; Losos 1990 & 2009; Schluter and McPhail 1992; Pritchard 1998; Martin and Pfennig 2011). Comparatively fewer well-supported studies of character displacement exist in plants (Levin 1985; Muchhala and Potts 2007; Muchhala 2008; Hopkins and Rausher 2011; Beans, 2014).

Of the available research on character displacement in plants, the majority (if not all) is focused on competition for pollinators, *e.g.*, the evolution of floral morphology and color as a response to competition for pollinators (Armbruster 1985 & 1986; Muchhala and Potts 2007; Muchhala 2008; Smith and Rausher 2008; Beans 2014). Other plant traits contribute to fitness and mediate plant-plant competition, however, and these remain largely unstudied as targets of character displacement. For example, the belowground root system plays a critical role in the acquisition of minerals and water from the soil (Fitter,1987; Fitter, 2002) and in mediating belowground plant-plant competition (Casper and Jackson 1997, Kroon et al. 2003 and Schenk 2006; Ravenek et al. 2016). Thus, the belowground root system could likely respond to selection *via* competition between closely related, co-occurring species, potentially leading to character displacement in root traits. However, research examining the potential for character displacement on the root system remains a significant gap in evolutionary ecology.

The belowground root system is a complex organ composed of diverse and developmentally interdependent traits that are often cataloged into ‘functional groups’ that influence resource uptake in different ways (Fig. 3-1; Fitter 1987; Lynch 1995; Bucksch et al. 2014). Phenotypic diversity in the traits within functional groups can influence how plants explore the soil and acquire nutrients – *e.g.,* the morphology of individual root traits (*e.g.,* root diameter), how components of traits are arranged spatially (architecture, *e.g.,* angles), the size of these traits and the volume they take up (root system size) and the distribution of these elements over space (root system topology; Fig. 3; Lynch and Brown, 2001; Fitter et al. 2001; Lynch 2005; Nguyen and Stangoulis 2019; Canales et al. 2019). Despite the important ecological and functional role of the root system, only a few studies have explicitly investigated the potential that natural selection can lead to the phenotypic evolution of root traits (Ferguson et al. 2016; Murren et al. 2020; Colom and Baucom 2020). Even, fewer have considered the role that belowground competition may play in the evolution of the root system (Colom and Baucom 2020).

Previously, we demonstrated that competitive belowground interactions can act as an agent of selection on root traits in *Ipomoea purpurea* and *I. hederacea*, two closely related species of morning glory that are found to co-occur, and compete, in agricultural fields and other areas of high disturbance (Colom and Baucom 2020). We found evidence of genetic variation underlying traits associated with both root morphology and architecture (*i.e.*, primary root length, angle and width), and additionally showed that competition between the two species influenced the pattern of selection on root traits. Specifically, when in competition with *I. purpurea*, *I. hederacea* individuals with shallower root architectures -- a trait associated with increased topsoil foraging (Fitter 1987; Lynch 1995) -- exhibited higher fitness. In contrast, belowground competition from *I. hederacea* altered the pattern of selection on root system size in *I. purpurea*. When *I. purpurea* was grown in the absence of *I. hederacea* competitors, selection favored individuals with larger root systems, whereas there was no detectable selection on root system size in *I. purpurea* in the presence of competition with *I. hederacea* (Colom and Baucom, 2020). These findings indicate that root traits can respond to selection, and that belowground competition acts as an agent of selection, potentially influencing the evolution of root traits.

Here, we build on our previous research and ask, “Can belowground competition between closely related species potentially result in character displacement of their root system traits?” We addressed this question by growing maternal lines of *I. purpurea* in the presence and absence of *I. hederacea* and determining if there is a relationship between fitness and the phenotypic distance of multivariate measures of root topology, architecture, size and morphology between these two species. We adopted part of the criteria used to evaluate evidence for the *pattern* of character displacement (McPhail and Schluter, 1992; Losos 2000) and focused on testing evidence for the *process* of character displacement. We examined the following core components of character displacement: Criterion 1) belowground competition influences fitness, Criterion 2) traits under selection must have a genetic basis, Criterion 3) belowground competition generates non-random fitness differences as a function of phenotypic variation (*i.e.,* competition is the agent of selection on phenotype), and Criterion 4) when in competition, the fitness of individuals increases with greater phenotypic distance in root traits compared to their competitor. Criterion 4 is the hallmark prediction of character displacement.

Materials and Methods

Study system--We used the closely related morning glory species, *I. purpurea* (L.) Roth and *I. hederacea* (L.) Jacquin (Convolvulaceae) as our experimental system. These *Ipomoea* species commonly co-occur in eastern United States (*personal* observation, RS Baucom). Historical records indicate the presence of *I. purpurea* as early as 1700’s (Pursh 1814) and the presence of *I. hederacea* since mid 1800’s (Bright 1998). *I. hederacea* and *I. purpurea* are both annual, self compatible weedy climbing vines that reside in similar habitats (*e.g.,* sides of train tracks, agricultural fields) and exhibit high within species morphological diversity in aboveground and belowground traits (Baucom et al. 2011; *personal* observation Colom and Baucom). Both species germinate between the months of May and August, begin to flower about six weeks after germination and continue to flower until they are killed at first frost. In this experiment, we used *I. purpurea* as our focal species to have a sufficiently high number of replicates to examine the potential for maternal line and thus genetic variation in root traits in the field while maintaining a feasibly sized experiment.

Field design and planting --We obtained ten maternal lines of *I. purpurea*, and six maternal lines of *I. hederacea* from a single population located in Pennsylvania and selfed them for one generation in greenhouse conditions to reduce maternal effects. We planted once-selfed seeds of *I. purpurea* in both the presence of interspecific competition with *I. hederacea,* hereafter, ‘competition’ treatment, and in the absence of competition, hereafter, ‘alone’ treatment at a 42m

× 40m field plot located at the Matthaei Botanical Gardens, Ann Arbor, MI on June 2nd of 2018. The field plot was tilled one week prior to planting. For the competition treatment, we used ten maternal lines of *I. purpurea* paired with each combination of six maternal lines of *I. hederacea* to yield 60 'unique combination pairings' which allowed us to obtain variation in phenotypic distance between individuals in competition. We replicated each of these pairings sixteen times. For the alone treatment, we replicated each *I. purpurea* maternal line sixteen times.

All seeds were planted in a complete random block design in which we arrayed four replicates of each unique pairing and maternal lines grown alone in each of four 10 m × 30 m spatial blocks. Blocks were separated from each other by approximately two meters, and seeds planted in the competition treatment were placed three inches away from each other. All plant pairs in the competition treatment and individual plants in the alone treatments were planted 1 m^2 apart from one another. We placed 1 m tall bamboo stakes next to each experimental seedling and later trained them to grow onto the bamboo sticks to prevent experimental plants from tangling and competing aboveground, following Colom and Baucom, 2020. Although both species may respond to selection given belowground competition, our focal species in this experiment was *I. purpurea*, and thus our data collection and analysis centered on this species with *I. hederacea* as the closely related competitor.

We watered seeds and recorded germination daily for the first two weeks, and subsequently, relied on rainfall to water our plants. Vole herbivory, which killed some plants during the course of the experiment, was recorded. We kept the soil within a six-inch radius around each experimental plant free of weeds and removed any non-experimental morning glories from the field. One month after planting we counted leaves of each *I. purpurea* plant to serve as a proxy of plant size.

Root excavation and phenotyping--When experimental plants were reproductively mature, we excavated a subsample of individuals to obtain root phenotype data from individuals grown alone and in competition. We sampled between four to eight biological replicates of each maternal line of *I. purpurea* in the alone treatment, and four to eight biological replicates of *I. purpurea* and *I. hederacea* planted in competition for each of the unique 60 combination pairings. In total we excavated and phenotyped 511 plants. We adopted the shovelomics method for root excavation (Colombi et al. 2015) as previously described (Colom and Baucom 2020) and imaged their roots with a high resolution camera, Canon EOS Rebel XSi 12.2 MP (18-55mm IS Lens).

Each of the images was imported to DIRT (Das et al, 2015), a fully automated online program designed to phenotype various basic and complex root traits from plants sampled in field conditions. DIRT enables users to phenotype monocot and dicot root structures and analyze either the whole root system or single excised roots. We removed traits from this output that were not applicable for our study system, such as monocot root traits, as well as highly redundant (*i.e.*, represented a mathematical combination of two or more traits). All trait measurements computed by DIRT rely on estimates of root length, diameters, branching angles, density and spatial root distribution that are quantified from the pixels of an image mask of the root system (binarized image of the root system) and a structural description of the root system or ‘skeleton’. The structural description (‘root tip path’ in DIRT) of the root system is a curve representation of the root system based on different samples points that allows the program to capture multiple measurements (‘skeleton’ traits in DIRT) that are otherwise occluded or confounded—*e.g.*, the network of a mature root system occludes its interior and smaller roots may bind together and appear as a single root.

Specific root traits analyzed--We examined a total of 33 traits, which we *a priori* classified into the four functional classes of root architecture, morphology, size and topology, *e.g.*, root angle and horizontal/vertical length, lateral root number and diameter, total root system surface area and maximum width of the root system for a given soil depth, respectively (*see* Table S2-1 and Fig. S2-1).

Fitness data--We began to collect mature fruit of experimental *I. purpurea* in September and continued to do so until all plants senesced in mid-October. We sampled between three to eight replicates for each maternal line per *I. purpurea* in the alone treatment, and between two and nine replicates of *I. purpurea* for every unique pairing in competition; in total we sampled seed from 429 *I. purpurea* (Num. alone = 62 and Num. in competition = 367).

Statistical analysis

All statistical analyses were performed in R version 3.0 (R core team 2018).

Modular root traits-- Because preliminary assessment of trait correlations uncovered significant and strong pairwise correlations ($r > 0.8$, *p-value* < 0.5) we elected to perform a principal component analysis (PCA) to reduce the high dimensionality/redundancy of our root phenotypes (see *Root system traits* section). we performed the PCA with the correlation matrix of 33 root traits rendered by DIRT (Table S2-1) using the 'PCA' function from the 'factoMiner' package (Le et al, 2008). Prior to PCA we mean centered each trait to zero, scaled the standard deviation to a value of one, and applied a box cox transformation to reduce skewness in the data with the 'preProcess' function of the 'caret' package (Khun, 2019). Since preliminary visualization of the PC's showed strong grouping by block, we performed the PCA on the indexed residuals of each root trait after controlling for block in a one-way ANOVA. A scree plot performed on the output of this PCA showed that each of the first four PC's explained at least 10.0% of the total phenotypic variation (Fig. S2-2). Therefore, we focused our analysis on the first four PC's as modular root traits. To evaluate how each individual root trait contributed to each PC (PC1-4), we calculated the proportion of squared loading coefficients to the sum of squares with the 'fviz_contrib' function in the 'factoextra' package (*see* Fig S2-2; Kassambara and Mundt, 2017. We found that the first four PCs were associated generally by traits that describe topological, architectural, size and morphological aspects of the root system, respectively, (*see* Fig. 3-3; Table S2-1). As such, we hereafter refer to PC1, PC2, PC3 and PC4 as root topology, architecture, size and morphology, respectively.

Evidence of belowground competition—To test whether belowground competition influences fitness, we performed a linear mixed model where we used the observed seed number as our response variable, block, treatment and block × treatment as fixed effects, and maternal line and treatment by maternal line interaction as a random effects. We excluded treatment by maternal line interaction in our final model because we found that the inclusion of this term was not significant in a preliminary analysis, nor did it improve akaike information criterion (AIC) when we compared it to a model that lacked this interaction term. Because preliminary analysis showed a strong correlation between leaf number, a proxy for plant size, and seed number, we also included leaf number as a covariate in our model. We did *F*-tests with type three sums of squares using Satterthwaite's method to evaluate the significance of fixed effects, and log-likelihood ratio χ2 tests to test for the random effect using the ‘anova’ and ‘ranova’ functions of the ‘lmerTest’ package (Kuznetsova et al, 2017), respectively. We estimated the least square means of seed number for each treatment averaged across block and block × treatment interaction with the ‘emmeans’ function as above (Length, 2019).

Maternal line variation of root traits—To determine if there was evidence of maternal line variation in modular root traits of plants grown in the field, as well as how phenotypes differed between competition treatments, we performed separate linear mixed models for each of the four PC’s. We ran separate models for each PC as block, treatment and treatment by block interaction as fixed effects, and maternal line and maternal line × treatment interaction were random effects. Because we found that maternal line × treatment and block × treatment interactions did not have a significant effect on any of the PC’s examined, nor did their inclusion improve the AIC when we compared it to a model that lacked these interaction terms, we removed these factors from our final models. *F*-tests with type three sums of squares using Satterthwaite's method and log-likelihood ratio χ2 tests were performed to assess the significance of fixed and random effects for each model using the ‘anova’ and ‘ranova’ functions of the ‘lmerTest’ package (Kuznetsova et al, 2017), respectively. We also evaluated evidence for block, treatment and maternal line variation on eight individual root morphology traits and two individual root architecture traits *post hoc* within *I. purpurea* because we detected evidence for selection on these traits in our focal species (see *Selection on root traits* below).

Calculating standardized relative fitness—To test for selection on root traits and that fitness increases with phenotypic distance, we used standardized relative fitness as our response variable. For our calculation of standardized relative fitness, we divided the observed seed number by the mean seed number for *I. purpurea,* within each competition treatment, (*e.g.*, Relative fitness = $\frac{n_{obs.\ competition}}{\bar{X}_{competition}}$, where *n* represents the number of observed seeds from each individual in competition, and $\bar{X}$ represents the mean seed number of plants in competition). Then we averaged the output by maternal line and treatment for the selection analysis and averaged the output by maternal line, and unique combination pairing for the test that fitness increases with phenotypic distance. Then we standardized values of average relative fitness to control for block and plant size (*i.e.*, leaf number) by using the residuals of a two-way ANOVA that included only block and leaf number as explanatory variables of average relative fitness.

Testing for selection on root traits--To examine if competition imposes selection on root architecture, topology and morphology as modular traits, we performed genotypic selection analysis (Lande and Arnold, 1983). To do so, we averaged the PC scores (block standardized) of root architecture, topology and morphology by maternal line and treatment, and then performed separate regressions for each root system trait onto standardized relative fitness (averaged by treatment and maternal line) for each treatment. We elected to exclude root size (PC3) from this and subsequent analysis because we did not find evidence for maternal line variation or directional selection on this trait in the presence of competition in previous work (Colom and Baucom 2020), or in the preliminary analysis of the present research. Preliminary assessment of quadratic selection on individual PCs did not reveal any significant effect of those terms, indicating little evidence for either stabilizing or divergent selection, and thus we report only linear terms. To test whether the pattern of directional selection differed between treatments, we combined both treatments and performed ANCOVAs for each PC, wherein treatment, trait and treatment × trait interaction were included as our independent variables, and standardized relative fitness was our response variable.

Because not all root traits necessarily contribute to fitness, the effect of selection on any individual trait contributing to a PC can be obscured (Mitchell-Olds and Shaw 1987; Chong et al. 2018). Therefore, we performed ‘PC back regression’, which is a linear transformation technique where we can input PC’s of interest --*i.e.*, root topology, architecture, and morphology--and their corresponding eigenvectors to recover the selection gradients acting on specific root traits in their original trait space. More specifically, selection gradients on the original root traits are reconstructed by projecting the regression coefficients from our selection analysis onto their corresponding eigenvectors (Jolliffe 2002, p. 169; Chong et al. 2019). We used a matrix with the eigenvectors of PC1, PC2, and PC4 (*i.e.*, root topology, architecture and morphology) standardized for block, and a vector of their corresponding selection gradients (R script available in supplementary). We performed matrix multiplication as shown by the formula, $\beta = EA$, where β represents a vector of the reconstructed selection gradients on the original root traits, E is the matrix of the three PC's eigenvectors standardized by block, and A is a vector of the regression coefficients obtained from regressing relative fitness on these PC scores. We calculated reconstructed βs for individuals of *I. purpurea* grown alone and in competition separately. To test the uncertainty that the reconstructed selection gradients are significantly different from zero, we first calculated a standard error for each reconstructed trait by taking the square root of the difference between the squared standard errors obtained from the regression of PC's onto relative fitness and their eigenvectors squared for each treatment. We estimated confidence intervals for each β at an alpha of 0.05% based on plus or minus two standard errors from each β (*i.e.*, $\beta \pm 1.96 \times \text{SE}$). If the confidence interval of β did not include zero, we interpreted those slopes as different from zero and reported it (Table 3-2). We evaluated whether the confidence intervals in the absence of competition and in the presence of competition did not overlap to infer if slopes differed according to treatment. We interpreted those selection gradients that did not lie within the 95% confidence interval of the other treatment as evidence that belowground competition imposes selection on that trait. The traditional approach to compare whether selection gradients differ between treatments would be to apply an ANCOVA and calculate an *F-statistic*. However, we compared slopes in this manner since PC back regression method applied to a subspace results in a loss of information and consequently impacts our ability to estimate the degrees of freedom required to calculate its corresponding *F-statistics* (personal communication, J. Stinchcombe).

Fitness increases with phenotypic distance--To test whether the phenotypic distance between root system traits between competitors positively associated with fitness in *I. purpurea*, we regressed of *I. purpurea* standardized relative fitness on the phenotypic distance between root traits of competing plants. For each PC we calculated the Euclidean distances between competitors with the 'cdist' function from the 'rdist' R package (Blaser, 2018). Specifically, the calculation of phenotypic distance was done by finding the linear distance within the same modular root system traits:

$\sqrt{PC^2\ (focal) - PC^2(competitor)}$, where PC represents the prinicpal component of a given axis), for phenotypic distances of root topology, architecture and morphology respectively). We also evaluated phenotypic distance between different types of traits--*e.g.,* phenotypic distance between PC1 and PC2--but did not uncover any evidence that different trait combinations influenced fitness, and thus we do not include these results here. Each metric of phenotypic distance was averaged according to each unique combination pairing and regressed onto our values of standardized relative fitness (averaged by maternal line, treatment and combination). We used F-statistics to ascertain whether the slope was significantly different from zero.

We also evaluated evidence for the prediction of character displacement on eight individual root morphology traits and three individual root architecture traits post hoc (within *I. purpurea*) because we detected evidence for selection on these traits at the individual level--*i.e.,* $\sqrt{Root\ trait^{2}\ (focal) - Root\ trait^{2}(competitor)}$), see *Selection on root traits* above.

Because we had multiple biological replicates per sample point in this analysis (N= 2-6 pairwise comparisons between maternal lines of each species), and our main goal was to examine changes in relative fitness given phenotypic distance in root traits, we elected to retain all samples to evaluate the relationship between fitness and phenotypic distance of modular and specific root traits. Preliminary analysis indicated that two such pairings may be outliers, however, we retained them in the final analysis because we found that they had a low amount of variation around the mean (*see* Fig. S2-4), indicating that these points are not biased by an extreme phenotypic value.

Results

Describing the root system as modular root traits--PCA showed that the first four PC's contributed to 22.5%, 20.0%, 13.7% and 10.9% of the total variation, respectively. Because the traits driving the variation in PC1, PC2, PC3 and PC4 were mainly descriptors of root topology, architecture, system size and morphology, respectively, we refer to them as corresponding modular root traits. For PC1 we found that accumulated root width per soil depth explained most of the variation on this axis ('root topology'; Fig. 3-3A). Since each measure of accumulated root width per soil depth loaded positively on this axis, higher scores correspond to a root system with greater root width per soil depth.

Higher scores for PC2 were associated with broader stems, root tips emerging from deeper in the soil, wider and more shallow root system and a decrease in vertical root growth.
In general, these results indicate that a higher score correspond to a root system that tends to grow more narrowly near the soil surface and indicate a trade-off in the spatial arrangement of the root system, where the ability to grow deeper is constrained to individuals with a narrower root system and vice versa.

For PC3 (‘root system size’; Fig. 3-3C) we found that the total surface area (*i.e.,* total number of root derived pixels) of the root system (‘projected root area’) explained most of the variation and loaded positively on this axis, therefore indicating that higher scores correspond to an overall larger root system. Multiple traits that describe overall root system morphology (*e.g.,* root diameter and root tip count) contributed mainly to PC4 (‘root morphology’; Fig. 3-3D). Overall, higher scores on the morphology axis correspond to a root system that has multiple lateral roots and smaller lateral root diameter (*i.e.,* thinner lateral roots) and exhibits a greater range in the rooting angles relative to the soil surface and to the tap root (*i.e.,* develops roots that grow both obtuse and acute relative to soil surface and tap root). As such, within *I. purpurea*, individuals that produce many lateral roots tend to produce smaller roots with less diverse rooting angles (*i.e.,* lateral roots grow mainly at an acute angle or mainly at an obtuse angle).

Evidence of belowground competition--We found a significant effect of treatment ($F_{1,370.05}$ = 3.98, *p-value* = 0.046) with *I. purpurea* producing 18% fewer seeds when in the presence of competition with *I. hederacea* compared to growing alone. We also uncovered a significant treatment × block interaction ($F_{3,371.54}$ = 2.62, *p-value* = 0.05). These results indicate that *I. purpurea* competed with *I. hederacea* belowground and that the intensity of competition was environmentally dependent.

Maternal line variation in root traits--From our linear mixed model ANOVA on each of the PCs, we uncovered evidence for maternal line variation in root morphology (χ^2 = 6.31, *p-value* = 0.01; Table 3-1) but no evidence for maternal line variation in root topology, architecture or size (Table 3-1). In addition, all four modular traits differed with environment (block), but not with competition (Table 3-1).

In addition, we performed *post hoc* linear mixed models on eight individual root morphology traits and three individual root architecture traits because we found evidence that belowground competition altered selection on these traits (see results below). This analysis uncovered significant maternal line variation for soil tissue angle range (χ^2 = 4.66, *p-value* = 0.03; Table S2-3), root tissue angle range (χ^2 = 4.22, *p-value* = 0.04; Table S2-3) and maximum soil tissue angle (χ^2 = 5.17, *p-value* = 0.02; Table S2-3), indicating that these individual root traits can potentially respond to selection. The block effect explained a significant proportion of variation in all these specific traits while competition did not.

Testing for selection on root traits--Selection analysis on the modular root traits showed evidence for negative directional selection on root morphology (PC4) (β = -0.17, *p-value* = 0.03; Table S2-3; Fig. 3-4) when *I. purpurea* was grown in the presence of competition, and positive (albeit non-significant) directional selection on root morphology in the absence of competition (β = 0.12, *p-value* = 0.10; Table S2-3; Fig. 3-4). ANCOVA revealed a significant treatment × trait interaction ($F_{1,16}$ = 5.33, *p-value* = 0.03; Table S2-3), providing evidence that the pattern of selection on root morphology was altered by belowground competition. These results indicate that belowground competition is generating selection for root systems that exhibit smaller root morphology (*i.e.*, a decrease in overall lateral root production with an increase in lateral root diameter along with selection for a decreased range of root angles). We did not find evidence for selection on root topology, or architecture (*i.e.*, PC1 and PC2) in either the presence or absence of competition (Table S2-3), suggesting that these traits are not under selection regardless of the competitive environment, or alternatively, that the signal of selection on specific traits contributing to each PC was diluted.

We next evaluated evidence for selection on individual root traits via PC back regression since the absence of selection at the modular level does not necessarily reflect absence of selection on specific root traits. In the absence of competition, we uncovered positive selection on skeleton node number, root tip count, the number of adventitious roots, and negative selection on average root density (Table 3-2). In the presence of competition, however, we found evidence for positive selection on mean root tip diameter, hypocotyl diameter, tap root diameter and maximum diameter 90-100% soil depth, and negative selection on soil tissue angle range, maximum soil tissue angle and root tissue angle range within *I. purpurea* (Table 3-2). Although maximum soil root tissue angle and soil and root tissue angle range describe spatial characteristics of the root system, they contribute mainly to PC4 (root morphology) (Fig. 3-3; Table S2-1), therefore contributing to selection acting on root morphology in the presence of competition. The 95% confidence intervals on selection gradients each of these traits did not overlap between treatments, indicating that, with the exception of skeleton node number, belowground competition altered the pattern of selection on these traits.

Test of character displacement (i.e. Fitness increases with phenotypic distance)--We found a negative linear relationship between phenotypic distance in root architecture (PC2) and standardized relative fitness (β = -0.06, *p-value* = 0.03; Table 3-3; Fig. 3-5), suggesting that competitor individuals with similar root architectures (*i.e.,* more shallow root architecture with decreased maximum root width or more narrow root architecture with increased maximum root width) exhibited higher fitness than competitor individuals with more divergent architectural traits (*i.e.,* character convergence rather than displacement). We found no evidence of a linear relationship between standardized relative fitness and phenotypic distances in root topology (PC1) or morphology (PC4) (Table 3-3), and did not evaluate character displacement in root size (PC3) since previous work found no evidence for selection on size in the presence of competition nor evidence of genetic variation underlying this trait.

Finally, since we found evidence that belowground competition altered selection on a handful of individual root traits (*see* Testing for selection on root traits *above*), we performed *post hoc* tests to examine the pattern of character displacement on eight individual root morphology traits and three individual root architecture traits. However, we found no evidence for a significant linear relationship between phenotypic distance between these traits and relative fitness (results not shown).

Discussion

Our research examined the potential that root traits may evolve *via* the process of character displacement. We performed a field experiment where we grew *I. purpurea* (focal species) in the presence and absence of *I. hederacea* and determined if the process of character displacement could influence the evolution of root traits by testing four key criteria. We found that *I. purpurea* grown in the presence of *I. hederacea* experienced a significant reduction in fitness, thus providing evidence that these species compete belowground, a result that is in alignment with our previous field study (Criterion 1; Colom and Baucom, 2020). We uncovered evidence for genetic variation in the modular trait root morphology and for three individual traits (soil root tissue angle range, root tissue angle range and maximum soil tissue angle). Therefore, that multiple root traits represent viable targets of selection (Criterion 2). Further, we found that belowground competition imposed selection on root morphology as a modular trait and on multiple individual root traits, indicating that belowground competition can act as an agent of selection (Criterion 3, also in alignment with previous work). Most importantly, our test for the hallmark prediction of character displacement (Criterion 4) revealed a significant linear association between plant fitness and phenotypic distance for root architecture as a modular trait. However, this analysis did not show evidence for the potential for character displacement as we hypothesized--instead, we found evidence for the potential for character *convergence* in root architecture. Below, we expand on the implications of our findings and our interpretations in light of current experimental and theoretical work in root trait biology and ecology.

Genetic variation in root traits suggests evolutionary potential

Our finding of significant maternal line and thus genetic variation in root morphology as a modular trait and in individual root traits shows that these traits exhibit the potential to respond to selection and evolve (Criterion 2). These results are in line with previously reported evidence for maternal line variation in root traits associated with root system architecture and morphology in both *I. purpurea* and *I. hederacea* (Colom and Baucom, 2020). Interestingly, these previous results were based on measurements taken from individuals that were grown in greenhouse conditions, where environmental conditions are fairly simple. That we also uncovered maternal line variation for specific root architecture traits, and root morphology as a modular trait under field conditions strengthens support for these traits as viable targets of selection that can potentially evolve given selection from belowground competition.

Belowground competition generates selection on root traits

Although we found evidence that multiple root traits have the potential to respond to selection, evidence that interspecific competition alters the pattern of selection on these same root phenotypes (Criterion 3) is necessary for making a strong case that belowground competition can lead to character displacement. To this end, we examined selection on root traits using two approaches: selection at the modular level (*i.e.,* selection on PC's), and selection on specific root traits. Selection analysis on each PC is appropriate for studying the root system given that many root traits are strongly correlated (Chong et al. 2018). However, if traits that load strongly on a single PC axis do not contribute to fitness, the signal for selection on traits that may contribute to that PC could go undetected (Mitchell-Olds and Shaw 1987; Chong et al. 2018). Therefore, we also performed PC back regression, a linear algebra transformation technique that allows us to project selection gradients back into original trait space, and compute estimates of selection coefficients on specific traits (Chong et al. 2018). We found that directional selection acted on root morphology as a modular trait, and that the direction of selection was altered according to competitive context. Specifically, our results show a pattern of negative selection on root morphology in the presence of competition, and positive (albeit nonsignificant) selection on root morphology in the absence of competition. This result indicates that competition was selecting on thicker but fewer number of lateral roots, whereas in the absence of competition we did not uncover any evidence of selection.

One potential reason we uncovered a pattern of selection for smaller values of root morphology in the presence of competition may be due to specific foraging strategies that provide a benefit in this environment. For example, the production of fewer and thicker lateral roots has been linked with resource conservation, suggesting that selection is favoring individuals of *I. purpurea* that may acquire nutrients efficiently (Eissenstat and Yanai 2002; Paula and Pausas 2011). This explanation is in line with theoretical models of belowground plant-plant competition which predict that when soil resources are low, efficient root foraging phenotypes are favored over exploitative ones because the 'per-root' costs are high relative to resource uptake (Hutchings and

John 2007; O'Brien et al. 2007; McNickle and Brown, 2012). Our results also suggest that belowground competition is selecting for a wider root system (*i.e.*, decrease in the angle formed between the soil surface and a given lateral root; *see* sketch of 'soil tissue angle' in Fig. S2-1) and decrease in the rooting angle range relative to the soil surface and tap root. The finding of negative selection on these root angle traits may reflect increased competition for topsoil resources when *I. purpurea* and *I. hederacea* grow close to each other.

In previous work we found evidence for selection for shallower root systems in *I. hederacea* as a response to belowground competition from *I. purpurea*, but no evidence of selection when considering *I. purpurea* as the focal species in competition with *I. hederacea* (Baucom and Colom 2020). At first glance it appears the results presented here contradicts our previous work, however, in our present study we measured more and different architectural traits than in Colom and Baucom (2020). In our present study we found evidence of negative directional selection acting on a similar trait, the maximum angle formed between lateral roots and the soil surface (*i.e.*, maximum soil tissue angle) when *I. purpurea* was grown in the presence of competition but not in the absence of competition. This result implies that belowground competition from *I. hederacea* is generating selection for a decrease in the maximum rooting angle formed across lateral roots relative to the soil surface, or a more *shallow* root system. Collectively, our past and present results indicate that root architecture plays an important role in how these plants compete and access to belowground resources and it indicates that competition for topsoil resources is strong between these two *Ipomoea* species.

Consistent with our results for selection on root morphology at the modular level, PC back regression analysis revealed evidence that belowground competition altered selection on traits that contribute mainly to this axis, including: soil root tissue angle range, maximum soil root tissue angle and root tissue angle range and multiple root diameter and lateral root number traits (Table S2-2). Therefore, selection on these individual root traits are driving the patterns of selection observed on root morphology at the modular level. In contrast, we did not detect evidence for selection on specific root traits that contributed to root topology or architecture at the modular level, which is consistent with the lack of evidence for selection on root topology and architecture as modular traits.

Character convergence but not displacement on root traits

Traditional hypotheses of character displacement predict that when two co-occurring, closely related species overlap in their resource associated traits, selection should favor divergence as that would lead to lower resource overlap between species, thus reducing the harmful effects of competition (Losos 2000; Pfennig and Pfennig 2009). As such, we predicted that fitness should increase with increasing phenotypic distance to a competitor (Criterion 4). However, we actually found the reverse result, indicating evidence for character *convergence* rather than displacement. If root architecture influences soil exploration and what resources are readily available for uptake, why did we find support for character convergence instead of divergence?

For plants, phenotypic plasticity in root architecture has been argued to represent an adaptive strategy that allows plants to access and compete for key nutrients, and further, root architecture has been shown to respond plastically to nutrient availability across multiple plant species (Fitter et al. 1991; Nicotra and Davidson 2010; Yu et al. 2014). Therefore, one plausible explanation behind our result of character convergence in root architecture is that *I. purpurea* individuals capable of recognizing and responding plastically to both their immediate resource environment and to the presence of a competitor individual would be able to maximize fitness, whereas individuals less capable of sensing and responding to these environmental constraints would exhibit lower fitness. Soils are complex and heterogeneous, and plants may be selected to respond plastically to very local soil conditions. If both *I. purpurea* and I. *hederacea* benefit from similar plastic responses to a given local soil environment, we might expect to see patterns of trait convergence associated with higher fitness. In short, the local environment may constrain morning glories into expressing convergent phenotypes. Such constraints operating on behavior and morphology are well known from studies of competition among animal species (Gibson 1980; Hunter and Willmer 1989; Hunter et al. 1997).

Moreover, it is well established that plant root growth can respond to the presence of competitors (Cahill et al 2010), with neighbor recognition hypothesized to be due to either sensing of root exudates (Bais et al. 2006; Biedrzyckie et al. 2010; *reviewed in* Pierik et al. 2013; Semchenko and Lepik 2014) or more simply from feedback given the internal nutrient status of the plant (*reviewed in* Pierik et al. 2013; McKnickle and Brown, 2014), *i.e.*, the plant is capable of maximizing the balance between the costs and benefits of root production given the availability of nutrients (McKnickle and Brown 2012). Consistent with the idea that individuals can respond to the presence of a neighbor, supplementary analysis showed that the root architecture of *I. purpurea* varied depending on the presence of specific *I. hederacea* competitors after the removal of block effects (Table S2-5). Additionally, while we focused here on fitness in *I. purpurea*, we found preliminary evidence for a negative linear trend between plant size and phenotypic distance in root architecture within *I. hederacea* (Fig. S2-5). Given that plant size is often a strong correlate of fecundity (Aarssen and Taylor, 1992), this result suggests that the pattern of convergence is potentially present in both species, and perhaps that both species may modify their root architecture to acquire and compete for varying limiting resources.

Phenotypic plasticity of root traits can have important implications for the evolution of the belowground root system. For example, phenotypic plasticity can obscure selection from acting on phenotypes that are genetically variable, and hence, impede traits from *responding* to selection thus leading to character displacement. However, reaction norms of functional traits can be genetically variable, and therefore, phenotypic plasticity itself can represent an important target of selection that can evolve in response to different environmental stressors (*e.g.*, competition; Via and Lande 1985; Schlichting 1986; Scheiner 1993). Testing whether plasticity in root traits is a viable target of selection and whether belowground competition-imposed selection on phenotypic plasticity in root traits was beyond the scope of our current research. However, literature in the field of plant breeding has demonstrated that plasticity in root architecture can be genetically variable (*reviewed in* Jung and McCouch 2013), opening up the possibility that phenotypic plasticity in root architecture is a viable target of selection. Whether it is possible that belowground plant-plant competition can promote the evolution of phenotypic plasticity in root architecture remains an elusive and unaddressed question.

Conclusion

In summary, our study shows that belowground competition results in a pattern of character convergence rather than divergence in root architecture between competing pairs of *I.*

purpurea and *I. hederacea*. Moreover, we found evidence to suggest that root architecture responds plastically to its specific competitive environment (*i.e.*, effect of competitor; Table S2-5), which may reflect an adaptive mechanism that allows plants to compete for multiple key nutrients. Additional research will be required to assess whether phenotypic plasticity in root architecture can potentially evolve as a response to belowground competition and result in patterns of character displacement or convergence in plasticity. Although our work did not show evidence that character displacement can influence the evolution of root traits, it emphasizes the importance of belowground competition to potentially influence the evolution of the root system and considers the complex and integrated nature of the root system. Therefore, we encourage other researchers to examine the potential for character convergence/displacement in different study systems/environments, and further, to consider phenotypic plasticity as a target of selection. Future work and experimental replication will allow us to understand how widespread and viable these evolutionary processes are in nature; *e.g.*, it could depend on the natural history of species and their specific environmental conditions (temporal and spatial nutrient availability and substitutability).

Data availability statement

The R code is available at https://github.com/SaraMColom/CharacterDisplacement, and the data will be uploaded to the Dryad Digital Repository.

Acknowledgments

We graciously thank Deborah Goldberg, Mark Hunter and members of the Baucom Lab for their helpful comments on earlier versions of this article. We thank John Stinchcombe for his advice on statistical analysis, and Alexander Bucksch for his assistance in interpreting the root traits quantified in our study. We thank Andres Ibarra, Jazlyn Marcos, Yoav Jacob and Donàa Williams for their invaluable assistance with planting, maintenance of the field site, and sample collection and processing. We thank employees of Matthaei Botanical Gardens, especially Michael Palmer, Paul Girard and Jeremy Moghtader for their expertise and loan of field equipment and machinery. This work was made possible with financial support of internal grants at the University of Michigan.

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Figures and Tables

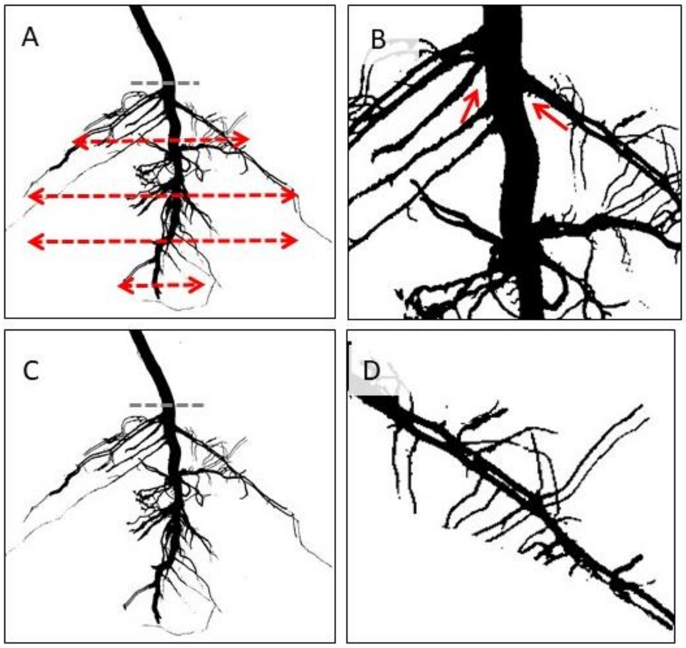

Figure 3-1 A depiction of four distinct functional root trait classes, (A) root topology, (B) architecture, (C) size, and (D) morphology. *Root topology* describes the general shape of the root system--*e.g.,* root system width with soil depth as indicated with the dashed red arrows of varying lengths beneath the soil line, shown by the grey dashed line. *Root architecture* is a suite of traits that describe the spatial arrangement of the root system including root angle formed between tissues (‘Root tissue angle’, indicated with the red arrows), overall root system width and length, and branching patterns (distances between lateral root nodes). *Root size* encompasses root traits such as root surface area and volume of the root system beneath the soil line (indicated with grey dashed line). *Root morphology* is a suite of traits that describes characteristics of individual root traits (*e.g.,* lateral root length, root diameter), and the relative number of individual root traits such as lateral root number and diameter. These traits are emphasized here with a close up depiction of a single lateral root where root length, diameter and number of second order branching roots are more readily visible.

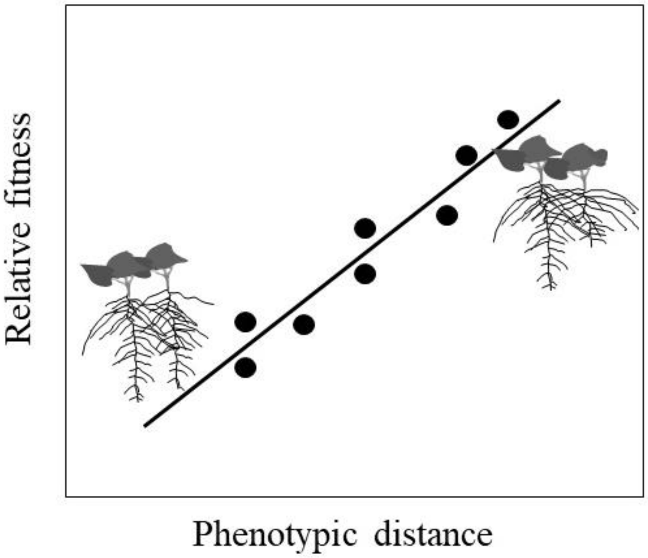

Figure 3-2 A positive linear relationship between phenotypic distance of competitors' root traits and relative fitness would support the hypothesis that character displacement can influence the evolution of root traits. Superimposed on the plot are sketches of two pairs of competitors with low (left pair) and high (right pair) phenotypic distance, respectively.

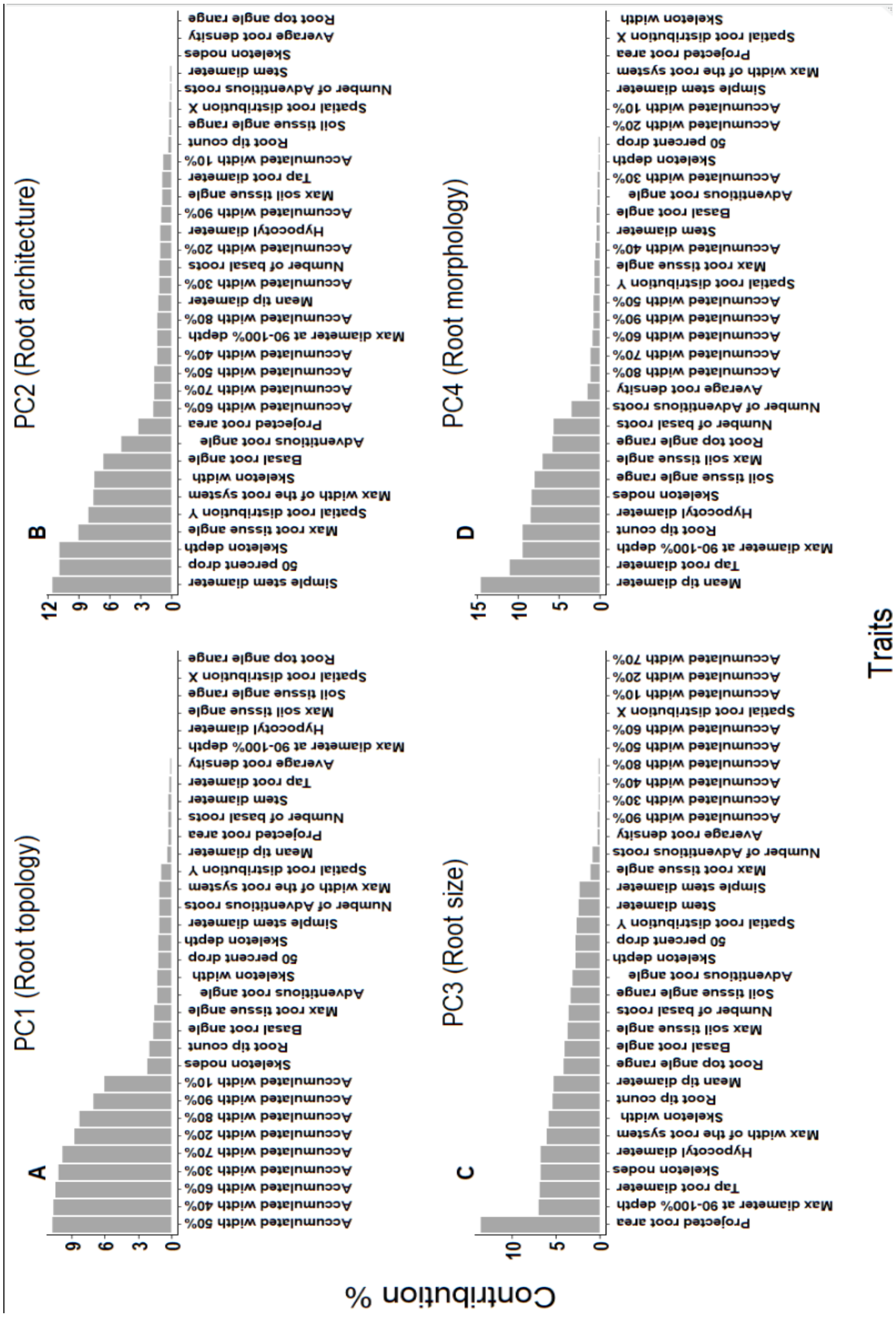

Figure 3-3 Bar graphs demonstrating the contribution of individual root traits to the first four PC's (A-D) after removing Block effects. We refer to these four PC's as topology (PC1), root architecture (PC2), root size (PC3) and root morphology (PC4). Individual traits that contribute to each modular root trait are defined in Table S1.

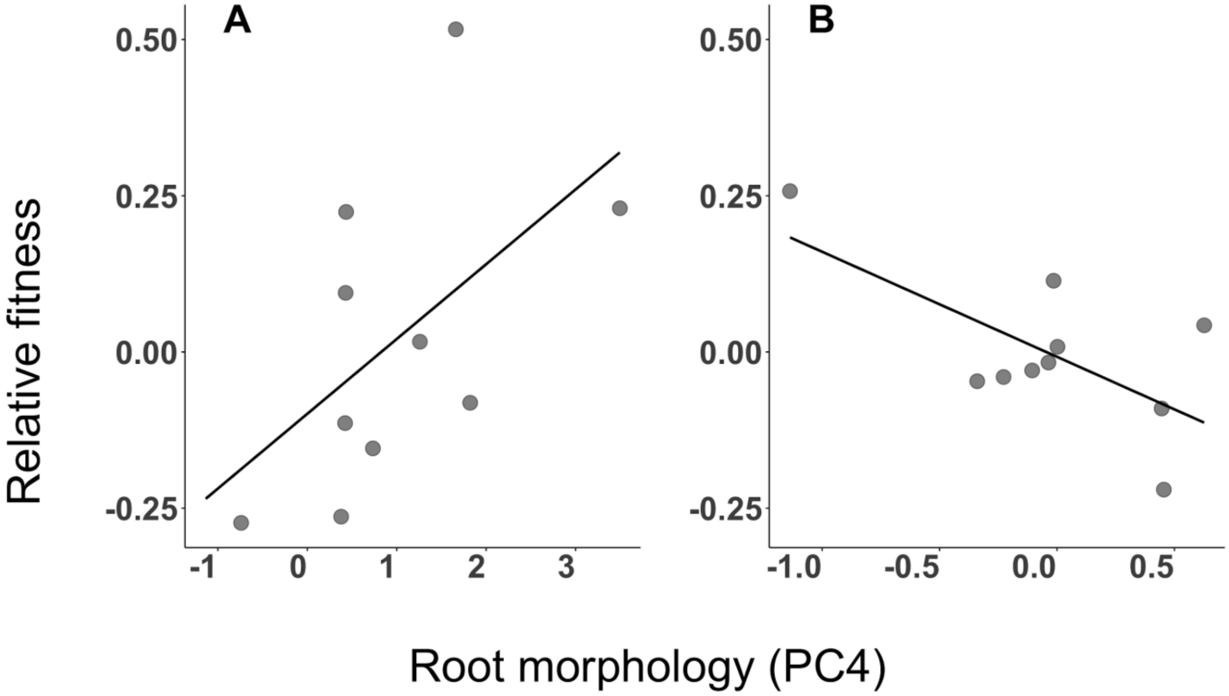

Figure 3-4 Linear regression of root morphology onto relative fitness by treatment. Root morphology was mean standardized and averaged by maternal line and treatment. There is nonsignificant positive selection on root morphology when *I. purpurea* is in the absence of competition (A) ($\beta = 0.12$, *p-value* = 0.10), and a significant negative selection (B) ($\beta = -0.17$, *p-value* = 0.03) when *I. purpurea* is in competition with *I. hederacea*. ANCOVA showed that the Treatment × root morphology is significant ($F_{1,16} = 5.33$, *p-value* = 0.03; Table S4), indicating that competition influences the pattern selection on root morphology as a modular trait.

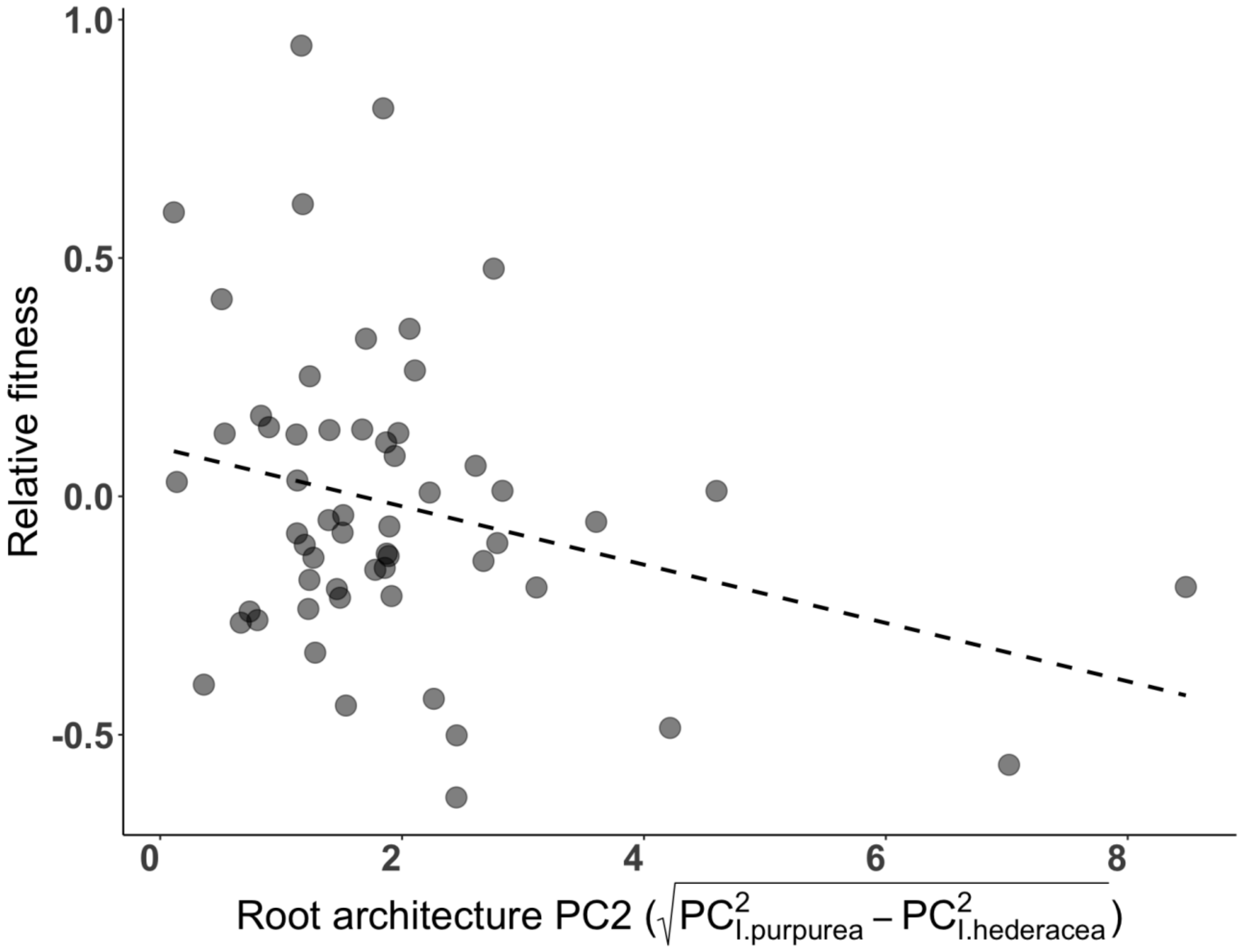

Figure 3-5 Negative relationship (β = -0.06 ± 0.03, *p-value* = 0.04; Table 4) between phenotypic distance of root architecture (PC2) and standardized relative fitness for *I. purpurea* when in competition with *I. hederacea*. The phenotypic distance of root architecture was calculated as the Euclidean distance in PC2 between competing pairs of *I. purpurea* and *I. hederacea* after the removal of Block effects, and then averaged by maternal line and species by maternal line combination type. Each point represents two to eight biological replicates.

	F-statistics		χ^2
Trait	Block DF = 3	Treatment DF = 1	Maternal Line DF = 1
Root topology (PC1)	1660.90 (2e-16)	0.77 (.38)	-6.82e-13 (.99)
Root architecture (PC2)	23.66 (1.51e-13)	1.42 (.23)	2.22 (.14)
Root size (PC3)	5.04 (.002)	2.32 (.13)	0.48 (.49)
Root morphology (PC4)	5.50 (0.001)	0.08 (.78)	**6.31** **(.01)**

Table 3-1 Linear mixed model results for the modular root system traits obtained from the first four principal components within *I. purpurea*. *F-statistics* and χ^2 values show the effects of Block, Treatment, and Maternal Line variation, respectively. *p-values* of fixed and random effects are reported within parentheses. Bolded values indicate *p-value* < 0.05.

Traits	Alone				Competition			
Trait description	β	SE	CI_U	CI_L	β	SE	CI_U	CI_L
Skeleton nodes (morphology)	**0.04**	**0.02**	**0.08**	**0.003**	-0.04	0.01	0.04	<0.001
Average root density (morphology)	**-0.02**	**0.01**	**-0.001**	**-0.03**	0.02	0.01	0.04	<-0.001
Mean tip diameter (morphology)	-0.05	0.03	0.001	-0.10	**0.07**	**0.02**	**0.13**	**0.02**
Root tip count (morphology)	**0.04**	**0.02**	**0.08**	**0.002**	-0.05	0.03	0.01	-0.09
Soil tissue angle range (architecture)	0.03	0.02	0.07	-0.002	**-0.05**	**0.01**	**-0.01**	**-0.09**
Maximum soil tissue angle (architecture)	0.03	0.02	0.07	-0.003	**-0.05**	**0.02**	**-0.02**	**-0.09**
Root tissue angle range (architecture)	0.03	0.02	0.06	-0.002	**-0.04**	**0.02**	**-0.01**	**-0.07**
Number of Adventitious roots (morphology)	**0.03**	**0.01**	**0.05**	**0.002**	-0.03	0.02	0.01	-0.06
Hypocotyl diameter (morphology)	-0.03	0.02	0.004	-0.07	**0.06**	**0.02**	**0.10**	**0.03**
Tap root diameter (morphology)	-0.04	0.02	0.001	-0.08	**0.06**	**0.02**	**0.11**	**0.02**
Maximum diameter at 90-100% percent depth (morphology)	-0.04	0.02	0.002	-0.08	**0.06**	**0.02**	**0.10**	**0.02**

Table 3-2 Results of PC back regression of selection gradient projected back onto original traits (β) and corresponding ± 1 standard error (SE) and upper and lower confidence intervals (CI) based on β ± 1.96 × SE. Bolded values indicate a confidence interval of 95% for β that does not include zero. Each root trait was cataloged into four functional trait classes indicated within parenthesis *a priori*.

Phenotypic distance ($\sqrt{PC_{I.purpurea}^2 - PC_{I.hederacea}^2}$)	β coefficient
Root topology (PC1)	0.03 ± 0.03 (.24)
Root architecture (PC2)	**-0.06 ± 0.06 (.03)**
Root morphology (PC4)	0.04 ± 0.07 (.62)

Table 3-3 Test for the hallmark prediction of character displacement in modular root system traits of *I. purpurea*. Phenotypic distance was calculated as the absolute Euclidean distance between competitor pairs of *I. purpurea* and *I. hederacea* for root topology (PC1), root architecture (PC2) and root morphology (PC4). Phenotypic distances were averaged by maternal Line and maternal line × species combination, and then regressed onto standardized values of relative fitness; ± 1 standard error is reported next to its corresponding linear regression slope (β coefficient). *p-values* are indicted within parentheses. Bolded values indicate a *p-value* < 0.05.

Chapter 4. Exploring Links Between the Rhizosphere Microbiome, Root Traits and Plant Fitness in Light of Belowground Competition

Abstract

Understanding if and how root phenotypes and the rhizosphere microbiome can influence each other and alter plant responses to belowground competition remains an important and elusive challenge in evolutionary ecology. Abundant research, however, has begun to indicate that plants may potentially shape the rhizosphere microbiome via their root traits and vice versa. Further, disparate lines of evidence suggest that specific root traits and the rhizosphere microbiome can play an important role in modulating belowground competition between plants. Research explicitly linking root phenotypes to variation in the rhizosphere microbiome, however, are limited. Moreover, research testing whether root phenotypes and rhizosphere community structure and composition alter plant fitness according to competitive environment, are lacking. To address this gap we asked, Does the rhizosphere bacterial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment? We used rhizosphere soil samples taken from our focal species, *Ipomoea purpurea*, that was grown in the presence and absence of competition with a closely related competitor, *I. hederacea*. We found evidence for linear associations between root traits and the rhizosphere microbiome, providing initial evidence that root phenotypes and the rhizosphere microbiome may influence each other. Further, our work uncovered a significant interaction effect between competitive environment and bacterial species richness on plant fitness. More specifically, we found that an increase in bacterial richness was associated with an increase in plant fitness when plants are grown in the presence of competition but found no evidence of a relationship in the absence of competition. Thus, this result indicates that rhizosphere bacterial richness may have a direct and positive impact on how *I. purpurea* competes for belowground competition. We discuss the ecological and evolutionary implications of our results and how future work can help uncover the underlying mechanisms behind our findings.

Introduction

A major and unresolved challenge in plant evolutionary ecology is understanding the relative role that plant-microbe interactions may play in the feedbacks between plant ecology and evolution. Evidence is accumulating that plant community structure and composition are driven by complex interactions between plant functional traits, the associated microbial communities of plants and environmental conditions (*e.g.,* soil quality, nutrient stress, or competitive interactions; Reynolds et al. 2014; Bever et al. 2012; Bardgett et al. 2014; Fitzpatrick et al. 2018). Recent research has indicated that belowground root traits may play an important role in shaping the root microbiome (Saleem et al. 2018), by significantly altering soil biophysical and edaphic properties (*e.g.,* aggregation, structure, pH and moisture). As a result, variation in root traits may promote variation in rhizosphere community structure and phenotypic differences in root traits may potentially lead to greater differentiation in the rhizosphere community between plants, *i.e.,* influence community composition. Further, recent research demonstrates that interactions between plant phenotypes and the microbial community can alter plant fitness linked traits (*e.g.,* flowering phenology) and therefore potentially alter plant evolution (Lau and Lennon 2011; Wagner et al. 2014; Panke-Buisse et al. 2015; *discussed in* Rebolleda-Gómez et al. 2019; Chaney and Baucom 2020). Despite the ecological and evolutionary implications of root-microbe interactions for plant systems, much of the published work examines root phenotypes of crop species (Roeland et al. 2012) and does not consider the interaction of root phenotypes and plant-plant competition, an important and ubiquitous agent of plant stress. Furthermore, evidence demonstrating that plant phenotypes can influence the root microbiome, and research reconciling the additive and synergistic effects of root phenotypes and the soil microbial community to feedback into belowground plant-plant competition (belowground competition hereafter) are lacking.

Due to its primary function in acquiring essential nutrients and water from the soil environment, a plant's root system plays a pivotal role in mediating competition for limiting resources belowground. The root system is a complex multicellular organ composed of many traits that can be broadly classified into four functional groups including traits that capture the spatial distribution of the root system, or root architecture (*e.g.,* angle formed between roots, root width and length) and traits that describe specific characteristics of individual roots, or root morphology (*e.g.,* root diameter, lateral root number). The root system can also be more coarsely described based on its overall size, including traits such as root surface area and/or biomass/volume and general shape, or topology (*e.g.,* root system width with soil depth). Because traits linked to each of these functional groups tend to behave in an integrated manner, their accumulated effects can therefore impact the resources that are readily available to plants and the extent to which plants can exploit and compete for them (*e.g.,* uptake efficiency; Lynch 1995; York et al. 2013). In recent work, we demonstrated that belowground competition can potentially influence the evolution of root traits and therefore may play an overlooked role in driving patterns of plant diversity and distributions (Colom and Baucom, 2020; Colom and Baucom In Prep). In our past work, however, we did not consider whether the microbial community in the root-soil interface (rhizosphere microbiome hereafter) was associated with root phenotypes nor did we test for evidence that the rhizosphere microbiome could alter plant fitness according to competitive environment.

The rhizosphere microbiome may influence plant function via facilitating plant nutrient uptake, stimulating plant growth, increasing tolerance to stressful environments and protecting against pathogens (Grichko and Glick 2001; Mayak et al. 2004; van der Heijden et al. 2008; Upadhyay et al. 2009; Verbon and Liberman, 2016; Jacoby et al. 2017; Kwak et al. 2018). In addition, the rhizosphere microbiome can also elicit phenotypic plasticity of root traits, potentially influencing root function (*discussed in* Friesen et al. 2011). Consequently, the rhizosphere microbiome may directly impact belowground competition by altering a plant's extended niche (*i.e.,* microbes may mediate resource partitioning between plants; *reviewed in* Reynolds et al. 2002 and Bever et al. 2010) and/or indirectly by modulating root phenotypes. Root traits, however, can also influence the rhizosphere microbiome indirectly through their effects on the immediate soil environment or directly through carbon turnover of root biomass (Stres et al. 2008; Bach et al. 2010; Brockett et al 2012; Peralta et al. 2013; Wang et al. 2013; Spohn et al. 2014; Van Horn et al. 2014; Yan et al. 2015; Erktan et al. 2018). Therefore, root traits and the rhizosphere microbiome may impose additive and/or synergistic effects on plant fitness and function. As a result, this may have important consequences on how plants compete for resources belowground and may potentially alter phenotypic selection on plant traits, linking ecology and evolution. However, research examining if and how root phenotypes and the rhizosphere microbiome can potentially influence each other is limited. Furthermore, research taking into account both root traits and variation in the microbial community structure and composition of the rhizosphere as important predictors of plant fitness in the context of competitive environment remains unexplored. Here, as a first step, we addressed the broad question, Does rhizosphere bacterial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment?

Here, we extended our previous analysis of belowground competition (Colom and Baucom in Prep) to that of the rhizosphere microbiome. We used rhizosphere soil samples taken from our focal plant species, *Ipomoea purpurea,* grown in the presence and absence of competition from its sister species, *I. hederacea,* and asked two main questions: (1) Does the rhizosphere microbiome vary with phenotypic variation in root traits? (2) Does plant fitness vary as a function of root trait and rhizosphere bacterial community structure and/or composition, according to competitive environment (presence vs absence of belowground competition)? Addressing the first question would provide initial evidence that root phenotypes and the rhizosphere microbiome may influence each other, whereas addressing the second question would provide preliminary evidence for the potential for root phenotypes and/or the rhizosphere microbiome to influence plant fitness in context of belowground competition. Together, answering these main questions would provide evidence for the potential that the structure of plant roots and their rhizosphere microbiome may feedback into competitive belowground dynamics.

Materials and methods

Field experiment, rhizosphere soil collection--We subsampled rhizosphere soil from individuals of *I. purpurea* and *I. hederacea* planted in the presence and absence of competition, with *I. purpurea* as the focal species in this experiment. For the competition treatment, we planted ten maternal lines of *I. purpurea* with six maternal lines of *I. hederacea*, for each possible maternal line by maternal line combination between species, which led to 60 unique competition pairings. We planted seeds 8 cm apart with 1 m^2 between experimental units. For the alone treatment, we planted a single replicate seed of the ten maternal lines of *I. purpurea* 1 m^2 apart. Each experimental unit was replicated sixteen times to yield a total of 2080 seeds. Seven weeks post planting, when plants began to show signs of reproductive maturity, we excavated a subset of individuals to quantify root system traits (Colom and Baucom, In Prep; **Chapter 3**). We sampled the rhizosphere soil from 173 plants; 27 plants grown alone and 146 plants grown in competition. We randomly selected between 2 and 4 biological replicates of each *I. purpurea* maternal line grown alone, and between 5-12 biological replicates of each *I. purpurea* maternal line grown in competition, with the exception of one maternal line that had only one biological replicate. To isolate the rhizosphere soil from plant roots, we gently shook the roots from the soil cores of excavated plants to remove loose soil, sampled a random lateral root with small pieces of soil (~25mg) attached to its immediate surface (~1mm) with a 15mL sterile plastic tube, separated it from the rest of the root system with a razor that was cleaned with 90% ethanol, stored the tube immediately on dry ice, and later transferred all tubes to a -80C freezer until further use.

DNA extraction and processing--We extracted DNA from approximately 0.25g of rhizosphere soil per plant per standard procedures of the DNeasy PowerSoil Kit (QIAGEN, Hilden Germany), and then randomized 1uL of the DNA samples across two 96 well plates. The bacterial V4 region of the 16S rRNA gene was amplified and barcoded at the University of Michigan Medical School, and pooled libraries were sequenced on Illumina MiSeq sequencer, using v2 chemistry 2 × 250 (500 cycles) paired-end reads. Sequence quality processing was performed with mothur v1.43.0 using the MiSeq standard operating protocol (accessed on 31 October 2019) for the generation of the operational taxonomic unit (OTU 97% sequence similarity). For sequence alignment and classification, we used the SILVA release taxonomy (v132, Quast et al 2013; accessed August 2019), and only bacterial sequences were retained.

Statistical analysis

All analyses were carried out in the statistical programming language R (R Core, 2019).

Calculation of rhizosphere microbiome community composition and structure--We aggregated our total OTU's (52,565) at the genus taxonomic level to reduce patchiness in our data with the 'tax_glom' function of the 'phyloseq' package (McMurdie and Holmes, 2013) which produced a total of 1,097 OTUs. We examined the distribution of sequencing depths of all our samples and filtered out extreme outliers (< 20,000 read counts) for all subsequent analyses. Sequence counts were used to compute different metrics of community composition including evenness, richness, Simpson diversity and Inverse Simpson diversity. Evenness quantifies how evenly represented different Bacterial taxa are as a proportion ranging from 0 to 1, where a value of 0 indicates lack of evenness in the community and towards 1 indicates a more even community. Richness is the total number of unique Bacterial taxa. Simpson diversity is a measure of community diversity that accounts for both species richness and their relative abundance. A Simpson Diversity measure of 0 indicates no diversity and increasing values indicates higher diversity within a given community. We also estimated the 'effective' species diversity (Inverse Simpson Diversity), a measure based on the Simpson Diversity at an order of 2 because it quantifies the effective number of different Bacterial taxa, wherein the weighted arithmetic mean is used to quantify average proportional abundance of types in the community. In practice, the Simpson Diversity Index can be used to measure the probability that two samples taken at random from the dataset represent the same taxon, whereas the Inverse Simpson Index can inform us the number of unique species weighted by their relative abundance. To estimate these metrics of ɑ-diversity, we used the function 'estimate_richness' from the phyloseq package (McMurdie and Holmes, 2013), and specified the 'measures' argument for the corresponding metrics above. For each metric of ɑ-diversity we rarified to the number of sequences in the smallest sample. Then we normalized our sequences based on OTU read count data scaled to the smallest library size (Denef et al. 2017) and used the scaled data to compute community composition with the Bray–Curtis dissimilarity inter-community metric with phyloseq's 'ordinate' function (McMurdie and Holmes, 2013).

Characterizing sources of variation in bacterial community composition and structure—We first examined how metrics of species structure and composition varied as a function of block, treatment and maternal line from rhizosphere microbiome collected from our focal species, *I. purpurea*. Preliminary histogram plots of bacterial species richness, evenness, Simpson Diversity, and Inverse Simpson Diversity showed normal distributions, hence, we elected to perform linear mixed model ANOVAs to test for these effects on our metrics of ɑ-diversity. We performed separate ANOVAs with the 'lmer' function from the lmerTest package (Kuznetsova et al. 2017), where we treated each ɑ-diversity metric as a response variable and included treatment and block as fixed effects and maternal line as a random effect. We excluded the interaction term between treatment and block because preliminary analysis did not show that these explained a significant portion of variation, nor did it improve the Akaike Information Criterion (AIC) of the model. Further, because none of our linear mixed models uncovered a significant maternal line effect on α-diversity, we excluded this term in our final model and report the results of the two-way ANOVA, α-diversity ~ Treatment + Block.

We performed a permutational ANOVA (PERMANOVA) to examine effects of block, treatment and maternal line on community composition using the 'adonis' function of the 'vegan' package (Oksanen *et al.* 2019) with default parameters and used 999 × permutations to access the significance of these variables for *I. purpurea* only. For this test, we treated community composition as our response variable and treatment, block and maternal line as fixed effects. Because preliminary analysis showed that maternal line did not explain a significant amount of variation in community composition, we excluded this term from our final model and report the results of the model, β -diversity ~ Treatment + Block.

Does bacterial community composition and structure vary with root traits? To examine if and how different metrics of the rhizosphere microbiome community composition and structure are associated with phenotypic variation in root traits, we performed separate linear regressions for root architecture, size, topology and morphology, onto each metric of α-diversity. We elected to focus on root architecture, size, topology and morphology because these traits can have direct impact on soil structure and plant resource uptake (Fitter 1987; Lynch 1995). To obtain our root traits, we applied multivariate statistics that transformed 33 root traits previously quantified from our experimental plants (Colom and Baucom, In Prep), into four modular traits. Specifically, we applied a Box-Cox transformation to all 33 root traits to normalize their distributions and standardized them by subtracting the mean and dividing by their standard deviations. Then we applied a PCA to their correlation matrix and elected to use the first four principal components (PCs) as our four modular traits because they each captured at least 10% of the total phenotypic variation each. We found that the first four PCs served as four important indicators of the root system: topology (PC1), or traits that describe the overall shape of the root system, architecture (PC2), or traits that capture the spatial arrangement of the root system (*e.g.,* different root tissue angle measurements, horizontal/vertical root distribution), size (PC3) (*e.g.,* root area) and morphology (PC4), or traits related to the individual characteristics of the root system (*e.g.,* root diameter estimates, basal root number and adventitious root number).

Briefly, a greater value of root topology (PC1), corresponds to a root system that exhibits a larger root width with a concomitant increase in soil depth. A greater value of root architecture (PC2) corresponds to a root system that grows more narrowly near the soil surface with an increase in the maximum root tissue angle. A greater value of root size (PC3) describes a root system that has a larger root surface area, and greater values of root morphology (PC4) correspond to a root system that has multiple lateral roots and smaller lateral root diameter and exhibits a greater range in the rooting angles relative to the soil surface and to the tap root. More details about how specific root traits contributed to each PC can be found in Chapter 3, *Describing the root system as modular root traits*).

To analyze the relationship between measures of bacterial community structure and phenotypic variation of root traits we performed separate linear regression analyses of root topology, architecture, size and morphology. We used our estimates of ɑ-diversity (*i.e.,* species richness and evenness and Simpson index, and Inverse Simpson index) as our predictor variables, and included treatment and block as covariates in all models. If we uncovered a significant linear relationship between a given root trait on ɑ-diversity, we also performed an ANCOVA using the ‘Anova’ function from the ‘car’ package (Fox and Weisberg, 2019) that included the interaction term of treatment by root trait. A significant root trait by treatment interaction would provide evidence that the competitive environment alters the relationship between a given root trait and measure of ɑ-diversity. We used *F-statistics* and Type III Sums of Squares to determine the statistical significance of fixed effects in the ANCOVAs.

Because root traits can significantly alter their immediate soil environment, we reasoned that greater phenotypic differentiation in root traits between plants could potentially promote greater differences in their corresponding rhizosphere communities. Accordingly, we evaluated whether greater phenotypic distance for a given root trait between individuals, was linearly linked to greater dissimilarity in their rhizosphere community composition. We used a Mantel test to evaluate evidence of a linear relationship between root phenotypes and community composition. For this analysis we calculated the Euclidean distance of root topology, architecture, size and morphology-- *i.e.*, 'phenotypic distances'--between all plant samples (*i.e.*, across treatment and species), and then regressed each phenotypic distance onto the untransformed Bray-Curtis dissimilarity matrix with the 'Mantel' function from the vegan package (Oksanen *et al.* 2019) with the Spearman correlation method and 999 permutations. Because our PERMANOVA above did not uncover significant treatment effects on community composition (Table 4-2), we ran this test across treatment within *I. purpurea* (Table 4-4).

Testing the effects of root traits and bacterial diversity measures on plant fitness– We performed an ANCOVA to evaluate whether root traits and/or measures of bacterial diversity have direct effects on the fitness of *I. purpurea* according to treatment. A model that includes all root traits and metrics of ɑ-diversity controls for their correlations and provides us with an estimate of their direct linear effect on plant fitness, respectively. To estimate relative fitness, we used values of observed seed number collected from *I. purpurea* plants that were maintained until senescence (Colom and Baucom, In Prep), and divided this by the mean seed number by treatment. Then we averaged the relative finesses by treatment, block and maternal line. Before analysis, we scaled our measures of ɑ-diversity to a mean of zero and standard deviation of one. We fit a linear model that included treatment, block, root traits and standardized measures of bacterial species evenness, richness and Inverse Simpson Diversity and each of their two-way interactions with treatment and block as explanatory fixed effects (Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block; Supplementary Information Table S3-1 for full model details). We did not include three-way interactions between treatment, block and root traits or between treatment, block and root traits and ɑ-diversity due to our limited sample size within block and treatment. Further, we excluded Simpson Diversity from this analysis as a predictor variable because it is strongly correlated to Inverse Simpson Diversity (r = 0.92, *p-value* < 0.001). We simplified our full model by doing a backwards model selection approach using the ‘stepAIC’ function from the MASS package (Venables and Ripley 2002) and retained the model with the lowest AIC (Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block; Table S3-1). We used *F-tests* with Type III Sums of Squares to evaluate the significance of interaction terms using the ‘Anova’ function from the ‘Car’ package (Fox and Weisburg, 2019). A significant root trait by treatment or ɑ-diversity by treatment term would provide evidence that belowground competition alters the direct effects of root trait or ɑ-diversity on plant fitness, respectively. Likewise, a significant root trait by treatment or ɑ-diversity by treatment term would provide evidence that the competitive environment influences the direct effects of root trait or ɑ-diversity on plant fitness, respectively.

Since our ANCOVA showed evidence of a significant treatment by richness interaction effect on relative fitness (see *Evidence of linear relationships between root traits and bacterial diversity* below), we performed a linear regression of relative fitness as a function of nontransformed richness values for each treatment separately. We used *t-tests* to assess the significance of the linear relationship.

Testing the effects of root traits and bacterial community composition on plant fitness–To evaluate whether relative fitness of *I. purpurea* varies with its rhizosphere bacterial community composition) and/or root traits, according to treatment, we performed a series of Mantel partial regressions. For each root trait we correlated the Bray-Curtis Dissimilarity matrix as a predictor variable and a vector of the Euclidean distances of a given root trait as a covariate (*i.e.*, ‘phenotypic distances’) onto a vector of the Euclidean distances of relative fitness of *I. purpurea*, for each treatment, separately. As above, analyzing community composition and root traits in the same model allow us to control for correlations between root traits and community composition and estimate their direct effects on plant fitness.

Results

Main effects of bacterial community composition and structure--ANOVAs demonstrated that block was the biggest source of variation in α-diversity metrics when examined across treatments (Richness: *F-value*$_{3,167}$ = 5.71, *p-value* < 0.01; Inverse Simpson: *F-value*$_{3,167}$ = 4.23, *p-value* < 0.01; Simpson: *F-value*$_{3,95}$ = 2.86, *p-value* = 0.03; Evenness: *F-value*$_{3,95}$ = 4.77, *p-value* < 0.001; Table 4-1). Likewise, PERMANOVAs showed that block explained the biggest proportion of variation in species composition (*F-value*$_{3,95}$ = 3.48, *p-value* < 0.001; Table 4-2). Competition treatment did not explain a significant proportion of the variation in α-diversity metrics (Table 4-1) or species composition (Table 4-2). Together, these results show that the immediate soil environment is the main driver underlying the community composition and structure of the rhizosphere microbiome.

Evidence of linear relationships between root traits and bacterial diversity—We uncovered a significant negative linear relationship between root architecture and bacterial species richness (R^2 = 0.18, B = -5.54 ± 2.24, *p-value* = 0.02; Table 4-3, Figure 4-1A), and significant positive relationships between root architecture and species evenness (R^2 = 0.12, B = 7.29 e-05 ± 3.28 e-05, *p-value* = 0.03; Table 4-3, Figure 4-1B) and root morphology and Inverse-Simpson diversity (R^2 = 0.10, B = 2.08 ± 1.05, *p-value* = 0.053; Table 4-3, Figure 4-2). We also uncovered a significant positive relationship between root morphology and Simpson diversity (R^2 = 0.14, B = 2.41 e-03 ± 8.98 e-03, *p-value* < 0.01), however, visual inspection revealed an outlier (Simpson diversity = 0.94) that was driving the linear trend between these two variables, and after removing the point the relationship was no longer significant (R^2 = 0.01, B = 0.001 ± 0.001, *p-value* 0.42; Table 4-3). These results provide evidence that an increase in traits associated with root architecture (*e.g.*, the maximum root tissue angle, basal root angle, root system width and root system length) is linked to a reduction in bacterial richness, and that an increase in these traits likewise is linked to increased bacterial evenness in the rhizosphere. Further, it shows that an increase in traits associated with root morphology, including traits such as root diameter, basal root number and adventitious root number, is linked to an increase in the Inverse-Simpson diversity. Interestingly, we uncovered no evidence that the presence of a competitor changed the direction of the relationship between root architecture and species richness and evenness or root morphology and Inverse Simpson diversity.

We conducted Mantel tests to examine the potential for a linear relationship between Bray-Curtis Dissimilarity matrix, *i.e.*, community composition, and phenotypic distance in root topology, architecture, size and morphology. We did not find evidence of significant correlations between phenotypic distances of these root traits and community composition. However, we found a weak and marginally significant correlation between root architecture and community composition ($r^2 = 0.07$, *p-value* = 0.07; Table 4-4). These results indicate that while differences in root topology, size and morphology are not linearly associated with greater differences in the bacterial community composition, root architecture may be.

Bacterial community structure effects plant fitness according to treatment--We found evidence for a treatment by bacterial species richness interaction effect ($F\text{-}value_{1,57} = 7.70$, *p-value* = 0.01; Table 4-5) on fitness, indicating that the bacterial community composition plays a significant role in the outcome of competition in *I. purpurea*. We found a significant positive relationship in the presence of competition between relative fitness and bacterial species richness ($\beta = 0.003 \pm 0.001$, *p-value* = 0.03; Figure 4-3), but no evidence of a relationship in the absence of competition ($\beta = 0.004 \pm 0.003$, *p-value* = 0.25; Figure 4-3). This suggests that an increase in bacterial species richness may have a positive effect on plant fitness when *I. purpurea* is grown in the presence of competition, but there is no evidence for such an effect when *I. purpurea* is grown in the absence of competition. Furthermore, we found evidence of an interaction between block and root topology ($F\text{-}value_{3,57} = 5.02$, *p-value* = 0.04; Table 4-5) and root morphology ($F\text{-}value_{3,57} = 6.14$, *p-value* = 0.001; Table 4-5), indicating that that the direct effects of root topology and morphology on plant fitness depend on environmental context. We likewise found a significant interaction between block and bacterial species richness ($F\text{-}value_{3,57}$ = 2.73, *p-value* = 0.05; Table 4-5), and evenness ($F\text{-}value_{3,57} = 2.69$, *p-value* = 0.05; Table 4-5) indicating that the direct effects the rhizosphere community structure on plant fitness depend on environmental context.

We found no evidence of direct effects of root traits and measures of bacterial community composition, or β-Diversity, on relative fitness between treatments (results not shown).

Discussion

The main goal of our work was to evaluate the potential for a relationship between modular root traits and the rhizosphere microbiome and to determine the relative impact of the rhizosphere bacterial community on plant fitness in context of belowground competition. Our findings reveal that multiple metrics of α-diversity (bacterial richness, evenness and Inverse Simpson diversity) were linearly associated with different root traits, and that bacterial species richness may play an important role in belowground competition, as indicated by evidence for a significant two-way interaction effect between bacterial richness and competitive treatment on the relative fitness of *I. purpurea*. We also found a significant influence of block on the rhizosphere microbiome community composition and structure, but no evidence for an effect of competition, indicating that the community structure and composition of the rhizosphere microbiome in this species is influenced largely by the environment. Below we expand on the interpretation of our main findings and discuss their eco-evolutionary implications and directions for future research

Associations between root traits and the rhizosphere microbiome

The belowground root system of plants can play a major role in altering the physical and chemical profile of its surrounding soil environment (Orwin et al. 2010; Bodner et al. 2014) and therefore may serve as a passive filter of the bacterial community assemblage in the rhizosphere. For instance, lateral root type, seminal or nodal roots, has been found to influence the composition of rhizosphere bacterial communities in *Brachypodium* (Kawasaki et al., 2016). In turn, microbes residing in the rhizosphere can alter phenotypic plasticity of root traits by producing growth stimulating molecules and/or altering the chemical profile of the soil environment (*discussed in* Friesen et al. 2011). As such, we reasoned that root traits and the rhizosphere microbiome community are likely to influence each other, which may potentially impact downstream effects on plant function and fitness. In line with this broad expectation, we found a significant positive linear relationship between root architecture – a modular trait that captures the spatial arrangement of the root system – and bacterial evenness, and likewise a negative linear relationship between root architecture and bacterial richness. These results suggest that narrower, but deeper-growing root systems (*i.e.,* increased values of 'root architecture'), are linked to a decrease in the presence of rare bacterial taxa (and vice versa). This would explain the simultaneous increase in bacterial evenness and decrease in bacterial richness with an increase in a more narrow/deep root system. Consistent with these results, we also found evidence of linear relationships between community composition and root traits, with a marginally significant positive correlation between community composition and root architecture, suggesting that specific root architectures in *I. purpurea* may play a role in differentiating the rhizosphere community between plants.

While we have identified these relationships between root architecture and bacterial richness and evenness, we have yet to test their mechanism. One plausible explanation for these findings is that root architecture influences its rhizosphere microbiome indirectly by altering soil moisture and/or access to nutrients, since root architecture can impact mineral aggregation and water flow in the soil (*reviewed in* Ghestem et al. 2011). Regardless of mechanism, our result that rhizosphere microbiome diversity varies with root architecture is in line with research from other plants, where research has uncovered associations between root system architecture traits and variation in rhizosphere bacterial communities (Szoboszlay et al. 2015; *discussed in* Saleem et al. 2018). For example, one study that compared the root system architecture and rhizosphere bacterial community of *Balsas teosinte* (progenitor of maize, *Zea mays* subsp. *Parviglumis*) and two domesticated corn cultivars, showed concurrent differences in rooting length and rhizosphere bacterial richness, composition and structure (Szoboszlay et al. 2015). In addition to bacterial community associations with architecture traits, we also found a significant positive relationship between Inverse Simpson Diversity and root morphology, indicating that root systems with an increase in lateral root number and decrease in overall root diameter (*i.e.,* thinner roots), support an increase in bacterial richness and relative abundance in the rhizosphere (and vice versa). This result may possibly reflect an increase in bacterial diversity through an increase in the available source of organic carbon in the soil from root litter (*discussed in* Reeder et al. 2001; Wardle et al. 2004; Bardgett et al. 2014), since thinner roots tend to have higher turn-over rates.

Consistent with this hypothesis, multiple studies have shown that root derived sources of carbon can alter soil bacterial community composition and structure (*discussed in* Reeder et al. 2001; Allison et al. 2006; Steenwerth et al. 2007) and some studies have reported positive associations between the abundance of particular bacteria (*e.g.*, Bacteroidetes) to thin root phenotypes in wild accessions of bean (Brown et al. 2012; Filippo et al. 2010; Pérez Jaramillo et al. 2017). These associations between root traits and rhizosphere bacterial communities, however, could also be due (at least partially) to rhizosphere linked microbes eliciting phenotypic plasticity of root architecture and/or morphology. For instance, many microbial taxa have been shown to influence root system architecture and morphological traits by synthesizing molecules that modulate the auxin pathway, *e.g.*, the production of phytohormones enhancing lateral root branching by plant growth promoting rhizobacteria (*reviewed in* Ortíz-Castro et al. 2009, Vacheron et al. 2013 and Sukumar et al. 2013; Bailly et al. 2014). Further, these patterns are also likely driven to some extent by microenvironmental changes in soil conditions because it can trigger both phenotypic plasticity of root traits and alter microbial niches and influence microbial communities (Bonser et al. 1996; Hodge 2004; Gruber et al. 2013; Tian et al. 2014; Yu et al. 2014; Bach et al. 2010; Brockett et al. 2012; Zhalnina et al. 2015).

Evidence for the potential of the rhizosphere microbiome to impact belowground competition

Given that the primary role of root traits is to acquire nutrients and water from the soil environment, and that the rhizosphere microbiome can strongly influence the bioavailability of key resources and thus plant fitness, we hypothesized that root traits and/or bacterial diversity may influence how plants respond to the stress of competition. We found that bacterial species richness had a significant positive linear relationship with plant fitness in the presence of competition, but no relationship in the absence of competition, suggesting that an increase rhizosphere species richness improves *I. purpurea*'s fitness when in competition. Thus, while belowground competition negatively impacts *I. purpurea*'s fitness (Colom and Baucom 2020; results in Chapter 3), our findings perhaps indicate that bacterial richness can ameliorate the negative effects of plant competition. However, we also identified a relationship between rhizosphere diversity metrics and root phenotypes, meaning that the effect on fitness we have identified here could simply be due to the effect of root phenotypic traits on plant fitness. To delineate the importance of the root phenotype versus metrics of rhizosphere diversity on plant fitness, we would need to assess the fitness of plants with different root architectures while experimentally altering **both** the bacterial diversity in the soil and the competitive environment.

If bacterial richness does indeed influence plant fitness while in competition, one possible explanation is that the bacterial community may lead to an increase in the bioavailability of essential nutrients *via* an increase in bacterial functional richness. For instance, Singh and others (2015) performed a controlled greenhouse experiment where they grew *Ocimum sanctum* (basil) plants in potting soil that was inoculated with different levels of bacterial species diversity and richness and found that richness was an important predictor of increased plant biomass. Further, they found that the functional group richness of bacterial species was positively associated with plant biomass, suggesting the potential for increase in rhizosphere bacterial richness to promote plant growth via an increase in bacterial function. While research examining the influence of both root traits and the rhizosphere microbiome on plant fitness remains scarce, multiple studies have shown that altering the soil microbial community can alter plant performance according to competitive environment (Callaway et al. 2004; Lankau 2010; *discussed in* Bever et al. 2010; Larios et al. 2015), highlighting the importance of plant-microbial interactions to influence belowground competition.

Conclusion

Understanding how root traits and their associated microbial communities may influence belowground competition and feedback into plant ecology and evolution is an elusive challenge in evolutionary ecology. As a first step, we demonstrated here that root traits and the rhizosphere microbiome are related, providing initial evidence that root phenotypes and the rhizosphere bacterial community may influence each other. We also found evidence that an increase in bacterial species richness can have a positive impact on plant fitness when plants experience belowground competition, suggesting that the rhizosphere microbiome can potentially mitigate the harmful effects of belowground competition. Therefore, our work provides preliminary evidence that interactions between root traits and the rhizosphere bacterial community may perhaps feedback into belowground competition thus potentially alter plant ecology and evolution. We emphasize, however, that the underlying mechanisms producing many of the patterns we uncovered are yet to be determined because we did not manipulate the rhizosphere microbial community and/or root traits. Furthermore, we also found that unmeasured aspects of the environment (*i.e.*, block effects) significantly influence the rhizosphere microbiome. Therefore, future work that manipulates the rhizosphere microbiome, soil conditions and/or root traits will be essential for disentangling different ecological factors and drawing causal inferences.

While our work serves as a first step towards understanding the potential for plants and their rhizosphere microbiome to feedback into dynamics of belowground competition, we are considerably limited in that fungal organisms were not evaluated as part of the rhizosphere microbiome here. As a result, we excluded many functionally relevant species that contribute to plant resource use and fitness (Jonsson et al. 2001; Bassirad 2005; van der Heijden et al. 2006 and 2008; Jacoby et al. 2017). Thus, consideration of both bacterial and fungal species in future work will be required in order to develop a more realistic view on how root traits and the rhizosphere microbiome may potentially feed back into processes that shape plant evolution and diversity.

Data availability statement:

The R code is available at GitHub at https://github.com/SaraMColom/Microbiome_2018 and the data will and the data will be uploaded to the Dryad Digital Repository.

Acknowledgements

We graciously thank Patrick Schloss, Mark Hunter and members of the Baucom Lab for their helpful comments on earlier versions of this manuscript. We also thank Nia Johnson, Eliot Jackson and Yoav Jacob with their invaluable help collecting and processing samples in the field. This work was made possible with financial support of internal grants at the University of Michigan.

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Figures and Tables

ɑ-Diversity	Treatment *df* = 1	Block *df* = 3
Richness	0.86 (0.36)	**5.71 (<0.01)**
Inverse Simpson	<0.01 (0.95)	**4.23 (<0.01)**
Simpson	0.36 (0.55)	**2.86 (0.04)**
Evenness	0.58 (0.45)	**4.77 (<0.001)**

Table 4-1 Results from separate ANOVAs to test for Treatment effects on different alpha diversity metrics (ɑ-Diversity Metric) of the rhizosphere microbiome of *I. purpurea* (Num. *I. purpurea* in competition = 73; Num. *I. purpurea* alone = 27). *F-values* are reported with their corresponding *p-values* in parentheses. Each model[ǂ] evaluated metrics of ɑ-Diversity as response variables, and Treatment and Block as fixed effects. Values in bold indicate a significant *p-value* < 0.05.

[ǂ] *ɑ-diversity* ~ Treatment + Block

Effect	DF	SS	MeanSS	*F-value*	R^2	*p-value*
Treatment	1	0.03	0.02	0.85	0.01	0.55
Block	3	0.32	0.10	3.48	0.10	<0.001

Table 4-2 Results of PERMANOVA of Bray-Curtis distances (community composition) to test for Treatment effects on community composition of the rhizosphere microbiome of *I. purpurea* (Num. *I. purpurea* in competition = 73; Num. *I. purpurea* alone = 27). The model[ǂ] included community composition as a response variable, with Treatment and Block as fixed effects.

[ǂ] *Community composition* ~ Treatment + Block

Linear Association Between Rhizosphere Microbiome α-Diversity and Root Traits				
α-Diversity	Root topology (PC1)	Root architecture (PC2)	Root size (PC3)	Root morphology (PC4)
Inverse Simpson	1.60 ± 1.42	-0.67 ± 0.72	0.87 ± 0.86	**2.08*** **± 1.05**
Simpson	1.50 e-03 ± 1.24 e-03	-8.49 e-04 ± 6.27 e-04	-8.49 e-04 ± 6.27 e-04	**2.41 e-03**** **± 8.98 e-04**
Richness	5.74 ± 0.23	**-5.54*** **± 2.24**	3.18 ± 2.79	2.69 ± 3.54
Evenness	-5.47 e-05 ± -6.74 e-05	**7.29 e-05*** **± 3.28 e-05**	-2.22 e-05 ± 4.09 e-05	4.47 e-05 ± 5.12 e-05

Table 4-3 Results of separate linear regression between different metrics (α-Diversity Metric) of the rhizosphere microbiome (Inverse Simpson, Simpson, Richness and Evenness) and four root traits (Root topology, Root architecture, Root size and Root morphology) examined in *I. purpurea*. α-Diversity metrics were treated as response variables for each root trait, and Block and Treatment were included in the final model[ǂ] as fixed main effects. Linear regression coefficient slopes (*B*) are reported with ± 1 standard error.

p-value < 0.05 *; *p-value* <0.01 **; *p-value* <0.001***; *p-value* <0.08 ^

[ǂ] Model: α-Diversity ~ Root trait + Block +Treatment

Table 4-4 Mantel test Bray-Curtis and root phenotypes within *I. purpurea*		
Root trait	r^2	*p-value*
Root topology	-0.04	0.77
Root architecture	0.07	0.07^
Root size	0.07	0.12
Root morphology	-0.04	0.76

p-value <0.08 ^

Fixed effect	**DF**	**SS**	**F-value**	**P-value**
Treatment	1	0.006	0.10	0.48
Block	3	0.82	4.51	**0.01**
Treatment × Block	3	0.27	1.48	0.23
Root topology × Treatment	1	0.19	3.16^	0.08
Root size × Treatment	1	0.13	2.20	0.14
Richness × Treatment	1	0.46	7.70	**0.01**
Evenness × Treatment	1	0.20	3.26	0.08
Root topology × Block	3	0.91	5.06	**0.003**
Root architecture × Block	3	0.23	1.29	0.29
Root size × Block	3	0.24	1.35	0.27
Root morphology × Block	3	1.11	6.14	**0.001**
Richness × Block	3	0.49	2.73	**0.05**
Inverse Simpson diversity × Block	3	0.45	2.49	0.07
Evenness × Block	3	0.49	2.69	**0.05**

Table 4-5 Results of ANCOVA to test the effects of root traits (root topology, architecture, size and morphology, respectively), measures of alpha diversity of the rhizosphere microbial community, and their two way interactions with Treatment and Block on relative fitness of *I. purpurea* (N = 100). Degrees of Freedom (DF), sum of squares (SS) and *F-values* and corresponding *p-value* in parentheses are reported for each fixed effect. For this analysis, we extrapolated observed values of root traits and alpha diversity metrics scaled to a mean of zero and standard deviation of one, onto relative fitness of *I. purpurea* averaged by maternal line and treatment. The final model included all the Fixed Effects listed in the table regressed onto relative fitness and *F-tests* with Type III Sums of Squares were used to estimate their statistical significance. *p-value* < 0.05 *; *p-value* <0.01 **; *p-value* <0.001***; *p-value* <0.11 ^

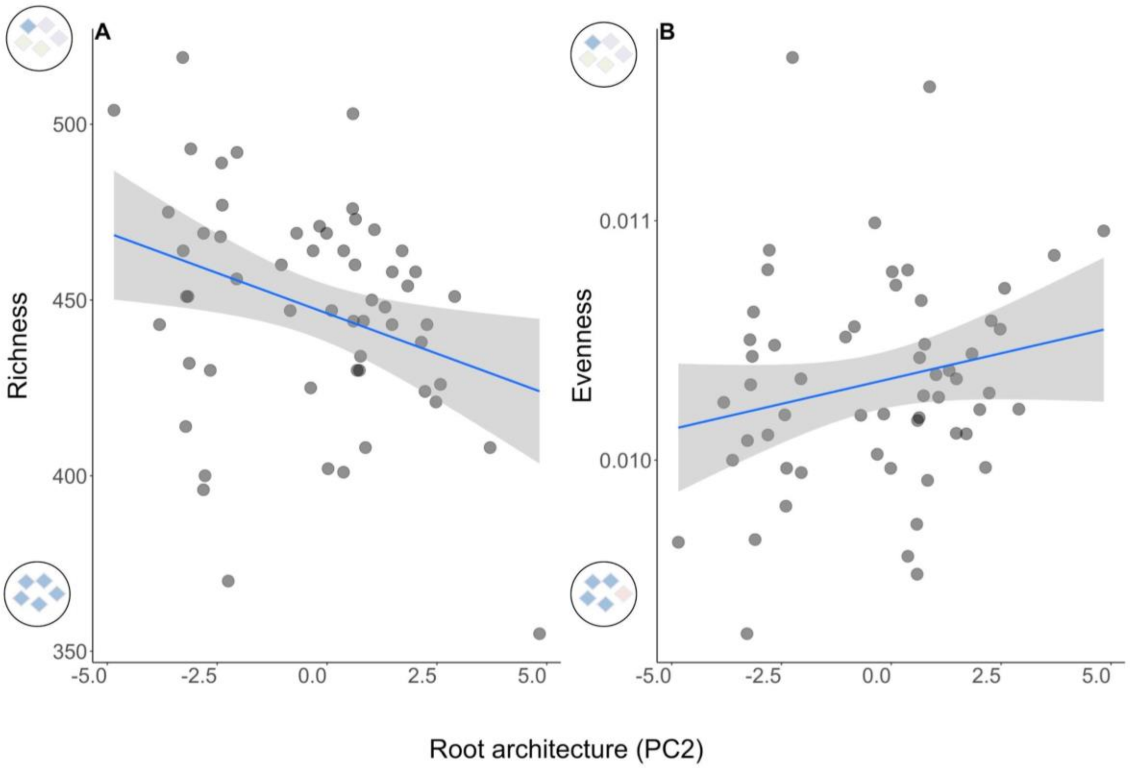

Figure 4-1 Linear regression plots between root architecture, and rhizosphere bacterial richness (A) and evenness (B). Plotted linear regression line, colored blue, and grey shading represent the linear regression slope and ± 1 standard error of the linear regression between root trait (predictor X variable) and alpha diversity metric (response Y variable). We found a significant negative relationship between root architecture as a modular trait (PC2, *i.e.*, collective increase in root tissue angle traits, horizontal/vertical distribution, root system width and root system length) and Species richness ($R^2 = 0.18$, $B = -5.54 \pm 2.24$, *p-value* = 0.02; Table 4-3), and a significant positive relationship with Species evenness ($R^2 = 0.12$, $B = 7.29$ e-05 ± 3.28 e-05, *p-value* = 0.03; Table 4-3). Near the Y-axis are schematic representations of the corresponding community composition variable according to low (bottom) and high (top) values, respectively, where each diamond represents an OTU, and the color a unique OTU.

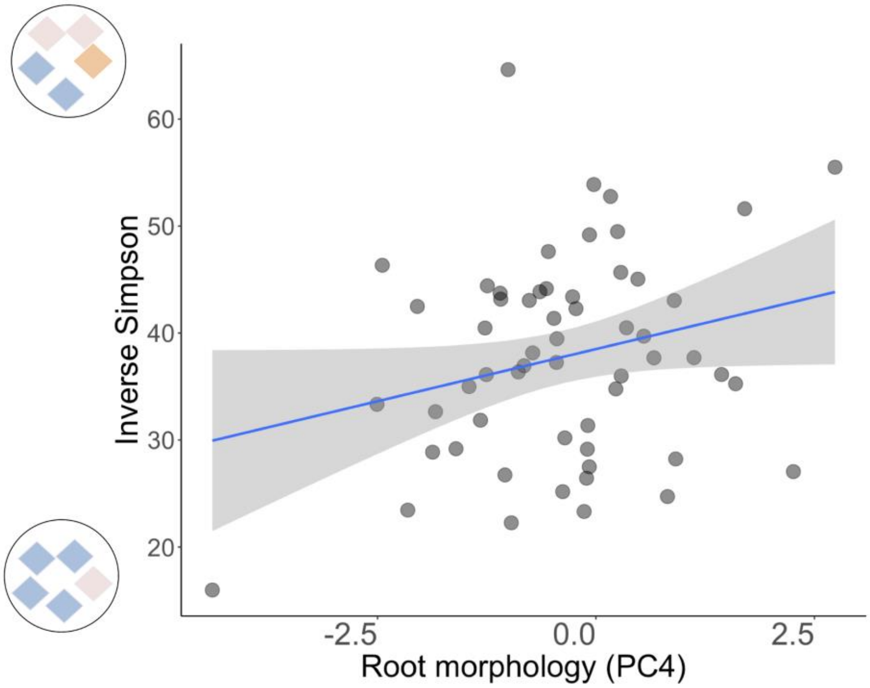

Figure 4-2 Linear regression plot between root morphology, and rhizosophere bacterial Inverse Simpson diversity indices. Plotted linear regression line, colored blue, and grey shading represent the linear regression slope and ± 1 standard error of the linear regression between root trait (predictor X variable) and alpha diversity metric (response Y variable). We found a significant positive relationship between root morphology as a modular trait (PC4, *i.e.*, collective increase in root diameter, basal root number and adventitious root number) and Inverse Simpson diversity ($R^2 = 0.10$, $B = 2.08 \pm 1.05$, *p-value* = 0.053; Table 4-3). Near the Y-axis are schematic representations of the corresponding community composition variable according to low (bottom) and high (top) values, respectively, where each diamond represents an OTU, and the color a unique OTU.

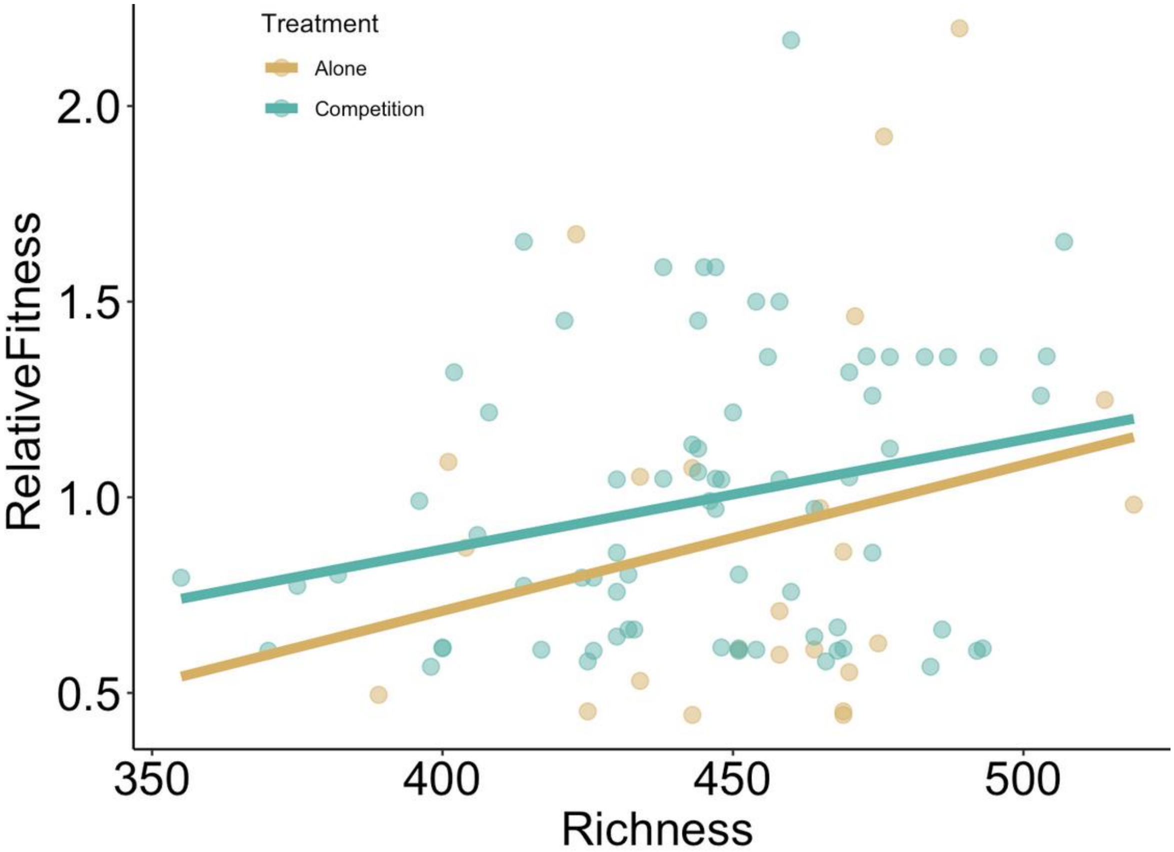

Figure 4-3 Linear regression plot between relative fitness and untransformed values of microbial species Richness from *I. purpurea* grown in the absence of competition, or 'Alone', (golden points) and in the presence of competition, or 'Competition', (green points) treatments, respectively. The gold and green line shows the corresponding linear regression slope between Richness and relative fitness estimated within the Alone and Competition treatments, respectively. We uncovered a significant positive relationship between Richness and relative fitness in the Competition treatment (*B* = -0.28 ± 0.17, *p-value* = 0.08), and no evidence of a significant linear relationship in Alone treatment (*B* = -0.47 ± 0.75, *p-value* = 0.54). ANCOVA demonstrated that the regression slope between Richness and relative fitness was significantly different according to treatment ($F\text{-}value_{1,57}$ = 3.26, *p-value* = 0.08; Table 4-5).

Discussion and Future Directions

The overarching goal of my dissertation was to evaluate the potential for character displacement to drive phenotypic evolution in root traits. I tested for the core criteria needed for the process of character displacement to occur using the closely related morning glories, *Ipomoea purpurea* and *I. hederacea*, as my study system. The findings of my dissertation demonstrate that belowground competition is a potentially overlooked agent of natural selection acting on root traits, and that root traits themselves are viable targets of selection (**Chapter 2** and **Chapter 3**). Therefore, satisfying two major criteria for character displacement. My evaluation for the hallmark prediction of character displacement (fitness increases with phenotypic distance in root traits) did not uncover evidence for character displacement as I expected, but evidence for the pattern of character *convergence* in root architecture (modular trait). Moreover, my findings indicate support that phenotypic plasticity in root architecture may be an important mechanism for how plants adapt to mitigate the harmful effects of belowground competition.

Although the main focus of my dissertation was to explore whether or not character displacement can lead to the evolution of the belowground root system, the microbial community that interacts at the immediate root-soil interface (‘rhizosphere microbiome’), can play an important role in how plants acquire and compete for belowground resources (Glick 2012; Olanrewaju et al. 2017; Rodriguez et al. 2019). Further, the rhizosphere microbiome can influence root traits and vice versa and result in complex plant-microbial interactions that can feedback into ecological and evolutionary processes (*e.g.*, belowground competition, character displacement; Fitzpatrick et al. 2018). To this end, for my third data chapter I explored the potential for the rhizosphere microbiome and root traits to influence each other and alter plant fitness in context of belowground competition (**Chapter 4**).

In summary, my dissertation is the first to examine evidence for the potential for character displacement to influence the evolution of root traits as a response to belowground competition.

In addition, because studying belowground root traits as potential targets of selection is nascent in the field evolutionary ecology, this dissertation also represents a novel framework for addressing important questions pertaining to root trait evolution. In addition, my dissertation provides a basis for considering the potential for root-microbe interactions to influence each other and belowground competition. Thus, each of my chapters merits discussion on how to improve our outstanding gap in root trait evolution as a response to plant-plant competition, whether competition between closely related plants is may result in character displacement and if/how the rhizosphere microbiome can feedback into these processes. Addressing these gaps will prove invaluable to our general understanding of the mechanisms that drive species diversity.

A closer look at root traits as a target of selection for belowground competition

It is widely accepted that belowground competition can have drastic effects on plant fitness (Cahill and McNickle 2011) but can and does this influence the evolution of root traits, and if so, what are its implications on species diversity? Research from **Chapter 2** suggests that belowground competition can potentially alter the evolution of root traits, specifically root angle (a specific root architecture trait), because this trait was genetically variable (*i.e.,* exhibit maternal line variation), and the pattern of selection that acted on this trait was altered according to competitive treatment (absence versus presence of competitor). Although I did not find belowground competition imposed selection on primary root length and root system width I did find that these traits were viable targets for evolution by natural selection--*i.e.,* they exhibited maternal line variation. These findings provide support that belowground competition may lead to the evolution of root architecture and size in weedy annual plants. Notwithstanding, my research is the first to my knowledge to test for belowground competition as a causal agent of selection on root traits and provide evidence of root traits as viable targets of selection. Hence, in order to determine its generalization across other plant species and environments merits more investigation. In this vein, abundant theoretical and empirical work has shown the existence of trade-offs between rapid acquisition of resources and resource conservation according to plant species, therefore I can expect selection to act differently according to a plant’s natural and evolutionary history. For example, Warembourg and others (2006) compared root traits linked to resource uptake between plants of different life histories (annuals and perennials) and families

(Fabaceae, Asteraceae and Poaceae) and found significant differences between life histories in root morphology (*e.g.*, specific root length and root tissue density) but no differences in root topology, as well as differences between plant family in root topology. Moreover, because selection from belowground competition will ultimately depend on what resources are limiting in that environment (*e.g.*, water, phosphorus, nitrogen) and other ecological factors (*e.g.*, herbivory), testing for the potential for belowground competition to impose selection should be repeated in different field settings. The former tests will provide insight on how general selection from belowground competition is across different plant species, whereas the latter will elucidate how different environmental contexts influence the strength and direction of selection on root traits. In short, replication and adaptation of my research with plants from different natural histories and taxonomic representation is essential in order to gauge the evolutionary repercussions of belowground plant-plant competition on plant diversity. Whereas, performing experiments in different environments will inform us on how differences in the ecology may impact the former.

Closing the gap of character displacement vs convergence as a driver in the evolution of root traits

Despite the evidence indicating that root traits are critical for mediating plant-plant competition and that they can potentially respond to selection, our understanding of whether character displacement has played an important role in the evolution of root traits remains largely unknown (Beans 2014). Here, my dissertation addressed this overall gap in the literature of evolutionary ecology. **Chapter 3** is very similar to **Chapter 2** in that they both examine for evidence of selection from belowground competition. In contrast to **Chapter 2**, **Chapter 3** focuses more on testing the main prediction of character displacement--*i.e.*, phenotypic distance between plant competitors positively associated with increase in plant fitness--while **Chapter 2** demonstrates evidence for the potential for belowground competition to ensue phenotypic evolution on root traits more generally which are prerequisites for character displacement. Further, **Chapter 3** uses *I. purpurea* as the focal species, and reconciles the complex and integrated nature of the root system by evaluating 33 root traits with multivariate statistics to transform them into a few ‘modular traits’ and then emphasizing a few specific root traits. We also investigate for evidence of maternal line variation in our root traits using mature field grown plants. Whereas

Chapter 2 examined both *I. purpurea* and *I. hederacea*, considered four specific root traits and measured greenhouse grown seedlings to uncover a basis for maternal line variation. The fact that I found maternal line variation in adult plants grown in the field, and evidence of selection from belowground competition for both specific and modular root traits in **Chapter 3**, corroborates our findings in **Chapter 2**. Together, these findings strengthen the general argument that belowground competition can lead to phenotypic evolution in root traits. Notably and most importantly, however, is that I did not find evidence for the hallmark prediction of character displacement as I expected. Instead I found evidence for a pattern of character *convergence* on root architecture a modular trait.

A finding for a pattern of character *convergence*, however, may be expected if there are constraints imposed by the local environment that favor a particular phenotype. In context of belowground plant-plant competition, competing species would benefit from a similar phenotypic response to a local soil environment. In **Chapter 3** I expand on this line of thought and discuss how a pattern for character convergence in root architecture may reflect evidence that plants are responding plastically to resources differences and the presence of a particular competitor, where individuals that can respond to both nutrients and competitors may be able to maximize fitness. Unfortunately, characterizing and manipulating the soil environment was beyond the scope of my overarching goal, and hence, I cannot draw any mechanistic inferences underlying my findings for character convergence. Future experimental work such as a competition field experiment where soil nutrients are manipulated, can help uncover if and how limiting resources influences the potential for character displacement (or convergence) to alter the evolution of root traits. Studying the reaction norms of root traits across environmental gradients and between competitive treatments, can help us understand the potential for phenotypic plasticity in root traits itself to undergo character displacement.

While demonstrating evidence for the *process* of character displacement/convergence to influence phenotypic evolution of root traits can implicate that belowground competition can drive plant species diversity (**Chapter 2** and **Chapter 3**), it is insufficient to make claims about whether character displacement/convergence is actually impacting plant diversity. As such, researchers should strive to test for both process and pattern in future work.

Considering relationships between the rhizosphere microbiome and root traits and their potential to influence belowground competition

The rhizosphere microbiome can alter plant function and fitness and potentially feedback into plant ecology and evolution (Berendsen et al. 2012; Backer et al. 2018). Further, the rhizosphere microbiome may influence root phenotypes and vice versa. Consequently, interactions between the rhizosphere microbial community and the plant root system may influence how plants compete belowground and adapt to competitive stress. Research testing if/how root traits and the rhizosphere microbiome vary with each other and impact plant fitness in context of belowground competition, however, is lacking. As such, for **Chapter 4** I addressed the broad question, *Does rhizosphere bacterial community composition and structure vary with root phenotypes and what are their relative effects on plant fitness according to competitive environment?* I used individuals of *I. purpurea* grown in the presence and absence of competition from *I. hederacea* and extended my analysis from **Chapter 3** to consider if variation in the rhizosphere microbiome was linked with variation in multiple modular root traits and relative fitness. For this study I focused on the bacterial microbiome and found that multiple metrics of bacterial community structure were linked with different root traits, and evidence that bacterial species richness may have a positive impact when plants experience belowground competition. I also demonstrated that community structure and composition of the rhizosphere microbiome is influenced mainly by the environment. Together, my work provides preliminary evidence that interactions between root traits and the rhizosphere microbial community may perhaps feedback into belowground competition thus potentially altering plant ecology and evolution.

Due to limitations to my experimental design, however, I could not draw causal conclusions about the significant patterns I found between metrics of the rhizosphere microbiome and host plant fitness and root phenotypes (*discussed* in **Chapter 4)**. Therefore, as a future step, I recommend that researchers evaluate for potential causal effects driving the linear patterns between root traits and microbial community structure in the rhizosphere, *e.g.*, manipulating the rhizosphere microboime and or root traits. For example, one could sterilize their experimental seeds and grow them in soil where the microbial community has been disrupted via autoclaving or other sterilizing methods. Then, compare how the relationship between root phenotypes and the rhizosphere microbiome vary according to different levels of soil disruption. Albeit, controlling for root phenotypes will be more difficult than manipulating the soil environment because it would require knowing *a priori* plant genotypes for different root phenotypes, and altering the genes underlying the root trait(s) of interest. If one has access to nearly isogenic lines (NILs) in respect to genes that code for root trait(s) of interest, however, one can take advantage that NIL's share a common genetic background to evaluate the effect of different root traits on the rhizosphere microbiome (Ambrose and Purugganan, 2012). In this vein, NIL's have been developed for different root traits including, rooting depth, root angle and branching, number (Shen et al. 2001; Loudet et al. 2005; Tuberosa et al. 2011).

Closing remarks

My dissertation shows that research on character displacement merits further investigation in order to fully expand our understanding on the mechanisms driving plant diversity and evolution. Namely, researchers should begin to consider how differences in soil resources influence competition between closely resembling plants, and in turn, how changes in resource availability alters selection on root traits and the propensity for character *divergence* versus *convergence* to transpire as an evolutionary driver. Likewise, it demonstrates that combining greenhouse and field experiments can serve as a two-step approach to studying root traits in non-model organisms, and test for the main criteria of character displacement. Finally, incorporating information about the root associated microbiome, namely the rhizosphere microbiome, into studies of character displacement in root traits will prove invaluable for elucidating if and how the microbial community may affect how plants compete and adapt as a response to belowground competition, *i.e.,* influence the potential for character displacement to drive phenotypic evolution of root traits. And, in turn, shed light on how the eco-evolutionary feedback between plants and microbes' shapes plant diversity as a result of plant-plant competition for limiting resources belowground.

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Appendices

Appendix S1 Supplementary Figures and Tables to Chapter 2

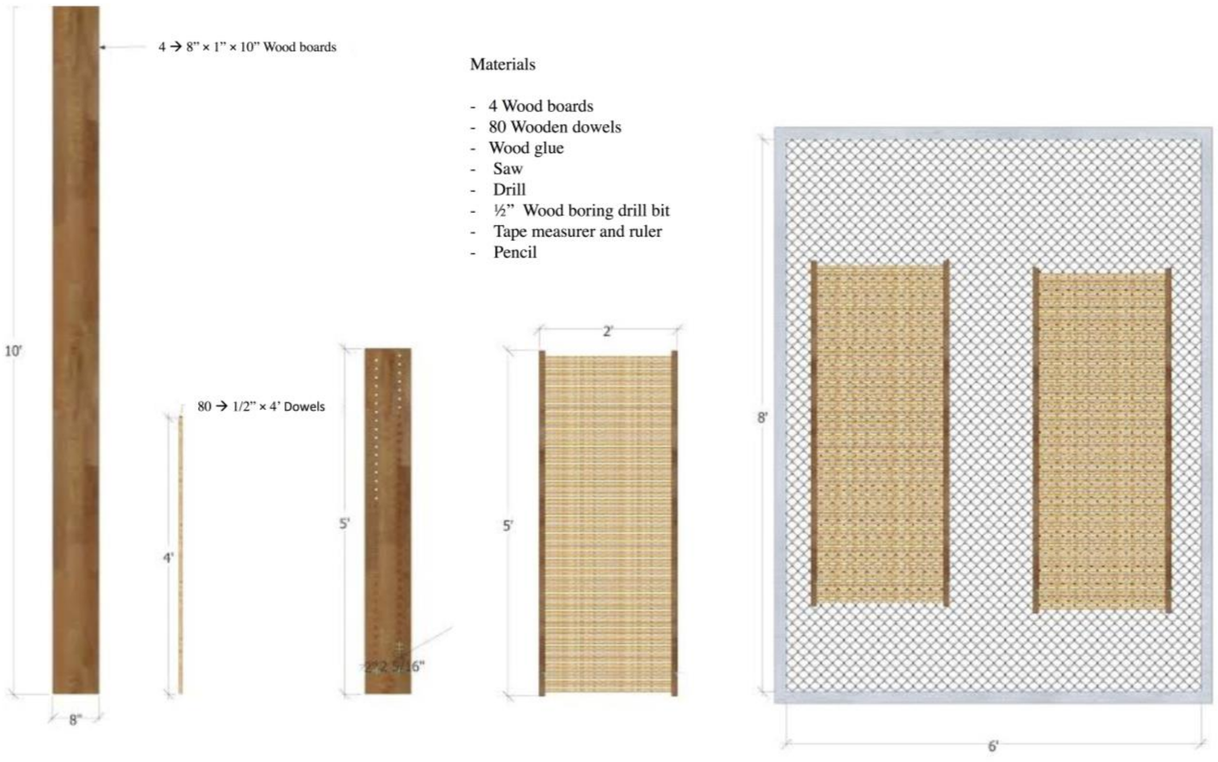

Figure S1-1 Procedure and materials required for construction of rhizotron frames. Four wood boards of 8" *x* 1" *x* 10', 80 ½" *x* 4' wooden dowels, wood glue, power drill, measuring tape, and a pencil will be required to build a total of four rhizotron frames. First saw each 10' wooden board in half to obtain a total of eight 5' boards and then saw each 4' wooden dowel in half to obtain a total of 160 2' dowels. Measure and draw out 40 evenly spaced dots on the 10' board approximately two inches from the top edge. For every of the 40 evenly spaced dots, draw a dot 4" beneath it at 30 degrees relative to the dots above. Drill a ½" hole where each dot is located. Make sure that each board looks identical with their holes matching up. Finally, line up two boards 2' apart and insert the ends of the dowels on the corresponding holes of the boards using wood glue at the tips to make sure they stay in place. Repeat this process until dowels have been placed in all the holes in order to create the frame.

Figure S1-2 The rhizotron greenhouse experiment. Each rhizotron was maintained at a thirty degree angle with the use of custom built wooden frames.

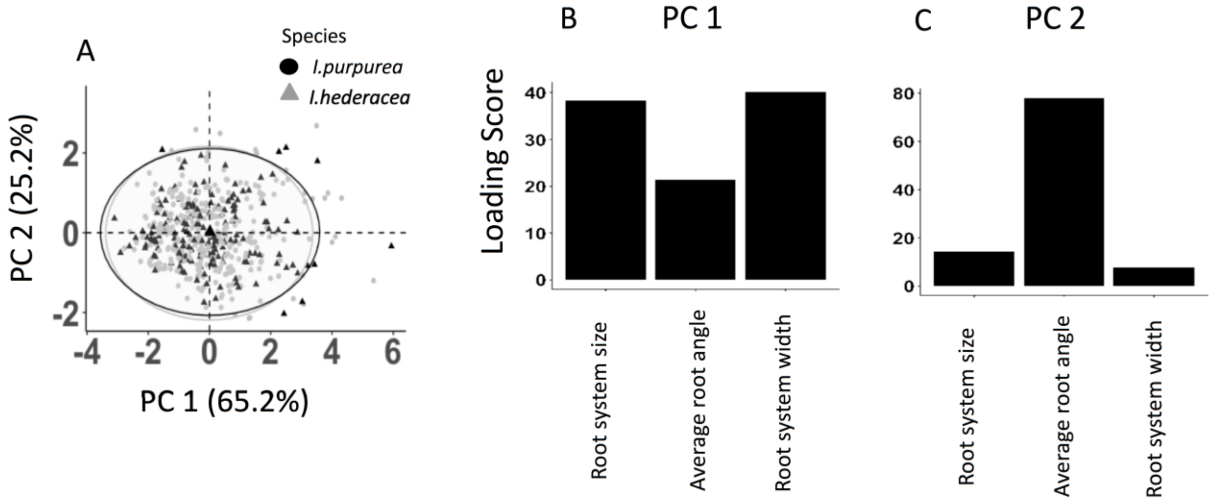

Figure S1-3 PCA summarizing root trait space between *I. hederacea* and *I. purpurea* from population PA4 grown in field conditions where 65.2 % and 25.2% of the phenotypic variation is explained by PC 1 and PC 2, respectively. Bar graphs (B & C) show the loading scores of each root trait for PC 1 and PC 2. B) root system size and root system width load heaviest in PC 1, C) and average root angle loads heaviest in PC 2.

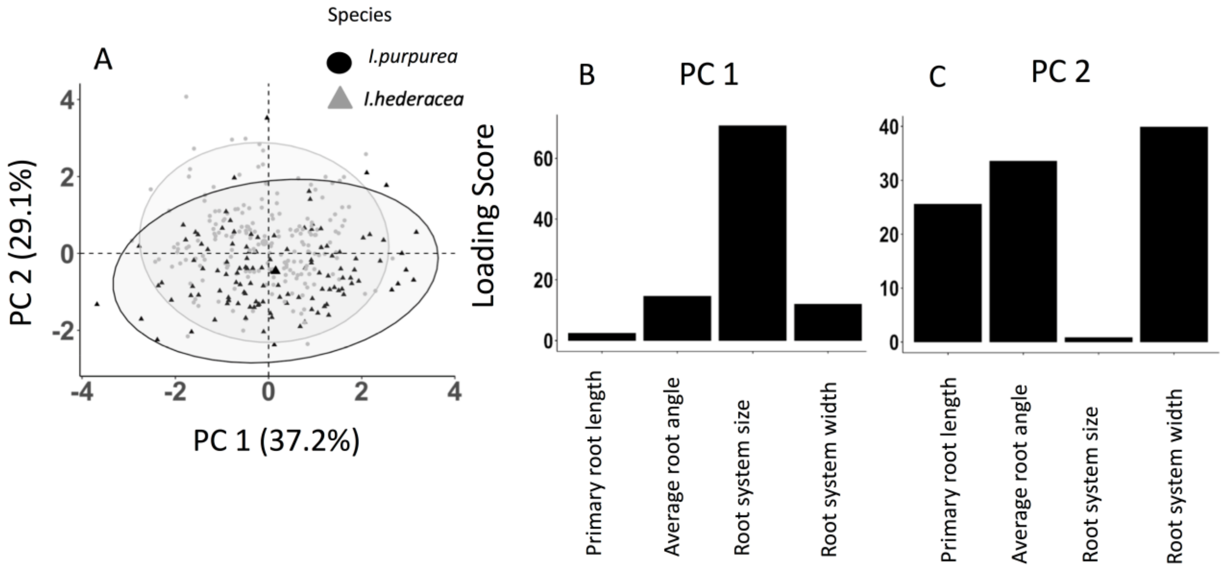

Figure S1-4 PCA results summarizing the variation of all the root traits measured in the greenhouse experiment (primary root length, root angle, root system size and root system width) between *I. hederacea* and *I. purpurea*. A) The PCA plot shows the first two PCs' and how individuals of *I.purpurea* (black solid circles) and *I.hederacea* (grey solid triangles) vary in this trait space. A) 37.2% and 29.1% of the phenotypic variation is explained by PC 1 and PC 2, respectively, and the overlapping ellipses representing the 95 % confidence intervals of each species indicates high phenotypic similarity between *I.purpurea* and *I.hederacea*. Bar graphs (B & C) show the loading scores that each root trait contributes to PC 1 and PC 2, respectively. B) Root angle, root system width and primary root length load strongest in PC 1, C) whereas root system size loads strongest in PC 2. PC 1 can be used as an indicator of root system architecture and PC 2 can serve as an indicator of root size.

Table S1-1: INFLUENCE OF COMPETITIVE TREATMENT ON I. PURPUREA AND I. HEDERACEA ROOT TRAITS WHEN GROWN IN THE FIELD

	Block	Species	Competition	ML
Trait	*F*	*F*	*F*	χ^2
Root system width (cm)	1.50	0.87	0.76	5.81*
Root system size (cm^2)	29.91	0.04	2.33	0.02
Root angle (degrees)	2.54	0.06	2.04	0.07
Seed number	9.41***	0.30	9.81***	5.43*

NOTE.—F-statistics showing the effects of block, species and competitive environment, and chi statistics(χ^2) showing maternal line variation on plant phenotypes. **Degrees of freedom for the linear mixed model the following:** Block: 3; Species: 1; Competition: 2; Maternal line: 1**.** Maternal line is abbreviated as ‘ML’.

***** $p < .05$
****** $p < .01$
*** $p < .001$

Table S1-2 GENETIC CORRELATION MATRIX BETWEEN ROOT TRAITS FOR *I. PURPUREA* (ABOVE DIAGONAL) AND FOR *I. HEDERACEA* (BELOW DIAGONAL)

	Root angle	Root width	Root size
Root angle	—	-0.01	0.1
Root system width	0.59***	—	0.85***
Root system size	0.60***	0.80***	—

NOTE.—Pearson correlations were calculated on the family means of non-transformed root traits for each species separately, and across treatments. Significant correlations (p-value < 0.05) are indicated in bold.

*** $p < .001$

Table S1-3 INFLUENCE OF INTERSPECIFIC AND INTRASPECIFIC COMPETITIVE ENVIRONMENT ON SELECTION GRADIENTS (MULTIVARIATE ANALYSES) IN *I. PURPUREA* AND *I. HEDERACEA*

	I. purpurea		*I. hederacea*		
Trait	Alone	Competition (Inter)	Alone	Competition (Inter)	Competition (Intra)
Root system width (cm)	-0.24 ±0.21	0.37 ±0.21	0.16 ±0.12	-0.22 ±0.16	-0.03 ±0.13
Root system size (cm^2)	0.56^ ±0.29	-0.15 ±0.22	-0.09 ±0.13	0.07 ±0.11	0.03 ±0.17
Root angle (degrees)	0.02 ±0.10	-0.09 ±0.08	0.01 ±0.08	0.23 ±0.09*	-0.27 ±0.24

NOTE.**—**Standard errors of the estimate presented as ±1 SE. Values in bold indicate significant selection gradient.

^ =/< 0.10
***** *p*< .05

Table S1-4 INFLUENCE OF INTERSPECIFIC COMPETITION WITHIN *I. PURPUREA* AND INTERSPECIFIC AND INTRASPECIFIC COMPETITION IN *I. HEDERACEA*, ON THE PATTERN OF SELECTION ACTING ON SPECIFIC ROOT TRAITS IN THESE SPECIES

	I. purpurea		*I. hederacea* (alone-inter)		*I. hederacea* (alone-intra)	
Root system width (cm)	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
Competition	2.51	0.12	0.84	0.36	1.34	0.25
Block	1.27	0.30	0.28	0.84	0.45	0.72
Competition × Block	4.80**	<0.01**	0.17	0.92	0.55	0.65
Root system size (cm^2)	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
Competition	4.88*	0.03*	0.81	0.37	1.14	0.29
Block	0.45	0.72	0.36	0.78	0.09	0.97
Competition × Block	1.00	0.40	0.13	0.94	0.29	0.84
Root angle	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
Competition	1.14	0.29	4.37*	0.04*	0.01	0.91
Block	0.46	0.71	1.45	0.24	3.18*	0.03*
Competition × Block	2.20^	0.10	2.28^	0.09	0.08	0.97

NOTE.—F-statistics from the ANCOVA testing the effects of competition, block, and root trait × competition on selection gradients are shown. Degrees of freedom for the ANCOVA are the following: Competition: 1, Block: 3; Competition × Block: 3.

^ =/< 0.10
***** $p < .05$
****** $p < .01$

Appendix S2 Supplementary Figures and Tables to Chapter 3

Trait name	**Functional Class**	**Description**	**Trait Code**	**PC Contribution (%)**
Accumulated width*	Topology	Percentage of width accumulation at 10-90 % depth	D10-D90	PC1: 7.00-10.69 %
Skeleton width	Architecture	Width calculated from the medial axis of the root system.	SKL WIDTH	PC2: 7.50 %
Skeleton depth	Architecture	Depth calculated from the medial axis of the root system.	SKL DEPTH	PC2: 10.84 %
Maximum soil tissue angle	Architecture	Maximum soil tissue angle measured over all root tips.	STA MAX	PC4: 6.94 %
Maximum width of the root system	Architecture	Maximum root system width measured from first to last horizontal foreground pixel.	WIDTH MAX	PC2: 7.53 %
Maximum root tissue angle	Architecture	Maximum root tissue angle measured over all root tip paths.	RTA MAX	PC2: 9.02 %
Root top angle range	Architecture	Range of root tissue angles present in the root system.	RTA RANGE	PC4: 5.73 %

Spatial root distribution X	Architecture	Spatial distribution of the root shape in the x-axis. This is the x component of the vector pointing from the center of the bounding box of the root shape to the center of mass of the root shape.	RDISTR X	PC2: 0.22 %
Spatial root distribution Y	Architecture	Spatial distribution of the root shape in the y-axis. This is the x component of the vector pointing from the center of the bounding box of the root shape to the center of mass of the root shape.	RDISTR Y	PC2: 8.01 %
Adventitious root angle	Architecture	Adventitious root angle estimated from the paths detected in the number of adventitious roots.	ADVT ANG	PC2: 4.83 %
Basal root angle	Architecture	Basal root angles estimated from the paths detected in the number of basal roots.	BASAL ANG	PC2: 6.61 %
50 percent drop	Architecture	Depth value where 50% of the root tip paths emerged from the central path.	DROP 50	PC2: 10.85 %
Projected root area	Size	Number of foreground pixels belonging to the root system.	AREA	PC3: 13.49 %
Skeleton nodes	Morphology	Nodes calculated from the medial axis of the root system.	SKL_NODES	PC4: 8.25 %

Stem diameter	Morphology	Stem diameter derived from medial axis.	DIA STM	PC3: 2.31 %
Simple stem diameter	Morphology	Simple stem diameter calculated in root estimator for shovelomics	DIA STM SIMPLE	PC2: 11.51 %
Average root density	Morphology	Ratio of foreground to background pixels within	AVG DENSITY	PC4: 1.43 %
Mean tip diameter	Morphology	Mean tip diameter estimated from the medial circle at the tips.	TD AVG	PC4: 14. 53 %
Root tip count	Morphology	Overall number of tips detected in the image	RTP COUNT	PC3: 9.37 %
Number of Adventitious	Morphology	Number of root tip paths emerging from root segment 1.	ADVT COUNT	PC4: 3.47 %
Number of basal roots	Morphology	Number of basal roots estimated as emerging root tip bundles from root segment 2.	BASAL COUNT	PC4: 5.64 %
Basal root angle	Architecture	Basal root angles estimated from the paths detected in the number of basal roots.	BASAL ANG	PC2: 6.61 %
Hypocotyl diameter	Morphology	Hypocotyl diameter estimated over detected hypocotyl region as the average diameters of medial circles.	HYP DIA	PC4: 8.46 %

Tap root diameter	Morphology	Tap root diameter estimated over detected taproot region as the average diameters of medial circles.	TAP DIA	PC4: 10.97 %
Maximum diameter at 90-100% percent depth	Morphology	Maximum diameter found in the interval of 90-100% rooting depth	MAX DIA 90	PC4: 9.42 %

Table S2-1 Lists the full root trait name and its corresponding abbreviated name ('Code') assigned by DIRT software for the 33 traits that were analyzed in the study. Each root trait was cataloged into four functional trait classes indicated within parenthesis *a priori*. We report the PC axis where a given individual root trait contributed the most variance and report its percent contribution to that axis ('PC Contribution'). Traits with '*' indicate multiple traits and a corresponding range in their percent (%) contribution to a given PC axis.

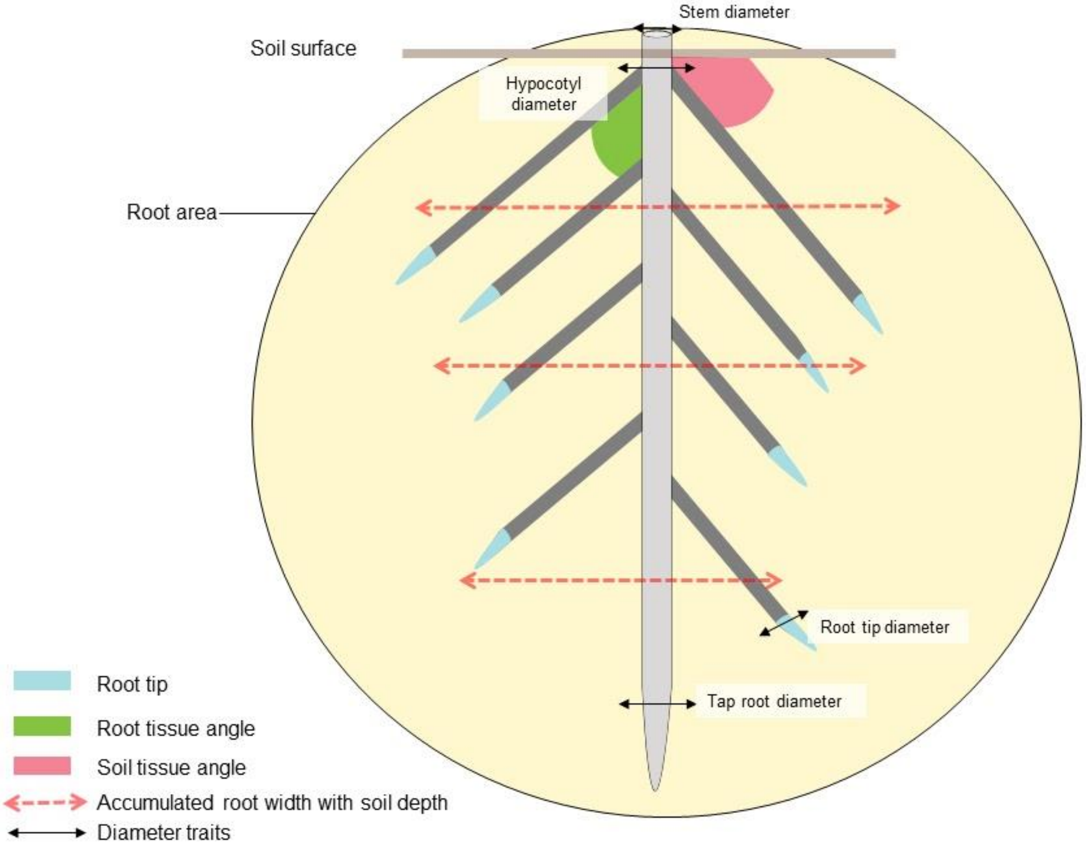

Figure S2-1 Sketch for some root traits captured by DIRT. Root area represents the ‘AREA’ trait, estimated as the total number of pixels of the root system as indicated by a light yellow circle encapsulating the entire sampled root system. Accumulated root width with soil depth is captured with the two-way dashed light red arrows (‘D%’; Table S1). Different diameter traits are indicated with two-way solid black arrows including, tap root diameter (‘TAP_DIA’), root tip diameter (‘TD_AVG’; Table S1), stem diameter (‘DIA_STEM’; Table S1) and hypocotyl diameter (‘HYP_DIA’; Table S1). Root tips are colored in light blue, and an example of soil root tissue angle and root tissue angle are shown by the pink and green shaded regions, respectively.

Trait	Selection gradient		*F-values*
	Alone	Competition	Treatment × Trait
Root topology (PC1)	0.05 ± 0.04 (.27)	0.01 ± 0.11 (.92)	0.04 (.85)
Root architecture (PC2)	-0.002 ± 0.05 (.97)	0.08 ± 0.07 (.28)	0.50 (.51)
Root morphology (PC4)	0.12^ ± 0.06 (.10)	**-0.17 ± 0.07** **(.03)**	**5.33** **(.03)**

Table S2-2 Results of linear selection gradient for root topology (PC1), architecture (PC2), and morphology (PC4), for each Treatment is presented with their respective linear regression slopes (β), its corresponding ± 1 standard error and *p-value* in parentheses. *F-values* and their corresponding *p-values* from ANCOVA are also shown to indicate the effect of Treatment on the pattern of selection for each trait (Treatment × Trait). Bolded values indicate a *p-value* < 0.05 and '^' indicates a marginally significant *p-value* = 0.10.

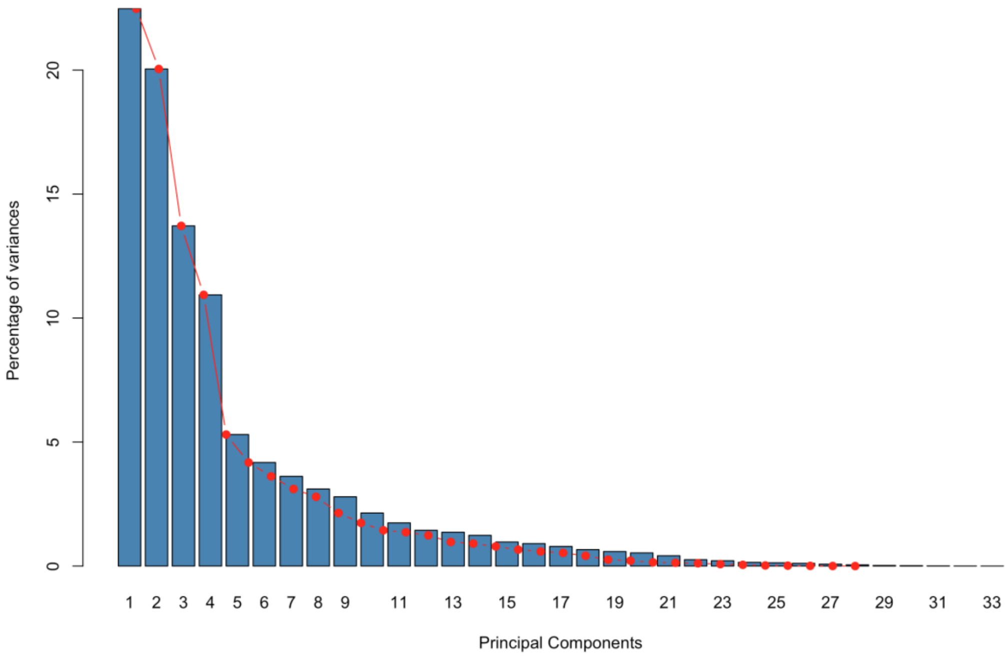

Figure S2-2 Scree plot demonstrating the percentage of variance explained by each principal component computed on the correlation matrix of the transformed root traits after the removal of Block effects. The first four principal components contribute to more than 10.0% of the total variation.

Traits	*F-statistics*		χ^2
Trait description	Block DF = 3	Treatment DF = 1	Maternal Line DF = 1
Skeleton nodes (morphology)	**130.59 (<.001)**	2.13 (.15)	0.47 (.50)
Maximum diameter at 90-100% percent depth (morphology)	**1011.34 (<.001)**	1.62 (.20)	<0.001 (.99)
Average root density (morphology)	**113.94 (<.001)**	0.02 (.88)	0.13 (.72)
Mean tip diameter (morphology)	**1208.57 (<.001)**	2.09 (.15)	<0.001 (.99)
Root tip count (morphology)	**13.60 (<.001)**	1.31 (.25)	1.66 (.20)
Soil tissue angle range (architecture)	**5.70 (.001)**	2.78 (.10)	**4.66 (.03)**
Maximum soil tissue angle (architecture)	**7.33 (<.001)**	2.36 (.13)	**5.17 (.02)**
Root top angle range (architecture)	2.29 (.08)	1.82 (0.18)	**4.22 (.04)**
Number of Adventitious roots (morphology)	**1.29 (.28)**	0.01 (0.91)	<0.001 (.99)
Hypocotyl diameter (morphology)	**1116.61 (<.001)**	3.47 (.06)^	<0.001 (.99)
Tap root diameter (morphology)	**840.17 (<.001)**	0.52 (.47)	<0.001 (.99)

Table S2-3 Results for post hoc linear mixed model analysis on a subset of individual root traits. *F-values* and their corresponding *p-values* from ANOVA are also shown to indicate the effect of Block and Treatment on original root traits. Bolded values indicate a *p-value* < 0.05 and ^ indicates *p-value* < 0.07.

Root trait	Competitor DF = 5	Combination Pairing DF = 54
Root topology (PC1)	1.55 (.17)	1.33 ^ (.07)
Root architecture (PC2)	2.09^ (.07)	**1.38 (.04)**
Root size (PC3)	**2.55 (.03)**	**1.75 (.001)**
Root morphology (PC4)	0.65 (.66)	**1.47 (.02)**

Table S2-4 Supplementary results for linear model ANOVA on root topology, architecture and morphology to evaluate the effect of Competitor and Combination Pairing on root architecture standardized for Block effects. Root trait was treated as the response variable and Competitor and Combination Pairing were fixed effects, we ran separate models for each root trait (root topology, architecture, size and morphology), respectively. *F-values* and their corresponding *p-values* are shown to indicate the effect of Combination Pairing and Treatment on root architecture. Bolded values indicate a *p-value* < 0.05.

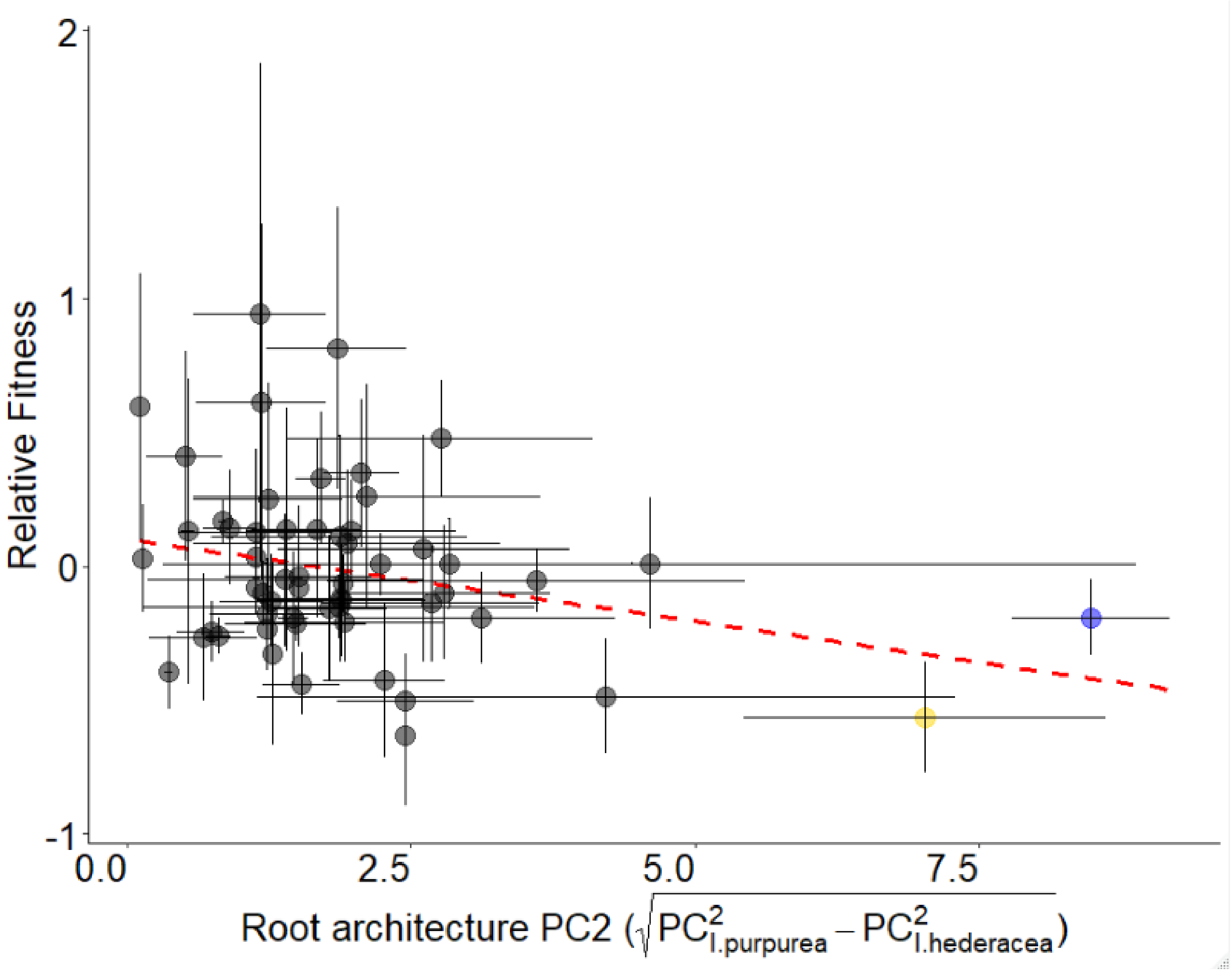

Figure S2-3 Negative relationship (β = -0.06 ± 0.03, *p-value* = 0.04; Table 4) between phenotypic distance of root architecture (PC2) and standardized relative fitness for *I. purpurea* when in competition with *I. hederacea*. The phenotypic distance of root architecture was calculated as the Euclidean distance in PC2 between competing pairs of *I. purpurea* and *I. hederacea* after the removal of Block effects, and then averaged by maternal line and species by maternal line combination type. Each point represents two to eight biological replicates. For each point, error bars were drawn based on observed values of relative fitness (Y-axis) and root architecture (X-axis) for a given maternal line within competition treatment, respectively. Colored points (yellow and blue) indicate two outliers that were maintained in our final analysis because of their low intraspecific variation.

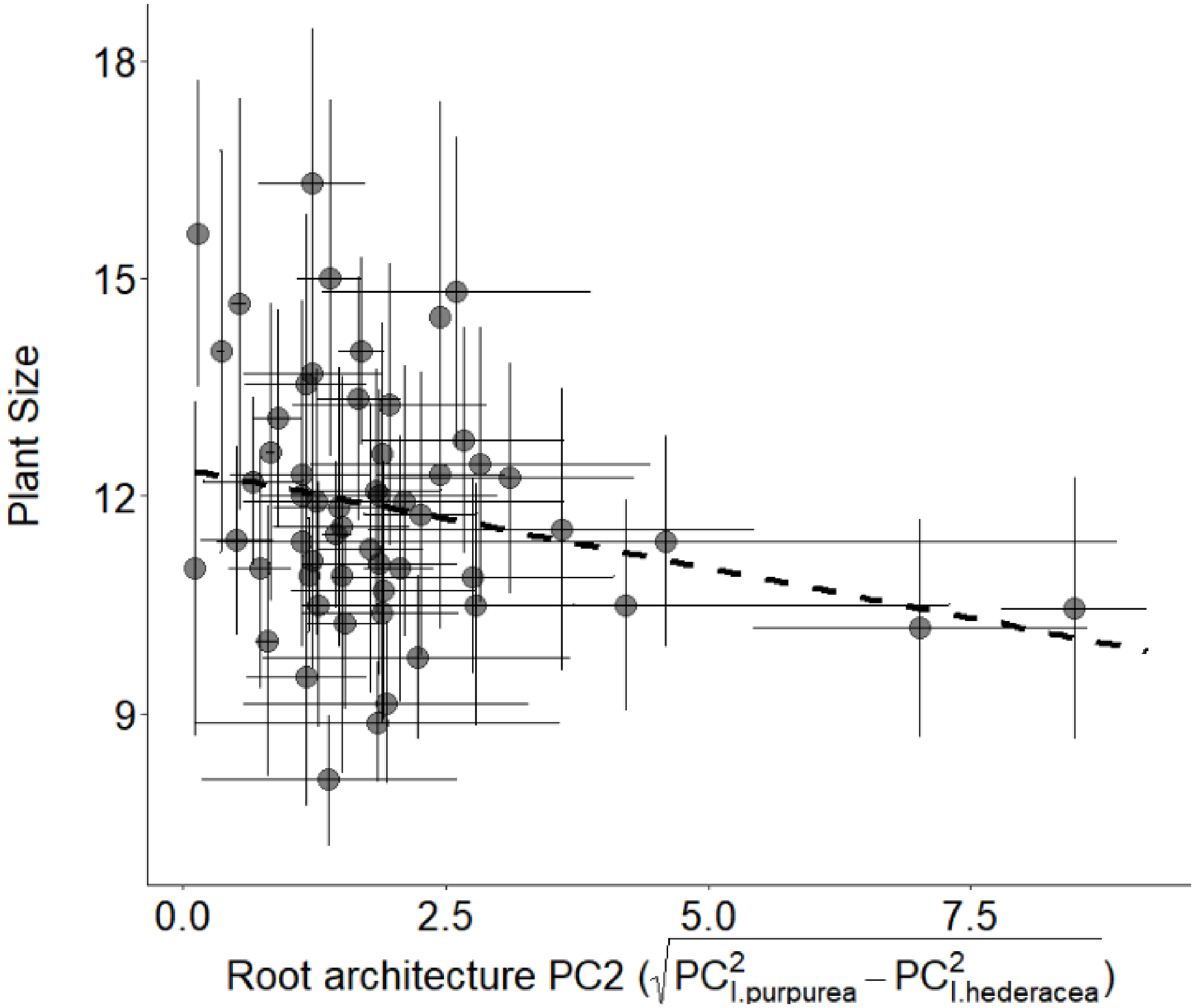

Figure S2-4 Evidence of a marginally significant negative linear relationship (β = -0.27 ± 0.15, *p-value* = 0.07) between phenotypic distance of root architecture (PC2) and plant size (*i.e.,* leaf number averaged by combination pairing and maternal line) for *I. hederacea* when in competition with *I. purpurea*. The phenotypic distance of root architecture was calculated as the Euclidean distance in PC2 between competing pairs of *I. purpurea* and *I. hederacea* after the removal of Block effects, and then averaged by maternal line and species by maternal line combination type. Each point represents two to eight biological replicates. For each point, error bars were drawn based on observed values of relative fitness (Y-axis) and root architecture (X-axis) for a given maternal line within competition treatment, respectively, for *I. hederacea*.

Traits		*F-statistics*		χ^2
Trait description	Trait Code	Block DF=3	Treatment DF=1	Maternal Line DF=1
Skeleton nodes (morphology)	SKL_NODES	**130.59** **(<.001)**	2.13 (.15)	0.47 (.50)
Stem diameter (morphology)	DIA_STM	**619.97** **(<.001)**	0.03 (.85)	1.56 (.21)
Average root density (morphology)	AVG_DENSITY	**113.94** **(<.001)**	0.02 (.88)	0.13 (.72)
Mean tip diameter (morphology)	TD_AVG	**1208.57** **(<.001)**	2.09 (.15)	<0.001 (.99)
Root tip count (morphology)	RTP_COUNT	**13.60** **(<.001)**	1.31 (.25)	1.66 (.20)
Soil tissue angle range (architecture)	STA_RANGE	**5.70** **(.001)**	2.78 (.10)	**4.66** **(.03)**
Root top angle range (architecture)	RTA_RANGE	2.29 (.08)	1.82 (0.18)	**4.22** **(.04)**
Number of Adventitious roots (morphology)	ADVT_COUNT	**1.29** **(.28)**	0.01 (0.91)	<0.001 (.99)
Number of basal roots (morphology)	BASAL_COUNT	**10.53** **(<.001)**	3.43 (.06)	**3.71** **(.054)**
Tap root diameter (morphology)	TAP_DIA	**840.17** **(<.001)**	0.52 (.47)	<0.001 (.99)

Table S2-5 Results for post hoc linear mixed model analysis on a subset of individual root traits. *F-values* and their corresponding *p-values* from ANOVA are also shown to indicate the effect of Block and Treatment on original root traits. Bolded values indicate a *p-value* < 0.05.

Appendix S3 Supplementary Figures and Tables to Chapter 4

Step	Deviance Residual	DF	AIC
-Root morphology × Treatment	0.004	55	-240.78
-Inverse Simpson × Diversity Treatment	0.005	56	-242.65
- Root architecture × Treatment	0.02	57	-244.06

Table S3-1 Backwards model selection statistics of the 'dropped' variables based on a full multivariate linear model^A of relative fitness of *I. purpurea* where we included Treatment, Block, all root traits and scaled values of microbial species richness, evenness, Inverse Simpson Diversity and their two-way interactions with Treatment and Block as predictor variables. We used the 'stepAIC' function of the 'MASS' package (Venagles and Ripley, 2002) to perform the backwards model selection computation and obtain a reduced model^B with the lowest AIC score.

^{A}**Full model:** Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block

^{B}**Reduced model:** Relative fitness ~ Treatment + Block + Root topology + Root architecture + Root morphology + Sp. Richness + Sp. Inverse Simpson Diversity + Treatment × Block + Root topology × Treatment + Root architecture × Treatment + Root morphology × Treatment + Sp. Richness × Treatment + Sp. Inverse Simpson Diversity × Treatment + Root topology × Block + Root architecture × Block + Root morphology × Block + Sp. Richness × Block + Sp. Inverse Simpson Diversity × Block