2008-04-27

Arbuscular Mycorrhizal Fungi Enhance the Acquisition of Mineral Nutrients from Leaf Litter by Morella cerifera

Catalina Aristizabal
University of Miami, cataristizabal@gmail.com

UNIVERSITY OF MIAMI

ARBUSCULAR MYCORRHIZAL FUNGI ENHANCE THE ACQUISITION OF MINERAL NUTRIENTS FROM LEAF LITTER BY MORELLA CERIFERA

By

Catalina Aristizábal

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2008

UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

ARBUSCULAR MYCORRHIZAL FUNGI ENHANCE THE ACQUISITION OF MINERAL NUTRIENTS FROM LEAF LITTER BY MORELLA CERIFERA

Catalina Aristizábal

Approved:

_____ Dr. David P. Janos Associate Professor of Biology	_____ Dr. Terri A. Scandura Dean of the Graduate School
_____ Dr. Leonel da Silveira Lobo Sternberg Professor of Biology	_____ Dr. Donald L. DeAngelis Professor of Biology

_____
Dr. Bruce Schaffer
Professor of Plant Physiology
University of Florida

ARISTIZABAL, CATALINA (Ph.D., Biology)

Arbuscular Mycorrhizal Fungi Enhance the Acquisition of Mineral Nutrients from Leaf Litter by *Morella cerifera* (May 2008)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Associate Professor David P. Janos
No. of pages in text: (136)

Morella cerifera (L.) Small, the Wax Myrtle, forms both arbuscular mycorrhizas and cluster roots which generally are regarded as alternative adaptations for phosphorus acquisition. But whether or not arbuscular mycorrhizal fungi (AMF) provide any benefit to *M. cerifera* is not known. Nevertheless, AMF can proliferate extensively within the litter leaves that accumulate beneath *M. cerifera*. The main objective of this study was to determine if AMF are beneficial to *M. cerifera* host plants in the presence of leaf litter.

In the field, I examined leaf traits that affect the colonization of leaf litter by AMF. I compared AMF colonization of labile versus recalcitrant leaves, and that of leaf pieces with obstructed versus non-obstructed veins. In pot experiments, I examined if labile or recalcitrant litter influences the potential benefit of AMF to *M. cerifera*, and if nitrogen (N) or phosphorus (P) fertilization influences that benefit in the presence of recalcitrant leaf litter.

I found that AMF extensively colonize both labile and recalcitrant leaves, but that they colonize labile leaves more rapidly than recalcitrant leaves. I found significantly less colonization in leaf pieces with obstructed veins than in those with non-obstructed veins which suggests that penetration by the fungi primarily is mechanical and not

enzymatic. The pot experiments showed that AMF are parasitic on *M. cerifera* except when recalcitrant leaf litter is present, and that the effects of AMF on *M. cerifera* are indirect and mediated through effects of AMF on N-fixing nodule dry weight. In both pot experiments, AMF enhanced litter decomposition and may have enhanced plant P-nutrition. AMF benefited *M. cerifera* growth in the absence of N fertilization but negatively affected *M. cerifera* growth when N was added. In the presence of litter, inoculation with AMF increased cluster root formation, suggesting that these two adaptations may be complementary in extremely nutrient-poor soils. Overall, this study shows that *M. cerifera* does benefit from association with AMF, and it suggests that AMF play a more important role in the acquisition of mineral nutrients from leaf litter than previously recognized.

Dedication

I would like to dedicate this dissertation to my family, my husband, and Fabiola Serna.

My family has always been there for me.

My husband not only assisted me with lab and field work, but also gave me the day to day support, encouragement, and love that helped me complete this dissertation.

Fabiola taught me to appreciate, love, and respect nature; she's probably the reason why I am a biologist.

Acknowledgements

I am extremely grateful to my advisor, Dr. David P. Janos for his guidance, time, and support during my years as a graduate student at the University of Miami (UM). He cared enough about my work to help it reach its full potential, and I am forever grateful for that. I could've not had a better mentor. I also greatly thank the members of my Ph.D. committee Drs. Leo Sternberg, Don DeAngelis, and Bruce Schaffer for all of their help with my Ph.D. research, and for their comments and suggestions on how to improve my dissertation. I am also very grateful to Emma Lucia Rivera, my undergraduate advisor in Colombia, who not only got me interested in mycorrhizas, but her work was the source of inspiration for my Ph.D. dissertation.

I am very grateful to Alejandro Hoyos, Bray Beltran, Sisy Noal, Rubymel Jijon, Mike Gutierrez, Michelina de LaMaza, and Tanya Wyss, for their help with field and lab work, and for their friendship. I would also like to thank Josie Hagel, Kristin Byrne, Pattie Terrell, Samira Ghazal, and Lucero Sevillano, who have been like a family to me here in Miami, and gave me a lot of encouragement and support through this process. I am very grateful to Dr. Maria Llabre for her help with Structural Equation Modeling. I would also like to thank the members of the "Mycorrhiza Discussion Group" for all of their comments on my dissertation, and for giving me great feedback on how to improve my dissertation seminar.

I would like to thank Sigma Xi, and the UM's Tropical Biology Fellowship, and Curtis and Smathers' grants for funding my work.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Plants that grow in nutrient-poor ecosystems exhibit a wide array of root system adaptations to efficiently acquire and recycle mineral nutrients. Root system adaptations to acquire mineral nutrients from nutrient-poor soils include the formation of cluster roots (a term used to encompass both proteoid cluster roots and cluster roots found in plant families other than the Proteaceae), and symbiotic associations with mycorrhizal fungi and nitrogen fixing bacteria. In nutrient-poor ecosystems plants produce low quality litter that decomposes slowly (Richards, 1996) and accumulates on the surface of the mineral soil. Because litter is a main source of mineral nutrients for plant growth in such ecosystems (Jordan *et al.*, 1979), most of the root system adaptations to acquire and recycle mineral nutrients occur within the litter layer. The extensive proliferation of absorptive roots within the litter layer forms what have been denominated root mats (Stark & Spratt, 1977).

Because of their position amidst litter, both arbuscular mycorrhizal fungi (AMF) and cluster roots are thought to directly acquire and transport mineral nutrients released by decomposition, before they enter the mineral soil. In nutrient impoverished ecosystems, AMF and cluster roots are thought to be involved in the tightening, or closure (i.e. reduction of mineral nutrient losses from the soil solution because of leaching and immobilization), of mineral nutrient cycles. That AMF colonize (i.e. penetrate and proliferate within) decomposing leaves, recently reported by Rivera &

Guerrero (1998) and Aristizábal *et al.* (2004), suggests that these fungi are even better positioned to scavenge and recycle mineral nutrients resulting from litter decomposition than previously thought.

Morella cerifera (L.) Small ("Wax Myrtle", formerly *Myrica cerifera*), is an example of a plant that grows in oligotrophic ecosystems and is very well adapted to acquire mineral nutrients. Several species in this genus have been found to form cluster roots and to associate with both AMF and *Frankia* sp. (a nitrogen-fixing actinomycete). Because *Morella* spp. form sclerophyllous leaves that are high in lignin and decompose slowly, it is common to observe litter mats, 1-20 cm thick, underneath the canopies of these plants. Within these leaf litter mats the fine roots of *Morella* spp. proliferate. AMF colonization of the fine roots and of the decomposing leaves within the litter of *M. parvifolia* Benth, *M. pubescens* Humb. & Bonp. ex Willd and *M. cerifera* has been reported (Rivera & Guerrero, 1998; Aristizábal *et al.*, 2004).

Even though there are several reports of AMF root and leaf litter colonization for several species in the Myricaceae (Poole & Sylvia, 1990; Koske *et al.*, 1992; Hurd & Schwintzer, 1997; Aristizábal *et al.*, 2004), it is still controversial whether or not AMF provide any benefit to these plants (Berliner & Torrey, 1989; Poole & Sylvia, 1990). The main reason for this controversy is that these plants form cluster roots, and cluster roots are regarded as a functional replacement for AMF (Brundrett & Abbott, 1991; Hetrick, 1991). Additionally, experimental evidence shows no beneficial effects of AMF inoculation on *M. cerifera* growth (Poole & Sylvia, 1990). These results however, may simply reflect the highly facultatively mycotrophic nature of this plant. Facultatively

mycotrophic plants may benefit from AMF under some conditions, but are able to grow without AMF under others (Janos, 2007).

As my Ph.D. dissertation research, I sought to determine in an ecologically realistic manner whether or not AMF provide benefit to *M. cerifera*. I provisioned mycorrhizal plants growing in nutrient-poor soils with entire dead leaves. Whether or not the ability of these plants to associate with AMF influences the efficiency with which they recycle nutrients from litter has not been determined, but could potentially influence their growth and survival in the nutrient impoverished soils they colonize.

Background

Role of arbuscular mycorrhizal fungi in plant mineral nutrition and tropical nutrient cycling

AMF are symbiotic fungi in the phylum Glomeromycota (Schussler *et al.*, 2001). The hyphae of these fungi colonize the cortices of plant roots, and in exchange for photosynthate, translocate mineral nutrients from the soil to their hosts. AMF extraradical hyphae branch in the mineral soil, forming absorptive hyphal networks that are involved in the acquisition of relatively immobile nutrients such as P, Zn, and Cu (Smith & Read, 1997). In addition to providing a large surface area for absorption of mineral nutrients, AMF hyphae grow beyond zones of nutrient depletion that develop around roots, and thus they improve the uptake of ions that diffuse slowly through the soil (Hetrick, 1991). Because hyphae may have a higher affinity than roots for phosphorus (Bolan, 1991), and phosphorus has low mobility in acid tropical soils, the importance of AMF for plant phosphorus nutrition in the tropics usually is emphasized.

In contrast, the role of AMF in recycling nutrients from litter is unclear. Because root and AMF hypha densities are high within leaf litter in nutrient-poor ecosystems (Went & Stark, 1968) and because AMF hyphae proliferate in response to decomposing organic matter (St. John *et al.*, 1983) it is thought that AMF may close mineral nutrient cycles. Although evidence supporting this contention is scant, it repeatedly has been suggested (Janos, 1983; Richards, 1996). Four different, but not mutually exclusive ways in which AMF may close nutrient cycles have been postulated. The first and most generally accepted way is by being strategically positioned within litter to acquire mineral nutrients released by decomposer microorganisms before those nutrients reach the mineral soil (Janos, 1983). A second way is by interconnecting plants. Because AMF hyphae can simultaneously colonize the root systems of different individuals, they are well positioned to scavenge nutrients from dying fine roots and to transport them to neighboring plants (Johansen & Jensen, 1996).

In a third way of closing mineral nutrient cycles, AMF could partially bypass decomposers by acquiring organic forms of macronutrients, such as phosphorus and nitrogen from soil organic matter (SOM). Although AMF (specifically *Glomus intraradices*) can hydrolyse organic phosphate under controlled conditions (Koide & Kabir, 2000), whether or not AMF acquire biologically significant amounts of organic phosphorus or nitrogen compounds in natural systems is still an open question. In phosphorus-limited tropical rainforests where there probably is intense competition among plants and soil microorganisms for phosphorus, the ability of AMF to release it from litter would be of tremendous ecological value (Read & Perez-Moreno, 2003). Hawkins *et al.* (2000) similarly have suggested that in nutrient poor sites (often

mineralization-limited) where considerable quantities of amino acids are present in the soil, their uptake by AMF could have substantial effects on host nitrogen nutrition. Bardgett *et al.* (2003) and Nasholm (2001) demonstrated that plants growing in mineralization-limited systems can acquire significant amounts of organic nitrogen, but whether or not AMF or other mycorrhizal fungi mediated this uptake was not determined.

The "Direct Nutrient Cycling Hypothesis", describes the fourth way in which AMF might close tropical nutrient cycles, and it has generated the most controversy. This hypothesis states that endomycorrhizas with the ability to digest cellulose and lignin "cycle nutrients directly from dead organic matter to the living roots" (Went & Stark, 1968). Because of the limited production of hydrolytic enzymes by AMF (Varma, 1999), inability of AMF to grow in the absence of a host, and inability of AMF to acquire carbon from decomposing SOM (Nakano *et al.*, 1999), however, it is highly unlikely that AMF are major agents of decomposition.

That AMF colonize dead leaves (Rivera & Guerrero, 1998; Aristizábal *et al.*, 2004) provides support for the contention that these fungi are involved in the closure of nutrient cycles in nutrient-poor ecosystems. Whether or not AMF actively can mineralize litter, however, is uncertain, and may be unlikely. Although penetration by AMF of sclerophyllous leaves, such as those of *Morella* spp., might suggest that AMF produce lignolytic or cellulolytic enzymes, these fungi instead may penetrate leaves mechanically through leaf veins, not enzymatically (Aristizábal *et al.*, 2004). Even if AMF themselves cannot mineralize litter, AMF within dead leaves are very well positioned to scavenge mineral nutrients released by the activity of saprophytic microorganisms (in either inorganic or organic form).

Although evidence such as that of Nakano *et al.* (1999) suggests that AMF lack saprophytic capability, a recent study has raised the question anew. Hodge *et al.* (2001) demonstrated that AMF can accelerate the decomposition of finely-ground leaves and acquire nitrogen from them. In explanation of this result, Hodge *et al.* (2001) suggested that AMF have a weak saprophytic capacity. Their results, however, could be explained by enhancement of decomposer microorganism activity by AMF. As an indirect effect of improved host nutrition, and as a direct consequence of enhanced photosynthesis, AMF have been found to stimulate root exudation (Azaizeh *et al.*, 1995). Such exudates provide a "quick" carbon source for decomposer microorganisms, resulting in an increase in their activity and potential enhancement of decomposition.

The results of Hodge *et al.* (2001) highlight our limited understanding of the role of AMF in nutrient cycling. If AMF do contribute to host acquisition of nutrients from organic matter, why have only Hodge *et al.* (2001) observed such an effect? The answer may be that most experiments have used either ground leaves or leaves that are very labile which disappear quickly (1-2 months). Hodge's (2003) results suggest that AMF do not increase nutrient uptake from labile organic matter. She grew *Lolium perenne* and *Plantago lanceolata* in intra- or interspecific competition, either with discrete or dispersed organic matter patches, and with or without AMF. Overall, she found that plants acquired more nitrogen from dispersed organic matter than from discrete patches, and that AMF did not influence nitrogen acquisition. She attributed this to nitrate-N being very mobile in soil and directly acquirable by roots.

Where mineralization of nitrogen is rapid, except perhaps when plants are competing intensely (Bowen, 1980), AMF may be unimportant for plant nitrogen

nutrition**.** But in ecosystems where mineralization of litter is inhibited by low temperatures, seasonal drought, or low litter quality, AMF might play an important role in the acquisition of ammonium or simple organic nitrogen compounds (Hawkins *et al.*, 2000).

Are cluster roots an alternative to AMF for **Morella** *species?*

Cluster roots were first described in the Proteaceae, a plant family with a large proportion of non-mycorrhizal species (Brundrett & Abbott, 1991). It frequently has been suggested that cluster roots are an alternative to AMF (Hetrick, 1991). Experiments involving plants in other families (i.e. Fabaceae) that form cluster roots have found no positive growth response to AMF inoculation (Trinick, 1977; Berliner & Torrey, 1989).

That cluster roots are an alternative to AMF also is suggested by the overlap in function and site of occurrence of these two nutrient acquisition adaptations. Cluster roots typically occur in humus-rich surface soil horizons (Lamont, 2003), and AMF have been found preferentially associated with decomposing OM (St. John *et al.*, 1983). When cluster roots occur among litter leaves, their concentration is greatest in the actively decomposing (A_0) layer (Lamont, 2003). Similarly, a recent report has shown that the presence of AMF extra-radical hyphae was greatest in the actively decomposing layer (Aristizábal *et al.*, 2004). The large surface: volume ratio of both cluster roots and AMF hyphae is thought to improve plant nutrition by increasing absorptive surface area. Although cluster roots secrete exudates (e.g. carboxylates, phenolics, protons, enzymes, organic acids) that are involved in the solubilization of mineral nutrients, the same has not been demonstrated unequivocally for AMF (Read & Perez-Moreno, 2003). The secretion of mucopolysaccharides that bind cluster roots to soil particles is important for

acquisition of mineral nutrients, especially in soils with low bulk density (Lamont, 2003). Whether or not secretion of glomalin by AMF extra-radical hyphae binds them to decomposing OM and plays a role in the acquisition of nutrients from decomposing OM by AMF is unresolved.

In spite of the overlap in function of AMF and cluster roots, several plant species exhibit both adaptations. If these adaptations occur in response to different soil conditions, or if cluster roots are formed when mycorrhizal inoculum is scant is not known. Reddell *et al.* (1997) attempted to answer the latter question. Although they found that at low phosphorus availabilities (< 25 mg P kg^{-1}) *Casuarina cunninghamiana* plants inoculated with *Glomus* sp. were significantly larger than controls, they did not find a negative correlation between AMF and cluster root formation. Only at 10 mg P kg^{-1} did controls have more cluster roots than AMF-inoculated plants, but this difference was not significant.

Another species that associates with AMF and forms cluster roots is *M. cerifera*. Whether or not there is a negative correlation between AMF colonization and cluster root formation in this species has yet to be determined. Cluster root formation is thought to be triggered by an internal message (e.g., foliar applications of phosphorus have suppressed cluster root formation; Louis *et al.*, 1990), and AMF usually improve host phosphorus nutrition. Therefore, after *M. cerifera* plants become colonized by AMF their phosphorus-acquisition might switch from cluster roots to AMF. That the construction and maintenance of cluster roots is more costly than AMF (Pate & Watt, 2002) suggests why there might be such a switch.

That AMF probably provide benefit to *M. cerifera* plants is supported by their sustaining colonization as high as 44% (of their root length) in the field (Poole & Sylvia, 1990). Moreover, the results of an experiment which purported to show that AMF do not provide benefit to *M. cerifera* may only reflect one end of the spectrum of responses that this facultatively mycotrophic species has to AMF. Poole and Sylvia (1990) tested the effect of AMF on the growth of 1 year-old, naturally nodulated, *M. cerifera* seedlings. They found that *M. cerifera* seedlings failed to become colonized unless they were grown with a companion plant, and that when colonization did occur there was no positive effect of inoculation on root or shoot growth. Their experiment lasted only 7 weeks, however, and they used loamy sand with a pH 6 and 26 mg P kg^{-1} as their growth medium. Additionally, their plants were fertilized once, after 3 weeks, with 10 ml modified Hoagland's solution containing KH_2PO_4 at 10 μM P. Thus their contention that the "failure of *M. cerifera* to become colonized when grown without a VAM companion plant and the absence of arbuscules when *M. cerifera* became colonized in the presence of a VAM companion plant suggest that the VAM association of *M. cerifera* is nonfunctional" (Poole & Sylvia, 1990) probably does not reflect what occurs when these plants are growing in nutrient impoverished soils.

Frankia*, cluster roots and AMF*

Biological nitrogen fixation is a process that is expensive of energy. It involves the break down of the very stable triple covalent bond of atmospheric N_2 (Taiz & Zeiger, 2002). Because of the high energy requirements of biological nitrogen fixation, nodules are a strong sink for phosphorus (Marschner, 2002). It has been demonstrated that phosphorus fertilization enhances nodulation and nitrogen fixation (Marschner, 2002). Because of

their importance in phosphorus acquisition by plants, AMF also have been found to stimulate nodulation in symbioses of legumes with *Rhizobium* (Chalk *et al.*, 2006) and in actinorrhizal plants (plants that associate with the actinomycetes, such as *Frankia* spp.; Fraga-Beddiar & Le Tacon, 1990; Jha *et al.*, 1993). Indeed, AMF may be more effective in enhancing *Frankia* sp. nodulation than is phosphorus fertilization (Fraga-Beddiar & Le Tacon, 1990). In addition to AMF, cluster roots, also may enhance nodulation and N-fixation. Because most of the species that form cluster roots also form nodules, it has been hypothesized that root clusters evolved in N-fixing species to supply phosphorus for nodule formation or to sustain fixation (Adams *et al.*, 2002).

Effects of litter quality and root proliferation on the colonization of litter by AMF

Because the colonization of decomposing leaves by AMF is a phenomenon only recently reported, there is no information regarding the effects of litter quality on it. The limited production of hydrolytic enzymes by AMF (Varma, 1999) suggests that soft, labile leaf litter may be colonized more quickly than tough, recalcitrant leaf litter. It is not known, however, if there is a positive relationship between colonization rate and the proportion of nutrients acquired by AMF from decomposing leaves. Indirect evidence suggests that there is no such relationship. High quality litter is quickly colonized by decomposer microorganisms, but because plant roots and AMF may require several days to colonize it, mineral nutrients may be susceptible to leaching loss, or temporary immobilization by soil bacteria or fungi. In contrast, low quality leaf litter does not provide a "rich" substrate for microbial growth. AMF may have a competitive advantage in colonizing it because they receive carbohydrates directly from their hosts.

Because AMF depend on their hosts for carbon, and are limited in the distance they can spread beyond plant roots, the colonization of OM by AMF might be affected by the ability of roots to respond to the presence of OM. The response of plant roots to OM patches often is slow (Tibbett & Sanders, 2002). If the colonization of leaf litter by AMF is dependent upon its distance from a host's roots, by the time AMF could colonize OM of high quality, it might be mostly gone. High plant and fungus investment would lead to little nutrient acquisition.

Objectives

The main objective of my Ph.D. research was to determine if AMF provide benefit to *M. cerifera* plants under conditions of litter accumulation. I specifically addressed the following two general questions:

I. In the field, what leaf traits affect the colonization of leaf litter by AMF?
II. What root system adaptations are involved in the acquisition and recycling of mineral nutrients by *M. cerifera*?

These two general questions, which represented the two phases of my Ph.D. dissertation research, were answered by investigating several component questions. The first phase (Phase I) involved experimentally determining how different factors influence the extent to which AMF colonize decomposing leaves. Although a wide array of factors potentially influence AMF decomposing leaf colonization, I focused on those which might explain why the colonization of *M. cerifera* leaf litter is so high. In Chapter 2 I test whether AMF differentially colonize labile (i.e., those that decompose rapidly) versus recalcitrant (i.e., those that decompose slowly) leaves, and whether or not AMF

preferentially penetrate decomposing leaves through primary veins, as has been suggested by Aristizábal *et al.* (2004).

The second phase (Phase II) of my Ph.D. work involved experimentally determining what root system adaptations are involved in the acquisition and recycling of nutrients by *M. cerifera*. Because *M. cerifera* plants in the field tend to accumulate litter beneath their canopies, and AMF colonization has been reported under field conditions, in Chapter 3 I examine the effect of AMF on the growth of *M. cerifera* when litter leaves are present in the soil. I specifically examined if any benefit AMF provide to *M. cerifera* changes in the presence labile versus recalcitrant leaf litter. Because the value of AMF is minimized in soils with ample soluble mineral nutrients and labile leaves quickly release their nutrients, AMF may be unimportant for the acquisition of nutrients from labile leaves. Moreover, AMF may have limited opportunity to colonize labile litter leaves and to enhance acquisition of mineral nutrients from them before they decompose. In contrast, conditions that limit abundance or movement of mineral nutrients in the soil such as slow mineralization may make acquisition of nutrients from litter by AMF important.

In Chapter 4, I investigate the conditions of soil nitrogen and phosphorus availability under which the acquisition of nutrients from leaf litter by AMF may be beneficial to *M. cerifera* seedlings. Soil solution phosphorus and nitrogen availability are likely to affect the potential benefit to hosts from AMF and dead leaves. Many studies have shown that host benefit from phosphorus uptake by AMF depends upon soil solution phosphorus availability being low (Janos, 2007). Indeed, soil phosphorus availability to host plants can determine if the arbuscular mycorrhizal symbiosis is

mutualistic or parasitic (Johnson *et al.*, 1997; Schroeder & Janos, 2004; Janos, 2007). Even though AMF are not thought to usually contribute to plant nitrogen nutrition (but see Cliquet *et al.*, 1997) nitrogen deficiency has been found to stimulate root colonization by AMF (Blanke *et al.* 2005) which suggest that AMF might sometimes function in nitrogen acquisition. Additionally, because *M. cerifera* associates with *Frankia*, soil nitrogen availability will likely directly influence the Actinomycete (e.g. Kohls & Baker, 1989), which may indirectly affect AMF because both symbionts depend upon host carbon. Finally, altering soil solution nitrogen and phosphorus availability may provide insights on what mineral nutrient or nutrients AMF may be acquiring from litter leaves.

Chapter 2

Leaf traits influence leaf litter colonization by arbuscular mycorrhizal fungi

The roots of most wild plants form mutualistic associations with arbuscular mycorrhizal fungi (AMF; Smith & Read, 1997). AMF enhance plant growth principally by acquiring relatively immobile inorganic mineral nutrients, such as phosphate, from the soil solution (Smith & Read, 1997). Generally, AMF are assumed incapable of mobilizing significant quantities of mineral nutrients from litter for supply to their hosts (Dighton, 1991; Read & Perez-Moreno, 2003) even though litter is a major source of mineral nutrients in most natural ecosystems (Swift *et al.*, 1979). AMF are obligate biotrophs that produce only small amounts of lignolytic and cellulolytic enzymes (Azcon-Aguilar et al., 1999; Varma, 1999), and hence, likely have limited ability to degrade organic substrates. For that reason, AMF are thought to predominate and to play a significant role in plant mineral nutrition principally in ecosystems in which plant growth is not limited by slow mineralization of organic matter (Read & Perez-Moreno, 2003).

Even though AMF produce scant quantities of hydrolytic enzymes, they can colonize other plant tissues than roots, and they frequently are found associated with dead organic matter (OM). AMF hyphae and vesicles have been reported in scale-like leaves along the rhizomes of *Zingiber officinale* (Taber & Trappe, 1982), *Dictyostega orobanchoides* (Imhof, 2001), and *Paepalanthus* sp. (Aristizábal *et al.*, 2004). AMF also

colonize and sporulate within dead seeds (Taber, 1982a; Taber, 1982b), oribatid mites (Rabatin & Rhodes, 1982), and other AMF spores (Koske, 1984). Rabatin (1980) found abundant *Glomus tenuis* hyphae within a senescent moss fragment. Proliferation of AMF hyphae in the presence of OM has been demonstrated experimentally (St. John *et al.*, 1983; Albertsen *et al.*, 2006). Even though most researchers tend to remove litter before sampling roots and soil to assess AMF colonization, Rose & Paranka (1987) found significantly higher AMF colonization in roots among litter than in roots from the soil beneath. Close examination of leaf litter among which roots and AMF hyphae proliferated revealed that litter leaves can be abundantly colonized by AMF (Rivera & Guerrero, 1998; Aristizábal *et al.*, 2004). Aristizábal *et al.* (2004) reported that AMF colonization of leaf litter can be high (up to 97.7 %), and in late stages of leaf decomposition can comprise as much as 68.6 % vesicles (estimated as the percentage of 1.63 mm^2 leaf sample areas occupied by vesicles).

That AMF colonize and proliferate within dead OM seems paradoxical given their lack of degradative enzymes. This paradox has led researchers to re-evaluate the two commonly held tenets that AMF lack saprophytic ability, and that AMF do not enhance host acquisition of mineral nutrients from organic sources. Nakano *et al.* (1999, 2001) confirmed that AMF lack saprophytic capability, but in contrast, Hodge *et al.* (2001) found that AMF accelerated the decomposition of finely ground leaves and acquired nitrogen from them. In consequence, Hodge *et al.* (2001) suggested that AMF have weak saprophytic capacity. The Hodge *et al.* (2001) finding of accelerated decomposition, however, might be explained by AMF enhancing decomposer microorganism activity (Read & Perez-Moreno, 2003). Regardless of the uncertainty surrounding saprophytic

ability of AMF, the fungi may play a more important role in capturing mineral nutrients from organic sources than previously recognized. AMF within litter probably are positioned strategically to scavenge mineral nutrients released by saprophytic microorganisms and to reduce potential nutrient losses to leaching and immobilization, thereby tightening nutrient cycles (Janos, 1983; Richards, 1996).

Although the colonization of leaf litter by AMF may greatly affect ecosystem nutrient cycling, the dynamics of colonization are unknown. For example, Aristizábal *et al.* (2004) did not determine how much time AMF required to attain the high levels of colonization they reported. Even though root colonization dynamics have been modeled (e.g. Buwalda *et al.*, 1982; Walker & Smith, 1984), those models may not apply to leaf colonization because the plant-fungus communication that regulates colonization of roots (Giovannetti *et al.*, 1994) does not apply for dead leaves.

Physical constraints primarily might influence the rate of spread of AMF within different types of dead leaves. Aristizábal *et al.* (2004) suggested that AMF might mechanically colonize tough sclerophyllous leaves such as those of *Morella* (formerly *Myrica*) species by passing through leaf vascular tissue. Nevertheless, even if vascular tissue does facilitate initial leaf colonization by AMF, leaf toughness may hinder spread beyond veins. On the other hand, although soft, labile leaves might be easily penetrable by AMF, whether or not they can occupy such a potentially ephemeral substrate likely depends upon their ability to respond quickly to OM with hypha proliferation and to compete effectively for space – and possibly mineral nutrients – with saprophytic microorganisms.

Just as the dynamics of litter leaf colonization by AMF are very little understood, so too is how geographically widespread the phenomenon may be. To date, it has been reported only in nutrient impoverished tropical montane ecosystems (Aristizábal *et al.*, 2004). Even though other types of mycorrhizas are expected in such ecosystems in which complex organic sources of mineral nutrients prevail (Read & Perez-Moreno, 2003), AMF can predominate in them (Kottke *et al.*, 2004). This might point to the AMF species of tropical montane ecosystems having unique adaptations to allow them efficiently to acquire mineral nutrients from organic matter. If so, those specific adaptations might restrict colonization of dead leaves by AMF to tropical montane ecosystems.

In this study, I ask if the indigenous AMF of a relatively fertile, lowland, sub-tropical hardwood hammock can colonize dead leaves of five different plant species. I conducted two experiments to examine the dynamics of colonization of dead leaves by AMF and the influence of leaf traits on colonization. Experiment 1 investigated two questions: "in a South Florida hammock are different types of leaves colonized by AMF" and "do AMF rates of spread in different types of leaves reflect leaf decomposition rate?" Experiment 2 investigated the question: "do leaf veins facilitate penetration of dead leaves by AMF?"

Methods

Experiments 1 and 2 were set up in a South Florida sub-tropical hardwood hammock located at the University of Miami's Four Fillies Farm. Sub-tropical hardwood hammocks are closed canopy forests containing a diverse assemblage of evergreen and semi-deciduous broadleaved trees and shrubs. Hammock soils are shallow, mostly

organic, and are underlain by limestone (Snyder *et al.*, 1990). In an average year, rainfall ranges from 1400 to 1525 mm. Rainfall is seasonal, with a dry season from October through May and a wet season from June through September. Average temperatures are 27˚C for July, and range from 18 to 21˚C in January (Snyder *et al.*, 1990). Although there are occasional ectomycorrhizal *Quercus virginiana* Miller in this community, most of the species form arbuscular mycorrhizas (Fisher & Jayachandran, 2005)**.** Experiment 1 began in June 2002 at the start of the rainy season just as there was a flush of fine roots within the litter layer. Experiment 2 began in July 2003.

Experiment 1

In the hammock, I established twenty-four stations along two 36 m parallel transects separated by 4 m. The distance between adjacent stations along each transect was 3 m. At each station, I buried five 100 cm^2 60-µm mesh bags, each containing a different leaf species. The leaves were from *Morella cerifera* (L.) Small (formerly *Myrica cerifera*), *Q. virginiana*, *Rhapis excelsa* (Thunb.) A. Henry ex Rehder, *Averrhoa carambola* L., and *Costus spicatus* (Jacq.) Sw. I placed the mesh bags on the surface of the mineral soil just beneath the litter layer. Within a station, mesh bags were equally spaced around the circumference of a 50 cm diameter circle. *M. cerifera* leaf bags were on the west side of the circle with *A. carambola*, *R. excelsa*, *Q. virginiana* and *C. spicatus* mesh bags following clockwise.

Each mesh bag contained approximately 2.5 g of dried leaves. I removed the leaves from plants at the University of Miami's Gifford Arboretum, and dried them at 60° C for 72 h. Leaves removed from plants were green, fully mature, and similar in size. *M. cerifera* (wax myrtle), *Q. virginiana* (live oak) and *R. excelsa* (lady palm) leaves are

tough and stiff, and *A. carambola* (starfruit) and *C. spicatus* (spiral ginger) leaves are soft and pliable. *R. excelsa* and *C. spicatus* leaves have parallel venation, and *M. cerifera*, *Q. virginiana*, and *A. carambola* have reticulate venation. Initial percentage of C and N in leaves was determined with a Carlo-Erba NC2100 analyzer (Table 2.1).

I collected mesh bags containing *M. cerifera*, *Q. virginiana*, *R. excelsa*, and A. *carambola* leaves 70,100, 170, and 540 d after placement. Each collection included mesh bags from six randomly selected stations. After 70 d, *C. spicatus* leaves had almost completely decomposed, so I collected the remaining bags of this species after 78, 86, and 100 d. For each collection, leaves were removed from the bags and weighed then dried at 60° C for 72 h after a sub-sample of approximately 1 g was removed, cleared, and stained using a method modified from Phillips & Hayman (1970). I weighed dried leaves to the nearest 0.0001 g.

To assess mycorrhizal colonization of leaves, I treated each sub-sample as follows. Depending on their state of degradation, I immersed leaves in 10-15% KOH at room temperature for 1-4 weeks, acidified them overnight in 1% HCl, and stained them in 0.05% acid Trypan blue for 6 h. I scored leaves for AMF hyphae (%) and vesicle (%) colonization following the procedure of Aristizábal *et al.* (2004). Briefly, cleared and stained leaf pieces were cut into approximately 2 x 5 mm long strips, and haphazardly selected strips were mounted roughly perpendicular to the long axis of a microscope slide. For each slide, six parallel longitudinal transects were scored for AMF colonization at their intersections with strips as viewed through a compound microscope at x100. AMF colonization was not determined for *A. carambola* leaves collected after 540 d because the leaves were very fragmented, and they disintegrated when immersed in KOH.

Additional sub-samples of each species were cleared and immunostained using a monoclonal antibody MAb 32B11 against glomalin, a glycoprotein produced by AMF (Wright, 2000).

Experiment 2

I collected green, fully mature *R. excelsa* leaves from the University of Miami's Gifford Arboretum, cut them into 3 x 3 cm squares and dried them to constant weight at 60˚C. I obstructed the parallel veins by coating opposite edges of leaf squares with exposed vein ends with nail polish and PlastiDip®. The same procedure was repeated for non-obstructed controls by coating leaf edges without exposed vein ends. Obstructed and non-obstructed leaf pieces were paired, placed atop one another, and deposited in 3.5 cm^2 60-µm mesh bags. I buried these mesh bags in the centers of the 50 cm diameter circular stations of Experiment 1. Four of the stations still had mesh bags from Experiment 1, and I was careful not to disrupt soil and litter around them. To ensure that AMF had an equal chance of colonizing both obstructed and non-obstructed leaf fragments, I buried half of the mesh bags with their obstructed leaf fragment on the bottom, and the other half of the mesh bags with their non-obstructed fragment on the bottom. I retrieved all of these bags after 130 d based upon the *R. excelsa* results of Experiment 1. In Experiment 1 after 100 d, AMF colonization was relatively extensive, but leaves were not so fragmented as to render obstructed veins available for colonization. After collection of the Experiment 2 bags, I assessed AMF colonization of leaf pieces as described for Experiment 1.

Data analysis

Prior to analysis, data were screened for normality and homogeneity of variances. When necessary, leaf dry weight remaining and AMF colonization data (% hyphae and % vesicles) of Experiments 1 and 2 were arcsine transformed. For ease of understanding, data shown in the tables and figures are not transformed. All statistical analyses were performed with SPSS v. 14.

Experiment 1

I compared leaf dry weight remaining among species after 70, 100, 170, and 540 d using one-way ANOVA and Tukey's HSD post hoc test. I estimated decomposition constants for all leaf species (except *C. spicatus* because it had almost completely decomposed after 70 d) by fitting the leaf dry weight data to Bunnel and Tait's double-exponential decay model (Wieder & Lang, 1982):

$$X = A\, e^{-k_1 t} + (1 - A) e^{-k_2 t} \quad (1)$$

in which X is the proportion of the initial mass that remains at time t; A is the leaf fraction that decomposes easily (i.e., the labile fraction), 1-A is the leaf fraction that decays slowly (i.e., the recalcitrant fraction), k_1 and k_2 are the exponential decomposition constants for the labile and recalcitrant leaf fractions, respectively, and t is time in days (Wieder & Lang, 1982).

I compared AMF hyphae and vesicle colonization among leaf species after 70, 100, 170, and 540 d using one-way MANOVA and Tukey's HSD post hoc test. To test whether or not the ratio of vesicle to total AMF colonization changed as a function of time for the different species I used one-way ANOVA and Tukey's HSD post hoc test.

I estimated AMF leaf colonization constants by fitting AMF colonization data to a form of the logistic equation suggested by D. DeAngelis (personal communication):

$$C_t = C_p / [1 + [(C_p - N_0)/N_0] * e^{-st}] \quad (2)$$

in which C_t is total (hyphae + vesicles) AMF colonization at time t, C_p is the maximum AMF colonization attained (the curve's asymptote), N_0 is AMF colonization at time 0, s describes the curve's steepness (i.e., how quickly the leaves reach asymptotic colonization), and t is time in days. This equation is a modification of the equation proposed by McGonigle (2001). In contrast to McGonigle's (2001) equation, this equation can be constrained to approach zero at time zero. This is accomplished by choosing an $N_0 << C_p$ (e.g. $0.02 * C_p$). The point of inflection, i, (the point around which the curve is symmetric, which occurs at 50% of the asymptotic colonization) can be estimated with the equation:

$$i = 1/s * \ln [(C_p - N_0)/N_0] \quad (3)$$

I did not fit the logistic equation to *A. carambola* and *C. spicatus* data because AMF colonization of those species' leaves occurred very quickly and was near its maximum value at the time of the first collection. I used linear regression to determine if AMF colonization in *A. carambola* and *C. spicatus* changed as a function of time.

Experiment 2

I used paired t-tests to compare AMF hyphae and vesicle colonization in obstructed and non-obstructed leaf fragments.

Results

AMF colonized all leaf species (Figure 2.1a-e). Coarse aseptate hyphae typical of AMF became immunostained (Figure 2.1f) but not adjacent septate, melanic fungal hyphae. At the 70 d collection, AMF colonization in tough *M. cerifera* and *Q. virginiana* leaves was restricted to, or adjacent to leaf veins (Figure 2.1a, b). In contrast, AMF colonization in soft *A. carambola* and *C. spicatus* leaves was widely spread throughout the leaf tissue (Figure 2.1d, e).

At each collection time, species differed significantly with respect to the percentage of leaf dry weight remaining (Table 2.2). Because I had to collect *C. spicatus* leaves relatively quickly, only two harvests of this species are shown in Table 2.2. At two additional harvests, mean dry weight ± SE of *C. spicatus* after 78 d was 29.56 ± 1.60 g and after 86 d was 30.76 ± 2.59 g. After 70 d *C. spicatus* and *A. carambola* had the lowest percent dry wt remaining. *A. carambola* leaves initially lost weight quickly and then decomposed slowly (Table 2.2). At the 100 d collection, the percent dry weight remaining of *A. carambola* leaves was not significantly different from that of *R. excelsa* and *Q. virginiana* leaves. At the 170 d collection, the percent dry weight remaining of *A. carambola* was similar to that of *R. excelsa*, and at the 540 d collection, it did not differ significantly from *R. excelsa* or *Q. virginiana*. *M. cerifera* consistently had the highest percent dry wt remaining.

Best-fit double-exponential decay curves are shown in Figure 2.2. The double-exponential decay model fit my data better than a single-exponential model, explaining at least 91% of the variance in leaf decomposition for each species. Note that these values may be artificially high, however, because I have no intermediate harvests between 170

and 540 days. Constants estimated using the double-exponential decay model are shown in Table 2.3. Even though their standard errors are too high to reveal statistical differences among species, general patterns can be discerned which correspond well to those revealed by ANOVA. The fraction of *M. cerifera* leaves that was labile was small, and it decomposed slowly. *Q. virginiana* leaves had a similarly small labile fraction, but it decomposed at a faster rate than *M. cerifera*'s labile fraction. *R. excelsa* and *A. carambola* both had high proportions of labile material. This material decomposed relatively slowly for *R. excelsa* and very quickly for *A. carambola*.

At the different collection times, leaf species had significant effects on AMF hypha and vesicle colonization (Figure 2.3). At the 70 d collection, *A. carambola* and *C. spicatus* leaves had the highest hyphal colonization, and *M. cerifera* leaves had the lowest. Hyphal colonization of *R. excelsa* leaves was intermediate between that of *A. carambola*/*C. spicatus* and *Q. virginiana* leaves, and hyphal colonization of *Q. virginiana* was intermediate to that of *R. excelsa* and *M. cerifera*. At 70 d, vesicle colonization of the different leaf species showed a similar pattern to hyphal colonization, with *A. carambola* having the highest percent colonization and *M. cerifera* the lowest (Figure 2.3).

Mean AMF hyphae and vesicles (% ± SE) in *C. spicatus* leaves (not shown in Figure 2.3 because they were collected earlier than other leaf species) are: 78 d hyphae = 25.32 ± 4.64, vesicles = 69.01 ± 6.67; 86 d hyphae = 32.69 ± 3.05, vesicles = 55.66 ± 4.34.

After 100 d, differences in vesicle colonization among the leaf species ceased to be evident (Figure 2.3), whereas hyphal colonization remained highest in *C. spicatus* and

lowest in *M. cerifera* and *Q. virginiana*. At the 170 d collection, hyphal colonization was highest in *A. carambola*, and lowest in *M. cerifera*. At the 540 d collection, hyphal colonization was highest in *R. excelsa* and lowest in *M. cerifera* and *Q. virginiana*.

The ratio of vesicle to total AMF colonization of leaves changed as a function of time only for *M. cerifera* ($F_{3,20} = 3.61$, $P = 0.031$). *M. cerifera* leaves collected after 70 d had a significantly lower proportion of vesicles than leaves collected after 540 d (Tukey's HSD). The proportion of vesicles in these leaves at 100 and 170 d was intermediate between that at 70 and 540 d.

For all species, except *A. carambola* and *C. spicatus*, total AMF colonization increased as a function of time (linear regression for *A. carambola* and *C. spicatus* respectively, $r^2 = 0.00$, $P = 0.95$, and $r^2 = 0.04$, $P = 0.35$). Best-fit logistic curves and their corresponding coefficients of determination are shown in Figure 2.4. In Table 2.4, I show colonization parameters estimated for Eq. (2) and those obtained for the equation presented by McGonigle (2001). Eq. (2) explains at least 62 % of the variance of total AMF leaf colonization. Constants estimated for the two different equations are similar for *M. cerifera* and *Q. virginiana*, but differ for *R. excelsa*. Because the standard errors of the estimated constants are large, I did not statistically compare colonization constants among species. The data suggest that these three species reach similarly high asymptotes (C_p), and that *M. cerifera* has the lowest curve steepness (s) and the longest time to inflection.

Mean AMF hyphal and vesicle colonization was significantly lower for *R. excelsa* leaf fragments with obstructed veins than for those with non-obstructed veins (Figure 2.5; hyphae: $t = 2.27$, $df = 21$, $P = 0.03$; vesicle $t = 2.09$, $df = 21$, $P = 0.05$).

Discussion

I found AMF to penetrate and proliferate within dead leaves of five species with a variety of different traits in a sub-tropical, hardwood hammock. This demonstrates that colonization of dead leaves by AMF is not restricted to highly oligotrophic montane tropical ecosystems. I also have observed high levels of AMF colonization of litter leaves in *Morella faya* (Ait.) Wilbur at Hawaii Volcanoes National Park (Aristizábal, unpublished data), and in *Guzmania monostachia* (L.) Rusby ex Mez. at the La Selva Biological Station in Costa Rica (Aristizábal & Morris, 2002). That I have found AMF colonizing litter leaves wherever I have found mycorrhizal roots in intimate association with litter, suggests that the phenomenon is widespread in tropical and sub-tropical ecosystems.

In this study, AMF spread most rapidly within soft leaves that decomposed relatively quickly, and overall AMF colonization of different leaf species inversely closely followed leaf mass remaining after 70 and 100 d. *A. carambola* and *C. spicatus* had the lowest remaining mass after 70 d and the highest percent hyphae and vesicles (Table 2.2, Figure 2.3). After 100 d, mass remaining in *R. excelsa* was similar to that in *A. carambola* and so was percent hyphal colonization. *M. cerifera*, which was the slowest to decompose, took the longest to attain high levels of AMF colonization. The rank ordering of species by decomposition rate corresponds well to the rank order of AMF colonization suggesting that decomposition rate is a good predictor of the rate of spread of AMF within leaves. I hypothesize that similar traits influence both the rate of leaf decomposition and how quickly AMF spread within leaves. Because the decomposition constants of the recalcitrant leaf fraction (k_2) are very similar, differences

in leaf decomposition seem to be influenced mainly by the size of the labile fraction (A) and by how quickly this labile fraction (k_1) decomposes (Table 2.3).

AMF produce limited amounts of hydrolytic and lignolytic enzymes (Azcon-Aguilar et al., 1999; Varma, 1999), and tough plant cell wall components, such as cellulose and lignin, may hinder AMF spread in leaves. Even though I found that after 70 d, AMF had colonized all types of leaves that I placed in the hammock, colonization of tough *Q. virginiana* and *M. cerifera* leaves was very low, and was restricted primarily to veins (Figure 2.1). Both these species had a relatively small labile fraction that decomposed slowly (Table 2.3). Nevertheless, that AMF colonization is present in tough leaves at 70 d but increases slowly, suggests that AMF inoculum was not lacking, but instead the difficulty of spreading within these leaves limited colonization. For tough leaves, attainment of asymptotic colonization may require at least 200 d (Figure 2.4).

Aristizábal *et al.* (2004) suggested that AMF initiate leaf colonization through leaf vascular tissue. Occurrence of AMF colonization mostly in the veins of tough, recalcitrant leaves at early collections during Experiment 1 supports this suggestion. The results of Experiment 2, that obstructing leaf veins significantly limits AMF colonization (Figure 2.5), provides direct support. Obstruction of veins did not completely prevent AMF from penetrating the leaves, however, because additional points of entry through veins probably became available as the leaves decomposed. As leaves are increasingly degraded through time, it might become easier for AMF to penetrate them mechanically. Both increased ease of penetration of cells, and breakdown and increased exposure of veins, may enhance colonization by AMF as decomposition proceeds.

I did not observe a lag in the formation of vesicles in either soft or tough leaves (Figure 2.3). AMF colonization and vesicle production within leaves that have decomposed very little suggests that the carbon stored in those vesicles originates from host roots. Because the release of simple carbon compounds from extremely recalcitrant leaves is delayed, it is improbable that tough leaves in initial stages of decomposition are a source of carbon for AMF. Moreover, the proportion of vesicles in leaves did not increase as decomposition proceeded, except for *M. cerifera*, the most recalcitrant species studied. In accordance with my observations, most available evidence suggests that AMF hyphae and spores proliferating in organic matter obtain carbon exclusively from their host plant (Nakano *et al.*, 1999; Nakano *et al.*, 2001).

Although I could not fit the logistic equation to AMF colonization of leaves that decomposed at high rates, it fitted AMF colonization of tough leaves well (Table 2.4, Figure 2.4). Estimates of the constants C_p and i calculated using the logistic equation proposed by McGonigle (2001) and calculated using Eq. (2) are very similar for *M. cerifera* and *Q. virginiana*, and only slightly different for *R. excelsa* (Table 2.4). In contrast, the estimates of s (the steepness of the curve) obtained using the two different equations differed greatly. That is a consequence of constraining the colonization of leaves to near zero at time zero in Eq. (2). Because the leaves had no colonization when placed in the field, I think this constraint is appropriate.

The pattern of AMF colonization of slowly decomposing leaves is similar to that observed for roots, which also has been modeled with the logistic equation. The logistic model indicates that colonization increases exponentially until saturation of the available substrate (i.e., roots or leaves) is approached, rates of colonization diminish to zero, and

colonization plateaus (Pattinson & McGee, 1997). Compared to values observed for root colonization (Pattinson & McGee, 1997; McGonigle, 2001), however, AMF colonization of tough leaves increased slowly as indicated by their long times to inflection (*i*) and low *s* coefficients (Table 2.4). For roots, *i* values range from 2.4 to 63.2 d (McGonigle, 2001), whereas my similarly-calculated values for dead leaves ranged from 111.3 to156.3 d. Curve steepness values (*s*) are lower for tough leaves (ranging from 0.015 to 0.024) than for roots (ranging from 0.067 to 0.765; McGonigle, 2001). Colonization constants for soft leaves, however, may be similar to those of roots, but that needs experimental verification because in my study the colonization of soft leaves already had reached an asymptote at 70 d.

Although the rates of colonization by AMF of dead leaves of different species differ, leaves with a wide variety of traits attain similarly high asymptotic levels of AMF colonization. Presumptively high nutrient contents of my experimental leaves might have induced artificially high levels of colonization, but field observations suggest otherwise. Aristizábal *et al.* (2004) reported similarly high levels of AMF colonization in natural leaf litter of three different plant species collected in three different tropical montane ecosystems in Colombia. I surmise, however, that high levels of colonization of litter leaves only may occur in ecosystems in which roots are intimately associated with litter. Because AMF depend on their hosts for carbon and thereby may be limited in the distance they can spread beyond plant roots, the ability of roots to respond to the presence of litter might affect the colonization of litter by AMF. In ecosystems where roots are not responsive to or closely associated with litter, the ability of AMF to colonize litter leaves,

especially those that are labile and quickly colonized by saprophytic microorganisms, might be diminished.

Overall, the results of this study suggest that AMF may play a greater role in recycling nutrients from leaf litter than previously recognized. Although AMF have been thought to tighten mineral nutrient cycles in nutrient-impoverished tropical ecosystems (Went & Stark, 1968; Janos, 1983; Richards, 1996), my findings in a relatively fertile, sub-tropical hardwood hammock suggest that this function of AMF may not be restricted to oligotrophic ecosystems. Even if AMF do not directly enhance litter decomposition, abundant AMF hyphae and vesicles in leaves might indirectly influence decomposition. AMF presence in litter leaves most likely changes leaf resource quality. C:N ratios of fungi are lower than those of litter (Killham, 1994), and because nitrogen often limits litter decomposition, fungus tissue within leaves ultimately might enhance decomposition. Fed directly by roots, AMF may have a strong competitive advantage over saprophytic microorganisms in colonizing litter leaves, and AMF thereby may be able to compete effectively for nutrients from them.

Table 2.1. Carbon and nitrogen concentrations of leaves from different plant species used for Experiment 1. Values represent the average of two sub-samples.

Species	% C	% N	C:N
M. cerifera	46.77	1.71	27.35
Q. virginiana	44.02	2.01	21.90
R. excelsa	46.08	2.13	21.63
A. carambola	43.43	2.25	19.30
C. spicatus	39.11	1.92	20.37

Table 2.2. Mean (± SE) dry weight remaining of leaves from different plant species that were placed beneath the litter of a South Florida hammock for 70, 100, 170, and 540 days (Experiment 1). At all collection times, plant species had a statistically significant effect on percent dry weight remaining, as determined by one-way ANOVA. Means within a collection time followed by the same letter do not differ significantly at $P < 0.05$ (Tukey's HSD).

	Collection time			
Species	70 days	100 days	170 days	540 days
M. cerifera	81.25 (1.63) a	73.09 (2.38) a	70.06 (2.83) a	52.11 (2.38) a
Q. virginiana	68.45 (2.73) b	67.04 (3.92) ab	58.99 (1.65) b	39.23 (2.23) b
R. excelsa	69.77 (4.50) ab	58.77 (3.62) b	48.83 (0.77) c	27.58 (3.73) c
A. carambola	52.81 (1.77) c	54.67 (2.84) b	48.92 (2.44) c	29.48 (2.32) bc
C. spicatus	34.35 (2.81) d	30.02 (2.09) c	-	-
	$F_{4,25} = 39.83$	$F_{4,24} = 25.89$	$F_{3,19} = 24.45$	$F_{3,19} = 18.17$
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Table 2.3. Constants (± SE) and coefficients of determination (r^2) obtained by fitting decomposition data of leaves from Experiment 1 to the double-exponential decay model (Equation 1). A= labile fraction; (1-A)= recalcitrant fraction; k_1= decomposition constant for labile fraction; k_2= decomposition constant for recalcitrant fraction.

Species	A (%)	(1-A) (%)	k_1 (d^{-1})	k_2 ($d^{-1} \times 10^{-4}$)	r^2
M. cerifera	0.232 (0.072)	0.768	0.018 (0.012)	7.15 (2.23)	0.910
Q. virginiana	0.268 (0.046)	0.732	0.041 (0.163)	11.64 (1.88)	0.925
R. excelsa	0.509 (0.128)	0.491	0.011 (0.004)	10.73 (7.08)	0.924
A. carambola	0.399 (0.021)	0.601	0.335[1]	12.93 (1.89)	0.960

[1]Because *A. carambola* leaves had almost completely decomposed at the first collection (after 70 days) a unique solution for k_1 could not be estimated.

Table 2.4. Estimates (±SE) of the constants C_p (curve's asymptote), s (abruptness of the curve), and i (point of inflection) obtained by fitting logistic equation (2) and the equation $C = C_p / \{1 + [e^{-s(t-i)}]\}$ (McGonigle, 2001) to the total AMF colonization data for different leaf species that were buried in a South Florida hammock for 70, 100, 170, and 540 days (Experiment 1). I set N_0 in Eq. (2) = 0.02.

Equation/ Species	C_p	s	i	r^2
Eq (2)				
M. cerifera	89.0 (8.6)	2.53×10^{-2} (2.60×10^{-3})	154[1]	0.659
Q. virginiana	78.4 (6.3)	3.62×10^{-2} (3.76×10^{-3})	108	0.729
R. excelsa	82.8 (7.4)	4.01×10^{-2} (4.63×10^{-3})	97	0.623
$C = C_p / 1 + [e^{-s(t-i)}]$				
M. cerifera	89.4 (8.3)	1.46×10^{-2} (4.98×10^{-3})	156 (25)	0.712
Q. virginiana	81.7 (7.0)	2.38×10^{-2} (6.80×10^{-3})	111 (14)	0.751
R. excelsa	92.8 (8.2)	1.70×10^{-2} (4.95×10^{-3})	112 (18)	0.715

[1]Because i was calculated using Eq. (3), no SE was estimated for this parameter.

Figure 2.1(a)-(f). AMF hyphae, vesicles, and spores within leaves of different plant species that were buried in a South Florida hammock for 70, 100, 170, and 540 days (Experiment 1). **(a)** *M. cerifera* leaf; bar 70 µm, **(b)** *Q. virginiana* leaf; bar 50 µm, **(c)** *R. excelsa* leaf; bar 50 µm, **(d)** *A. carambola* leaf; bar 100 µm, **(e)** *C. spicatus* leaf; bar 50 µm, and **(f)** Immunostained AMF hypha (arrow opposite an unilateral angular projection) within *Q. virginiana* leaf; bar 25 µm.

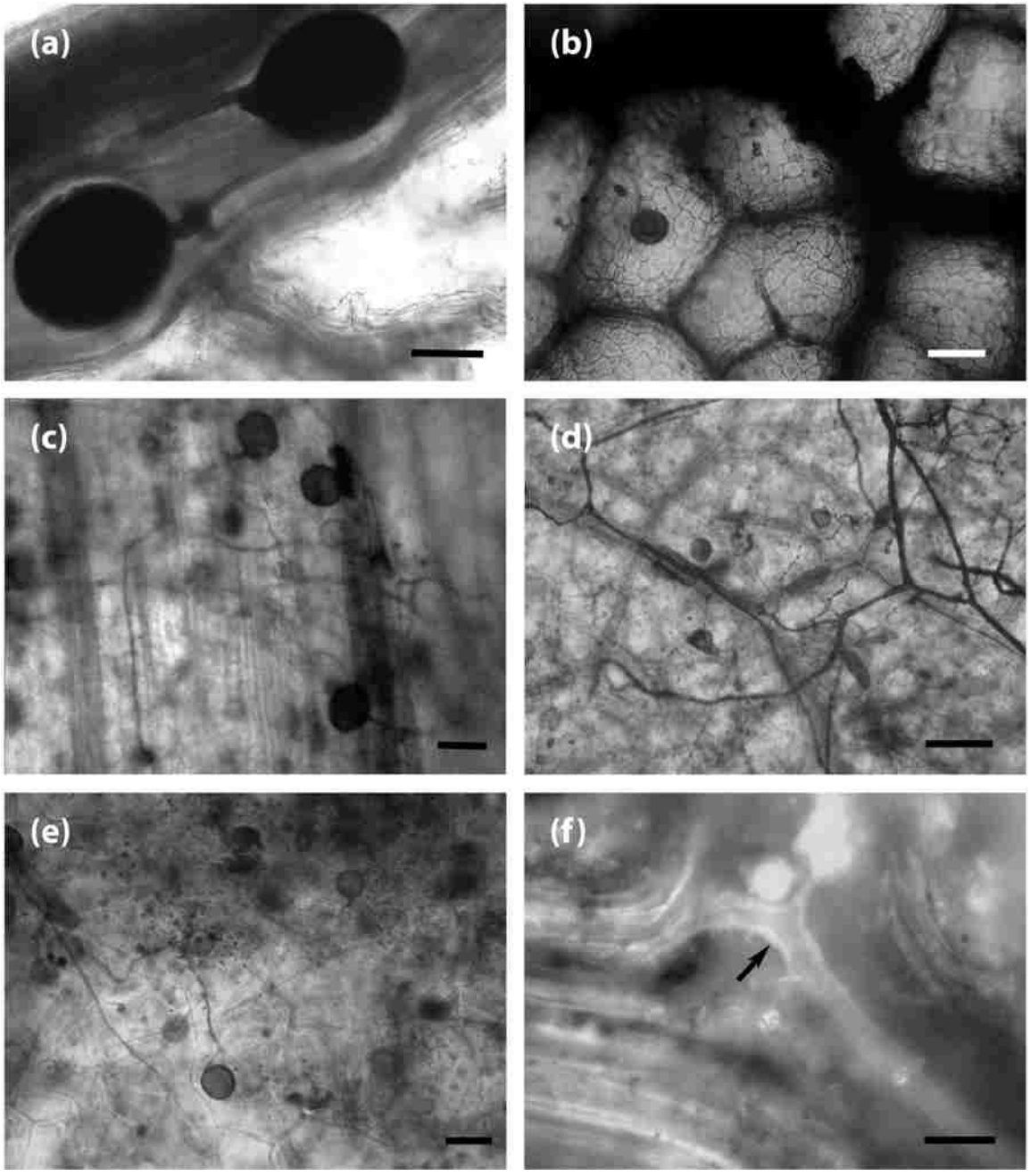
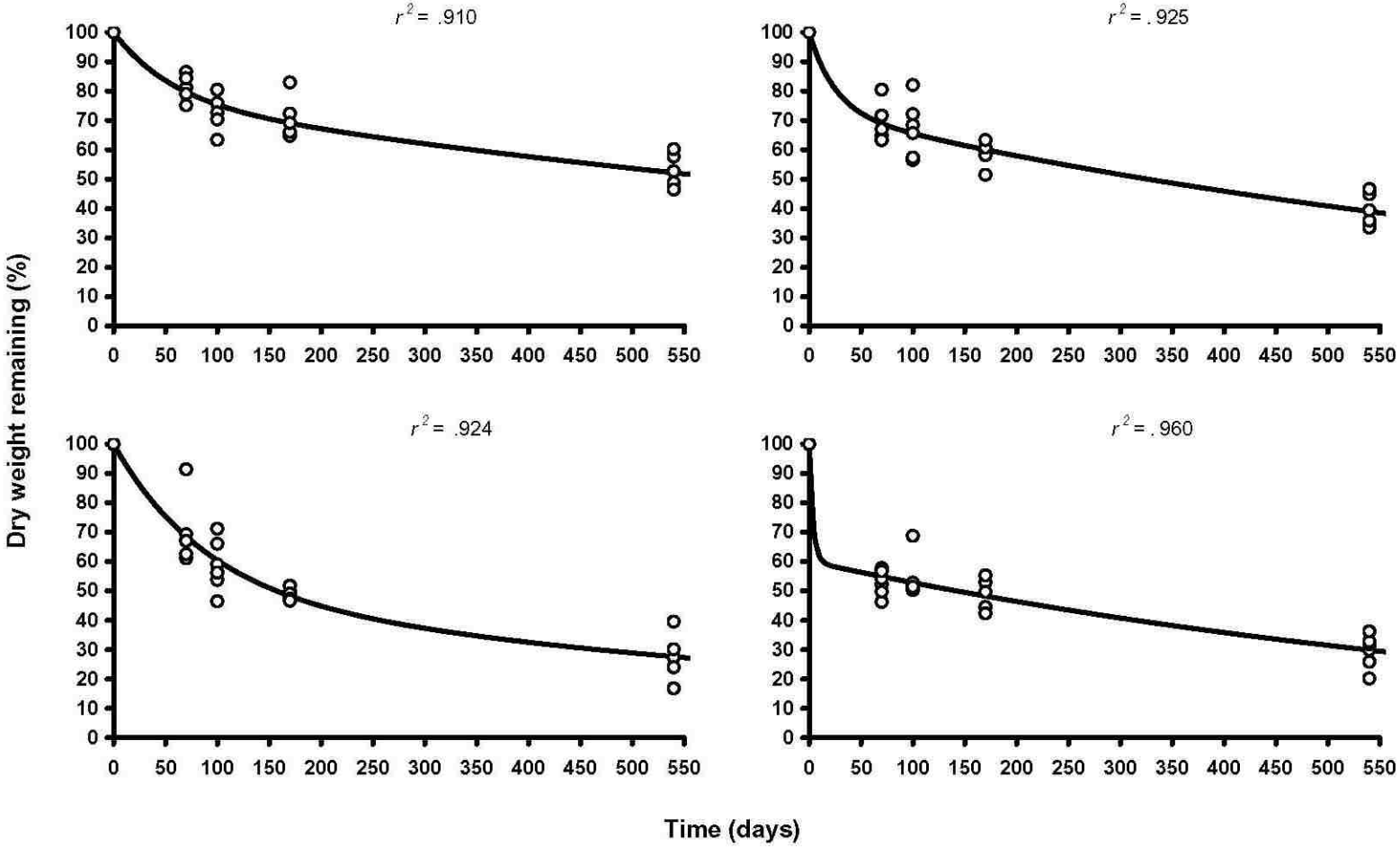


Figure 2.2(a)-(d). Decomposition curves and data points for leaves of (a) *M. cerifera*, (b) *Q. virginiana*, (c) *R. excelsa*, (d) *A. carambola*, that were buried in a South Florida hammock for 70, 100, 170, and 540 days (Experiment 1). Curves were estimated by non-linear regression using Eq. (1) for leaf dry weight remaining (%). Regressions are significant at $P < 0.001$. Estimated parameters are shown in Table 2.3.



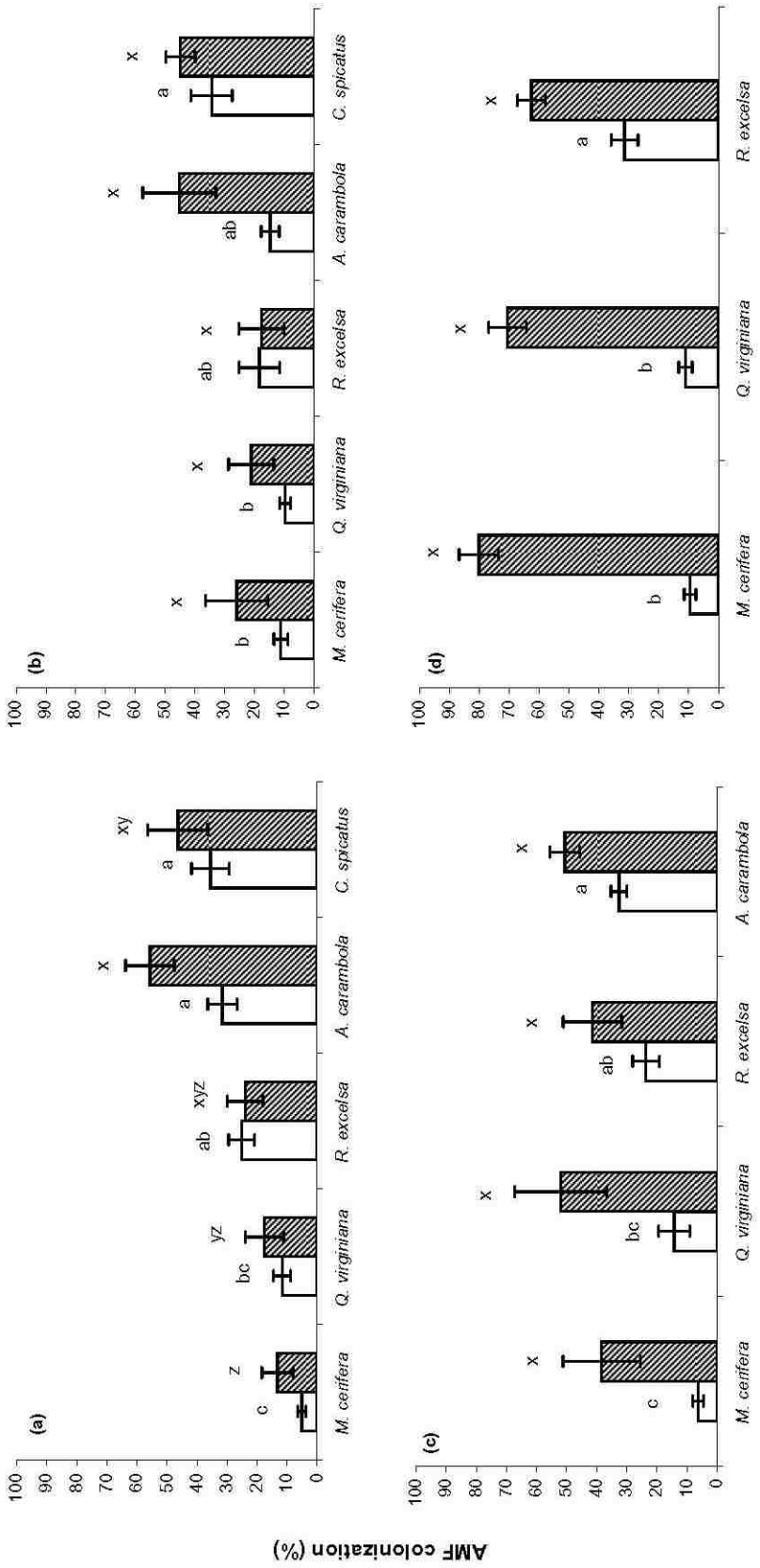


Figure 2.3. Mean percent (± SE) AMF colonization by hyphae (open bars) and vesicles (hatched bars) in different leaf species used for Experiment 1, collected after (a) 70 days, (b) 100 days, (c) 170 days, and (d) 540 days. Bars of the same pattern topped with the same letter do not differ significantly at $P < 0.05$ (Tukey's HSD).

Figure 2.4(a)-(c). AMF colonization curves and data points for different leaf species that were buried in a South Florida hammock for 70, 100, 170, and 540 days. Curves were estimated by non-linear regression using Eq. (2) for total AMF colonization (%) in leaves of (a) *M. cerifera*, (b) *Q. virginiana*, and (c) *R. excelsa*. Regressions are significant at $P < 0.001$. Estimated parameters are shown in Table 2.4.

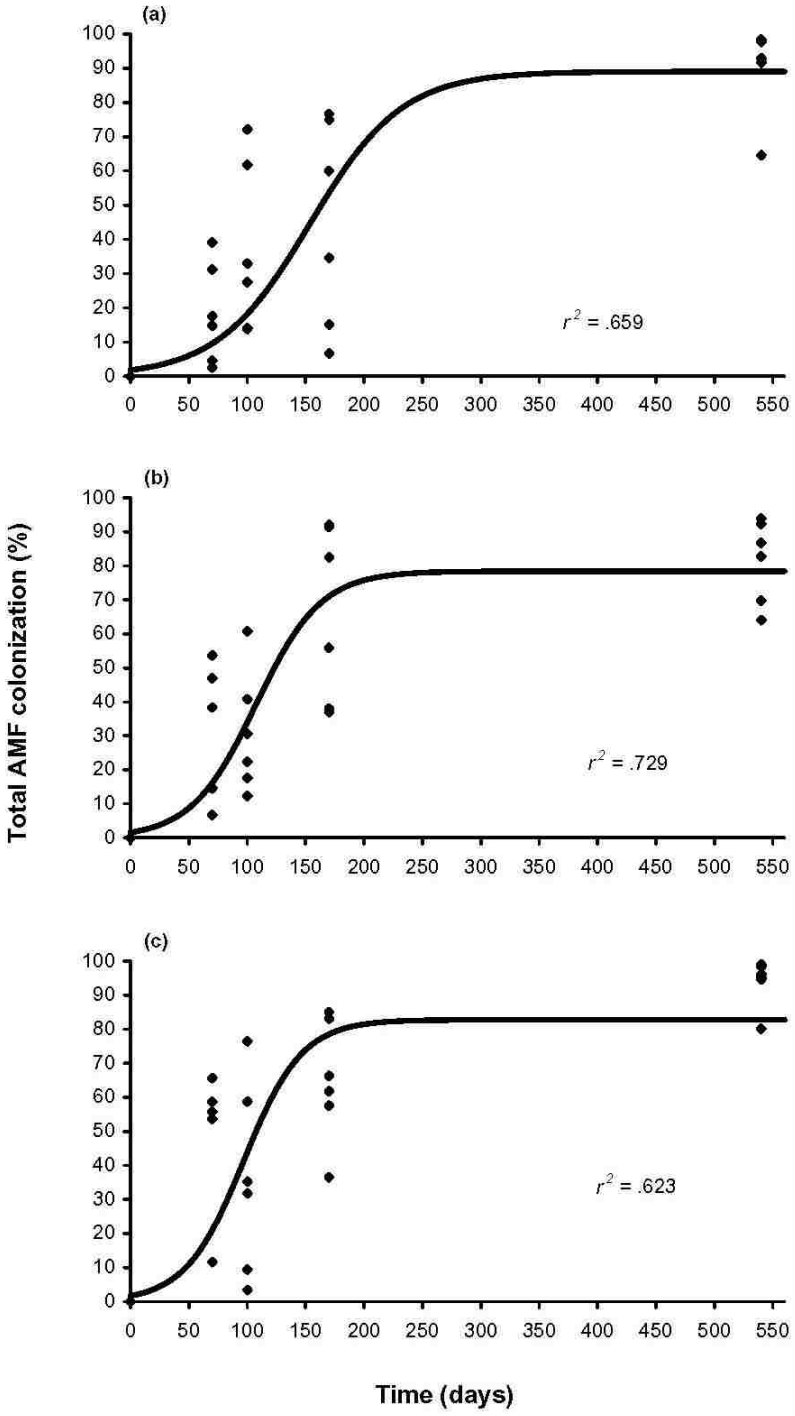
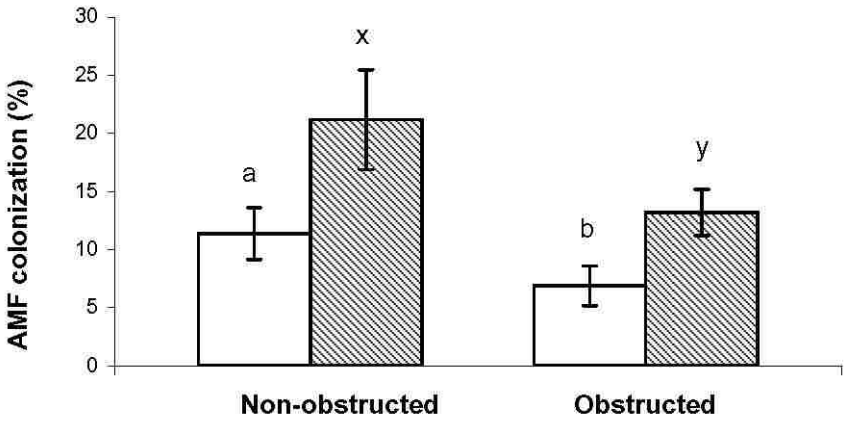


Figure 2.5. Mean (± SE) hyphae (open bars) and vesicles (hatched bars) in obstructed and non-obstructed leaf fragments (Experiment 2). Bars of the same pattern topped with different letters differ significantly at $P < 0.05$ (paired t-test).



Chapter 3

Leaf litter influences *Morella cerifera* responses to root symbionts

Most plants form symbiotic associations with arbuscular mycorrhizal fungi (AMF) (Smith & Read, 1997). The hyphae of these glomeromycotan fungi colonize the cortices of plant roots, and in exchange for photosynthate, translocate relatively immobile mineral nutrients such as phosphorus from the soil solution to plants (Smith & Read, 1997). Even though AMF may be energetically costly to maintain (Fitter, 1991), in nutrient impoverished soils many plants rely on AMF to grow and reproduce (Janos, 1980a).

Although associating with AMF is regarded as the primitive condition of land plants (Malloch *et al.*, 1980), several species have developed alternative adaptations to acquire mineral nutrients that limit their growth. Among these adaptations are cluster roots, which resemble a bottle brush and have been defined as "a region of the parent root where many short rootlets are produced in a compact grouping" (Skene, 1998). They are commonly found in litter and humus layers that accumulate on the surface of the soil (Lamont, 1993), and have been shown to increase P, Fe, and Mn uptake (Hurd & Schwintzer, 1997). Because of their higher surface area to volume ratio, AMF are thought to be less costly to form and maintain than are alternative P-acquisition root adaptations (Pate & Watt, 2002). In addition, AMF hyphae grow beyond zones of nutrient depletion that develop around roots, and thus they improve the uptake of ions that diffuse slowly through the soil (Hetrick, 1991). Even though AMF may be more efficient at acquiring phosphorus than alternative P-acquisition root adaptations, these

adaptations have appeared secondarily in colonizer species that grow in habitats where AMF may not always be present in the soil (Janos, 1980b).

Even though some species that have developed highly specialized P-acquisition adaptations, such as cluster roots, are not colonized by AMF (Brundrett & Abbott, 1991), many still are (e.g. Koske *et al.*, 1992). This suggests that AMF and alternative P-acquisition adaptations may provide differential benefits under different soil and environmental conditions. Which of these two root adaptations predominates at a given moment probably is determined by the soil conditions and the availability of the symbiont. In an environment where mineral nutrients are very scarce, or are available for a very limited period of time, it might be possible to observe high levels of both adaptations in the same root system (e.g. Schroeder & Janos, 2004). This could maximize absorption of a limiting mineral nutrient.

Morella cerifera (L.) Small is a small tree or shrub that is usually found growing in disturbed, or early successional ecosystems, with low soil N and P concentrations. It is an example of a plant species that has a wide array of root adaptations to acquire nutrients. It forms cluster roots and associates with AMF and *Frankia*, a nitrogen-fixing actinomycete. It has been demonstrated that AMF and *Frankia* can be synergistic (e.g. Tian *et al.*, 2002). Nitrogen fixation is an expensive energetic process, requiring high amounts of phosphorus, and AMF are highly specialized to acquire and transport P to plants. In contrast, because AMF and cluster roots are both involved in P acquisition, it has been repeatedly hypothesized that cluster roots are an alternative adaptation to AMF (Hetrick, 1991). For this reason, it has been stated that *M. cerifera* forms a non-functional symbiosis with AMF. In a pot experiment, Poole and Sylvia (1990) found no

effect of AMF on the growth of *M. cerifera* seedlings. Hurd & Schwintzer (1997) concluded that *M. pensylvanica* lacks functional mycorrhizae because they did not find arbuscules in its root system, even though they found AMF hyphae and vesicles in all root samples. Abundant AMF colonization in the field has also been observed for *M. cerifera* plants (as high as 44%; Poole & Sylvia, 1990).

AMF may, however, provide benefit to *M. cerifera* under conditions of litter accumulation. In the field *M. cerifera* plants tend to accumulate dead leaves underneath their canopy. It has been hypothesized that the AMF of *M. cerifera* are involved in recycling nutrients from litter (Aristizábal *et al.*, 2004). The objective of this study was to determine whether AMF enhance the growth of *M. cerifera* in the presence of labile (fast decomposition) and recalcitrant (slow decomposition) leaf litter in a nutrient impoverished substrate. I hypothesized that AMF would be beneficial to *M. cerifera* only in the presence of recalcitrant litter. Because labile leaves decompose quickly, and the value of AMF relative to roots is reduced when ample soluble mineral nutrients are present in the soil, AMF may be unimportant in acquisition of mineral nutrients from labile litter. Additionally, I examined the effect of AMF, and labile and recalcitrant leaf litter on *M. cerifera* cluster root production, nodule dry weight, nitrogen nutrition (as assessed by foliar $\delta^{15}N$ and N), and discrimination of ^{13}C ($\Delta^{13}C$).

Methods

To test how AMF and quality of leaf litter influence the growth of *M. cerifera* seedlings, I used a two-factor, complete factorial experiment (+/- AMF × 3 litter treatments: labile leaves, recalcitrant leaves, and no leaves = 6 treatments). Each of the treatment

combinations was replicated 10 times, for a total of 60 pots, each containing a single seedling.

I collected *M. cerifera* seeds from 5 different reproductive individuals in Monroe County, South Florida. I mechanically scarified the seeds with sand paper, and germinated them in Petri dishes on moist filter paper. I transferred emergent seedlings to 2 cm diameter Ray Leach Conetainers filled with coarse silica sand. After two months, to initiate the experiment the seedlings were transplanted into 10 cm diameter, 500 ml pots filled with a 2:1 mixture of autoclaved coarse (L 6-20) and fine grained (L 30-65) silica sand (Standard Sand and Silica Co., Miami, FL). This mixture was free of organic matter, nitrogen, and phosphorus.

Prior to seedling transplant, I half-filled the litter treatment pots with sand mixture and placed alternating layers of non-overlapping dried leaves and sand above that. The final layer of leaves was topped by 1 cm depth of fine sand. The litter treatment leaves were collected fresh, and were dried at 60 °C for 72 h. Twenty pots, comprising the labile leaf treatment, received 1 g of *Piper auritum* Kunth dried leaves which had been cut into 4 x 2 cm pieces prior to drying. *P. auritum* leaves are soft, and pliable, characteristics that typically indicate a rapid decomposition rate. Another twenty pots, comprising the recalcitrant leaf treatment, received 1 g of *M. cerifera* dried, whole leaves. In Chapter 2, I found that tough *M. cerifera* leaves take more than two years to completely decompose, indicating that they are very recalcitrant. The remaining twenty pots did not receive any dried leaves.

I inoculated +AMF seedlings with a mixture of unidentified *Glomus* and *Gigaspora* species that originated from a South Florida native hardwood hammock soil.

The AMF mixture had been proliferated on *Sorghum* sp. host plants from which I isolated spores by wet-sieving (Brundrett *et al.*, 1996). Each pot received 1.35 g of the material retained by a 45 μm sieve after passage through a 250 μm sieve. I coated transplanted seedling roots with the inoculum before depositing the remainder in the planting hole. Non-inoculated plants received the same volume of autoclaved (twice for 1 h, 24 h apart) sievings and 20 ml of an AMF-free microbial filtrate that I made by overnight infusion of sievings in distilled water which was then vacuum-filtered through Whatman #1 filter paper.

While growing my "stock" seedlings in Conetainers filled with sterile sand, I noticed that some of them suddenly grew at a rapid rate and their leaves turned dark green. Examination of the roots of these seedlings revealed incipient nodulation, indicating that they had been colonized spontaneously by *Frankia* sp. To reduce error variance, for my experiment I only used seedlings that showed none of the signs of *Frankia* sp. colonization at the outset. One week after transplant (May, 2005), each *M. cerifera* seedling received 10 ml of a *Frankia* sp. infusion. I prepared this infusion by surface sterilizing field-collected *M. cerifera* nodules, pureeing them in a blender (five 2 sec pulses) with distilled water, soaking them overnight, and then filtering the infusion through Whatman #1 filter paper.

I randomized the pots on benches in the University of Miami's ambient greenhouse. Pots were re-randomized once, 5 months after planting, during the one year (May 2005-May 2006) growth period. I manually watered plants daily as necessary to keep the well-drained, sand substrate moist, but I never fertilized the plants. I measured plant height, stem diameter, and length of the longest leaf approximately monthly.

Immediately before harvest, I assessed "leaf greenness" (which is an indirect indicator of leaf chlorophyll content) with a Minolta SPAD 502 meter**.**

At harvest, I excised plant shoots and separated leaves from stems. These were oven-dried at 60 °C to constant weight, and weighed. Leaves were ground, and 5 mg sub-samples were analyzed in an isotope-ratio gas mass spectrometer (Isoprime, Thermo Electron, Manchester, UK) with an Eurovector elemental analyzer (Milan, Italy) for N and C concentration, and for ^{15}N and ^{13}C abundance. Stable isotope abundance was calculated according to the formula:

$$\delta^{15}N \text{ or } \delta^{13}C\ (‰) = [(R_{sample}/R_{standard}] - 1) \times 1000 \quad (1)$$

in which R_{sample} and $R_{standard}$ represent the ratio of the heavy isotope to the light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$) in the sample and standard, respectively (Taiz & Zeiger, 2002). I used PDB (a marine chalk of the Pee Dee-formation, USA) and atmospheric nitrogen as carbon and nitrogen standards, respectively. Foliar N contents were calculated as the product of foliar N concentration and leaf dry weight. To estimate plant discrimination of ^{13}C ($\Delta^{13}C$) I subtracted plant $\delta^{13}C$ from atmospheric $\delta^{13}C$ (-7.8).

Nodules were removed from roots, oven-dried, and weighed. Roots were washed free of sand and scanned using a WinRhizo ® system to determine root length. Fine (< 2 mm diameter) and coarse roots (≥ 2mm diameter) were separated manually, air dried, and weighed. Sub-samples of both diameter classes were oven-dried and weighed to allow calculation of total root dry weight. Additional sub-samples were cleared in KOH, acidified, and stained with 0.05% Trypan Blue (Phillips & Hayman, 1970) to confirm colonization by AMF. I printed scanned root images, and used a gridline intersect

method (Brundrett *et al.*, 1996) to estimate the percentage of the root system that had cluster roots.

At harvest I manually collected what remained of recalcitrant litter leaves from the layers in which they had been placed. Any root fragments that adhered to them were removed manually. The recovered leaf fragments were washed free of sand, oven-dried, and weighed. No leaf fragments could be recovered from the labile leaf treatment.

Data analysis

To assess the effects of AMF inoculation, litter addition, and their interaction on *M. cerifera* growth I used a two-way, repeated measures MANOVA. I used two-way MANOVAs to determine the effect of AMF inoculation, litter addition, and their interaction on *M. cerifera* root variables (root dry wt., root:shoot ratio, and root length), and on foliar N concentration, N content, and $\delta^{15}N$. I used two-way ANOVAs to determine the effect of AMF inoculation, litter addition, and their interaction on percent cluster roots of *M. cerifera* and foliar $\Delta^{13}C$. I used a t-test to examine the effect of AMF on litter weight remaining in the recalcitrant leaf treatment. Levene's Test was used to test for homogeneity of variance. If necessary, data were square root transformed.

I used regression analysis to determine whether or not foliar N concentration is a good predictor of "leaf greenness", and to determine whether or not nodule dry weight is a good predictor of foliar N concentration, N content, and $\delta^{15}N$. Within each response variable, significance was Bonferroni-corrected for the number of comparisons made ($P < 0.017$ reflects 0.05/3). All the aforementioned statistical analyses were performed with SPSS v. 14.

To examine if treatment effects on shoot and root growth were direct, or if they were mediated through effects on *Frankia* sp. nodulation, I tested a structural equation model (Figure 1) with Mplus v. 4.2. I dummy coded the different treatments (AMF, RL = recalcitrant litter, and LL = labile litter), and incorporated their interactions in the model as exogenous variables AMF × RL and AMF × LL (Figure 3.1).

Structural Equation Modeling has been defined as the use of two or more linear equations to model multivariate relationships (Grace, 2006). Structural Equation Models are a combination of measurement and path models (Kline, 2005). Measurement models use "observed variables as indicators of underlying factors", which are called latent variables (Kline, 2005). Path models allow a researcher to test complicated causal relationships among variables (Mitchell, 2001). Because path analysis estimates the effect of a independent variable on a dependent variable controlling for the effects of other variables on it, if offers statistical control (Grace, 2006).

Prior to analysis of the structural equation model, I screened shoot and root data for normality. The distributions of the variables root length, stem diameter, height, and length of longest leaf were not normal, so these variables were square-root transformed. The variable nodule dry weight was log transformed. The variables that measured shoot growth (height, stem diameter, and length of longest leaf) were combined to create a latent factor. I also tried to combine the variables that measured root growth (root dry wt., root length, number of tips) to create a latent factor, but the fit of the latter measurement model was very poor, so I used only root length to represent root growth. I combined the measurement model for shoot growth that included the variables stem diameter, height, and length of longest leaf, with the variable root length, and then assessed overall fit.

After obtaining a good fit of the measurement model [$X^2 = 1.237$, $P = 0.539$, Comparative Fit Index (CFI) = 1, Root Mean Squared Error of Approximation (RMSEA) < 0.001, and Standardized Root Mean squared Residual (SRMR) = 0.007], I estimated parameters for the structural equation model (Figure 3.1).

Results

Labile and recalcitrant leaf litter decomposed at different rates and likely differentially elevated mineral nutrient availability in the highly infertile, sand substrate. By the end of my one year long experiment, all labile leaf pieces had decomposed to amorphous organic matter, but recoverable fragments of the recalcitrant leaves still were present. After a year, the weight of recalcitrant leaves buried in the pots that had been inoculated with AMF was significantly lower (Figure 3.2) than that in non-inoculated pots.

Between-Subjects MANOVA showed a significant main effect of litter on aboveground *M. cerifera* growth parameters (Table 3.1). Within-Subjects MANOVA showed a marginally significant effect of AMF inoculation, and significant effects of litter treatment and AMF × litter interaction, on the growth of *M. cerifera* seedlings over time (Table 3.1). Between-Subjects univariate repeated-measures tests show significant effects of litter on "height", "stem diameter", and "length of longest leaf". Within-Subjects univariate repeated-measures tests show significant effects of litter on "height" and "stem diameter"(Table 3.2). Plants that received labile leaves were larger for all three parameters than those in the other two litter treatments which did not differ from one another (Figure 3.3). AMF inoculation negatively influenced the growth of *M. cerifera* seedlings in the no litter and labile leaf treatments, but produced no difference in any parameter for the recalcitrant leaf treatment (Figure 3.3).

Belowground growth of *M. cerifera* seedlings was significantly affected by litter treatment and by the AMF × litter interaction (Table 3.3). Univariate tests show significant effects of main treatments on root dry weight and root length (Table 3.4). Plants that were inoculated with AMF had a significantly lower root dry weight, and a marginally significantly smaller root length than non inoculated plants. Plants that received labile leaves had significantly higher (Tukey's HSD at $P \leq 0.05$) root dry weight and longer root length than those in the other two litter treatments which did not differ from one another (Figure 3.4).

AMF and Litter treatments did not significantly affect percent cluster roots (Figure 3.5) of *M. cerifera* plants (two-factor ANOVA: AMF: $F_{1,53} = 2.479$, $P = 0.121$; Litter: $F_{2,53} = 1.429$, $P = 0.249$). There was a marginally significant effect of AMF × litter interaction on cluster root formation ($F_{2,53} = 2.520$, $P = 0.090$).

Even though all *M. cerifera* plants received the *Frankia* sp. infusion, total nodule dry weight was highly variable among individuals, ranging from no nodules to 0.46 g. Nodule dry weight was significantly associated with foliar nitrogen content only in the no litter and recalcitrant leaf treatments. Nodule dry weight was not associated significantly with N concentration or $\delta^{15}N$ enrichment for any litter treatment (Figure 3.6). Foliar nitrogen concentration was a good predictor of "leaf greenness"($r^2 = 0.571$, $P < 0.001$).

Overall, foliar N concentration, N content, and $\delta^{15}N$ were significantly affected by litter treatment (Table 3.5). Univariate tests show a significant effect of litter treatments on $\delta^{15}N$ (Table 3.6). Plants that received labile leaves had significantly higher (Tukey's HSD at $P \leq 0.05$) foliar $\delta^{15}N$ values than those in the other two litter treatments which did not differ from one another.

Foliar $\delta^{13}C$ of *M. cerifera* plants (Figure 3.7) was significantly reduced by AMF (two-factor ANOVA: $F_{1,53} = 6.598$, $P = 0.013$), but was not affected by litter treatment ($F_{2,53} = 0.393$, $P = 0.677$) or by AMF × litter interaction ($F_{2,53} = 2.100$, $P = 0.133$).

The fit of the structural model depicted in Figure 3.1 was good, with $X^2 = 15.622$, $P = 0.337$, CFI = 0.996, RMSEA = 0.044, and SRMR = 0.027. Table 3.7 provides the direct (i.e., path coefficients), indirect, and total effects of the model depicted in Figure 3.1. The model explained 27% of the variance in nodule dry weight, 57 % of the variance in root length, and 72% of the variance in shoot growth. Standardized parameter estimates for only those path coefficients that are significantly different from zero are shown in Figure 3.8.

The parameterized structural equation model (Figure 3.8) suggests that seedlings that were inoculated with AMF or that received recalcitrant leaves had lower nodule dry weights than those seedlings not receiving those treatments. Thereby, AMF inoculation and recalcitrant leaves had negative indirect effects on root length and shoot growth (Table 3.7 and Figure 3.8). Nodule dry weight itself, however, had large positive direct effects on root length and shoot growth (Figure 3.8). Root length and shoot growth were positively correlated (Figure 3.8). Labile leaf addition had a direct, positive effect on shoot growth. Notwithstanding the negative effects of AMF inoculation and recalcitrant leaves alone on nodule dry weight, when AMF were present the addition of recalcitrant leaves (AMF × RL) had a positive effect on nodule dry weight.

Discussion

The results of this study show that in a nutrient impoverished soil AMF can negatively affect the growth of *M. cerifera* plants, except when a source of recalcitrant litter is present, where the effect of AMF on *M. cerifera* growth is neutral. That the outcome of the interaction between AMF and their host plant can shift from mutualistic to parasitic has been observed in multiple experiments (Johnson, 1993; Schroeder & Janos, 2004). It has been hypothesized that the outcome of a mutualistic interaction varies in space and time, and is dependent on how ecological conditions affect net costs and net benefits of the interaction for each partner (Bronstein, 1994). The ecological conditions under which AMF can be parasitic have been discussed extensively (Janos, 1985; Janos, 1987; Johnson *et al.*, 1997; Jones & Smith, 2004; Janos, 2007). AMF are usually thought to be parasitic when high levels of phosphorus (Schroeder & Janos, 2004) are applied to the soil. Even though AMF are thought be beneficial to plants at low levels of soil fertility, in this experiment I show that in highly oligotrophic soils AMF can be parasitic, as has been discussed by Janos (2007).

That the negative effects of AMF on plant growth only appeared after several months in the no litter and labile litter treatments suggests that either AMF root colonization was slow, or that initially the cost of AMF was equal in magnitude to the benefit they provided to *M. cerifera* plants. In the no litter treatment the negative effects of AMF on aboveground growth started to appear after six months and first affected the length of the longest leaf. In the labile litter treatment the negative effects of AMF appeared after 5 months and first affected plant height (Figure 3.3). Although I did not fertilize the plants, the soil I added in conjunction with the AMF inoculum likely

provided P and other mineral nutrients necessary for plant growth, and maintenance of N fixation. It is likely however, that when the nutrients in the pot became depleted, AMF were still a cost to plants and did not provide any benefit, hence the symbiosis shifted from neutral to parasitic. Plants without mycorrhizas probably did not have to allocate additional photosynthate to AMF, and could use that photosynthate to grow and to maintain their other symbiont, *Frankia* sp. The results of my structural equation model support this hypothesis. They suggest that the negative effects of AMF on *M. cerifera* growth (both shoot and root growth) are indirect, and are mediated by the negative effect AMF have on plant nodule dry weight.

Even though AMF and *Frankia* are generally regarded as synergistic (e.g. Yamanaka *et al.*, 2005), it has been hypothesized that these symbionts may compete in plant roots for space or for photosynthates**.** Sempavalan *et al.* (1995) did not find evidence supporting the competition for space hypothesis, while Bethlenfalvay *et al.* (1985) and Gardner (1986) did. Gardner (1986) demonstrated that initial colonization of a plant by AMF inhibits later colonization of the roots by *Frankia*. Orfanoudakis *et al*. (2004) found evidence supporting the idea that these symbionts can compete in plant roots for photosynthates. Because nitrogen is the nutrient that most limits the growth of *M. cerifera* plants (see Chapter 4), any reduction in nodule biomass will have a large negative effect on plant biomass (both root length and shoot growth).

I found that litter addition affects aboveground and belowground growth of *M. cerifera* plants. The plants in the labile litter treatment were larger than the plants in the no litter and recalcitrant litter treatments, which did not differ from each other. As Figure 3.3 illustrates, for the first four months the growth rate of plants in the labile litter

treatment was higher than that of plants in the no litter and recalcitrant litter treatments, but then slowed down. This suggests that nutrients in the labile litter became depleted at that time. That at harvest I could not recover any leaves from this treatment supports this idea. There was no interaction between AMF and labile leaves, meaning that plant roots could access these nutrients with or without mycorrhizas. This result is in accordance with what has been reported in other experiments (e.g. Hodge, 2003), that AMF do not enhance the capture of mineral nutrients in systems that are not mineralization limited (i.e. where decomposition rate is fast).

Although *M. cerifera* aboveground and belowground growth in the no litter and recalcitrant litter treatments did not differ significantly, the results of the structural model suggest that recalcitrant litter had a negative effect on nodule dry weight. That recalcitrant leaves typically immobilize nutrients from the soil before they start to decompose, may explain this result. My model also suggests that when recalcitrant litter is present in the soil AMF have a positive effect on nodule dry weight and a marginally significant positive indirect effect on shoot and root growth. Although it probably took several months for nutrients in recalcitrant leaves to become available to plants, similarly to the other treatments, the nutrients I provided in the inoculum probably made AMF be neutral until then. It is possible that when plants in the no litter and labile litter treatments had exhausted the nutrients available in the inoculum, mineral nutrients from the recalcitrant leaves became available. That AMF were neutral or slightly positive (as suggested by the marginally significant positive effect on shoot and root growth) to *M. cerifera* plants in the presence of recalcitrant leaves could be explained by an increase in the decomposition rate of these leaves directly or indirectly influenced by AMF, coupled

with better capture of slowly released nutrients by AMF than by roots alone. That the recalcitrant leaves in the pots inoculated with AMF had a significantly lower biomass than that of the controls (Figure 3.2) supports this contention. Hodge *et al.* (2001) also found that AMF accelerate the decomposition of OM, and I found similar effects of AMF on litter decomposition in another experiment (Chapter 4). AMF penetrate and proliferate within litter leaves, suggesting that they may be positioned advantageously to acquire mineral nutrients resulting from litter decomposition (Aristizábal *et al.*, 2004).

Nodule dry weight was not a good predictor of foliar N concentration, and the labile litter treatment did not affect N concentration, suggesting that the concentration of N in *M. cerifera* leaves is relatively constant. That nodule dry weight was a good predictor of foliar N content suggests that in this experiment the growth of *M. cerifera* plants was limited by nitrogen. The relationship between nodule dry weight and foliar N content is not observed in the labile leaf treatment. This suggests that these leaves provided a source of N for these plants. That the labile leaf treatment was significantly different in $\delta^{15}N$ than the two other treatments supports this idea. That the $\delta^{15}N$ of no-litter and recalcitrant litter treatments did not differ from each other suggests that these plants obtained their N from a similar source, likely atmospheric N via nitrogen fixation.

Similarly to what happened aboveground, AMF reduced the belowground growth of *M. cerifera* plants in the no litter and labile leaf treatments, and had no effects on the recalcitrant leaf treatment. I found a similar pattern for foliar ^{13}C discrimination ($\Delta^{13}C$) in *M. cerifera* plants. Even though the AMF × Litter interaction is only marginally significant for this variable, AMF plants had a significantly higher $\Delta^{13}C$ than non inoculated plants. Carbon isotope discrimination (Δ) is a function of the foliar internal to

external CO_2 concentration ratio (C_i/C_a) and isotopic fractionations associated with stomatal diffusion (*b*) and the Calvin cycle carboxylation enzyme (*a*): $\Delta = b + (a - b)$ C_i/C_a (Farquhar *et al.*, 1982). Therefore, under a constant external CO_2 concentration, a higher internal CO_2 concentration causes a greater discrimination. Several processes occurring in the leaf tissue may have produced the AMF carbon isotopic effect. The effect of AMF in increasing stomatal conductance is well documented (Auge, 2001). An increase in stomatal conductance without a concomitant increase in biochemical assimilation will result in higher C_i and therefore higher discrimination against ^{13}C during photosynthesis. I hypothesize that this may have occurred in plants where AMF were parasitic, i.e. AMF increased stomatal conductance without any substantial increase in the biochemical assimilation. On the other hand the beneficial AMF association with the recalcitrant litter, in addition to increasing stomatal conductance, could have caused a compensating increase in biochemical assimilation which maintains C_i constant. This would result in no change in discrimination with AMF as observed in plants treated with recalcitrant litter.

Even though not statistically significant, my data suggest that AMF reduced cluster root formation in the no litter treatment, but did not affect it in the labile or recalcitrant leaf treatments. In spite of the overlap in function of AMF and cluster roots, my experiment shows that a plant can simultaneously exhibit both adaptations, suggesting that the hypothesis that cluster roots are formed only when mycorrhizal inoculum is scant is not true under conditions of litter accumulation. Reddell *et al.* (1997) found that at low P levels (< 25 mg P kg^{-1}) *Casuarina cunninghamiana* plants inoculated with *Glomus* sp. were significantly larger than controls, but those authors did

not find a negative correlation between AMF and cluster root formation. Both Reddell *et al.*'s (1997) experimental results, and the finding that in the field plants are both colonized by AMF and form cluster roots (Aristizábal *et al.*, 2004), suggests that these two adaptations might not be performing the same service in the same way and are not mutually exclusive, but might complement each other.

Overall, my results underscore the idea that the conditions in which a plant grows can determine how its associated symbionts interact, and what benefits, if any, they provide to it. In my experiment the presence of recalcitrant litter shifted the effect of AMF on the growth of *M. cerifera* plants from parasitic to neutral by affecting nodule dry weight. I hypothesize that AMF affected nodule dry weight by increasing the decomposition of recalcitrant litter, augmenting available nutrients in the pot, and allowing *M. cerifera* plants to support higher levels of *Frankia* sp. These results and the results of another experiment I conducted in which I found beneficial effects of AMF on the growth of *M. cerifera* plants (Chapter 4), in addition to AMF frequently being found in the roots of these plants in the field, suggest that the symbiotic association between AMF and *M. cerifera* is functional but is probably shifted easily from mutualistic to parasitic by environmental conditions. *M. cerifera* forms tripartite symbiotic associations, but has been found to grow without AMF under experimental conditions, suggesting that it is a facultatively mycotrophic species (sensu Janos, 2007). Bronstein (1994) predicted that the outcomes of facultative mutualisms and of mutualisms in which a third species is intimately involved are more variable than the outcomes of obligate mutualisms. This suggests that for this species the shift from mutualism to parasitism can occur easily, as was observed in this study.

Table 3.1. Two-factor repeated-measures MANOVA showing the effects of different experimental treatments (AMF×Litter), and their interaction, on the growth (height, stem diameter, and length of the longest leaf) of *M. cerifera* plants for one year. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Factor	Wilks' λ	*df*	*F*	*P*
Between subject effects				
AMF	0.891	3, 51	2.076	0.115
Litter	0.445	6, 102	8.381	**< 0.001**
AMF× Litter	0.832	6, 102	1.634	0.145
Within subject effects				
Time	0.006	34, 20	104.293	**< 0.001**
Time × AMF	0.237	34, 20	1.894	0.067
Time × Litter	0.011	68, 40	5.083	**< 0.001**
Time × AMF × Litter	0.026	68, 40	3.088	**< 0.001**

Table 3.2. (Table is shown on following page). Univariate tests from two-factor (AMF×Litter) repeated-measures MANOVA of the growth data (height, stem diameter, and length of the longest leaf) of *M. cerifera* plants for one year. Greenhouse-Geisser Epsilon values were used to determine significance of within subject effects. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Only effects of factors that were significant in the multivariate tests are shown in bold to indicate significance.

Source	Dependent Variable	*df*	*F*	*P*
Between subject effects				
AMF	Height	1, 53	3.630	0.062
	Stem diameter	1, 53	5.942	0.018
	Length of longest leaf	1, 53	5.467	0.023
Litter	Height	2, 53	25.997	**< 0.001**
	Stem diameter	2, 53	29.490	**< 0.001**
	Length of longest leaf	2, 53	18.346	**< 0.001**
AMF × Litter	Height	2, 53	1.824	0.171
	Stem diameter	2, 53	.084	0.920
	Length of longest leaf	2, 53	.754	0.475
Within subject effects				
Time	Height	1.9, 82.4	179.276	**< 0.001**
	Stem diameter	1.6, 99.1	251.887	**< 0.001**
	Length of longest leaf	1.8, 97.3	102.991	**< 0.001**
Time × AMF	Height	1.9, 82.4	2.627	0.081
	Stem diameter	1.6, 99.1	3.893	0.034
	Length of longest leaf	1.8, 97.3	3.479	0.038
Time × Litter	Height	3.7, 82.4	5.266	**0.001**
	Stem diameter	3.1, 99.1	5.339	**0.002**
	Length of longest leaf	3.7, 97.3	1.303	0.276
Time × AMF × Litter	Height	3.7, 82.4	2.355	0.063
	Stem diameter	3.1, 99.1	.781	0.512
	Length of longest leaf	3.7, 97.3	1.576	0.191

Table 3.3. Two-factor MANOVA showing the effects of different experimental treatments (AMF×Litter), and their interaction, on root dry weight, root: shoot ratio, and root length of *M. cerifera* plants harvested after one year. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Factor	Wilks' λ	*df*	*F*	*P*
AMF	0.896	3, 51	1.982	0.128
Litter	0.621	6, 102	4.570	**< 0.001**
AMF × Litter	0.754	6, 102	2.572	**0.023**

Table 3.4. Univariate tests from two-factor (AMF×Litter) MANOVA of the root data (root dry wt, root:shoot ratio, and root length) of *M. cerifera* plants for one year. Only effects of factors that were significant in the multivariate tests are shown in bold to indicate significance.

Source	Dependent Variable	*df*	*F*	*P*
AMF	Root dry wt	1	5.791	0.020
	Root:shoot ratio	1	1.866	0.178
	Root length	1	3.666	0.061
Litter	Root dry wt	2	11.376	**<0.001**
	Root:shoot ratio	2	1.105	0.339
	Root length	2	5.571	**0.006**
AMF × Litter	Root dry wt	2	1.693	0.194
	Root:shoot ratio	2	2.058	0.138
	Root length	2	0.381	0.685

Table 3.5. Two-factor MANOVA showing the effects of different experimental treatments (AMF and Litter), and their interaction, on foliar N concentration, N content, and $\delta^{15}N$ of *M. cerifera* plants harvested after one year. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Factor	Wilks' λ	*df*	*F*	*P*
AMF	0.895	3, 51	1.999	0.126
Litter	0.421	6, 102	9.215	**< 0.001**
AMF × Litter	0.884	6, 102	1.084	0.377

Table 3.6. Univariate tests from two-factor (AMF×Litter) MANOVA of the foliar data (N concentration, N content, and $\delta^{15}N$) of *M. cerifera* plants for one year. Only effects of factors that were significant in the multivariate tests are shown in bold to indicate significance.

Source	Dependent Variable	*df*	*F*	*P*
AMF	N concentration	1	3.395	0.071
	N content	1	5.846	0.019
	$\delta^{15}N$	1	0.514	0.477
Litter	N concentration	2	0.598	0.554
	N content	2	2.824	0.068
	$\delta^{15}N$	2	20.349	**< 0.001**
AMF × Litter	N concentration	2	0.705	0.498
	N content	2	1.834	0.170
	$\delta^{15}N$	2	1.405	0.254

Table 3.7. Direct, indirect and total effects of different experimental treatments [AMF and the addition of recalcitrant (RL) and labile (LL) leaves], on nodule dry weight, root length, and shoot growth. Z-statistic values are shown in parentheses. Direct effects = path coefficients shown in Figure 3.8. NA = not applicable. Coefficients that are significantly different from zero ($Z \geq 1.96$ to be significant at $P < 0.05$) are shown in bold.

	Effect on								
	Nodule weight			Root length			Shoot growth		
Variable	Direct	Indirect	Total	Direct	Indirect	Total	Direct	Indirect	Total
AMF	**-0.45**	NA	**-0.45**	-0.07	**-0.30**	-0.37	-0.09	**-0.34**	**-0.43**
	(-2.35)		(-2.35)	(-0.43)	(-2.22)	(-1.89)	(-0.68)	(-2.25)	(-2.22)
RL	**-0.35**	NA	**-0.35**	0.22	-0.24	-0.02	0.21	-0.27	-0.06
	(-1.96)		(-1.96)	(1.55)	(-1.88)	(-0.09)	(1.69)	(-1.90)	(-0.32)
LL	0.28	NA	0.28	0.16	0.19	0.35	**0.26**	0.21	**0.47**
	(1.50)		(1.50)	(1.12)	(1.47)	(1.85)	(2.05)	(1.48)	(2.49)
AMF × RL	**0.40**	NA	0.28	-0.10	0.27	0.17	0.01	0.30	0.32
	(1.96)		(1.50)	(-0.61)	(1.88)	(0.82)	(0.10)	(1.90)	(1.54)
AMF × LL	0.16	NA	0.28	0.06	0.11	0.17	-0.08	0.12	0.04
	(0.78)		(1.50)	(0.38)	(0.78)	(0.80)	(-0.57)	(0.78)	(0.21)
Nodule weight	NA	NA	NA	**0.68**	NA	NA	**0.76**	NA	NA
				(6.78)			(7.97)		

Figure 3.1. (Figure is shown on following page). Structural equation model depicting the potential effects of different experimental treatments (AMF×Litter) on nodule biomass, root length and shoot growth of *M. cerifera* plants. This model tests if the effect of experimental treatments on root length and shoot growth are direct, or indirect and mediated through their effect on nodule biomass. AMF = arbuscular mycorrhizal fungi, RL = recalcitrant leaf treatment, LL = labile leaf treatment, AMF × RL = combination of AMF and recalcitrant leaf treatments, AMF × LL = combination of AMF and labile leaf treatments, E = measurement error associated with a given indicator**,** D = disturbance or unexplained variance (1- r^2 = D).

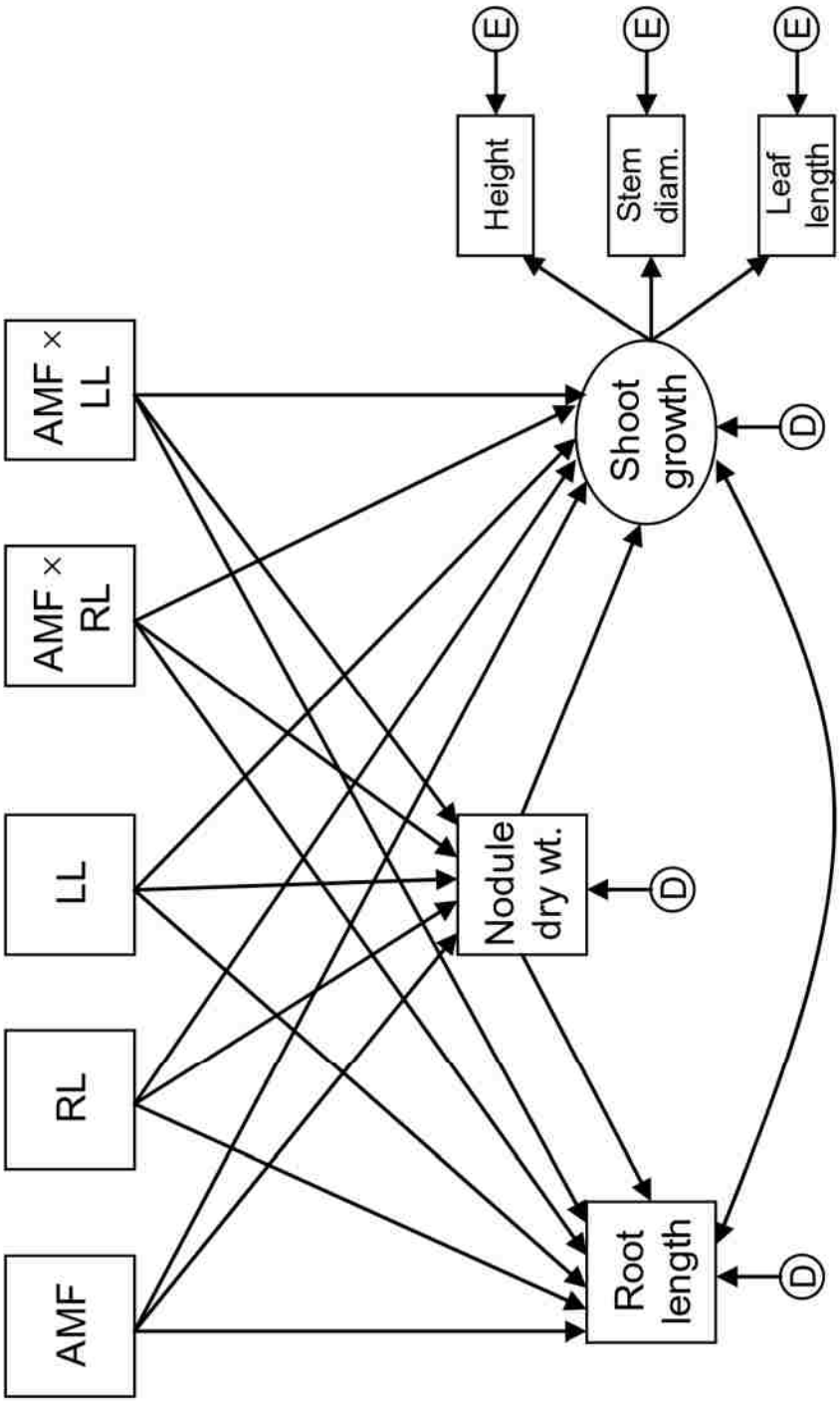

AMF
RL
LL
AMF × RL
AMF × LL
Nodule dry wt.
Root length
Shoot growth
Height
Stem diam.
Leaf length
D
D
D
E
E
E

Figure 3.2. Average weight (± SE) of recalcitrant leaves that were recovered from pots in which *M. cerifera* plants grew for one year. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. The weight of leaves in pots that were inoculated with AMF was significantly lower than that in pots of non-inoculated plants (t = -3.038, df = 18, P = 0.007).

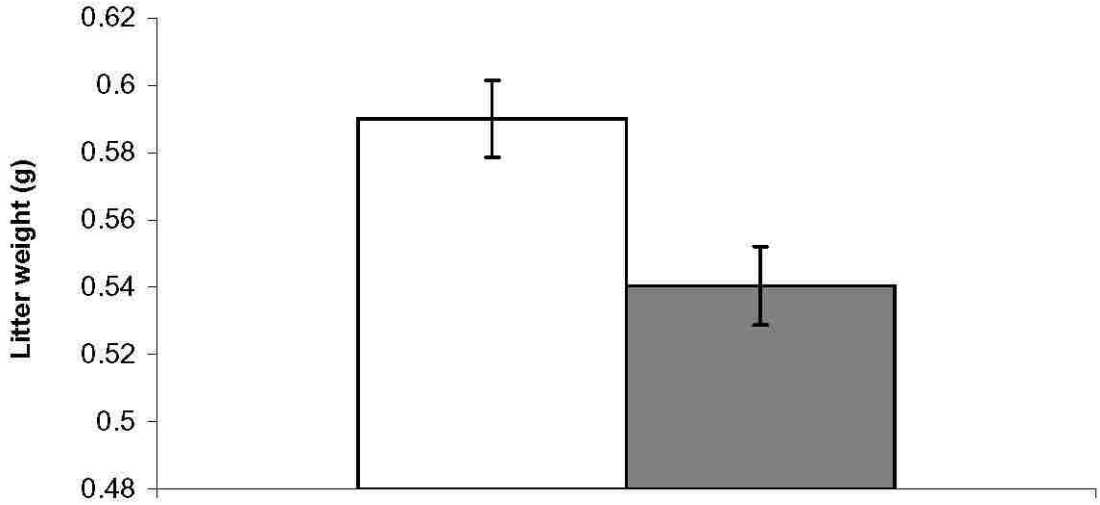


Figure 3.3. (Figure is shown on following page). Mean (± SE) change in height, stem diameter, and length of longest leaf of *M. cerifera* plants exposed to different AMF and litter treatments for one year, beginning in May, 2005. Closed circles denote AMF plants, open circles denote non-inoculated plants.

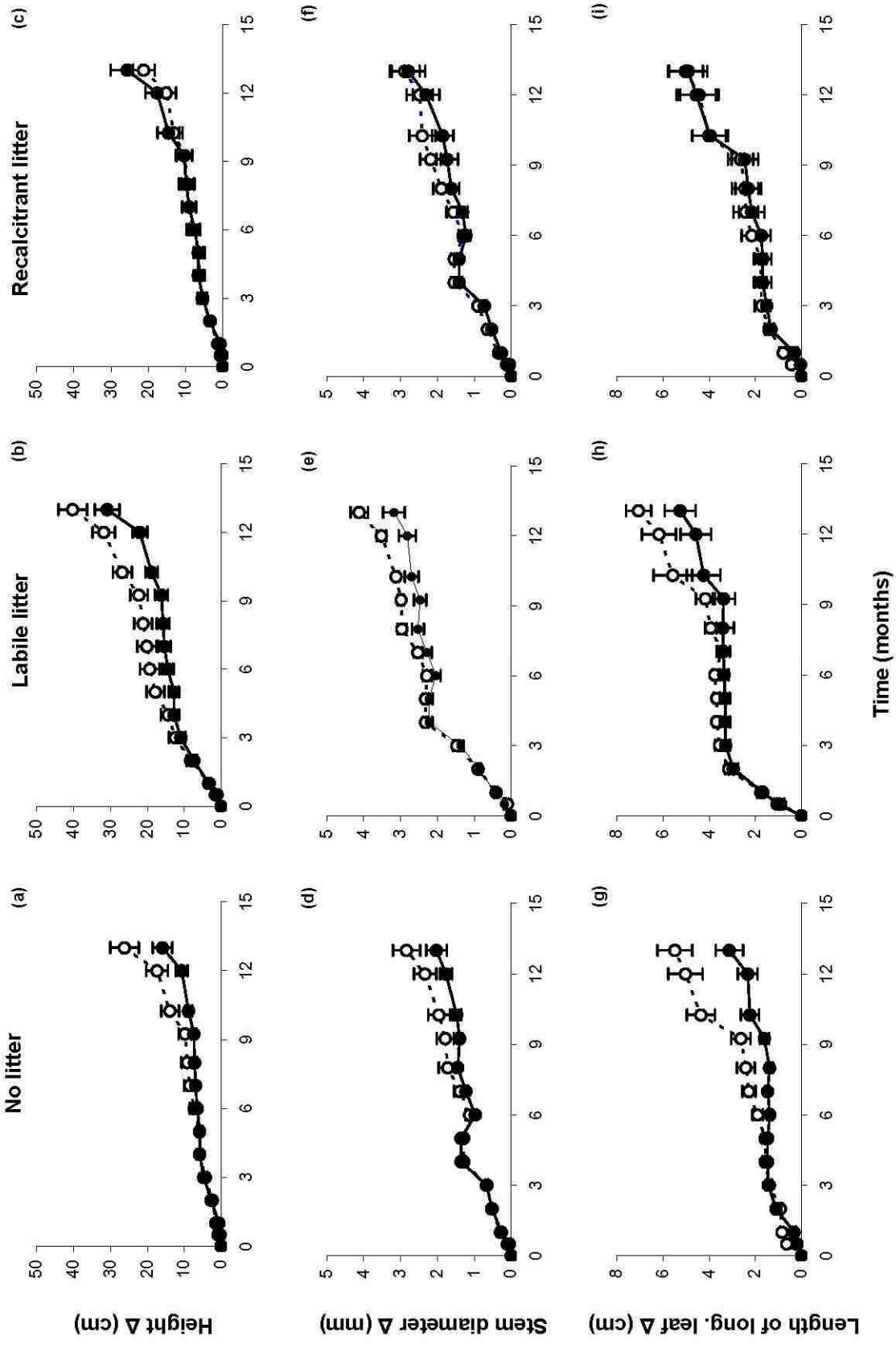

No litter
Labile litter
Recalcitrant litter
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Height Δ (cm)
Stem diameter Δ (mm)
Length of long. leaf Δ (cm)
Time (months)

Figure 3.4. Average (± SE) root dry weight (a), root:shoot ratio (b), and root length (c) of *M. cerifera* plants exposed to no-litter, labile leaf litter, or recalcitrant leaf litter. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. Significant effects from tests of between-subject effects from two-factor (AMF×Litter) MANOVA are shown (see Table 3.4). Bars topped with the same letter do not differ significantly at $P < 0.05$ (Tukey's HSD).

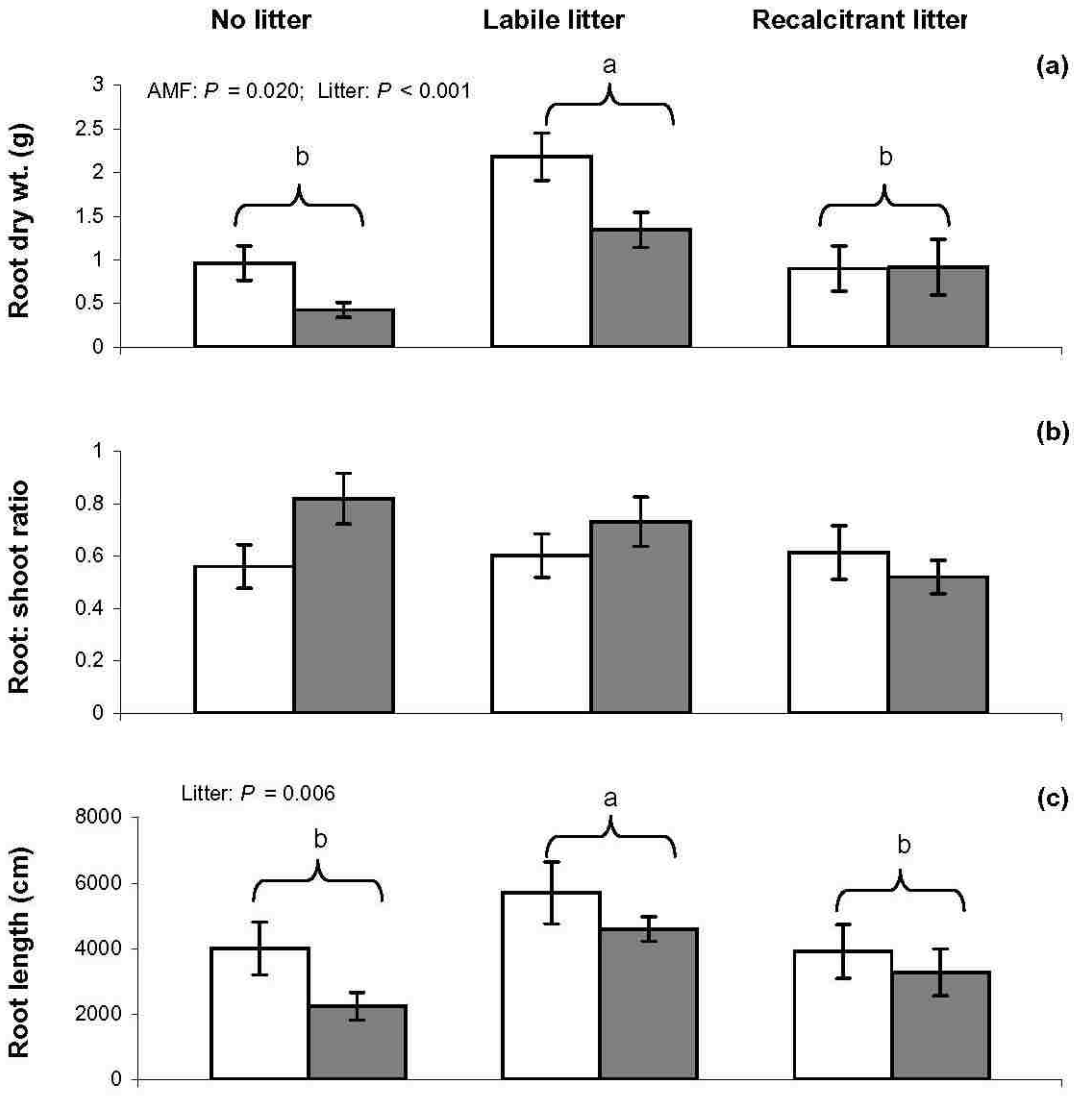


Figure 3.5. Average (± SE) cluster roots of *M. cerifera* plants exposed to different AMF and litter treatments for one year, beginning in May, 2005. Shaded bars denote AMF plants, and open bars denote non-inoculated plants.

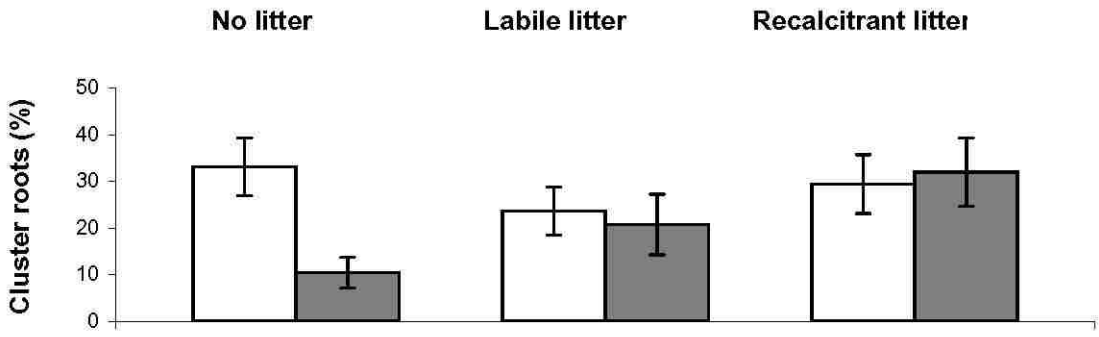


Figure 3.6. (Figure is shown on following page). Nodule biomass versus leaf nitrogen concentration (%), nitrogen content, and $\delta^{15}N$ (‰) of *M. cerifera* plants. Closed symbols denote AMF plants, open symbols denote non-inoculated plants. Circles denote no-litter plants, triangles denote labile leaf litter plants, and squares denote recalcitrant leaf litter plants. Linear regression r^2 values, and associated *P* value are shown. Best-fit lines are only shown for significant regressions.

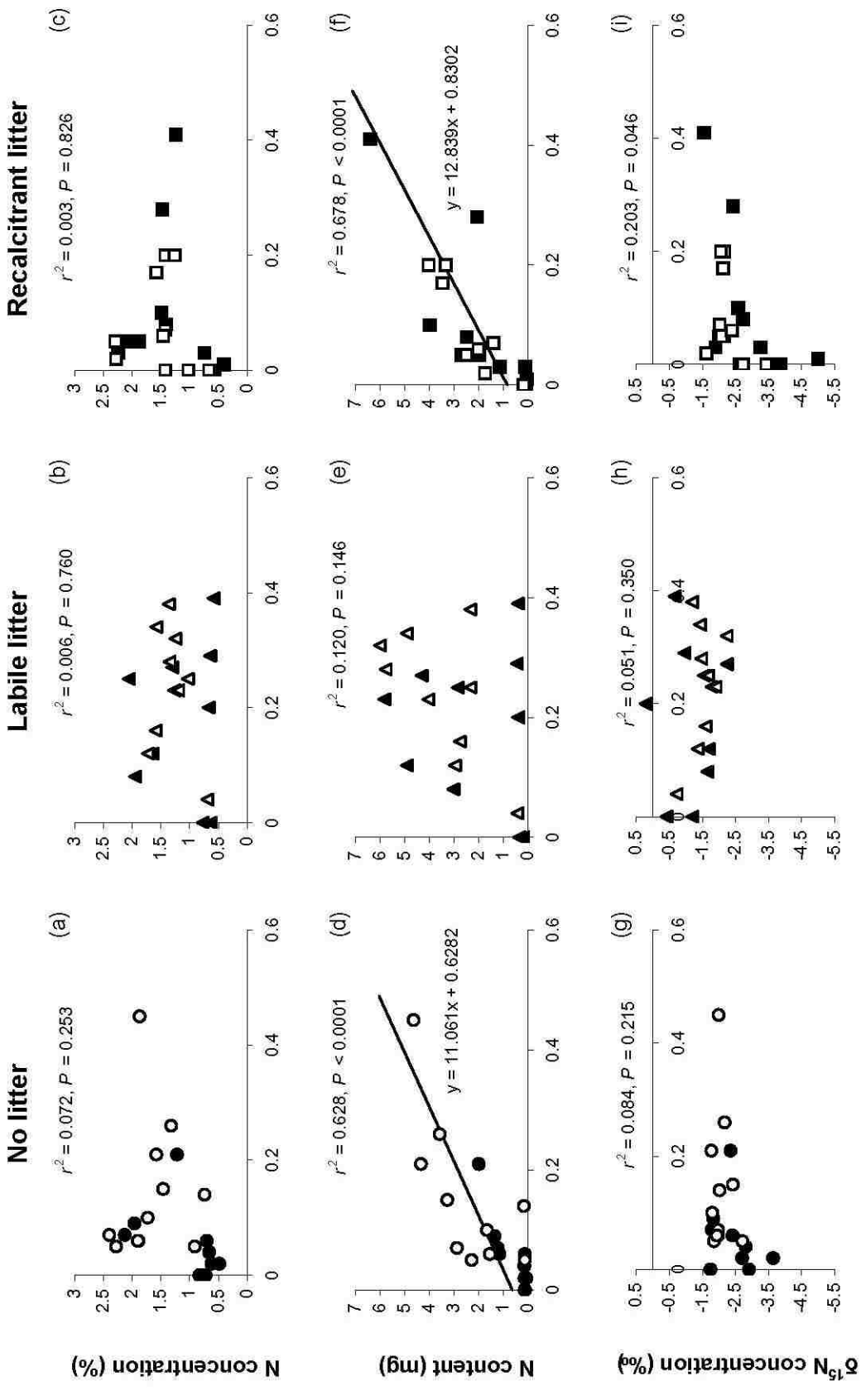

No litter
Labile litter
Recalcitrant litter
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
r^2 = 0.072, P = 0.253
r^2 = 0.006, P = 0.760
r^2 = 0.003, P = 0.826
r^2 = 0.628, P < 0.0001
y = 11.061x + 0.6282
r^2 = 0.120, P = 0.146
r^2 = 0.678, P < 0.0001
y = 12.839x + 0.8302
r^2 = 0.084, P = 0.215
r^2 = 0.051, P = 0.350
r^2 = 0.203, P = 0.046
N concentration (%)
N content (mg)
δ15N concentration (‰)
Nodule dry weight (g)

Figure 3.7. Average (± SE) $\Delta^{13}C$ (‰) of leaf tissue of *M. cerifera* plants exposed to no-litter, labile leaf litter, and recalcitrant leaf litter. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. Test of between-subject effects from two-factor (AMF×Litter) MANOVA showed that AMF significantly increased ^{13}C discrimination ($\Delta^{13}C$).

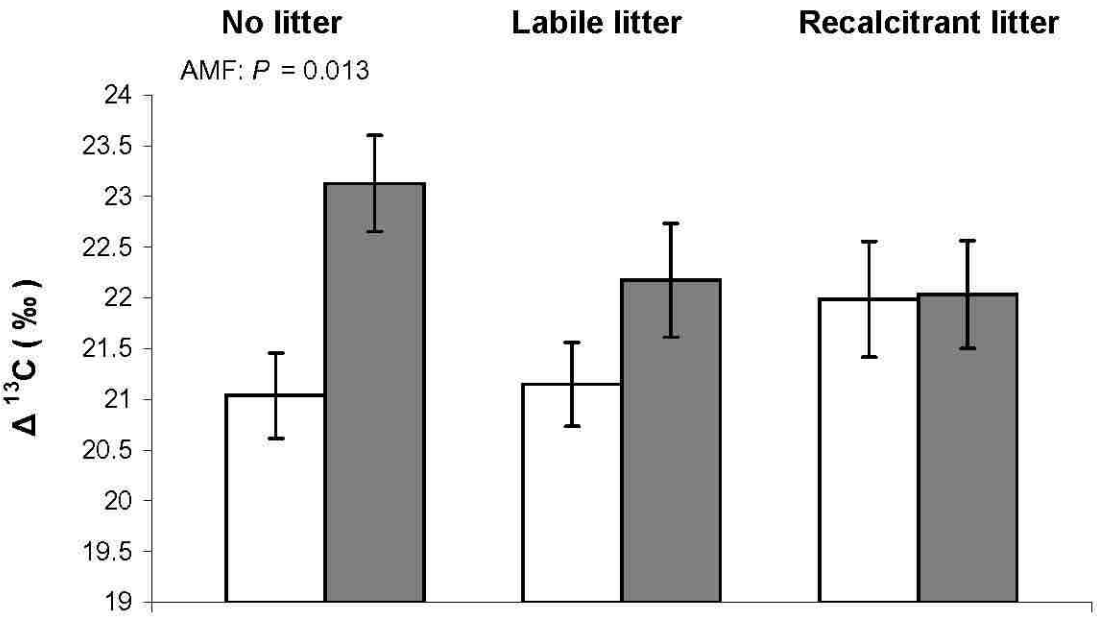
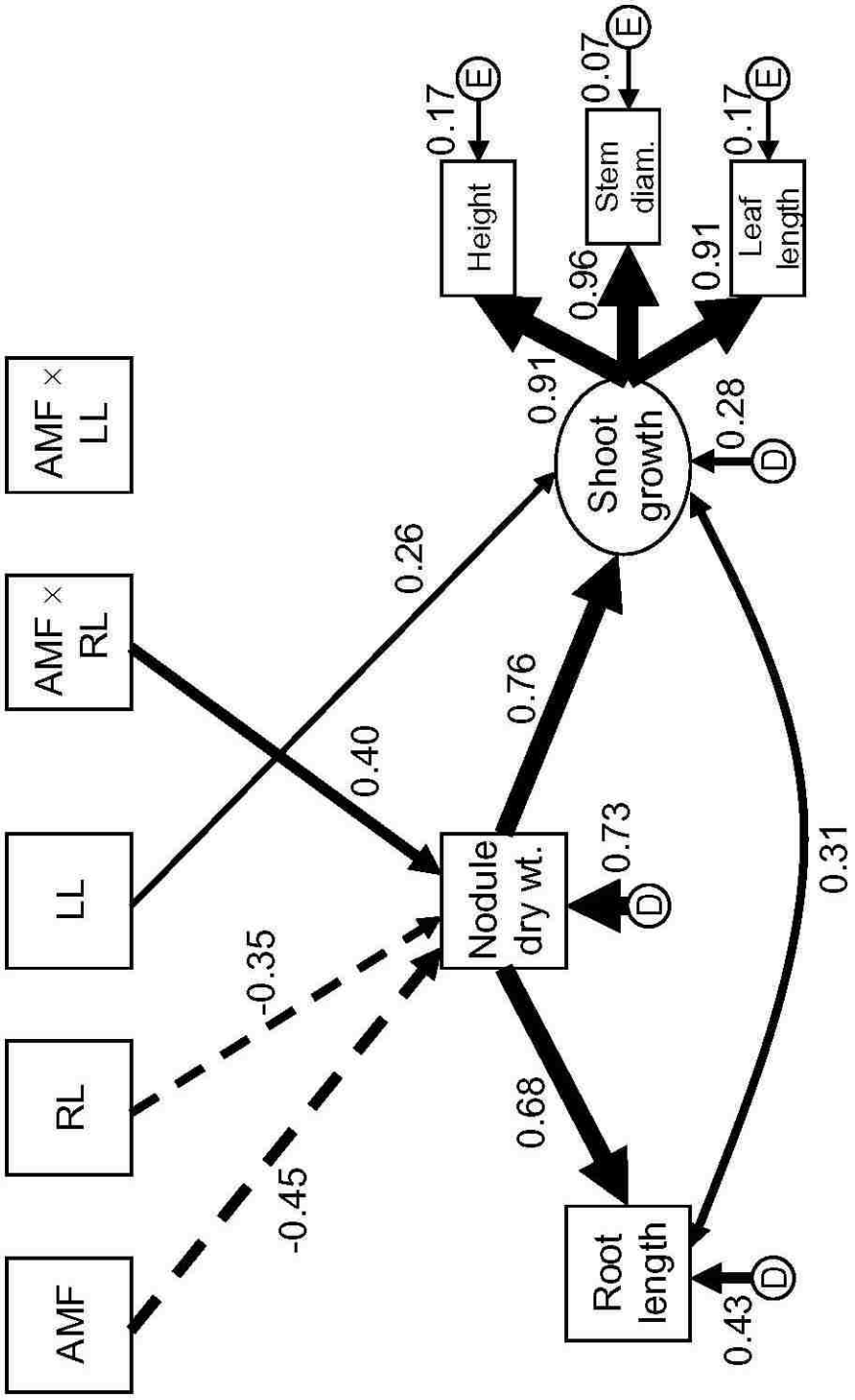


Figure 3.8. (Figure is shown on following page). Standardized parameter estimates for Structural model depicted in Figure 1. Only path coefficients that are significantly different from zero ($Z \geq 1.96$ to be significant at $P < 0.05$) are shown. One-headed arrows denote the effect of an independent variable on a dependent variable. Two-headed arrows denote correlations between variables. Positive effects are shown by solid lines, and negative effects by dashed lines. Arrow widths are proportional to the magnitude of the path coefficients. For estimates of non-significant path coefficients, see direct effects in Table 3.7. For abbreviations, see Fig 3.1.


AMF
RL
LL
AMF × RL
AMF × LL
-0.35
-0.45
0.40
0.26
Nodule dry wt.
0.73
D
0.76
0.68
Shoot growth
0.91
0.28
D
Root length
0.43
D
0.31
Height
0.17
E
Stem diam.
0.07
E
Leaf length
0.17
E
0.96
0.91

Chapter 4

Arbuscular mycorrhizal fungi enhance *Morella cerifera* growth under conditions of litter accumulation

To acquire mineral nutrients in infertile soils, plants have developed a wide array of root adaptations. Some of these root adaptations are morphological; others involve the association of roots with symbiotic microorganisms, such as nitrogen fixing bacteria and mycorrhizal fungi. Mycorrhizal fungi colonize plant roots, and in exchange for photosynthate, translocate mineral nutrients from the soil solution to plants (Smith & Read, 1997). The symbiotic association of plant roots with arbuscular mycorrhizal fungi (AMF) in the fungal phylum Glomeromycota (Schussler *et al.*, 2001), is regarded as the most primitive of all root adaptations. Because early land plants probably had coarse root systems which are limited in their capacity to absorb relatively immobile mineral nutrients such as phosphorus from the soil, it has been hypothesized that association with AMF helped plants to colonize the land (Malloch *et al.*, 1980; Brundrett, 2002). Many plants have developed highly specialized root systems, and some have secondarily lost the ability to associate with AMF. Among the adaptations that are regarded as a functional replacement for arbuscular mycorrhizas in phosphorus acquisition by plants are cluster roots and the production of highly branched root systems, such as those observed in grasses (Brundrett, 2002). These adaptations have appeared in colonizer

species that grow in habitats were AMF may not always be present in the soil (Janos, 1980b).

Morella cerifera (L.) Small ("Wax Myrtle", formerly *M. cerifera*) is an example of a plant that has a wide variety of root adaptations to acquire mineral nutrients. It grows in early successional or disturbed ecosystems in which availability of nitrogen and phosphorus typically is low. To enhance nitrogen and phosphorus acquisition it associates with *Frankia* sp. (a nitrogen fixing actinomycete) and forms cluster roots. Even though *M. cerifera* has been found to harbor AMF root colonization in the field, it has been suggested that it forms a non-funtional symbiosis with AMF (Poole & Sylvia, 1990). Poole & Sylvia (1990) found no effect of AMF inoculation on root and shoot growth of *M. cerifera* plants.

Several lines of evidence, however, suggest that AMF do provide benefit to *M. cerifera* under conditions of litter accumulation. *M. cerifera* may sustain as high as 44% of their root length colonized by AMF in the field (Poole & Sylvia, 1990). In natural ecosystems it is common to observe high litter accumulation beneath the canopy of *M. cerifera* plants. I have recorded litter depth underneath a plant in the Myricaceae in an early successional site as much as 18.5 cm (Aristizábal, unpublished results). In the field, AMF have been found to proliferate within the litter leaves of *M. cerifera* (Aristizábal, 2002), and within the litter leaves of several other species in the genus (Aristizábal *et al.*, 2004). That AMF have been found both amidst and within the litter of *M. cerifera* plants suggests that AMF could play a role scavenging mineral nutrients from relatively intact dead leaves.

The results of Chapter 3 in which I found that AMF had negative effect on the growth of *M. cerifera* except when a source of recalcitrant litter was present, also suggest that AMF provide a benefit to *M. cerifera* plants under conditions of litter accumulation. Similarly to the results of Hodge *et al.* (2001), the results of Chapter 3 show that AMF associated with *M. cerifera* plants accelerated the decomposition of litter leaves. Because the soils in which *M. cerifera* grows are very nutrient-poor, enhancement of decomposition and acquisition of mineral nutrients from recalcitrant litter are likely to affect plant growth and survival.

M. cerifera is a highly facultatively mycotrophic plant of which the response to AMF may change when environmental conditions change (Janos, 2007). These changes in the response to AMF may even be more pronounced when another symbiont, like *Frankia*, is involved (Bronstein, 1994). Consequently, the results of the experiment (Poole & Sylvia, 1990) that show that AMF do not provide benefit to *M. cerifera* may not reflect what occurs when these plants are growing in nutrient impoverished soils. Because soil nitrogen and phosphorus availability probably limit the growth of *M. cerifera* in its natural ecosystems, these nutrients may drive changes in the response of *M. cerifera* to AMF. Soil nitrogen and phosphorus availability may directly affect the plant, or may affect it indirectly by affecting its associated symbionts: AMF and *Frankia* sp. The objective of this study was to determine, under conditions of litter accumulation, the effect of AMF, N, and P on *M. cerifera* growth, nodulation, and cluster root formation. I hypothesized that: 1.) AMF would be beneficial to *M. cerifera* plants in the presence of litter when no nitrogen or phosphorus were added, 2.) AMF and phosphorus fertilization

would reduce cluster root formation, and, 3.) Nitrogen fertilization would reduce *Frankia* sp. nodulation.

Methods

To test how AMF, N, and P and their interaction influence the growth of *M. cerifera* in the presence of litter, I used a three-factor, complete factorial experiment (+/- AMF × +/- N × +/-P = 8 treatments). Each of the treatment combinations was replicated 12 times, for a total of 96 pots, each containing a single seedling.

I collected *M. cerifera* seeds from 5 different reproductive individuals in Monroe County, South Florida. To control for genetic effects, seeds from different reproductive individuals were evenly distributed among treatments. I mechanically scarified the seeds with sandpaper, and germinated them in Petri dishes on moist filter paper. I transferred emergent seedlings to 2 cm diameter Ray Leach Conetainers ® filled with coarse silica sand. After three months, to initiate the experiment the seedlings were transplanted into 10 cm diameter, 500 ml pots filled with a 2:1 mixture of autoclaved coarse (L 6-20) and fine grained (L 30-65) silica sand (Standard Sand and Silica Co., Miami, FL). This mixture was free of organic matter, nitrogen, and phosphorus.

Prior to seedling transplant, I half-filled the pots with sand mixture and then placed alternating layers of non-overlapping *Rhapis excelsa* (Thunb.) A. Henry ex Rehder dried leaf pieces (1 g) and sand above that. The final layer of leaves was topped by 1 cm depth of fine sand. I used *R. excelsa* leaves in this experiment based on the results of Chapter 2 in which I found that decomposition and AMF vesicle colonization of these leaves occurred at an intermediate, low rate (49 % dry weight remaining, and

41% AMF vesicle colonization after 170 days). The *R. excelsa* litter leaves were collected fresh at the University of Miami Gifford Arboretum. *R. excelsa* (common name "lady palm") has palmate leaves with blades that range in length from 20- 30 cm. I removed leaf blades from the rachis, selected those that were approximately 2.5 cm wide, cut them into 2.5 cm^2 pieces, and dried them at 60 °C for 72 h.

I inoculated +AMF seedlings with a mixture of unidentified *Glomus* and *Gigaspora* species that originated from a South Florida native hardwood hammock soil. The AMF mixture had been proliferated on *Sorghum* sp. host plants from which I isolated spores by wet-sieving (Brundrett *et al.*, 1996). Each pot received 1.35 g of the material retained by a 45 μm sieve after passage through a 250 μm sieve. I coated transplanted seedling roots with the inoculum before depositing the remainder in the planting hole. Non-inoculated plants received the same volume of autoclaved (twice for 1 h, 24 h apart) sievings and 20 ml of an AMF-free microbial filtrate that I made by overnight infusion of sievings in distilled water which was then vacuum-filtered through Whatman #1 filter paper.

While growing my "stock" seedlings in Conetainers filled with sterile sand, I noticed that some of them suddenly grew quickly and their leaves turned dark green. Examination of the roots of these seedlings revealed incipient nodulation, indicating that they had been colonized spontaneously by *Frankia* sp. To reduce error variance, for my experiment I only used seedlings that showed none of the signs of *Frankia* sp. colonization at the outset. One week after transplant (June, 2005), each *M. cerifera* seedling in my experiment received 10 ml of a *Frankia* sp. infusion. I prepared this infusion by surface sterilizing field-collected *M. cerifera* nodules, pureeing them in a

blender (five 2 sec pulses) with distilled water, soaking them overnight, and then vacuum filtering the infusion through Whatman #1 filter paper.

I allowed AMF root colonization to develop for 108 days, before initiating fertilization treatments. Plants were fertilized fortnightly with 50 ml of Hewitt's Standard Nutrient solution lacking N and P (Hewitt, 1952). Four fertilizer treatments were imposed with ¼ strength P (10.31 mg P L^{-1}) and full-strength N (27.99 mg N L^{-1}) as: no addition (no NP treatment), plus P (P treatment), plus N (N treatment), and plus N and P (NP treatment). Even though P fertilization can make AMF parasitic on their host, prior experiments with other plant species (Schroeder & Janos, 2004) have shown that ¼ strength P Hewitt Standard Nutrient solution does not have this effect. The nutrient solutions that received no N were supplemented with calcium and potassium sulfates (272.3 mg $CaSO_4$ L^{-1} and 575.1 mg K_2SO_4 L^{-1}) to compensate for elimination of KNO_3 and $Ca(NO_3)_2$ (Hewitt, 1952).

I randomized the pots on benches in the University of Miami's ambient greenhouse. Pots were re-randomized once, 5 months after planting. I manually watered plants daily as necessary to keep the well-drained, sand substrate moist. I measured plant height, stem diameter, and counted the number of leaves approximately monthly.

At harvest, eight-and-a-half months after initiating the fertilization treatments, I excised plant shoots and separated leaves from stems. These were oven-dried at 60 °C to constant weight, and weighed. Nodules were removed from roots, oven-dried, and weighed. Roots were washed free of sand and scanned using a WinRhizo ® system to determine root length. Fine (< 2 mm diameter) and coarse roots (≥ 2mm diameter) were separated manually, air dried, and weighed. Sub-samples of both diameter classes were

oven-dried and weighed to allow me to calculate total root dry weight. Additional sub-samples were cleared in KOH, acidified, and stained with 0.05% Trypan Blue (Phillips & Hayman, 1970) to confirm colonization by AMF. I printed scanned root images, and used a gridline intersect method (Brundrett *et al.*, 1996) to estimate the percentage of the root system that had cluster roots.

At harvest, I manually collected what remained of the litter leaves from the layers in which they had been placed. Any root fragments that adhered to them were manually removed. The recovered leaf fragments were washed free of sand, oven-dried, and weighed. A sub-sample of litter leaves from each treatment combination was cleared and stained as described by Aristizábal *et al.* (2004), to determine if they were colonized by AMF.

Data analysis

To assess the effect of AMF inoculation on *M. cerifera* aboveground growth prior to the start of fertilization treatments I used a one-way, repeated measures MANOVA. To assess the effects of AMF inoculation, N and P additions, and their interaction on *M. cerifera* aboveground growth I used a three-way repeated measures MANOVA. To correct for differences in plant size attributable to the effects of AMF inoculation prior to the initiation of fertilization treatments, plant sizes at the onset of fertilization were subtracted from all subsequent measurements. Repeated measures data were examined for sphericity. When sphericity was present Greenhouse-Geisser Epsilon values were used to determine significance.

I used three-way ANOVAs to determine the effects of AMF inoculation, N and P additions, and their interaction on *M. cerifera* shoot and root variables (shoot dry weight, root dry weight, root length, root: shoot ratio, nodule dry weight, relative nodule dry weight, and percent cluster roots) and on litter weight loss. I relativized nodule weight by dividing nodule dry weight by total plant dry weight. Significance was Bonferroni-corrected for the number of comparisons made ($P < 0.007$ reflects 0.05/7).

Levene's Test was used to test for homogeneity of variance. If necessary, data were log or square root transformed. No transformation successfully eliminated heteroscasticity of the variables shoot dry weight, root dry weight, and root: shoot ratio. Because there is no non-parametric counterpart for a three-way ANOVA, the results of the tests for these variables should be interpreted with caution because the assumption of homogeneity of variance was violated. All the aforementioned statistical analyses were performed with SPSS v. 14.

In addition to the fertilization treatments, nodulation and litter decomposition may have influenced aboveground and belowground plant growth by providing mineral nutrients. To examine if treatment effects on root length and height were direct, or indirect and mediated through effects on *Frankia* sp. nodulation, or litter weight loss, I tested the path diagram shown in Figure 4.1 with Mplus v. 4.2.

I examined the effects of experimental treatments only on height and root length for several reasons. First, the distribution of root and shoot weight was heteroscedastic. Second, height responded quickly to the fertilization treatments, and overall seemed to be a good indicator of plant growth. I dummy coded the different treatments (AMF, P, N),

and I incorporated their interactions in the model as exogenous variables P×N, AMF×P, AMF×N, and AMF×P×N (Figure 4.1).

Prior to analysis of the path diagram, I screened shoot and root data for normality. The distributions of the variables nodule dry weight, litter weight remaining, root length, and height, were not normal, so these variables were square-root transformed.

Results

Aboveground growth of *M. cerifera* seedlings prior to the initiation of fertilization treatments was significantly affected by AMF inoculation (Table 4.1). Overall, plants that were inoculated with AMF were significantly smaller for all morphometric parameters measured than non-inoculated plants (Figure 4.2). Univariate repeated-measures tests show significant effects of AMF inoculation on height and stem diameter, but not on number of leaves (Table 4.2).

Between-Subjects MANOVA showed significant main effects of P and N, and significant AMF×P, AMF×N, P×N interactions on aboveground *M. cerifera* growth parameters. Within-Subjects MANOVA also showed that aboveground growth of *M. cerifera* seedlings was significantly affected by P and N fertilization, and by AMF×P and P×N interactions (Table 4.3). Within-subjects univariate repeated-measures tests show significant effects of P on height change and of N on height change, stem diameter change, and number of leaves change (listed as “height”, “stem diameter”, and “number of leaves”, respectively; Table 4.4). Plants that received P were slightly, but significantly larger than those that did not receive P. N had a strong positive effect on all plant growth parameters (Figure 4.3). The combination of N and P made plants even larger than

adding just P or N. Even though AMF had positive effects on plant growth in the no NP, and P treatments, AMF had negative effects on *M. cerifera* growth in the N and NP treatments (Figure 4.3).

The results of the three-way ANOVAs indicate that N has a strong positive effect on *M. cerifera* shoot (Table 4.5) and root weight (Table 4.6, Figure 4.4a), and on root length (Table 4.7, Figure 4.4b). They also indicate that AMF inoculation had a positive effect on shoot and root weight, except when nitrogen was applied, where the effects of AMF inoculation became negative (Figure 4.4a and 4.4b). Nitrogen addition significantly decreased *M. cerifera* root: shoot ratios (Table 4.8, Figure 4.4c).

There was a significant effect of AMF on litter weight loss. Relative to non-inoculated plants, AMF inoculation significantly accelerated litter decomposition in pots (Table 4.9, Figure 4.5). I found AMF hyphal and vesicular colonization in these litter leaves. Total AMF colonization (hyphae + vesicles) in these litter leaves was as high as 55% (see Aristizábal *et al.*, 2004 for a description of how leaf colonization was quantified).

Even though I tried to reduce error variance by inoculating all *M. cerifera* plants with a *Frankia* sp. infusion, total nodule dry weight was highly variable among individuals, ranging from no nodules to 0.986 g. P addition had a marginally significant effect on nodule dry weight (Table 4.10, Figure 4.6a). AMF inoculation had a marginally significant effect on relative nodule dry weight (Table 4.11, Figure 4.6b). Percent cluster roots was significantly affected by P and N (Table 4.12, Figure 4.6c). N fertilization significantly increased percent cluster roots in *M. cerifera* plants. In contrast, P fertilization significantly reduced cluster root formation (Figure 4.6c).

The fit of the path diagram depicted in Figure 4.1 was good, with $X^2 = 0.411$, $df = 1$, $P = 0.521$, Comparative Fit Index = 1, Root Mean Squared Error of Approximation < 0.001, and Standardized Root Mean squared Residual = 0.007. Table 4.13 provides the direct (i.e., path coefficients), total indirect, specific indirect (mediated by either litter weight loss or nodule dry weight), and total effects of the model depicted in Figure 4.1. The model explained 17 % of the variance in nodule dry weight, 43 % of the variance in litter weight remaining, 71 % of the variance in root length, and 74 % of the variance in height. Standardized parameter estimates for only those path coefficients that are significantly different from zero ($Z \geq 1.96$ to be significant at $P < 0.05$) are shown in Figure 4.7.

The parameterized path diagram (Figure 4.7) suggests that nitrogen addition had a large direct, positive effect on *M. cerifera* seedling root length and height. Phosphorus fertilization also had a positive, but much smaller, effect on height. The AMF and AMF×N treatments had direct negative effects on plant height. Nodule dry weight had large positive effects on root length and height (Figure 4.7). Root length and plant height were positively correlated (Figure 4.7).

Even though the AMF treatment had no direct effect on root length, and had a negative effect on height, seedlings that were inoculated with AMF had higher nodule dry weights and higher litter weight loss than those seedlings not inoculated with AMF. Through its effect on nodule dry weight, AMF inoculation had a positive indirect effect on height and root length (see specific indirect effects of nodules in Table 4.13).

AMF inoculation and P fertilization positively affected litter weight loss. In contrast, the P×N treatment combination reduced litter weight loss.

Although not shown on the path diagram (because the path coefficients were only marginally significant) the AMF×N treatment combination had a negative direct effect on litter weight loss, and on nodule dry weight. Through its effect on nodule dry weight, the AMF×N treatment combination also had a marginally significant negative indirect effect on height and root length (see specific indirect effects of nodules in Table 4.13).

Discussion

Effect of AMF, N, and P on M. cerifera growth

This study shows that in the presence of leaf litter, application of exogenous N shifts the effect of AMF on the growth of *M. cerifera* from positive to negative. Even though AMF inoculation prior to the initiation of fertilization treatments negatively affected all the *M. cerifera* growth parameters that I measured (Figure 4.2), several months after the initiation of fertilization treatments this shifted in the no NP and P treatments. In these two treatments AMF inoculation had clear beneficial effects on the growth of *M. cerifera* (Figure 4.3). In contrast, in the N and NP treatments, AMF remained disadvantageous for *M. cerifera* growth (Figure 4.3). Multiple studies have demonstrated that changes in the availability of mineral nutrients in the soil can shift the outcome of the symbiotic association between AMF and their host from mutualistic to parasitic (Johnson, 1993; Johnson *et al.*, 1997; Janos, 2007). Because AMF are thought to mainly affect plant phosphorus nutrition (Smith & Read, 1997), most research has focused on how changes in soil phosphorus availability affect the dynamics of the symbiosis (e.g. Schroeder & Janos, 2004). Even though the extent to which AMF contribute to plant nitrogen nutrition is still controversial (Smith & Read, 1997), nitrogen deficiency has been found

to stimulate root colonization by AMF (Blanke *et al.* 2005) which suggests that AMF might sometimes function in nitrogen acquisition.

I found a similar positive effect of AMF on belowground growth parameters of *M. cerifera* in the no NP and P treatments, and a negative effect of AMF in the N and NP treatments (Figure 4.4, Tables 4.6, 4.7, and 4.8). Even though in the no NP and P treatments AMF significantly increased root weight and root length, these effects of AMF are a consequence of increased plant growth and not an increase in root proliferation because of AMF. This can be clearly seen in Figure 4.4c where the AMF×N interaction had no effect on root: shoot ratios.

All of my results show effects of N and P on *M. cerifera* aboveground and belowground growth. Even though the magnitude of the effect of N fertilization on the growth of *M. cerifera* was larger than that of P fertilization, both of these mineral nutrients significantly increased *M. cerifera* growth. N addition also reduced root: shoot ratios suggesting that when N was provided plants allocated more photosynthate to aboveground growth, than to belowground growth. Overall, these results suggest that availability of N and P limited *M. cerifera* growth in this experiment and that *M. cerifera* needs a higher proportion of N than P to grow. I also observed a significant N×P interaction, showing that *M. cerifera* plants grow at a faster rate when both N and P are added, than with either of them alone.

Effect of AMF, N, and P on litter decomposition

That the positive effects of AMF on *M. cerifera* growth only appeared after several months, when mineral nutrients from moderately recalcitrant litter likely started to become available, suggests that AMF enhance host acquisition of nutrients from litter. In

this experiment AMF greatly enhanced litter decomposition (Figure 4.5), and likely elevated mineral nutrient availability in the pots, supporting this contention. AMF have been found to associate with decomposing organic matter (St. John *et al.*, 1983), and to penetrate and proliferate within litter leaves (Aristizábal *et al.*, 2004), suggesting that they are strategically positioned to acquire mineral nutrients resulting from litter decomposition. AMF colonization of *R. excelsa* leaf litter in the field, where AMF inoculum potential is probably higher than it was in my experiment, occurs at a relatively slow rate, reaching an asymptote after approximately 7 months (Chapter 2). This might also explain the delay in observing beneficial effects of AMF on *M. cerifera* growth.

Even though AMF are not thought to play role in litter decomposition because of their limited production of hydrolytic enzymes (Azcon-Aguilar *et al.*, 1999), Hodge *et al.* (2001) recently demonstrated that they can stimulate litter decomposition. Although the mechanism through which AMF enhance litter decomposition is still unknown, AMF have been found to stimulate carbon exudation from roots (Azaizeh *et al.*, 1995), which could in turn stimulate the activity of decomposer microorganisms. In addition to the possibility of increasing exudation of carbon from roots, AMF may have stimulated litter decomposition in my experiment by increasing the availability of phosphorus in the substrate. AMF have been found to hydrolyze organic phosphate (Koide & Kabir, 2000), which supports this idea. That in addition to AMF, P fertilization enhanced litter mass loss in this study (Figure 4.5 and Figure 4.7) also supports this contention. A final way by which AMF may enhance litter decomposition is by mechanically disrupting the leaf tissue by proliferation within it. This mechanical disruption may make litter more

susceptible to colonization by decomposer microorganisms, thus enhancing its rate of decomposition.

Even though AMF enhanced litter decomposition in my study, the path analysis failed to detect a direct effect of litter weight loss on plant height or root length. That may have been a consequence of inadequate statistical power. Additionally, the result may be consistent with AMF not directly decomposing leaf litter such that sequestration of nutrients from leaf litter by AMF is independent of litter weight loss.

P fertilization enhanced litter mass loss in this study, indicating that litter decomposition was limited by P. If litter decomposition is limited by P, then in the absence of exogenous P, P from litter may become immobilized by decomposer microorganisms thereby negatively affecting plant growth. That recalcitrant litter initially immobilizes P may explain why in another experiment (Chapter 3) I found that *M. cerifera* seedlings provided with recalcitrant litter had a lower nodule weight than those without litter. In the same experiment, plants provided recalcitrant leaf litter but inoculated with AMF had a higher mean nodule weight than those without AMF suggesting that AMF may mobilize P from litter (Chapter 3).

In contrast, litter mass loss was negatively affected by the P×N treatment combination. Even though it is generally thought that nitrogen increases litter decomposition, it was recently proposed that "microbial nitrogen mining" may be negatively affected by nitrogen addition (Craine *et al*., 2007). This may explain why addition of N eliminated the effects of P on litter mass loss.

Effect of AMF, N, and P on nodulation

Even though the enhancement of litter decomposition by AMF did not directly translate into improved plant growth (Fig 4.7, Table 4.13), litter decomposition may have influenced plant phosphorus nutrition. The results of the path analysis suggest that the positive effects of AMF on *M. cerifera* growth are not direct, but are indirect and are mediated by the effect AMF have on nodule dry weight (Figure 4.7). Because nodulation highly requires phosphorus (Marschner, 2002) I infer that AMF may have aided in acquisition of phosphorus from litter in this study. That P had a marginally significant effect on nodule dry weight supports this idea. It has been demonstrated that AMF enhance nodulation by improving plant phosphorus nutrition in leguminous species that associate with *Rhizobium* (Chalk *et al.*, 2006) and also in species that associate with *Frankia* sp. such as *Alnus* (Fraga-Beddiar & Le Tacon, 1990; Jha *et al.*, 1993).

The plants that were both inoculated with AMF and fertilized with P were larger and tended to have greater nodule dry weight (although not significantly so) than those which only received P (Figure 4.4a and Figure 4.6a), suggesting that AMF helped capture P that plant roots, even cluster roots, could not access. The AMF plus P plants showed the positive effects of AMF sooner than those not fertilized with P, suggesting that AMF enhanced host acquisition of P, which likely amortized the cost of AMF during the time before phosphorus from litter became available. In the plus P treatment, AMF inoculation had positive effects on all growth parameters, and these effects started to become apparent approximately after 3 months. In the no NP treatment positive effects of AMF took approximately 5 months to appear.

In this experiment I observed clear negative effects of AMF on *M. cerifera* growth when N fertilizer was added. Application of N fertilizer likely makes plants less reliant on nitrogen fixation to acquire nitrogen to grow. Because the only positive effect AMF had on plant growth was indirect, by enhancing nodulation, in the treatments where N was applied AMF would still be a cost to the host and would not provide a benefit. Acquiring nitrogen through fixation is an expensive energetic process. Nitrogen fertilization can reduce nodulation (Kohls & Baker, 1989), and even though N did not significantly reduce relative nodule weight in this experiment, it is likely that a large portion of the nitrogen requirements of the plant were met by fertilization. My having applied soluble N fortnightly, may explain why N did not eliminate nodulation. Because nitrogen is highly mobile and leaches easily, the pulses of high nitrogen may have been too transient to suppress nodulation. However, both in the N, and NP treatments relative nodule weight in AMF inoculated and non-inoculated plants was not different, suggesting that there was no benefit of AMF inoculation in nodule formation, and still a carbon cost to the host. Additionally, there was still a carbon cost of *Frankia* sp. which were not likely supplying as great a proportion of the plants' nitrogen.

Effect of AMF, N and P on cluster roots

The results of this study also suggest that AMF and cluster roots are complementary and not mutually exclusive root adaptations. Contrary to what I expected, in this experiment AMF did not reduce cluster root formation. This result however can be explained by the highly infertile substrate that I used. That AMF enhanced the decomposition rate of litter, and likely increased available nutrients in the pots, may explain why AMF did not suppress cluster root production. Having both adaptations may have maximized

acquisition of nutrients in this infertile substrate. Reddell *et al.* (1997) also failed to find a negative correlation between AMF inoculation and cluster root formation by *Casuarina cunninghamiana*.

In this experiment I observed that P fertilization reduced cluster root formation, but N fertilization increased it (Figure 4.6, Table 4.12). That P fertilization reduces cluster root formation has been repeatedly observed (e.g. Louis *et al.*, 1990). This is in accordance with what is thought to be the main function of cluster roots: P-acquisition. Because cluster roots are expensive to produce, addition of phosphorus reduces them. In contrast to P, N increased cluster root formation in my experiment. The positive effect of N on cluster root formation can be explained by the increase in growth N typically promoted. Once the N limitation was overcome, and the plants started to grow, the nutrient that next most limits growth of *M. cerifera* will become limiting. That *M. cerifera* plants fertilized with N became limited by P, may explain why they formed more cluster roots. Additionally, cluster roots with their great increase in root surface area may also aid in the acquisition of a highly pulsed nitrogen supply.

The results of this experiment, and those reported in Chapter 3 suggest that AMF mediate acquisition of nutrients from leaf litter. They also suggest that the benefit AMF provide to *M. cerifera* is indirect, and mediated by the effect of AMF on nodulation. *M. cerifera* typically colonizes early successional ecosystems, where AMF may be absent (Janos, 1980b). Under these conditions, *M. cerifera* probably can acquire phosphorus from the soil via cluster roots, and can associate with *Frankia* sp. to acquire nitrogen. As succession advances, and these plants accumulate litter beneath their canopy, it is increasingly likely that they will encounter AMF. AMF in this situation can help make

mineral nutrients, especially P, available from litter. Even though *M. cerifera* could be expected to reduce cluster root production in the presence of AMF, both I and Reddell *et al.* (1997) have not observed that to be the case. Because the sites in which these plants grow are very nutrient deficient, these plants may require both adaptations to acquire limiting mineral nutrients. This may explain why in the field it is common to observe plants forming cluster roots, nodulating, and associating with AMF.

Table 4.1. One-way repeated-measures MANOVA showing the effects of AMF on growth (height, stem diameter, and number of leaves) of *M. cerifera* plants for three months prior to initiation of fertilization treatments. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Factor	Wilks' λ	*df*	*F*	*P*
Between subjects effects				
AMF	0.563	3, 91	23.530	**< 0.001**
Within subject effects				
Time	0.160	6, 88	77.181	**< 0.001**
Time × AMF	0.650	6, 88	7.890	**< 0.001**

Table 4.2. Univariate tests (Greenhouse-Geisser) from one-way (AMF) repeated-measures MANOVA of growth data (height, stem diameter, and number of leaves) for *M. cerifera* plants for three months prior to fertilization treatments. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Source	Dependent Variable	*df*	*F*	*P*
Between subject effects				
	Height	1, 93	46.237	**< 0.001**
	Stem diameter	1, 93	35.340	**< 0.001**
AMF	Number of leaves	1, 93	0.516	0.474
Within subject effects				
Time	Height	1.1, 105.9	258.560	**< 0.001**
	Stem diameter	1.8, 170.8	218.357	**< 0.001**
	Number of leaves	1.4, 128.2	36.985	**< 0.001**
Time × AMF	Height	1.1, 105.9	8.020	**0.004**
	Stem diameter	1.8, 170.8	2.318	0.106
	Number of leaves	1.4, 128.2	10.599	**< 0.001**

Table 4.3. Three-factor repeated-measures MANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on growth (height, stem diameter, and number of leaves) of *M. cerifera* plants for eight and a half months after initiation of fertilization treatments. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant effects are shown in bold.

Factor	Wilks' λ	*df*	*F*	*P*
Between subject effects				
AMF	0.946	3, 84	1.612	0.193
P	0.821	3, 84	6.100	**0.001**
N	0.395	3, 84	42.855	**< 0.001**
AMF × P	0.911	3, 84	2.724	**0.049**
AMF × N	0.853	3, 84	4.831	**0.004**
P × N	0.888	3, 84	3.521	**0.018**
AMF × P × N	0.984	3, 84	0.450	0.718
Within subject effects				
Time	0.072	18, 69	49.478	**< 0.001**
Time × AMF	0.737	18, 69	1.366	0.177
Time × P	0.571	18, 69	2.882	**0.001**
Time × N	0.216	18, 69	13.876	**< 0.001**
Time × AMF × P	0.670	18, 69	1.885	**0.032**
Time × AMF × N	0.713	18, 69	1.540	0.103
Time × P × N	0.592	18, 69	2.641	**0.002**
Time × AMF × P × N	0.756	18, 69	1.236	0.259

Table 4.4. Univariate tests (Greenhouse-Geisser) from three-factor (AMF × P × N) repeated-measures MANOVA on growth data (height, stem diameter, and number of leaves) of *M. cerifera* plants for eight and a half months after the initiation of fertilization treatments. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Only effects of factors that were significant in the multivariate tests are shown in bold to indicate significance.

Source	Dependent Variable	*df*	*F*	*P*
Between subject effects				
AMF	Height	1, 86	0.100	0.752
	Stem diameter	1, 86	0.545	0.462
	Number of leaves	1, 86	0.538	0.465
P	Height	1, 86	12.372	**0.001**
	Stem diameter	1, 86	0.981	0.325
	Number of leaves	1, 86	1.945	0.167
N	Height	1, 86	121.880	**< 0.001**
	Stem diameter	1, 86	104.003	**< 0.001**
	Number of leaves	1, 86	63.426	**< 0.001**
AMF×P	Height	1, 86	1.012	0.317
	Stem diameter	1, 86	1.037	0.311
	Number of leaves	1, 86	0.058	0.811
AMF×N	Height	1, 86	10.630	**0.002**
	Stem diameter	1, 86	11.799	**0.001**
	Number of leaves	1, 86	13.620	**< 0.001**
P×N	Height	1, 86	3.954	**0.050**
	Stem diameter	1, 86	0.007	0.932
	Number of leaves	1, 86	0.052	0.821

AMF×P×N	Height	1, 86	1.361	0.247
	Stem diameter	1, 86	0.911	0.342
	Number of leaves	1, 86	0.630	0.429
Within subject effects				
Time	Height	1.2, 104.3	456.935	**< 0.001**
	Stem diameter	1.6, 139.9	374.529	**< 0.001**
	Number of leaves	1.4, 121.1	152.044	**< 0.001**
Time × AMF	Height	1.2, 104.3	0.397	0.570
	Stem diameter	1.6, 139.9	0.162	0.807
	Number of leaves	1.4, 121.1	0.307	0.658
Time × P	Height	1.2, 104.3	9.537	**0.001**
	Stem diameter	1.6, 139.9	1.089	0.329
	Number of leaves	1.4, 121.1	0.691	0.455
Time × N	Height	1.2, 104.3	57.177	**< 0.001**
	Stem diameter	1.6, 139.9	61.683	**< 0.001**
	Number of leaves	1.4, 121.1	24.871	**< 0.001**
Time × AMF × P	Height	1.2, 104.3	0.414	0.560
	Stem diameter	1.6, 139.9	0.733	0.456
	Number of leaves	1.4, 121.1	0.233	0.712
Time × AMF × N	Height	1.2, 104.3	6.371	0.009
	Stem diameter	1.6, 139.9	8.034	0.001
	Number of leaves	1.4, 121.1	8.028	0.002
Time × P × N	Height	1.2, 104.3	2.300	0.127
	Stem diameter	1.6, 139.9	0.135	0.831
	Number of leaves	1.4, 121.1	0.339	0.637

Time × AMF × P × N	Height	1.2, 104.3	0.906	0.362
	Stem diameter	1.6, 139.9	0.374	0.645
	Number of leaves	1.4, 121.1	0.588	0.500

Table 4.5. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on shoot dry weight of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	0.014	0.906
P	1, 87	2.928	0.091
N	1, 87	64.796	**< 0.001**
AMF × P	1, 87	0.009	0.923
AMF × N	1, 87	9.713	**0.002**
P × N	1, 87	0.587	0.446
AMF × P × N	1, 87	1.296	0.258

Table 4.6. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on root dry weight of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	0.984	0.324
P	1, 87	1.874	0.175
N	1, 87	136.288	**< 0.001**
AMF × P	1, 87	0.168	0.683
AMF × N	1, 87	17.530	**< 0.001**
P × N	1, 87	0.849	0.359
AMF × P × N	1, 87	0.960	0.330

Table 4.7. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on root length of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	0.338	0.563
P	1, 87	0.429	0.514
N	1, 87	98.293	**< 0.001**
AMF × P	1, 87	0.107	0.744
AMF × N	1, 87	5.462	0.022
P × N	1, 87	0.047	0.830
AMF × P × N	1, 87	0.001	0.977

Table 4.8. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on root: shoot ratios of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	0.504	0.480
P	1, 87	0.740	0.392
N	1, 87	17.691	**< 0.001**
AMF × P	1, 87	1.541	0.218
AMF × N	1, 87	1.446	0.232
P × N	1, 87	0.096	0.757
AMF × P × N	1, 87	< 0.001	0.985

Table 4.9. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on litter mass loss of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	48.708	**< 0.001**
P	1, 87	3.748	0.056
N	1, 87	2.106	0.150
AMF × P	1, 87	0.534	0.467
AMF × N	1, 87	3.471	0.066
P × N	1, 87	4.622	0.034
AMF × P × N	1, 87	0.265	0.608

Table 4.10. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on nodule dry weight of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	2.207	0.141
P	1, 87	5.332	0.023
N	1, 87	0.767	0.384
AMF × P	1, 87	0.072	0.789
AMF × N	1, 87	2.721	0.103
P × N	1, 87	1.504	0.223
AMF × P × N	1, 87	0.256	0.614

Table 4.11. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on relative nodule weight of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	6.868	0.010
P	1, 87	0.412	0.523
N	1, 87	1.962	0.165
AMF × P	1, 87	0.070	0.792
AMF × N	1, 87	2.769	0.100
P × N	1, 87	2.187	0.143
AMF × P × N	1, 87	0.526	0.470

Table 4.12. Three-way ANOVA showing the effects of different experimental treatments (AMF, P, and N), and their interaction, on cluster roots of *M. cerifera* plants for eight and a half months. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significance level was Bonferroni-corrected ($P < 0.007$, for acceptance).

Source of variation	*df*	*F*	*P*
AMF	1, 87	5.972	0.017
P	1, 87	24.227	**< 0.001**
N	1, 87	26.166	**< 0.001**
AMF × P	1, 87	0.362	0.549
AMF × N	1, 87	0.452	0.503
P × N	1, 87	0.187	0.666
AMF × P × N	1, 87	0.358	0.551

Table 4.13. (Table is shown on following page). Direct, indirect (total and specific) and total effects of different experimental treatments (AMF and the addition of P and N), on nodule dry weight, litter weight remaining, root length, and height. Z-statistic values are shown in parentheses. Direct effects = path coefficients shown in Figure 4.7. Ind. = indirect effects. NA = not applicable. Coefficients that are significantly different from zero ($Z \geq 1.96$ to be significant at $P < 0.05$) are shown in bold.

Effect on:	Litter	Nodule	Height					Root length				
Variable	Direct (=Total)	Direct (=Total)	Direct	Total Ind.	Specific Ind. (litter)	Specific Ind. (nodules)	Total	Direct	Total Ind.	Specific Ind. (litter)	Specific Ind. (nodules)	Total
AMF	**0.574**	**0.308**	**-0.155**	**0.183**	0.012	**0.171**	0.028	-0.098	**0.143**	0.003	**0.140**	0.045
	(7.385)	(3.285)	(-2.294)	(2.706)	(0.308)	(3.113)	(0.377)	(-1.359)	(2.280)	(0.071)	(3.008)	(0.644)
P	**0.153**	0.111	**0.182**	0.065	0.003	0.062	**0.247**	-0.108	0.051	0.001	0.050	-0.057
	(1.969)	(1.186)	(3.399)	(1.215)	(0.305)	(1.178)	(3.353)	(-1.904)	(1.152)	(0.071)	(1.172)	(-0.817)
N	0.115	0.086	**0.556**	0.050	0.002	0.048	**0.606**	**0.670**	0.040	0.001	0.039	**0.710**
	(1.482)	(0.916)	(10.506)	(0.948)	(0.302)	(0.912)	(8.232)	(11.920)	(0.906)	(0.071)	(0.909)	(10.171)
P×N	**-0.173**	0.143	0.058	0.076	-0.004	0.079	0.134	-0.051	0.064	-0.001	0.065	0.013
	(-2.220)	(1.527)	(1.070)	(1.403)	(-0.306)	(1.508)	(1.814)	(-0.893)	(1.416)	(-0.071)	(1.496)	(0.182)
AMF×P	-0.057	0.013	-0.068	0.006	-0.001	0.007	-0.063	-0.039	0.005	0.000	0.006	-0.034
	(-0.736)	(0.134)	(-1.311)	(0.110)	(-0.285)	(0.134)	(-0.852)	(-0.708)	(0.126)	(-0.071)	(0.134)	(-0.486)
AMF×N	-0.144	-0.167	**-0.113**	-0.095	-0.003	-0.092	**-0.209**	-0.094	-0.076	-0.001	-0.075	**-0.170**
	(-1.855)	(-1.777)	(-2.102)	(-1.774)	(-0.304)	(-1.748)	(-2.835)	(-1.643)	(-1.697)	(-0.071)	(-1.729)	(-2.440)
AMF×P×N	0.041	-0.008	-0.061	-0.004	0.001	-0.004	-0.065	0.015	-0.003	0.000	-0.004	0.012
	(0.527)	(-0.086)	(-1.173)	(-0.069)	(0.266)	(-0.086)	(-0.880)	(0.271)	(-0.081)	(0.071)	(-0.086)	(0.166)
Litter	NA	NA	0.021	NA	NA	NA	NA	0.005	NA	NA	NA	NA
			(0.309)					(0.071)				
Nodule	NA	NA	**0.554**	NA	NA	NA	NA	**0.453**	NA	NA	NA	NA
			(9.728)					(7.484)				

Figure 4.1. Path diagram depicting the effects of different experimental treatments (AMF, P, and N) on nodule dry weight, litter weight loss, root length and height of *M. cerifera* plants. This model tests if the effects of experimental treatments on root length and height are direct or indirect and mediated through effects on nodule dry weight or litter weight remaining. AMF = arbuscular mycorrhizal fungi, P = phosphorus, N = nitrogen, P × N = combination of P and N, AMF × P = combination of AMF and P, AMF × N = combination of AMF and N, AMF × P × N = combination of AMF, P, and N, D = disturbance or unexplained variance (1- r^2 = D).

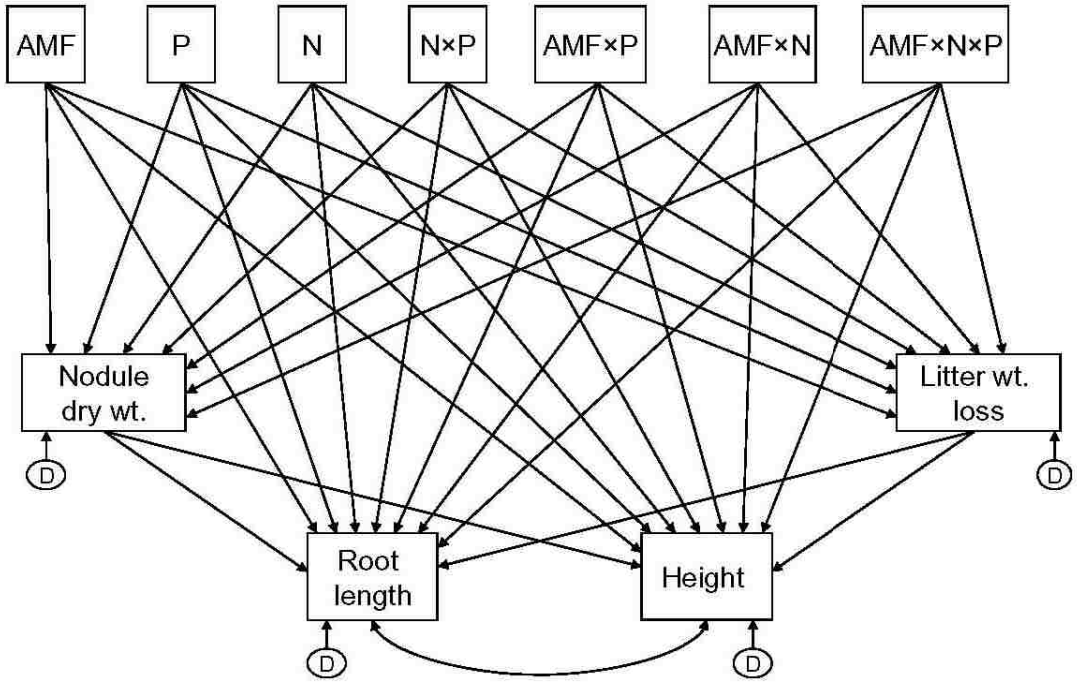


Figure 4.2. Mean (± SE) change in height (a), stem diameter (b), and number of leaves (c) of *M. cerifera* plants exposed to AMF for three months (beginning in June, 2005) prior to initiation of fertilization treatments. Closed circles denote AMF plants, open circles denote non-inoculated plants.

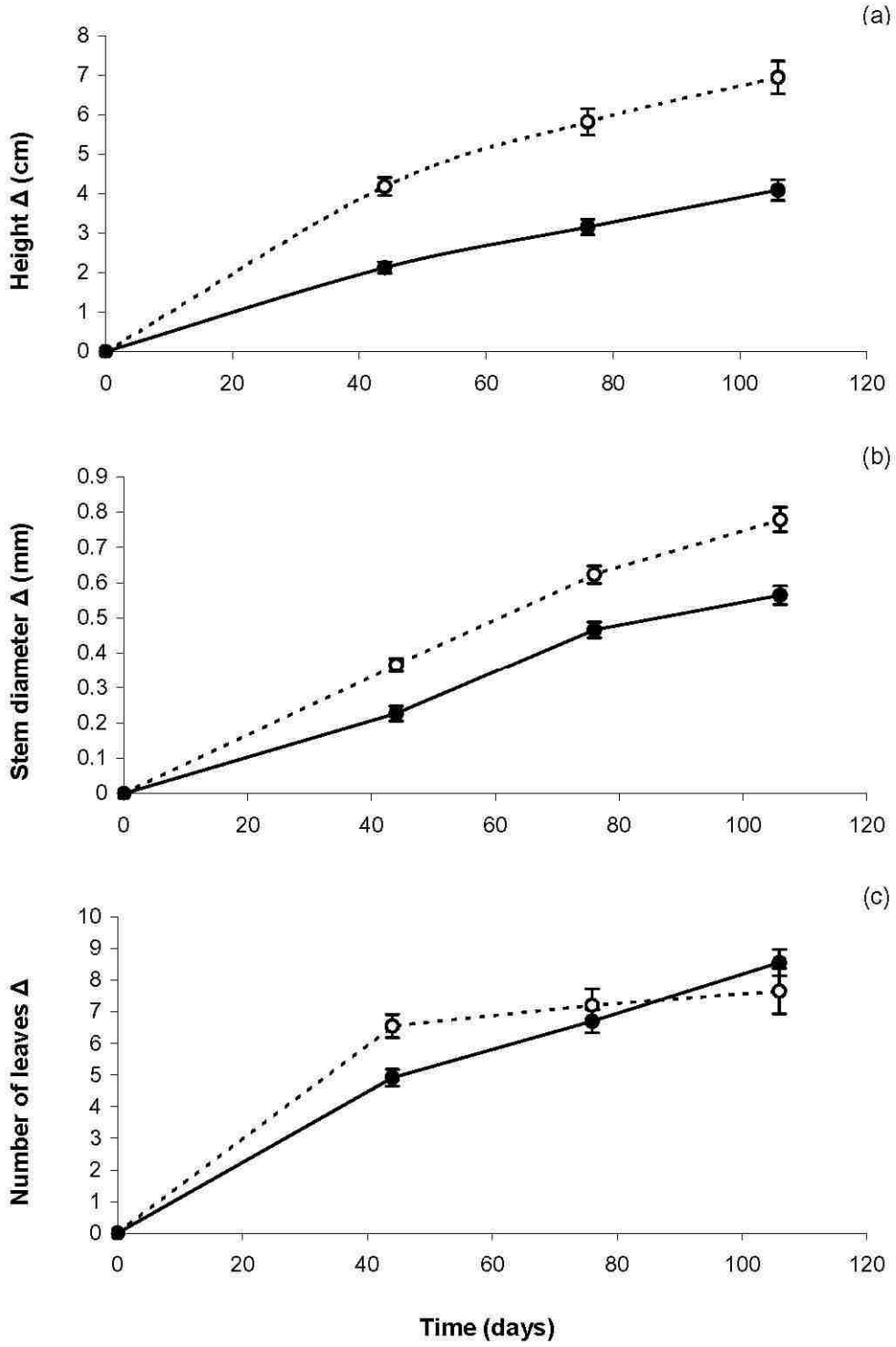


Figure 4.3. (Figure is shown on following page). Mean (± SE) change in height (a-d), stem diameter (e-h), and number of leaves (i-l) of *M. cerifera* plants exposed to AMF, P, and N factorial treatment combinations for eight and a half months. Closed circles denote AMF plants, open circles denote non-inoculated plants.

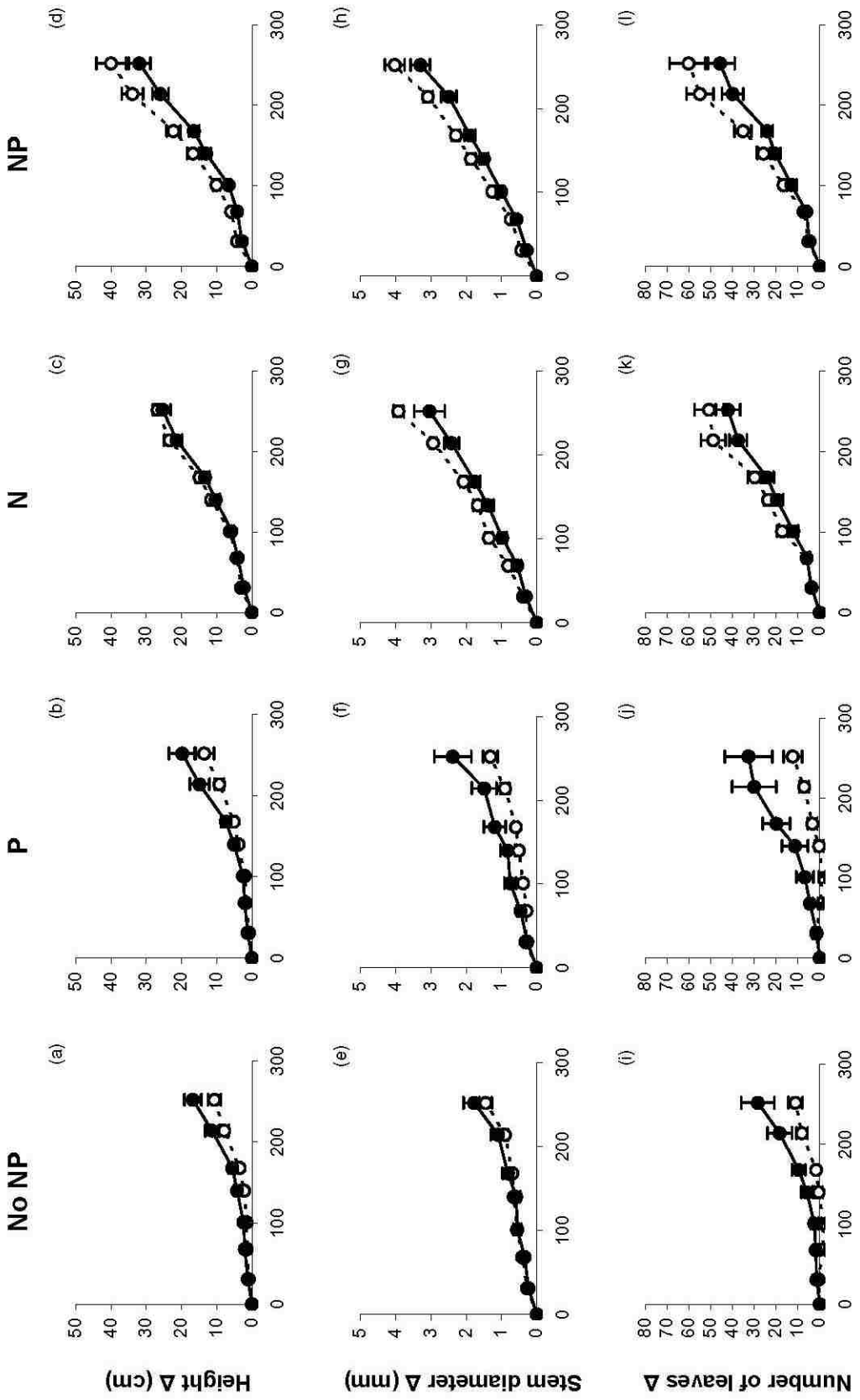

No NP
P
N
NP
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Height Δ (cm)
Stem diameter Δ (mm)
Number of leaves Δ
Time (days)

Figure 4.4. (Figure is shown on following page). Average (± SE) shoot and root dry weight (a), root length (b), and root:shoot ratios (c), of *M. cerifera* plants exposed to AMF, P, and N factorial treatment combinations for eight and a half months. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. In (a) root dry weight is shown as positive values below the x-axis. See Tables 6, 7, and 8 for three-way ANOVA results. Results for the variables shoot weight and root:shoot ratio should be interpreted with caution, as the assumption of homogeneity of variance for this test was not met (see methods section).

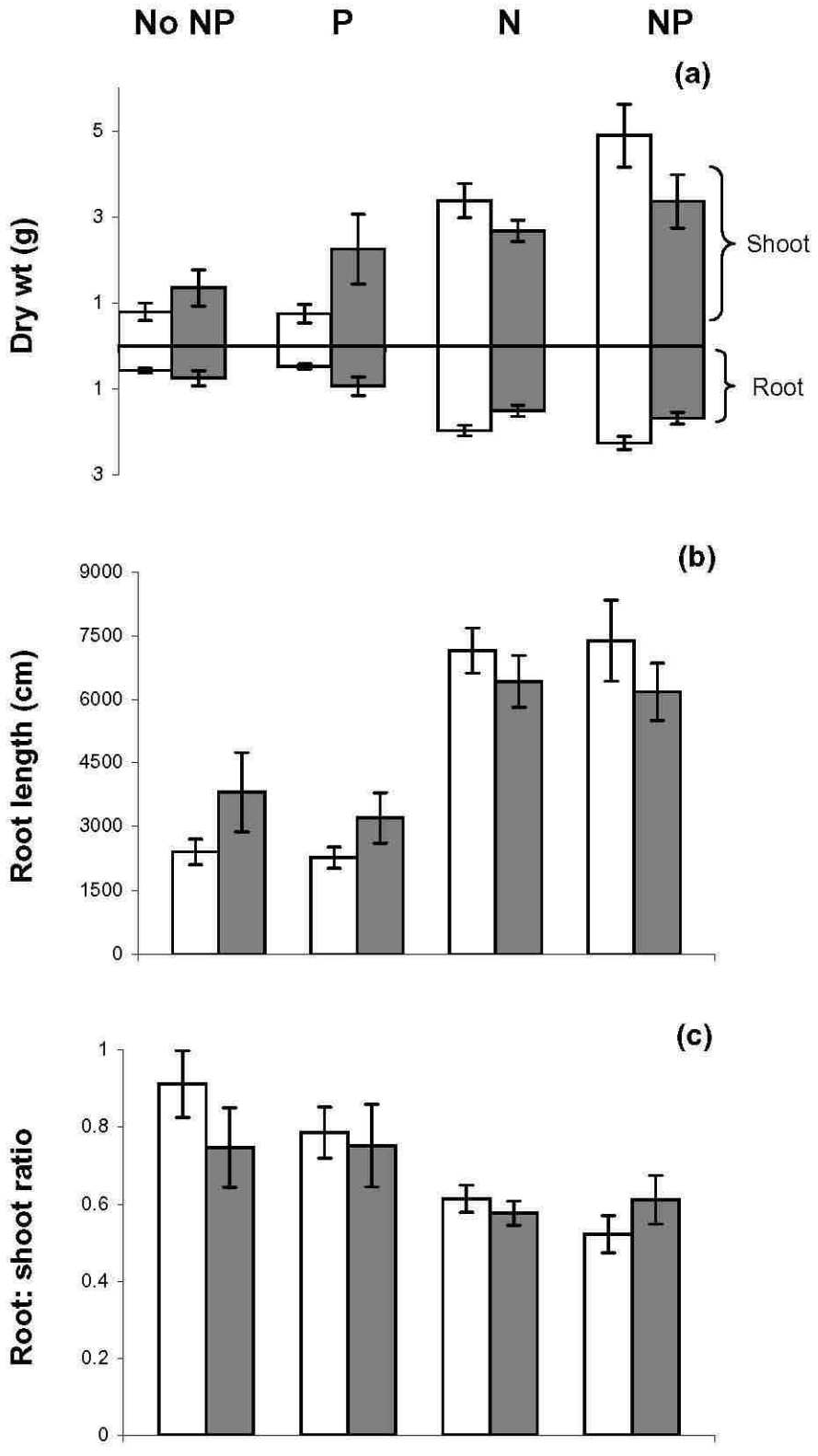

No NP
P
N
NP
(a)
Dry wt (g)
5
3
1
1
3
Shoot
Root
(b)
Root length (cm)
9000
7500
6000
4500
3000
1500
0
(c)
Root: shoot ratio
1
0.8
0.6
0.4
0.2
0

Figure 4.5. Average weight loss (± SE) of litter leaves that were deposited in pots where *M. cerifera* plants grew for one year. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. See Table 4.9 for three-way ANOVA results.

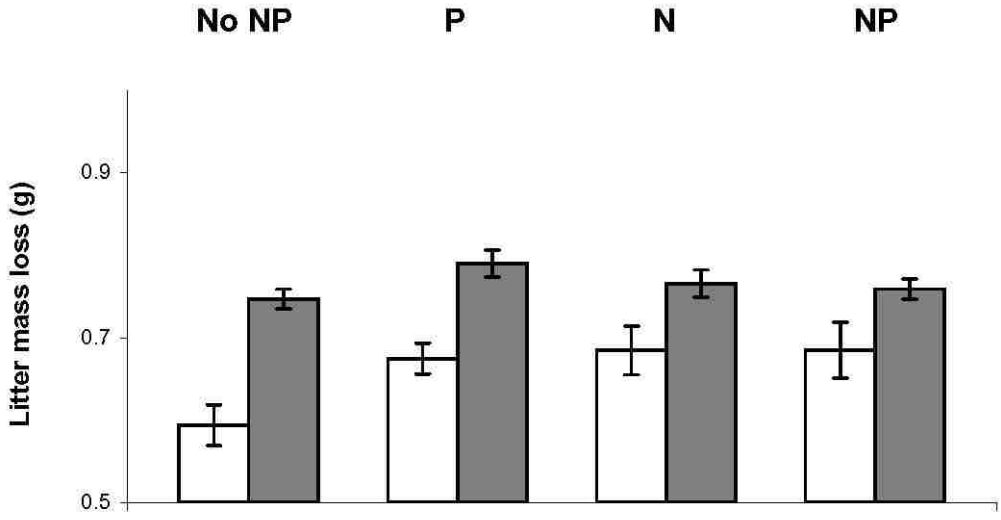


Figure 4.6. (Figure is shown on following page). Average (± SE) nodule dry weight (a), relative nodule dry weight (b), and percent cluster roots (c) of *M. cerifera* plants exposed to AMF, P, and N factorial treatment combinations for eight and a half months. Shaded bars denote AMF plants, and open bars denote non-inoculated plants. See Tables 10, 11, and 12 for three-way ANOVA results.

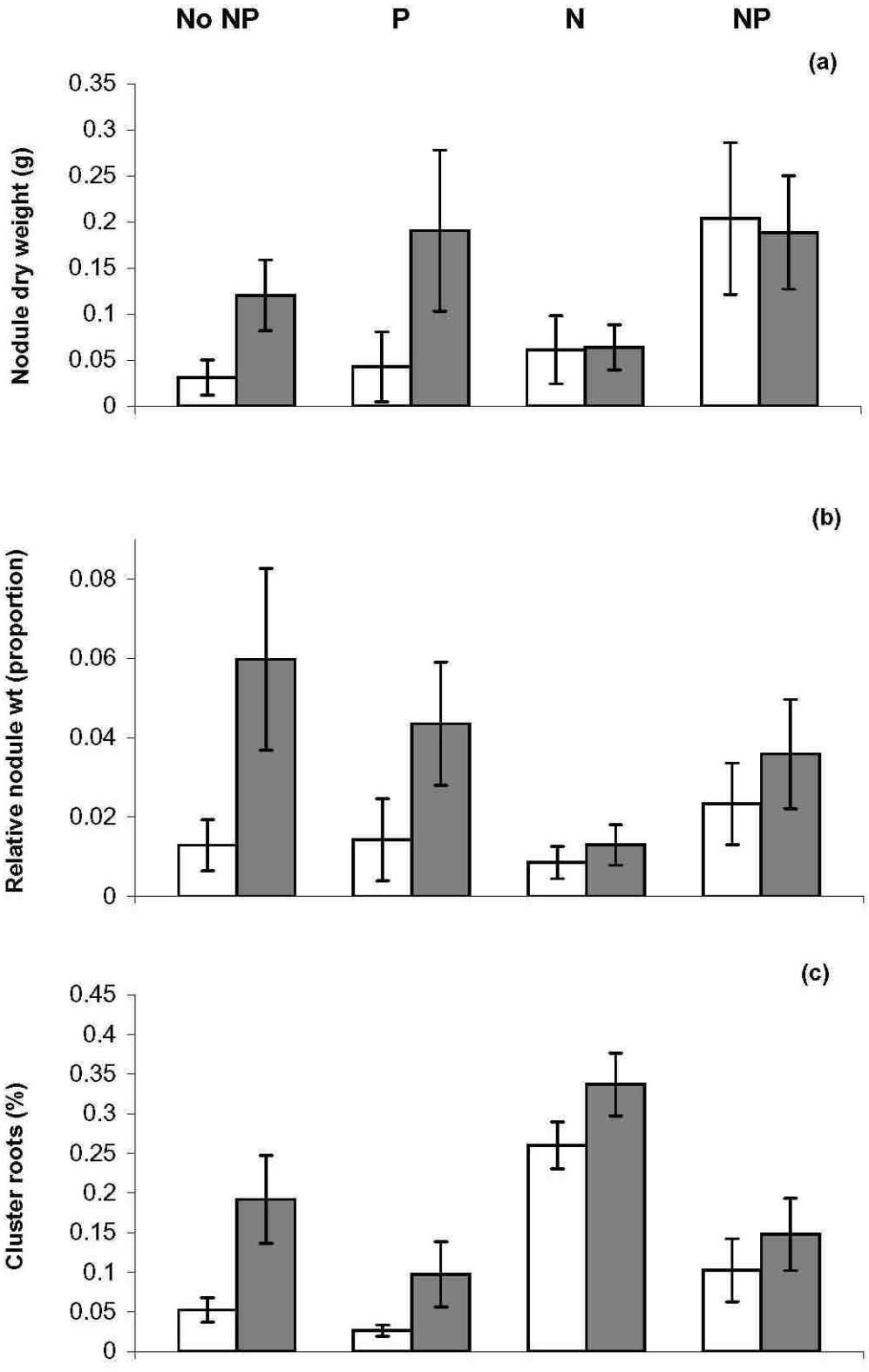
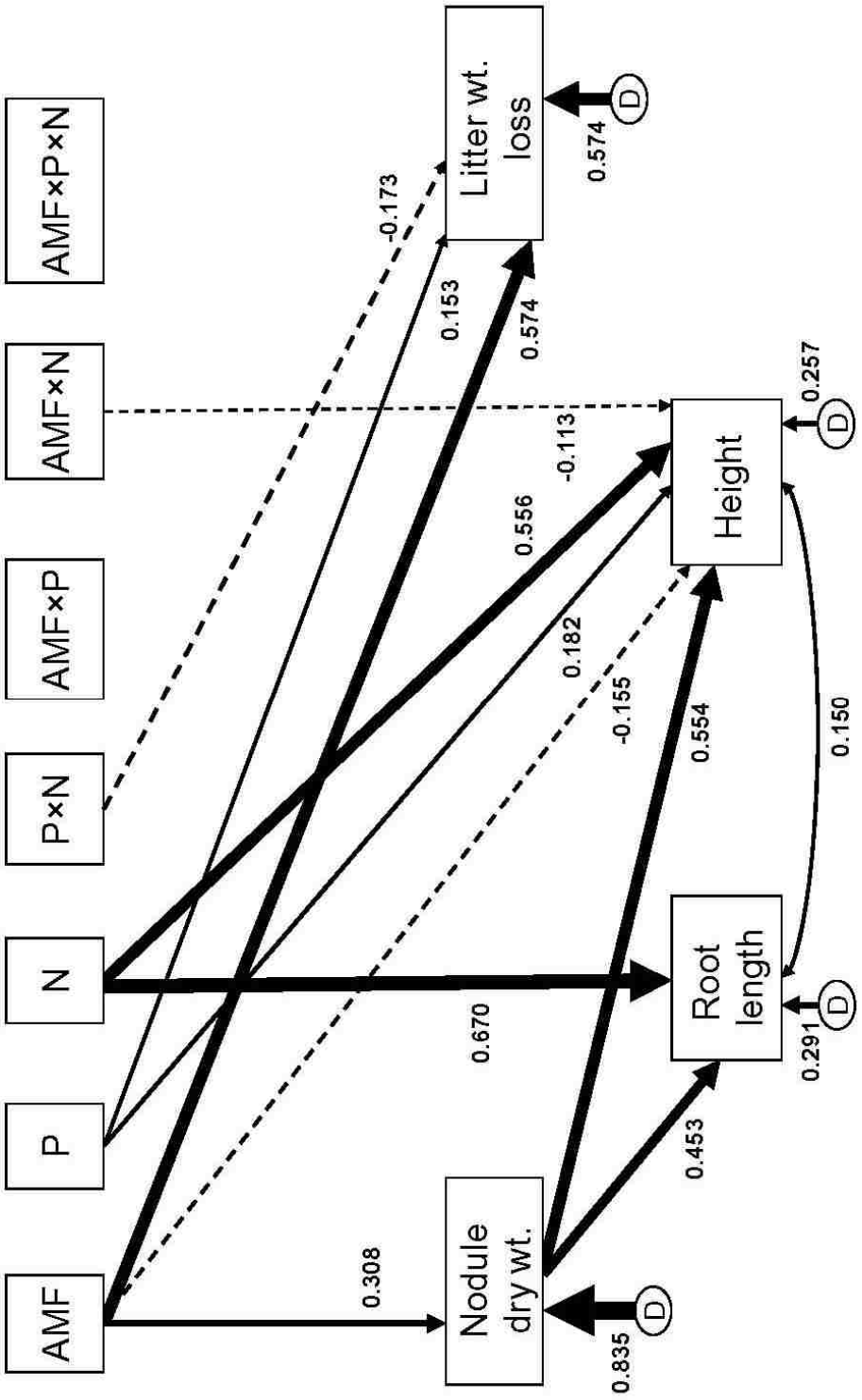

No NP
P
N
NP
(a)
Nodule dry weight (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(b)
Relative nodule wt (proportion)
0.08
0.06
0.04
0.02
0
(c)
Cluster roots (%)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

Figure 4.7. (Figure is shown on following page). Standardized parameter estimates for the path diagram depicted in Figure 4.1. Only path coefficients that are significantly different from zero ($Z \geq 1.96$ to be significant at $P < 0.05$) are shown. One-headed arrows denote the effect of an independent variable on a dependent variable. Two-headed arrows denote correlations between variables. Positive effects are shown by solid lines, and negative effects by dashed lines. Arrow widths are proportional to the magnitude of the path coefficients. For estimates of non-significant path coefficients, see direct effects in Table 4.13. For abbreviations, see Fig 4.1.


AMF
P
N
P×N
AMF×P
AMF×N
AMF×P×N
Nodule dry wt.
Root length
Height
Litter wt. loss
D
0.308
0.670
0.453
0.554
0.556
0.182
-0.155
-0.113
0.153
0.574
-0.173
0.574
0.257
0.150
0.291
0.835

Chapter 5

Conclusions

In this study, I examined the effects of AMF on the growth of *Morella cerifera* plants under conditions of litter accumulation. The first phase (Phase I) of my dissertation research involved determining what leaf traits affect the colonization of leaf litter by AMF. To test this, and to find out if the AMF litter colonization phenomenon occurs in the lowlands and sub-tropics, in Chapter 2 I examined whether or not AMF colonize different types of litter leaves, including those of *M. cerifera*, in a sub-tropical hardwood hammock. The second phase (Phase II) of my dissertation involved determining what root system adaptations are involved in the acquisition and recycling of mineral nutrients from litter by *M. cerifera*. In Chapters 3 and 4 I examined whether or not AMF are beneficial to *M. cerifera* under ecologically realistic conditions, by provisioning seedlings in a nutrient-poor substrate with simulated litter leaves. In Chapter 3 I compared benefits to *M. cerifera* from labile and recalcitrant leaves in the presence or absence of AMF. In Chapter 4 I compared benefits to *M. cerifera* in the presence of recalcitrant leaves and AMF, when soluble nitrogen and phosphorus were added.

In Chapter 2 (Phase I) I determined that in a sub-tropical hardwood hammock AMF colonize a wide variety of simulated litter leaves (*M. cerifera*, *Quercus virginiana*, *Rhapis excelsa*, *Averrhoa carambola*, and *Costus spicatus*). Because the colonization of litter leaves by AMF was only recently reported in three species (*Morella parvifolia*,

Morella pubescens, and *Paepalanthus* sp.) in two montane tropical ecosystems in Colombia (see Rivera & Guerrero, 1998; Aristizábal *et al.*, 2004) not much is known about this phenomenon, including whether or not it occurrs in other ecosystems. The results of Chapter 2, and my finding of colonization in montane tropical ecosystems in Hawaii and Costa Rica and in epyphitic bromeliads in a Costa Rican lowland rainforest, all suggest that AMF may colonize a wide variety of litter leaves in tropical and sub-tropical ecosystems.

The results of Chapter 2 also show how leaf litter traits influence the colonization of litter leaves by AMF. Although AMF colonized all leaf species extensively, AMF spread more rapidly within soft, labile leaves with high initial weight loss than within tough, recalcitrant leaves. My results suggest that soft, labile leaves attain asymptotic colonization after 70 days, and recalcitrant leaves attain asymptotic colonization after 200-335 days. Compared to values observed for root colonization, AMF colonization of tough, recalcitrant leaves increases slowly as indicated by their long times to inflection (*i*) and low *s* coefficients. For roots, *i* values range from 2.4 to 63.2 d (McGonigle, 2001), whereas my similarly-calculated values for dead leaves ranged from 111.3 to156.3 d. Curve abruptness values (*s*) are lower for tough leaves (ranging from 0.015 to 0.024) than for roots (ranging from 0.067 to 0.765; McGonigle, 2001). The rank ordering of species by decomposition rate corresponds well to the rank order of AMF colonization, suggesting that decomposition rate is a good predictor of the rate of spread of AMF within leaves. These results suggest that the same traits that influence leaf decomposition also influence how quickly AMF spread within leaves.

In an early successional montane ecosystem in Costa Rica I found that *M. cerifera* litter leaves had higher levels of colonization by AMF than did other, more labile, litter leaves (Aristizábal, 2002). Even though one of the possible explanations for this observation is that the litter traits of *M. cerifera*, particularly its recalcitrance, positively influence AMF leaf colonization, the results of Chapter 2 show that this is not the case because other species also attained similarly high levels of colonization. Other factors, such as the formation of root mats by *M. cerifera*, may explain the high levels of AMF leaf litter colonization of this species.

The results of Chapter 2 support the hypothesis of Aristizábal *et al.* (2004) that colonization of recalcitrant leaves begins in leaf veins. I found significantly less AMF colonization in leaf pieces with obstructed veins than in those with non-obstructed veins. This result provides a mechanism to explain how AMF with a low production of hydrolytic enzymes (Varma, 1999) penetrate and proliferate within tough recalcitrant leaves.

Combined, all the results of Chapter 2 suggest that leaf traits influence how quickly AMF spread within leaves. If AMF are found to substantially enhance acquisition of mineral nutrients from the leaves they colonize, these results will help us to understand how different litter traits may influence within-plant nutrient cycling, and how the litter characteristics of functional groups such as colonizer species can exert indirect controls over ecosystem-level nutrient cycling. Additionally, information about the factors that positively influence the colonization of decomposing leaves by AMF is of potential use in agroecology, where the achievement of synchrony (i.e. the matching through time, of mineral nutrient availability and plant uptake; Myers *et al*. 1997) is a

major aim. Although research on how to achieve synchrony through the management of litter quality factors such as lignin and polyphenols (Myers *et al.*, 1997) is extensive, the potential role AMF might play in synchronizing nutrient release and uptake has been little recognized or exploited.

Even though in Chapter 2, I found that AMF spread more rapidly within labile leaf litter than within recalcitrant leaf litter, the results of Chapter 3 (Phase II) allowed me to determine that AMF might only enhance host acquisition of mineral nutrients from recalcitrant leaf litter. I found that in a substrate devoid of mineral nutrients, AMF negatively affected the growth of *M. cerifera* except when a source of recalcitrant litter was present, when they were neutral. The results of a structural equation model suggested that the effects of AMF are indirect, and are mediated by the positive effect AMF have on *Frankia*-nodule dry weight in the presence of recalcitrant litter. This result can be explained by the enhancement of litter decomposition by AMF, which likely elevated nutrient availability in the pots. In contrast, when no recalcitrant litter was present in the substrate, AMF negatively affected nodulation. These results are in accordance with what has been hypothesized by Orfanoudakis *et al.* (2004) and Bethlenfalvay *et al.* (1985), that AMF and *Frankia* sp. may compete in plant roots, and that the presence of one symbiont can reduce or preclude the presence of the other in a plant's root system. A nutrient poor soil might exacerbate such inter-symbiont competition. The results of the ^{13}C analysis (Chapter 3), that AMF increased plant ^{13}C discrimination ($\Delta^{13}C$) in the no-litter and labile litter treatments, suggested that those AMF plants were biochemically suppressed.

The results of Chapter 3 also show that AMF had no effect on acquisition of N or ^{15}N in any of the treatments, suggesting that AMF did not mediate acquisition of N from leaves, as found by Hodge *et al.* (2001). In my experiment, nodule dry weight was a good predictor of foliar N content in the no-litter and recalcitrant litter treatments. This suggests that plant growth was limited by nitrogen, and that *Frankia* sp. provided most of this mineral nutrient to *M. cerifera*. A relationship between nodulation and foliar N content, however, was not observed in the labile litter treatment. Additionally, the plants in the labile litter treatment had a different ^{15}N signature than those in the no-litter and recalcitrant litter treatments, suggesting that these plants acquired N from labile litter. That the AMF×labile litter interaction was not significant suggests that the plants could access mineral nutrients from labile leaves with or without mycorrhizas, and supports the idea that AMF do not enhance the capture of mineral nutrients in substrates that are not mineralization limited (Hawkins *et al*., 2000).

Because cluster roots are a more costly P-acquiring adaptation than are AMF (Pate & Watt, 2002), I expected to find that *M. cerifera* plants would form cluster roots only in the absence of AMF at very low P levels. Although not statistically significant, I found that when no litter was added, cluster root formation declined in the presence of AMF, supporting that contention. Contrary to what I expected, however, in the presence of both labile and recalcitrant litter and AMF, cluster root formation was not affected by AMF. Because decomposing litter was likely the primary source of mineral nutrients in the extremely deficient substrate, both adaptations may have aided in mineral nutrient acquisition complementarily.

The results of Chapter 4 (Phase II) allowed me to determine under which conditions of soil N and P availability AMF, *Frankia*, and cluster roots predominate, and to assess the benefits they provide to *M. cerifera* under those conditions. I found that AMF and nitrogen enhanced cluster root formation by *M. cerifera* plants, and P fertilization decreased it. I also found that in the presence of simulated litter, addition of N shifted the effect of AMF from positive to negative. In the P or no N or P treatments, AMF enhanced *M. cerifera* growth, both aboveground and belowground. In contrast, AMF negatively affected *M. cerifera* aboveground growth when N was added. Similarly to Chapter 3, the results of a path analysis suggested that the positive effect of AMF on *M. cerifera* growth was indirect, and was mediated by the positive effect AMF have on nodule dry weight in the presence of litter. The negative effects of AMF when N was added can be explained by the cost of maintaining two symbionts, AMF and *Frankia* sp., that were not providing any benefit in N acquisition when fertilizer N was available.

Similarly to what has been found in other studies, in this study I found that both P and AMF stimulated nodulation (Fraga-Beddiar & Le Tacon, 1990; Jha *et al.*, 1993). Because P typically enhances nodulation, I hypothesize that AMF enhanced nodulation by enhancing acquisition of P from litter. That AMF highly enhanced litter decomposition in this study supports my hypothesis. My results suggest that AMF aid in the acquisition of P from litter, something that has not been studied extensively, but could be of tremendous ecological significance (Read & Perez-Moreno, 2003). In addition to the probability of aiding in P acquisition from litter, my results suggest that in the P treatment, AMF helped acquire P that cluster roots and non-colonized roots could not acquire.

The results of Chapters 3 and 4 show negative, neutral, and positive effects of AMF on *M. cerifera*. Bronstein (1994) predicted that the outcome of facultative mutualisms and of mutualisms in which a third species is intimately involved are highly variable. The results of Chapters 3 and 4 clearly illustrate this. That in Chapter 3 AMF were neutral to *M. cerifera* plants in the presence of especially recalcitrant *M. cerifera* litter, but in Chapter 4 they positively affected plant growth in the presence of less recalcitrant *R. excelsa* litter, may be explained by the results of Chapter 2. The decomposition and AMF colonization of especially recalcitrant leaves, such as those of *M. cerifera*, takes a long time. Delay in decomposition and AMF colonization of litter of *M. cerifera* may explain delayed beneficial effects.

Even though most evidence suggests that AMF themselves do not directly break down litter, the results of Chapters 3 and 4 show that AMF have a large stimulatory effect on litter decomposition. These results are similar to the findings of Hodge *et al.* (2001). There may be several explanations for how AMF might accelerate decomposition, including that the AMF inoculum had a higher concentration of saprophytic microorganisms, that the community of decomposer microorganisms associated with the AMF treatments was different from those in the no-AMF treatment, and that AMF may have increased host root exudation, thereby stimulating the decomposer community. Even though the effects of AMF on litter decomposition were likely indirect, AMF did enhance host growth in my experiments, most likely by enhancing uptake of mineral nutrients from litter in the extremely mineral-poor sand substrate that I used.

Overall, the results of my research show that *M. cerifera* does obtain benefits from its association with AMF. Thus it does not fail to form a functional symbiosis with

AMF as has been suggested (Poole & Sylvia, 1990). My results also underscore that experiments with highly facultative plants that only explore one set of environmental conditions do not reveal the multiplicity of responses of facultatively mycotrophic species. In the field, *M. cerifera* typically accumulates litter beneath its canopy, and its fine roots, associated AMF hyphae, and cluster roots proliferate amidst that litter. AMF profusely colonize *M. cerifera* litter leaves, and my work illustrates that AMF may aid in the acquisition and efficient recycling of nutrients from those dead leaves. The results of my experiments show that in a nutrient-poor substrate, AMF enhance the growth of *M. cerifera* plants only in the presence of recalcitrant litter leaves. My results also suggest that AMF enhance host growth indirectly by influencing *Frankia* nodule weight. My results suggest that cluster roots and AMF are not alternative P-acquisition root adaptations for this species. That AMF enhance the growth of *M. cerifera* plants in the presence of litter suggests that these fungi have a more important role recycling nutrients from organic matter than previously recognized.

References

Adams MA, Bell TL, Pate JS. 2002. Phosphorus sources and availability modify growth and distribution of root clusters and nodules of native Australian legumes. *Plant, Cell and Environment* **25**: 837-850.

Albertsen A, Ravnskov S, Green H, Jensen D, Larsen J. 2006. Interactions between the external mycelium of the mycorrhizal fungus *Glomus intraradices* and other soil microorganisms as affected by organic matter. *Soil Biology & Biochemistry* **38**: 1008-1014.

Aristizábal C. 2002. Comparison of decomposing leaf colonization by arbuscular mycorrhizal fungi in *Myrica cerifera* and other colonizer species. In: *Organization for tropical studies course book 02-3*. Durham, NC, USA: Organization for Tropical Studies, 45-50.

Aristizábal C, Morris M. 2002. Arbuscular mycorrhizal colonization in *Guzmania monostachia* in a lowland tropical rainforest. In: *Organization for tropical studies course book 02-3*. Durham, NC, USA: Organization for Tropical Studies, 213-219.

Aristizábal C, Rivera EL, Janos DP. 2004. Arbuscular mycorrhizal fungi colonize decomposing leaves of *Myrica parvifolia*, *M. pubescens* and *Paepalanthus* sp. *Mycorrhiza* **14**: 221-228.

Auge RM. 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* **11**: 3-42.

Azaizeh HA, Marschner H, Romheld V, Wittenmayer L. 1995. Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza* **5**: 321-327.

Azcon-Aguilar C, Bago B, Barea JM. 1999. Saprophytic growth of arbuscular mycorrhizal fungi. In: Varma A, Hock B, eds. *Mycorrhiza: Structure, function, molecular biology and biotechnology, 2nd edition.* Berlin, Heidelberg, Germany: Springer-Verlag, 391-408.

Bardgett RD, Streeter TC, Bol R. 2003. Soil microbes compete effectively with plants for organic-nitrogen inputs to temperate grasslands. *Ecology* **84**: 1277-1287.

Berliner R, Torrey JG. 1989. On tripartite *Frankia*-mycorrhizal associations in the Myricaceae. *Canadian Journal of Botany* **67**: 1708-1712.

Bethlenfalvay GJ, Brown MS, Stafford AE. 1985. Glycine-*Glomus-Rhizobium* symbiosis. ii. Antagonistic effects between mycorrhizal colonisation and nodulation. *Plant Physiology* **79**: 1054-1058.

Blanke V, Renker C, Wagner M, Fullner K, Held M, Kuhn AJ, Buscot F. 2005. Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. *New Phytologist* **166**: 981-992.

Bolan N. 1991. A critical-review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* **134**: 189-207.

Bowen GD. 1980. Mycorrhizal roles in tropical plants and ecosystems. In: Mikola P, ed. *Tropical mycorrhiza research*. New York, USA: Oxford University Press, 165-190.

Bronstein JL. 1994. Conditional outcomes in mutualistic interactions. *TREE* **9**: 214-217.

Brundrett MC, Abbott LK. 1991. Roots of Jarrah Forest plants. I. Mycorrhizal associations of shrubs and herbaceous plants. *Australian Journal of Botany* **39**: 445-457.

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N. 1996. *Working with mycorrhizas in forestry and agriculture*. Canberra, Australia: Australian Centre for International Agricultural Research.

Brundrett MC. 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist* **154**: 275-304.

Buwalda JG, Ross GJS, Stribley DP, Tinker PB. 1982. The development of endomycorrhizal root systems. iii. The mathematical representation of the spread of vesicular-arbuscular mycorrhizal infection in root systems. *New Phytologist* **91**: 669-682.

Chalk PM, Souza RD, Urquiaga S, Alves BJR, Boddey RM. 2006. The role of arbuscular mycorrhiza in legume symbiotic performance. *Soil Biology & Biochemistry* **38**: 2944-2951.

Cliquet JB, Murray PJ, Boucaud J. 1997. Effect of the arbuscular mycorrhizal fungus *Glomus fasciculatum* on the uptake of amino nitrogen by *Lolium perenne*. *New Phytologist* **137**: 345-349.

Craine JM, Morrow C, Fierer N. 2007. Microbial nitrogen limitation increases decomposition. *Ecology* **88**: 2105-2113.

Dighton J. 1991. Acquisition of nutrients from organic resources by mycorrhizal autotrophic plants. *Experientia* **47**: 362-369.

Farquhar GD, O'leary MH, Berry JA. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9**: 121-137.

Fisher JB, Jayachandran K. 2005. Presence of arbuscular mycorrhizal fungi in South Florida native plants. *Mycorrhiza* **15**: 580-588.

Fitter AH. 1991. Costs and benefits of mycorrhizas: Implications for functioning under natural conditions. *Experientia* **47**: 350-355.

Fraga-Beddiar A, Le Tacon F. 1990. Interactions between VA mycorrhizal fungus and *Frankia* associated with Alder (*Alnus glutinosa* (L.) Gaetn.). *Symbiosis* **9**: 247-258.

Gardner IC. 1986. Mycorrhizae of actinorhizal plants. *MIRCEN Journal* **2**: 147-160.

Giovannetti M, Sbrana C, Logi C. 1994. Early processes involved in host recognition by arbuscular mycorrhizal fungi. *New Phytologist* **127**: 703-709.

Grace JB. 2006. *Structural equation modeling and natural systems*. New York, USA: Cambridge University Press.

Hawkins HJ, Johansen A, George E. 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil* **226**: 275-285.

Hetrick BAD. 1991. Mycorrhizas and root architecture. *Experientia* **47**: 355-362.

Hewitt EJ. 1952. *Sand and water culture methods used in the study of plant nutrition*. Farnham Royal, Bucks, England: Commonwealth Agricultural Bureaux.

Hodge A, Campbell CD, Fitter AH. 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413**: 297-299.

Hodge A. 2003. Plant nitrogen capture from organic matter as affected by spatial dispersion, interspecific competition and mycorrhizal colonization. *New Phytologist* **157**: 303-314.

Hurd TM, Schwintzer CR. 1997. Formation of cluster roots and mycorrhizal status of *Comptonia peregrina* and *Myrica pensylvanica* in Maine, USA. *Physiologia Plantarum* **99**: 680-689.

Imhof S. 2001. Subterranean structures and mycotrophy of the achlorophyllous *Dictyostega orobanchoides* (Burmanniaceae). *Revista de Biología Tropical* **49**: 239-247.

Janos DP. 1980a. Vesicular-arbuscular mycorrhizae affect lowland tropical rain forest plant growth. *Ecology* **61**: 151-162.

Janos D. 1980b. Mycorrhizae influence tropical succession. *Biotropica* **12**: 56-64.

Janos DP. 1983. Tropical mycorrhizas, nutrient cycles and plant growth. In: Sutton S, Whitmore T, Chadwick A, eds. *Tropical rain forest: Ecology and management*. Oxford, England, UK: Blackwell Scientific Publications, 327-345.

Janos DP. 1985. Mycorrhizal fungi: Agents or symptoms of tropical community succession? In: Molina R, ed. *Proceedings of the 6th NACOM*. Corvallis, OR, USA: Oregon State University, 98-106.

Janos D. 1987. VA mycorrhizas in humid tropical ecosystems. In: Safir G, ed. *Ecophysiology of va mycorrhizal plants*. FL, USA: CRC Press, 107-134.

Janos DP. 2007. Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. *Mycorrhiza* **17**: 75-91.

Jha DK, Sharma GD, Mishra RR. 1993. Mineral nutrition in the tripartite interaction between *Frankia*, *Glomus* and *Alnus* at different soil phosphorus regimes. *New Phytologist* **123**: 307-311.

Johansen A, Jensen ES. 1996. Transfer of N and P from intact or decomposing roots of pea to barley interconnected by an arbuscular mycorrhizal fungus. *Soil Biology & Biochemistry* **28**: 73-81.

Johnson NC. 1993. Can fertilization of soil select less mutualistic mycorrhizae? *Ecological Applications* **3**: 749-757.

Johnson NC, Graham JH, Smith FA. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* **135**: 575-586.

Jones MD, Smith SE. 2004. Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? *Canadian Journal of Botany* **82**: 1089-1109.

Jordan CF, Todd RL, Escalante G. 1979. Nitrogen conservation in a tropical rain forest. *Oecologia* **39**: 123-128.

Killham K. 1994. *Soil ecology*. Great Britain: Cambridge University Press.

Kline RB. 2005. *Principles and practice of structural equation modeling. Second edition.* New York, USA: The Guildford Press.

Kohls SJ, Baker DD. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorrhizal plants. *Plant and Soil* **118**: 171-179.

Koide RT, Kabir Z. 2000. Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. *New Phytologist* **148**: 511-517.

Koske R. 1984. Spores of vesicular-arbuscular mycorrhizal fungi inside spores of vesicular-arbuscular mycorrhizal fungi. *Mycologia* **76**: 853-862.

Koske RE, Gemma JN, Flynn T. 1992. Mycorrhizae in Hawaiian angiosperms - a survey with implications for the origin of the native flora. *American Journal of Botany* **79**: 853-862.

Kottke I, Beck A, Oberwinkler F, Homeier J, Neill D. 2004. Arbuscular endomycorrhizas are dominant in the organic soil of a neotropical montane cloud forest. *Journal of Tropical Ecology* **20**: 125-129.

Lamont BB. 1993. Why are hairy root clusters so abundant in the most nutrient-impoverished soils of Australia? *Plant and Soil* **156**: 269-272.

Lamont BB. 2003. Structure, ecology and physiology of root clusters - a review. *Plant and Soil* **248**: 1-19.

Louis I, Racette S, Torrey JG. 1990. Occurrence of cluster roots on *Myrica cerifera* L. (Myricaceae) in water culture in relation to phosphorus-nutrition. *New Phytologist* **115**: 311-317.

Malloch DW, Pirozynski KA, Raven PH. 1980. Ecological and evolutionary significance of mycorrhizal symbioses in vascular plants (a review). *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **77**: 2113-2118.

Marschner H. 2002. *Mineral nutrition of higher plants. Second edition.* San Diego, CA, USA: Elsevier Academic Press.

McGonigle TP. 2001. On the use of non-linear regression with the logistic equation for changes with time of percentage root length colonized by arbuscular mycorrhizal fungi. *Mycorrhiza* **10**: 249-254.

Mitchell RJ. 2001. Path analysis: Pollination. In: Scheiner SM, Gurevitch J, eds. *Design and analysis of ecological experiments*. New York, USA: Oxford University Press, Inc., 217-234.

Myers RJK, Noordwijk Mv, Vityakon P. 1997. Synchrony of nutrient release and plant demand: Plant litter quality, soil environment and farmer management options. In: Cadisch G, Giller K, eds. *Driven by nature: Plant litter quality and decomposition*. Wallingford, UK: CAB International, 215-238.

Nakano A, Takahashi K, Kimura M. 1999. The carbon origin of arbuscular mycorrhizal fungi estimated from delta C-13 values of individual spores. *Mycorrhiza* **9**: 41-47.

Nakano A, Takahashi K, Kimura M. 2001. Effect of host shoot clipping on carbon and nitrogen sources for arbuscular mycorrhizal fungi. *Mycorrhiza* **10**: 287-293.

Nasholm T, Huss-Danell K, Hogberg P. 2001. Uptake of glycine by field grown wheat. *New Phytologist* **150**: 59-63.

Orfanoudakis MZ, Hooker JE, Wheeler CT. 2004. Early interactions between arbuscular mycorrhizal fungi and *Frankia* during colonisation and root nodulation of *Alnus glutinosa*. *Symbiosis* **36**: 69-82.

Pate J, Watt M. 2002. Roots of *Banksia* spp. (Proteaceae) with special reference to functioning of their specialized proteoid root clusters. In: Waisel Y, Eshel A, Kafkafi U, eds. *Plant roots: The hidden half*. New York, USA: Marcel Dekker, Inc.

Pattinson GS, McGee PA. 1997. High densities of arbuscular mycorrhizal fungi maintained during long fallows in soils used to grow cotton except when soil is wetted periodically. *New Phytologist* **136**: 571-580.

Phillips J, Hayman D. 1970. Improved procedure for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**: 158-161.

Poole B, Sylvia D. 1990. Companion plants affect colonization of *Myrica cerifera* by vesicular-arbuscular mycorrhizal fungi. *Canadian Journal of Botany* **68**: 2703-2707.

Rabatin S. 1980. The occurrence of the vesicular-arbuscular-mycorrhizal fungus *Glomus tenuis* with moss. *Mycologia* **72**: 191-195.

Rabatin S, Rhodes L. 1982. *Acaulospora bireticulata* inside oribatid mites. *Mycologia* **74**: 859-861.

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytologist* **157**: 475-492.

Reddell P, Yun Y, Shipton W. 1997. Cluster roots and mycorrhizae in *Casuarina cunninghamiana*: Their occurrence and formation in relation to phosphorus supply. *Australian Journal of Botany* **45**: 41-51.

Richards PW. 1996. *The tropical rain forest*. Great Britain, UK: Cambridge University Press.

Rivera EL, Guerrero E. 1998. Ciclaje directo de nutrientes a través de endomicorriza. Un complemento del proceso de mineralización? In: *Congres Mondial de Science du Sol, August 20-26, 1998*. Montpellier, France.

Rose S, Paranka J. 1987. The location of roots and mycorrhizae in tropical forest litter. In: Sylvia D, Hung L, Graham J, eds. *Mycorrhizae in the next decade: practical applications and research priorities*. Gainesville, Florida, USA: Institute of Food and Agricultural Sciences, 165.

Schroeder MS, Janos DP. 2004. Phosphorus and intraspecific density alter plant responses to arbuscular mycorrhizas. *Plant and Soil* **264**: 335-348.

Schussler A, Schwarzott D, Walker C. 2001. A new fungal phylum, the Glomeromycota: Phylogeny and evolution. *Mycological Research* **105**: 1413-1421.

Sempavalan J, Wheeler CT, Hooker JE. 1995. Lack of competition between *Frankia* and *Glomus* for infection and colonization of roots of *Casuarina equisetifolia* (l.). *New Phytologist* **130**: 429-436.

Skene K. 1998. Cluster roots: Some ecological considerations. *Journal of Ecology* **86**: 1060- 1064.

Smith S, Read D. 1997. *Mycorrhizal symbiosis*. San Diego, California, USA: Academic Press, Inc.

Snyder JR, Herndon A, Robertson Jr. WB. 1990. South Florida Rockland. In: Myers RL, Ewel JJ, eds. *Ecosystems of Florida*. Orlando, Florida, USA: University of Central Florida Press, 230-277.

St. John T, Coleman D, Reid C. 1983. Association of vesicular-arbuscular mycorrhizal hyphae with soil organic particles. *Ecology* **64**: 957-959.

Stark N, Spratt M. 1977. Root biomass and nutrient storage in rain forest oxisols near San Carlos de Rio Negro. *Tropical Ecology* **18**: 1-9.

Swift MJ, Heal OW, Anderson JM. 1979. *Decomposition in terrestrial ecosystems*. Berkeley, California, USA: University of California Press.

Taber R. 1982a. Ocurrence of *Glomus* spores in weed seeds in soil. *Mycologia* **74**: 515-520.

Taber R. 1982b. *Gigaspora* spores and associated hyperparasites in weed seeds in soil. *Mycologia* **74**: 1026-1031.

Taber R, Trappe J. 1982. Vesicular-arbuscular mycorrhiza in rhizomes, scale-like leaves, roots, and xylem of ginger. *Mycologia* **74**: 156-161.

Taiz L, Zeiger E. 2002. *Plant physiology*. Sunderland, MA, U.S.A.: Sinauer Associates, Inc.

Tian C, Xingyuan H, Zhong Y, Chen J. 2002. Effects of VA mycorrhizae and *Frankia* dual inoculation on growth and nitrogen fixation of *Hippophae tibetana*. *Forest Ecology and Management* **170**: 307-312.

Tibbett M, Sanders FE. 2002. Ectomycorrhizal symbiosis can enhance plant nutrition through improved access to discrete organic nutrient patches of high resource quality. *Annals of Botany* **89**: 783-789.

Trinick M. 1977. Vesicular-arbuscular infection and soil phosphorus utilization in *Lupinus* spp. *New Phytologist* **78**: 297-304.

Varma A. 1999. Hydrolytic enzymes from arbuscular mycorrhizae: The current status. In: Varma A, Hock B, eds. *Mycorrhiza: Structure, function, molecular biology and biotechnology. 2nd edition.* Berlin Heidelberg: Springer-Verlag, 373-389.

Walker NA, Smith SE. 1984. The quantitative study of mycorrhizal infection. ii. The relation of rate of infection and speed of fungal growth to propagule density, the mean length of the infection unit and the limiting value of the fraction of the root infected. *New Phytologist* **96**: 55-69.

Went F, Stark N. 1968. The biological and mechanical role of soil fungi. *Proceedings of the National Academy of Sciences* **60**: 497-504.

Wieder RK, Lang GE. 1982. A critique of the analytical methods used in examining decomposition data obtained from litter bags. *Ecology* **63**: 1636-1642.

Wright SF. 2000. A fluorescent antibody assay for hyphae and glomalin from arbuscular mycorrhizal fungi. *Plant and Soil* **226**: 171-177.

Yamanaka T, Akama A, Li C, Okabe H. 2005. Growth, nitrogen fixation and mineral acquisition of *Alnus sieboldiana* after inoculation of *Frankia* together with *Gigaspora margarita* and *Pseudomonas putida*. *J For Res* **10**: 21-26.