THE ROLE OF MYCORRHIZAL FUNGI AND SOIL NUTRIENTS IN TROPICAL REFORESTATION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Forestry – Doctor of Philosophy
Ecology, Evolutionary Biology and Behavior – Doctor of Philosophy

2016

PUBLIC ABSTRACT

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Forests are vital to human health and livelihood through timber production, air quality, and water purification; yet large scale tropical deforestation threatens these goods and services and underlines an urgent need to understand how to reforest these landscapes. Forest growth is highly dependent upon the availability of soil nutrients, but due to high amounts of rainfall and deeply weathered soils, most tropical soils are nutrient poor. Mycorrhizae fungi infect tree roots and can improve tree growth and reforestation efforts by increasing soil nutrient uptake in exchange for tree sugar. Generally, there are two types of mycorrhizal fungi (arbuscular mycorrhizae and ectomycorrhizae) that may differ in their ability to break down complex soil materials and acquire nutrients for trees in nutrient-poor soils. More diverse fungal communities also may be important to reforestation since combinations of different fungal species may take up more soil nutrients. So, reforestation success may depend upon differences in fungal type, fungal diversity, and soil nutrient levels.

In this dissertation, I combined field and greenhouse-based experiments to examine fungal-tree-soil interactions and test the role of mycorrhizal fungi in restoring tropical trees in nutrient-poor soils in southern Costa Rica. I tested: (1) the importance of fungal type on tree growth, (2) the role of fungal type in nutrient uptake under different soil nutrient availabilities, and (3) the effect of fungal diversity on tree growth.

My results showed that fungi can both increase and decrease tree growth, depending on tree species, fungal type, and soil nutrient status. In the field, I found that differences in growth

and tissue nutrient concentrations among four tree species were greater than differences between fungal types after two years of growth, highlighting the importance of tree species selection in reforestation. Fertilizer treatments and initial soil nutrient levels across sites also influenced tree growth and tissue nutrient differences. After separating fungal from tree species effects in the greenhouse, I did not find consistent effects of fungal type on tree growth and tissue nutrient levels across tree species; one tree species' growth responded more to the ectomycorrhizal fungal type, whereas another tree species had greater tissue nutrient content with the arbuscular mycorrhizal fungal type. In restored agricultural lands, I found that increases in fungal diversity and spore numbers reduced tree growth after 5 to 7 years, suggesting that more diverse and productive fungal communities could consume more tree sugar. In general, this dissertation provided greenhouse and field-based support for both positive and negative tree growth responses to mycorrhizal fungi and demonstrated the importance of mycorrhizal fungi in reforestation efforts. This research also highlights that understanding specific fungal-tree relationships and soil nutrient status are crucial to making broader management recommendations for reforestation success.

ABSTRACT

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With increasing awareness of large scale tropical deforestation and the ecological importance of mycorrhizae, incorporating fungal-plant symbioses into tropical restoration strategies has attracted considerable attention in recent years. In nutrient-poor landscapes, mycorrhizal fungi can help trees alleviate soil nutrient constraints to growth, and in particular, ectomycorrhizal fungi (EMF) may be more advantageous for nutrient capture than arbuscular mycorrhizal fungi (AMF) due to EMF's saprophytic capabilities and potential for increased nutrient uptake for their host trees. Establishing diverse mycorrhizal fungal communities is considered to be important to forest recovery, yet mycorrhizae may have complex effects on tree growth depending on the fungal composition of species present. Thus, the relative benefits that mycorrhizal fungi confer to host trees may be sensitive to changes in fungal type, fungal diversity and/or composition, and the soil nutrient environment.

In this dissertation, I combined field and greenhouse-based experiments to examine fungal-tree-soil interactions and test the role of mycorrhizal fungi in restoring tropical trees in nutrient poor soils in southern Costa Rica. I tested: (1) the importance of fungal type (AMF vs. EMF) on tree growth, (2) the role of fungal type in nutrient acquisition (as reflected in tissue nutrient concentrations) under different soil nutrient availabilities, and (3) the effect of AMF diversity and fungal composition on tree growth in multiple reforested sites.

My results showed variation in tree responses to fungal symbionts, depending on tree species, fungal type, and soil nutrient status. In the field, I found that differences in growth and

tissue nutrient concentrations among four tree species were greater than fungal type differences (AMF vs. EMF) after two years of growth, highlighting the importance of tree species selection in reforestation. Soil nutrient availability via site variation (i.e., initial base cation availability) and nutrient treatments also were associated with differences in tree growth. After isolating fungal from tree species effects in the greenhouse, I did not find consistent effects of fungal type on plant growth and tissue nutrient content; AMF had greater tissue nutrient content compared to EMF- or non-inoculated plants in one tree species whereas another tree species' growth responded more to EMF symbioses. In reforested agricultural lands, I found that increasing AMF diversity and spore numbers negatively correlated with reduced tree growth after 5 to 7 years, suggesting that more diverse and productive fungal communities could consume more tree carbon. In general, this dissertation provided greenhouse and field-based support for both positive and negative tree growth responses to mycorrhizal fungal community and demonstrated the importance of mycorrhizal fungi in reforestation efforts. This research also highlights that understanding specific fungal-tree symbioses, soil nutrient status, and site effects are crucial to making broader management recommendations for reforestation success.

This work is dedicated to my son, Edison,
for whom I hope my research may assist future
reforestation efforts and make the world a better place;
and to my husband, Todd,
thank you for being my rock throughout this journey and beyond.
I cannot wait to see where life leads us next!

ACKNOWLEDGEMENTS

A sincere thank you is extended to Richard Kobe for his guidance, support, and advice throughout my graduate work. The skills I have learned from him will undoubtedly assist me in my future endeavors both professionally and personally. Also, I would like to offer my sincere appreciation to Lars Brudvig, Catherine Gehring, G. Philip Robertson, and Michael Walters who have dedicated time and energy as members of my graduate committee. Their advice and insights were instrumental to my dissertation research, and their emotional and technical support was invaluable. A special thank you to Karen Holl and Zak Zawahi for the use of their research plots, all their great insights, and their support these past few years. And thanks to F. Lynn Carpenter who first ignited the idea of studying mycorrhizal fungal type in tropical reforestation.

I also would like to extend my great appreciation to Claudio Murillo for his invaluable field assistance and friendship; Ellen Cole, Michael Cook, Sarah Neumann, and Phu Nguygen for technical advice; Brian Graff and the MSU Agronomy farm, Sophan Chhin, David Rothstein, and Pascal Nzokou for the use of equipment; Carolyn Malmstrom for the use of her molecular facilities; Paul Bloese for answering multiple questions relating to my greenhouse experiment; and Georgia Bayerl, Tyler Harris, and Nikole Vannest for numerous hours spent assisting me in the laboratory. Thank you to the Organization of Tropical Studies and Las Cruces Biological Station staff for their support during my field work, especially Rodolfo Quiros. And I would like to express a great appreciation for Michigan State's Forestry department; Ecology, Evolutionary Biology and Behavior program; and Environmental Science and Policy program in helping me become a better scientist. I have learned a great deal during my time at Michigan State University.

Finally, I would like to thank my family and friends for their continual encouragement and support, and my husband, Todd Robinson, for his listening ear, constant understanding, and loving support. It is the encouragement and support of all of these wonderful people and organizations that has made this dissertation possible.

I was fortunate to receive received numerous small grants to fund my research, provided by the National Science Foundation (NSF-DEB 0918112), P.E.O. Scholar Award, MSU Environmental Science and Policy Program Fellowship, MSU Rasmussen Fellowship, MSU Center for Latin American and Caribbean Studies Fellowship, Tinker Field Research Grant, and Organization of Tropical Studies Research Grant. Additionally, I was supported by NSF Graduate Research, MSU Plant Sciences, Carol C. Gustafson and Gary S. Murphy Endowed Fellowship, and Dissertation Completion Fellowships while contributing to my dissertation. Thank you to these funding organizations for believing in me and my research.

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CHAPTER I:

INTRODUCTION

Large scale deforestation and degradation underline an urgent need to understand how to restore ecosystem functions in tropical landscapes (Lamb et al. 2005). Mycorrhizal fungal-plant symbioses can improve ecosystem functioning through maintaining soil aggregation (Rillig 2004), increasing nutrient cycling (Read and Perez-Moreno 2003), and improving plant growth and survival (Janos 1980). Globally approximately 80% of plant species form symbioses in which fungal-acquired soil nutrients are exchanged for plant-produced carbon (C) (Smith and Read 2008). Yet, surprisingly, little is known about fungal abundance and diversity in tropical soils (Alexander and Selosse 2009), and even less about their promising role in ecological restoration (Kardol and Wardle 2010).

A majority of tropical plants establish symbioses with either arbuscular mycorrhizal fungi (AMF) or ectomycorrhizal fungi (EMF), and a few species may form associations with both fungal types (Smith and Read 2008). Morphologically, EMF differentiate from AMF by forming a thick sheath of fungal mycelium around its host plants' roots and a hyphal network (i.e., Hartig net) that extends between root cells, while AMF penetrate into the root cells of its host plants and characteristically form arbuscules (branch-like structures that are key sites for nutrient exchange between the fungus and host plant). Functionally, EMF may have a competitive advantage over AMF in nutrient acquisition and uptake in nutrient-poor soils. EMF can directly break down leaf litter (Malloch et al. 1980), secrete enzymes that break down complex substrates (Hodge et al. 1995; Blum et al. 2002), stimulate microbial nitrogen (N) mineralization in the soil (Chalot and Brun 1998), and produce more hyphae than AMF in nutrient-poor soils (Jones et al. 1998) which

can increase the explored soil volume for nutrients and the absorbing soil surface area. AMF generally do not have saprophytic capabilities (but see Ezawa et al. 2005; Leigh et al. 2009). In exchange for fungal-acquired soil nutrients, up to 20% of a plant's total C budget may be allocated to its AMF symbiont and up to 30% to its EMF symbiont (Rygiewicz and Andersen 1994).

Whereas it is commonly assumed that a diverse mycorrhizal fungal community will enhance tree growth in forest restoration, the effect of fungal community composition on tree growth is complex. Studies have shown increases in plant productivity with greater fungal diversity (van der Heijden et al. 1998; Vogelsang *et al.* 2006), commonly attributed to functional differences among fungal species (Maherali and Klironomos 2007, 2012). Differences in relative plant benefits are generally associated with different life history strategies in AMF species via the amount of C they extract from their hosts (Olsson et al. 2010), their ability to acquire nutrients (Smith et al. 2000), and fungal nutrient storage capacity (Kiers et al. 2011). Host plants also can preferentially allocate more C to particular symbionts that favor their growth (Bever et al. 2009; Kiers et al. 2011).

The nutrient status of a particular site also is critical to determining the relative benefits derived from fungal-plant symbioses. Under nutrient-limiting conditions, host plants can benefit from the increased uptake of soil nutrients by their fungal symbionts (Smith and Read 2008), and plant dependence on fungal-acquired nutrients may increase after high nutrient depletion events such as erosion or land use changes (e.g. from forest to annual row crop production). However, to manage infertile tropical soils and improve tree growth, fertilization is a common practice in tropical restoration (Bloomfield et al. 1982; Bradshaw 2004). Under these nutrient-rich conditions, fungal abundance may decrease due to plants allocating C elsewhere (Read 1991;

Treseder 2004) or fungal symbionts acting as C drains on plant resources (Johnson et al. 1997; Allen et al. 2003). The cost:benefit ratio of fungal-plant symbioses may differ depending upon the limiting soil nutrient. Phosphorus, for example, can be fairly immobile in tropical soils (Sollins et al. 1988; Ticconi and Abel 2004), while plant available N is more mobile and may more easily be taken up by the plant roots. Mycorrhizal fungal-plant symbioses also may respond more to the ratio of nutrients than to the overall quantity of each nutrient (Hoeksema et al. 2010; Johnson 2010; Johnson et al. 2015).

Despite strong links among mycorrhizal fungi, soil nutrient status, and plant growth, few studies have tested their interactions in tropical restoration (Alexander and Selosse 2009; Kardol and Wardle 2010). Since 2000, only about 3% of mycorrhizal fungal studies have been conducted in tropical forests (Alexander and Selosse 2009), and even fewer examined mycorrhizae in tropical restoration. Different land uses (e.g., pasture, cropland, secondary forest) can affect the fungal community not only in terms of species diversity but also the frequency and relative abundance of sporulating species (Sturmer and Siqueira 2011). The conversion of a mature tropical forest to pasture also can alter the taxonomic composition of fungal communities, and planted seedlings in pastures may form fungal associations different from those in nearby forests or by pasture grasses (Aldrich-Wolfe 2007). Thus, incorporating fungal-plant symbioses into successful tropical restoration efforts requires a better understanding of how changes in fungal type, fungal diversity and/or species composition, and environmental conditions alter the benefits that fungi confer to trees in restoration plantings.

Organization of dissertation

In this dissertation, I examined fungal-tree-soil interactions to test the role of mycorrhizal fungi in restoring tropical trees in nutrient-poor and enriched (i.e., fertilized) soils in southern Costa Rica. This region is classified as a tropical montane rain forest (Holdridge 1967), but due to a history of agricultural land use over the past 60 years has largely been deforested (approximately 28% is forested today compared to 98% in the late 1940's; Zahawi et al. 2015). My combination of field and greenhouse-based experiments tested: (1) the importance of fungal type on tree growth, (2) the role of fungal type in nutrient acquisition (as reflected in tissue nutrient concentrations) under different soil nutrient availabilities, and (3) the effect of fungal diversity and specific fungal species on tree growth in multiple reforested sites.

In Chapter II, I examined different mycorrhizal fungal types in relation to tree growth, tissue nutrient concentrations, and soil nutrient availability on previously agricultural landscapes. I found that variation in tree growth and tissue nutrient concentrations was influenced more by tree species, site effects, and soil nutrient availability than mycorrhizal fungal type. In Chapter III, I utilized tree species that could host both AMF and EMF (dual fungal-plant symbioses) to test the effects of fungal type on tree growth and tissue nutrient content under different soil nutrient levels. While AMF and EMF influenced nutrient acquisition and tree growth, I did not find consistent effects of fungal type across tree species. In Chapter IV, I examined the effects of AMF fungal community diversity and abundance on the variable growth of four tree species in eight 5 to 7 year old reforested sites. AMF diversity and spore density across sites negatively correlated with reduced tree growth. In the final chapter, I provided a general summary of the entire dissertation and insights into future research directions.

CHAPTER II:

TREE SPECIES AND SOIL NUTRIENTS DRIVE TROPICAL REFORESTATION MORE THAN ASSOCIATIONS WITH MYCORRHIZAL FUNGAL TYPE

ABSTRACT

Mycorrhizal fungi could assist reforestation efforts in degraded tropical landscapes by increasing soil nutrient uptake and improving tree growth. The relative benefits that mycorrhizal fungi confer to host plants and reforestation efforts may differ due to fungal type (arbuscular (AMF) or ectomycorrhizal fungi (EMF)) and changes in soil nutrient status. This research's objective was to examine the interactive effects of mycorrhizal fungal type (AMF vs. EMF) and soil nutrient availability on host tree nutrient acquisition (i.e., growth and tissue nutrient concentration) after two years. Four tree species, two associated with EMF (*Pinus caribaea* and *Quercus insignis*) or AMF (*Terminalia amazonia* and *Swietenia macrophylla*), were planted across eight sites and subjected to five nutrient treatments (control, nitrogen (N), phosphorus (P), N + P, and N + P + base cations) in southern Costa Rica. After two years of growth, there were strong growth differences among tree species but not according to fungal type (EMF or AMF). Site variation, specifically initial base cation availability, and nutrient treatments also influenced tree growth and tissue nutrient concentrations. In a complementary greenhouse experiment that isolated fungal from tree species effects, the ratio of N to P in nutrient treatments was more important to the growth response of tree species to fungal symbionts than individual nutrients. These results show that changes in soil nutrient availability may be more important to tree species' nutrient acquisition than mycorrhizal fungal type. This study also highlights the importance of tree

species selection and replication across multiple sites with different ratios of soil nutrients to make management recommendations for reforestation success.

INTRODUCTION

Tropical trees associate with two types of mycorrhizal fungi: arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF). Generally, AMF dominate tropical ecosystems (Read 1991), but some tropical forests are EMF dominated (McGuire et al. 2008; Peay et al. 2010), and a few tropical tree species may form associations of both types (Smith and Read 2008). Both mycorrhizal fungal types can increase soil nutrient uptake and improve host plant growth, processes especially important to reforestation in nutrient-poor tropical systems. The relative benefits that AMF and EMF symbionts confer to host plants may vary based on functional differences between fungal types and soil nutrient status. Carpenter et al. (2004) suggested that differences between fungal types may assist reforestation efforts in degraded agricultural lands in Costa Rica. While a majority of mycorrhizal fungal research focuses on either AMF or EMF, relatively few have contrasted fungal types in a single study (but see Phillips and Fahey 2006; Phillips et al. 2013; Dickie et al. 2014; Yin et al. 2014) and, to my knowledge, never in a tropical system. In tropical ecosystems where over 350 million hectares have been deforested and another 500 million hectares have been degraded (Lamb et al. 2005), understanding the role of mycorrhizal fungal type in reforestation could be critical to reforestation success.

Land use changes can adversely alter physiochemical properties in tropical soils already characteristically infertile and deeply weathered (Sahani and Behera 2001). Tropical soils generally are considered phosphorus (P)-limited (Porder et al. 2007; Celentano et al. 2010) due to their tendency to bind to and immobilize P (Sollins et al. 1988; Ticconi and Abel 2004); at

higher elevations, tropical soils also may be nitrogen (N)-limited (Tanner et al. 1990) due to high diffusion rates of nitrate and ammonium and current land-use changes (e.g. forest to agricultural) that lead to high N loss through nitrification and denitrification (Robertson and Tiedje 1988). Base cations (e.g., calcium, magnesium, potassium (K)) also may be important in theses soils since they are prone to leaching (Hedin et al. 2003) and recent tropical research has highlighted their constraints to tropical seedling growth (Holste et al. 2011; Wright et al. 2011). To manage these infertile soils and improve tree growth, a common practice in tropical reforestation is to surface-supply critical plant macronutrients (N-P-K) through fertilization (Bloomfield et al. 1982; Bradshaw 2004). But this practice can be costly, prone to nutrient loss from targeted sites and accumulation in sensitive areas, and disruptive of fungal-tree relationships.

Mycorrhizal fungi, particularly EMF, may help alleviate nutrient limitation constraints to tropical plant growth. Generally, EMF associations may have a competitive advantage over AMF in nutrient-poor landscapes due to their direct role in breaking down leaf litter (Malloch et al. 1980), their capability to secrete enzymes that break down complex substrates such as mineral P (e.g., acid phosphatase enzyme; Blum et al. 2002) and organic N (Hodge et al. 1995), and their stimulation of microbial N mineralization in the soil (Grayston et al. 1996; Chalot and Brun 1998). AMF generally do not have these capabilities (but see Leigh et al. 2009; Verbruggen et al. 2016) but can still deliver up to 80% of their host tree's P requirements and 25% of their N requirements (Marschner and Dell 1994). EMF also may increase nutrient capture by producing more hyphal length that can explore a greater soil volume than AMF in nutrient-poor soils (Jones et al. 1998). Conversely, under increasing nutrient enrichment (e.g., fertilization), relative fungal type benefits may disappear due to host plants decreasing fungal associations (Read 1991).

However, there has been wide variation in fungal-plant interactions with nutrient additions (Treseder 2004; Hoeksema et al. 2010).

Despite strong links among mycorrhizal fungi, nutrient availability, and plant growth, few studies have tested their interactions in the context of Neotropical forest restoration (Alexander and Selosse 2009; Kardol and Wardle 2010). Many studies examining mycorrhizal fungi are conducted under controlled greenhouse conditions that do not accurately reflect the complex environment that fungal-tree symbioses encounter in nature such as climatic variability, complex soil substrates, intact soil fauna and microbial communities, and disturbances. In addition, relatively few Neotropical restoration studies are conducted across multiple sites (Piotto et al. 2003; Calvo-Alvarado et al. 2007; Holl et al. 2011), even though tree growth can be highly variable, especially on a fine spatial scale.

In this study, I examined two EMF and two AMF associated tree species planted across eight sites in southern Costa Rica with previous deforestation and agricultural or pastoral land-use histories and different initial soil nutrient concentrations (Table 2.1). I used five nutrient treatments (control, N, P, N + P, and N + P + base cations) to test the interactive effects of nutrient enrichment on mycorrhizal fungi, tree growth, and tissue nutrient concentrations after two years. In addition, I conducted a complementary greenhouse experiment to isolate the effects of fungal type vs. tree species on plant growth and nutrient acquisition. In these nutrient-poor landscapes, I hypothesized that: (**H1**) trees associated with EMF have greater nutrient uptake (i.e., as reflected in higher tissue nutrient concentration and tree growth) than trees associated with AMF; (**H2**) EMF associated trees have greater rhizosphere effects (i.e., alterations to the chemical or biological characteristics of soil around roots) than AMF-trees, such as greater acid phosphatase activity and microbial N production; and (**H3**) percent fungal

Table 2.1. Characteristics for each of the eight sites before transplanting seedlings. Site names reflect the properties' landowners. Soil nutrient values are means ± standard errors, and letters represent significant differences (P<0.05) between sites.

Site	Average Elevation (meters asl)	Maximum Slope (degrees)	Time since abandonment	Type of Previous Land-Use	Nitrogen Mineralization (ug/g/d)	Phosphate (ppm)	Sum of Base Cations (ppm)
BA	1150	19	<1 year	Pasture	$55.4^{bc} \pm 1.6$	$0.4^{a} \pm 0.06$	$32.6^{a} \pm 1.8$
BB	1140	<1	<1 year	Pasture	$36.5^{a} \pm 1.7$	$0.8^{bc} \pm 0.05$	$36.8^{a} \pm 1.5$
CM	1170	19	<1 year	Crop Production	$62.6^{cd} \pm 3.1$	$0.7^{bc} \pm 0.06$	$92.8^{b} \pm 4.0$
HE	1260	22	<1 year	Pasture	$49.9^{b} \pm 2.7$	$0.5^{ab} \pm 0.07$	$27.9^{a} \pm 2.1$
IP	1190	19	1-5 years	Crop Production	$34.6^{a} \pm 1.8$	$0.8^{bc} \pm 0.06$	$64.1^{c} \pm 2.5$
WG	1210	24	5-10 years	Coffee/Crop Production	$70.1^{d} \pm 2.5$	$1.1^{d} \pm 0.09$	$111.2^{d} \pm 3.9$
LG	1110	<1	5-10 years	Pasture	$40.7^{a} \pm 1.1$	$0.9^{cd} \pm 0.06$	$114.7^{d} \pm 2.2$
PC	1140	<1	5-10 years	Pasture	$99.8^{e} \pm 2.0$	$1.6^{e} \pm 0.08$	$76.1^{e} \pm 2.0$

colonization in both EMF and AMF associated trees decrease with nutrient additions (i.e., fertilization).

METHODS

Site description

The eight sites used in this research were established in Coto Brus county (8°47' N, 82°57' W) in southern Costa Rica. The forests primarily covering this region were classified as tropical premontane rainforests (Holdridge 1967), however due to a history of land use changes from forest to agriculture (e.g., coffee plantations, agricultural fields, and pasture) in the past 60 years, only about 28% of Coto Brus (approximately a 15 km radius) remains forested (Zahawi et al. 2015). Even though the previous dominant tree species in this area were not recorded, forests were mostly likely similar to the primary forests found in La Amistad Biosphere Reserve, in which the dominant tree groups include the Lauraceae family (e.g., *Ocotea, Persea, Nectandra,* and *Phoebe* spp.) and endemic oaks (*Quercus* spp.) (Hogan 2014), and the dominant fungal type is AMF.

Sites range in elevation from 1100 to 1300 m above sea level, receive approximately 4000 mm of rainfall annually with a dry season from December to March, have mean annual temperatures ranging from 13 to 21°C and up to a 24-degree slope. All sites were previously used for agriculture and were either recently abandoned pastures dominated by exotic forage grasses or coffee farms dominated by a mixture of forage and non-forage grasses (Table 2.1).

Site nutrient characteristics

The soils in this region are classified as lixisols (Centro Científico Tropical 2004) and typically have a clay-enriched subsoil, high base saturation, low P and exchangeable cations levels, and low pH (Celentano et al. 2011). Erosion is a major problem influencing soil fertility in this region due to steep land gradients, high annual rainfall amounts, and the conversion of the land to agricultural uses.

To create a soil resource map of each site, 50 soil samples were collected per site in May 2011 and analyzed for initial site nutrient levels (e.g. inorganic N, N mineralization, phosphate, and base cations (calcium, potassium, and magnesium); Table 2.1). Soil N was extracted with a 2M potassium chloride solution and analyzed colorimetrically with an ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc, Winooski, VT). Nitrogen mineralization was calculated as the change in inorganic N concentrations during a 28 day incubation period. Phosphate and base cations were extracted with a Mehlich III solution (Mehlich 1984) and analyzed either colorimetrically with an Absorbance Microplate Reader or spectrophotometrically using an Optima 2100DV Induced Coupled Plasma (ICP) Optical Emission Spectrometer (Perkin-Elmer, Shelton, CT).

Site preparation and transplanting

Two AMF associated species (*Swietenia macrophylla* King (Meliaceae) and *Terminalia amazonia* (J. F. Gmelin) Exell (Combretaceae)) and two EMF associated species (*Pinus caribaea* Morelet var. *hondurensis* (Barret & Golfari) (Pinaceae) and *Quercus insignis* (M. Martens & Galeotti) (Fagaceae)) were randomly transplanted in August 2011 into a regular grid pattern in each of eight sites (6 – 25 x 25 m; 1 – 15 x 40 m; and 1 – 20 x 25 m). Although *Quercus* spp. are

commonly classified as EMF associated (e.g., Trappe 1962; Wang and Qui 2006), they may form associations with both fungal types (Smith and Read 2008; Egerton-Warburton and Allen 2001). *P. caribaea* was the only species not native to Costa Rica but commonly found across the landscape and native to Latin America. *S. macrophylla, T. amazonia,* and *Q. insignis* are late-successional tree species, while *P. caribaea* is an early-successional species.

Seventy-two seedlings (18 per species) were transplanted into each site (72 x 8 sites = 576 seedlings in total), separated by a minimum of 2.8 m between seedlings. Transplanted seedlings were cultivated from seed in nursery grow bags for approximately two to four months prior to transplanting. Seeds were collected from around Coto Brus county (*T. amazonia* and *Q. insignis)* or plantations across Costa Rica (*S. macrophylla* and *P. caribaea).* Average seed weight was 0.42 g for *S. macrophylla*, 0.004 g for *T. amazonia,* 0.02 g for *P. caribaea*, and 57.50 g for *Q. insignis*. Initial plant height (height to apical bud) and basal diameter was measured for each seedling after transplanting. Following standard reforestation practices in the region, sites were initially prepared by machete clearing and one glyphosate herbicide treatment to reduce initial competition with exotic forage grasses during establishment; subsequently, competing vegetation was reduced (by machete and weed trimmer) throughout the study.

Mycorrhizal fungal inoculation

Both *Q. insignis* and *P. caribaea* were inoculated with approximately 100g of soil from under mature trees of the same species at the time of transplanting to ensure EMF symbioses. Initial site-level AMF density and diversity were analyzed to assess *S. macrophylla* and *T. amazonia's* capability to be colonized by AMF from the *in-situ* fungal community. Nine random soils samples per site were taken prior to transplanting (in August 2011) to assess the initial site-level

AMF community. Spores were extracted from the soil matrix using multiple sieves (250, 160, and 20 um) and the sucrose flotation method (Ianson and Allen 1986). To assist spore separation from the soil matrix in high clay content soils and to standardize the methods, sodium hexametaphosphate was used across all samples. Estimates of spore abundance and diversity were standardized to spores per one gram of soil. Spores were mounted on slides using a PVLG solution (polyvinyl alcohol, lactic acid, and glycerol) and then counted and identified to the genus level following Schenck and Perez (1990).

Nutrient treatments

To test the effects of nutrient enrichment on mycorrhizal fungi and tree growth, N and P treatments (control, N, P, N + P) were applied to seedlings with 32 seedlings/species across all sites. An extra treatment, N + P + base cations, was applied to an additional 16 seedlings/species to help clarify the effects of base cations (see Holste et al. 2011). Fertilizer application rates were the same across all sites. Nitrogen (NH_4NO_3) and calcium (CaOH) were applied annually at rates of 10 g/m^2, while phosphorus (P_2O_5), magnesium (MgOH) and potassium (K_2O_5) were applied at 5 g/m^2. Application rates were calculated from previous Neotropical fertilization studies (Wright et al. 2011; Yavitt et al. 2011) and nutrient availability as influenced by litter decomposition in the area (Celentano et al. 2011). Nutrient treatments were applied in a circular radius (0.5 m radius for year one; 1 m radius for year two) around each individual tree three times a year during the wet season.

Measurements of growth and nutrient acquisition

Tree height and basal diameter were measured every three months for two years (from August 2011 to August 2013). Basal diameter was used (i.e., 30 cm above the ground level; Chhetri and Fowler 1996) instead of diameter at breast height (DBH), since seedlings were initially below standard breast height (i.e., 1.30 m); 28% of individuals did not reach 1.30 m during the course of the study. After two years of growth, soil and plant tissue were collected for percent fungal colonization, nutrient analyses, and fungal enzyme activity to examine nutrient acquisition of fungal-tree symbioses.

The roots of each individual tree was measured for percent fungal colonization. Roots were traced from the base of the tree to their fine root extensions to ensure that the correct roots were collected, and only fine roots were used for fungal colonization analyses. Roots were cleared with a 10% sodium hydroxide solution and stained with Schaeffer's ink and vinegar method (Vierheilig et al. 1998). For AMF, percent root length colonized was scored using a modified gridline intersections method with approximately 20 cm of root per sample (McGonigle et al. 1990). For EMF, percent root tips colonized also was scored from about 20 cm of root per sample randomly selected and examined at 40X magnification for the number of root tips colonized (Gehring and Whitham 1991). Since *Quercus* seedlings also may form associations with AMF, *Q. insignis* roots were examined for both EMF and AMF independently (i.e., on separate root subsets).

To characterize nutrient availability and acquisition, soil and plant tissue was analyzed. Three soil subsamples were collected at a depth of approximately 10-15 cm and within a 1 m radius of each tree, composited, and analyzed for inorganic N, N mineralization, phosphate, and base cations to acquire an estimate of soil nutrient availability. A subset of leaves, stems, and

roots were collected from 16 trees per tree species and nutrient treatment (320 samples) to determine how much of the available soil nutrient pools were amassed within each tissue component. Portions of each component were collected and pooled to obtain one composite sample per tree. When possible, new, fully expanded leaves were collected from each tree for foliage, and roots were traced from the base of the tree to their fine extensions to ensure that the correct roots were collected. All samples were oven dried and then finely ground using a ball-bearing mill (Kleco, Visalia, CA) or grinding mill (Christy & Norris Laboratory mill, Christy Turner Ltd., Ipswich, Suffolk, UK). Ground samples were either analyzed with an Elemental Combustion CHNS-O analyzer (ECS 4010, Costech Analytical Technologies, Valencia, CA) for total N and C or digested in 5% nitric acid using a block digester (AIM600 Block Digestion System, A.i. Scientific Pty Ltd., Clontarf, Queensland, Australia) and analyzed with an ICP Spectrometer for P, Ca^{+2}, K^{+}, and Mg^{+2}.

Rhizosphere soil was used to evaluate relative differences between fungal-tree symbioses in terms of their acid phosphatase activity and stimulation of microbial activity via N mineralization rates. Subsamples of rhizosphere soil were taken from the soil adhering to the fine roots of each tree. For acid phosphatase activity (using methods described in Phillips and Fahey (2006)), the rhizosphere soil was mixed with toluene, a universal buffer, and a *p*-nitrophenol phosphate solution; incubated for an hour; mixed with calcium chloride and sodium hydroxide to stop the reaction; and then centrifuged and filtered. For each sample, a second control sample was incubated and extracted using the same procedure to correct for color development from the background soil matrix. Acid phosphatase enzyme activity was estimated by calculating the amount of *p*-nitrophenol released by phosphatase activity (determined colorimetrically with an Absorbance Microplate Reader at an absorbance of 405 nm) after correcting for the background

soil matrix. Microbial N mineralization samples were extracted with a 2M potassium chloride solution, analyzed colorimetrically with an Absorbance Microplate Reader, and calculated as the change in nitrate and ammonium during a 10 day incubation period.

Complementary greenhouse experiment

Since it is difficult to isolate and eliminate fungi in the field, a complementary greenhouse experiment was conducted to test tree species with and without their associated mycorrhizal fungi. One tree species from each fungal type (*S. macrophylla* for AMF; *P. caribaea* for EMF) were grown in a complete randomized design of two fungal treatments (non-mycorrhizal, and AMF or EMF) and four nutrient treatments (low N and P, high N and P, low N:P, and high N:P). Plants were grown for approximately three months to allow for unconstrained root growth, and then all plant components (i.e., leaves, stems, and roots) were harvested, dried (60^0C), and weighed. Analogous to the field, soil and plant tissue were collected from greenhouse individuals and analyzed for the growth and tissue nutrient concentrations of fungal-tree symbioses.

Ninety-six individuals of each species (12 replicates per treatment) were planted in 2.8-L pots (Tall-One Treepots, Stuewe & Sons, Inc., Tangent, OR) with 85% commercial soil mix containing peat moss, perlite, and vermiculite (Fafard #2, BFG Supply Co., Kalamazoo, MI) plus 15% sterilized sand, based on a ratio obtained from water infiltration tests conducted across Coto Brus county, Costa Rica (EKH, unpublished data, May 2012). Seedlings were grown under full light conditions (similar to that experienced in the field's abandoned agricultural sites) and watered to field capacity as required. Daylight was kept at a constant twelve hour day schedule (6am-6pm, EST) and temperatures ranged between 18 to 24^0C, similar to what seedlings would

experience in southern Costa Rica. A modified Hoagland's solution was applied in a diluted dose twice a week with two levels of N (100 and 250 mg N per liter) and P (12 and 62 mg P per liter). Plastic bags enclosed the bottom of the pots and sterile crushed granite was layered on top of the pots to reduce nutrient loss and escaping spores and to prevent cross contamination between nutrient and fungal treatments (Brundrett et al. 1996).

For the mycorrhizal fungal inoculum, AMF and EMF were trapped in the roots of bait plants cultivated in a combination of two-thirds field soil (collected from under mature *Q. costaricensis* or *E. grandis* trees around Coto Brus canton and near Cerro de la Muerte, Costa Rica and kept separate by species) and one-third sterilized sand (to facilitate root harvesting) for the fungal inoculum. Similar to the methods described in Meinhardt and Gehring (2012), these root trap cultures were grown for about four months with plant species that formed only one type of mycorrhizal fungal association (*Zea mays* and *Allium* sp. bait plants for AMF; *Pinus caribaea* and *Pinus oocarpa* for EMF), allowing field soil to be used to create AMF- and EMF-only inoculum treatments. Bait plants were checked for fungal colonization and potential contamination prior to use as inoculum for the tree species. Approximately 2.5 g of bait plant root fragments from the appropriate trap culture (either AMF or EMF inoculated roots) were added to pots. For the control treatment, about 2.5 g of autoclaved root fragments from AMF or EMF bait plants were added.

Data analysis

Tree growth was defined as volume, which included both height and basal diameter to more closely approximate tree biomass. Volume was calculated using the geometric equation for a cone, since the trees were grown in full light conditions.

$$\text{Volume} = 0.33\left(\pi * \left(\frac{\text{Basal Diameter}^2}{4}\right) * \text{Height}\right) \quad (1)$$

Both height and basal diameter values of individual trees accounted for initial size effects using a compound interest formula similar to that used to calculate growth in Holste et al. (2011). Although I used basal diameter instead of the standard DBH, volume measurements were standardized across all tree species so that relative differences remained constant.

Comparisons of mycorrhizal fungal type, nutrient treatment, and tissue nutrient concentrations (i.e., reported as a percentage) were tested with one way analyses of variance (ANOVA) with a randomized block design using the R project computing software (R version 3.0.1, The R Foundation for Statistical Computing, http://www.r-project.org). Multivariate analyses of variance (MANOVA) were performed where there were multiple dependent variables (e.g., foliar, stem, and root nutrient concentrations) with Bonferroni corrections made to p-values with multiple comparisons. Calcium, potassium, and magnesium were combined into a composite metric of sum of base cations (SBC) expressed in charge equivalents due to their high correlations to each other and to avoid multicollinearity effects in both soil and plant tissues. To better understand site effects, linear models of initial specific-site soil nutrients were tested against fungal variables (e.g. type and percent root length colonized), tree species growth, and tissue nutrient concentrations.

Since *Q. insignis* was dual-colonized by both fungal types, *Q. insignis'* EMF colonization was examined as a function of *Q. insignis'* AMF colonization. *Q. insignis* individuals above an identity line (i.e., 1:1 line) were considered more EMF associated while those below the line were more AMF, thus dividing *Q. insignis* individuals into "more EMF" or "more AMF." The residuals of the EMF vs. AMF relationship were used test fungal type hypotheses against tree growth and tissue nutrient concentrations within the same species.

In the greenhouse experiment, growth was defined as plant biomass. Host plant responsiveness to mycorrhizae (%) (i.e., relative plant biomass growth as defined by Janos (2007)) was calculated as a ratio of the total dry weight gain from fungal symbiosis (leaf + stem + root in inoculated plant minus non-inoculated plant biomass) to the control (non-inoculated) plant biomass, as calculated in Plenchette et al. (1983) and Egerton-Warburton and Allen (2001):

[(dry wt. AMF or EMF – dry wt. Control)/dry wt. Control] x 100 (2)

Tissue nutrient content (i.e., calculated using nutrient concentrations x dry weight plant mass) was modeled as functions of root or shoot mass to account for tree species differences in plant mass (see Kobe et al., 2010). To test the effects of tree species and fungal type, slope estimates were compared and considered significantly different ($P<0.05$) when they had <29% overlap in 95% support (Austin and Hux, 2002). However, tissue nutrient content was not calculated on field tissue since whole trees were not harvested.

RESULTS

Field experiment

Nutrient treatments were effective in raising soil nutrient levels. Soil N availability was three- to six-fold higher under N, NP, and NPBC treatments ($F_{4,542}=43.59$, $P<0.001$) than the control or P treatments. Likewise, soil P availability was 50 to 60% greater under P, NP, and NPBC treatments ($F_{4,544}=8.37$, $P<0.001$) than the control or N treatments. Soil SBC levels were 10 to 20% higher in the NPBC treatment than the N, NP, and P treatments ($F_{4,544}=6.48$, $P<0.001$). Site effects influenced initial (before transplanting; Table 2.1) and final (after two years of growth) soil nutrient levels, and there was a nutrient treatment by site interaction for final soil N ($F_{28,474}=3.19$, $P<0.001$) and P levels ($F_{28,476}=2.23$, $P<0.001$).

Contrary to **H1**, tree growth did not differ by fungal type but instead by tree species and sites ($F_{3,545}$=70.39, P<0.001; $F_{7,545}$=8.62, P<0.001, respectively). Generally, trees grew well, averaging 60 to 90 cm per year in height and 1.4 to 2.6 cm per year in basal diameter growth across tree species and sites, and a majority of trees survived the two years (96%). *P. caribaea* volume growth was, on average, two-to three-fold higher than the *Q. insignis, S. macrophylla,* and *T. amazonia* ($F_{3,545}$=70.39, P<0.001; Fig. 2.1). Site effects could be partially attributed to the influence of soil SBC on tree growth for *P. caribaea*, *T. amazonia,* and *S. macrophylla,* with greater growth positively correlated with greater SBC levels (Fig. 2.2).

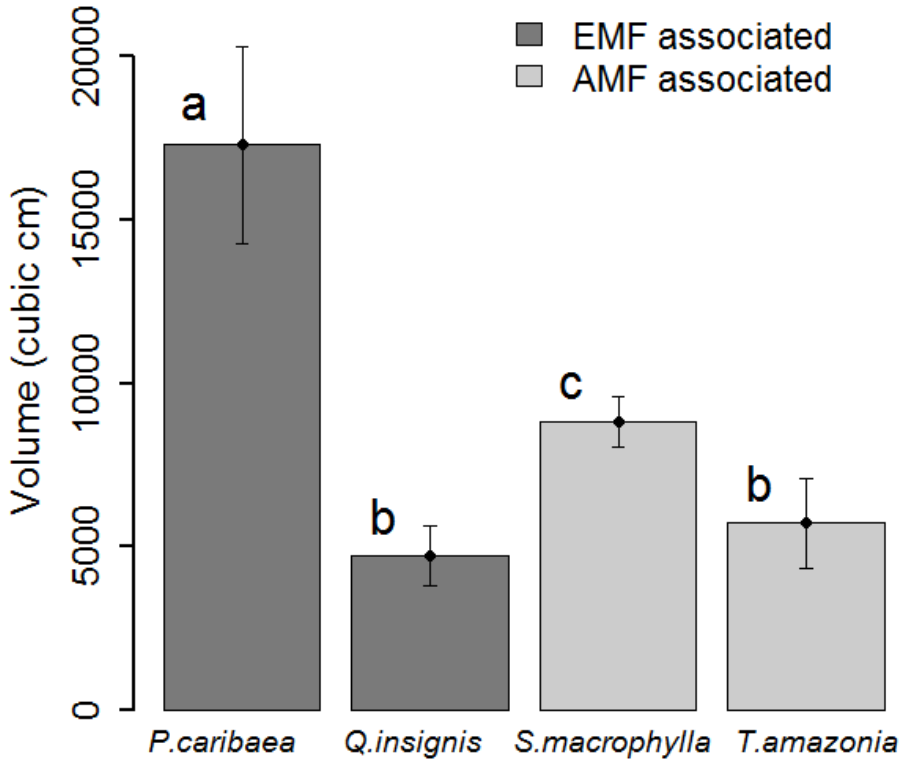

Figure 2.1. Volume differences (accounting for initial height and basal diameter) by species. Lower-case letters represent significant growth differences (P<0.05) between species.

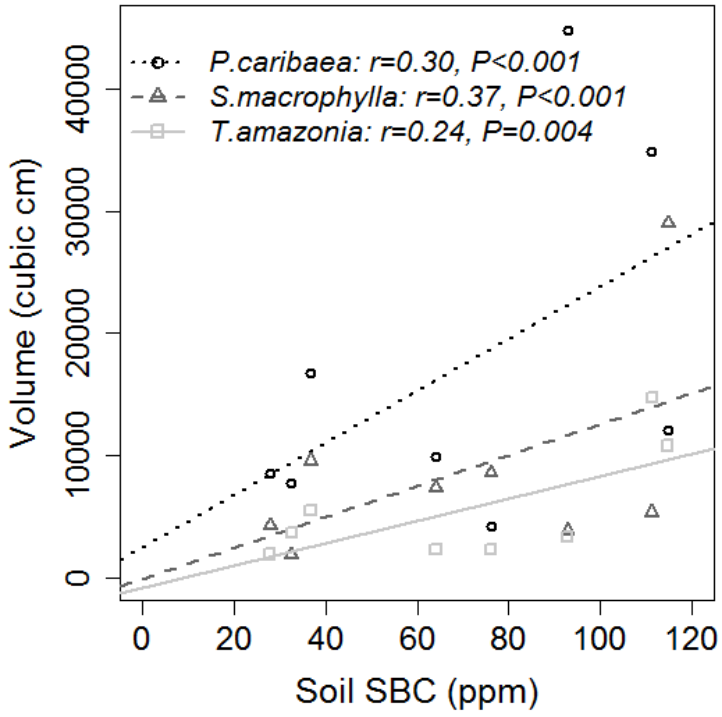

Figure 2.2. Tree species volume as a function of initial soil sum of base cation (SBC) **at each site.**

Tree species, site effects, and nutrient treatments also influenced foliar, stem, and root nutrient concentrations (Table 2.2), but fungal type (**H1**) did not. *S. macrophylla* had approximately 10 to 45% higher foliar N, foliar P, and stem SBC concentrations than the other tree species, and *T. amazonia* has 20 to 80% greater stem N and root SBC concentrations. *P. caribaea* had approximately 50 to 60% higher root P concentrations, whereas *P. caribaea* and *T. amazonia* had 15% greater root N than *Q. insignis* and *S. macrophylla*. *S. macrophylla* and *T. amazonia* had 20 to 120% higher foliar SBC and stem P concentrations than *P. caribaea* and *Q. insignis.* Stem and root concentrations also were influenced by site effects (Table 2.2). In addition, root N concentrations were marginally ($\alpha<0.10$) greater (up to 20%) in N fertilized

trees (Table 2.2), stem P concentrations were 15 to 20% greater under P fertilized trees (Table 2.2), and stem and root N:P concentrations were 25 to 35% greater under N fertilized trees than P fertilized ($F_{4,299}$=2.89, P=0.023; $F_{4,301}$=2.87, P=0.023, respectively).

Table 2.2. MANOVA effects of species, site, and nutrient treatments on tissue nutrient concentrations. P-values in bold type are significant (P<0.05) with a Bonferroni adjustment for multiple comparisons, while P-values with an asterisk (*) are marginally significant (P<0.10).

Tissue	Tree Species		Site		Nutrient Treatment	
nutrient	F-value	P-value	F-value	P-value	F-value	P-value
Nitrogen						
Foliar	11.72	**<0.001**	0.79	1.000	0.99	1.000
Stem	11.70	**<0.001**	0.85	1.000	2.39	0.154
Root	6.82	**<0.001**	4.75	**<0.001**	2.82	* 0.077
Phosphorus						
Foliar	7.66	**<0.001**	1.08	1.000	0.50	1.000
Stem	21.07	**<0.001**	5.29	**<0.001**	3.60	**0.007**
Root	13.80	**<0.001**	8.49	**<0.001**	2.64	0.174
Base Cations						
Foliar	118.58	**<0.001**	2.07	0.140	0.11	1.000
Stem	50.33	**<0.001**	3.56	**0.003**	0.41	1.000
Root	27.41	**<0.001**	1.62	0.386	1.62	0.507

Q. insignis was colonized by both fungal types, providing another opportunity to test fungal type differences without the confounding of tree species host. For Q. insignis, EMF colonization positively correlated with AMF on roots (r=0.78, P<0.001; Fig. 2.3). In contrast to **H1**, *Q. insignis* trees associated with more EMF did not correlate with greater volume growth (r=0.08, P=0.332) or higher tissue nutrient concentrations (Table A2.1).

Acid phosphatase activity and microbial N mineralization did not differ by fungal type, contrary to expectation (**H2)**. Instead, acid phosphatase activity varied up to five fold among sites ($F_{7,546}$=140.5, P<0.001).

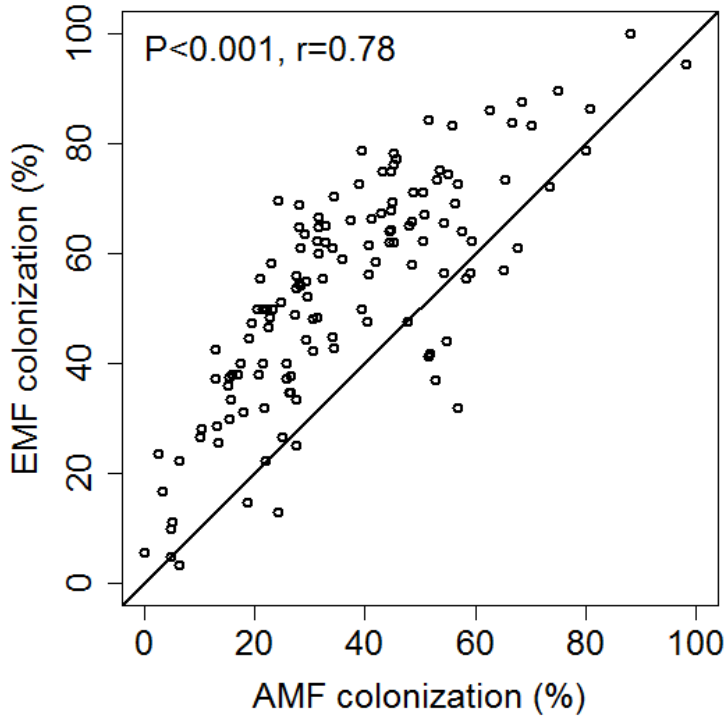

Figure 2.3. Linear relationship between AMF and EMF colonization on the roots of *Q. insignis.* Individuals above the 1:1 line represent plants with greater EMF colonization, whereas individuals below the line are colonzied more by AMF.

Counter to **H3**, nutrient treatments did not influence percent fungal colonization. Tree species affected percent colonization ($F_{3,517}$=86.23, P<0.001) with over half of all trees' roots colonized (*P. caribaea*: 80.9 ± 13.4%; *Q. insignis:* 53.3 ± 20.1% (for EMF) and 36.4 ± 19.2% (for AMF); *S. macrophylla:* 53.2 ± 19.1%; *T. amazonia:* 51.3 ± 18.7%). Across all tree species, the amount of root length colonized was not proportionate to tree size. Sites also influenced percent colonization ($F_{7,517}$=5.26, P<0.001), and specifically AMF colonization ($F_{7,389}$=3.34, P=0.002). Before transplanting, the average number of spores per site ranged between 35 and 92 spores per gram of soil. Five AMF genera were identified across all sites: *Glomus, Acaulospora, Archaeospora, Gigaspora,* and *Scutellospora*. *Glomus* was the most commonly found genus across all sites averaging 45 spores per gram of soil, while *Acaulospora* was the second most

abundant with 12 spores per gram of soil. Percent fungal colonization, specifically AMF, also weakly correlated with tissue nutrient concentrations, with higher colonization positively correlated with greater root N (r=0.17, P=0.002) and root P (r=0.11, P=0.061) concentrations and negatively correlated with lower root SBC (r= -0.26, P<0.001) concentrations.

Complementary greenhouse experiment

Contrary to the field experiment, *S. macrophylla* plants (AMF and non-inoculated) had greater total plant biomass than *P. caribaea* plants (EMF and non-inoculated; $F_{3,119}$=18.46, P<0.001; **H1**). *S. macrophylla* plants also had higher shoot N and higher SBC levels but lower root N concentrations than *P. caribaea* plants at a given level of plant mass, which supported that tree species' differences in N and SBC acquisition was stronger than fungal effects (Fig. 2.4, Table A2.2). No differences were found between fungal type and acid phosphatase activity (**H2**) or percent fungal colonization and nutrient treatments (**H3**). Nevertheless, the growth of *S. macrophylla* plants negatively responded (in biomass growth) to their AMF symbionts under most nutrient treatments (in ascending order of N:P applied: -136% for low N:P; -52% for high N and P; 2% for low N and P; and -16% for high N:P; Fig. 2.5), whereas *P. caribaea* plant growth generally positively responded to EMF (low N to P: 31%; high N and P: 88%, high N to P: -47%; low N and P: 203%; Fig. 2.5). Root biomass was greater in *S. macrophylla* under the low N:P treatment ($F_{3,68}$=2.89, P=0.042), but plants did not differ in root to shoot ratios among mycorrhizal associated and non-inoculated. Mean host plant responsiveness to mycorrhizae in both species was a humped-shaped relationship (as best supported by the data according to AIC)

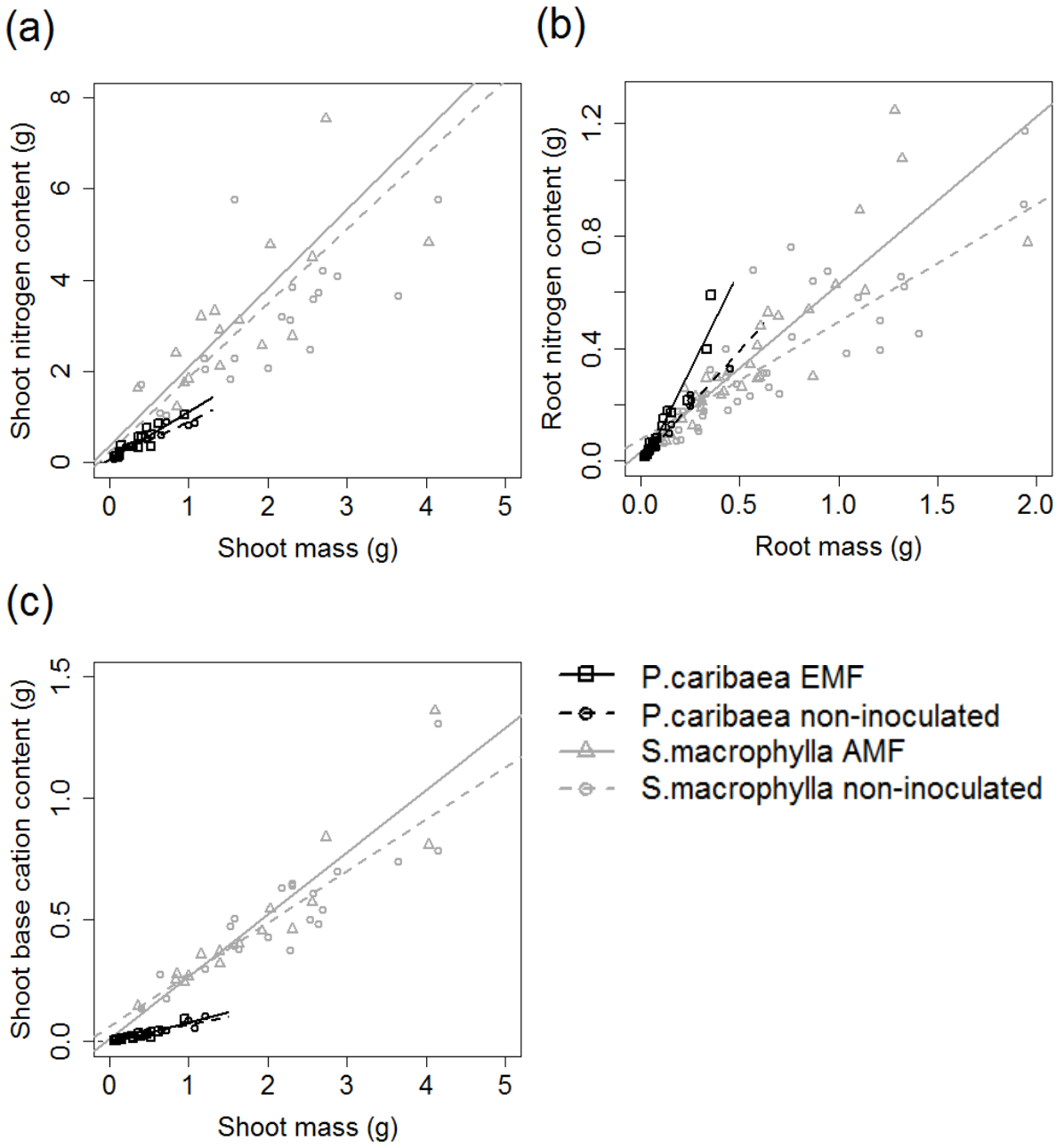

Figure 2.4. Greenhouse comparisons of tissue nutrient content as functions of plant mass by mycorrhizal fungal type and their respective non-inoculated controls.

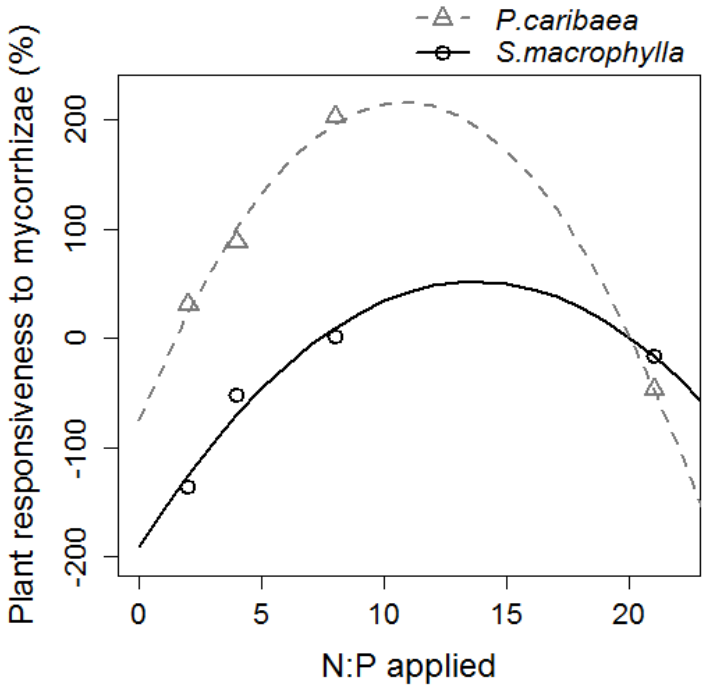

Figure 2.5. Greenhouse comparisons of mean plant responsiveness to mycorrhizae as a function of nutrient treatments by their ratio of nitrogen (N) to phosphorus (P) applied across species. Positive plant responsiveness indicates that plants utilized their fungal symbiont for greater plant growth, whereas negative plant responsiveness denotes smaller fungal associated plants compared to non-inoculated plants.

with the ratio of N to P in nutrient treatments (r=0.87, P=0.210 for *S. macrophylla*; r=0.98, P=0.090 for *P. caribaea*; Fig. 2.5). Greenhouse fungal colonization was 25-30% of field root length colonized in *S. macrophylla* and *P. caribaea*.

DISCUSSION

Contrary to this study's hypotheses, no support was found for mycorrhizal fungal type influencing nutrient uptake through aboveground tree growth and tissue nutrient concentrations (**H1**) or rhizosphere effects (**H2**) in both the field and greenhouse. Instead, changes in soil nutrient status (i.e., site effects and nutrient treatments) influenced tree species' nutrient

acquisition. I expected EMF associated trees to have greater nutrient uptake due to their saprophytic capabilities compared to AMF-trees; however, the advantage of EMF symbioses may be dependent upon the organic matter content of sites. In addition, EMF associated trees may produce lower foliar nutrient concentrations and greater litter recalcitrance than AMF-trees (Read 1991; Read and Perez-Moreno 2003; Phillips et al. 2013), but this is based upon evidence from temperate forests where a disproportionate number of EMF-trees are coniferous (Cornelissen et al. 2001; Hobbie et al. 2006; Averill et al. 2014; Dickie et al. 2014). In the tropics, where the quantity of organic matter is generally low (Ross 1993) and EMF associated trees are not dominated by conifers (Alexander and Hogberg 1986; McGuire et al. 2008), changes in soil nutrient availability may be more important to tree species' nutrient acquisition than mycorrhizal fungal type. Thus, from this study, I conclude that mycorrhizal fungal type (EMF vs. AMF) would not influence tropical reforestation; I cannot, however, directly evaluate the overall importance of fungal-tree symbioses (inoculated vs. non-inoculated trees) in reforestation.

Although *Quercus sp.* are generally considered EMF (Trappe 1962; Wang and Qui 2006; but see Egerton-Warburton and Allen 2001), dual colonization by both fungal types (mean colonization of 53% for EMF and 36% for AMF) in *Q. insignis* roots provided another test of fungal type effects on tree growth and tissue nutrient concentrations, without the confounding effects of differences in tree species hosts. I acknowledge that single inoculations of EMF- and AMF-only would more effectively separate fungal type differences from tree species effects; however, maintaining pure or dominant colonization by one fungal type would be extremely challenging under field conditions. Nonetheless, within *Q. insignis*, the relative colonization by fungal type did not influence tree growth (**H1**) or tissue nutrient concentrations (**H2**). Previous

studies found that dual colonization varied in its effects on percent colonization of both fungal types (compared to AMF- or EMF-only), nutritional effects, and plant growth (Lodge and Wentworth 1990; Egerton-Warburton and Allen 2001; Founoune et al. 2002; Kariman et al. 2012), but I could not determine whether dual-colonization improved or depressed *Q. insignis* growth since I did not isolate the specific fungal types. Additionally, since *Q. insignis* received EMF soil inoculum, which also could have contained AMF, *Q. insignis* roots may have been exposed to a different AMF community than this research's designated AMF-tree associations (Aldrich et al. 2007), which could have affected *Q. insignis'* AMF-tree relationship (Klironomos 2003).

Counter to **H3,** mycorrhizal fungi, both EMF and AMF, did not respond to nutrient treatments in the field and greenhouse. Although reduced root colonization with fertilization is commonly assumed, increased or no changes in fungal colonization also have been found (Treseder 2004; Treseder 2013). Changes in root colonization are typically interpreted as alterations to host plants' C allocation and, subsequently, to reliance on fungi for nutrient uptake. Yet empirical evidence for nutrient effects on colonization is weak, suggesting that colonization is most likely governed by both plants and fungi (Maherali and Klironomos 2007; Kiers et al. 2011).

Tree growth and tissue nutrient concentrations did respond to nutrient treatments. In the field, approximately 20% increases in tissue nutrient concentrations occurred under fertilization, but the lack of growth response suggests that fertilization levels were inadequate to relieve nutrient constraints to growth. In the greenhouse, on the other hand, the growth of both EMF and AMF associated tree species responded to the ratio of N to P in nutrient treatments. As the ratio of N to P applied decreased (i.e., more N-limited), tree growth response to fungal symbionts

was more negative (Fig. 2.5), which could indicate greater competition between the fungus and plant for plant C. This is consistent with previous research that suggests that N-limitation reduces plant photosynthetic capacity and consequently C supply to fungi (Hoeksema et al. 2010; Johnson 2010), and thus plants may experience negative growth (Reynolds et al. 2005). However, when trees were no longer limited by N (i.e., high N:P), unnecessary mycorrhizae also were less likely to provide plant growth benefits (Fig. 2.5; Johnson 2010). This concept of a hump-shaped relationship with the ratio of N to P is consistent with prior studies (e.g., Treseder and Allen 2002; Grman and Robinson 2013), in which plant growth attributed to fungal symbionts was low in the nutrient treatment with the lowest N:P ratio, increased with greater N:P additions, but then decreased again at the highest N:P ratio. Thus the ratio of N to P experienced by these symbiotic relationships may be more important to tree growth than the available quantities of particular nutrients particular nutrients.

In addition, this study highlights site effects on tree growth, tissue nutrient concentrations, acid phosphatase activity, and fungal colonization more than hypothesized fungal-tree symbioses. Strong site-specific differences are common in reforestation studies that have been conducted across several sites (Calvo-Alvarado et al. 2007; Wishnie et al. 2007; Holl et al. 2011). Tree growth differences among sites could be partly explained by variation in base cations, which is consistent with previous studies that found increased seedling growth with greater base cation availability (Holste et al. 2011; Wright et al. 2011; Yavitt et al. 2011). Base cations are important in many chemical and physical processes affecting tree growth, such as magnesium as a structural component of chlorophyll, calcium as a correlate of photosynthetic rates (St. Clair and Lynch 2005), and the roles of potassium and calcium in signal transduction and nutrient uptake (Stevens et al. 1993). However, since these results were correlative, I was

unable to attribute site effects on growth to base cations. The results demonstrate the need to replicate research across multiple sites with different soil nutrient availabilities to better understand site effects on reforestation.

Differences in phosphatase activity across sites also are consistent with site-specific variation found in previous multi-site studies (Phillips and Fahey 2006; Yin et al. 2014). Though site-specific differences in rhizosphere effects are typically attributed to nutrient availability and mycorrhizal fungal associations of the dominant trees (Phillip et al. 2013), in this study, phosphatase activity was not correlated with measured soil nutrient concentrations or fungal type. Although it is unclear which site factors contributed to differences in phosphatase activity, the strong differences in phosphatase activity among sites (R^2=0.64) may have masked any potential differences between EMF- and AMF-tree symbioses.

Variation among particular fungal-tree combinations similarly may have obscured fungal type differences. EMF can differ in their mycelial foraging strategies (Cairney 1999) and enzyme production (Conn and Dighton 2000; Baxter and Dighton 2005), which can affect the ability to acquire nutrients for host trees. Variation in plant growth responses to AMF also can arise from particular AMF-plant associations that differ in benefits (Bever 2002; Klironomos 2003). Thus, mycorrhizal fungal and plant species groupings could provide greater resolution on growth effects than fungal type.

One limitation to this study's field comparisons of AMF- and EMF-tree symbioses was that fungal effects were confounded by tree species effects, since I used different tree species with different growth and nutritional physiology. Typically, gymnosperms are thought to have faster growth than angiosperms (but see Cernusak et al. 2008). Of the four tree species, *P. caribaea* was the only gymnosperm and its growth was ten-fold higher than the other three

species. Surprisingly, the overall growth of *P. caribaea* relative to *S. macrophylla* in the field was opposite to greenhouse measurements; however, plants in the greenhouse were allowed to grow for only three months versus two years in the field and ontogeny could confound greenhouse-field differences.

In the greenhouse, species differences in initial seed size (Allsop and Stock 1995; Gehring 2004) could explain species differences in plant growth responses to mycorrhizae. Seedlings with large seed reserves tend to have lower growth responses to fungal symbionts than small-seeded species, especially under nutrient-limited conditions, because they have sufficient nutrient reserves for early seedling growth without mycorrhizae (Siqueira et al. 1998; Zangaro et al. 2000). *S. macrophylla* seed weight was approximately 21 times greater than *P. caribaea,* and *S. macrophylla* growth responsiveness to AMF during early seedling growth was lower than the growth of *P. caribaea* to EMF across all nutrient treatments (Fig. 2.5). Moreover, a majority of *P. caribaea* plants responded positively to their fungal symbiont except under the highest N:P nutrient treatment, while *S. macrophylla* growth responded negatively to their mycorrhizae (Fig. 2.5), implying that smaller-seeded *P. caribaea* plants were more dependent upon their fungal symbionts under nutrient-limiting conditions than *S. macrophylla*.

Conclusions

In order to effectively incorporate mycorrhizal fungi into a reforestation strategy, a better understanding of the interactions among fungi, tree growth, and nutrient uptake is needed. In this study, I found that tree species and site nutrient conditions drove differences in aboveground tree growth rather than mycorrhizal fungal type. *P. caribaea*, in particular, had the highest growth across sites, suggesting that it may be important in initial forest cover and facilitating the

establishment of late-successional tree species (e.g., Ashton et al. 1997). While EMF symbioses may possess numerous processes for increased nutrient uptake compared to AMF, their benefit to reforestation was not supported here. Similar to variability in species-specific tree growth, fungal symbionts can vary in their nutrient acquisition capabilities (Burgess et al. 1993), and we cannot generalize that trees associated with a particular fungal type will similarly increase nutrient uptake and growth. This study highlights the importance of tree species selection and replication across multiple sites with different soil nutrient availabilities to better understand the potential for success in restoring degraded Neotropical landscapes.

CHAPTER III:

PLANT SPECIES DIFFER IN EARLY SEEDLING GROWTH AND TISSUE NUTRIENT RESPONSES TO ARBUSCULAR AND ECTOMYCORRHIZAL FUNGI

ABSTRACT

The relative benefits that mycorrhizal fungi confer to host plants may be sensitive to changes in fungal type (arbuscular (AMF) or ectomycorrhizal fungi (EMF)) and environmental conditions. In order to understand the role of fungal type on plant growth and nutrient acquisition, experiments with species that can host both AMF and EMF (dual fungal-plant symbioses) are critical to separating fungal from plant species effects. This research's objective was to examine the effects of fungal type in relation to the growth and tissue nutrient content of two dual inoculated tree species (*Eucalptus grandis* and *Quercus costaricensis*) grown under four nutrient treatments in the greenhouse. I did not find consistent effects of fungal type on plant growth and tissue nutrient content. In *E. grandis,* fungi had a positive effect on belowground plant biomass but negative effect on aboveground biomass, and AMF-plants had greater foliar phosphorus, root nitrogen, and root base cation content compared to EMF- or non-inoculated plants. Conversely, *Q. costaricensis* growth responded more to EMF symbioses. These results demonstrate that mycorrhizal fungal type may differ in their influence on tree performance. However, tree species' characteristics, especially during early seedling establishment, can affect how AMF and EMF interact with their host plants.

INTRODUCTION

The majority of trees establish symbiotic relationships with either arbuscular mycorrhizal fungi (AMF) or ectomycorrhizal fungi (EMF), and a few tree species may form dual-associations with both fungal types (Smith and Read 2008). These fungal-plant symbioses, primarily based upon the transfer of plant carbon (C) and mineral nutrients, are important for plant growth in many nutrient-poor ecosystems (van der Heijden et al. 2008). Yet the relative benefits that AMF and EMF symbionts confer to host plants may differ. While a considerable amount of mycorrhizal fungal research has focused on the effects of either AMF or EMF on plant growth, relatively few studies have contrasted fungal types (but see Jones et al. 1998; Chen et al. 2000; Egerton-Warburton and Allen 2001; van der Heijden and Kuyper 2001). Moreover, isolating the effects of the fungal symbiont from the plant species is difficult, especially under complex field conditions. Dual fungal-plant symbioses can help to separate and clarify the similarities and differences of AMF and EMF on plant growth and nutrient acquisition, without the confounding effects of differences in plant species hosts.

Although both EMF and AMF can improve nutrient acquisition to host plants, the benefits are not necessarily equal. Morphologically, EMF differentiate from AMF by forming a thick sheath of fungal mycelium around their host plants' roots and a hyphal network (i.e., Hartig net) that extends between root cells, while AMF penetrate into the root cells of its host plants and characteristically form arbuscules (branch-like structures that are key sites for nutrient exchange between the fungus and host plant). Functionally, EMF may have a competitive advantage over AMF in nutrient acquisition and uptake under nutrient-poor conditions. EMF can directly break down leaf litter (Malloch et al. 1980), secrete enzymes that break down complex substrates such as mineral or organic nutrients (e.g., acid phosphatase enzyme; Hodge et al. 1995, Blum et al.

2002), stimulate microbial N mineralization in the soil which may facilitate greater nutrient availability to its host plants (Chalot and Brun 1998), and produce up to seven times more hyphae than AMF in nutrient-poor soils (Jones et al. 1998) which can increase the explored soil volume for nutrients and the absorbing soil surface area. AMF generally do not have saprophytic capabilities (but see Ezawa et al. 2005; Leigh et al. 2009) but can still deliver up to 80% of their host plant's P and 25% of their N requirements (Marschner and Dell 1994).

The nutrient status of a particular site also is critical to determining the relative benefits derived from fungal-plant symbioses. Under nutrient-limiting conditions, host plants generally benefit from the increased uptake of soil nutrients by their fungal symbionts (Smith and Read 2008), and plant dependence on fungal-acquired nutrients may increase after nutrient depletion events such as erosion or land use change (e.g. from forest to annual row crop production). Thus, host plants theoretically should allocate less C to root biomass with increases in fungal-acquired nutrients, since increases in nutrient availability generally decreases plant belowground allocation (Hermans et al. 2006). Under nutrient-rich conditions, host plants may decrease unnecessary fungal associations (Treseder 2004) or fungal symbionts may become parasitic and act as C drains on plant resources (Johnson et al. 1997; Allen et al. 2003). Since up to 20% of a plant's total C budget may be allocated to its AMF symbiont and up to 30% its EMF symbiont (Rygiewicz and Andersen 1994), declines in EMF abundance may increase plant growth more than a release from AMF symbioses (Read 1991). Mycorrhizal fungal-plant symbioses, on the other hand, may respond more to the ratio of nutrients than to the overall quantity of each nutrient (Hoeksema et al. 2010; Johnson 2010; Johnson et al. 2015), which could determine whether or not symbiotic benefits outweigh their costs. However, there has been wide variation in fungal-plant interactions with nutrient additions (Treseder 2004; Hoeksema et al. 2010).

Mycorrhizae may be particularly important to nutrient uptake and tree growth in tropical areas, where large-scale deforestation and land degradation threaten many forests (Lamb et al. 2005) and reduce soil nutrient availability (Sahani and Behera 2001). In certain areas of Costa Rica, for example, mycorrhizal fungi have improved tree growth and the rehabilitation of deforested lands (Carpenter et al. 2001; Allen et al. 2003). Hence, with up to 36% of tropical and subtropical forests expected to be lost by 2050 (Wright 2010), understanding this symbiotic relationship may be critical to future tropical reforestation efforts.

In this study, two tree species (*Quercus costaricensis* and *Eucalptus grandis)* were grown in a greenhouse without mycorrhizal fungi, with AMF, or with EMF and under four different nutrient treatments to test the relative effects of fungal type and soil nutrient status on host plant growth and tissue nutrient content during early seedling establishment. These tree species were chosen because both oaks (*Quercus* spp.) and eucalyptuses (*Eucalpytus* spp.) may associate with AMF and EMF (Smith and Read, 2008) and are ubiquitous in tropical ecosystems. Since N and P are generally considered the two most limiting nutrients for tree growth (Vitousek 2004), the amount of N and P applied varied (low vs. high concentrations) by nutrient treatment and represented four different N:P ratios. I hypothesized that: (**H1**) plants associated with mycorrhizal fungi (EMF or AMF) allocate less biomass to roots, due to fungal symbionts assisting in nutrient acquisition, and (**H2**) percent fungal colonization in both EMF and AMF decrease with increasing soil concentrations of N and/or P. I also hypothesized that (**H3a**) EMF associated plants have (**1**) greater nutrient uptake (i.e., as reflected in higher tissue nutrient content and/or tree growth) and (**2**) greater acid phosphatase (enzyme) activity than AMF-plant symbioses. Alternatively, (**H3b**) AMF associated plant growth could be greater than EMF-plants due to higher C costs associated with EMF-plant symbioses.

METHODS

Experimental design

I used a complete randomized design of 3 fungal treatments (non-inoculated (control), AMF, EMF) x 4 nutrient treatments (2 nitrogen levels (low and high) x 2 phosphorus levels (low and high)). Seeds were surface-sterilized (10% bleach solution) and planted in 85% commercial soil mix containing peat moss, perlite, and vermiculite (Fafard #2, BFG Supply Co., Kalamazoo, MI, USA) plus 15% sterilized sand. One hundred forty-four seeds of each tree species (12 replicates per treatment) were planted in sterilized 2.8-L pots (Tall-One Treepots, Stuewe and Sons, Inc., Tangent, OR) to allow for unconstrained root growth. Seed weights were recorded, and seeds were allowed to germinate in the pots for 1-2 months before the start of the experiment (November-December 2012).

Seedlings were grown for approximately three months in the greenhouse (January-March 2013) at Michigan State University (42°42'7" N, 84°28'56" W) in full light and watered to field capacity as required. Daylight was kept at a constant twelve hour day schedule (6am-6pm, EST) and temperatures ranged between 18 to 24°C, similar to conditions that seedlings would experience in Costa Rica. A modified Hoagland's solution was applied in a diluted dose twice a week (to ensure nutrient levels throughout the experiment) with two levels of N (100 and 250 mg N per liter) and P (12 and 62 mg P per liter) in four different nutrient treatments: 1) low N and P, 2) high N and P, 3) low N:P ratio (low N + high P), and 4) high N:P ratio (high N + low P). Plastic bags enclosed the bottom of the pots to reduce nutrient loss and prevent cross contamination between nutrient and fungal treatments (Brundrett et al. 1996).

Tree species

Two tree species were chosen for this study: *Quercus costaricensis* Liebm. (Fagaceae) and *Eucalptus grandis* (W. Hill) ex Maiden (Myrtaceae). Species of *Quercus* and *Eucalyptus,* although predominately EMF associated as mature trees, also may form AMF symbioses during the seedling stage (Egerton-Warburton and Allen 2001; Adams et al. 2006). *Q. costaricensis* is a species of oak endemic to the montane forests of Costa Rica. *Eucalyptus* spp. are native to Australia, but these tropical species are found throughout Costa Rica in plantations and on private properties. *Quercus* seeds were collected in Costa Rica at Los Nimbulos Biological Station, near Cerro de la Muerte (9.5667° N, 83.7500°W), and *Eucalyptus* seeds were obtained from a university-based seed bank in Costa Rica (Centro Agronómico Tropical de Investigación y Enseñanza's Banco de Semillas Forestales; http://www.catie.ac.cr/). Average seed weight was 0.023 mg for *E. grandis* (calculated as the average from 1000 seeds) and 16.60 ± 5.31 g for *Q. costaricensis.*

Fungal inoculum

I trapped AMF and EMF in the roots of bait plants cultivated in a combination of two-thirds field soil (collected from under mature *Q. costaricensis* or *E. grandis* trees around Coto Brus canton and near Cerro de la Muerte, Costa Rica and kept separate by species) and one-third sterilized sand (to facilitate root harvesting) for the fungal inoculum. These root trap cultures were grown in a greenhouse at Las Cruces Biological Station in Coto Brus, Costa Rica (8°47'7" N, 82°57'32" W) with plant species that formed only one type of mycorrhizal fungal association (*Zea mays* and *Allium* sp. for AMF; *Pinus caribaea* and *Pinus oocarpa* for EMF), allowing field soil to be used to create AMF- and EMF-only inoculum treatments similar to the methods

described in Meinhardt and Gehring (2012). Bait plants were allowed to grow for about four months (May-October 2012) and were checked for mycorrhizal fungal colonization and potential contamination prior to use as inoculum for the tree species. Fresh roots with mycorrhizal fungi were kept cool during transport to Michigan State University.

Approximately 2.5 g of bait plant root fragments from the appropriate trap culture (either AMF or EMF inoculated roots) were immediately added to pots with germinating seeds in a category 3 ("complete containment") greenhouse at Michigan State University (November 2012). For the control treatment, about 1.25 g of autoclaved root fragments from both AMF and EMF bait plants were added. Sterile crushed granite was layered on top of the pots to reduce escaping spores and avoid cross contamination between treatments (Brundrett et al. 1996).

Tree growth, fungal, and tissue nutrient content measurements

All components of the seedlings (i.e., leaves, stems, and roots) were harvested after three months (April 2013) due to the roots beginning to experience space constraints. Root systems were carefully hand washed with deionized water to preserve fine root mass and remove all soil material. A portion of the fresh roots were subsampled, cleared with a 10% sodium hydroxide solution, and stained with Schaeffer's ink and vinegar method (Vierheilig et al. 1998) for fungal colonization analyses. For AMF, percent root length colonized was scored using a modified gridline intersections method from approximately 20 cm of root per sample, when available (McGonigle et al. 1990). For EMF, percent root tips colonized was scored from about 20 cm of root per sample randomly selected and examined at 40X magnification for the number of EMF root tips (Gehring and Whitham 1991). Non-inoculated plants were examined for potential fungal contamination.

Leaves, stems, and the remaining roots (not subsampled for percent colonization) were dried at 65^0C and dry mass was measured for each component. To account for the root tissue used for percent colonization in the total plant root mass, the subsampled root dry weight was calculated by multiplying the dry weight ratio for each individual plant by the fresh mass and then summed to obtain the total dried root mass. The remaining roots as well as all leaves and stems were pulverized into a fine powder via a ball-bearing mill (Kleco, Visalia, CA) or grinding mill (Christy and Norris Laboratory mill, Christy Turner Ltd., Ipswich, Suffolk, UK). Leaf, stem, and root C and nutrient content (N, P, calcium (Ca^{+2}), potassium (K^{+}), and magnesium (Mg^{+2})) were analyzed in each sample to determine available soil nutrients accumulated within each component. All tissue concentrations were expressed as oven-dried mass (i.e., nutrient content). Total C and N in each component was analyzed using the dry combustion method (Elemental Combustion CHNS-O analyzer, ECS 4010, Costech Analytical Technologies, Valencia, CA). Phosphorus and base cations (Ca^{+2}, K^{+}, and Mg^{+2}) were extracted from each tissue component with a 5% nitric acid solution and block digester (AIM600 Block Digestion System, A.i. Scientific Pty Ltd., Clontarf, Queensland, Australia), and then analyzed spectrophotometrically using an Optima 2100DV Induced Coupled Plasma (ICP) Optical Emission Spectrometer (Perkin-Elmer, Shelton, CT).

Soil nutrients and enzyme activity

Soil was collected at the time of harvest for nutrient analyses and to estimate fungal enzyme activity. Total extractable P and base cations were extracted using Mehlich III, a weak acidic solution (Mehlich 1984), and analyzed using the ICP Spectrometer. Nitrate and ammonium were extracted with a 2M potassium hydroxide solution and analyzed colorimetrically using a

continuous flow auto-analyzer (ELx808 Absorbance Microplate Reader, BioTek Instruments, Inc, Winooski, VT). Fungal enzyme activity was analyzed using rhizosphere soil, the soil adhering to the roots. This soil was collected from the roots of each individual seedling and the concentration of *p*-nitrophenol released by acid phosphate activity was determined colorimetrically with the Absorbance Microplate Reader via the methods described in Phillips and Fahey (2006). For each sample, a second control sample was incubated and extracted using the same procedure to correct for color development from the background soil matrix. Acid phosphatase activity was estimated by calculating the amount of *p*-nitrophenol released after correcting for the background soil matrix.

Data analysis

Tree growth was defined as the dry weight biomass. Host plant responsiveness to mycorrhizae (%) (i.e., relative plant biomass growth as defined by Janos (2007)) was calculated as a ratio of the total dry weight gain from fungal symbiosis (leaf + stem + root in inoculated plant minus non-inoculated plant biomass) to the control (non-inoculated) plant biomass, as calculated in Plenchette et al. (1983) and Egerton-Warburton and Allen (2001): [(dry wt. AMF or EMF – dry wt. Control)/dry wt. Control] x 100.

I used an analysis of variance (ANOVA) to analyze the effects of fungal treatment and nutrient treatment on plant growth and tissue nutrient content using the R project computing software (R version 3.0.1, The R Foundation for Statistical Computing, http://www.r-project.org). Where there were multiple dependent variables (e.g., leaf, stem, and root nutrient content), multivariate analyses of variance (MANOVA) were performed. As a result of low germination and survival rates, not all treatments had equal numbers of seedlings, creating an

unbalanced design; type I and III sum of squares were compared to ensure good model fits. Iused linear regression to analyze the effects of percent fungal colonization and acid phosphatase activity on tissue nutrient content and plant growth. To avoid multicollinearity effects, calcium, potassium, and magnesium concentrations were combined into a composite metric of sum of base cations (SBC) expressed in charge equivalents in both soil and plant tissues. I modeled tissue content variables as functions of root or shoot mass to account for fungal treatment differences in plant mass (see Kobe et al. 2010). To test the effects of fungal type, I compared slope estimates; slopes were considered significantly different ($P<0.05$) when they had <29% overlap in 95% support (Austin and Hux 2002).

RESULTS

Contrary to **H1**, fungal associated *E. grandis* (AMF and EMF) had greater root but lower shoot biomass than non-inoculated controls (Fig. 3.1, Appendix 3.A), but allocation to roots vs. shoots in *Q. costaricensis* did not differ ($F_{2,127}=0.10$, $P=0.902$).

Percent colonization was not affected by nutrient treatments, in contrast to **H2**. Percent fungal colonization rates were low (approximately 12% for AMF and 6% for EMF) across species, and control plants were not colonized by either fungal type. Additionally, nutrient treatments did not influence tree growth but affected tissue P levels, with higher P in the low N:P (low N + high P) treatment than the high N:P (high N + low P) and low N and P treatments in *E. grandis* leaves ($F_{3,117}=5.65$, $P=0.001$) and *Q. costaricensis* stems ($F_{3,95}=5.90$, $P=0.001$). Interactions between nutrient treatments and fungal treatments resulted in marginally ($\alpha<0.10$) higher leaf P under the low N:P ratio and AMF treatments for *E. grandis* ($F_{6,117}=1.93$, $P=0.082$). Host plant responsiveness to mycorrhizae also was marginally negatively correlated with the N:P

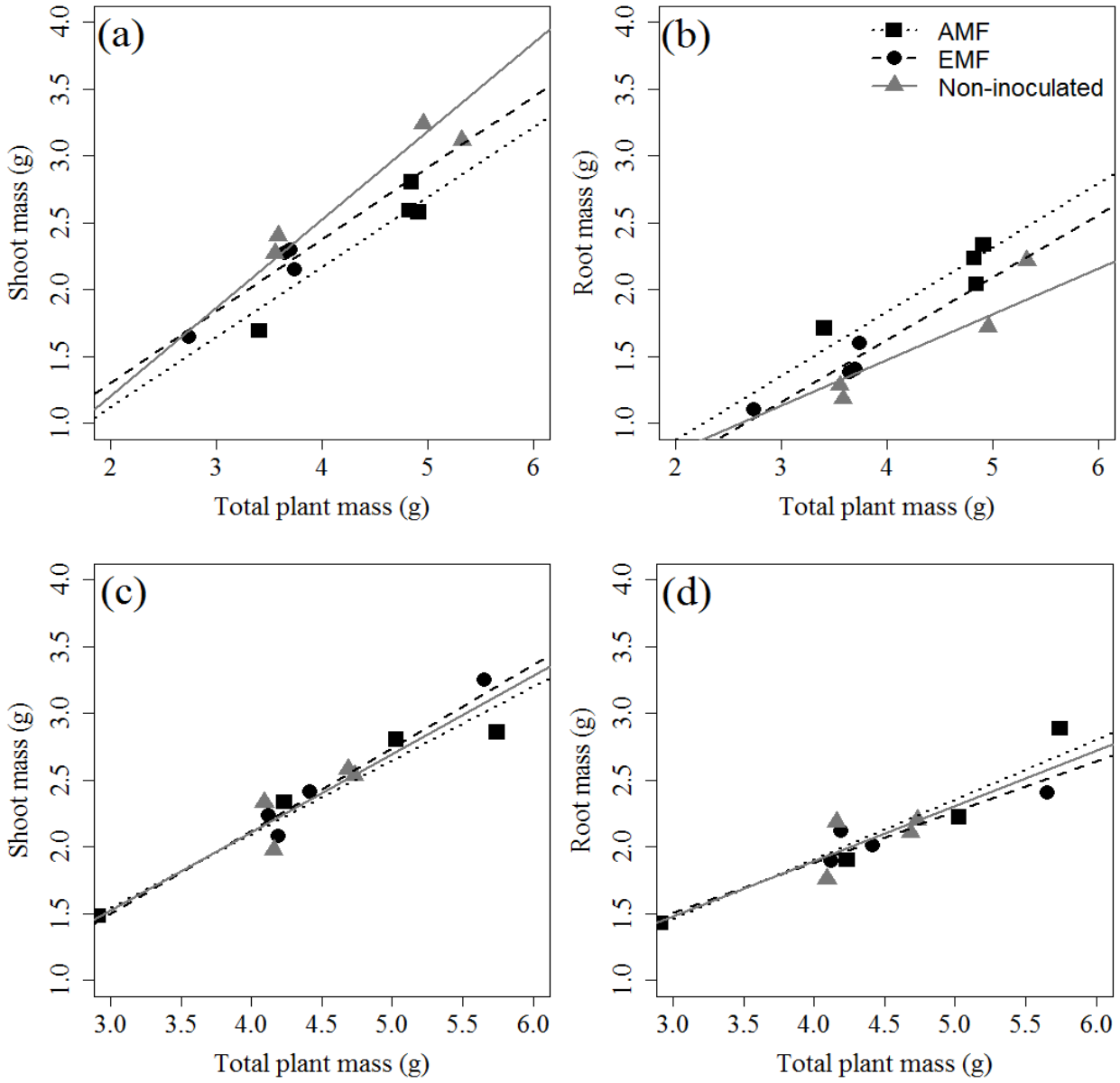

Figure 3.1. Shoot and root mass as functions of total plant mass by mycorrhizal fungal treatment and tree species, whereas (a) *Eucalyptus grandis* shoot:total mass, (b) *E. grandis* root:total mass, (c) *Quercus costaricensis* shoot:total mass, and (d) *Q. costarciensis* root:total mass. For purposes of clarity, each point represents the mean mass by nutrient and fungal treatments, and lines represent the best fit linear relationship across all points.

ratio of nutrient additions (r = -0.44, P=0.095) across species. Plant responsiveness was calculated without reference to particular nutrient availability (as suggested in Janos 2007), since this study's nutrient treatments did not influence tree growth.

Counter to **H3a,** AMF associated *E. grandis* had greater overall tissue P content compared to EMF- or control plants, mainly driven by higher foliar P ($F_{2, 126}$=4.50, P=0.012; Fig. 3.2). AMF associated *E. grandis* also had greater root N ($F_{2, 138}$=7.79, P<0.001) and root SBC ($F_{2,133}$=8.57, P<0.001) mass (Fig. 3.2). Due to variation in biomass allocation by fungal treatments, all *E. grandis* tissue nutrient contents were checked for differences in allometric relationships (Appendix 3.B). In *Q. costaricensis*, tissue nutrient contents were not correlated with fungal treatments.

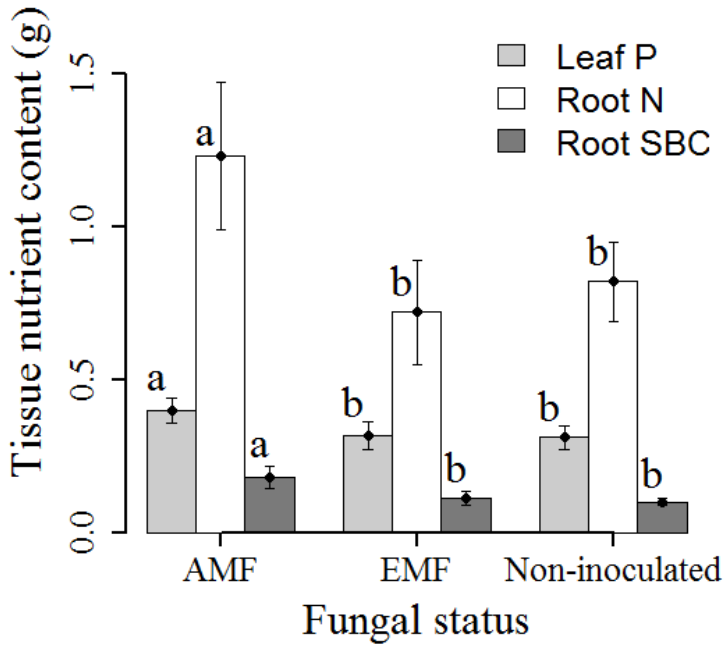

Figure 3.2. Leaf phosphorus, root nitrogen, and root sum of base cation content by fungal status in *Eucalyptus grandis*. Bars represent standard errors and letters indicate significant differences between fungal treatments (P<0.05).

Although average total plant biomass of *E. grandis* and *Q. costaricensis* was similar ($F_{1,269}$=2.18, P=0.141), plant species differed in their responses to fungal symbionts. Total plant biomass in *E. grandis* was lower in EMF-plants than non-inoculated and AMF-plants ($F_{2,138}$=3.59, P=0.030), in concurrence with **H3b.** *E. grandis* also demonstrated a negative responsiveness to EMF (-19.91%) but a positive plant responsiveness with AMF (5.32%). Conversely, total plant biomass in *Q. costaricensis* was similar across all fungal treatments ($F_{2,127}$=0.10, P=0.902), but plants inoculated with EMF had a larger positive responsiveness (5.00%) than AMF-plants (1.59%), consistent with **H3a**. In both species, neither plant growth nor tissue nutrient levels were correlated with acid phosphatase activity (**H3a**).

DISCUSSION

I did not find consistent effects of mycorrhizal fungal type (AMF or EMF) on plant growth and tissue nutrient content across species. Instead, plant responses to fungal symbionts differed between plant species and between types of fungal symbionts, reinforcing the highly context-dependent nature of the symbiosis (Klironomos 2003; Bever et al. 2009). Fungal associated *E. grandis* plants had a positive effect on belowground biomass (approximately 30% greater) but negative effect on aboveground biomass (smaller by 10%). *E. grandis'* AMF-plants, in particular, had approximately 20% greater tissue P, 30% greater tissue SBC, and 15-50% greater tissue N contents compared to EMF- or non-inoculated plants. Conversely, *Q. costaricensis* plants associated with EMF, on average, had greater biomass response to their symbiont (3%) than AMF plants. Previous studies with *Eucalyptus* spp. (Jones et al. 1998; Chen et al. 2000) or *Quercus* sp. (Egerton-Warburton and Allen 2001) found that EMF-plants had the greatest growth and foliar P content compared to AMF and non-inoculated plants. However, recent studies

comparing EMF and AMF did not find uniform trends in foliar traits with fungal type across plant species (Koele et al. 2012; Dickie et al. 2014), and plant growth differences associated with EMF or AMF have been shown to vary even within the same host species (Klironomos 2003; Karst et al. 2008).

Contrary to the first hypothesis (**H1**), fungal-plant symbioses increased partitioning to root biomass in *E. grandis.* According to optimal partitioning theory, plants should allocate more biomass to the organ that will alleviate resource limitation (Bloom et al. 1985). Since fungal symbionts are more efficient scavengers for soil nutrients than plant roots (Allen 1991), the expectation was that root mass allocation would be lower with mycorrhizal fungi as they would substitute for root uptake. Previous studies, however, also have found inconsistencies in biomass allocation in fungal-plant symbioses (see meta-analysis by Veresoglou et al. 2012).

Increased partitioning to roots in fungal-plant symbioses could have arisen from the inclusion of fungal biomass in root measurements, changes in root morphology, and/or initial plant C investment of fungal symbionts. EMF biomass can constitute up to 40% of root biomass (Ekblad et al. 1995; Colpaert et al. 1996), which could confound root biomass measurements. Increases in lateral root length, branching, and fine root length with AMF and EMF colonization (Hooker et al. 1992; Yao et al. 2009; Comas et al. 2014) could contribute to greater absorptive surface area for nutrient capture, increased space for root symbionts, and overall greater root biomass in fungal-plant symbioses. This study's fungal-plant symbioses also reduced shoot biomass compared to non-inoculated plants, which is commonly attributed to higher C allocation to fungal symbionts especially in early seedling development (Johnson et al. 1997). Hence, increased root biomass could be a result of *E. grandis* seedlings initially investing more in fungal symbionts and nutrient acquisition than shoot growth.

Inconsistent with **H2**, fungi did not respond to nutrient treatments. Although reduced root colonization with fertilization is commonly assumed, increased or no changes in fungal colonization also have been found (Treseder 2004; Treseder 2013). Changes in root colonization are typically interpreted as alterations of C allocation to fungi, even though empirical evidence is weak and percent colonization is most likely determined by both plants and fungi (Maherali and Klironomos 2007; Kiers et al. 2011).

Plant nutrient tissue content responded to the ratio of N to P applied. Higher shoot P content and no growth responses in both species to the low N:P treatment could indicate plant N-limitation. Previous studies have hypothesized that N-limited plants would benefit the least from fungal symbionts, since N limitation reduces plant photosynthetic capacity and consequently C supply to fungi (Hoeksema et al. 2010; Johnson 2010). Moreover, N-limited plants tend to compete with fungi for C, and thus may experience no or negative growth (Reynolds et al. 2005). However, if plants were experiencing N-limitation, I would have expected to see an increase in plant biomass or N tissue content at the high N:P treatment, yet neither response occurred. This was most likely due to N levels not being large enough to relieve N-limited plants. Even though the ratio of N to P between low to high N:P treatments was 10-fold higher, plants and fungi can require 15 times more N than P (Johnson 2010). Thus, it is probable that N-limitation inhibited the growth of this study's fungal-plant symbioses, but I cannot discount that plant growth also may have been limited by other factors such as water, base cations or micronutrients.

The positive effects of AMF on *E. grandis* tissue nutrient content may be a result of its ability to rapidly colonize plant roots and acquire soil nutrients, instead of EMF's better mobilization of soil nutrients (**H3a**). AMF symbionts have been shown to be early colonizers of *Eucalyptus* spp. (Chen et al. 2000), whereas root colonization by EMF can take at least 8-12

weeks (Lilleskov and Bruns 2003). Rapidly colonizing AMF could mean that *E. grandis* plants benefited from AMF symbioses earlier than with EMF.

On the other hand, consistent with **H3b**, the negative effects of EMF on overall *E. grandis* growth could be attributed to seedlings' large initial C investment in EMF's fungal tissue (Miller 1989, Colpaert et al. 1996). EMF commonly form dense hyphal tissue (Rygiewicz and Anderson 1994), potentially creating higher initial respiratory costs during the early stages of colonization to approximately 90 days after (Cairney et al. 1989; Cairney and Alexander 1992; Durell et al. 1994). Since seedlings were grown for approximately 90 days, the EMF-plants may still have been experiencing a drain on its C resources. Yet higher hypothesized EMF respiratory costs did not seem to affect *Q. costaricensis* growth responses.

Variation in plant responses to fungal symbionts could be caused by differences in initial seed size (Allsop and Stock 1995; Gehring 2004). In this study, *Q. costaricensis'* seed weight was approximately 16 thousand times greater than *E. grandis*. Previous studies have found that *Quercus* seedlings can rely heavily on cotyledon reserves in their early development (Villar-Salvador et al. 2010; Yi and Wang 2015), especially in nutrient-poor environments (Milberg et al. 1998). Ifound positive correlations between *Q. costaricensis'* seed size and final seedling biomass under all nutrient treatments ($r=0.40$, $P<0.001$) except the treatment with the highest N:P ratio, and many *Q. costaricensis* seedlings still had seed reserves at the time of harvest. Thus, I speculate that *Q. costaricensis* seedlings utilized cotyledon reserves for most early seedling growth, potentially diluting mycorrhizal fungal effects on growth.

Relatively low percentages of fungal colonization also could have obscured differences in the effects of AMF versus EMF. I used root trap cultures in an attempt to better represent a natural, field fungal community, yet average fungal colonization was half to two-thirds lower

than field averages of *E. grandis* percent colonization found in plantations at various plant ages (Campos et al. 2011). Previous studies that isolated AMF vs. EMF effects have used one or two fungal taxa in high concentrations to ensure colonization (Jones et al. 1998; Chen et al. 2000; van der Heijden and Kuyper 2001), but these studies also may have overestimated the effects of highly infective fungi or highlighted functional fungal differences (Courty et al. 2010; Smith and Smith 2011). Conversely, the use of root trap cultures as fungal inoculum may have underestimated the effects of mycorrhizal fungi on tree growth. In addition, bait plants may have trapped fungal species different than those that would commonly associate with this study's plant species, thus creating a cultivation bias attributed to the selective effects of taxonomically different bait plant species (Sykorova et al. 2007).

Conclusions

Dual fungal-plant symbioses can be important for separating fungal and plant effects from the complex environment that they experience in nature. I found that mycorrhizal fungal types may differ in their influence on tree performance and that unique tree species' characteristics can affect how AMF and EMF interact with their host plants, especially during early seedling growth. Across two study species, AMF-plant symbioses improved tissue nutrient content and plant growth, while plants associated with EMF had more variable growth responses which may have arisen from greater initial C investment. These results highlight the highly context-dependent nature of the symbiosis and the importance of identifying specific fungal-plant combinations that improve plant growth, which, for example, could be of crucial importance for tropical restoration efforts.

CHAPTER IV:

REDUCED ABOVEGROUND TREE GROWTH ASSOCIATED WITH HIGHER ARBUSCULAR MYCORRHIZAL FUNGAL DIVERSITY IN TROPICAL RESTORATION

ABSTRACT

Establishing diverse mycorrhizal fungal communities is considered important for forest recovery, yet mycorrhizae may have complex effects on tree growth depending on the composition of fungal species present. In an effort to understand the role of mycorrhizal fungi community in forest restoration in southern Costa Rica, I sampled the arbuscular mycorrhizal fungal (AMF) community across eight sites that were planted with the same species (*Inga edulis, Erythrina poeppigiana, Terminalia amazonia,* and *Vochysia guatemalensis*) but varied two- to four-fold in overall tree growth rates. The AMF community was measured in multiple ways: as percent colonization of host tree roots, by DNA isolation of the fungal species associated with the roots, and through spore density, volume, and identity in both the wet and dry seasons. The majority of fungal species belonged to the genus *Glomus* and genus *Acaulospora*, accounting for more than half of the species and relative abundance found on trees roots and over 95% of spore density across all sites. Contrary to previous literature findings, increases in AMF species diversity and spore densities were correlated with lower tree growth, which may have arisen from greater carbon (C) consumption by more diverse and productive AMF communities. Larger C requirements of particular AMF (i.e., *Gigaspora* spp. and *Scutellospora* spp.) only found in low growth sites also may have contributed to a greater drain on host trees' C resources and reduced tree growth. These results highlight the importance of AMF in forest recovery and suggest that fungal community dynamics could have important consequences for tree C balance and growth.

INTRODUCTION

Incorporating mycorrhizal fungi into tropical restoration efforts requires an understanding of multiple ecological processes relating belowground organisms, aboveground plant performance, and site-specific environmental variables (Heneghan et al. 2008). Mycorrhizal fungal-plant symbioses are important for maintaining soil aggregation (Rillig 2004), increasing nutrient cycling (Read and Perez-Moreno 2003), and most importantly to reforestation efforts, for improving plant growth and survival (Janos 1980). Globally, approximately 80% of plant species form a symbiotic mycorrhizal fungal relationship, and in many tropical forests, the predominant fungal type is arbuscular mycorrhizal fungal (AMF). Despite their ubiquity and importance to ecosystem structure and function, surprisingly little is known about the abundance and diversity of AMF in tropical soils (Alexander and Selosse 2009), and even less about their role in ecological restoration (Kardol and Wardle 2010).

The impact of changing from a high diversity, woody-species dominated habitat to a low diversity, graminoid pasture or mono-dominant cropland can have profound effects on the mycorrhizal fungal community's diversity, composition and relative abundances (Aldrich-Wolfe 2007; Stumer and Siqueira 2011; Mueller et al. 2014). Moreover, decreases in soil nutrient availability due to land use conversions may increase AMF root inoculation as plants become more dependent on their fungal symbionts for nutrient acquisition (Smith and Read 2008).

Whereas it is commonly assumed that a diverse mycorrhizal fungal community will enhance tree growth in forest restoration, actual AMF effects could be more complex. Fungal species richness can associate with both increases (van der Heijden et al. 1998; Vogelsang et al. 2006) and decreases in plant productivity (Hiiesalu et al. 2014). More phylogenetically diverse AMF are likely to be functionally different (Maherali and Klironomos 2012), and functional

diversity among fungal taxa could yield distinctive impacts on plant growth (Munkvold et al. 2004). For example, AMF families may improve plant growth differently by either providing protection against fungal pathogens (Glomeraceae) or enhancing plant phosphorus (P) uptake (Gigasporaceae) (Maherali and Klironomos 2007). AMF species also may vary in their tolerance to their environment and susceptibility to disturbance which can affect their relationship with plants (Jasper et al. 1991).

Plant growth is likely affected more by AMF composition than by diversity, as particular AMF-plant associations appear to have a greater impact on the growth of specific plant species than others (Klironomos 2003). Differences in benefits are generally associated with life history strategies in AMF species via the amount of C extracted from their hosts (Olsson et al. 2010), their ability to acquire nutrients (Smith et al. 2000), and fungal nutrient storage capacity (Kiers et al. 2011). Bever et al. (2009) found that host plants can preferentially allocate photosynthates to more beneficial fungal partners and thus may "choose" symbionts that increase their growth. Hence, changes in AMF diversity and abundance due to land use conversion may have profound effects on plant growth and restoration efforts.

I examined the relationships between AMF community abundance and diversity and the growth of four tree species in eight 5 to 7 year old reforested sites in southern Costa Rica (Table 4.1, Fig. 4.1). Previous research tested past land use, soil nutrients, soil compaction, and understory cover as potential causes for differences in tree growth, but only the ranked length of pasture use explained a significant amount of variation (Holl et al. 2011; Holl and Zahawi 2014). Since past land use intensity can strongly affect soil microbial communities (Carpenter et al. 2001; Oehl et al. 2010), I investigated whether differences in mycorrhizal fungal communities could help explain the influence of prior pasture use on tree growth. AMF abundance and

Table 4.1. Average tree growth (height and diameter at breast height (DBH)) and soil characteristic for all sites.

Site [a]	DBH Growth (cm yr^{-1})	Height Growth (m yr^{-1})	Year Planted	Ranked duration of pasture use [b]	pH	Organic Matter (%)	C (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Total Exchange capacity (meq 100 g^{-1})
Site 1	0.82	0.48	2004	6	5.1	15.77	6.55	0.54	2	88	623	167	9.58
Site 2	0.96	0.54	2004	5	5.5	11.76	5.49	0.42	3	170	1788	294	18.78
Site 3	1.17	0.53	2006	6	4.8	13.88	5.35	0.55	2	55	316	70	5.70
Site 4	1.30	0.65	2005	4	5.1	17.86	6.09	0.55	7	71	429	58	5.81
Site 5	1.63	1.32	2005	2	5.4	22.38	9.46	0.72	2	57	1050	147	11.20
Site 6	1.81	1.35	2005	2	5.4	22.72	9.59	0.79	3	68	1296	146	13.23
Site 7	1.90	1.39	2004	3	4.9	14.59	7.72	0.53	2	88	802	139	12.24
Site 8	2.22	1.48	2006	1	5.6	24.81	10.93	0.83	5	67	1183	185	11.54

[a] Site numbers are ranked based on the average DBH growth, where 1 = lowest average DBH growth and 8 = highest.
[b] 1 = shortest amount of time land was in pasture and 6 = longest.

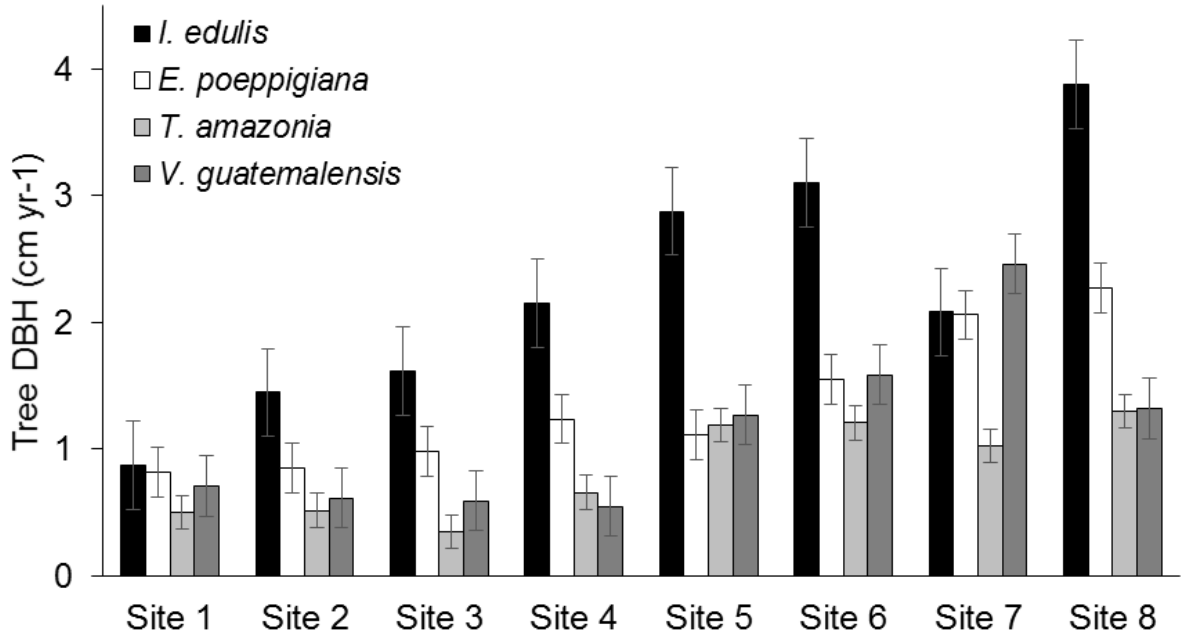

Figure 4.1. Mean annual tree diameter at breast height (DBH) growth grouped by species and site (±1 SE).

diversity were characterized in three ways: percent colonization of host tree roots; identification of the fungal species associated with trees roots through DNA isolation; and spore density, volume, and identity in both the wet and dry seasons. Although soil nutrients explained little of the variation in tree growth, nutrient availability can alter AMF abundance and diversity (Camenzind et al. 2014); thus data on soil attributes were collected to better evaluate the mechanisms underlying the site, tree and fungal differences. Specifically, I hypothesized that: **H1**) AMF abundance (i.e., percent fungal colonization) and species diversity are positively correlated with tree growth; **H2**) AMF spore production is positively correlated with tree growth, **H3**) relative abundances of specific AMF species are related to tree growth, and **H4**) AMF abundance, diversity, and spore production are negatively correlated with soil characteristics.

METHODS

Site Description

Eight sites (50 × 50 m) distributed across a 100 km^2 area were established between 2004 and 2006 (these eight sites were a subset of sites from Holl et al (2011) with the highest and lowest tree growth). Sites were located near the town of Agua Buena (8^0 44' 36" N, 82^0 58' 04" W) and Las Cruces Biological Station (8^0 47' 7" N, 82^0 57' 32" W) in Coto Brus county in southern Costa Rica. This region is classified as a tropical montane rain forest (Holdridge 1967), but due to a history of agricultural land use over the past 60 years, has largely been deforested. Estimates show that approximately 28% (~13 km radius) is forested today, compared to 98% in the late 1940s (Zahawi et al. 2015). All sites were used for at least 18 years for agriculture and were either recently abandoned pastures dominated by exotic forage grasses or abandoned coffee farms dominated by a mixture of forage and non-forage grasses, forbs, and *Pteridium arachnoidum* (Kaulf.) Maxon (see Holl et al. 2011 for more detailed site descriptions).

The soils are classified as lixisols (Centro Científico Tropical 2004). In August 2007 and July 2012, 25 soil cores were taken across each site, composited, and analyzed for soil pH, organic matter (OM), percent C and nitrogen (N), P, cations, and micronutrients following standard procedures at Brookside Laboratories, Knoxville, OH (see http://www.blinc.com/worksheet_pdf/SoilMethodologies.pdf for details on protocols).

Tree species

Four tree species were planted in each of the eight (50 × 50 m) sites between 2004 and 2006: two native species (*Terminalia amazonia* (J.F. Gmel.) Exell (Combretaceae) and *Vochysia guatemalensis* Donn. Sm. (Vochysiaceae)) and two naturalized, N-fixing species (*Erythrina*

poeppigiana (Walp.) Skeels and *Inga edulis* Mart. (both Fabaceae)). A total of 313 seedlings were planted in rows into each site (see Holl et al. 2011). Seedlings were acquired from a local nursery, and no mycorrhizal fungal inoculation was used at the time of planting. Height and diameter at breast height (DBH) of each tree was measured annually. Across sites, growth rates ranged from 0.8 to 2.2 cm yr^{-1} for DBH and 0.5 to 1.5 m yr^{-1} for height between the time of planting to 2011 (Holl et al. 2011), and the two measurements were highly correlated ($r=0.95$, $P=0.0003$). The eight sites were numbered based on average DBH growth (Table 4.1), where sites 1-4 had the lowest tree growth and sites 5-8 had the highest.

Mycorrhizal fungal percent colonization

To assess mycorrhizal fungal differences by site and species, ten root samples per site were randomly collected from each of the four tree species in the eight plantation sites in July 2011 (10 samples × 8 sites = 80 per tree species). Roots were examined and traced back to the adult tree to ensure that they originated from the correct tree species. The roots were subsampled for percent colonization analyses. Roots were cleared with a 10% sodium hydroxide solution and stained with a Schaeffer's ink and vinegar method (Vierheilig et al. 1998). Percent root length colonized was scored using a modified gridline intersections method with approximately 20 cm of root per sample (McGonigle et al. 1990).

AMF species identification

Root tips from the same roots collected for the percent colonization analyses were used to identify the particular fungal species that associated with each tree species at each site. AMF DNA from approximately 25 mg of root tips were extracted with MoBio PowerSoil isolation kits

(MO BIO Laboratories, Inc. Carlsbad, CA), according to the manufacturer's instructions. Root tips from ten individual trees from each site (10 trees × 8 sites = 80 per tree species) were extracted and pooled into one sample per tree species per site. The roots and DNA extracts were kept frozen or cool during transport prior to PCR amplification and sequencing. Amplification of DNA and Roche 454 sequencing were performed by the Research and Testing Laboratory, Lubbock, TX (http://www.researchandtesting.com/).

The 18S rDNA genes in the DNA extracts, commonly used genetic markers for AMF identification, were amplified for pyrosequencing using forward and reverse fusion primers (developed from Dumbrell et al. 2011). The fusion primers used were: Forward 5'-**GCCTCCCTCGCGCCATCAG** (10 bp MID) CAGCCGCGGTAATTCCAGCT-3' and Reverse 5'-**GCCTTGCCAGCCCGCTCAG** GTTTCCCGTAAGGCGCCGAA-3'. The forward primer was constructed (5'-3') with the Roche A linker (in bold type), a 10-bp barcode, and the WANDA primer (Dumbrell et al. 2011), which is a universal eukaryotic primer internal to NS31 (Simon et al. 1992). The reverse fusion primer was constructed (5'-3') with the Roche B linker (in bold type) and the AM1 reverse primer (Helgason et al. 1998) which excludes plants and amplifies AMF families.

Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA) with the following thermal profile: 95°C for 5 minutes, then 35 cycles of 94°C for 30 seconds, 54°C for 40 seconds, 72°C for 1 minute, followed by one cycle of 72°C for 10 minute and 4°C hold. Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled equimolar and each pool was cleaned and size selected using Agencourt AMPure XP

(BeckmanCoulter, Indianapolis, IN) following Roche 454 protocols (454 Life Sciences, Branford, CT). Size selected pools were then quantified and diluted to be used in emPCR reactions, which were performed and subsequently enriched. Samples were sequenced with a Roche 454 GS-FLX+ system (454 Life Sciences, Branford, CT) following established manufacture protocols.

In order to determine the identity of each sequence, sequences were clustered into operational taxonomic units (OTUs) with 100% identity (0% divergence) and compared to the GenBank database for taxonomic identification (http://www.ncbi.nih.gov/Genbank/) using BLASTn+ (KrakenBLAST, http://www.krakenblast.com). Sequences were then classified into the appropriate taxonomic levels based upon greater than 97% sequence similarity at the species level, and 95-97% at the genus level. Nine samples failed to identify any AMF on trees roots or yield PCR product and were excluded from further analyses.

Spore production

Five soil samples were randomly collected in the wet and dry season (July 2011, February 2012) per site, to estimate the fungal spore community since not all AMF species sporulate at the same time (Lovelock et al. 2003). Each soil sample was a homogenized composite of three subsamples within a 1 m^2 area. Spores were extracted from 20g of the fresh soil matrix using multiple sieves (250, 160, and 20 um) and the sucrose flotation method (Ianson and Allen 1986). Sodium hexametaphosphate was used to standardize the methods and assist spore separation from the soil matrix in high clay content soils. Spores were mounted on slides using a solution of polyvinyl alcohol, lactic acid, and glycerol, and then microscopically counted and identified to the genus level *sensu* Schenck and Perez (1990). I was able to identify the spores only to genus-

level probably because a majority of the fungi associated with the roots, according to the molecular analyses, were unidentifiable or new species. Spore production was characterized by spore density and total spore volume. Spore density provides an estimate of the total number of spores produced, whereas spore volume takes in account size and germination differences and provides an estimate of the C resources used in spore production (Koske 1987). Spore volume was calculated assuming a spherical shape and the measured diameter of each spore, and then adding together the spore volumes for a particular genera or site. Estimates of spore density and volume were standardized to spores per gram of soil.

Data Analysis

I used a mixed model analysis of variance (ANOVA) to analyze the relationship between tree species and AMF abundance (i.e., percent colonization, spore density and volume) using the R project computing software (R version 3.2.3, The R Foundation for Statistical Computing, http://www.r-project.org). I used multiple linear regression to analyze the relationships between site-level AMF community variables (i.e., percent colonization; fungal species diversity, richness, and evenness; spore identity, density, and volume) and site-level tree growth (i.e., DBH). Outliers were tested with Grubbs' test for outliers (Grubbs 1950).

I modeled fungal species accumulation curves (R package vegan; Oksanen et al. 2013) based on individual trees and sites to determine whether I adequately sampled roots for AMF and to estimate species richness (Gotelli and Colwell 2010). Generally, the number of individuals that must be sampled to reach an asymptote in these curves can be extremely large in the tropics (Chao et al. 2009), where species diversity is high and most species are rare. Therefore, I used jackknife estimators to improve accuracy and reduce bias in species richness (Palmer 1990). I

calculated species evenness to determine if the distribution of species richness was biased (Buzas and Hayek 2005) and used Simpson's diversity index (Simpson 1949) due to its robustness to sample size and sensitivity to rare species (R package vegan).

Testing multiple individual soil characteristics (i.e., pH, OM, macro- and micronutrients) for plant-soil associations would result in many comparisons and inflate type I errors. So I obtained orthogonal composite variables by computing principle components (PCs) of all soil variables (averaged at the site-level) using a principal component analysis (PCA; R packages labdsv and FactoMineR; Roberts 2013; Husson et al. 2015). I chose the first three PCs, because the other PCs explained less than 10% of the variation (Table A4.1). I used Pearson correlation coefficients (r) to characterize the relationships between ranked length of pasture use, soil variables (PCs), site-level AMF variables (i.e., percent colonization; species diversity; spore production), and tree growth (R package Hmisc; Harrell et al. 2015).

The composition of the AMF community across tree species and sites was compared using non-metric multi-dimensional scaling (NMDS; R package vegan), as it is robust to non-linear relationships and zero inflation (Clark et al. 1993). NMDS was applied to a dissimilarity matrix calculated from the relative abundances of the fungal species using the Bray-Curtis dissimilarity coefficient. To test the effect of tree species identity on the AMF community composition, the community dataset was analyzed using permutational multivariate analysis of variance (PERMANOVA, Anderson 2001), which is robust to correlations and heterogeneous variances in the dataset (Anderson and Walsh 2013).

RESULTS

Contrary to the first hypothesis **(H1)**, percent colonization was not correlated with site-level tree growth (r=0.36, P=0.3789) but varied by site ($F_{7, 263} = 4.82$, P<0.0001; Fig. B4.1) and species ($F_{3, 263} = 7.96$, P<0.0001; *E. poeppigiana*: 39.9 ± 4.2%, *I. edulis*: 48.4 ± 4.9, *T. amazonia:* 51.6 ± 3.7, *V. guatemalensis*: 53.0 ± 4.1%). Also in contrast to **H1**, the roots of low tree growth sites tended to have greater species richness (r = -0.72, P=0.0427; Fig. 4.2A) and fungal species diversity (i.e., Simpson's diversity index; r = -0.41, P=0.3094 (with outlier); r = -0.91, P=0.0033 (excluding Site 4 outlier which had roots dominated by non-AMF; Fig. 4.2C), but marginally ($\alpha < 0.10$) lower species evenness (r = 0.64, P=0.0862; Fig. 4.2B), than high growth sites. First- and second order jackknife estimates of species richness by the number of sites were higher than observed species richness, but there was a trend ($\alpha \leq 0.10$) for estimated richness differing by site-level tree growth (r = -0.70, P=0.0537; r = -0.69, P=0.0620; see Table A4.2). The roots of the four tree species contained 22 AMF taxa (17 species and 5 identifiable only to the genus; per taxonomic classification of Schussler and Walker 2010). Although the dominant AMF taxa differed across sites (Fig. 4.3), three AMF taxa *(Acaulospora* sp.1, *Glomus* sp.1, and *Rhizophagus clarus*) constituted 67-100% of AMF tree associations across all sites while the other 19 taxa were rare. Over 75% of *Glomus* spp. and over 99% of *Acaulospora* spp. were unidentifiable/new species. *Gigaspora*, *Scutellospora,* and *Diversispora* spp. were only found in low growth sites and comprised less than 2% of all AMF (Table A4.3). At two sites (Sites 4 and 7), non-AMF (mainly pathogenic and plant litter decomposing fungi; e.g., *Exophiala salmonis*, *Metacordyceps chlamydosporia*, *Myrothecium cinctum,* and *Volutella ciliata*) dominated the tree roots, with AMF species accounting for less than 20% of fungal inoculations.

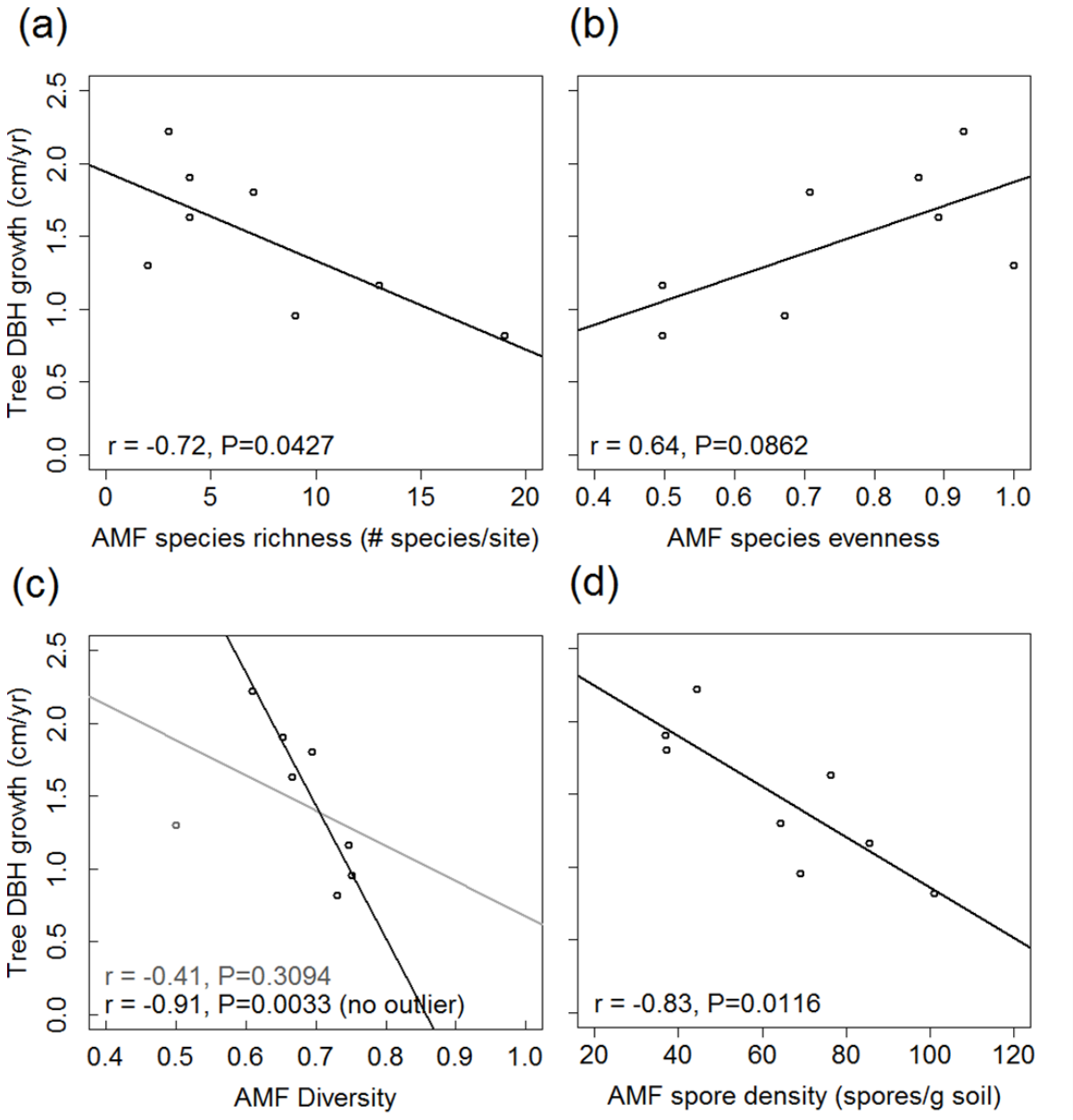

Figure 4.2. Site-level tree diameter at breast height (DBH) growth per year as functions of (a) arbuscular mycorrhizal fungi (AMF) species richness, (b) AMF species evenness, (c) AMF diversity (grey line and text represent the diversity-DBH relationship with all data points, while the black line is without one outlier (Site 4)), and (d) AMF spore density. AMF species richness, evenness and diversity (calculated from Simpson's diversity index) are representative of host trees' roots across all four species. AMF spore density is characterized by the number of spores per gram of soil across both wet and dry seasons.

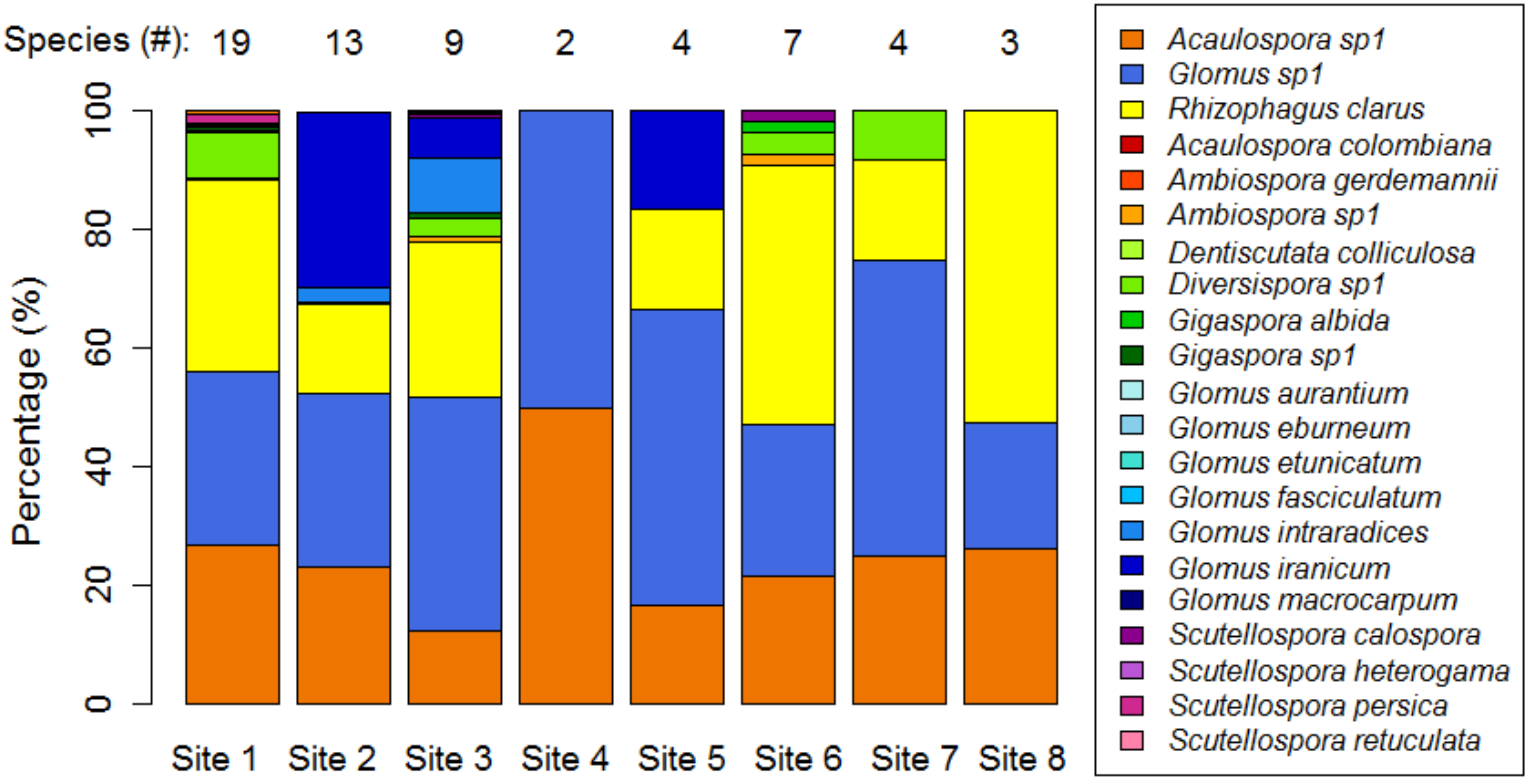

Figure 4.3. Relative abundance of arbuscular mycorrhizal fungi (AMF) within each site when omitting non-AMF across all tree species. The number of AMF species (i.e. species richness) for each site is indicated above the respective bar.

Low growth sites had a two-fold higher density of spores per gram of soil in dry (r = -0.81, P=0.0161) and wet (r = -0.67, P=0.0677) seasons than high growth sites (Fig. 4.2D), counter to **H2**, but site-level tree growth was not associated with the total spore volume (Table 4.2). Spore density did not differ between seasons ($F_{1,13}$=1.97, P=0.1845), and dry season spore volume was marginally ($\alpha \leq 0.10$) greater than wet season ($F_{1,13}$=3.17, P=0.0985). Similar to the root results, the majority of the spores by number and volume were members of the genus *Glomus* (95%; 61%, respectively) and genus *Acaulospora* (5%; 32%, respectively). *Glomus* had higher spore density in low growth sites, regardless of season (r = -0.84, P=0.0101), while *Gigaspora*, in the dry season, had marginally higher spore density (r = -0.62, P=0.1054) and volume (r = -0.69, P=0.0552) in low growth sites but was less than 1% of overall spore numbers and volume.

Inconsistent with expectations (**H3**), tree growth was not correlated with the relative abundance of specific AMF taxa (*I. edulis*: $F_{1,7}$=0.68, P=0.6078; *E. poeppigiana*: $F_{1,7}$=0.82, P=0.5341; *T. amazonia*: $F_{1,7}$=0.58, P=0.6856; *V. guatemalensis*: $F_{1,7}$=0.22, P=0.9146). The relative abundances of the AMF community also were not influenced by host tree identity ($F_{3,22}$=1.19, P=0.3003; Fig. B4.2), and particular fungi did not associate with only one tree species. *V. guatemalensis* associated with the most AMF taxa (17), followed by *E. poeppigiana* (15), *I. edulis* (14), and *T. amazonia* (12). First- and second order jackknife estimates of species richness by individual trees were slightly higher than actual species richness but similar across tree species (see Table A4.2). In *T. amazonia* and *I. edulis*, fungal taxa from the genus *Glomus* dominated (58 and 57% of AMF, respectively), whereas *R. clarus* comprised 49% of *V. guatemalensis* fungal-tree symbioses. A combination of *Glomus* and *Acaulospora* made up 33 and 27%, respectively, of symbioses with *E. poeppigiana*.

Table 4.2. Pearson correlation coefficients (r) for tree growth (i.e., DBH) and ranked duration of pasture use with AMF variables and soil principle components (PCs) across sites. The loadings for the soil PCs from Principal Component Analyses are in Table A4.1. Correlations and p-values in bold type are significant (P<0.05).

Variables	Tree growth Correlation (r)	Tree growth P-value	Ranked duration of pasture use correlation (r)	Ranked duration of pasture use P-value
Ranked duration of pasture use	**-0.92**	**0.0013**	**----**	**----**
AMF diversity	-0.41	0.3094	0.45	0.2651
AMF diversity (no outlier)	**-0.92**	**0.0033**	**0.88**	**0.0091**
AMF species richness	**-0.72**	**0.0427**	**0.77**	**0.0246**
AMF species evenness	0.64	0.0862	**-0.73**	**0.0381**
AMF percent colonization	0.36	0.3789	-0.25	0.5554
AMF spore density	**-0.83**	**0.0116**	**0.75**	**0.0303**
AMF spore volume	-0.35	0.3946	0.24	0.5748
Soil PC1	0.19	0.6530	<0.01	0.9947
Soil PC2	**-0.80**	**0.0173**	**0.92**	**0.0013**
Soil PC3	<0.01	0.9970	0.10	0.8147

AMF species diversity was negatively correlated with OM, C, and N concentrations (PC 2; Table A4.1) (r=0.78, P=0.0394 (without outlier)) consistent with predictions (**H4**) but not spore density (r= -0.02, P=0.1563) or volume (r=0.55, P=0.9641). Soils were acidic (pH ~5.2), had a clay-enriched subsoil, low to moderate levels of exchangeable cations (calcium, magnesium, and potassium), and low P levels; soil OM also was high (> 10%; Table 4.1). Lower OM, C, and N concentrations (PC 2) also were correlated with higher site-level fungal colonization on only *V. guatemalensis* roots (r= -0.73, P=0.0389).

Additionally, sites with longer duration of previous pasture use had higher AMF species diversity (without outlier), higher species richness, higher spore density, lower soil OM, C, and N concentrations (PC 2) and lesser species evenness, but pasture duration was not related to fungal colonization or spore volume (Table 4.2). Longer duration of pasture use and greater AMF diversity were similarly strongly correlated with tree growth, whereas higher spore densities and lower soil OM, C and N were less strongly correlated with tree growth (Table 4.2).

DISCUSSION

Contrary to the hypotheses (**H1** and **H2**) and to previous studies (van der Heijden et al. 1998, Vogelsang et al. 2006), tree growth negatively correlated with greater AMF species richness, diversity and spore density in restored pastures (Fig. 4.2). AMF richness, diversity and spore density also positively correlated with longer duration of pasture use and could explain some influence of prior land use on tree growth. The majority of fungal species and spores belonged to *Glomus* and *Acaulospora,* consistent with their ability to tolerate soil disturbances (Boddington and Dodd 2000) and similar to prior tropical restoration research (Allen et al. 2003, Haug et al. 2010). Conversely, negative correlations between AMF diversity versus soil organic

matter (OM), carbon (C), and nitrogen (N) (**H4**) was in accordance with previous research (Egerton-Warburton and Allen 2000; Camenzind et al. 2014) as well as soil relationships with pasture use and tree growth (see Guariguata and Ostertag 2001). For this study, I cannot distinguish whether soil attributes (OM, C, and N) or mycorrhizal diversity directly influenced tree growth, but there could be a role for both.

The relationship between greater AMF diversity and increased spore densities at low growth sites could represent a positive productivity-diversity response within the AMF community (see Koch et al. 2012), even though typically productivity-diversity relationships are examined in plants (Tilman et al. 1997) or between microbial and plant communities (van der Heijden et al. 1998; Vogelsang et al. 2006). In this scenario, the ecosystem is the tree roots, productivity is spore production, AMF diversity is measured by Simpson's diversity index, and decreases in tree growth are measurements of AMF resource use (i.e., plant C). While I have good estimates of spore production as an indicator of AMF productivity, I acknowledge that this study is lacking a complete picture of AMF biomass. Nonetheless, from this perspective, the tree and AMF symbionts are competing for C.

Examining the mechanisms driving the productivity-diversity relationship from an AMF perspective may help explain their relationship with plant growth across sites. As applied to AMF, functional complementarity, a common productivity-diversity mechanism, is the co-existence of several fungal species occupying different niches on the same roots which could result in more efficient extraction of resources and thus higher productivity for AMF and lower plant growth. Trees roots across all sites were dominated by *Glomus* spp. and *Acaulospora* spp., which can have different niche space on roots (Maherali and Klironomos 2007, 2012), which is consistent with this mechanism. Another mechanism is the sampling or portfolio effect; that is,

more diverse AMF communities had a higher chance of including highly productive species that dominated the community, which could be driving the positive productivity-diversity response among the AMF taxa. In this study, the dominant AMF (i.e., *Glomus* and *Acaulospora*; as measured by relative abundance on roots) also were the most productive genera (as measured by spore production) across higher and lower growth sites, negating sampling effects across sites. A third potential mechanism is facilitation, in which encounters between species benefit at least one but does not harm either (e.g., Thonar et al. 2014). Across sites, I found that the occurrence of *R. clarus* on trees roots increased more in the presence of *Ambiospora gerdemannii*, suggesting facilitative effects among AMF. These examples suggest that positive productivity-diversity relationships within the AMF community could constitute greater utilization of C resources and a potential avenue for AMF to affect plant growth.

One explanation for the negative AMF-plant growth relationship could be due to AMF species differing in C utilization through greater fungal biomass or higher sporulation. Larger C requirements of particular fungal taxa (i.e., *Gigaspora* or *Scutellospora*) may result in a greater drain on host plants' C resources and reduced plant growth (Allen et al. 2003; Lendenmann et al. 2011), while greater spore production could constitute a major drain on host plants' C resources (Bago et al. 2000). Whereas some AMF species can increase plant growth, others can depress growth (Klironomos 2003; Hart et al. 2013). Only the low tree growth sites had *Gigaspora* and *Scutellospora* colonization and greater densities of *Gigaspora* spores. Thus, I suggest that more diverse and productive AMF communities resulted in greater C utilization and reduced plant growth, since sites with greater fungal diversity and sporulation may have included species that drained more C from their associated trees, but note that I cannot directly infer causality.

Another potential explanation for the observed negative relationship between AMF and plant growth is that trees in low growth sites associated with multiple, inefficient fungi. The destruction of soil structure, specifically from disturbances such as longer pasture duration in low growth sites, can promote the proliferation of less mutualistic fungi (Bever et al. 2009). Since not all AMF species are equally beneficial to host plants, C allocated to multiple, less efficient fungi could result in reduced plant growth (Kiers et al. 2011). Although plants may preferentially allocate C to more beneficial AMF (Kiers et al. 2011), they may not strongly control the initial stages of AMF colonization (David Schwartz et al. 2003; Akiyama et al. 2005), so that less efficient fungi can colonize roots before more beneficial AMF.

The negative AMF-plant growth relationship also may have arisen from soil attributes causing variation in both tree growth and AMF diversity. Trees can facultatively increase their associations with fungal symbionts under degraded environmental conditions (Johnson et al. 1997; Smith et al. 2009), such that AMF richness (Egerton-Warburton and Allen 2000), AMF abundance (Tresder 2004), and plant C allocation to AMF structures (Johnson et al. 2003) may increase with decreasing soil fertility. Consistent with predictions (**H4**) AMF diversity was negatively correlated with soil characteristics (i.e., OM, C, and N), but AMF abundance or spore production did not vary (Table 4.2). Higher levels of soil OM, C and N also strongly associated with greater tree growth and shorter pasture use, even though soil attributes in previous studies (i.e., Holl et al. 2011; Holl and Zahawi 2014) explained very little of the variation in tree growth potentially due to differences in data analysis (PCA composites vs. regression with Bonferroni corrections). The more diverse AMF communities at low growth and soil fertility sites could indicate those trees' greater need for fungal symbionts. But as AMF diversity increased at low growth sites, the overall amount of fungal root colonization did not vary with site-level tree

growth (**H1**), which is the characteristic measurement for plant allocation to fungal structures. Thus, these results do not provide evidence for facultative fungal-tree relationships in the sites.

Methodological considerations

Although more individual species were found at low growth sites (Fig. 4.2A), there were a few common species and many rare ones, as evidenced from species evenness measurements (Fig. 4.2B) and the narrow range of diversity indices (Fig. 4.2C) across sites. Uneven species abundances could have been a consequence of measuring only fungal structures internal to the trees roots (obtained by fungal DNA isolation from root fragments), which may have underestimated the influence and presence of rare species. Whereas the diffuse internal hyphae and sparse external structures of *Glomus* and *Acaulospora* would be adequately represented by the relative abundance measurements, *Gigaspora* tends to produce densely aggregated internal hyphae and long external hyphae which would be poorly represented by internal root measurements (Hart and Reader 2002). Thus, the many "rare" species could be underestimated and may be more "common" than represented in this study. This limitation, combined with many fungal individuals unidentifiable to species level, constrains my ability to fully evaluate the relationship between specific AMF taxa and tree growth (**H3**).

Greater AMF diversity in low growth sites may have been a consequence of greater root sampling. Although aboveground tree growth was lower in low growth sites, I cannot know if root biomass differed among sites since it was not measured. Lower aboveground growth could suggest smaller biomass belowground, but it also might indicate greater resource allocation to roots. Since all trees roots were similarly subsampled, I may have sampled more roots from low growth sites if those sites also had lower root biomass.

I also did not measure the fungal composition of sites prior to tree planting. Although higher AMF diversity and spore density were associated with reduced tree growth 5 to 7 years after transplanting, I do not know if the AMF community changed during that time. Land-use changes (i.e., forest to pasture) can alter the taxonomic composition of AMF communities (Aldrich et al. 2007) but not necessarily species richness and abundance (Leal et al. 2013).

Conclusions

Although numerous studies have found a positive link between the AMF community and plant growth, this study's results of a negative plant growth-fungal diversity relationship may represent one scenario in which the presumed mutualist fungal-plant symbiosis may create a barrier to restoration efforts. While AMF are thought to be facilitative symbionts, greater species diversity with increased spore densities may indicate greater utilization of AMF resources (i.e., plant C) and provide evidence for a positive productivity-diversity relationships within the AMF community that could influence tree C dynamics and growth. A more diverse AMF community may include more species that have larger C requirements and greater sporulation, resulting in a greater drain on host plants' C resources and reduced plant growth. Soil attributes also may influence both AMF diversity and tree growth. This research highlights the importance of considering mycorrhizal symbionts in the growth of tropical trees, especially in a restoration context.

CHAPTER V:

CONCLUSIONS

Mycorrhizal fungal symbioses are presumably beneficial to tropical restoration efforts since sites are predominantly nutrient-poor. Numerous restoration studies have directly manipulated fungal symbionts in an effort to improve restoration results through the addition of fungal spores or soil (Fischer et al. 1994, Brundrett et al. 1996, Allen et al. 2003, Duponnois et al. 2005). Yet incorporating mycorrhizal fungi requires an understanding of multiple ecological processes relating belowground organisms, aboveground individuals, and site-specific environmental variables (Heneghan et al. 2008). In this dissertation, I found variation in the influence of fungal symbionts (both arbuscular (AMF) and ectomycorrhizal (EMF) fungi) on tree growth, thus complicating whether mycorrhizae should be used to enhance reforestation success.

In my first field experiment (Chapter II), I showed that variation in growth and tissue nutrient concentrations among four tree species were greater than fungal type differences (AMF vs. EMF) in nutrient acquisition. Site variation, specifically initial base cation availability, and nutrient treatments also were associated with differences in tree growth. While EMF symbioses may possess numerous processes for increased nutrient uptake compared to AMF, their benefit to reforestation was not supported, and we cannot generalize that trees associated with a particular fungal type will similarly increase nutrient uptake and growth. Instead this research highlights the importance of both tree species selection and replication across multiple sites with different soil nutrient availabilities in reforestation efforts.

In the greenhouse (Chapter III), I did not find consistent effects of fungal type on plant growth and tissue nutrient content after isolating fungal from tree species effects. In *Eucalyptus grandis*, fungal associated plants had positive effects on belowground biomass but negative

effects on aboveground biomass, and AMF associated plants, in particular, had greater tissue nutrient content than EMF- or non-inoculated plants. Conversely, *Quercus costaricensis* growth responded more to EMF symbionts. These results demonstrate that mycorrhizal fungal type may differ in their influence on tree performance, especially during early seedling establishment, and that tree species' characteristics can affect how AMF and EMF interact with their host plants.

Finally in my second field experiment (Chapter IV), I found that increases in AMF species diversity and spore densities were correlated with lower tree growth after 5 to 7 years in reforested agricultural lands. In addition, greater AMF diversity and spore production represented a positive productivity-diversity response within the AMF community, which may have contributed to lower tree growth as more diverse and productive AMF communities could consume more tree carbon. This study's negative fungal diversity-plant productivity relationship could represent one scenario in which the presumed mutualist fungal-plant symbiosis may have important consequences for tree carbon balance and growth and reforestation efforts.

In general, my dissertation highlights the importance of understanding specific fungal-tree symbioses, soil nutrient status, and site effects to make management recommendations for reforestation success. In order to effectively incorporate mycorrhizal fungi into a reforestation strategy, a better understanding of the interactions between fungi, trees, and soil nutrient availability is needed. Further research on specific fungal-tree combinations that promote positive growth responses is crucial. My results also show that applying mycorrhizal fungal strategies will require knowledge about initial site conditions, such as soil nutrient availability and prior land use. Finally, we need to understand the links between the specific fungal species that constitute positive AMF productivity-diversity relationships and their effect on aboveground restoration success (i.e., host plant growth).

APPENDICES

APPENDIX A. Tables

Table A2.1. *Q. insignis* tissue nutrient concentrations as functions of trees associated with more EMF (as calculated from the residuals of EMF vs. AMF colonization) **across sites.** P-values are corrected with a Bonferroni adjustment for multiple comparisons and considered significant at $P<0.05$.

Tissue Nutrient	Leaf		Stem		Root	
	r	P-value	r	P-value	r	P-value
Nitrogen	0.04	1.000	0.02	0.835	0.03	1.000
Phosphorus	0.09	1.000	0.15	0.651	0.04	1.000
Sum of Base Cations	0.20	0.285	0.07	1.000	0.24	0.141

Table A2.2. Parameter estimates of tissue nutrient content to plant mass (shoot or root) **components.** Slope estimates, 95% confidence intervals, and associated statistics for components as a function of plant mass based on linear regression models. Letters next to slope estimates designate significant differences (Austin and Hux 2002) among shoot or root tissue nutrient content.

Tissue Nutrient	Species	Fungal treatment	Shoot mass ± 95% CI	Shoot R^2	Root mass ± 95% CI	Root R^2
Nitrogen	*S. macrophylla*	AMF	1.73 ± 0.58 [a]	0.71	0.60 ± 0.10 [a]	0.79
	S. macrophylla	Non-inoculated	1.63 ± 0.91 [b]	0.43	0.42 ± 0.09 [b]	0.72
	P. caribaea	EMF	1.05 ± 0.27 [b]	0.83	1.42 ± 0.22 [c]	0.93
	P. caribaea	Non-inoculated	0.83 ± 0.19 [b]	0.92	0.78 ± 0.08 [d]	0.97
Phosphorus	*S. macrophylla*	AMF	0.32 ± 0.11 [a]	0.74	0.08 ± 0.02 [a]	0.71
	S. macrophylla	Non-inoculated	0.25 ± 0.10 [ab]	0.61	0.08 ± 0.02 [a]	0.75
	P. caribaea	EMF	0.30 ± 0.08 [a]	0.80	0.15 ± 0.09 [a]	0.73
	P. caribaea	Non-inoculated	0.16 ± 0.05 [b]	0.89	0.12 ± 0.08 [a]	0.80
Sum of Base Cations	*S. macrophylla*	AMF	0.26 ± 0.06 [a]	0.85	0.26 ± 0.06 [a]	0.85
	S. macrophylla	Non-inoculated	0.21 ± 0.05 [a]	0.77	0.21 ± 0.06 [a]	0.77
	P. caribaea	EMF	0.08 ± 0.02 [b]	0.84	0.08 ± 0.02 [a]	0.84
	P. caribaea	Non-inoculated	0.06 ± 0.03 [b]	0.83	0.06 ± 0.02 [a]	0.85

Table A3.1. Parameter estimates of shoot or root mass as functions of total plant mass. Slope estimates, 95% confidence intervals, and associated statistics for components as a function of plant mass based on linear regression models. Letters next to slope estimates represent significant differences (Austin and Hux 2002) among shoot or root mass allocation within tree species.

Tree species	Fungal treatment	Shoot mass ± 95% CI	Shoot R^2	Root mass ± 95% CI	Root R^2
E. grandis	AMF	0.52 ± 0.08 [a]	0.81	0.48 ± 0.08 [a]	0.79
	EMF	0.54 ± 0.04 [a]	0.94	0.46 ± 0.08 [a]	0.93
	Non-inoculated	0.66 ± 0.07 [b]	0.88	0.34 ± 0.07 [b]	0.66
Q. costaricensis	AMF	0.55 ± 0.06 [a]	0.89	0.45 ± 0.06 [a]	0.85
	EMF	0.62 ± 0.07 [a]	0.88	0.38 ± 0.07 [a]	0.73
	Non-inoculated	0.59 ± 0.06 [a]	0.90	0.41 ± 0.06 [a]	0.82

Table A3.2. Parameter estimates of tissue nutrient content as functions of plant mass (shoot or root) **components.** Slope estimates, 95% confidence intervals, and associated statistics based on linear regression models. Letters next to slope estimates designate significant differences (Austin and Hux 2002) among shoot or root tissue nutrient contents within tree species.

Tissue nutrient	Tree species	Fungal treatment	Shoot mass ± 95% CI	Shoot R^2	Root mass ± 95% CI	Root R^2
Nitrogen	*E. grandis*	AMF	1.63 ± 0.49 [a]	0.61	0.64 ± 0.09 [a]	0.81
	E. grandis	AMF (no outlier)	1.39 ± 0.53 [a]	0.51	0.05 ± 0.02 [b]	0.46
	E. grandis	EMF	0.69 ± 0.33 [b]	0.39	0.52 ± 0.08 [c]	0.81
	E. grandis	Non-inoculated	1.40 ± 0.34 [a]	0.71	0.46 ± 0.12 [d]	0.59
	Q. costaricensis	AMF	0.75 ± 0.09 [a]	0.93	0.47 ± 0.09 [a]	0.73
	Q. costaricensis	EMF	0.81 ± 0.16 [a]	0.82	0.39 ± 0.12 [a]	0.52
	Q. costaricensis	Non-inoculated	0.55 ± 0.43 [b]	0.81	0.45 ± 0.10 [a]	0.67
Phosphorus	*E. grandis*	AMF	0.09 ± 0.05 [a]	0.29	0.05 ± 0.02 [a]	0.46
	E. grandis	AMF (no outlier)	0.07 ± 0.06 [a]	0.19	0.05 ± 0.02 [a]	0.46
	E. grandis	EMF	0.13 ± 0.06 [a]	0.41	0.05 ± 0.01 [a]	0.67
	E. grandis	Non-inoculated	0.13 ± 0.04 [a]	0.66	0.06 ± 0.02 [a]	0.57
	Q. costaricensis	AMF	0.12 ± 0.02 [a]	0.91	0.10 ± 0.03 [a]	0.56
	Q. costaricensis	EMF	0.13 ± 0.03 [a]	0.74	0.11 ± 0.05 [a]	0.35
	Q. costaricensis	Non-inoculated	0.10 ± 0.04 [a]	0.60	0.12 ± 0.04 [a]	0.51
Sum of Base Cations	*E. grandis*	AMF	0.06 ± 0.01 [a]	0.75	0.10 ± 0.01 [a]	0.90
	E. grandis	AMF (no outlier)	0.06 ± 0.02 [a]	0.69	0.10 ± 0.01 [a]	0.90
	E. grandis	EMF	0.07 ± 0.01 [a]	0.83	0.07 ± 0.01 [b]	0.90
	E. grandis	Non-inoculated	0.08 ± 0.02 [a]	0.81	0.06 ± 0.01 [b]	0.74
	Q. costaricensis	AMF	0.08 ± 0.01 [a]	0.91	0.05 ± 0.01 [a]	0.92
	Q. costaricensis	EMF	0.10 ± 0.02 [a]	0.86	0.04 ± 0.01 [a]	0.75
	Q. costaricensis	Non-inoculated	0.08 ± 0.02 [a]	0.79	0.05 ± 0.01 [a]	0.84

Table A4.1. Soil variable loadings for the eight sites from Principal Component Analyses (PCA)**.** Variance values indicate the percentage of the total variance accounted for by each PC. Other PCs explained less than 10% of the variation. Individuals in bold type were the significant variables in each PC.

Soil Variables	**PC 1**	**PC 2**	**PC 3**
pH	-0.236	-0.293	-0.127
Organic Matter	---	**-0.405**	---
C:N	-0.205	-0.159	---
C	---	**-0.418**	---
N	0.101	**-0.405**	---
P	---	---	**-0.639**
K	**-0.337**	0.173	---
Ca	**-0.335**	-0.167	---
Mg	**-0.369**	---	---
Total Exchange Capacity	**-0.353**	---	---
S	**0.297**	---	-0.412
Na	0.171	0.265	0.201
Fe	-0.180	**0.346**	---
Mn	-0.165	0.293	-0.327
Cu	---	-0.151	-0.386
Zn	**-0.307**	---	0.115
Al	**0.342**	---	0.224
Variance (%)	41	32	11

Table A4.2. Estimates of species richness for each tree species and site based on species accumulation curves.

Variable	Observed species richness	First-order jackknife species richness estimate ± standard error	Second-order jackknife species richness estimate
Tree species			
E. poeppigiana	15	23.8 ± 7.6	30.0
I. edulis	14	19.3 ± 4.1	21.8
T. amazonia	12	19.0 ± 7.4	23.7
V. guatemalensis	17	24.9 ± 6.6	30.3
Sites			
Site 1	19	26.5 ± 6.1	30.2
Site 2	10	13.8 ± 4.2	15.9
Site 3	13	14.5 ± 1.1	14.2
Site 4	3	4.5 ± 1.1	5.2
Site 5	5	8.0 ± 2.6	9.7
Site 6	8	12.5 ± 5.1	14.8
Site 7	5	7.3 ± 1.8	8.4
Site 8	4	5.5 ± 1.6	5.8

Table A4.3. Relative abundance of each AMF within each site when omitting non-AMF. Individuals in bold type only were identifiable to the genus level.

Genera	AMF Species	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
Acaulospora	***Acaulospora* sp1**	26.82	23.17	12.31	50.00	16.67	21.82	25.00	26.32
Acaulospora	*Acaulospora colombiana*	--	0.27	--	--	--	--	--	--
Acaulospora	*Acaulospora rehmii*	--	0.41	--	--	--	--	--	--
Ambiospora	***Ambiospora* sp1**	0.05	--	0.15	--	--	--	--	--
Ambiospora	*Ambiospora gerdemannii*	0.12	--	0.76	--	--	1.82	--	--
Dentiscutata	*Dentiscutata colliculosa*	7.88	0.07	3.04	--	--	3.64	8.33	--
Diversispora	***Diversispora* sp1**	0.09	--	--	--	--	1.82	--	--
Gigaspora	***Gigaspora* sp1**	0.01	--	--	--	--	--	--	--
Gigaspora	*Gigaspora albida*	0.61	--	1.04	--	--	--	--	--
Glomus	***Glomus* sp1**	29.16	29.37	39.43	50.00	50.00	25.45	50.00	21.05
Glomus	*Glomus aurantium*	0.05	--	--	--	--	--	--	--
Glomus	*Glomus eburneum*	0.04	--	--	--	--	--	--	--
Glomus	*Glomus etunicatum*	0.03	--	--	--	--	--	--	--
Glomus	*Glomus fasciculatum*	--	2.39	9.03	--	--	--	--	--
Glomus	*Glomus intraradices*	0.40	29.37	6.73	--	16.67	--	--	--
Glomus	*Glomus iranicum*	0.01	--	0.02	--	--	--	--	--
Glomus	*Glomus macrocarpum*	0.02	0.03	0.75	--	--	1.82	--	--
Rhizophagus	*Rhizophagus clarus*	32.51	14.92	26.28	--	16.67	43.64	16.67	52.63
Scutellospora	*Scutellospora calospora*	0.15	--	--	--	--	--	--	--
Scutellospora	*Scutellospora heterogama*	1.44	--	0.30	--	--	--	--	--
Scutellospora	*Scutellospora persica*	0.01	--	--	--	--	--	--	--
Scutellospora	*Scutellospora reticulata*	0.57	--	0.15	--	--	--	--	--

APPENDIX B. Figures

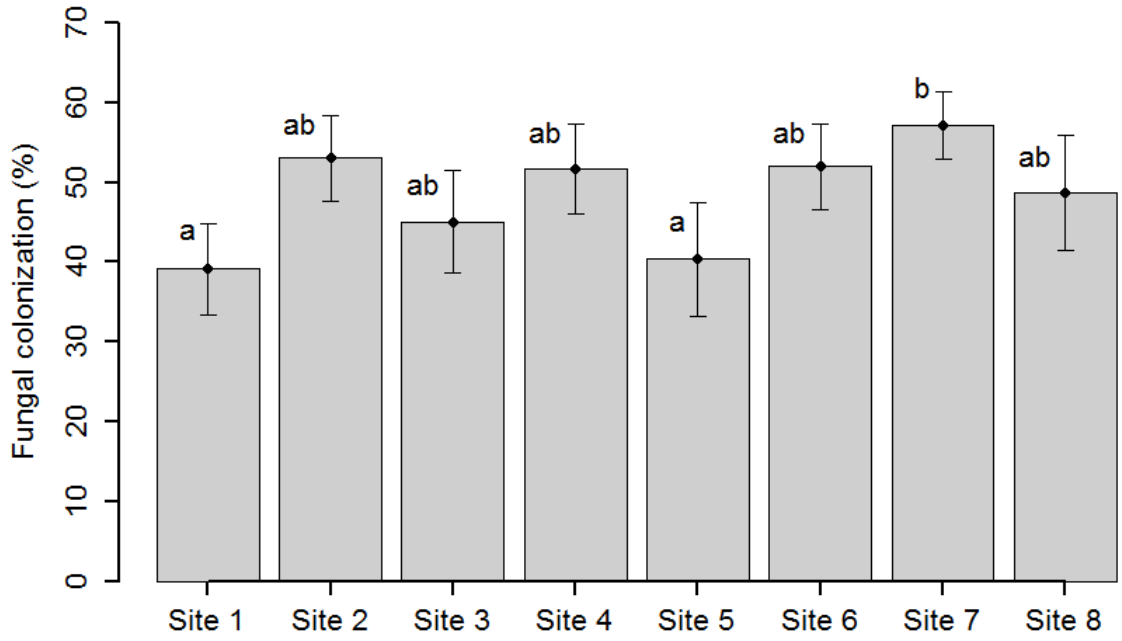

Figure B4.1. Percent AMF colonization for each site across all four tree species. Bars represent standard errors, and letters denote significant differences ($P<0.05$) between sites.

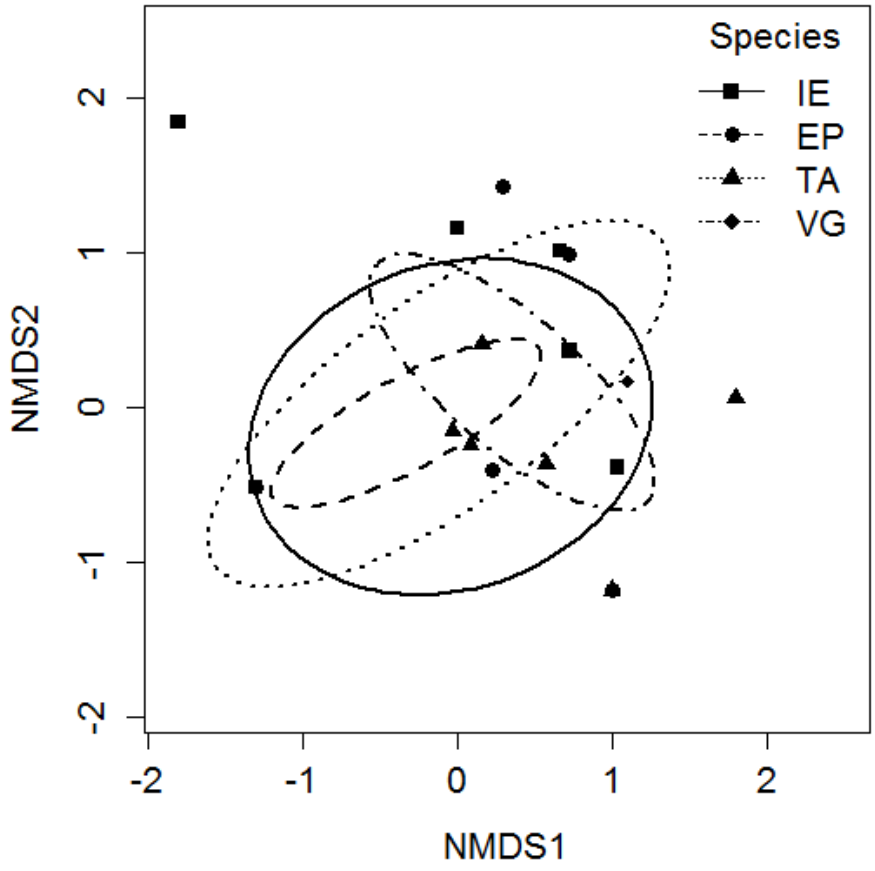

Figure B4.2. Non-metric dimensional scaling (NMDS) **plot** (2 Dimensional NMDS, Stress=0.07, R^2=0.995) **of the AMF community structure classified by tree species identity** (IE=*I. edulis*, EP= *E. poeppigiana*, TA=*T. amazonia*, VG=*V.guatemalensis*)**.** Each point represents a composite of the AMF community of a given tree species per site, and ellipses show the overlap in the fungal community across tree species (confidence area of ellipses = 0.95). PERMANOVA tests showed no differences in the AMF community between tree species (P=0.3003).

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