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# Pinus elliottii var. densa Seedling Performance Reflects Ectomycorrhizas, Soil Nutrient Availability and Root Competition

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UNIVERSITY OF MIAMI

*PINUS ELLIOTTII* VAR. *DENSA* SEEDLING PERFORMANCE REFLECTS  
ECTOMYCORRHIZAS, SOIL NUTRIENT AVAILABILITY AND ROOT  
COMPETITION

By

Tania Wyss Lozano Hoyos

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

December 2010

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*Pinus elliottii* var. *densa* Seedling Performance  
Reflects Ectomycorrhizas, Soil Nutrient  
Availability and Root Competition

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Ectomycorrhizas generally improve seedling mineral nutrition and growth, so I hypothesized that decline of the Florida native pine variety *Pinus elliottii* var. *densa* Little & Dorman is related to deficiency of appropriate ectomycorrhizal (ECM) fungi in the pine's native flatwoods. At Archbold Biological Station I examined how quickly ECM fungi colonize *P. elliottii* var. *densa* seedlings and I compared the effect of local absence versus presence of adult pines on ECM colonization and pine seedling performance. Under controlled greenhouse conditions, I investigated how a wide range of ECM colonization and spread of extraradical mycelium throughout a large volume of relatively infertile, flatwoods soil enhance the mineral nutrition and growth of pine seedlings.

In a field bioassay, I transplanted two-month-old pine seedlings to three flatwoods sites with low (4 pines/400 m<sup>2</sup>), medium (9 pines/400 m<sup>2</sup>), and high (19 pines/400 m<sup>2</sup>) adult pine densities. I subsequently excavated seedlings every two weeks for four-and-a-half months and determined their ECM colonization, response to shade, and response to surrounding grass density. Across all sites, pine seedlings in high shade had a higher mean chlorophyll concentration and lower stem dry weight than in full sun. Competition

with grass reduced seedling survival and stem dry weight. Initial colonization was rapid and not different among sites, with 5.4 % of roots colonized 15 days after transplant. Pine seedlings had midpoint means of 29.5 %, 18.1 % and 21.3 % ECM root tips in low, medium and high adult pine density sites, respectively, suggesting that pine seedlings establishing in flatwoods encounter sufficient ECM fungi to support their growth, regardless of adult pine density.

In a field experiment, I determined in the presence versus absence of adult pines if pine seedlings had higher ECM colonization and consequent improved survival, mineral nutrition, and growth. Within and beyond pine stands, I transplanted seedlings into intact or drilled, hyphae in-growth pipes buried in the ground. I placed autoclaved or fresh ECM root inoculum in two sets of intact pipes, and autoclaved inoculum in drilled pipes into which mycorrhizal hyphae could extend from the surrounding vegetation. Seven-and-a-half months after transplant, ECM hyphae had penetrated the drilled pipes and colonized pine seedlings, but roots from the surrounding vegetation also penetrated pipes. Extraneous roots reduced the survival of seedlings both within and beyond pine stands, but extraneous roots reduced seedling growth only beyond pine stands. Because percentage ECM root tips was higher in the presence (53 %) than in the absence (38.8%) of adult pines, pine stands might benefit the competitive ability of seedlings by increased ECM colonization and possibly by common mycorrhizal networks connecting seedlings to adults.

Because beneficial effects of ECM in the field were small, I also examined ECM effects on pine seedlings in a greenhouse experiment. I manipulated ECM fungus colonization and the volume of flatwoods soil to which extraradical mycelium had access. In a small volume of soil (220 mL), fresh ECM root inoculum promoted the mycorrhizal

colonization of seedlings versus those receiving autoclaved roots, but seedling growth and uptake of Mg, Ca, and Zn was lower with fresh than with autoclaved root inoculum. Growth and mineral nutrient uptake likely was enhanced by a pulse of nutrients from autoclaved roots, but for inoculated plants may have been reduced because of nutrient retention by saprotrophic microorganisms degrading fresh ECM roots and because of mineral nutrient retention by ECM fungi. Ectomycorrhizal seedlings with extraradical mycelium access to a large soil volume had higher mean chlorophyll concentration than those in a small soil volume. Weekly disturbance of the extraradical mycelium, however, reduced foliar contents of Mn, K, P, N, and Zn by one-third to one-half, and reduced needle dry weight of seedlings by one-third, demonstrating the importance of extraradical mycelium accessing a large volume of soil when it is nutrient-poor.

My research demonstrates that ECM fungi are widespread in flatwoods and rapidly colonize pine seedlings. ECM fungus inocula are greater in the presence than in the absence of adult pines, and ECM or seedlings' connections to a common mycorrhizal network improve seedlings' belowground competitive ability. ECM especially enhance seedling mineral nutrition and growth when undisturbed, extraradical mycelium extends throughout a large volume of soil. Populations of *Pinus elliotii* var. *densa* might best regenerate in flatwoods if seedlings recruit near adult pines and where there is little competition for light, water, and mineral nutrients.

## **Dedication**

To Oscar,

For his love, support and immense patience.

To Heinz, Ruth and Cindy,

For their constant encouragement and good cheer.

To Anne-Claude, Catalina and Lucero,

For being precious friends.



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# Chapter 1

## Introduction

Mycorrhizal fungi – mutualistic fungi that benefit plants by associating with plant roots – play a major role in seedling establishment, in interactions of plants with their environment, and in the persistence of plant populations. Examples of mycorrhizas benefiting plant survival (Richard et al. 2009), growth (Quoreshi et al. 2008), mineral nutrition (Dickie et al. 2002), water transfer (Plamboeck et al. 2007; Warren et al. 2008) and competitive ability (Booth and Hoeksema 2010) of seedlings are numerous (Smith and Read 2008). Once seedlings establish, mycorrhizas affect interactions among co-occurring individuals (van der Heijden et al. 2003; Schroeder-Moreno and Janos 2008), sometimes contributing to the diversity of plant communities (van der Heijden et al. 1998).

Many different species of mycorrhizal fungi associate with land plants, resulting in different types of associations depending on both the fungus and plant species involved (Smith and Read 2008). Arbuscular mycorrhizas are formed between fungi in the phylum Glomeromycota (Schussler et al. 2001) and the great majority of land plants (Wang and Qiu 2006). Plant species belonging to families such Fagaceae, Dipterocarpaceae and Pinaceae, however, associate with ectomycorrhizal (ECM) fungi (Wang and Qiu 2006). Many fewer plant species form ectomycorrhizas than arbuscular mycorrhizas, but ECM plant species, especially those belonging to the Pinaceae such as *Pseudotsuga* spp, *Picea* spp. and *Pinus* spp. not only have great commercial value, but are important canopy trees in many ecosystems of the northern hemisphere (Richardson and Rundel 1998).

*Pinus elliottii* var. *densa* Little & Dorman is a pine variety native to central and south Florida (Abrahamson and Hartnett 1990). It is a keystone species in native ecosystems such as flatwoods and pine rocklands (Snyder et al. 1990) because its flammable leaf litter favors fire return. As a member of the Pinaceae, this species forms ectomycorrhizas, but the effects of ECM fungi on seedling performance have not been determined. Ectomycorrhizas might be particularly important for the maintenance of *P. elliottii* var. *densa* in the nutrient-poor and seasonally water limited ecosystems of Florida. In particular, flatwoods occur on dry, sandy, nutrient poor soils, and are maintained by frequent fires (Monk 1968; Abrahamson 1984). Because of human activities, however, areas occupied by *P. elliottii* var. *densa* are greatly reduced today compared to pre-settlement times (Ross 1995; cited in Ford and Brooks 2003). To my knowledge, no formal report on pine population decline exists, but many observers anecdotally have reported that pine populations presently are in decline. The decline might be associated with poor seedling establishment. Thus, because of the important role of ectomycorrhizas in seedling performance, I studied the ECM colonization, survival and growth of *P. elliottii* var. *densa* seedlings in central Florida flatwoods, and in flatwoods soil in the greenhouse.

## **Background**

Here, I briefly review seedling recruitment, in particular that of conifers; I describe mycorrhizas and their effects on seedling establishment and performance; I describe my study species and system; and finally, I present my research objectives.

### *Seedling recruitment*

Seedling recruitment in plant communities depends both upon seed arrival and upon the availability of suitable sites for seedling survival and growth (Clark et al. 2007). The relative importance of these factors for seedling establishment can differ among plant species (e.g. Norghauer and Newbery 2010) or with seedling age (Paine and Harms 2009), and can influence the distribution of plant populations (e.g. Albrecht and McCarthy 2009; Duncan et al. 2009). Seed arrival is affected by limited production of seeds by adults because of abiotic or biotic factors (Goheen et al. 2010), limited seed dispersal (Golley et al. 1994), predation on seeds (Zwolak et al. 2010) or low persistence of seeds in the soil seed bank (Salazar Parra 2010). Once a seed germinates and exhausts its reserves, a seedling depends upon the suitability of the establishment site for its survival and growth (Clark et al. 2007). The suitability of a site is affected by many factors, including water availability (Iacona et al. 2010), soil properties, such as pH, organic matter content and nutrient availability (Jones and del Moral 2009; Breen and Richards 2008), light availability (Matlaga and Horvitz 2009), presence versus absence of understory plants and the strength of their competition (Maher et al. 2005; Hagenah et al. 2009), disturbances such as fire (Zwolak et al. 2010) or presence versus absence of mycorrhizal fungi (Collier and Bidartondo 2009; Thiet and Boerner 2007).

Both the availability of seeds and of suitable sites affects the recruitment of conifer seedlings. For example, the presence of large adults increased the density of recruiting *Pinus canariensis* Chr. Sm. ex DC. seedlings after fire (Otto et al. 2010). At low fire severity, abundant unburned litter reduced the recruitment of *P. canariensis* seedlings, while no seedlings recruited after high fire severity because the crowns of adult

pinus were too damaged to produce seeds. Consequently, greatest seedling recruitment was found at intermediate fire severity (Otto et al. 2010). In *Pinus ponderosa* Laws. forests, seedling recruitment was limited by seed source availability when adult trees died (Keyser et al. 2008). Recruitment of several conifer species (*Pinus sylvestris* L., *Larix decidua* Mill. and *Picea abies* (L.) Karst.) was negatively correlated with distance to seed source in a burned site in the Swiss alps and seedling recruitment was limited by dense litter or by moisture deficit at low altitudes (Moser et al. 2010). Reduction of the organic layer by wildfire in Canadian boreal forests favored the germination and survival of seedlings of *Picea mariana* (Mills.) BSP and *Pinus banksiana* Lamb. (Greene et al. 2007). In French grasslands, grasses competed with pine seedlings for light, soil water, and soil nitrogen, reducing performance of transplanted *Pinus sylvestris* seedlings compared to those transplanted to bare soil (Picon-Cochard et al. 2006). In pine plantations in southern Spain, recruitment of *Pinus pinaster* Ait. and *Pinus nigra* Arnold. seedlings was virtually absent at high adult pine densities (above 1500 pines/ha), but was greatest at moderate adult density, suggesting that adults facilitated seedling recruitment at moderate canopy cover (potentially by buffering extreme temperatures and reducing evapotranspiration), but that at high densities they competed against seedlings, in particular for water (Gomez-Aparicio et al. 2009). Competition with grass likewise reduced survival and growth of *Pinus radiata* D. Don. seedlings, but the response of pine seedlings to grass competition depended upon seedling genotype (Mason 2006). Clearly, the recruitment of conifer seedlings is not only influenced by seed arrival, but also is influenced by factors such as the depth of organic litter, water and nutrient availability, and competition or facilitation by co-occurring plants. Seedling establishment is

facilitated by co-occurring adults, particularly if those adults support appropriate mycorrhizal fungi at the germination site (e.g. Simard 2009; Harvey et al. 1980).

### *Mycorrhizas*

By associating with obligately biotrophic fungi very early in land plant evolution, plants formed mycorrhizas to overcome the difficulties of terrestrial life (Redecker et al. 2000). Mycorrhizas comprise two main parts: the interface for exchanges between the fungus and the plant in the roots, and the extraradical mycelium of the fungus extending into the soil. There are several types of mycorrhizas, including arbuscular mycorrhizas, ectomycorrhizas and ericoid mycorrhizas. In the arbuscular mycorrhizas of most land plants (including herbaceous and tree species) (Wang and Qiu 2006; Smith and Read 2008), fungus hyphae penetrate plant roots and form a highly branched structure between the cell wall and the cell membrane of root cells. This structure, called an arbuscule, is believed to be the primary interface for exchanges between the plant and the fungus (Smith and Read 2008). Ectomycorrhizal (ECM) fungi of the Basidiomycota or Ascomycota, on the other hand, predominantly colonize tree species (Wang and Qiu 2006). In most ECM associations, dense hyphae surround plant root tips, forming a mantle. Hyphae penetrate the root tips and also surround the first layers of cortical cells, forming the Hartig net, believed to be the site of exchange between the fungus and the plant (Smith and Read 2008). A third type of mycorrhizas of woody plants, ericoid mycorrhizas, formed by plant species belonging to the Ericaceae, is found mostly in highly organic, acidic soils with low decomposition rates (Leake et al. 1990). In all types of mycorrhizas, the fungus hyphae extend into the soil, forming an extensive extraradical mycelium (e.g. Colpaert et al. 1992). The extraradical mycelium enhances nutrient

acquisition beyond the roots' nutrient depletion zone, such as for phosphorus uptake by arbuscular mycorrhizas (e.g. Sanders and Tinker 1971) and both phosphorus and nitrogen uptake by ectomycorrhizas or ericoid mycorrhizas (e.g. Brandes et al. 1998).

Plant species usually are colonized by only one type of mycorrhizal fungi, for example either arbuscular mycorrhizal (AM) or ECM fungi, but some species can form both types of associations, such as members of the Salicaceae (van der Heijden 2001) and *Eucalyptus* spp. (Chilvers et al. 1987). In *Eucalyptus* spp., seedlings form arbuscular mycorrhizas and gradually switch to ectomycorrhizas as they age, with both types of mycorrhizas enhancing phosphorus nutrition (Jones et al. 1998). Plants in the Pinaceae and in the genus *Quercus* were thought to be exclusively colonized by ECM fungi, but Pinaceae and *Quercus* plants can have arbuscular mycorrhizas too (Smith et al. 1998; Horton et al. 1998; Dickie et al. 2001). In most cases, those plants are colonized by AM fungi when an AM host grows nearby (Smith et al. 1998). In *Quercus agrifolia* Nee. trees, the predominance of either AM or ECM colonization depended upon soil moisture (Querejeta et al. 2009). Dual colonization of *Q. agrifolia* resulted in the lowest growth and survival of seedlings, indicating that dual colonizations can represent a high carbon cost for seedlings (Egerton-Warburton and Allen 2001). AM colonization of *Quercus rubra* L. seedlings negatively affected their growth and N tissue concentration (Dickie et al. 2001). In contrast, for *Pseudotsuga menziesii* (Mirb.) Franco seedlings, phosphorus nutrition was improved in AM versus non-AM seedlings (Smith et al. 1998). In those associations, however, only hyphae or storage vesicles, but not arbuscules, were observed in roots. Overall, the significance of AM fungi in typically ECM plant species is unclear (Cázares and Trappe 1993).

Different types of mycorrhizas not only occur within the same root system, but they can also co-occur within ecosystems on different plant species (e.g. McHugh and Gehring 2006). In many temperate forests, canopy trees form ectomycorrhizas and understory herbaceous plants form arbuscular mycorrhizas. Co-occurring mycorrhizal types might compete for the same belowground resources, but they appear to occupy different microniches in the soil (Bruns 1995; Erland and Taylor 2002; Neville et al. 2002). Following the hypothesis of Read (1991), ECM predominate in organic-matter-rich horizons because of their ability to utilize organic molecules (Abuzinadah and Read 1986) and because of their proteolytic activity (Zhu et al. 1990). On the other hand, AM fungi predominate in horizons with mainly inorganic P availability. Thus, by partitioning soil resources, the two types of mycorrhizas coexist in a single ecosystem (Read and Perez-Moreno 2003). The presence and species composition of mycorrhizal fungi in ecosystems has been demonstrated to influence plant community composition in a number of ways (Francis and Read 1995; van der Heijden et al. 2003), including modifying competitive interactions between mycorrhizal and non-mycorrhizal plants (e.g. Janos 1980; Francis and Read 1995) and among plants with different mycorrhizal types (e.g. McHugh and Gehring 2006; Pedersen et al. 1999), by affecting the interactions between plants and other symbiotic organisms (Aristizábal 2008), and by affecting nutrient cycling (Aerts 2002). Feedback between plants, fungi, biotic and abiotic conditions (Hoeksema et al. 2010; Pivato et al. 2007; Grime et al. 1987; Rillig 2004) influence seedling establishment, and thus plant population regeneration and distribution (e.g. Collier and Bidartondo 2009; Janos 1996).

The importance of ECM fungi for uptake and transfer to host plants of poorly mobile nutrients such as phosphorus (P), potassium (K), zinc (Zn) and boron (B) has been



demonstrated in many greenhouse studies (e.g. Table 2.1. in Simard et al. 2002; Lehto et al. 2004), as has ECM acquisition and transfer of nitrogen (N) from inorganic and organic sources (Turnbull et al. 1996). ECM fungi even can improve host plant P supply by weathering minerals such as apatite (Wallander et al. 1997). ECM fungi not only enhance the nutrient status of seedlings, but also enhance their water status, as observed in microcosms in which a water-soluble fluorescent dye moved from a compartment containing mycorrhizal *Arctostaphylos viscida* Parry to root tips of *Pseudotsuga menziesii* and *Pinus lambertiana* Doug. via a mycorrhizal fungus (Plamboeck et al. 2007). Deuterium-enriched water was transferred from a compartment in a microcosm to water-stressed *Quercus agrifolia* Nee. seedlings via ectomycorrhizal connections to other oak seedlings that had access to the deuterated-water compartment (Egerton-Warburton et al. 2007). Hydraulic lift, the process of water redistribution by plant roots from a wet to a dry part of the soil, not only favors water-stressed ECM seedlings, but also the persistence of ECM hyphae in the soil during drought (Querejeta et al. 2003). Ectomycorrhizal fungi not only confer nutritional and water-related benefits, but enhance resistance to pathogenic and parasitic organisms (Mueller and Gehring 2006).

Colonization of seedlings by ECM fungi can be derived from dispersed spores, but also can develop from hyphae of ECM fungi already present in the soil of the seed germination site. The presence of mycorrhizal adults not only enhances mycorrhizal colonization of establishing seedlings (e.g. Cline et al. 2007; Harvey et al. 1980), but also sustains a diverse ECM fungus community in the soil (Luoma et al. 2006). ECM fungus diversity on roots of *Pseudotsuga menziesii* seedlings decreased with distance from mature trees (Cline et al. 2005), while the percentage of active ECM root tips and the performance of seedlings also were greatest near mature trees (Cline et al. 2007). The

ECM community on *Betula papyrifera* Marsh. seedlings likewise was richer within than outside the rooting zone of mature trees (Kranabetter 1999). Invasion of open barrens or prairies by the ECM invasive species *Pinus virginiana* L. was favored close to forests from which ECM hyphae extended into the open areas (Thiet and Boerner 2007). Not only conspecific, but also congeneric or unrelated ECM adult plants enhance mycorrhizal colonization of neighboring establishing seedlings (Bai et al. 2009; Richard et al. 2009; Dickie et al. 2002).

Disturbances can negatively affect regeneration of plant populations if mycorrhizal adults have been eliminated from a community. Although ECM fungus spores or hyphae extending from dying roots can serve as inoculum for seedlings shortly after a clearcut (Bâ et al. 1991; Brundrett 1991; Taylor and Bruns 1999), viable ECM fungus propagules may lose infectivity after some time without a suitable plant host. Sclerotia (fungus vegetative bodies resistant to environmental stress) of the ECM fungus *Hebeloma sacchariolens* Quèl. that were air-dried had reduced infectivity after 40 weeks without a host, while non-dried sclerotia were still infective after 40 weeks (Fox 1986b). Nevertheless, sclerotia of *Cenococcum geophilum* Fr. were still infective after several years without a host (Shaw and Sidle 1982; cited in Fox 1986b). Studies performed as greenhouse or field bioassays of ECM inoculum potential after clear cuts show conflicting results with either higher or lower percentage ECM root tips on seedlings in soil from clear cuts than from intact forests (see Table 2 in Jones et al. 2003), but the ECM communities on seedling roots clearly differ between clear-cut and intact forest soil (Jones et al. 2003). Human-made and natural disturbances such as logging, wildfires and hurricanes (Platt et al. 2002) limit the potential for plant population regeneration, not only

through reduced seed availability, but also through altered availability and community structure of beneficial fungal mutualists.

The extraradical mycelium of ECM fungi can densely colonize soil, contributing a high proportion of microbial biomass (Högberg and Högberg 2002). Because ECM fungi can associate with multiple hosts and can colonize adjacent plants' roots (Brownlee et al. 1983), mycorrhizal plants are interconnected by a common mycorrhizal network in the field (e.g. Simard and Durall 2004). By using molecular markers, Beiler et al. (2010) found that single genotypes of the ECM fungi *Rhizopogon* spp. simultaneously colonized several *Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco trees of different ages in the field, with large trees having more mycorrhizal connections than small trees to other trees. The span of individual genotypes reached 20 meters. In that study, the tree with the highest number of connections was connected to 47 other trees through 11 different *Rhizopogon* spp. genotypes. The ability of ECM fungus species to inhabit the roots of different plant species (e.g. Horton and Bruns 1998), as well as the spatial and temporal dynamics of the ECM community on a single plant (Pickles et al. 2010), offer a huge potential for connections among conspecific and unrelated co-occurring plants.

Depending upon the directionality of nutrient flows through mycorrhizal networks, mycorrhizal networks have important effects on seedling performance. In a microcosm experiment, nitrogen fixed by symbiotic *Frankia* spp. colonizing the roots of *Alnus glutinosa* L. Gaertn. was transferred to seedlings of *Pinus contorta* Doug. ex. Loud via an ECM fungus connecting both the pine and the alder (Arnebrant et al. 1993). In the field, carbon was transferred from *Betula papyrifera* Marsh. to *Pseudotsuga menziesii* seedlings, with amplified transfer when *P. menziesii* seedlings grew in deep shade (Simard et al. 1997b). In a greenhouse experiment, however, transfer of carbon between

*P. menziesii* and *B. papyrifera* seedlings seemed bidirectional (Simard et al. 1997a), although the authors could not distinguish whether the carbon was transferred by hyphal links or via soil pathways.

By providing otherwise unavailable mineral nutrients or carbon to establishing seedlings, mycorrhizal networks enhance seedling competitive ability (Simard et al. 2002). In a temperate forest, ectomycorrhizal networks had a negative effect on the survival of *Acer rubrum* L. seedlings (an arbuscular mycorrhizal species), a neutral effect on the survival of several ECM species including *Betula allegheniensis* Britton, and a positive effect on the growth of *P. strobus* seedlings in absence of root competition (Booth 2004). Improved growth of *Betula allegheniensis* seedlings connected to ectomycorrhizal networks, however, attracted deer browsing (Booth 2004). By using a combination of trenching and mesh barrier treatments, Booth and Hoeksema (2010) found that ectomycorrhizal networks somewhat counteracted the negative effect of competing roots on the survival of *Pinus radiata* D. Don. seedlings, in particular because of enhanced water status. For *Tsuga heterophylla* Raf. seedlings, root competition was the major determinant of seedling performance, while mycorrhizal networks with adult *T. heterophylla* did not counteract the negative effects of root competition. In addition, *T. heterophylla* seedlings grew very slowly, indicating light limitation (Kranabetter 2005). The diversity of ECM fungi on seedlings affects the outcome of competitive interactions with co-occurring plants. In a microcosm experiment, competitive interactions between *Pseudotsuga menziesii* and *Pinus ponderosa*, planted either in monoculture or mixed, were influenced by which ECM fungi were added to pots (Perry et al. 1989).

Recently, van der Heijden and Horton (2009) reviewed the importance of mycorrhizal fungus networks for facilitation in terrestrial ecosystems. They found that

seedlings of most plant species benefited from connection to mycorrhizal networks, apparently more so for ectomycorrhizal than for arbuscular mycorrhizal plant species, although they did not specifically address this question in the context of root competition. They speculated that mycorrhizal networks in ecosystems mostly prevent nutrient leaching, promote recycling of nutrients from dying roots or plants, and promote rapid mycorrhizal colonization of seedlings. On the other hand, they indicated that the significance of mycorrhizal networks for nutrient and water transfers among plants and for facilitation of seedling establishment still is not clear.

#### *Study species and system*

Several pine species, such as *Pinus palustris* Mill., *Pinus clausa* (Chapm.) Vasey or *Pinus elliottii* Engelm., are native to Florida, and other pine species are planted for commercial purposes (Arny and Flinchum 2006). *Pinus elliottii* is represented by two varieties, var. *elliottii* (slash pine) and var. *densa* (south Florida slash pine), of which var. *densa* is endemic to peninsular Florida and the Florida Keys (Little and Dorman 1952; 1954; Abrahamson and Hartnett 1990). *Pinus elliottii* var. *elliottii* is common in pine plantations in Florida, but also is planted outside the United States, such as in China (Chen et al. 2006) and in Australia (Izumi et al. 2008). In Australia, *P. elliottii* var. *elliottii* can account for as much as 7 % of pine plantation areas (Bastias et al. 2007). So, several studies have been conducted on *P. elliottii* var. *elliottii* because of its economic importance (e.g. Zhao et al. 2008; Burger and Pritchett 1988), but much less is known about *P. elliottii* var. *densa*.

*Pinus elliottii* is a monoecious, wind-pollinated tree species, producing mature cones usually in September in its native habitats (Lohrey and Kossuth 1990) and seeds

that are wind-dispersed starting in October. Most seeds fall within 50 m of a mother tree (Lohrey and Kossuth 1990), resulting in a clumped distribution of seedlings and saplings (Teague 2003). The natural range of *Pinus elliottii* var. *elliottii* extends along the United States southeastern coast, from South Carolina to eastern Louisiana and to central Florida. *Pinus elliottii* var. *densa* ranges from central Florida to the Florida Keys (Abrahamson and Hartnett 1990). The ecosystems in which *P. elliottii* occurs are subject to flooding and fire. *Pinus elliottii* var. *densa*, however, is more fire-tolerant than the *elliottii* variety. Fire adaptations of the *densa* variety include a seedling grass-stage similar to that of *P. palustris* (with densely grouped needles, a short and thick trunk and a deep tap root (Keeley and Zedler 1998)), a thick, insulating bark, and shedding of low branches (Little and Dorman 1954; Moyroud 1996), but it usually lacks a seed bank. When growing in seasonal ponds subject to flooding, seedlings in the grass-stage took a median 7 years to reach a height of 1 meter (Menges and Marks 2008). In the grass-stage, mortality was high when seedlings were completely submersed by flooding or were burned by an intense fire, but once seedlings reached a height of 1 meter, they better survived several months of flooding or low intensity fires (Menges and Marks 2008). In dendroecological studies, some of the oldest trees recruited 80-100 years prior (Higuera and Menges, unpublished results; Ford and Brooks 2003; 2002), indicating that *Pinus elliottii* var. *densa* trees can become 100 or more years old (Platt et al. 2000).

In Florida, *Pinus elliottii* var. *densa* occurs in ecosystems such as pine rocklands, xeric sandhills, or flatwoods (Abrahamson et al. 1984; Snyder et al. 1990; Abrahamson and Hartnett 1990). Pine rocklands occur in south Florida on limestone outcroppings, with poorly developed soils. Flatwoods are characterized by nutrient-poor, acidic soils with low water holding capacity. Differences in water availability associated with small

topographic differences determine the distribution of pines and other plants in flatwoods. For example, *Pinus palustris* is found in dry, elevated sites, while *P. elliotii* is found in mesic, low sites (Abrahamson and Hartnett 1990; Foster and Brooks 2001). *Pinus elliotii* var. *densa*

As in many other ecosystems in which *Pinus* spp. occur (Agee 1998), fire naturally disturbs ecosystems dominated by *P. elliotii* var. *densa* (e.g. Wade et al. 1980; Menges and Deyrup 2001), with intervals of about 1-10 years between fires (Breininger et al. 2002). Lightning ignites the highly flammable organic litter produced by plants in those ecosystems (Mitchener and Parker 2005; Breininger et al. 2002; Behm et al. 2004), usually at the start of the wet season. *P. elliotii* var. *densa* seedlings experience particularly high mortality in intense fires when they are smaller than 1 meter (Menges and Marks 2008). Although adults have fire-adaptations, survival of adults after a fire depends upon several factors, including fire intensity, char height, and beetle infestation. Survival of pines was maximized when char height was low and a high percentage of needles remained green (Menges and Deyrup 2001). Recently burned sites, and spring or summer fires also provided higher pine survival than sites long unburned (more than 25 years) or those burned in the fall. Intense fires favored infestation by bark or wood-boring beetles that exacerbated mortality after a fire (Menges and Deyrup 2001). In bayheads (associations of broadleaf evergreen trees on acidic, organic soils in depressions in central and south Florida), survival of *P. elliotii* var. *densa* was likewise negatively related to char height, but positively related to stem diameter of the pines, possibly because of reduced crown damage or because of thicker insulating bark in trees with wide stems (Matlaga et al. 2010). Interestingly, more pines recruited near the edges of

bayheads than in their interior, but pine survival after a fire was best in the interior (Matlaga et al. 2010).

Since European settlement, fire regimes in Florida have been altered (Wade et al. 1980). In particular, fire suppression in pine rocklands can lead to invasion of pinelands by less fire-tolerant hammock plant species (Snyder et al. 1990; Wade et al. 1980), or by exotic species such as *Schinus terebinthifolius* Raddi. (Stevens and Beckage 2010), while very frequent fires favor the release of seeds of *Melaleuca quinquenervia* Cav. Blake (Sevillano García Mayeya 2010). When fire returns after many years of fire suppression, the intensity of the fire can be so high that pines are eliminated, resulting in increased spread of *Serenoa repens* (Wade et al. 1980; Maliakal et al. 2000). Prescribed fires presently are used to manage pine rocklands and flatwoods in Florida. If prescribed fire management is not carefully planned, however, it can change the density and size-class structure of pine stands (Doren et al. 1993; Menges and Deyrup 2001). In pine rocklands in the Everglades, size-class structure in a non-logged/non-managed pine stand was much more heterogeneous than in two fire-managed and/or logged pine stands. In particular, the size-class structure of the fire managed sites was homogeneous, with no trees in small or large size-classes, in contrast to pines in the non-logged/non-managed pine stand (Doren et al. 1993).

In central and south Florida, rain mostly falls in summer while the winter and spring months are particularly dry. Low rainfall combined with high insolation in the spring results in high water stress for the vegetation (Chen and Gerber 1990). On the other hand, high rainfall in the summer (particularly during tropical storms and occasional hurricanes) can result in flooding. Humans not only have altered fire regimes, but through drainage and construction of dams and roads have altered hydrological patterns in



Florida. By using dendrochronological techniques, Ford and Brooks (2002) determined the effects of human-related increases in water level along the Myakka River on the growth of pines in flatwoods. They found that stands of *Pinus elliottii* var. *densa* experienced high mortality after water level increase. Even after the alteration of the river level, however, stem growth of pines was positively correlated with yearly rainfall, indicating that pine growth benefits from increased water availability. Tree-ring growth of *Pinus elliottii* var. *densa* was particularly enhanced by high water availability during the dry months in spring (Ford and Brooks 2003). Wet sites such as seasonal ponds favor the recruitment of juvenile pines (Menges and Marks 2008). Flooding decreased seedling survival in seasonal ponds, however, when seedlings were totally submersed, while adults can resist several weeks to months in flooded areas (Menges and Marks 2008).

In rocklands, where soil is poorly developed, trees and shrubs, including *P. elliottii* var. *densa*, predominantly utilize water from deep water sources, even during the wet season (Ewe et al. 1999). Young pine seedlings that do not have access to deep water sources yet, however, particularly suffer from limited water availability, especially during years when rainfall is affected by climatic phenomena such as La Niña, that decreases precipitation over Florida, and may suffer from flooding during El Niño, that increases precipitation (Sun and Furbish 1997). Complex interactions among multiple factors influence the recruitment, growth and distribution of *Pinus elliottii* var. *densa*. Wet sites seem to favor its germination. Its survival and growth are positively correlated with water availability up to the point that too much water negatively affects performance. Fire favors the maintenance of adults by excluding competing plants, while intense fires can kill both juveniles and adults. Human activities disturbing such complex interactions

might be responsible for the decline of *Pinus elliottii* var. *densa* and for its current low rate of establishment.

By enhancing seedling performance, ectomycorrhizal fungi colonizing *Pinus elliottii* may play an important role in the maintenance of this species in the nutrient poor and disturbance-prone ecosystems of Florida. Research on ectomycorrhizas of *Pinus elliottii* has mostly been conducted on the *elliottii* variety, in particular in plantations, with much less conducted regarding its ecology in natural stands. In addition, there is no published research on ectomycorrhizas of the *densa* variety. Early research indicated that visible ectomycorrhizas on nursery *P. elliottii* (unspecified variety) seedlings favored survival after outplanting to a sandy plantation site, even on a particularly dry site (Jorgensen and Shoulders 1967). Later, Lamb and Richards (1970) identified several ECM fungus species on the roots of *Pinus elliottii* var. *elliottii* from a plantation in Australia, including species belonging to *Rhizopogon* spp., as well as *Suillus granulatus* and *Cenococcum graniforme*, genera that often colonize conifer roots (Smith and Read 2008). By comparing a native eucalypt forest and an adjacent *P. elliottii* var. *elliottii* plantation in Australia, Bastias et al. (2007) found that the soil fungal communities were different between the two types of forests, although they did not determine the contribution of ECM fungi to the total fungal community in the soil. This might indicate that *Pinus elliottii* var. *elliottii* harbors a different set of ECM fungus species than eucalypts in native Australian ecosystems (Bastias et al. 2007). In a greenhouse experiment, Chen et al. (2006) found that several species of *Scleroderma*, which are ECM fungi, colonized the roots of *Pinus elliottii* seedlings (variety not specified) three months after spore inoculation, resulting in different percentages of mycorrhizal roots. Interestingly, some *Scleroderma* species enhanced seedling growth (e.g. *S. citrinum*)

while others decreased seedling growth (e.g. *S. cepa*) relative to that of non-inoculated seedlings (Chen et al. 2006). The roots of *P. elliotii* var. *elliotii* seedlings transplanted to a former pine plantation on sandy soil in northern Florida were dominated by three species of ECM fungi, comprising a *Thelephora* spp., a *Cenococcum* spp. and a white, rhizomorph-forming fungus (Sylvia and Jarstfer 1997). In addition, pine seedlings had reduced root and mycorrhiza development when growing with co-occurring weeds (Sylvia and Jarstfer 1997). Ectomycorrhizas may have the potential to improve the survival, growth, and competitive ability of *Pinus elliotii* var. *densa* seedlings, but the occurrence of mycorrhizas and their significance for pine seedling performance and recruitment in flatwoods have not been determined. Accordingly, the research questions that I shall answer in the following chapters are:

1. How quickly are transplanted *Pinus elliotii* var. *densa* seedlings colonized by ECM fungi in flatwoods in central Florida? Does ECM colonization of seedlings differ among pine stands with different densities of adults?
2. Do pine seedlings have higher ECM colonization and consequent improved survival, mineral nutrition and growth when transplanted in the presence versus the absence of adult pines in flatwoods?
3. In flatwoods soil under greenhouse conditions, do ectomycorrhizal seedlings have better mineral nutrition and growth than non-mycorrhizal seedlings? Do ECM hyphae with access to a large volume of soil confer greater benefits to seedlings than hyphae with access only to a limited volume of soil?

## Chapter 2

### **Pine seedling bioassay of ectomycorrhiza formation in flatwoods with different adult pine densities**

Establishment of plants in terrestrial ecosystems is affected by many abiotic and biotic factors, including mycorrhizal fungi (Janos 1980; Read and Perez-Moreno 2003; McGuire 2007). For example, arbuscular mycorrhizal (AM) fungus species influence the co-existence and allocation of nutrients among plant species in microcosms (van der Heijden et al. 2003), and species diversity of ectomycorrhizal (ECM) fungi promotes the productivity of *Betula pendula* Roth. at low soil fertility (Jonsson et al. 2001). In turn, established vegetation affects the distribution of mycorrhizal fungi in the soil (Haskins and Gehring 2005; Collier and Bidartondo 2009).

Ectomycorrhizal fungi provide several benefits to establishing seedlings. ECM fungi enhance mineral nutrition (Smith and Read 2008), and protect against pathogens (Kope and Fortin 1989). A high diversity of ECM fungus associates may help plants to forage for nutrients from patches in a heterogeneous soil (Bruns 1995; Erland and Taylor 2002), and thereby increase seedling biomass (Jonsson et al. 2001). ECM fungi enhance host plant water status (Parke et al. 1983; Plamboeck et al. 2007), which may be important in ecosystems subject to drought.

Successful seedling establishment is fundamental to forest regeneration, and successful establishment depends upon the presence of ECM inocula in the soil. Many studies have shown that the presence of an established host provides ECM inoculum for seedlings (e.g. Dickie et al. 2002; McGuire 2007; Thiet and Boerner 2007) and can

enhance newly establishing seedling performance (Simard et al. 1997c; Dickie et al. 2002). Fungal inocula, however, can be reduced by disturbance (Hagerman et al. 1999). Consequently, after disturbances such as clear-cutting or wildfires, establishment and regeneration of some plant species is limited by a lack of mycorrhizal inocula (Dahlberg et al. 2001; Simard 2009).

Adult trees facilitate establishment of ECM seedlings in additional ways. By providing shade, they protect against heat and favor a moist environment in the understory (Simard 2009). Because ECM fungi can colonize several host plants simultaneously, mycorrhizal networks can form among plants. Nitrogen and even carbon can be transferred among plants connected through ECM networks (Simard et al. 1997a; He et al. 2006). To a seedling, an ECM fungus that also is connected to an adult might represent less of a carbon drain than if the seedling had to support the fungus by itself. The sometimes-observed negative effect of ECM on seedling growth in greenhouse experiments results from a high carbon cost to the seedling of being the sole support for its ECM fungi (Colpaert et al. 1992). Van der Heijden and Horton (2009) reviewed the effect of mycorrhizal networks on seedlings, and found that in most cases, the presence of an ECM adult benefited seedlings.

The positive effects of an adult on a seedling's mycorrhizal status and growth might depend upon the distance between the adult and the seedling. Although ECM species richness can be higher at short distances than far from established trees (Cline et al. 2005; Teste et al. 2009), Teste and Simard (2008) found that performance of *Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco seedlings improved with distance that reduced competition with the adult for light and soil resources. Adult density might act

similarly to distance from an adult tree, as has been proposed by Dickie et al. (2002) for *Quercus rubra* L. seedlings germinating in stands of *Quercus montana* Willdenow.

In central Florida, some flatwoods ecosystems are characterized by an overstory dominated by *Pinus elliottii* var. *densa* Little & Dorman which forms ectomycorrhizas, a few shrubby *Quercus* species which also form ectomycorrhizas, and an understory dominated by the palm *Serenoa repens* (Bartr.) Small which forms arbuscular mycorrhizas (Fisher and Jayachandran 1999; Abrahamson et al. 1984). *Pinus elliottii* var. *densa* is native to central and south Florida. The canopy of pines in flatwoods can range from relatively dense to very open, with some areas that are treeless (Wade et al. 1980; Abrahamson and Hartnett 1990). Flatwoods are disturbed naturally by wildfires. *Pinus elliottii* var. *densa* is adapted to survive low intensity fires, but mortality of pines after severe fires is high (Menges and Deyrup 2001). For the last few decades, populations of *P. elliottii* var. *densa* have been in decline in Central Florida, mainly because of low seedling establishment possibly resulting from an altered fire regime. Because ectomycorrhizas play an important role in pine seedling establishment, I investigated the effect of adult pine density on ectomycorrhizal colonization of *P. elliottii* var. *densa* seedlings. Specifically, I determined how adult pine density in flatwoods affected the time-course of ECM colonization and ECM fungus diversity on young pine seedlings in the field. I expected that pine seedlings transplanted within a dense stand of adult pines would have higher ECM colonization and higher ECM fungus diversity than pine seedlings transplanted to a site with few pines.

## Materials and Methods

### *Field sites*

To study ectomycorrhiza formation by transplanted pine seedlings, I selected three experimental sites at Archbold Biological Station (Archbold), Highlands County, Florida, USA (27°11'N lat., 81°21'W long.) within flatwoods ecosystems. The climate at Archbold is characterized by hot, wet summers and mild, dry winters (Chen and Gerber 1990). During the experiment (from July 20 to December 2, 2009), the mean air temperature was 24.5°C, with a maximum of 36.5°C on August 2 at 4:15 pm and a minimum of 1°C on November 28 at 1:15 am. The total rainfall during that period was 343.6 mm. At the station, all soils are deep, usually acidic and sandy, with low available water holding capacity (Abrahamson et al. 1984).

The different density sites were not replicated because of limited resources. The three sites selected for different adult *P. elliotii* var. *densa* densities were located within a single fire management unit. Between 1967 and 2009, the sites were affected by fire in August 1987, May 1989, July 1998, June 2002 and July 2002. In each site, adult pines were counted in an area of 20 × 20 m. The low pine density site had 4 adult pines/400 m<sup>2</sup>, the medium pine density site had 9 adult pines/400m<sup>2</sup>, and the high pine density site had 19 adult pines/400 m<sup>2</sup>. Dominant plant species of each site are listed in Table 2.1. Although I did not measure plant cover quantitatively, the low, spreading *Serenoa repens* was conspicuously more abundant in the low pine density site than at the other two sites.

### *Pine seedling transplant and subsequent harvest*

Seeds of *P. elliottii* var. *densa* were donated by the Andrews Nursery (Florida Division of Forestry, Chiefland, FL, USA; seeds were collected from mature trees in South Florida). Seeds were soaked in distilled water for 24 hours and incubated at 4°C for 7 days. Seeds were germinated in individual containers in coarse silica sand (L 6-20, Standard Sand and Silica Co., Miami, FL, USA) in an ambient temperature shadehouse, and were watered daily.

Non-mycorrhizal, two-month-old pine seedlings were transplanted to the field sites on July 20 to 24, 2009. In the center of the 20 × 20 m area within each site, I established a 6.75 m × 6.75 m grid into which 100 seedlings were planted in a 10 × 10 arrangement, each separated by about 0.75 m in both directions from its neighbors (Fig. 2.1a, b and c). The position of a seedling sometimes needed to be adjusted by a few centimeters because of the presence of thick pine roots or palm rhizomes. A 2 cm-diameter hole was made in the soil with a soil corer to insert the bare roots of the seedling, and the hole was refilled with the soil from the core. Each seedling was protected from herbivores with a 0.6 cm-mesh hardware-cloth cage. During the experiment, seedlings were watered 3 times a week with tap water when there was no rainfall for 3 or more consecutive days.

Approximately every 2 weeks for 4.5 months, 12 seedlings were randomly selected for harvest at each site. Before excavation, the amount of shade and density of potentially competing grasses surrounding each pine seedling was estimated visually by classifying the shade or grass density into categories (for shade: no shade, low, medium, or high; for grass density: no grass, medium, or high density). Pine seedlings were



harvested manually by shovel within a block of surrounding soil taking care to excavate the entire root system. Seedlings and their block of soil were placed individually into plastic bags for prompt transport to the laboratory. The last seedlings were excavated on December 2, 2009 (135 days after transplant).

#### *Soil properties at the sites*

Soil properties were determined for the native soil surrounding the roots of excavated seedlings. The soil from all seedlings excavated at a single harvest was pooled and mixed by site. Soil samples from 15, 56, 99, and 135 days after transplant (*i.e.*, collected on August 4, September 14, October 27, and December 2, 2009) were sent to A & L Southern Agricultural Laboratories, Inc. (Deerfield Beach, FL, USA) for analysis.

#### *Seedling performance and ectomycorrhiza formation*

In the laboratory, all needles were separated from the shoot and weighed fresh. Chlorophyll concentration in needles was measured by cutting 0.2 g of needles into 0.5 cm-pieces, and incubating them for 48 hours in 80 % acetone in the dark (Proctor 1981). The concentrations of chlorophyll *a* and *b* were determined by measuring absorbance of the resulting solution at 645 and 663 nm (Porra 2002). The remaining needles and the stem were dried at 50°C for 1 week, and then weighed (the total dry weight of needles was determined by adjusting for the removal of needles for chlorophyll analysis). The root system of each seedling was gently washed in tap water. After examination for ECM, root systems were dried and weighed.

ECM colonization was assessed by examination of root tips with a dissecting microscope by employing the gridline intersect method as described by Brundrett et al.

(1996). Different morphotypes were distinguished based on attributes of the ectomycorrhiza mantle (Agerer 1991).

### *Statistical analyses*

Soil properties were tested for differences among sites by separate ANCOVAs for each property, with pine density as the main, fixed factor and days after transplant as the covariate, with Bonferroni correction for testing multiple response variables.

Concentrations of Ca, H<sup>+</sup>, K, Mg, and P, pH, and cation exchange capacity were homoscedastic, but the concentration of N had to be log-transformed for homoscedasticity. Because some pine densities had concentrations of Fe without variance, as also did concentrations of H<sup>+</sup> and pH for days after transplant, their heteroscedasticity could not be remedied. Therefore, analyses of those variables are approximate.

Chi-square tests were used to determine if the distributions of seedlings experiencing different categories of shade and grass density differed among sites. Because grass density and shade were categorical, mean differences among sites were assessed with non-parametric, Kruskal-Wallis analyses of variance.

Chi-square tests were used to determine if survival of seedlings differed among sites and categories of shade and grass density. Effect of site on plant biomass (total, needle, stem, and root dry weights, root/shoot ratio, total number of root tips) and chlorophyll (total chlorophyll concentration and content, chlorophyll *a* and *b* concentrations, chlorophyll *a/b* ratio) were analysed with separate ANCOVAs with pine density as the main, fixed factor and days after transplant as the covariate, with Bonferroni correction for testing multiple response variables. Total, needle, stem, and

root dry weight, number of root tips, and chlorophyll *a/b* ratio were log-transformed. Chlorophyll content still was heteroscedastic after log-transformation, but no other transformation provided homoscedasticity, so ANCOVA of log-transformed chlorophyll content is considered approximate. The effects of shade and grass density on non-transformed response variables were tested with separate Kruskal-Wallis analyses of variance, with Bonferroni corrections separately for shade and grass density.

Proportions of seedlings with versus without ECM among sites were tested with Chi-square analyses for harvests 15, 39, and 56 days after transplant (after which, all seedlings had ectomycorrhizas). Percent ECM root tips was arcsine-square root transformed for statistical analysis. Differences in percentage ECM root tips (excluding non-mycorrhizal seedlings of the first three harvests) and in number of different ECM morphotypes (square-root transformed) were tested with ANCOVA, with pine density as the main, fixed factor and days after transplant as the covariate. I also tested the effect of pine density and days after transplant on the number of different ECM morphotypes per ECM root tip (log-transformed). The effects of shade and grass density on percentage ECM root tips were tested with Kruskal-Wallis analyses of variance.

Least-squares, linear regression with transformed variables was used to determine the contribution of ECM to total dry weight, as well as to determine differences in the contributions of ECM to total dry weight among different categories of shade.

After significant ANCOVAs or Kruskal-Wallis analyses of variance, differences among group means were detected by post-hoc Tukey HSD tests. Back-transformed means and standard errors are shown in tables and figures for those variables that required transformation.

## Results

### *Site characteristics*

Over four collection dates spanning 4.5 months, all sites had similar, low mean soil pH of 4.32 to 4.40. They also had similar cation exchange capacity and soil concentrations of Ca, Fe, H<sup>+</sup>, K, and total N. Concentrations of Mg and P, however, differed significantly among sites (Table 2.2). The high pine density site had almost double the Mg concentration of the medium and low density sites. The low pine density site had 1.6 and 1.9 times higher mean P concentration than the high and medium pine density sites, respectively (Table 2.2).

Soil pH increased with time, as it was lower at 15 and 56 days after transplant ( $4.20 \pm 0.00$  and  $4.20 \pm 0.03$ , respectively) than at 99 and 135 days after transplant ( $4.46 \pm 0.03$  and  $4.56 \pm 0.05$ , respectively). As soil pH increased, H<sup>+</sup> concentration and cation exchange capacity decreased. H<sup>+</sup> concentration was highest 15 days after transplant ( $2.73 \pm 0.46$  meq/100g) and lowest 135 days after transplant ( $1.16 \pm 0.06$  meq/100g), while it was intermediate at 56 and 99 days after transplant ( $2.30 \pm 0.00$  and  $1.63 \pm 0.06$  meq/100g, respectively). Averaged across all three sites, cation exchange capacity was highest 15 days after transplant ( $3.60 \pm 0.64$  meq/100g), lowest 135 days after transplant ( $1.96 \pm 0.17$  meq/100g), and intermediate at 56 and 99 days after transplant ( $3.16 \pm 0.15$  and  $2.73 \pm 0.33$  meq/100g, respectively). The soil concentrations of Ca (mean across all dates =  $97.5 \pm 10.55$  mg/kg), Fe ( $6.5 \pm 0.41$  mg/kg), K ( $27.41 \pm 3.10$  mg/kg), Mg ( $26.41 \pm 2.55$  mg/kg), P ( $4.75 \pm 0.47$  mg/kg) and total N ( $9.33 \pm 0.86$  mg/kg) did not change through time, as indicated by a non-significant effect of the days after transplant covariate (Table 2.2).

The category of shade and grass density experienced by pine seedlings was not independent of pine density. Seedlings in the medium pine density site were less shaded than those in the low and high pine density sites, with a higher frequency of seedlings exposed to no shade than at the other sites (Chi-square statistic = 9.69,  $P < 0.01$ ). The Kruskal-Wallis analysis of mean shade category among sites also indicates more shade in the low pine density than in the medium pine density site, while the high pine density site had intermediate shade (Kruskal-Wallis statistic = 7.44,  $P = 0.02$ ). Fewer seedlings in the low pine density site than at the other sites were exposed to high grass density (Chi-square statistic = 13.09,  $P = 0.05$ ). The Kruskal-Wallis analysis of variance, however, did not detect significant differences in mean grass density among sites (Kruskal-Wallis statistic = 2.97,  $P = 0.22$ ). Across all three sites combined, shade and grass density were independent of each other (overall Chi-square statistic = 12.85,  $P = 0.16$ ).

#### *Pine seedling performance*

Survival of seedlings until harvest was high and did not differ among sites (Chi-square statistic = 2.09,  $P = 0.3515$ ), with 92 % survival at low pine density, 88 % survival at medium pine density, and 90 % survival at high pine density.

All aspects of chlorophyll status of pine seedlings were affected by site (Table 2.3). Mean total chlorophyll concentration was lower at medium adult pine density than at low and high pine density. Chlorophyll *a* concentration followed a similar pattern to total chlorophyll concentration (Table 2.3). Chlorophyll *b* concentration differed among all three sites, being highest at high adult pine density, medium at low pine density and lowest at medium pine density (Table 2.3). Chlorophyll *a/b* ratio was higher at low and medium pine density than at high pine density. Total chlorophyll content was higher at

low pine density than at medium and high pine densities (Table 2.3). Days after transplant affected chlorophyll *a/b* ratio and total chlorophyll content, while concentrations of total chlorophyll, chlorophyll *a*, and chlorophyll *b* did not change through time (Table 2.3).

Pine seedlings transplanted to the low and medium pine density sites grew better than those at the high pine density site (Fig. 2.2). In the low and medium pine density sites, seedlings had higher mean needle dry weight, stem dry weight, root dry weight, total dry weight, and more root tips than seedlings in the high pine density site (Table 2.3). All seedlings, however, had similar root/shoot ratios of 0.42 (overall mean). Because the seedlings grew, days after transplant significantly affected needle dry weight, stem dry weight, root dry weight, total dry weight, and number of root tips, but not root/shoot ratio (Table 2.3).

Total chlorophyll, chlorophyll *a*, and chlorophyll *b* concentrations all were affected strongly by shade (Table 2.4), being lowest in no shade and increasing as shade increased (Fig 2.3). Total chlorophyll content was higher in medium and high shade than in low and no shade, and chlorophyll *a/b* ratio decreased as shade increased (Table 2.4).

Survival of seedlings was not affected by shade (Chi-square statistic = 5.0306,  $P = 0.1696$ ). Category of shade experienced by pine seedlings significantly affected mean stem dry weight which was higher in no shade than in low or high shade, while it was intermediate in medium shade (Table 2.4). The effect of shade on the relationship between percentage ECM root tips and total dry weight was assessed with linear regression including only seedlings that did not experience potential grass competition: total dry weight was positively, linearly related to percentage ECM root tips, with percentage ECM root tips explaining about 20 % of the variance in total dry weight ( $F_1$ ,

$_{114} = 29.62, P < 0.0001, R^2 = 0.2062$ ). The intercepts of this regression among the four different shade categories differed ( $F_{3,111} = 3.71, P = 0.0138$ ), decreasing with increasing shade, but the slopes of the regressions did not differ ( $F_{3,108} = 0.22, P = 0.8826$ ) (Fig. 2.4). Root/shoot ratio conspicuously was not affected by shade (Table 2.4).

Survival was significantly lower when seedlings experienced high grass density (78.4 % seedlings surviving) than medium grass density or no grass at all (91.1 % and 94.5 % survival, respectively; Chi-square statistic = 13.66,  $P = 0.0011$ ). After Bonferroni correction, density of grass surrounding seedlings had a significant effect only on mean stem dry weight but not on other dry weight variables. Stem dry weight was higher without grass than with high grass density around seedlings, while seedlings surrounded by medium grass density had intermediate mean stem dry weight (Table 2.5).

#### *Ectomycorrhiza status of seedlings*

Although all pine seedlings were without ECM at transplant, fifteen days after transplant, 45.4 %, 55.5 %, and 40.0 % of excavated seedlings had ECM in low, medium, and high adult pine density sites, respectively. On average across the three sites, 96.7 % and 93.9 % of seedlings were ectomycorrhizal by 39 and 56 days after transplant, respectively. There were no differences in proportions of mycorrhizal seedlings among sites at any date (e.g. Chi-square statistic at 15 days after transplant = 0.49,  $P = 0.79$ ). All harvested seedlings were ectomycorrhizal on the 71<sup>st</sup> day after transplant and thereafter.

Site ( $F_{2,247} = 12.57, P < 0.0001$ ) and days after transplant ( $F_{1,247} = 48.29, P < 0.0001$ ) significantly affected the percentage of root tips with ECM (Fig. 2.5).

Seedlings in the low pine density site had a higher percentage ECM root tips (29.50 % +1.79/-1.76) than seedlings at the high and medium pine density sites (21.33 % +1.64/-

1.59 and 18.08 % +1.53/-1.48, respectively; Table 2.3). Neither shade (Table 2.4) nor grass density (Table 2.5) affected percentage ECM root tips.

Site significantly affected the number of ECM morphotypes, while days after transplant had no effect on number of morphotypes (Table 2.3). The site effect also was supported by a Kruskal-Wallis analysis of variance (Kruskal-Wallis statistic = 30.38,  $P < 0.0001$ ). Seedlings at the high adult pine density site had significantly more ECM morphotypes than seedlings at the medium and low pine density sites (Table 2.3). Neither shade (Kruskal-Wallis statistic = 1.80,  $P = 0.61$ ) nor grass density (Kruskal-Wallis statistic = 2.18,  $P = 0.53$ ) affected the number of ECM morphotypes on seedling roots.

Site and days after transplant significantly affected the number of ECM morphotypes per ECM root tip. Seedlings at the high pine density site had the highest number of ECM morphotypes per ECM root tip, followed by seedlings at the medium and the low pine density sites (Table 2.3).

Across all sites, total dry weight of seedlings was positively, linearly related to percentage ECM root tips, but percentage ECM root tips explained only 7 % of the variance in total dry weight (model  $F_{1,249} = 19.46$ ;  $P < 0.0001$ ,  $R^2 = 0.0725$ ).

## **Discussion**

### *Site characteristics*

Flatwoods are one of the most nutrient poor ecosystems in Florida (Monk 1968). I found slightly higher concentrations of Ca, Mg, K, and P (Table 2.2) than the 69.2 mg/kg Ca, 15.8 mg/kg Mg, 4.2 mg/kg K, and 1.3 mg/kg P given for flatwoods soil by Monk (1968).



Even though my sites all had similar fire histories and vegetation, the high pine density site had a higher concentration of Mg than the medium and low pine density sites, while the low pine density site had the highest concentration of P.

### *Seedling performance*

Because I watered my seedlings when rainfall was lacking, and because I protected them against herbivores, seedling survival, around 90 %, was high in all three sites. Good survival enabled me to detect pine seedling growth and chlorophyll response differences among the sites. Pine seedlings grew the best in the low and medium pine density sites (Fig. 2.2), while their total chlorophyll concentrations were highest in the low and high adult pine density sites (Table 2.3). Although the high growth and chlorophyll concentrations of seedlings in the low pine density sites might reflect their high ECM colonization, because of the low proportion (7 %) of variance in total dry weight explained by ECM, other factors such as shade, surrounding grass density, and soil attributes also are likely to have influenced pine seedling performance which, in turn, constrained seedlings' ability to support ECM fungi.

Shade had a strong effect on the chlorophyll concentrations of pine seedlings. Total chlorophyll and chlorophyll *a* and *b* concentrations increased with increasing shade (Fig. 2.3), while the chlorophyll *a/b* ratio decreased with increasing shade (Table 2.4). Pine seedlings compensated for limited light availability by increasing chlorophyll concentration and by increasing the relative contribution of chlorophyll *b* to total chlorophyll. A reduced chlorophyll *a/b* ratio is a common response to shade, even within a single tree between sun leaves and shade leaves (Hansen et al. 2002). Similar to my seedlings, seedlings of *Pinus brutia* Ten. and *P. pinea* L. increased chlorophyll

concentration in response to shading (Awada et al. 2003). In those two pine species, however, the elevated chlorophyll concentration did not compensate for the reduction of photosynthesis caused by shading, resulting in reduced growth (Awada et al. 2003). Shade probably contributed to differences in needle chlorophyll concentrations among my three flatwoods sites. Because shade did not differ between my high and low adult pine density sites, however, the difference in seedling mean dry weights between those two sites is not likely to be attributable to shade.

Surrounding grass density reduced survival, as well as the mean stem dry weight of pine seedlings. It also consistently reduced other mean growth response variables, although not significantly after Bonferroni correction (Table 2.5). In grasslands and pine plantations, grasses reduce conifer seedling performance through competition for water or soil nutrients (Picon-Cochard et al. 2006; Smethurst et al. 1993), and it is likely they had a similar effect in my flatwoods experiment. Removal of competing plants from pinyon-juniper woodlands promoted the growth of adult *Pinus edulis* Engelm. (McHugh and Gehring 2006), supporting pine sensitivity to competition in spite of being ectomycorrhizal. In competition with the grass *Panicum chamaelonche* Trin., inoculation of *Pinus elliottii* Engelm. var. *elliottii* seedlings with the ECM fungus *Pisolithus arhizus* Scop. Per Pers. improved seedling growth and P uptake (Pedersen et al. 1999). In my experiment, however, seedling survival and performance diminished independently of ECM as the density of surrounding grasses increased (Table 2.5).

Although my seedlings were watered regularly when rainfall was insufficient, their reduced survival and mean stem dry weight at high grass density most likely was caused by competition for water in the well-drained flatwoods soils, exacerbated during the dry season. Indeed, wet sites enhance *P. elliottii* var. *densa* seedling growth and

survival (Menges and Marks 2008). Water addition, but not N fertilization, of a longleaf pine savanna in Georgia, an ecosystem similar to pine flatwoods, increased the species diversity of recruiting graminoid, forb, and legume seedlings (Iacona et al. 2010). Grasses might have competed for mineral nutrients, but potential competition for nutrients did not result in differences in chlorophyll concentration which is usually correlated with nutrient status (Chapter 3 & 4). In my study, the negative effect of grass density was not likely attributable to a shading effect of grass, because of the lack of effects on chlorophyll concentrations, and because grass density and shade categories overall were statistically independent. Because pine seedling growth in the low and medium pine density sites did not differ significantly (Table 2.3) even though the low adult pine density site had lower grass density than the other sites, differences in seedling growth among my sites cannot be attributed entirely to effects of grass density.

Soil fertility differences among sites probably played a minor role in affecting seedling performance. Within 4.5 months, *P. elliotii* var. *densa* seedlings can respond to NPK fertilization of flatwoods soil with increased growth under greenhouse conditions (G. Toledo et al., unpublished results). The low pine density site had significantly higher mean soil P than the medium and high adult pine density sites (Table 2.2), but because there were no significant growth differences between the low and medium pine density sites, P was unlikely to have strongly affected seedling growth in the field. Additionally, although the high pine density site had the highest soil Mg concentration (Table 2), seedlings there had the lowest total dry weight. Nevertheless, in combination with shade, high Mg and P concentrations in soil might have contributed to the high total chlorophyll, chlorophyll *a*, and chlorophyll *b* concentrations of seedlings in the low and high pine density sites (Table 2.3). High P concentration in soil, low grass competition for water,

and high seedling chlorophyll concentrations in the low adult pine density site all may have combined to favor continued growth there.

#### *Ectomycorrhiza formation*

Pine seedlings that were two months old at transplant formed ectomycorrhizas with similar time-courses in all three sites. At the first three harvests, the proportions of ECM and non-colonized seedlings did not differ among sites. Initial colonization was rapid, as indicated by an overall mean 5.35 % of root tips colonized just 15 days after transplant. The increase in percentage of root tips with ECM colonization remained rapid for the first 99 days after transplant, but appeared to plateau from 99 to 135 days after transplant (Fig. 2.5), with 30 % mean ECM root tips overall at the final harvest. Thus, provided that at least a few adult pines were present, seedlings were colonized quickly by ECM fungi in all three central Florida flatwoods sites that I studied.

My *P. elliotii* var. *densa* seedlings had a similar ECM colonization time-course to *Pinus contorta* Dougl. ex Loud. seedlings derived from seeds germinated in absence of adults in recently clear-cut pine stands (Bradbury 1998). *P. contorta* seedlings had 6.3 % and 30.4 % ECM colonization one and two months after germination, respectively (Bradbury 1998). In contrast, in recently burned sites, ECM colonization of *Pinus muricata* D. Don. seedlings was delayed until about 3 months after germination (Horton et al. 1998). At alpine tree-lines also, ECM colonization can be slow, as demonstrated by very few seedlings of *Picea engelmannii* Parry having ECM four months after germination (Germino et al. 2006).

Pine seedlings in the three flatwoods sites that I studied sustained different midpoint mean percentage ECM colonization. Seedlings transplanted to the low pine

density site had a higher percentage ECM root tips than those in the medium and high pine density sites (Fig. 2.5). The highest mean chlorophyll concentrations and the highest mean dry weight of seedlings were combined in the low adult pine density site, and that combination may have enabled seedlings to support more ECM there than in the medium and high pine density sites. Nevertheless, seedlings in the low and high pine density sites had statistically indistinguishable mean total chlorophyll concentrations, and seedlings in the low and medium pine density sites had statistically indistinguishable mean total dry weights (Table 2.3), so neither alone is likely to account for differences in mean percentage ECM root tips.

Shade and competition both can affect a plant's capacity to support ECM fungus associates. Shade reduced mycorrhizal colonization of ericaceous shrubs by reducing photosynthesis (Zijlstra (2006), cited in Olsrud and Michelsen (2009)). Removal of competing shrubs promoted ECM colonization of adult *Pinus edulis* (McHugh and Gehring 2006), but did so by increasing plant growth. Nevertheless, in my study, although shade and grass density reduced mean stem dry weight, neither one affected mean percentage ECM root tips (Table 2.4 and 2.5).

High ECM colonization at low adult pine density site might have been influenced by dominance of a particular ECM fungus species. Seedlings at the low pine density site had fewer ECM morphotypes than at the high pine density site. In the low pine density site, ECM root tips were dominated by a yellowish morphotype that seemed to spread aggressively. Although the yellowish morphotype also was found at the other two sites, increased abundance of other ECM fungus species might have interfered with its spread on seedling roots.

Pine seedlings showed the most ECM morphotypes in the high adult pine density site. In particular, brown-hairy and black-hairy morphotypes, which already had colonized seedlings at 15 days after transplant in the high adult pine density site, never were seen at the low pine density site. The black-hairy morphotype might correspond to *Cenococcum geophilum* Fr., a mycorrhizal fungus observed in many ecosystems on conifers (Bradbury 1998; Hasselquist et al. 2005). *C. geophilum* was one of the first ECM fungi to colonize *Pinus contorta* within one month of germination (Bradbury 1998). Colonization by *C. geophilum* especially might enhance pine seedling water status, as it did for *Picea engelmannii* following cessation of watering (Hasselquist et al. 2005).

In flatwoods, high adult pine density probably contributed to the high diversity of ECM fungi on pine seedlings. Other studies have found that ECM fungus diversity is highest when seedlings are grown close to ECM trees (Dickie et al. 2002; Teste et al. 2009) or in contact with ECM mycelium spreading from host trees (Simard et al. 1997c). Diverse ECM communities can contribute to host productivity at low soil fertility (Jonsson et al. 2001) and to increased whole plant P concentration (Baxter and Dighton 2001). In my high adult pine density site, however, ECM diversity did not contribute to higher seedling dry weight or higher total chlorophyll concentration than in the low adult pine density site. Notwithstanding, if I had not watered my seedlings, ECM diversity (Teste and Simard 2008) at the high adult pine density site might have promoted seedling survival.

The ECM status of seedlings can be influenced by soil mineral nutrient concentrations. For example, Kernaghan et al. (2003) found a positive relationship between cation exchange capacity and the abundance of *Cenococcum* and *Russula* ectomycorrhizas. Twieg et al. (2009) found that available P was negatively correlated

with fungus species richness on *Pseudotsuga menziesii* (Mirb.) Franco, because dominance of particular ECM taxa increased with increased P. Twieg et al. (2009), however, found no relationship between soil N and ECM fungus diversity. Neither were ECM communities correlated with soil N or P in *Pinus contorta* stands or in old-growth, mixed conifer stands (Douglas et al. 2005). In my study, site differences in P and Mg might have influenced ECM fungus diversity, especially the low diversity with high P in the low adult pine density site, similar to findings of Twieg et al. (2009), but the correlative nature of my non-replicated study only is suggestive.

In flatwoods, various plants other than *Pinus elliottii* var. *densa* might support ECM fungi and provide ECM fungus inocula for establishing pine seedlings. Several different plant species can share ECM fungus species (Arnebrant et al. 1993; Smith et al. 2009). In my study sites, *Quercus geminata* Small and *Quercus minima* (Sarg.) Small are likely to support ECM fungi (Wang and Qiu 2006), and oaks and pines can share some ECM fungus species in other low-elevation woodlands (Smith et al. 2009). I also found species in the Ericaceae, such as *Vaccinium myrsinites* Lam., *Lyonia ferruginea* (Walter) Nutt., *L. fruticosa* (Michx.) Torr. and *L. lucida* (Lam.) K. Koch in the flatwoods sites (Table 2.1). Although members of the Ericaceae usually form ericoid mycorrhizas with specific ascomycetous fungi (Smith and Read 2008), ECM fungi such as *C. geophilum* (which is an ascomycete) can colonize the roots of Ericaceae such as *Vaccinium* spp. (Vohnik et al. 2007) and can be shared by ericaceous and ECM hosts (Curlevski et al. 2009).

ECM fungi might have colonized my bioassay pine seedlings through hyphae extending from established hosts (Wu et al. 2001) and through mycelia derived from spores (Fox 1986a). Although I did not deliberately search for epigeous fruiting bodies, I

never encountered any in the sites during the 4.5 months of this study. So, the contribution of spores to ECM formation in my sites is unknown. In a greenhouse experiment (Chapter 4), however, ECM fungi readily colonized pine seedlings from propagules in extracted, homogenized flatwoods soil, suggesting that mycelia associated with adult trees are not the only form of inoculum present.

In conclusion, this study demonstrates that there is no lack of ECM inocula for nearly immediate seedling root colonization in three flatwoods sites with different adult pine densities at Archbold Biological Station. Moreover, young (6.5 month old or less) *P. elliotii* var. *densa* seedlings can sustain ECM fungi, although their ability to do so may be diminished by shade, for which increased chlorophyll concentrations cannot compensate, by competition with grasses, and by low soil P availability. Although high adult pine density may increase the diversity of ECM fungi associated with seedlings, it is unlikely that failure to form ECM limits regeneration of *Pinus elliotii* var. *densa* even in flatwoods with a low density of adult pines.



**Table 2-1** Dominant plant species at three flatwoods sites (Archbold Biological Station, Central Florida) with different *Pinus elliotii* var. *densa* adult densities into which *P. elliotii* var. *densa* seedlings were transplanted. X indicates a species' presence

Species	Low adult pine density 4 pines/400 m <sup>2</sup>	Medium adult pine density 9 pines/400 m <sup>2</sup>	High adult pine density 19 pines/400 m <sup>2</sup>
<i>Andropogon glomeratus</i> (Elliott) C Mohr	X	X	
<i>Aristida beyrichiana</i> Trin. & Rupr.	X	X	X
<i>Lachnocaulon anceps</i> (Walter) Morong		X	X
<i>Galactia elliotii</i> Nutt.		X	
<i>Hypericum</i> sp.	X	X	
<i>Lyonia ferruginea</i> (Walter) Nutt.			X
<i>Lyonia fructicosa</i> (Michx.) Torr.	X	X	X
<i>Lyonia lucida</i> (Lam.) K. Koch	X	X	X
<i>Panicum abscissum</i> Swallen	X	X	X
<i>Quercus geminata</i> Small	X		X
<i>Quercus minima</i> (Sarg.) Small	X	X	
<i>Sabal etonia</i> Swingle ex Nash		X	
<i>Serenoa repens</i> (Bartr.) Small	X	X	X
<i>Vaccinium myrsinites</i> Lam.	X		
<i>Xyris caroliniana</i> Walter			X

**Table 2-2** Soil properties of three different flatwoods sites with different *Pinus elliotii* var. *densa* adult densities at the Archbold Biological Station, Central Florida. For each site, values are mid-point means  $\pm$  SE of 12 soil samples after ANCOVA. Within a row, values followed by the same letter are not significantly different at  $P \leq 0.05$ . Probabilities in bold indicate a significant difference among groups at Bonferroni-corrected  $P \leq 0.05/9 = 0.0055$

Response variable	Low pine density	Medium pine density	High pine density	F/P effect of pine density	F/P effect of days after transplant (covariate)
Soil pH	4.32 $\pm$ 0.11 a	4.40 $\pm$ 0.09 a	4.35 $\pm$ 0.08 a	0.83 0.4706	39.99 <b>0.0002</b>
CEC* (meq/100g)	2.72 $\pm$ 0.34 ab	2.42 $\pm$ 0.27 b	3.45 $\pm$ 0.51 a	6.79 0.0189	25.78 <b>0.0010</b>
Ca (mg/kg)	72.25 $\pm$ 7.12 b	87.00 $\pm$ 19.05 ab	133.25 $\pm$ 11.62 a	5.01 0.0388	0.17 0.6946
Fe (mg/kg)	5.00 $\pm$ 0.00 b	7.00 $\pm$ 0.70 ab	7.50 $\pm$ 0.50 a	6.43 0.0216	0.27 0.6181
H <sup>+</sup> (meq/100g)	1.95 $\pm$ 0.37 a	1.65 $\pm$ 0.27 a	2.27 $\pm$ 0.45 a	3.91 0.0655	43.24 <b>0.0002</b>
K (mg/kg)	27.75 $\pm$ 6.04 a	20.25 $\pm$ 2.95 a	34.25 $\pm$ 5.26 a	3.91 0.0654	9.45 0.0153
Mg (mg/kg)	21.50 $\pm$ 1.19 b	20.75 $\pm$ 1.88 b	37.00 $\pm$ 3.24 a	14.61 <b>0.0021</b>	0.06 0.8090
N total (mg/kg)	9.25 $\pm$ 2.28 a	7.50 $\pm$ 0.64 a	11.25 $\pm$ 0.47 a	2.94 0.1106	3.24 0.1095
P (mg/kg)	6.75 $\pm$ 0.47 a	3.25 $\pm$ 0.25 b	4.25 $\pm$ 0.25 b	25.71 <b>0.0003</b>	0.41 0.5419

**Table 2-3** Ectomycorrhiza status, chlorophyll status, and weight of *Pinus elliotii* var. *densa* seedlings transplanted to three flatwoods sites with different adult pine densities at the Archbold Biological Station, Florida. Values are mid-point means  $\pm$  SE after ANCOVA. Within a row, values followed by the same letter are not significantly different at  $P \leq 0.05$ . All variables except root/shoot ratio were significantly affected by pine density at a Bonferroni-corrected  $P \leq 0.05/14 = 0.0036$  among groups at Bonferroni-corrected  $P \leq 0.05/9 = 0.0055$

Response variable	Low pine density	Medium pine density	High pine density	F/P effect of pine density	F/P effect of days after transplant (covariate)
ECM root tips (%)	29.50 + 1.79/-1.76 a	18.08 + 1.53/-1.48 b	21.33 + 1.64/-1.59 b	12.57 <0.0001	48.29 <0.0001
Number of ECM morphotypes	1.11 $\pm$ 0.04 b	1.08 $\pm$ 0.04 b	1.46 $\pm$ 0.05 a	18.67 <0.0001	0.51 0.4760
Number of morphotypes/ECM tip	0.033 + 0.004/-0.003 c	0.060 + 0.007/-0.006 b	0.095 + 0.001/-0.010 a	20.12 <0.0001	46.37 <0.0001
Total chlorophyll concentration ( $\mu\text{g/g}$ )	536.39 $\pm$ 15.89 a	455.20 $\pm$ 16.26 b	580.42 $\pm$ 16.07 a	15.40 <0.0001	0.32 0.5712
Chlorophyll $\alpha$ concentration ( $\mu\text{g/g}$ )	434.10 $\pm$ 11.93 a	368.85 $\pm$ 12.21 b	460.25 $\pm$ 12.07 a	15.00 <0.0001	0.02 0.8755
Chlorophyll $\beta$ concentration ( $\mu\text{g/g}$ )	102.41 $\pm$ 4.28 b	86.45 $\pm$ 86.45 c	120.30 $\pm$ 4.33 a	15.07 <0.0001	2.78 0.0969
Chlorophyll $\alpha/\beta$ ratio	4.44 $\pm$ 0.08 a	4.40 $\pm$ 0.08 a	3.95 $\pm$ 0.07 b	11.95 <0.0001	15.18 0.0001
Total chlorophyll content ( $\mu\text{g}$ )	146.68 + 6.94/-6.63 a	99.74 + 4.82/-4.59 b	108.26 + 5.18/-4.94 b	19.03 <0.0001	24.66 <0.0001
Needle dry weight (mg)	89.39 + 3.09/-2.99 a	80.74 + 2.87/-2.77 a	62.50 + 2.19/-2.12 b	28.73 <0.0001	51.04 <0.0001

Table 2-3 (Continued)

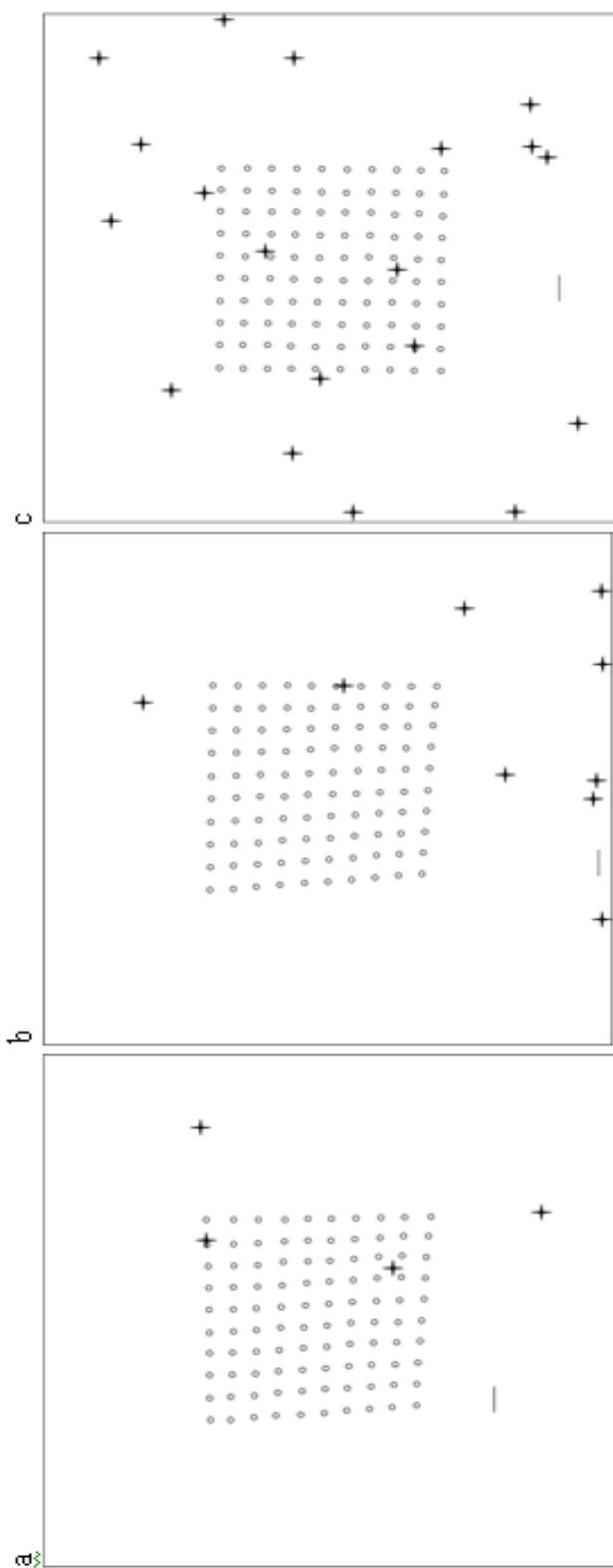
Response variable	Low pine density	Medium pine density	High pine density	<i>F/P</i> effect of pine density	<i>F/P</i> effect of days after transplant density (covariate)
Stem dry weight (mg)	20.50 + 0.68/-0.66 a	20.42 + 0.69/-0.67 a	15.75 + 0.53/-0.51 b	20.65 <0.0001	76.44 <0.0001
Root dry weight (mg)	35.04 + 1.42/-1.36 a	31.40 + 1.30/-1.25 a	24.46 + 1.00/-0.96 b	21.13 <0.0001	54.25 <0.0001
Total dry weight (mg)	146.18 + 4.82/-4.66 a	133.62 + 4.50/-4.35 a	103.82 + 3.45/-3.33 b	29.44 <0.0001	63.82 <0.0001
Root/shoot ratio	0.42 ± 0.01 a	0.42 ± 0.01 a	0.42 ± 0.01 a	0.06 0.9387	3.36 0.0679
Total number of root tips	127.17 + 6.05/-5.77 a	120.22 + 5.84/-5.56 a	81.62 + 3.90/-3.72 b	26.61 <0.0001	23.54 <0.0001

**Table 2-4** Ectomycorrhiza status, chlorophyll status, and weight of *Pinus elliottii* var. *densa* seedlings experiencing different categories of shade. Values are means  $\pm$  SE. Within a row, values followed by the same letter are not significantly different at  $P = 0.05$ . Probabilities in bold indicate a significant difference among shade categories at Bonferroni-corrected  $P \leq 0.05/13 = 0.0038$

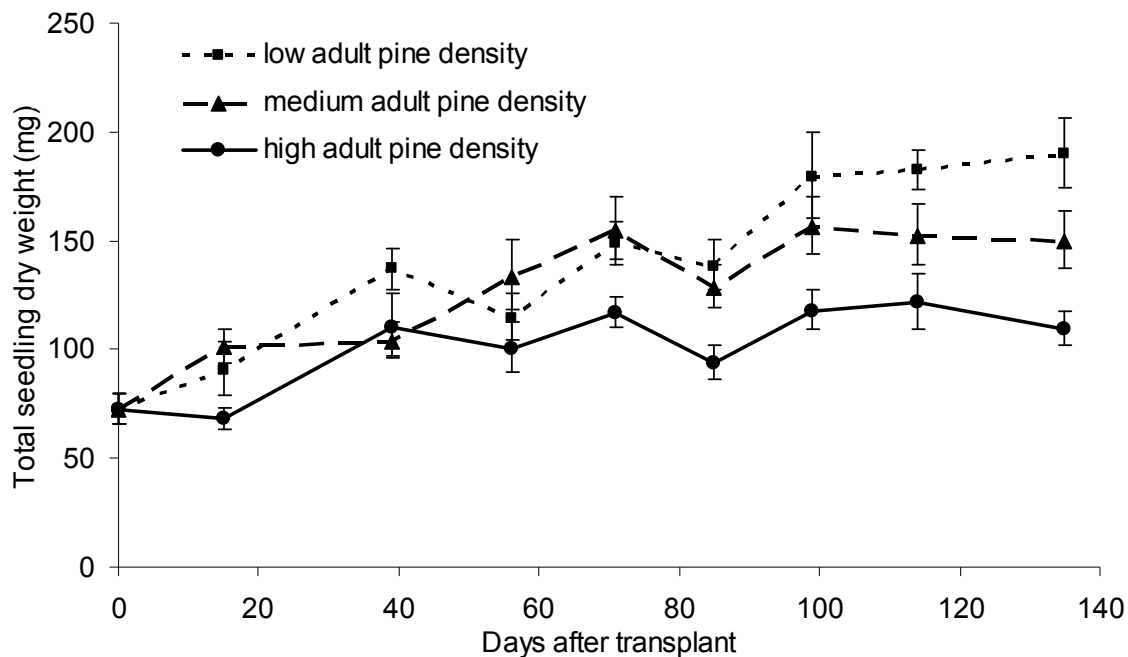
Response variable	No shade	Low shade	Medium shade	High shade	Kruskal-Wallis statistic / $P$
ECM root tips (%)	27.49 $\pm$ 3.05 a	21.54 $\pm$ 1.76 a	22.35 $\pm$ 1.79 a	25.63 $\pm$ 1.97 a	2.28 0.0798
Total chlorophyll concentration ( $\mu\text{g/g}$ )	412.80 $\pm$ 23.03 c	488.56 $\pm$ 15.03 bc	541.43 $\pm$ 16.54 b	634.26 $\pm$ 18.31 a	49.2597 <b>&lt;0.0001</b>
Total chlorophyll content ( $\mu\text{g}$ )	104.22 $\pm$ 7.82 b	118.24 $\pm$ 7.00 b	139.94 $\pm$ 6.28 a	152.65 $\pm$ 8.83 a	27.0823 <b>&lt;0.0001</b>
Chlorophyll <i>a</i> concentration ( $\mu\text{g/g}$ )	338.04 $\pm$ 18.33 c	395.86 $\pm$ 11.69 bc	434.18 $\pm$ 12.44 b	500.42 $\pm$ 12.98 a	46.6097 <b>&lt;0.0001</b>
Chlorophyll <i>b</i> concentration ( $\mu\text{g/g}$ )	74.84 $\pm$ 5.51 c	92.81 $\pm$ 3.90 bc	107.37 $\pm$ 4.42 b	133.98 $\pm$ 5.97 a	50.7703 <b>&lt;0.0001</b>
Chlorophyll <i>a/b</i> ratio	4.72 $\pm$ 0.14 a	4.45 $\pm$ 0.07 ab	4.26 $\pm$ 0.07 bc	3.96 $\pm$ 0.12 c	22.8090 <b>&lt;0.0001</b>
Needle dry weight (mg)	94.92 $\pm$ 4.84 a	80.36 $\pm$ 3.57 b	91.13 $\pm$ 7.96 ab	75.03 $\pm$ 3.03 b	11.5847 0.0089
Stem dry weight (mg)	24.3 $\pm$ 1.40 a	18.90 $\pm$ 0.72 b	20.76 $\pm$ 0.88 ab	18.29 $\pm$ 0.88 b	14.3015 <b>0.0025</b>
Root dry weight (mg)	41.49 $\pm$ 3.06 a	32.63 $\pm$ 1.66 ab	32.90 $\pm$ 1.39 ab	28.57 $\pm$ 1.38 b	13.0314 0.0046
Total dry weight (mg)	160.72 $\pm$ 8.65 a	131.89 $\pm$ 5.53 b	144.80 $\pm$ 8.89 ab	121.89 $\pm$ 4.82 b	13.2332 0.0042
Root/shoot ratio	0.45 $\pm$ 0.01 a	0.42 $\pm$ 0.01 a	0.42 $\pm$ 0.01 a	0.40 $\pm$ 0.01 a	4.3796 0.2233
Total number of root tips	152.94 $\pm$ 11.51 a	114.50 $\pm$ 6.23 ab	120.47 $\pm$ 5.97 ab	111.67 $\pm$ 6.01 b	8.8808 0.0309

**Table 2-5** Ectomycorrhiza status, chlorophyll status, and weight of *Pinus elliotii* var. *densa* seedlings experiencing different surrounding grass densities in flatwoods sites. Values are means  $\pm$  SE. Within a row, values followed by the same letter are not significantly different at  $P = 0.05$ . Probabilities in bold indicate a significant difference among grass densities at Bonferroni-corrected  $P \leq 0.05/13 = 0.0038$

Response variable	No grass	Medium grass density	High grass density	Kruskal-Wallis statistic / $P$
ECM root tips (%)	24.61 $\pm$ 1.26 a	23.86 $\pm$ 2.59 a	18.71 $\pm$ 2.13 a	4.4818 0.1064
Total chlorophyll concentration ( $\mu\text{g/g}$ )	519.72 $\pm$ 11.99 a	506.25 $\pm$ 25.30 a	561.71 $\pm$ 21.75 a	3.4105 0.1817
Total chlorophyll content ( $\mu\text{g}$ )	138.11 $\pm$ 5.16 a	114.70 $\pm$ 7.51 a	120.03 $\pm$ 7.49 a	6.3543 0.0417
Chlorophyll $\alpha$ concentration ( $\mu\text{g/g}$ )	419.03 $\pm$ 9.00 a	404.80 $\pm$ 19.01 a	447.53 $\pm$ 16.15 a	3.2753 0.1944
Chlorophyll $\beta$ concentration ( $\mu\text{g/g}$ )	100.81 $\pm$ 3.23 a	101.56 $\pm$ 6.63 a	114.30 $\pm$ 6.20 a	4.2819 0.1175
Chlorophyll $\alpha/\beta$ ratio	4.42 $\pm$ 0.06 a	4.21 $\pm$ 0.10 ab	4.12 $\pm$ 0.11 b	7.2369 0.0268
Needle dry weight (mg)	90.14 $\pm$ 0.60 a	77.97 $\pm$ 3.98 ab	74.26 $\pm$ 4.32 b	6.8978 0.0318
Stem dry weight (mg)	21.27 $\pm$ 0.60 a	19.30 $\pm$ 1.12 ab	17.10 $\pm$ 0.90 b	12.5615 <b>0.0019</b>
Root dry weight (mg)	34.66 $\pm$ 1.13 a	33.58 $\pm$ 2.29 ab	27.26 $\pm$ 1.64 b	10.5648 0.0051
Total dry weight (mg)	146.08 $\pm$ 5.46 a	130.85 $\pm$ 6.96 ab	118.62 $\pm$ 6.11 b	9.0556 0.0108
Root/shoot ratio	0.42 $\pm$ 0.008 a	0.44 $\pm$ 0.01 a	0.40 $\pm$ 0.01 a	4.5210 0.1043
Total number of root tips	127.35 $\pm$ 4.54 a	118.00 $\pm$ 8.05 ab	103.06 $\pm$ 7.62 b	6.3562 0.0417

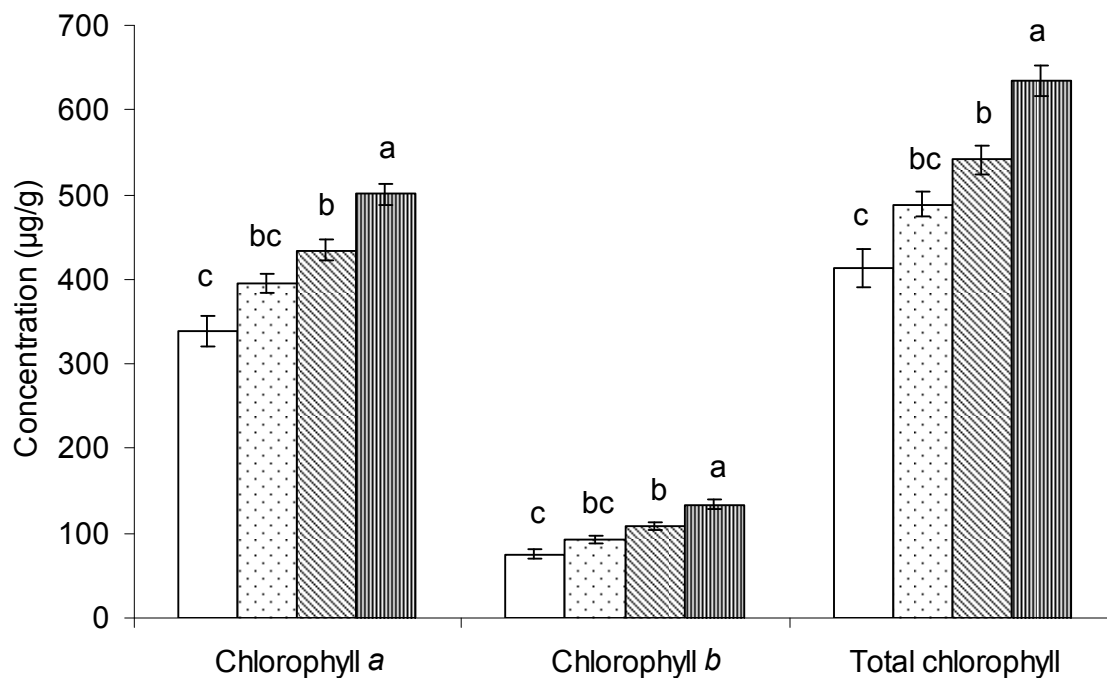


**Figure 2-1** Maps of *Pinus elliotii* var. *densa* seedlings (O symbols) transplanted to three flatwoods sites with different adult pine (✦ symbols) densities. Adult pines within a  $20 \times 20$  m square in which the planting grid was centered are shown. The scale bar in each map represents 1 m. (a) 4 adult pines/400 m<sup>2</sup>. (b) 9 adult pines/400 m<sup>2</sup>. (c) 19 adult pines/400 m<sup>2</sup>. (The grids in (a) and (b) were not perfectly square because of dense stands of *Serenoa repens* among which it was not possible to transplant pine seedlings)

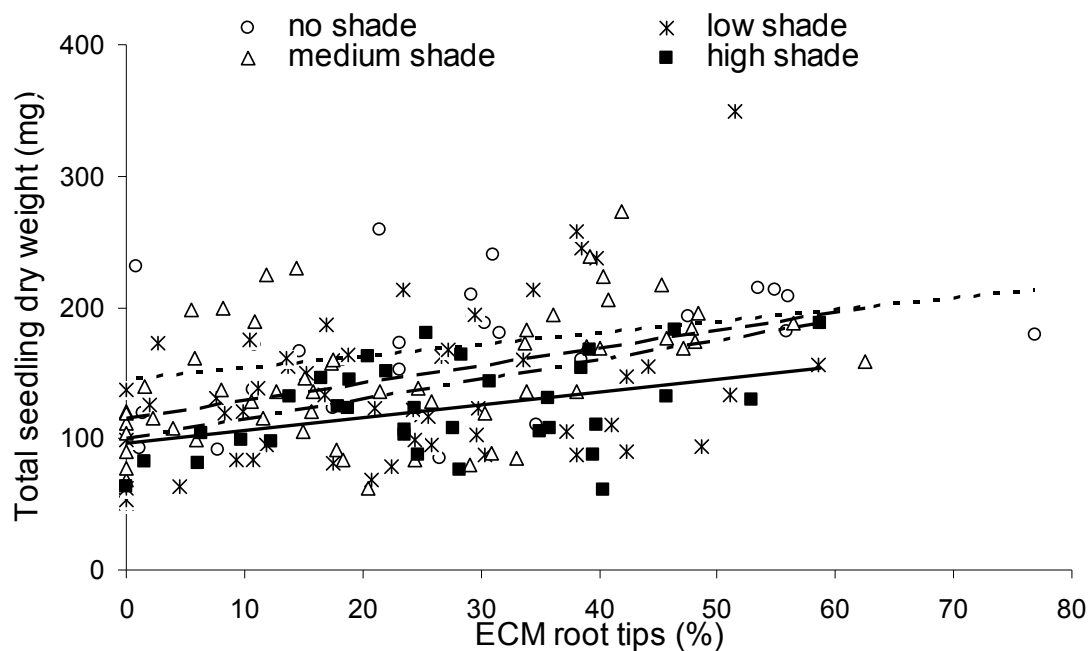


**Figure 2-2** Mean ( $\pm$  SE) total dry weight of *Pinus elliottii* var. *densa* seedlings transplanted to three Central Florida flatwoods sites with low (dotted line with squares), medium (dashed line with triangles) and high (solid line with circles) adult pine densities. Pine seedlings were excavated approximately every two weeks. The mid-point total dry weight after ANCOVA was higher in seedlings in the low and medium density sites than in the high adult pine density site ( $F_{2, 266} = 29.44$ ;  $P < 0.0001$ )

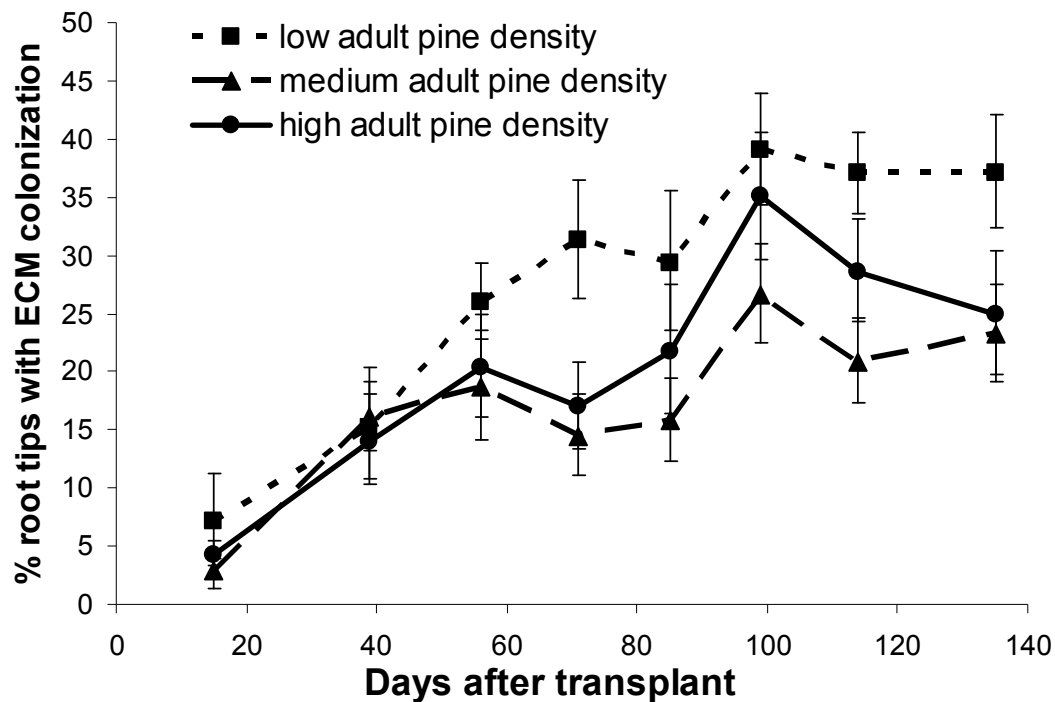




**Figure 2-3** Mean ( $\pm$  SE) chlorophyll *a*, chlorophyll *b* and total chlorophyll concentration of *Pinus elliottii* var. *densa* seedlings transplanted to flatwoods sites in which they experienced different categories of shade. Open bars = no shade; dotted bars = low shade; diagonal hatched bars = medium shade; vertical hatched bars = high shade. Within each response variable, bars topped by the same letter are not significantly different at Kruskal-Wallis  $P = 0.05$



**Figure 2-4** Linear regression of total dry weight of *Pinus elliotii* var. *densa* seedlings transplanted to flatwoods sites in which they experienced different categories of shade. Circles and dotted line = no shade; triangles and dotted-dashed line = low shade; stars and dashed line = medium shade; squares and solid line = full shade. Total dry weight is significantly associated with percentage ECM root tips ( $R^2 = 0.2062$ ; model  $F_{1, 114} = 29.62$ ,  $P < 0.0001$ ). The intercepts of the regressions of the four different shade categories differ ( $F_{3, 111} = 3.71$ ,  $P = 0.0138$ ), but the slopes of the regressions do not differ ( $F_{3, 108} = 0.22$ ,  $P = 0.8826$ )



**Figure 2-5** Mean ( $\pm$  SE) percent ectomycorrhizal (ECM) root tips of *Pinus elliotii* var. *densa* seedlings transplanted to three Central Florida flatwoods sites with low (dotted line with squares), medium (dashed line with triangles) and high (solid line with circles) adult pine densities. Pine seedlings were excavated approximately every two weeks. The mid-point percentage ECM root tips after ANCOVA was higher for seedlings in the low than in the medium and high adult pine density sites ( $F_{2, 247} = 12.57$ ;  $P$ -value  $< 0.0001$ )

## Chapter 3

### **Pine seedling ectomycorrhizas cannot ameliorate root competition in Florida flatwoods**

In plant communities, competitive and facilitative interactions with established vegetation influence the survival and establishment of tree seedlings or juveniles in the understory (Callaway and Walker 1997; Sthultz et al. 2007). For example, competition with the roots of surrounding adult trees for belowground resources reduces the growth of conifer seedlings (Kranabetter 2005). Canopy cover, by causing shade, negatively affects seedling growth (Dickie et al. 2005), but canopy cover also buffers extreme temperatures or help to maintain favorable soil moisture for seedlings (Maher et al. 2005; Nunez et al. 2009). In addition, by supporting mycorrhizal fungi, established vegetation provides beneficial, compatible mycorrhizal fungus inocula for establishing tree seedlings (Dickie et al. 2002).

In many terrestrial ecosystems, non-mycorrhizal, ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) plant species co-occur. Thus, in addition to ECM overstory plants, co-occurring non-mycorrhizal (Wolfe et al. 2008) or arbuscular mycorrhizal plants may affect establishing ECM plants (Sylvia and Jarstfer 1997). In a pine plantation, root length and ECM colonization of *Pinus elliottii* Engelm. var. *elliottii* was higher in the absence than in the presence of AM weeds (Sylvia and Jarstfer 1997). Similarly, the ECM species community on roots of *Pinus edulis* Engelm. was altered by neighbouring AM *Juniperus monopserma* (Engelm.) Sarg. but not by neighboring *Pinus ponderosa* P. and C. Lawson (Hubert and Gehring 2008). By altering the mycorrhizal status of

ectomycorrhizal plants, co-occurring AM plants reduce the ECM plants' performance (McHugh and Gehring 2006).

Several studies have shown that established vegetation enhancement of survival or growth of ECM seedlings is mediated by ectomycorrhizal fungi (van der Heijden and Horton 2009; McGuire 2007; Booth and Hoeksema 2010). *Quercus rubra* L. seedlings had higher ectomycorrhizal colonization when growing near established *Quercus montana* Willdenow. than near *Acer rubrum* L. Proximity to *Q. montana* also enhanced foliar N and P concentrations of *Q. rubra* seedlings (Dickie et al. 2002). Adults not only provide ECM inocula, but also may transfer C or nutrients to seedlings through inter-connecting mycorrhizal hyphae, which potentially is especially important for seedlings growing in shady understories (Simard et al. 1997b). Common mycorrhizal networks among adult conifers and seedlings enhanced the growth of *Pinus strobus* L. seedlings (Booth 2004). In a mature forest, however, mycorrhizal networks among adult conifers and seedlings of *Tsuga heterophylla* Raf. or *Picea glauca* × *Picea sitchensis* (Moench) Voss did not confer growth benefits to seedlings, because root competition was the most important determinant of seedling growth (Kranabetter 2005).

Because ECM fungi are important for plant performance (Read 1998; Smith and Read 2008), limited availability of mycorrhizal inocula can preclude the establishment of mycorrhizal seedlings. Early attempts to establish pines in AM grasslands failed unless ECM fungi were introduced (Allen 1991; Read 1998). ECM colonization of pine seedlings can be limited when AM plants dominate (Haskins and Gehring 2005; Dickie et al. 2002). Absence of appropriate mycorrhizal inocula also can limit invasion of heathlands by mycorrhizal plant species (Collier and Bidartondo 2009). At increasing

distances from adult trees, ECM colonization or ECM morphotype richness of young seedlings may be reduced (Dickie et al. 2005; Kranabetter 1999).

*Pinus elliottii* var. *densa* Little & Dorman, a pine species native to Central and South Florida, is a keystone species in several ecosystems such as pine rocklands and flatwoods (Snyder et al. 1990; Abrahamson and Hartnett 1990). The pine canopy in flatwoods ranges from areas of dense canopy to areas without pines (see Fig. 3.1). Changes in fire regimes caused by human activity have reduced pine survival in open areas (Wade et al. 1980). Those areas may have remained open because of little seed arrival (Golley et al. 1994) or possibly because of low availability of suitable sites for pine seedling recruitment, particularly if understory plants are dense (Wade et al. 1980).

In a previous study (Chapter 2), I found that ECM colonization of *Pinus elliottii* var. *densa* seedlings was not influenced by adult pine density, which suggests that ECM inocula are widespread in flatwoods. On the other hand, a complete absence of adult pines might conspicuously limit ECM inocula. Because oaks often are present in flatwoods devoid of pines, however, oaks might provide ECM inocula for recruiting pine seedlings. Indeed, unrelated plant species provided ECM fungi for seedlings of species such as pines (Bai et al. 2009) or oaks (Richard et al. 2009), thus enhancing their survival or growth. Other understory plants in flatwoods, however, such as arbuscular mycorrhizal or non-mycorrhizal plants may negatively affect recruiting pine seedlings through belowground competition.

The objective of this field study was to determine how the ECM fungus inocula available to pine seedlings in flatwoods in the presence versus the absence of adult pines affects pine seedling performance. Because of potential mycorrhizal connections among adults and seedlings within pine stands, and because of competition with arbuscular

mycorrhizal fungi beyond pine stands, I expected that seedlings transplanted within pine stands would perform better than seedlings transplanted beyond pine stands.

## **Materials and Methods**

### *Site description*

This experiment was conducted at Archbold Biological Station (Archbold), Highlands County, Florida, USA (27°11'N lat., 81°21'W long.). The climate in central Florida is characterized, by dry, mild winters and wet, hot summers (Chen and Gerber 1990).

During the experiment (from October 8, 2008 to November 2<sup>nd</sup>, 2009), the mean average air temperature was 21.5°C, and temperature ranged from a minimum of -9.5°C on January 22<sup>nd</sup>, 2009 at 7:15 am to a maximum of 37.4°C on May 9, 2009 at 5 pm. The total rainfall during the experiment was 896 mm. From October 8 to May 12, 2009 the rainfall was 183 mm, from May 13 to September 28, 2009 it was 702 mm, and from September 29 to November 2<sup>nd</sup>, 2009 it was 11 mm.

At Archbold, I selected eight stands within flatwoods ecosystems in a split-plot design. Four stands were selected within *Pinus elliottii* var. *densa* stands, and a paired area devoid of adult pines was selected 50-80 meters beyond each pine stand, for a total of 8 experiment stands (Fig. 3.1). All stands were chosen within the same fire management unit. Between 1967 and 2008, this unit burned in January 1980, March 1990, June 1995, February 2001, and July 2007. Dominant plant species composition of each stand is listed in Table 3.1.

### *Experiment design*

The availability of ECM inoculum and performance of *Pinus elliottii* var. *densa* seedlings in the absence or presence of adults was investigated in a split-plot, factorial, three-factor field experiment. The three factors consisted of the split-plot factor of presence/absence of adult pines, of three levels of ECM and AM fungus inocula (autoclaved root pieces, fresh ECM root pieces, ECM and AM hyphae from the surrounding vegetation), and two levels of soil origin (soil collected within or beyond pine stands). Each treatment combination was replicated 16 times for a total of 192 seedlings.

Pine seedlings were grown in 9 cm internal diameter, 20 cm-long PVC pipes. To allow for drainage through the pipes, a 30  $\mu$ m nylon mesh was glued across the bottom of each pipe. Four 5-cm diameter holes (45 % of the pipe wall area) were drilled into the sides of one-third of the pipes to allow for ingrowth of mycorrhizal hyphae from surrounding vegetation. Each hole was covered with a piece of GORE-TEX<sup>®</sup> (W. L. Gore & Associates Inc, Newark, DE, USA), a hydrophobic membrane through which mycorrhizal hyphae can grow but direct water and dissolved nutrient movement is prevented (Mäder et al. 1993). Intact PVC pipes were used for the autoclaved roots and for the fresh ECM root treatments.

At each stand with and without adult pines, 10 cm-diameter holes in the ground were dug with a post-hole digger, and intact or drilled PVC pipes were placed in the holes to a depth of 18 cm. Any space between the outside of the pipes and the soil was refilled with soil removed from the hole. Six pipes were arranged at equal distances around the circumference of a circle of 1 m diameter, with four such sets located at the corners of a 5  $\times$  5 m square. Treatments were arranged randomly around each circle (Fig. 3.2).



### *Soil*

Soil was collected to a depth of 15-20 cm either within or beyond four flatwoods pine stands at Archbold. These two soils were sifted separately through a 4 mm sieve, each homogenized, and autoclaved three times at 121°C and 1.4 kg/cm<sup>2</sup> for 1 hour, 24 hours apart. After autoclaving, the soils from within pine stands contained 2.3 % organic matter, 40.8 kg/A estimated nitrogen release, 2.0 mg/kg weak Bray P, 4.0 mg/kg K, 8.0 mg/kg Mg, 28.0 mg/kg Ca, 2.0 mg/kg Zn, 4.0 mg/kg Mn, 12.0 mg/kg Fe, 0.1 mg/kg Cu, had a cation exchange capacity of 0.2 meq/100g, and had a pH of 3.5. The soil from beyond pine stands contained 2.7 % organic matter, 44.5 kg/A estimated nitrogen release, 2.0 mg/kg P, 3.0 mg/kg K, 6.0 mg/kg Mg, 12.0 mg/g Ca, 1.3 mg/kg Zn, 2.0 mg/kg Mn, 12.0 mg/kg Fe, 0.1 mg/kg Cu, had a cation exchange capacity of 0.1 meq/100g, and had a pH of 3.5 (determined by the A & L Southern Agricultural Laboratories, Inc. Deerfield Beach, USA). Buried pipes were filled with 900 ml of either of the autoclaved soils, covered with an aluminium foil, and left for 6 months in the field prior to seedling transplant to allow fungus hypha ingrowth

### *Mycorrhiza inoculum and seedling transplant*

To serve as mycorrhizal inoculum, ectomycorrhizal roots from adult *Pinus elliottii* var. *densa* trees were collected at Archbold two weeks before pine transplant. Roots were collected from multiple trees in flatwoods by removing the litter layer close to the base of the tree, and searching for pine roots to a depth of 5 cm in the soil around each tree. Pine roots were heavily colonized by ECM fungi. Because the ECM root tips presented different mantle characteristics, the ECM community on the root inoculum likely was diverse. In the lab, all fresh ECM roots were mixed, washed in distilled water, and cut

into 1-2 cm long pieces. A portion of the roots was autoclaved three times at 121°C at 1.4 kg/cm<sup>2</sup> for 30 minutes, 24 hours apart, to serve as a control for the addition of organic matter to inoculated pipes. Autoclaved roots were added to intact pipes and to the drilled pipes. To restore microbial populations in pipes receiving autoclaved roots, a microbial filtrate was prepared by soaking 38.4 g of fresh ECM roots in 800 ml distilled water for 24 hours. To exclude fungal propagules, the microbial filtrate was filtered three times through Whatman #1 filter paper, once through a 41 µm Nylon Net filter (Millipore, Billerica, MA, USA), and once through an 11 µm Nylon Net filter (Millipore).

*Pinus elliotii* var. *densa* seeds, donated by the Andrews Nursery (Florida Division of Forestry, Chiefland, FL, USA), were soaked in distilled water for 24 hours, and incubated at 4°C for 7 days. Seeds were germinated in coarse silica sand (L 6-20, Standard Sand and Silica Co., Miami, FL, USA) in small individual containers in an ambient temperature shadehouse at the University of Miami. Emergent seedlings were watered daily with tap water and were not fertilized.

On March 19 2009, one 6 week-old pine seedling was transplanted to each PVC pipe in the field. A hole was made in the soil with a 2-cm diameter cork-borer and 0.3 g of ECM root fragments (either fresh or autoclaved) were deposited at the bottom of the hole. The bare roots of the seedling were placed atop the mycorrhizal root inoculum and covered with autoclaved soil. Six ml of microbial filtrate were added to seedlings that received autoclaved ECM roots. At the same time, seedlings inoculated with fresh roots received the same amount of distilled water. To ensure ectomycorrhizal colonization from fresh ECM roots, I added 0.18 g of fresh ECM pine roots 40 days after transplant by placing them in a 1 cm-deep hole near the stem of each seedling (again including appropriate autoclaved root controls and microbial filtrates).

All seedlings were protected against herbivores with a 0.6 cm-mesh hardware cloth cage fixed to bamboo stakes. Seedlings were watered with 250 ml of tap water, 2-3 times a week in the absence of rainfall for 3 or more consecutive days.

### *Seedling performance*

At the time of transplant, pine seedlings had an average stem diameter of  $0.71 \pm 0.01$  (SE) mm and an average longest needle length of  $3.44 \pm 0.03$  cm with no differences in these variables among treatments. After 30 weeks in the field, seedlings were harvested because I detected significant differences in stem diameter among treatments.

At harvest, all pipes were excavated and brought to the lab where they were emptied of soil in order to facilitate recovery of seedlings' entire root systems. Needles were separated from shoots and weighed fresh. Chlorophyll concentration in needles was measured by cutting 0.2 g of needles into 0.5 cm pieces, and incubating them for 48 hours in 80 % acetone in the dark (Proctor, 1981). The concentration of chlorophyll *a* and *b* was determined by measuring the absorbance at 645 and 663 nm (Porra, 2002). The remaining needles and the stems were dried at 50°C for 1 week, and their weights were determined (needle dry weight was adjusted for removal of needles for chlorophyll analysis). Dried needles were finely ground in liquid nitrogen and sent to A & L Southern Agricultural Laboratories for mineral nutrient analysis (N, P, K, Mg, Ca, Fe, Mn, B, Cu, Zn). Nutrients were extracted by dry ashing followed by dissolution in acid. Nitrogen was extracted with a modified Kjeldahl method. Phosphorus was measured colorimetrically, and other nutrients were measured by inductively coupled plasma spectroscopy. Because needle samples were small, the needles of seedlings within treatment groups were composited for mineral nutrient quantification based on similar

chlorophyll concentration. Root systems were gently washed to remove all soil, and were weighed fresh. The level of ECM colonization was determined by examination of root tips with a dissecting microscope by using the gridline intersect method as described in Brundrett et al. (1996). I did not morphotype ECM root tips. Roots were dried at 50°C to constant weight and weighed dry.

In a greenhouse experiment (Chapter 4), the hydrophobic membrane was an effective barrier against penetration by roots. In the present field experiment, however, roots from the surrounding vegetation penetrated the membrane and colonized many pipes. The amount of extraneous roots in each pipe was categorized into four categories: absent, low, medium, or high as pipes were emptied to remove seedlings.

#### *Statistical analyses*

Because extraneous roots penetrated the PVC pipes, the numbers of pipes of each treatment penetrated by roots were compared with Chi-square tests. Likewise, differences in seedling survival among treatments were compared with Chi-square tests.

Total chlorophyll concentration and chlorophyll *a* concentration were square root transformed for normality and homoscedasticity, while chlorophyll *b* concentration and chlorophyll content were  $\log_{10}$  transformed. No transformation of chlorophyll *a/b* created normality, and thus, analysis was performed on ranked chlorophyll *a/b* ratios (Conover and Iman 1981). Effects of treatments on seedling chlorophyll status were determined by separate split-plot ANCOVAs with two fully-crossed sub-plot factors with percentage ECM root tips as the covariate.

Needle dry weight, stem dry weight, shoot dry weight, root dry weight and total dry weight of seedlings were  $\log_{10}$  transformed for normality and homoscedasticity. No

transformation of root/shoot ratio created normality, and thus, analysis was performed on ranked root/shoot ratios. Treatment effects were determined by separate split-plot ANCOVAs with two fully-crossed sub-plot factors with percentage ECM root tips as the covariate. Effects of treatments on chlorophyll and growth response variables were considered significant at Bonferroni-corrected  $P = 0.05/11 = 0.0045$ .

Because of small sample size (because samples had to be composited) for mineral nutrient data, the mineral nutrient concentrations and contents of seedlings within treatment groups were averaged within each stand (= treatment  $\times$  stand averages,  $n = 48$ ). The effects of treatments on stand averages were then tested with separate split-plot ANCOVAs on ranked data with root dry weight as the covariate for mineral nutrient concentrations and percentage ECM root tips as the covariate for mineral nutrient contents. Effects of treatments on mineral nutrient concentrations and nutrient ratios were considered significant at Bonferroni corrected  $P = 0.05/13 = 0.00385$ , and at  $P = 0.05/10 = 0.005$  for mineral nutrient contents.

Spearman's rank correlations among percentage ECM root tips, needle dry weight, and total chlorophyll concentration were determined across all individual seedlings, while correlations among those response variables and nutrient concentrations were determined for treatment  $\times$  stand averages. Probabilities were Bonferroni-corrected for multiple correlations.

To distinguish between the effects of ectomycorrhizas and extraneous roots on seedling dry weight, least-squares linear regression between total dry weight and percentage ECM root tips or mean category of extraneous roots in pipes were performed on treatment  $\times$  stand averages. Least-squares linear regressions of ranked total dry weight, ranked percentage ECM root tips and ranked foliar P concentration against

ranked mean category of extraneous roots also were performed separately for each mycorrhizal inoculum treatment.

Several approaches were used to determine whether or not established adult pines, mycorrhizal inocula and soil treatments affected ECM colonization of seedlings. Because seedling size may influence the amount of ectomycorrhizas that a seedling can support, effects of treatments on percentage ECM root tips were tested with a split-plot, two-factor factorial ANCOVA with root dry weight as the covariate. The relationship between seedling root dry weight and percentage ECM root tips was examined with least-squares linear regression, separating seedlings into those transplanted within or beyond pine stands for comparison of regression lines. The effect of absence versus presence of adult pines on stand mean ECM colonization (ignoring mycorrhizal inoculum or soil origin treatments) also was determined by paired t-tests between the four stands with and the four stands without adult pines. Subsequently, three paired t-tests were used to compare ECM of seedlings in absence or presence of adult pines separately for each mycorrhizal inoculum level.

For all transformed response variables, back-transformed values are shown in tables and figures.

## **Results**

### *Extraneous roots in pipes*

At the time of harvest, pipes were penetrated by extraneous roots from the surrounding vegetation. Roots penetrated into intact pipes through the bottom mesh, while roots in drilled pipes penetrated the hydrophobic membrane. Neither soil type (Chi-square

statistic  $< 0.01$ ,  $P = 0.95$ ) nor absence versus presence of adult pines (Chi-square statistic = 0.18,  $P = 0.67$ ) affected the penetration of pipes by roots. Drilled pipes, however, were penetrated by extraneous roots much more than intact pipes (Chi-square statistic = 108.57,  $P < 0.0001$ ). Only 14.2 % of all intact pipes were penetrated by extraneous roots, versus 96.5 % of drilled pipes (Fig 3.3).

#### *Pine seedling survival and performance*

Presence versus absence of adult pines did not affect survival of seedlings (Chi-square statistic = 1.85,  $P = 0.17$ ), nor did soil type (Chi-square statistic = 1.78,  $P = 0.18$ ). Pine seedlings in the drilled pipes, however, had greater mortality than seedlings in intact pipes (Chi-square statistic = 9.95,  $P = 0.007$ ). In intact pipes, 100 % of seedlings survived, while 91.9 % of seedlings in drilled pipes survived until the harvest (Fig 3.4).

No treatment and no interaction among treatments significantly affected any chlorophyll parameter. Overall, pine seedlings had an average concentration of 253.22  $\mu\text{g/g} \pm 7.70$  (mean  $\pm$  SE) of chlorophyll *a*, 44.32  $\mu\text{g/g} \pm 1.49$  of chlorophyll *b*, 297.48  $\mu\text{g/g} \pm 9.13$  of total chlorophyll, a chlorophyll *a/b* ratio of  $5.76 \pm 0.04$  and a chlorophyll content of 593.61  $\mu\text{g} \pm 41.75$ . The ECM covariate, however, had a significant effect on chlorophyll *a* concentration ( $F_{1, 128} = 18.56$ ,  $P < 0.0001$ ), chlorophyll *b* concentration ( $F_{1, 128} = 10.50$ ,  $P = 0.0015$ ), total chlorophyll concentration ( $F_{1, 128} = 16.72$ ,  $P = 0.0001$ ), chlorophyll content ( $F_{1, 128} = 44.76$ ,  $P < 0.0001$ ) and chlorophyll *a/b* ratio ( $F_{1, 128} = 31.20$ ,  $P < 0.0001$ ). The regression relationship with percentage ECM root tips was positive for chlorophyll *a* concentration ( $F_{1, 174} = 14.18$ , Bonferroni-corrected  $P$ -value ( $P_{Bc}$ ) = 0.0022,  $R^2 = 0.0754$ ), chlorophyll *b* concentration ( $F_{1, 174} = 8.55$ ,  $P_{Bc} = 0.0429$ ,  $R^2 = 0.0469$ ), total chlorophyll concentration ( $F_{1, 174} = 12.63$ ,  $P_{Bc} = 0.0055$ ,  $R^2 = 0.0677$ ), chlorophyll *a/b*

ratio ( $F_{1,174} = 24.37$ ,  $P_{Bc} < 0.0011$ ,  $R^2 = 0.1229$ ) and chlorophyll content ( $F_{1,174} = 32.67$ ,  $P_{Bc} < 0.0011$ ,  $R^2 = 0.1581$ ), although relatively little variance was explained.

Total chlorophyll concentration was positively correlated with needle dry weight (Spearman's rank correlation coefficient ( $r_S$ ) = 0.3044,  $P_{Bc} < 0.0003$ ), K concentration ( $r_S$  = 0.5167,  $P_{Bc} = 0.0462$ ), N concentration ( $r_S = 0.6743$ ,  $P_{Bc} < 0.0033$ ) and P concentration ( $r_S = 0.7051$ ,  $P_{Bc} < 0.0033$ ).

Except for root/shoot ratio, all growth variables of seedlings were affected by mycorrhizal inocula (Table 3.2). Over all soil origin and adult pine treatments, needle dry weight was lower in drilled pipes (0.38 g +0.03/-0.02) than in intact pipes with either autoclaved (0.55 g +0.04/-0.03) or fresh ECM root inoculum (0.53 g  $\pm$  0.03). Stem dry weight, total shoot dry weight, and root dry weight also were all lower in drilled than in intact pipes (Table 3.3). An interaction between absence/presence of adult pines and mycorrhizal inocula significantly affected total dry weight of seedlings (Table 3.2;  $F_{2,128} = 7.03$ ;  $P = 0.0031$ ). Beyond pine stands, total dry weight was reduced in drilled pipes, while it was not reduced in drilled pipes within pine stands (Fig. 3.5). Root/shoot ratio was the only variable affected by soil origin (Table 3.2). Root/shoot ratio was higher in soil collected within (0.64  $\pm$  0.02) than beyond (0.54  $\pm$  0.02) pine stands. Split-plot ANCOVA detected no growth variable as significantly affected by absence versus presence of adult pines (Table 3.2), supported by a non-significant difference in total dry weight among sites after a paired t-test (t statistic = 1.23,  $P = 0.2298$ ). Paired t-tests indicated a tendency of seedling stems and shoots to be heavier in intact pipes beyond than within pine stands, but this was not significant after Bonferroni correction (stem dry weight: t-statistic = 3.25,  $P = 0.0140$ ; shoot dry weight: t-statistic = 2.38,  $P = 0.0487$ ).



Very few treatments affected foliar mineral nutrient concentrations.

Concentrations of N, K, Mg, Ca, Fe, B, Cu and Zn were not affected by treatments (Table 3.4). On average, seedling needles contained  $0.63\% \pm 0.03$  N,  $0.52\% \pm 0.01$  K,  $0.21\% \pm 0.003$  Mg,  $0.31\% \pm 0.01$  Ca,  $128.78\ \mu\text{g/g} \pm 7.46$  Fe,  $22.53\ \mu\text{g/g} \pm 1.90$  B,  $8.05\ \mu\text{g/g} \pm 0.71$  Cu and  $425.57\ \mu\text{g/g} \pm 9.77$  Zn. Mycorrhizal inocula had a significant effect on P concentration. Phosphorus concentration was higher in seedlings in drilled pipes ( $0.076\% \pm 0.004$ ) than in intact pipes with autoclaved or fresh ECM roots ( $0.056\% \pm 0.004$  and  $0.048\% \pm 0.003$ , respectively). Pine seedlings growing in soil collected within pine stands had higher Mn concentration ( $81.23\ \mu\text{g/g} \pm 2.66$ ) than in soil collected beyond pine stands ( $34.50\ \mu\text{g/g} \pm 2.06$ ). After Bonferroni correction, soil had a similar effect on foliar Mn content ( $F_{1,15} = 24.62$ ,  $P < 0.0001$ ), while absence/presence of adults significantly affected foliar Zn content (absence of adults:  $247.11\ \mu\text{g} \pm 10.64$ ; presence of adults:  $298.15\ \mu\text{g} \pm 11.22$ ;  $F_{1,3} = 99.81$ ,  $P = 0.0021$ ). Ratios of N:P, N:K and K:P were not affected by treatments (Table 3.4). Overall, pine seedlings had an N:P ratio of  $10.28 \pm 0.37$ , an N:K ratio of  $1.19 \pm 0.05$  and a K:P ratio of  $9.37 \pm 0.29$ .

The effect of extraneous roots on seedling performance was examined with least-squares linear regressions. When treatments were averaged within stands, total dry weight was not related to percentage ECM root tips (regression coefficient = 0.0307,  $P = 0.08146$ ; Fig. 3.6a) although it was significantly negatively associated with mean category of extraneous roots (regression coefficient = -0.5059,  $P = 0.0005$ ;  $R^2 = 0.2397$ ; Fig. 3.6b). Within intact pipes with autoclaved or fresh ECM roots, neither total dry weight ( $F_{1,30} = 0.72$ ,  $P = 0.4026$ ), percentage ECM root tips ( $F_{1,30} = 1.05$ ,  $P = 0.3142$ ) nor P concentration ( $F_{1,29} = 0.01$ ,  $P = 0.9235$ ) were associated with mean category of extraneous roots. In drilled pipes, however, stand mean total dry weight was negatively

associated with level of extraneous roots in pipes (regression coefficient = -1.7704,  $F_{1,14} = 14.00$ ,  $P_{Bc} = 0.0066$ ,  $R^2 = 0.5000$ ; Fig.3.7a). Stand mean percentage ECM root tips showed a similar relationship to total dry weight in drilled pipes (regression coefficient = -1.6482,  $F_{1,14} = 7.81$ ,  $P_{Bc} = 0.0429$ ,  $R^2 = 0.3580$ ; Fig. 3.7b). After Bonferroni correction, however, stand mean foliar P concentration was not associated with level of extraneous roots ( $F_{1,12} = 4.85$ ,  $P = 0.1440$ ,  $R^2 = 0.2877$ ; Fig. 3.7c).

### *Ectomycorrhiza formation*

A split-plot ANCOVA analysis with root dry weight as the covariate indicated that neither soil origin nor interactions among factors had significant effects on percentage ECM root tips (Table 3.5). Although pine seedlings in the presence of adult pines seemed to have a consistently higher percentage ECM root tips than seedlings in absence of adult pines (Fig. 3.8), the split-plot ANCOVA model did not detect a significant difference in percentage ECM root tips between stands with and without adult pines ( $F_{1,3} = 4.51$ ,  $P = 0.1238$ ), probably because of limited replication of sites. Overall, however, percentage ECM root tips of seedlings differed among mycorrhizal inocula (Table 3.5). Seedlings in drilled pipes had the highest mean percentage ECM root tips ( $55.35 \% \pm 2.18$ ), seedlings in intact pipes with fresh ECM root inoculum had an intermediate percentage ECM root tips ( $45.93 \% \pm 1.98$ ), and seedlings in intact pipes with autoclaved roots had the least ECM colonization ( $38.78 \% \pm 2.00$ ).

Across all seedlings, seedling root dry weight was positively associated with percentage ECM root tips (least-squares linear regression:  $F_{1,175} = 20.89$ ,  $P < 0.0001$ ), although root dry weight explained only 10 % of the variance in ECM ( $R^2 = 0.1067$ ). When the regressions were separated into pine seedlings in the absence versus presence

of adults, the slopes of both regressions did not differ (comparison of slopes:  $F_{1, 173} = 0.04$ ,  $P = 0.8370$ ), while the intercept of the regression was significantly higher in the presence (intercept = 71.57 %) than in the absence (intercept = 56.80 %) of adults (comparison of intercepts:  $F_{1, 174} = 26.83$ ,  $P < 0.0001$ ; Fig. 3.9).

After paired t-tests, the stand mean percentage ECM root tips of seedlings in the absence versus presence of adults was significantly different (t-statistic = -4.29, two-tailed  $P = 0.0003$ ). Over all soil origin and mycorrhizal inoculum treatments, seedlings beyond pine stands had  $38.75 \% \pm 2.10$  ECM root tips, while seedlings within pine stands had  $52.95 \% \pm 1.79$  ECM root tips. Within intact pipes with autoclaved roots, ECM fungi colonized  $30.68 \% \pm 3.22$  of seedling root tips beyond pine stands and  $47.29 \% \pm 2.80$  of root tips within pine stands (t-statistic = -2.77, two-tailed  $P = 0.0277$ ; Fig. 3.8). Within intact pipes with fresh ECM roots, percentage ECM root tips of seedlings did not differ beyond or within pine stands (absence of adults:  $41.06 \% \pm 3.19$ , presence of adults:  $50.67 \% \pm 2.79$ ; t-statistic = -1.27, two-tailed  $P = 0.2455$ ; Fig. 3.8). In drilled pipes, however, seedlings had a higher stand mean percentage ECM root tips within than beyond pine stands ( $49.48 \% \pm 3.56$  and  $60.90 \% \pm 2.80$ , respectively; t-statistic = -4.11, two-tailed  $P = 0.0045$ ; Fig. 3.8).

Across all seedlings, percentage ECM root tips was positively correlated with needle dry weight ( $r_S = 0.2458$ ,  $P_{Bc} = 0.0029$ ) and total chlorophyll concentration ( $r_S = 0.2737$ ,  $P_{Bc} = 0.0007$ ).

## Discussion

### *Extraneous roots*

Because pipes were left in the field for more than a year, the hydrophobic membrane covering openings in pipes was easily penetrated by roots from the surrounding vegetation, whether beyond or within pine stands. Thereby, the mycorrhizal inoculum factor potentially was confounded with belowground competition. *Serenoa repens* was the dominant understorey species in my flatwoods sites, but its roots, because they are coarse, did not penetrate the membrane. Extraneous roots were either woody with fine terminal roots, possibly from shrubs, or were graminoid roots probably from grasses. Within pine stands, pine roots also occurred in a few pipes, but they mainly penetrated pipes through the bottom mesh. Pine seedlings establishing naturally in flatwoods are likely to be exposed to competition with *S. repens* roots besides the interactions with roots observed in this study.

### *Pine seedling survival and performance*

Because I watered them regularly, pine seedlings had very high survival, with 97.25 % seedlings surviving overall. Survival of *Pinus elliottii* var. *densa* seedlings in normal field conditions can be lower. Indeed, one-year survival of naturally regenerating seedlings ranged from 4 % to 33 % within pine stands in flatwoods (Teague 2003). One hundred percent of pine seedlings survived in intact pipes, while fewer, 91.9 %, survived in drilled pipes (Fig. 3.4), probably because of a negative effect of extraneous roots potentially competing for water or mineral nutrients. Likewise, weeds competing for water, dramatically reduced seedling survival of *Quercus ilex* L. and *Pinus halepensis*

Mill. in Mediterranean ecosystems (Cuesta et al. 2010). In Mediterranean montane forests, maximum survival of *Quercus petraea* (Matt.) Liebl. and *Q. pyrenaica* Willd. seedlings three years after emergence was 8% (Rodriguez-Calcerrada et al. 2010). Because water availability can severely limit seedling survival in ecosystems subject to drought, root competition for water may exacerbate pine seedling mortality in flatwoods during dry periods.

No treatment or treatment interactions affected seedling chlorophyll parameters. Overall, pine seedlings had an average total chlorophyll concentration of 297  $\mu\text{g/g}$  (= 0.297 mg/g). In a study of *Pinus elliotii* in north Florida (variety not specified), newly emerged leaves on adult pines that were not fertilized had a chlorophyll concentration of 1.25 mg/g, while mature leaves from an NPK-fertilized plot had a chlorophyll concentration of 2.80 mg/g (Curran et al. 1995), values that are considerably higher than the those observed in my study. Six-month old seedlings grown in flatwoods soil in greenhouse conditions had about 320  $\mu\text{g/g}$  total chlorophyll concentration, while NPK fertilization increased it to nearly 700  $\mu\text{g/g}$  (Toledo et al. unpublished data). NPK fertilization clearly enhances both pine seedling and adult chlorophyll concentrations, also attested in this study by positive correlations between chlorophyll concentration and foliar N, P and K concentrations. Soil origin (soil collected from beyond pine stands contained slightly more organic matter and had a higher estimated N release), however, did not affect chlorophyll concentration. Although both the ANCOVA with percentage ECM root tips as a covariate and the Spearman's rank correlation suggested that ECM colonization elevates chlorophyll concentrations, percentage ECM root tips explained only from 4.69 to 15.81% of the variance in chlorophyll parameters. Even though shade (Chapter 2) and extensive ECM mycelia (Chapter 4) can affect pine seedling chlorophyll,

in this study none of the treatment factors or their interactions had a sufficiently strong effect to be detectable statistically.

Except for root/shoot ratio, all growth parameters of pine seedlings were lower in drilled than in intact pipes (Table 3.3). In particular, mean total dry weight was 29 % lower in drilled pipes than in intact pipes, a growth reduction that I attribute to the penetration of roots into the pipes. This was supported by a negative regression relationship between total dry weight and mean category of extraneous roots in pipes, both when the regressions were performed including all plot  $\times$  treatment averages (Fig. 3.6b) or only within drilled pipes (Fig. 3.7a). Thus, extraneous roots not only reduced survival of pine seedlings but also reduced their growth.

Belowground competition by co-occurring plant species commonly affects tree seedlings negatively, in particular in low fertility ecosystems, as indicated by an increase in seedling performance after release from root competition by trenching (Coomes and Grubb 2000; Coomes and Grubb 1998). Trenching also has favored the recruitment of conifer seedlings such as *Pinus strobus* L. and *Tsuga canadensis* L. (Toumey and Kienholz 1931; cited in Coomes and Grubb 2000). In a *Pinus elliottii* (variety not specified) plantation in Florida flatwoods, intensive plot preparation (in which the vegetation was burned and bladed, and the soil was harrowed and bedded) reduced competing vegetation biomass but enhanced soil water status as well as ammonium and nitrate availability thereby elevating foliar N concentration (0.93%) and improving stem growth of two-year-old *P. elliottii* seedlings (Burger and Pritchett 1988). Herbicide application similarly was an effective tool for enhancing productivity of *P. elliottii* plantations (Zhao et al. 2008). In young *P. elliottii* plantations especially, pine

productivity was more improved by herbicide application than by fertilizer application (Zhao et al. 2009), underscoring the importance of competition.

Foliar Mn concentrations of pine seedlings (34.50  $\mu\text{g/g}$  and 81.23  $\mu\text{g/g}$  Mn in soil from beyond and within pine stands, respectively) seemed to reflect a lower Mn concentration in soil collected beyond than within pine stands (although I did not replicate soil analyses). Foliar Mn concentrations in *P. elliotii* var. *densa* seedlings only slightly exceeded the general plant Mn deficiency level of 10-30  $\mu\text{g/g}$  (Kabata-Pendias and Pendias 2001; cited in Paschke et al. 2005), suggesting that availability of Mn in flatwoods soil, particularly beyond pine stands, can be low. Manganese plays an important role as an enzyme activator in plants, and is most available for uptake by plants in acidic soils (Marschner 1995). Although manganese can be phytotoxic if supplied at high concentration (Paschke et al. 2005), conifers seem rather tolerant of excess Mn (Ducic et al. 2008) and can show high variability in foliar Mn concentration when growing in different sites (Albaugh et al. 2010). Increased availability of Mn led to increased foliar Mn concentrations in two varieties of *Pseudotsuga menziesii*, while inoculation with a strain of the ECM fungus *Rhizopogon subareolatus* Smith did not affect Mn uptake by *P. menziesii* seedlings (Ducic et al. 2008). Manganese added as  $\text{MnSO}_4$  fertilizer was retained for several years in a *P. elliotii* var. *elliottii* plantation in northern Florida, resulting in improved stand productivity (Jokela et al. 1991). In *P. elliotii* var. *densa* seedlings, however, growth was not correlated with foliar Mn concentration, but I cannot exclude that high foliar Mn concentration might favor seedlings over a longer duration than that of this study.

All pine seedlings had similar concentrations of N, K, Mg, Ca, Fe, B, Cu and Zn. Average concentration of N was 0.63 %, and average concentration of K was 0.52 %. In

*Pinus elliotii* trees (variety not specified), critical concentrations (the concentration of a nutrient below which growth is restricted) of N and K are 10 mg/g (= 1%) and 3 mg/g (= 0.3%), respectively (Fisher and Binkley 2000; cited in Barron-Gafford et al. 2003). Thus, in my experiment, the foliar N concentration of pine seedlings was lower than the critical concentration, suggesting that N might have limited seedling growth. The low mean N:P of 10.28 and N:K of 1.19 in needles further support N limitation of seedling growth in the two flatwoods soils used (Koerselman and Meuleman 1996; Willby et al. 2001).

Consistent with probable N limitation, root/shoot ratio of seedlings was lowest in soil collected beyond pine stands (Table 3.2) because of possibly higher organic matter content and estimated N release in this soil than in that collected within pine stands. Root and shoot growth often respond differently to soil nutrient availability, as increased soil fertility commonly reduces plant root/shoot ratio, such as in young *Picea abies* (L.) Karst. trees in response to inorganic N supply (Seith et al. 1996). The foliar K concentration of my seedlings was above the critical concentration for *P. elliotii*. Potassium concentration was even higher in my experiment than in 3-year old *P. elliotii* var. *elliotii* grown in sandhills with NPKMgS fertilization (max. foliar K concentration was 0.38 % (Bengtson 1976)), suggesting that K availability was adequate for *P. elliotii* var. *densa* seedling growth. Although extraneous roots commonly penetrated drilled pipes, the concentrations of N and K, as well as N:P, were similar in seedlings in drilled and in intact pipes, suggesting that the reduced growth in drilled pipes did not result from competition for N or K.

Foliar P concentration was higher in seedlings in drilled pipes (0.076 %) than in intact pipes inoculated with either autoclaved roots (0.056 %) or fresh ECM roots (0.048%). Ectomycorrhizal fungi are well known to improve P uptake (Colpaert et al.



1999) and foliar P concentration of their host (Rousseau et al. 1994; Smith and Read 2008). Consequently, the high foliar P concentration observed in seedlings in drilled pipes might be attributable to their high percentage ECM root tips (Table 3.5 and Fig. 3.8). Although percentage ECM root tips was not correlated with foliar P concentration when treatment  $\times$  plot averages were used ( $r_s = -0.0423$ ;  $P = 0.8053$ ), probably because of low replication, foliar P and ECM were positively correlated when individual seedlings were used as the basis for the correlation ( $r_s = 0.3173$ ;  $P = 0.0008$ ). Likewise, ECM and foliar P concentration of *P. elliotii* var. *densa* seedlings grown in greenhouse conditions were positively correlated (Chapter 4), suggesting that ectomycorrhizas can improve the P nutrition of *P. elliotii* var. *densa* seedlings.

Ectomycorrhizal fungi can form extensive extraradical mycelia through which P is translocated (Finlay and Read 1986). ECM hyphae extending beyond drilled pipes across the membranes might have contributed to improved seedling P nutrition (as observed in Chapter 4), in particular if ECM fungus species colonizing seedlings formed extensive mycelia (Colpaert et al. 1999). Nevertheless, although P concentrations differed (Table 3.4), the P contents of seedlings in intact and drilled pipes did not differ. The high needle biomass in intact pipes (Fig. 3.5) might have ‘diluted’ P in needles, resulting in a ‘dilution effect’ of plant size on nutrient concentrations, a phenomenon commonly observed in plants (e.g. Hejerman et al. 2010). Phosphorus concentration of seedlings in drilled pipes was close to 0.09 %, the critical concentration for *P. elliotii* suggested by Fisher and Binkley (2000; cited in Barron-Gafford et al. 2003), but in intact pipes, seedling P concentration was lower than the critical concentration. Needles of three-year old *P. elliotii* var. *elliotii* growing in sandhills soil in the absence of competing vegetation contained a nearly three-times higher P concentration (0.14 % (Bengtson 1976)) than *P.*

*elliottii* var. *densa* seedlings in intact pipes, suggesting that P could have limited growth of pine seedlings in my experiment. Seedlings in drilled pipes with the highest P concentration, however, grew the least, resulting in ‘luxury accumulation’ of P, indicating that P was not growth-limiting.

Depending on whether ecosystems are subject to soil water and/or nutrient limitation, co-occurring vegetation competes against pines either for water (e.g. Kirongo et al. 2002) or nutrients (Smethurst et al. 1993), or for both at the same time (e.g. Burger and Pritchett 1988; Picon-Cochard et al. 2006). Because I did not detect a negative effect of competing roots on seedling nutrient concentrations in drilled pipes, and because of a lack of association between plot mean P and mean category of extraneous roots (Fig. 3.7c), the overall reduction of seedling growth and survival by extraneous roots in drilled pipes probably is not attributable to competition for P, N or K, but most likely reflects competition for water. Several species of pines are susceptible to water limitation. The growth and/or survival of species such as *Pinus sylvestris* L., *P. pinaster* Ait. and *P. lambertiana* Doug. was enhanced by increased water availability (Ruano et al. 2009; Legras et al. 2010; Dobbertin et al. 2010). During my experiment, the longest period without rainfall was 19 days, with an average daytime temperature of 26.6°C. In sandhills ecosystems at Archbold, the top 10 cm of soil dried (*i.e.* the soil matric potential dropped below -1.5 MPa) in 5-6 days without rainfall, while the top 20 cm of soil dried in about 10 days (P. Ellsworth, unpublished data). Although flatwoods usually occur on slightly less well-drained soils than sandhills (Abrahamson et al. 1984), surrounding plants probably competed greatly for the water I added to pipes in the absence of substantial rainfall.

### *Ectomycorrhiza formation*

In a greenhouse study of the effect of ectomycorrhizas on *Pinus elliottii* var. *densa* seedlings (Chapter 4), seedlings grown in autoclaved soil had very low levels of ECM root tips (6.25 %) after 9 months of growth, demonstrating that autoclaving was an effective way to reduce ECM inocula in flatwoods soil. In this field experiment, pine seedlings similarly were grown in soil autoclaved three times, but the lowest percentage ECM root tips achieved was 30.7 % in intact pipes with autoclaved roots beyond pine stands (Fig. 3.8). Those intact pipes likely were affected by any of several sources of ECM inoculum in the field. Shortly after burial of the pipes, some unknown animals dug in them possibly contaminating them with native soil. After that, even though pipes and seedlings were protected with hardware cloth cages, I sometimes observed carpenter ants, spiders or small toads in the pipes. Soil splash might occur during heavy rains and contribute to contamination. Finally, although I never found any basidiomata during my study, spores might disperse from fruiting ECM fungi.

All seedlings formed ectomycorrhizas to some extent within 7.5 months in the field, suggesting that ECM fungi are widespread in flatwoods at the Archbold Biological Station, consistent with my findings in Chapter 2. The mycorrhizal inoculum treatment, however, significantly affected the percentage ECM root tips of seedlings (Table 3.5). While mean percentage ECM was lowest in intact pipes with autoclaved roots (38.78 %; Fig. 3.8), the highest mean percentage ECM, 55.35 %, was found in drilled pipes. ECM fungus hyphae readily cross mesh barriers (Teste et al. 2006; Teste et al. 2009), so ECM fungi extending from surrounding vegetation likely contributed to the high percentage ECM root tips observed in drilled pipes.

The effect of absence versus presence of adult pines on ECM colonization was not significant according to ANCOVA, probably because of the low replication of plots. The intercept of the regression of percentage ECM root tips against root dry weight was higher within than beyond pine stands (Fig. 3.9), however, a difference that was supported by a paired t-test, indicating that the presence of adult pines did increase the ECM colonization of pine seedlings transplanted to flatwoods. In particular for drilled pipes, seedlings beyond pine stands had 49.5 % ECM root tips, while seedlings within pine stands had 60.9 % (Fig. 3.8). In the absence of conspecific adults, other ectomycorrhizal plants (e.g. Horton et al. 1999; Bai et al. 2009; Richard et al. 2009) and even ericoid mycorrhizal plant species (Vohnik et al. 2007; Curlevski et al. 2009) can provide mycorrhizal inoculum for seedlings. In flatwoods, *Quercus minima*, present at three out of the four plots without pines, *Vaccinium mysinites*, present in two plots, and *Lyonia lucida*, present in all plots without pines (Table 3.1) likely supported ECM fungi. Pines and oaks belong to different plant families, but they can share ECM fungus species. In California woodlands, 42 % of ECM taxa found on *Pinus sabiniana* Douglas roots also occurred on roots of either *Quercus douglasii* Hook & Arn. or *Quercus wislizeni* A. DC. (Smith et al. 2009). The dominant ECM taxa on *P. sabiniana*, however, appeared at low frequency on the root tips of either of the *Quercus* species (Smith et al. 2009). Plots without pines were selected 50-80 m beyond pine stands, but some isolated pines still were present within 19-25 m of transplanted seedlings. *Pseudotsuga menziesii* (Mirb.) Franco seedlings planted within 16-30 m of an adult *P. menziesii* still developed ectomycorrhizas, but had fewer active ECM root tips than seedlings planted within 2-6 m of an adult (Cline et al. 2007), suggesting that adult pines, besides co-occurring oaks and

ericoid plants, might have contributed to the ECM colonization of *P. elliotii* var. *densa* seedlings beyond pine stands.

My data indicate that the presence of adults can have a facilitative effect on ectomycorrhiza formation of *P. elliotii* var. *densa* seedlings, similar to other species (e.g. Dickie et al. 2002; Dickie et al. 2005; Haskins and Gehring 2005). By supporting a diverse fungus community, adults also enhance ECM richness on seedling roots (Cline et al. 2005), but I did not quantify ECM diversity in this study. The same ECM fungus species usually colonize conspecific adults and seedlings (Teste et al. 2009), providing the potential to form mycorrhizal networks between them (Teste and Simard 2008). In my study, seedlings in drilled pipes within pine stands had the highest mean percentage ECM root tips suggesting that ectomycorrhizal fungi from the roots of adults might have linked my seedlings to them through a common mycorrhizal network.

The performance of most conifer species depends strongly on ECM fungi (Read 1998). In my study, chlorophyll concentration and total dry weight also were associated with percentage of root tips colonized by ECM, but percentage ECM explained only a small amount of variance of either parameter. This resulted in a lack of significant differences in chlorophyll among treatments, in similar growth of seedlings with different percentages of ECM within and beyond pine stands, and in low seedling dry weights in drilled pipes even though they had the highest mean ECM colonization and mean foliar P concentration. In my study, extraneous roots were a stronger determinant of *P. elliotii* var. *densa* seedling performance than was ECM colonization. Moreover, the reduced performance of pine seedlings with increasing root competition have reduced their ability to support high levels of ECM colonization. Indeed, in drilled pipes overall, extraneous roots were negatively correlated with percentage ECM root tips (Fig. 3.7b). A similar

phenomenon was observed with *Pinus edulis* Engelm. for which interaction with roots of the arbuscular mycorrhizal species *Juniperus monosperma* (Engelm.) Sarg. decreased ECM root tips (Haskins and Gehring 2004). ECM colonization of *P. edulis* also was diminished by a high density of competing shrubs (McHugh and Gehring 2006).

Within pine stands, ectomycorrhizas might have contributed to maintain seedling performance in drilled pipes (Fig. 3.5). The absence of an effect of root competition within pine stands might be influenced by several phenomena. First, shade could have limited seedlings growth within pine stands, which precluded growth differences among pipes. Shade increases chlorophyll concentration and decreases stem dry weight in *P. elliotii* var. *densa* seedlings (Chapter 2). Here, chlorophyll did not differ with absence or presence of adults, but there was a tendency to higher growth beyond than within pine stands. Although I did not measure light availability, differences in shade might have existed, but there was no strong evidence of seedling limitation by shade within pine stands. Second, competition with extraneous roots might have been lower within than beyond pine stands. Although a Chi-square test suggested that extraneous roots did not differ beyond versus within pine stands, a paired t-test suggested that there might be less root competition within than beyond pine stands (t-statistic = 2.30,  $P = 0.0311$  not Bonferroni-corrected). Because seedling dry weight and percentage ECM root tips are positively associated (Chapter 4), a negative effect of root competition might have been compensated by a higher percentage ECM root tips in drilled pipes than in intact pipes. In addition, potential connections to adult pines through shared ECM mycelia might have enhanced the belowground competitive ability of pine seedlings against extraneous roots, (e.g. Booth and Hoeksema 2010; Nara 2006), in particular if nutrients or water were transferred to seedlings (Simard et al. 1997b; Arnebrant et al. 1993). Because I have

argued that water was growth limiting and that extraneous roots competed for water, connection to adults and/or high ECM colonization with extension of hyphae beyond drilled pipes probably enhanced pine seedling competitive ability through improved water status (Plamboeck et al. 2007), particularly if water was transferred from established *P. elliotii* var. *densa* with access to deep water sources (Ewe et al. 1999; Egerton-Warburton et al. 2007) to seedlings.

Adult pines facilitate pine seedling establishment by supporting needed ECM fungi, and by buffering extreme temperatures and soil moisture (Maher et al. 2005; Nunez et al. 2009), especially in stressful conditions (Maestre et al. 2009). Alternatively, if their own water and nutrient demands are high, adult *P. elliotii* var. *densa* might compete against establishing pine seedlings. Indeed, I occasionally found pine roots not belonging to seedlings in pipes within pine stands. In a study of facilitation and competition between adults and seedlings, Dickie et al. (2005) found that *Quercus macrocarpa* Michx seedling performance was best at intermediate distance from adult oaks where ECM inoculum still was high but competition from trees was lower than at close distance. Similarly, the growth of *P. palustris* seedlings within pine stands increased when competing roots were severed by trenching and/or understory plants were removed (Pecot et al. 2007). Competition can be especially high if adults are at high density. In a fertilized plantation on sandy soils in Georgia, stand stem productivity of *Pinus elliotii* Engelm. (variety not specified) did not increase linearly with increase in pine density, and foliar N and K decreased with increasing pine density, suggesting competition among pines for soil nutrients (Barron-Gafford et al. 2003). In a *Pinus palustris* Mill. plantation, pines considerably reduced the survival and growth of shade-tolerant shrubs such as *Callicarpa americana* L. and *Morella cerifera* (L.) Small, probably through belowground

competition for water and/or nitrogen (Hagan et al. 2009). While limiting understorey woody species by belowground competition, *P. palustris* trees also influenced understorey herbaceous species such as *Aristida stricta* (Michx.) by limiting light availability (Pecot et al. 2007).

In summary, the performance of *Pinus elliottii* var. *densa* seedlings growing for 7.5 months in flatwoods depends on several opposing factors. Seedling growth in the absence of adult pines might be rapid because of potentially higher N availability beyond pine stands, but growth beyond pine stands also can be reduced dramatically by competition with co-occurring plants. Pine stands facilitate seedling performance by Mn accumulation and by providing ectomycorrhizal fungi to seedlings.



**Table 3-1** Dominant plant species in flatwoods (Archbold Biological Station, Central Florida), within four stands of adult *Pinus elliottii* var. *densa* and four nearby paired stands lacking adult pines into which *P. elliottii* var. *densa* seedlings were transplanted. **X** indicates that the species was present

Species	Family	Pines Site 1	Pines Site 2	Pines Site 3	Pines Site 4	No pines Site 1	No pines Site 2	No pines Site 3	No pines Site 4
<i>Andropogon glomeratus</i> (Elliott) C Mohr	Poaceae	X	X	X	X	X		X	
<i>Aristida beyrichiana</i> Trin. & Rupr.	Poaceae	X	X		X	X	X	X	X
<i>Asimina reticulata</i> Shuttlew. ex. Chapm.	Annonaceae	X							
<i>Bejaria racemosa</i> Ventenat	Ericaceae		X		X				
<i>Dichantherium</i> sp.	Poaceae		X						
<i>Lachnocaulon anceps</i> (Walter) Morong	Eriocaulaceae	X	X	X	X				X
<i>Galactia elliottii</i> Nutt.	Fabaceae		X		X		X	X	X
<i>Gaylussacia dumosa</i> (Andrews) Torr. & A. Gray	Ericaceae				X				
<i>Hypericum edisonianum</i> (Small) Adams & Robson.	Clusiaceae					X	X		X
<i>Hypericum reductum</i> (Svenson) W.P. Adams	Clusiaceae	X		X		X		X	
<i>Ilex glabra</i> (L.) Gray	Aquifoliaceae	X							
<i>Lyonia fruticosa</i> (Michx.) Torr.	Ericaceae				X	X	X		X
<i>Lyonia lucida</i> (Lam.) K. Koch	Ericaceae	X		X		X	X	X	X
<i>Mimosa quadrivalvis</i> (Torr. & A. Gray)	Fabaceae	X						X	X
<i>Panicum abscissum</i> Swallen	Poaceae	X	X	X	X			X	
<i>Quercus chapmanii</i> Sarg.	Fagaceae		X						
<i>Quercus minima</i> (Sarg.) Small	Fagaceae		X		X			X	X
<i>Sabal etonia</i> Swingle ex Nash	Areaceae				X		X	X	X
<i>Serenoa repens</i> (Bartr.) Small	Areaceae	X	X	X	X	X	X	X	X
<i>Vaccinium darrowii</i> Camp	Ericaceae								
<i>Vaccinium myrsinites</i> Lam.	Ericaceae	X	X	X	X	X		X	X
<i>Ximelia americana</i> L.	Oleaceae				X				X

**Table 3-2** F statistics and associated probabilities of split-plot ANCOVAs of treatment effects on growth parameters of *Pinus elliottii* var. *densa* seedlings transplanted to flatwoods stands. Adults = absence/presence of adult pines; Myco = mycorrhizal inoculum source (autoclaved root inoculum, fresh root inoculum, hyphae extending from the surrounding vegetation); Soil = soil origin (soil collected either within or beyond pine stands). Numbers in parentheses indicate degrees of freedom of treatments. Bold *P*-values indicate significant effects of treatment at Bonferroni-corrected  $P = 0.05/11 = 0.0045$

Response variable	Adults (1)	Myco (2)	Soil (1)	Myco ×		Adults ×		Adults × Myco × Soil (2)	ECM covariate (1)
				Soil (1)	Soil (2)	Myco (2)	Soil (1)		
Needle dry weight	1.86	9.73	4.83	2.44	5.96	0.00	0.38	31.91	
	0.2658	<b>0.0006</b>	0.0358	0.1041	0.0066	0.9787	0.6847	< <b>0.0001</b>	
Stem dry weight	4.43	10.08	4.95	2.49	4.19	0.02	0.00	23.41	
	0.1260	<b>0.0004</b>	0.0337	0.0997	0.0248	0.8938	0.9957	< <b>0.0001</b>	
Shoot dry weight	2.47	10.97	5.26	2.39	6.32	0.00	0.19	33.12	
	0.2140	<b>0.0003</b>	0.0290	0.1092	0.0051	0.9843	0.8278	< <b>0.0001</b>	
Root dry weight	0.57	25.24	0.15	4.22	4.13	0.80	0.55	38.95	
	0.5054	< <b>0.0001</b>	0.7023	0.0242	0.0261	0.3782	0.5822	< <b>0.0001</b>	
Total dry weight	1.58	16.48	2.80	3.08	7.03	0.09	0.29	37.44	
	0.2980	< <b>0.0001</b>	0.1046	0.0607	<b>0.0031</b>	0.7673	0.7494	< <b>0.0001</b>	
Root/shoot ratio	16.63	1.11	12.67	2.49	5.05	0.32	0.09	2.32	
	0.0266	0.3428	<b>0.0013</b>	0.0996	0.0129	0.5745	0.9159	0.1298	

**Table 3-3** Growth of *Pinus elliottii* var. *densa* seedlings (midpoint mean  $\pm$  SE after ANCOVA) transplanted to flatwoods stands either in intact pipes with autoclaved or fresh ECM root inoculum or in drilled pipes, across all stands with and without adult pines. Within a row, values followed by the same letter are not significantly different at non-Bonferroni corrected Tukey's HSD  $P = 0.05$

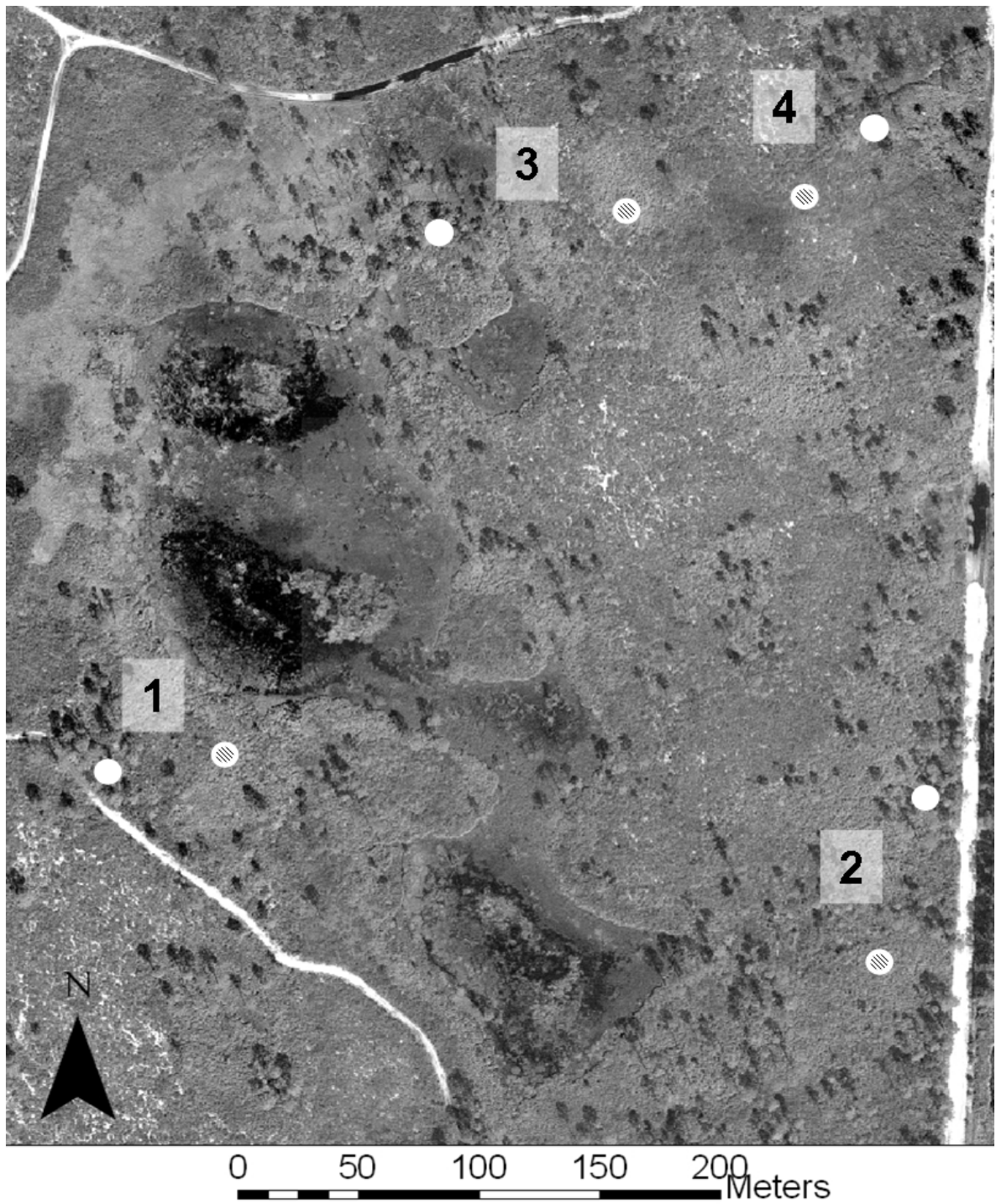
Response variable	Intact pipes + autoclaved roots	Intact pipes + fresh ECM roots	Drilled pipes
Needle dry weight (g)	0.55 + 0.04/-0.03 a	0.53 $\pm$ 0.03 a	0.38 + 0.03/-0.02 b
Stem dry weight (g)	0.11 $\pm$ 0.01 a	0.11 $\pm$ 0.01 a	0.07 $\pm$ 0.01 b
Shoot dry weight (g)	0.66 $\pm$ 0.04 a	0.65 $\pm$ 0.04 a	0.45 $\pm$ 0.03 b
Root dry weight (g)	0.40 + 0.02/-0.01 a	0.36 $\pm$ 0.01 a	0.27 $\pm$ 0.01 b
Total dry weight (g)	1.07 $\pm$ 0.05 a	1.02 $\pm$ 0.05 a	0.74 $\pm$ 0.04 b
Root/shoot ratio	0.60 $\pm$ 0.03 a	0.56 +0.03/-0.02 a	0.60 $\pm$ 0.03 a

**Table 3-4** F statistics and associated probabilities of split-plot ANCOVAs of treatment effects on ranked nutrient concentration of *Pinus elliotii* var. *densa* seedlings transplanted to flatwoods stands. Adults = absence/presence of adult pines; Myco = mycorrhizal inoculum source (autoclaved root inoculum, fresh root inoculum, hyphae extending from the surrounding vegetation); Soil = soil origin (soil collected either within or beyond pine stands). Numbers in parentheses indicate degrees of freedom of treatments. Bold *P*-values indicate significant effects of treatment at Bonferroni-corrected  $P = 0.05/13 = 0.00385$

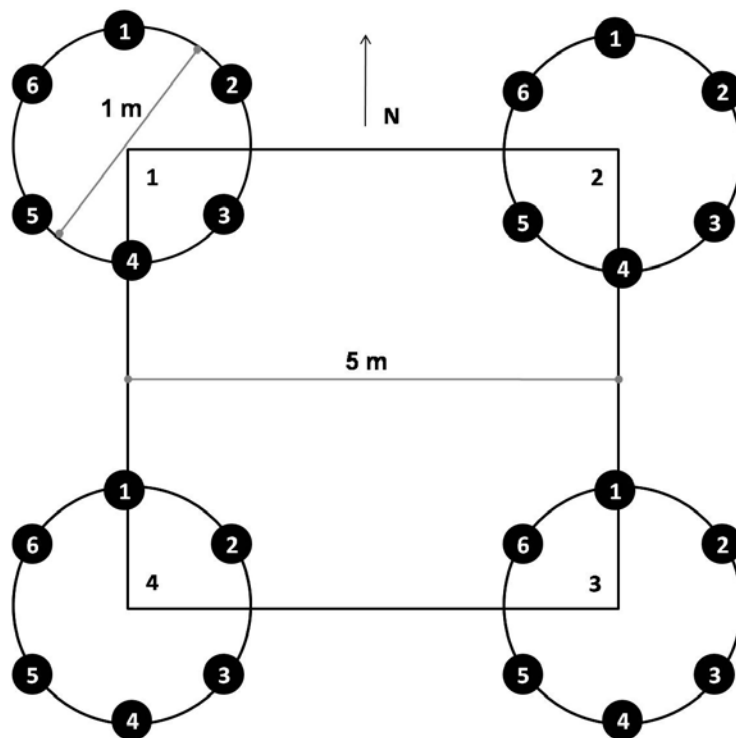
Response variable	Adults (1)	Myco (2)	Soil (1)	Myco × Soil (2)	Adults × Myco (2)	Adults × Soil (1)	Adults × Myco × Soil (2)	Root dry weight covariate (1)
B concentration	0.70	1.82	0.12	0.32	0.59	0.45	0.68	2.36
	0.4636	0.1825	0.7306	0.7278	0.5599	0.5087	0.5149	0.1362
Ca concentration	0.47	1.21	1.25	0.65	2.36	0.27	0.32	0.87
	0.5412	0.3142	0.2736	0.5281	0.1147	0.6049	0.7310	0.3587
Cu concentration	12.23	0.23	2.45	0.04	1.89	2.44	5.36	0.32
	0.0396	0.7927	0.1299	0.9642	0.1717	0.1304	0.0113	0.5789
Fe concentration	0.14	0.05	0.01	1.84	0.13	2.46	0.60	0.00
	0.7303	0.9484	0.9198	0.1796	0.8824	0.1290	0.5574	0.9794
K concentration	0.01	3.24	5.61	0.34	0.74	0.00	0.62	5.52
	0.9278	0.0553	0.0255	0.7135	0.4869	0.9544	0.5460	0.0266
Mg concentration	0.52	2.57	0.32	0.36	0.80	0.48	0.00	0.26
	0.5231	0.0962	0.5771	0.6986	0.4612	0.4954	0.9965	0.6171
Mn concentration	6.60	0.30	70.98	0.17	0.10	0.85	0.58	0.56
	0.0826	0.7447	< <b>0.0001</b>	0.8425	0.9013	0.3657	0.5678	0.4613
N concentration	11.44	0.95	2.44	0.29	2.22	0.52	1.12	0.13
	0.0430	0.4058	0.1369	0.7515	0.1395	0.4807	0.3483	0.7256
P concentration	3.51	10.79	0.69	0.13	3.21	0.97	0.91	6.76
	0.1576	<b>0.0004</b>	0.4135	0.8792	0.0566	0.3338	0.4169	0.0152
Zn concentration	0.25	3.18	0.77	0.23	1.03	1.17	0.22	3.38
	0.6526	0.0581	0.3870	0.7951	0.3728	0.2887	0.8008	0.0776
N:P	1.25	0.12	0.22	0.86	0.95	1.57	0.15	0.02
	0.3444	0.8852	0.6439	0.4394	0.4073	0.2266	0.8593	0.8864
N:K	17.10	0.37	0.00	0.49	1.43	0.19	1.72	0.18
	0.0257	0.6950	0.9599	0.6232	0.2676	0.6692	0.2086	0.6770
K:P	0.55	4.51	1.08	0.22	1.54	1.30	1.56	1.48
	0.5119	0.0208	0.3079	0.8044	0.2339	0.2642	0.2293	0.2343

**Table 3-5** Split-plot ANCOVA table of effects of mycorrhizal inoculum, soil origin and absence/presence of adult pines on percentage ECM root tips of *Pinus elliottii* var. *densa* seedlings transplanted to flatwoods stands. Significant effects at  $P \leq 0.05$  are indicated in bold

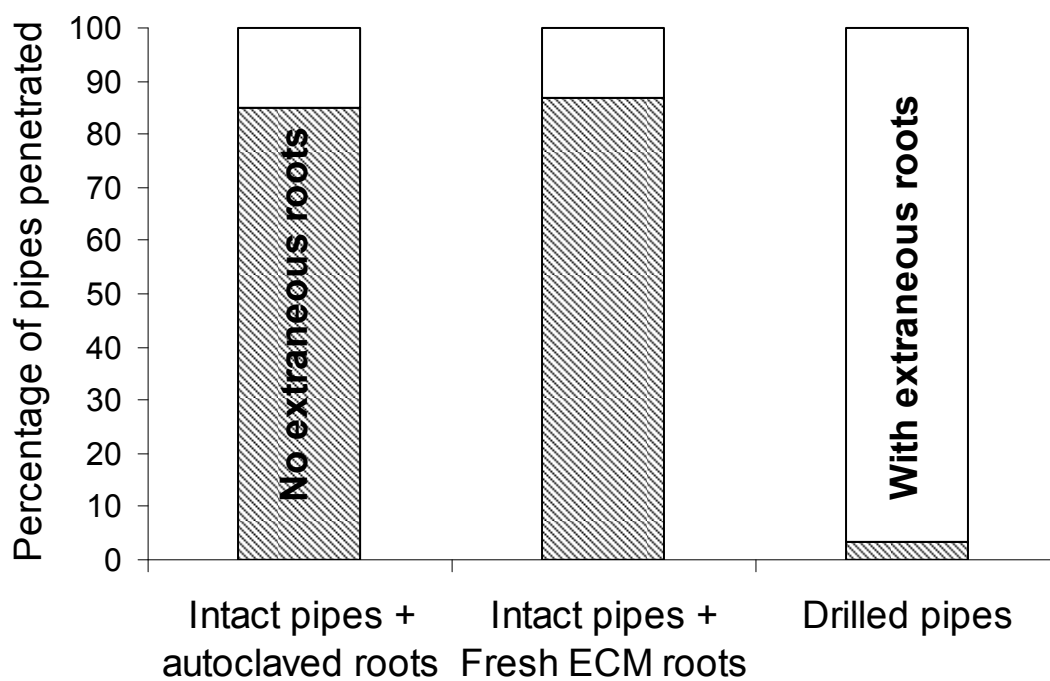
Source	<i>df</i>	<i>F-value</i>	<i>P</i>
Stand	3	0.23	0.8724
Adults	1	4.51	0.1238
Error stand × adults	3		
Mycorrhizal inoculum	2	14.95	< <b>0.0001</b>
Soil origin	1	0.06	0.8145
Mycorrhizal inoculum × soil origin	2	0.84	0.4427
Adults × Mycorrhizal inoculum	2	0.59	0.5598
Adults × soil origin	1	0.38	0.5429
Adults × mycorrhizal inoculum × soil origin	2	0.02	0.9820
Error stand × adults × mycorrhizal inoculum × soil origin	30		
Root dry weight covariate	1	38.95	< <b>0.0001</b>
Error covariate	128		
Total	176		



**Figure 3-1** Location of four pairs of field stands in flatwoods at Archbold Biological Station, Central Florida, on an aerial photo taken by Highlands County in 2005. Each pair consisted of a stand within (white circles) and a site beyond (hatched circles) adult pines

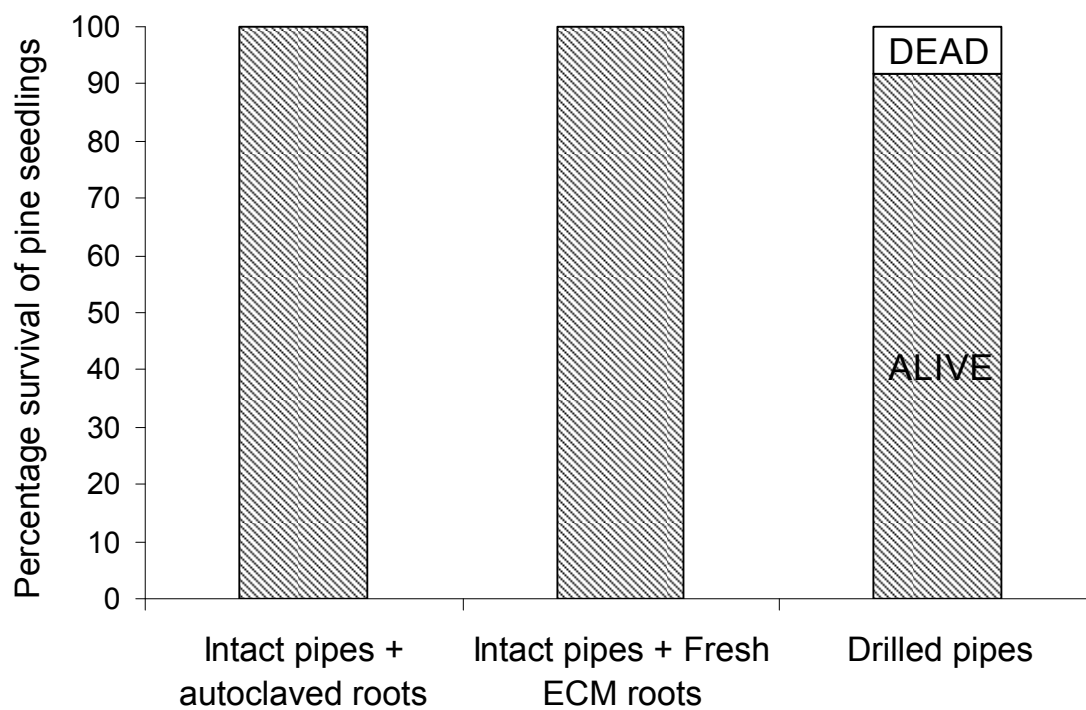


**Figure 3-2** To investigate the ectomycorrhizal colonization and performance of *Pinus Elliottii* var. *densa* seedlings at the Archbold Biological Station, Central Florida, seedlings were grown in PVC pipes arranged in a split-plot design, either within or beyond adult pine stands. An experimental split-plot consisted of six PVC pipes buried at equal distances around the circumference of a 1 m-circle at each corner of a 5 × 5 m square. Around each circle, the six treatments were arranged randomly

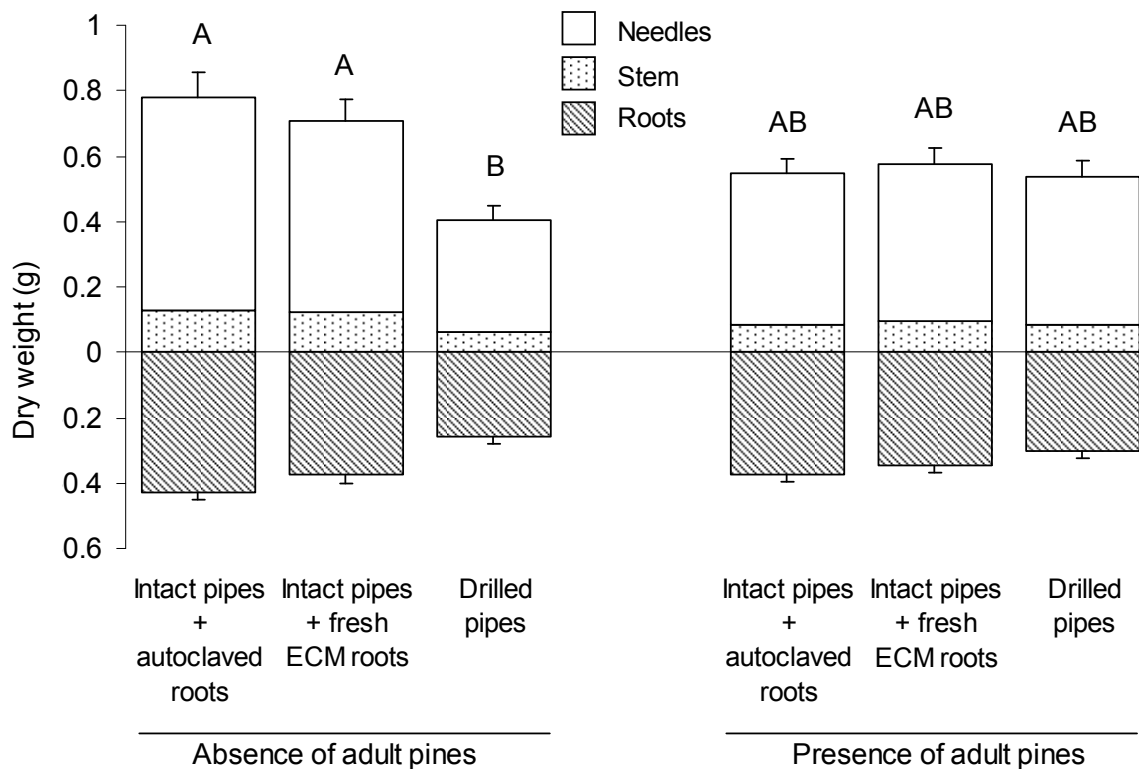


**Figure 3-3** Percentage of pipes (64 pipes of each type) buried in flatwoods stands at Archbold Biological Station, Central Florida, that were penetrated by extraneous roots from the surrounding vegetation. Hatched bars = no extraneous roots in pipes; white bars = pipes penetrated by extraneous roots

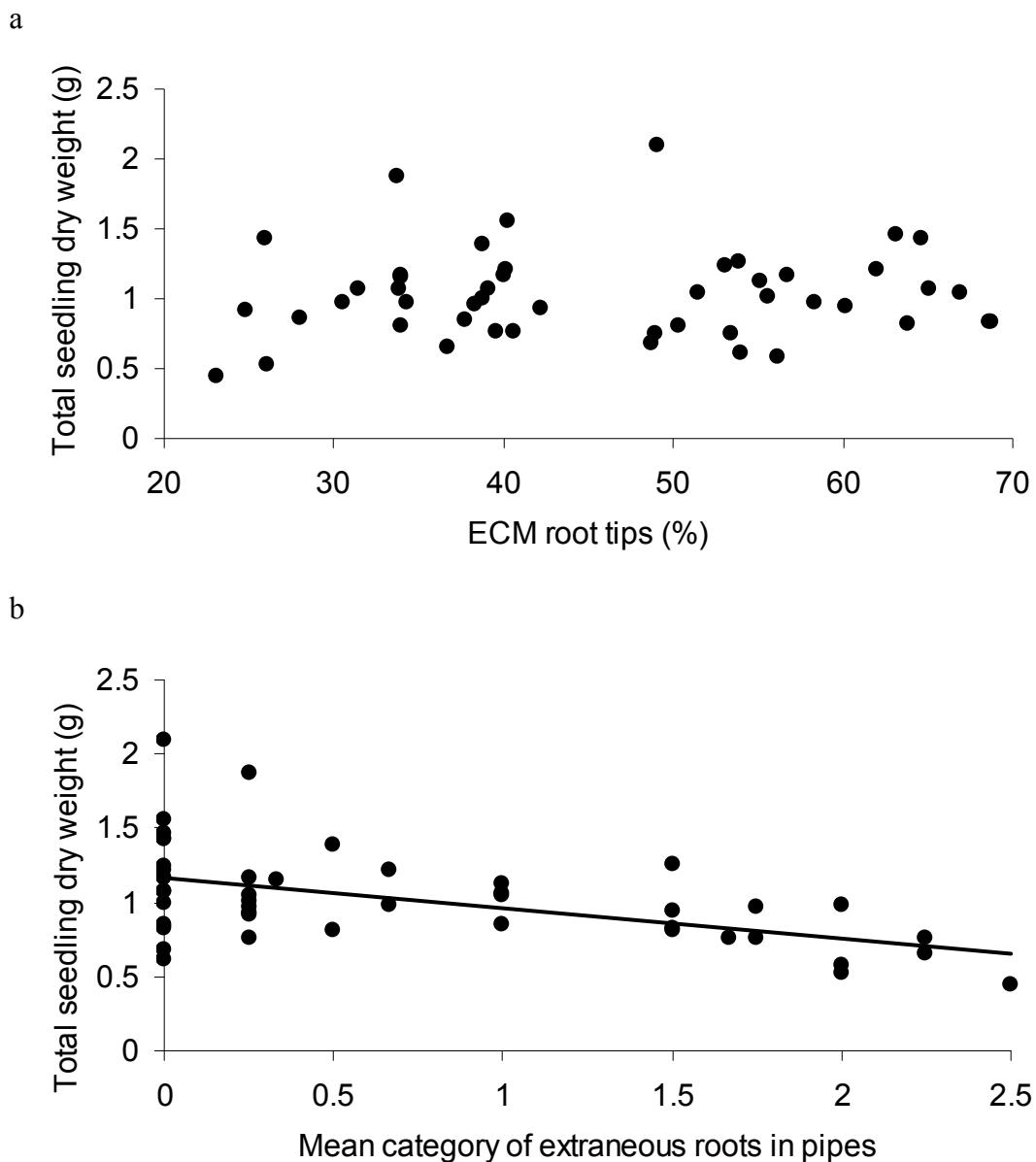




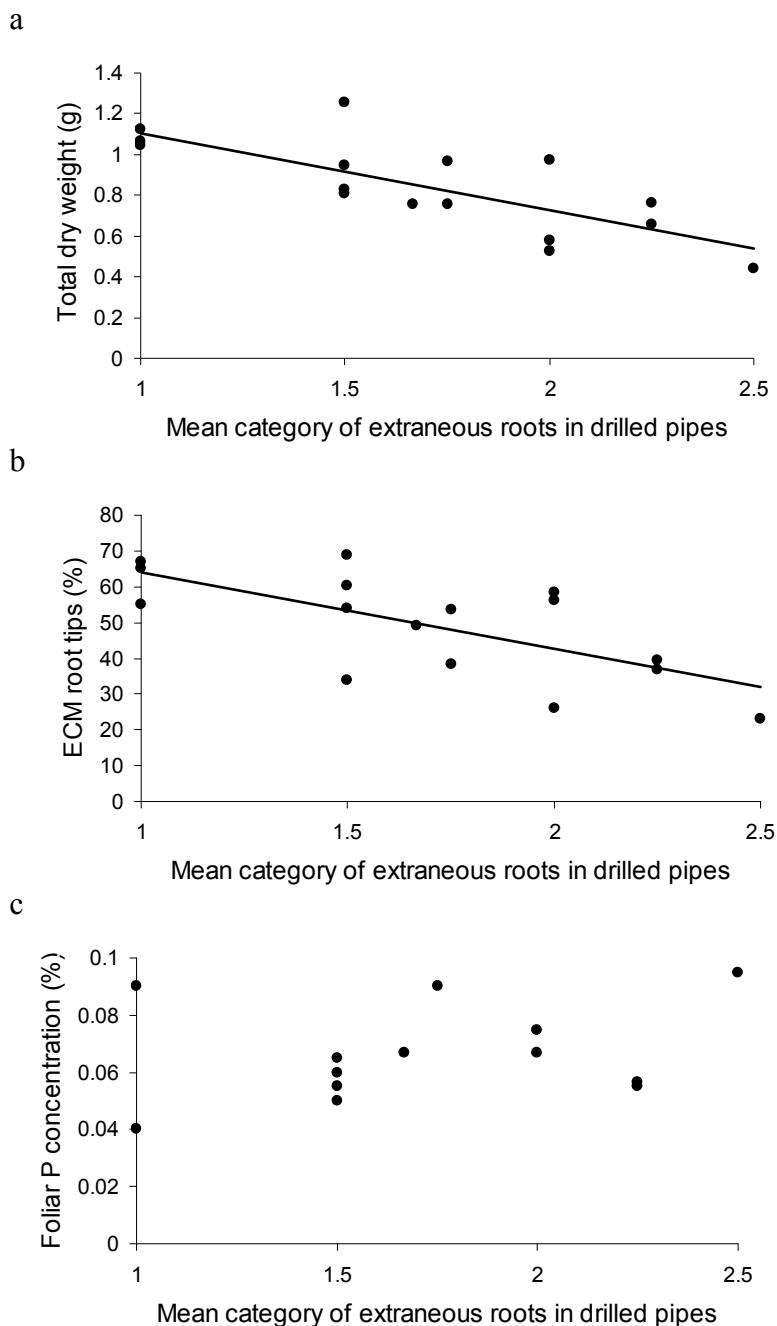
**Figure 3-4** Survival over 7.5 months of *Pinus elliottii* var. *densa* seedlings transplanted to intact or drilled PVC pipes in flatwoods stands at Archbold Biological Station, Central Florida. Hatched bars = alive seedlings; white bars = dead seedlings



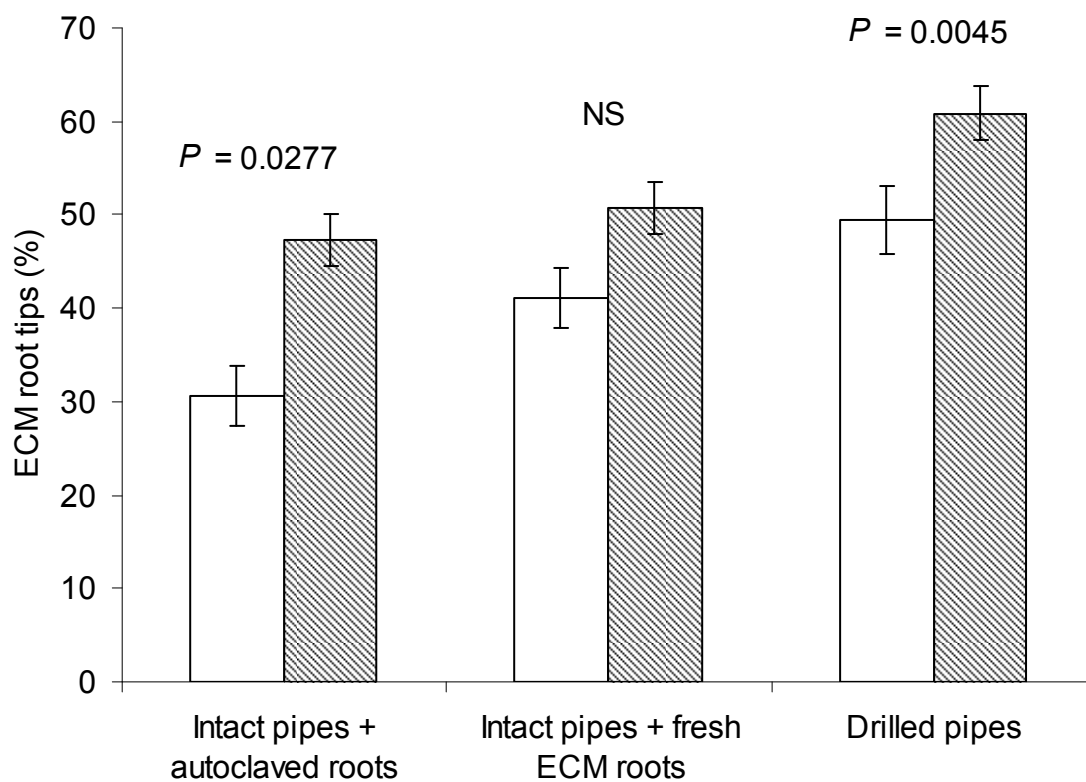
**Figure 3-5** Dry weight of needles (white bars), stems (dotted bars) and roots (hatched bars; represented as positive values below the X-axis) of *Pinus elliotii* var. *densa* seedlings transplanted to flatwoods in the absence versus presence of adult pines. Error bars are for total shoot dry weight and root dry weight. Bars topped by the same letter indicate that total seedling dry weight (needle + stem + roots) was not significantly different at  $P \leq 0.05$



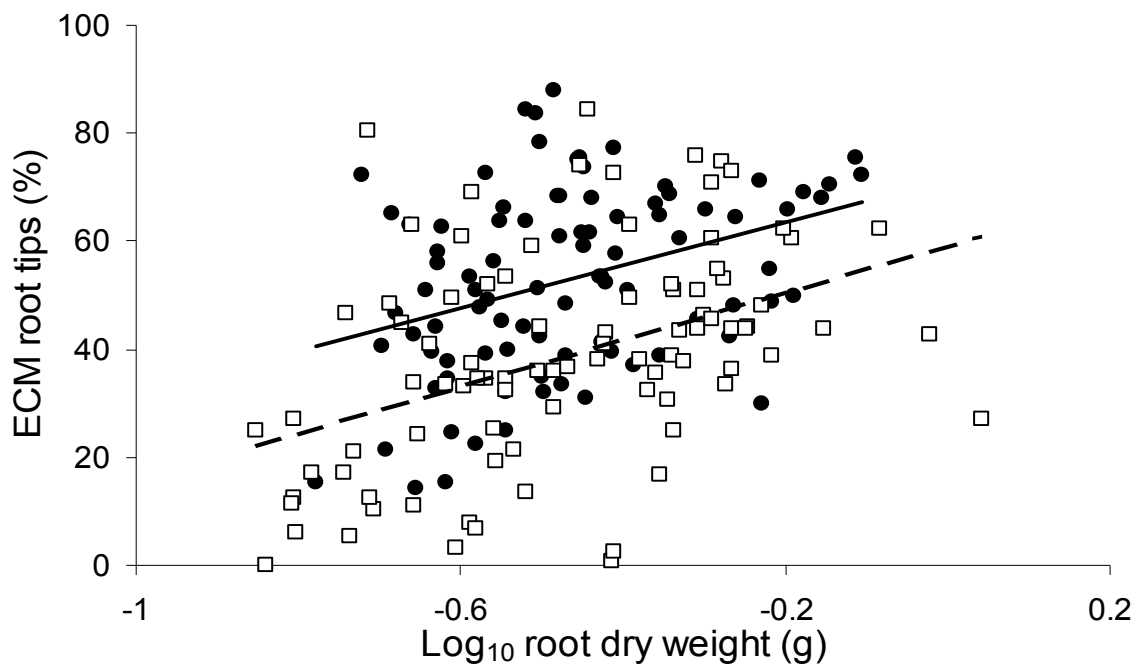
**Figure 3-6** Least-squares linear regressions of total seedling dry weight against percentage ectomycorrhizal (ECM) root tips of seedlings (a) or mean category of extraneous roots in pipes (b) for all treatment  $\times$  stand averages. Total dry weight was not associated with percentage ECM root tips (regression coefficient = 0.0307,  $P = 0.8146$ ) but it was negatively associated with mean category of extraneous roots (regression coefficient = -0.5059,  $P = 0.0005$ ;  $R^2 = 0.2397$ )



**Figure 3-7** Least-squares linear regressions of total dry weight (a), percentage ectomycorrhizal (ECM) root tips (b) and foliar P concentration (c) against mean category of extraneous roots for treatment  $\times$  stand averages for only drilled pipes. Total dry weight (regression coefficient = -1.7704, model  $F_{1,14} = 14.00$ , Bonferroni corrected  $P$  ( $P_{Bc}$ ) = 0.0066,  $R^2 = 0.5000$ ) and percentage ECM root tips (regression coefficient = -1.6482, model  $F_{1,14} = 7.81$ ,  $P_{Bc} = 0.0429$ ,  $R^2 = 0.3580$ ) were negatively associated with mean category of extraneous roots, while foliar P concentration was not significantly associated with mean category of extraneous roots (model  $F_{1,12} = 4.85$ ,  $P_{Bc} = 0.1440$ )



**Figure 3-8** Mean percentage ectomycorrhizal (ECM) root tips ( $\pm$  SE) after ANCOVA (with root dry weight as the covariate) of *Pinus elliotii* var. *densa* seedlings transplanted to pipes buried in flatwoods in the absence (white bars) or in the presence (hatched bars) of adult pines. Probabilities above bars indicate significant differences at  $P \leq 0.05$  after paired t-tests. NS = not-significant



**Figure 3-9** Least-squares regression ( $F_{1,175} = 20.89$ ,  $P < 0.0001$ ,  $R^2 = 0.1067$ ) of *Pinus elliotii* var. *densa* percentage ectomycorrhizal (ECM) root tips against  $\log_{10}$  of root dry weight of seedlings in the absence (open squares, dashed line) or presence (solid circles, solid line) of adult pines in flatwoods stands. Absence of adults: intercept = 56.80, slope = 36.71; presence of adults: intercept = 71.57, slope = 39.86

## Chapter 4

### **Extraradical ectomycorrhizal mycelium enhances the mineral nutrition and growth of *Pinus elliottii* var. *densa* seedlings**

Ectomycorrhizal (ECM) fungi are symbiotic colonists of the roots of plant species in diverse families such as *Pinaceae*, *Fagaceae*, *Myrtaceae*, and *Dipterocarpaceae* (Smith and Read 2008; Wang and Qiu 2006). ECM fungi offer several benefits to their host plants including enhanced mineral nutrition of nitrogen, phosphorus, and even boron (Lehto et al. 2004; Nilsson and Wallander 2003; Smith and Read 2008), enhanced water status, and resistance to drought (Kazantseva et al. 2009; Plamboeck et al. 2007). ECM fungi usually improve the growth of their hosts (Koide and Kabir 2001) but also can diminish the growth of some coniferous seedlings when the carbon cost of ECM fungi outweighs the benefits they provide, especially under greenhouse conditions (Colpaert et al. 1992; Conjeaud et al. 1996).

Extraradical mycelium that extends away from roots into soil is an indispensable component of the ECM symbiosis (Leake et al. 2004). Because ECM hyphae can extend several meters beyond their host plant (Cline et al. 2005), they can forage for mineral nutrients through large soil volumes. Such extensive ECM fungus mycelia may represent as much as one-third of all microorganism biomass in boreal ecosystem soils (Högberg and Högberg 2002).

The importance of the extraradical mycelium and the volume of soil to which it has access can be determined by growing mycorrhizal plants within in-growth cores that

are inserted in a large volume of soil and that are rotated to disconnect the extended mycelium of the fungus beyond the core (Johnson et al. 2001; Cheng et al. 2008; Rillig 2004). Disconnection of the extraradical mycelium is likely to have a strong effect on the host plant because of extensive spread of hyphae throughout the substrate (Colpaert et al. 1992; Leake et al. 2001). With arbuscular mycorrhizas (AM), rotation of an in-growth core can reduce mycorrhizal colonization of a plant in the core (Johnson et al. 2001), and the disconnection of the extraradical mycorrhizal mycelium reduces P nutrition of the host (Johnson et al. 2001). With ectomycorrhizas, the effects of extraradical mycelium have been studied by trenching (Booth 2004) more often than with in-growth cores (Hendricks et al. 2006).

*Pinus elliottii* var. *densa* Little & Dorman is a pine variety native to central and south Florida. *Pinus elliottii* var. *densa* is the sole canopy tree in native Florida ecosystems such as some pine flatwoods and pine rocklands (Snyder et al. 1990). In pine rocklands, pine populations are in decline because of human disturbance such as urban development and consequent changes in fire regime (Wade et al. 1980). The pine rocklands ecosystem in which *P. elliottii* var. *densa* is the keystone species, is endangered (Snyder et al. 1990). Flatwoods, on the other hand, are not formally designated as endangered, but their pine populations also are declining, possibly because of altered fire regimes (Menges and Deyrup 2001; Snyder et al. 1990). The decline of pine populations is thought to reflect diminished seedling establishment.

Flatwoods are nutrient-poor ecosystems in which adult *P. elliottii* var. *densa* associate with ectomycorrhizal fungi. The common understory plant species *Serenoa repens* (Bartr.) Small, however, forms arbuscular mycorrhizas (Fisher and Jayachandran 1999). So, pine seedlings in flatwoods often must establish in competition with *S. repens*



and other AM plants. Moreover, although pines typically are ectomycorrhizal, AM fungi can colonize the roots of some pine species either from spores or when an AM host grows nearby (Horton et al. 1998), and they might colonize *P. elliotii* var. *densa* seedlings with unknown consequences. Because successful survival and growth of seedlings is fundamental to pine population reestablishment, in the presence of an AM host plant in microcosms, I investigated the effects of ECM fungus colonization and of the volume of soil to which the ECM extraradical mycelium has access on the growth and mineral nutrition of *Pinus elliotii* var. *densa* seedlings.

My specific objectives were: 1) to ascertain if AM fungi from root inoculum or extending from a host plant colonize and directly affect pine seedlings, 2) to determine whether ectomycorrhizas enhance the mineral nutrition, chlorophyll concentration, and growth of pine seedlings in flatwoods soil, and 3) to examine with the rotated, in-growth core method how the volume of soil to which the ECM extraradical mycelium has access affects seedling performance. I expected that pine seedlings colonized by ECM fungi would have better mineral nutrition, higher chlorophyll concentration, and better growth than non-mycorrhizal seedlings, and that seedling performance would be highest when ECM fungi extend through a large volume of soil.

## **Materials and methods**

### *Experiment design*

I investigated the effects of mycorrhizal fungi on *P. elliotii* var. *densa* seedlings in a fully factorial, two-factor microcosm experiment. The first factor (ECM fungi factor) controlled the presence/absence of ectomycorrhizal fungi and comprised two levels of

ECM fungi: autoclaved versus fresh ECM root inoculum. The second factor (AM-pot factor) controlled both the presence/absence of AM fungi and the volume of soil to which ECM fungi had access, and comprised four levels: intact pot with autoclaved AM root inoculum, intact pot with fresh AM root inoculum; slotted, rotated pot, and slotted, static pot, both with autoclaved AM root inoculum (Fig. 4.1). Slotted pots embedded within microcosms allowed AM fungi extending from an AM host to penetrate the pots in which pine seedlings grew, and allowed ECM fungus hyphae to extend beyond the pots into the microcosms. Weekly rotation of slotted pots severed ECM hyphae extending beyond pots, effectively reducing the volume of soil to which they had access. All eight treatment combinations, each imposed on an individual pine seedling, were included singly in each microcosm. There were 25 replicate microcosms with a total of 200 pine seedlings.

In each microcosm, one *Tamarindus indica* L. was used as an AM host plant. *T. indica* seeds (The Banana Tree Inc., Easton, PA, USA) were soaked in distilled water for 24 hours and germinated in Petri dishes on Whatman #1 filter paper. Once the radicle had emerged, seedlings were transplanted into fresh soil collected to a depth of 10 cm from a lawn at the University of Miami's Gifford Arboretum. The radicle of germinating seeds was put in contact with 3 grams of fresh lawn grass roots collected from the Arboretum to enhance AM colonization of the tamarind seedlings. *Tamarindus indica* seedlings were grown in individual pots (DeePots, 5 cm diameter x 18 cm long, D16, Stuewe & Sons, Inc., Corvallis, OR, USA) in an ambient temperature and ambient daylight shadehouse at the University of Miami. They were watered 2-3 times a week with tap water, and not fertilized. Although I did not determine arbuscular mycorrhizal

colonization of tamarind seedlings, I suspect they were mycorrhizal because they were grown in fresh soil with freshly collected grass roots.

*P. elliotii* var. *densa* seeds were donated by the Andrews Nursery (Florida Division of Forestry, Chiefland, FL, USA). Seeds were soaked in distilled water for 24 hours and incubated at 4°C for 7 days. Seeds were germinated in coarse silica sand (L 6-20, Standard Sand and Silica Co., Miami, FL, USA). Seedlings were grown in a shadehouse, in small, individual containers of silica sand for six weeks, were watered daily with tap water and were not fertilized.

To avoid direct root competition between the AM host plant *T. indica* and *P. elliotii* var. *densa* seedlings during the experiment, the pine seedlings were transplanted to separate pots (DeePots, 5 cm diameter x 18 cm long, D16, Stuewe & Sons, Inc., Corvallis, OR, USA) embedded within the soil of microcosms. The microcosms consisted of expanded polystyrene coolers (37 cm wide, 44.5 cm long, 23.5 cm deep, and 3.7 cm thick). In the bottom of each, eight holes of 3.5 cm diameter at equal distances around the circumference of a circle of 21 cm diameter tightly held the bottoms of individual pots but allowed external drainage. An 8.5 × 3 cm slot was cut in the side of each “slotted” pot, and was covered with GORE-TEX<sup>®</sup> (W. L. Gore & Associates Inc, Newark, DE, USA), a hydrophobic membrane through which hyphae but neither water nor dissolved nutrients can pass (Mäder et al. 1993). Intact pots lacking the slot were used for treatments not intended to allow hypha passage.

Each microcosm was filled with 13 liters of fresh soil which I collected to a depth of 20 cm in flatwoods ecosystems at five different locations at Archbold Biological Station (Archbold), Highlands County, FL, USA. The soil was passed through a 2 mm sieve and homogenized. This acidic, very sandy soil had the following properties: pH

4.25, 2.8 % organic matter, 2.5 mg/kg P (weak Bray), 5.5 mg/kg P (strong Bray), 11 mg/kg K, 17 mg/kg Mg, 125 mg/kg Ca, 0.15 mg/kg Cu, 0.65 mg/kg Zn, 1 mg/kg Mn, and 16.5 mg/kg Fe (analyses not replicated; A & L Southern Agricultural Laboratories Inc., Deerfield Beach, FL, USA). A twenty-four week-old *T. indica* seedling was transplanted to the center of each microcosm. *T. indica* seedlings were allowed to establish in the microcosms for 2 months before the start of the experiment, at which time they averaged 55 cm tall (soil surface to shoot apex).

A portion of the flatwoods soil was autoclaved three times, 24 hours apart, at 121°C and 1.4 kg/cm<sup>2</sup> for one hour, and 220 ml autoclaved soil was used to fill each individual pot. The pots were moistened, covered with tin foil, and were not rotated for 2 months to allow growth of AM hyphae into them before pine seedling transplant.

#### *Mycorrhiza treatments*

Ectomycorrhizal roots were collected from adult *P. elliotii* var. *densa* trees at Archbold to serve as ECM inoculum. Roots were collected from multiple trees in flatwoods by removing the litter layer close to the base of the tree, and searching for pine roots to a depth of 5 cm in the soil around each tree. Pine roots were heavily colonized by ECM fungi. I did not identify the ECM fungus species colonizing adult pine roots, but because the ECM root tips presented different mantle characteristics, the ECM community on the root inoculum was likely diverse. In the lab, all fresh ECM roots were mixed, washed in distilled water and were cut into 1-2 cm pieces. At the time of transplant of six-week-old pine seedlings, 0.26 g of fresh ECM root fragments was added by depositing the ECM roots at the bottom of the planting hole. To ensure ECM colonization, 0.16 g of freshly-collected ECM pine root pieces was added again six weeks after the start of the

experiment by putting them in contact with pine roots in a 1-cm deep hole adjacent to each seedling. The hole was subsequently filled with autoclaved soil. ECM pine roots used as inoculum contained 0.8 % N, 0.05 % P, 0.11 % K, 0.1 % Mg, 0.27 % Ca, 889 mg/kg Fe, 8 mg/kg Mn, 14 mg/kg B, 8 mg/kg Cu, and 149 mg/kg Zn.

Because *Serenoa repens* is the main understory AM species in flatwoods, I used its roots as AM inoculum. *S. repens* roots were collected at Archbold by removing several blocks of soil with a shovel within two dense stands of *S. repens*. The roots of *S. repens* were separated from other roots, mixed and washed in distilled water. The roots were cut into 2 cm pieces, and 3.76 g were added to the *P. elliotii* var. *densa* seedlings by deposition at the bottom of the planting hole (together with the ECM root pieces) as required. *S. repens* roots contained 0.6 % N, 0.05 % P, 0.13 % K, 0.14 % Mg, 0.13 % Ca, 123 mg/kg Fe, 10 mg/kg Mn, 15 mg/kg B, 6 mg/kg Cu, and 2 mg/kg Zn. To control for the effect of addition of roots, non-inoculated controls received equal weights of ECM and/or AM roots autoclaved three times, 24 hours apart.

In order to re-establish microbe populations in the autoclaved soil in pots, a microbial filtrate was prepared by soaking 26 g of ECM roots or 564 g of AM roots in distilled water for 24 hours. To exclude fungus propagules, the microbial filtrates were vacuum filtered three times through Whatman #1 filter paper, once through a 41  $\mu$ m Nylon filter (Millipore, Billerica, MA, USA), and once through an 11  $\mu$ m Nylon Net filter (Millipore). Four milliliters of ECM microbial filtrate was added to pine seedlings that had received autoclaved ECM roots, and 10 ml of AM microbial filtrate was added to pine seedlings that had received autoclaved AM roots. At the same time, seedlings inoculated with fresh roots received the same amounts of distilled water.

Beginning two months after pine seedling transplant, mycorrhizal hyphae penetrating the GORE-TEX<sup>®</sup> membrane of slotted pots were severed by weekly rotating designated pots clockwise one-full revolution. To control for the effect of rotation, all pots except those designated as slotted, static pots, were rotated weekly. The microcosms were grown in an ambient temperature greenhouse at the University of Miami, with no temperature regulation and with ambient day light. The mean daily air temperature (as measured by the closest weather station located at the Miami International Airport) from the day of transplant of tamarinds in microcosms to the day of the final harvest was 24.4°C, ranging from a minimum of 3.3°C on February 5<sup>th</sup> 2009 to a maximum of 36.7°C on June 22<sup>nd</sup> 2009. Tamarinds were watered 2-4 times a week with 500 ml of tap water, while pine seedlings received 50 ml of tap water 2-4 times a week, depending on air temperature and how quickly the soil appeared to dry. Neither the tamarinds nor the pine seedlings were fertilized during the experiment. The microcosms were randomly rearranged in the greenhouse once a month to homogenize growing conditions.

#### *Seedling performance*

At the time of transplant, all pine seedlings had an average stem diameter of 0.93 mm  $\pm$  0.01 (SE) and an average longest needle length of 3.80 cm  $\pm$  0.05, with no differences in either variable among treatments. The experiment lasted for 39 weeks after pine seedling transplant. During that time, to detect possible differences among treatments, the stem diameter and length of the longest needle were measured every two to four weeks. At harvest, the needles were separated from the shoot and weighed fresh. Chlorophyll concentration in needles was measured by cutting 0.2 g of needles into 0.5 cm pieces that were immersed for 48 hours in 80 % acetone in the dark (Proctor 1981). The

concentration of chlorophyll *a* and *b* was determined by measuring the absorbance of the solution decanted from the needles at 645 and 663 nm (Porra 2002). The remaining needles and the stems were dried at 50 °C to constant weight, and their dry weight was determined and adjusted for the removal of needles for chlorophyll analysis. Dried needles were finely ground with a coffee grinder and sent to A & L Southern Agricultural Laboratories for mineral nutrient analysis (N, P, K, Mg, Ca, Fe, Mn, B, Cu, Zn). Nutrients were extracted by the lab by dry ashing followed by dissolution in acid. Nitrogen was extracted with a modified Kjeldahl method. Phosphorus was measured colorimetrically, and other nutrients were measured by inductively-coupled plasma spectroscopy. Root systems were gently washed to remove all soil, blotted dry, weighed fresh, and root length was determined by scanning followed by analysis with WinRHIZO software (Regent Instruments Inc., Canada). ECM colonization was determined by counting colonized root tips under a dissecting microscope following the gridline intersect method (Brundrett et al. 1996). The roots of one randomly-selected seedling per treatment were cleared and stained (Brundrett et al. 1996) to seek AM. Root systems not cleared and stained were dried at 50 °C to constant weight before weighing.

#### *Treatment group revisions and statistical analyses*

Survival of seedlings among treatments was tested with a Chi-square test. All above- and belowground response variables of pine seedlings were heteroscedastic, and no transformation produced homoscedasticity. Nevertheless, two-way parametric ANOVAs were performed to test for significant interactions among treatments. Interactions also were tested with two-way ANOVAs on ranked observations, an anti-conservative approach (Conover and Iman 1981).

Separate Kruskal-Wallis tests within intact pots given fresh AM root inoculum, within slotted, rotated pots, and within slotted, static pots showed that pines that were given fresh ECM root inoculum had similar percentages ECM colonization to pines that were given autoclaved ECM root inoculum (Fig. 4.2a). Therefore, pine seedlings given fresh or autoclaved ECM inoculum were combined within each of these three AM-pot treatments for one-way, non-parametric analyses (Fig. 4.2b). Addition of fresh ECM root inoculum increased the percentage ECM root tips of pines only in intact pots with autoclaved AM root inoculum (Fig. 4.2a), and so those two groups were retained. Thus, I statistically analysed differences of performance variables of pine seedlings assigned to five new groups (Fig. 4.2b) by using Kruskal-Wallis one-way analyses of variance followed by all pairwise comparison post-hoc tests to detect differences among means. Bonferroni corrections were applied for having individually tested multiple response variables.

Correlations among ECM colonization, dry weights, total chlorophyll concentration, and mineral nutrients in pine needles were determined by Spearman's rank correlations with Bonferroni corrections for multiple, post hoc analyses.

## **Results**

After 9 months in microcosms, differences among treatments in morphometric measurements were detected, so the seedlings were harvested. Among the initial 200 seedlings, 89.5% survived until harvest, with similar survival among all treatments (Chi-square statistic = 3.66,  $P = 0.8177$ ).

Among all response variables, I found only one significant interaction by an ANOVA on ranked observations ( $F_{3, 148} = 2.67$ ,  $P = 0.0497$ ) for total chlorophyll



concentration. Because the analysis is anti-conservative, however, I did not accept this probability as indicating significance. Because of the lack of significant interactions, all analysis of variance results presented are those of one-way, non-parametric Kruskal-Wallis analyses.

#### *Mycorrhizal colonization*

Although the experiment design required control of ECM colonization, very low ECM colonization was maintained only for seedlings in intact pots inoculated with autoclaved ECM and autoclaved AM roots (Fig. 4.2a) which averaged  $6.3 \% \pm 1.0$  (SE) ECM root tips. Addition of ECM roots increased the mycorrhizal colonization to  $25.7 \% \pm 2.9$  (Fig. 4.2a). In intact pots inoculated with fresh AM roots, pine seedlings had  $33.0 \% \pm 2.4$  ECM root tips. The highest percentage of ECM root tips was observed in slotted pots, regardless of the type of root inoculum (slotted, rotated pots:  $49.3 \% \pm 2.9$  ECM root tips; slotted, static pots:  $50.0 \% \pm 2.2$  ECM root tips; Fig. 4.2b).

Although I searched approximately 15 cm of cleared and stained fine roots per examined seedling, especially focusing on non-ECM root tips, I did not find any AM fungi in the roots of *P. elliotii* var. *densa* seedlings. The ubiquity of ECM fungus hyphae, however, made it difficult to detect possibly sparse AM colonization.

#### *Aboveground responses*

Addition of autoclaved or fresh mycorrhizal roots and pot type significantly affected mean shoot dry weight of seedlings (Fig. 4.3). The mean shoot dry weight of seedlings in intact pots with fresh AM and ECM root inoculum ( $1.12 \text{ g} \pm 0.05$ ) was significantly lower than that of all other treatments except seedlings given fresh ECM root inoculum.

Seedlings in slotted, static pots had the highest shoot dry weight ( $2.23 \text{ g} \pm 0.16$ ). For the slotted pots, seedlings in rotated pots ( $1.53 \text{ g} \pm 0.08$ ) had significantly lower shoot dry weight than seedlings in static pots. Seedlings in the slotted, rotated pots, however, had a similar shoot dry weight to seedlings in intact pots with all autoclaved roots ( $1.43 \text{ g} \pm 0.05$ ) and to those given fresh ECM roots ( $1.32 \text{ g} \pm 0.05$ ). Needle dry weight differences principally contributed to the differences in shoot dry weight (Table 4.1), because there were no significant differences among stem dry weights.

Mean total chlorophyll concentration was significantly different only between intact pots and slotted pots (Fig. 4.4), with seedlings in slotted pots having more than twice the chlorophyll concentration of seedlings in intact pots. Seedlings in slotted, rotated and slotted, static pots had similar chlorophyll concentrations (rotated:  $429.7 \text{ } \mu\text{g/g} \pm 25.4$ ; static:  $509.2 \text{ } \mu\text{g/g} \pm 22.7$ ). All seedlings in intact pots had statistically indistinguishable chlorophyll concentrations, regardless of mycorrhizal root inocula. Total chlorophyll concentration principally comprised chlorophyll *a* which was more than three-fold higher than chlorophyll *b* concentration. Both chlorophyll *a* and *b* concentrations followed the same trends as total chlorophyll, with chlorophyll *a* and *b* concentrations highest in slotted pots (Table 1). Although chlorophyll concentrations differed significantly among treatments, chlorophyll *a/b* ratios (ranging from 3.64 to 3.96) did not (Table 4.1).

Many mean foliar mineral nutrient concentrations and contents were affected significantly by treatments (Tables 4.2 and 4.3). In particular, all seedlings in slotted pots had higher concentrations of N, K, and Zn than seedlings in intact pots, but P concentrations did not differ among treatments. Accordingly, N:P and K:P were higher in seedlings in slotted than in intact pots. After sequential Bonferroni correction (Rice

1990), foliar concentrations of Ca and Mg (Table 4.2) were marginally significantly affected by treatments (threshold *P*-values for significance of Ca = 0.00417, and of Mg = 0.00455). The concentration of Ca was higher in seedlings in intact pots with autoclaved root inoculum than in slotted, static pots, while the other treatments were intermediate. Magnesium concentration followed a similar pattern, with Mg concentration higher in intact pots with autoclaved roots than in slotted, static and rotated pots. The concentration of Mn was lowest in intact pots with autoclaved roots, and significantly different from that in slotted pots. Seedlings in slotted, rotated pots had a similar Mn concentration to those in intact pots with fresh ECM and with fresh ECM and AM root inocula. Concentrations of Fe, Cu, and N:K did not differ among treatments. Although B concentration appeared to be affected by treatment according to its individual Kruskal-Wallis analysis (Table 4.2), the Bonferroni correction suggested that it did not differ among treatments (threshold *P*-value after sequential Bonferroni correction = 0.0056).

Mean foliar N and Zn contents of needles were significantly higher for seedlings in slotted, static pots than for those in any other treatment except slotted, rotated pots (Table 4.3). Nitrogen and Zn contents in slotted, rotated pots were similar to those in intact pots with autoclaved roots, while the N and Zn contents were lowest in intact pots with fresh ECM roots and with fresh AM and ECM root inocula. Phosphorus content was significantly higher in slotted, static pots than in all other treatments except intact pots with fresh ECM root inoculum which were intermediate to all other groups. The contents of K and Mn were higher in slotted pots than in intact pots. The contents of Mg and Ca were significantly higher in slotted, static pots than in intact pots with fresh ECM or with fresh AM and ECM root inoculum. Contents of B, Fe and Cu did not differ among treatments.

I compared the effect of rotation on mean dry weights and mineral nutrient contents of seedlings in slotted pots to assess the importance of extraradical mycelium beyond the pots. Even though all seedlings in slotted pots had similar ECM colonization, rotation significantly diminished needle dry weight by 32.5 % and diminished the contents of Mn, K, P, N, and Zn (Fig. 4.5). Static seedlings took up 47.9 % more Mn, 44.6 % more K, 41.1 % more P, 40.3 % more N, and 36.7 % more Zn than rotated seedlings.

Because seedlings in intact pots with autoclaved root inoculum had a 24.8 % higher needle dry weight than those in intact pots with fresh AM and ECM root inocula, I compared the mean nutrient contents of those two original treatments to assess the effects of all autoclaved versus all fresh root inocula. Although seedlings with all autoclaved inoculant roots had a low level of ECM colonization, they had significantly higher contents of Mg, Ca, and Zn than those inoculated with both fresh AM and ECM roots (Fig. 4.6). Seedlings grown with fresh AM and ECM roots took up 36.3 % less Mg, 35.5 % less Ca, and 33.3 % less Zn than those given autoclaved roots.

Total chlorophyll concentration was most strongly correlated with N concentration (Spearman's  $r(r_S) = 0.76$ , Bonferroni-corrected  $P(P_{Bc})$  for 33 correlations  $< 0.0033$ , Fig. 4.7), but also was correlated with concentrations of Zn ( $r_S = 0.71$ ,  $P_{Bc} < 0.0033$ ), K ( $r_S = 0.63$ ,  $P_{Bc} < 0.0033$ ), Mn ( $r_S = 0.38$ ,  $P_{Bc} < 0.0033$ ), P ( $r_S = 0.32$ ,  $P_{Bc} = 0.0033$ ), percent ECM root tips ( $r_S = 0.62$ ,  $P_{Bc} < 0.0033$ ), and needle dry weight ( $r_S = 0.55$ ,  $P_{Bc} < 0.0033$ ). Total chlorophyll concentration was negatively correlated with B concentration ( $r_S = -0.30$ ,  $P_{Bc} = 0.0066$ ).

Several response variables besides total chlorophyll were correlated with percent ECM root tips. Variables that were positively correlated with percent ECM root tips

include needle dry weight ( $r_S = 0.38$ ,  $P_{Bc} < 0.0033$ ), P concentration ( $r_S = 0.27$ ,  $P_{Bc} = 0.0297$ ), N concentration ( $r_S = 0.49$ ,  $P_{Bc} < 0.0033$ ), K concentration ( $r_S = 0.51$ ,  $P_{Bc} < 0.0033$ ), Mn concentration ( $r_S = 0.27$ ,  $P_{Bc} = 0.0198$ ), and Zn concentration ( $r_S = 0.56$ ,  $P_{Bc} < 0.0033$ ). B concentration ( $r_S = -0.27$ ,  $P_{Bc} = 0.0198$ ) was negatively correlated with percent ECM root tips.

In addition to being positively correlated with chlorophyll concentration and percent ECM root tips, needle dry weight also was positively correlated with concentrations of P ( $r_S = 0.27$ ,  $P_{Bc} = 0.0264$ ), N ( $r_S = 0.52$ ,  $P_{Bc} < 0.0033$ ), and Zn ( $r_S = 0.47$ ,  $P_{Bc} < 0.0033$ ). Needle dry weight was negatively correlated with B concentration ( $r_S = -0.46$ ,  $P_{Bc} < 0.0033$ ).

#### *Belowground responses*

Addition of autoclaved or fresh mycorrhizal roots and pot type significantly affected mean total root dry weight of pine seedlings, in a way similar to shoot dry weight (Fig. 4.3). I found the highest total root dry weight for seedlings with autoclaved root inocula and those in slotted, rotated and static pots ( $0.72 \text{ g} \pm 0.02$ ,  $0.73 \text{ g} \pm 0.03$ , and  $0.76 \text{ g} \pm 0.03$ , respectively). Seedlings with fresh AM and ECM root inocula had a total root dry weight ( $0.59 \text{ g} \pm 0.02$ ) significantly lower than all other treatments except seedlings given fresh ECM root inoculum. Primary radicle and fine root dry weights followed similar patterns to total root dry weight (Table 4.1).

Seedlings given fresh ECM root inoculum and fresh AM and ECM root inocula had the shortest mean root lengths ( $554.5 \text{ cm} \pm 38.10$ , and  $546.2 \text{ cm} \pm 24.2$ , respectively; Fig. 4.8a). Seedlings given autoclaved roots ( $840.1 \text{ cm} \pm 26.61$ ) and seedlings in the slotted, rotated ( $899.4 \text{ cm} \pm 40.79$ ) and static ( $838.4 \text{ cm} \pm 39.75$ ) pots had the greatest

mean root lengths. Specific root length followed a pattern similar to that of root length. Seedlings given fresh ECM root inoculum ( $8.66 \text{ m/g} \pm 0.64$ ) and given fresh AM and ECM root inocula ( $9.36 \text{ m/g} \pm 0.37$ ) had lower mean specific root lengths than seedlings given autoclaved roots ( $11.93 \text{ m/g} \pm 0.62$ ) and those in slotted, rotated pots ( $12.47 \text{ m/g} \pm 0.37$ ). Seedlings in slotted, static pots had specific root lengths intermediate to all other groups ( $10.91 \text{ m/g} \pm 0.39$ ; Fig. 4.8b).

The mean root-to-shoot ratio was lowest for seedlings growing in slotted, static pots ( $0.39 \pm 0.02$ ), but was not significantly different among the four other treatments which had a root-to-shoot ratio near 0.5 (Table 4.1).

## **Discussion**

*Pinus elliottii* var. *densa* seedlings had different ectomycorrhizal colonization, mineral nutrition, growth and chlorophyll production in response to treatments. In brief, I found that ECM extraradical mycelium having access to a large volume of soil conferred high mineral nutrition, growth and chlorophyll concentration to pine seedlings. In intact pots with limited soil volume, even though pine seedlings inoculated with fresh ECM roots had a higher percentage ECM root tips than seedlings inoculated with autoclaved roots, seedlings with high ECM colonization had lower mineral nutrient uptake and growth than seedlings with low ECM colonization.

### *Mycorrhizal colonization*

After 9 months of growth in microcosms, *P. elliottii* var. *densa* seedlings had well-developed ectomycorrhizas. High ECM colonization of non-inoculated seedlings in slotted pots versus those in intact pots indicates that the GORE-TEX<sup>®</sup> membrane was

easily penetrated by ectomycorrhizal fungus hyphae while being an effective barrier against tamarind and pine seedling roots.

Although I intended to produce different levels of ECM colonization, all slotted pot treatments had similar, high ECM colonization means of about 50 % ECM root tips (Fig. 4.2a). Ectomycorrhiza formation in those treatments might have come from two sources. The soil surrounding the pots in the microcosms was not autoclaved in order to provide AM fungus propagules for the *T. indica* host plants, and although it was collected away from adult pines, it may have contained ECM fungus propagules. Additionally, ECM fungi from inoculum added to half of the slotted pots might have spread into non-inoculated slotted pots through the microcosm soil. That the percentage ECM root tips did not differ statistically between slotted, rotated and slotted, static pots (Fig. 4.2b) suggests that all seedlings may have been colonized simultaneously, and favors the presence of ECM fungus propagules in the microcosm soil.

Pine seedlings that were inoculated with all autoclaved roots had 6.3 % ECM root tips at the time of harvest. Those ECM fungi might have come from several sources. First, some ECM fungi might have survived after autoclaving of root inoculum or soil and thus colonized the seedlings. Second, although I carefully watered the seedlings to avoid water splash, cross-contamination through splash might have happened. Finally, airborne fungus spores are often the cause of contaminations in mycorrhizal greenhouse experiments. Concurrent with this experiment, I conducted a mushroom survey fortnightly in a nearby pineland, and I found fruiting bodies of potentially ectomycorrhizal fungi only on very few days (Janos and Wyss, unpublished data). The paucity of fruiting bodies, together with very low spontaneous ECM colonization of pine

seedlings in another concurrent greenhouse experiment, suggest that airborne spore contamination is low in South Florida.

Overall, I observed few ECM morphotypes. The dominant morphotype was a yellowish morphotype, in particular in slotted pots, which might correspond to one single fungus species. Very occasionally, a black-hairy morphotype, which had similar characteristics to *Cenococcum geophilum* Fr. was observed on pine roots, but I did not quantify the occurrence of any of those morphotypes.

Some pine species can be colonized by AM fungi from spores or when an AM host grows nearby in the field (Horton et al. 1998; Smith et al. 1998), and I searched for AM structures in pine seedling roots but did not find any. Although AM fungi from *S. repens* root inoculum may have lacked the capacity to colonize pine roots, I anticipated that AM fungi sustained by the tamarind host plant were most likely to be competent to colonize the pine seedlings to which they had access in slotted pots. Unfortunately, those seedlings had the highest percentage ECM tips (Fig. 4.2b) which almost entirely precluded observation of AM fungi in pine seedlings roots. My experiment did not resolve whether *P. elliotii* var. *densa* seedlings can form AM or be affected directly by them.

#### *Aboveground responses in intact pots*

I added autoclaved ECM and AM roots and a microbial filtrate to control for the effects of adding fresh organic matter and accompanying microbes to inoculated pots.

Interestingly, when seedlings in intact pots are compared, addition of fresh AM and ECM root inocula decreased needle dry weight by 23 % versus addition of autoclaved roots of both types (Table 4.1, Fig. 4.3). Total belowground dry weight and stem diameter at



harvest (data not shown) also were reduced by the addition of fresh AM and ECM root inocula.

The difference in seedling growth between intact pots given all autoclaved or all fresh mycorrhizal root inocula might be explained by several phenomena that are not mutually exclusive. Autoclaved roots might have provided an early pulse of mineral nutrients to seedlings; saprotrophs might have immobilized more mineral nutrients while decomposing non-autoclaved, recalcitrant, woody roots; ECM fungi might have retained nutrients or otherwise failed to amortize their carbon cost, and parasites or pathogens might have been transmitted by fresh root inocula.

By physically disrupting cells, autoclaving of root inocula might have released a pulse of nutrients for pine seedlings immediately after transplant. Seedlings in intact pots that received autoclaved roots had higher mean Mg and Ca contents than those that received fresh AM and ECM roots (Table 4.3; Fig. 4.6). An early, slight increase in nutrient availability might have benefitted the early growth and photosynthesis of pine seedlings, which then provided a persistent advantage for subsequent growth.

Pine roots are woody and palm roots are coarse (Fisher and Jayachandran 1999), and fresh roots might have been difficult for saprotrophic microorganisms to degrade. In consequence, saprotrophic microorganisms might have immobilized more mineral nutrients released from fresh roots than from autoclaved roots. Because of the high C:N and C:P of plant litter, saprotrophic microorganisms decomposing plant litter immobilize nutrients such as N (Chapin III et al. 2002; Homyak et al. 2008) and P (Herbert et al. 2003) before net mineralization takes place. Saprotrophic microorganisms reduced the N content of *Pinus resinosa* Ait. seedlings, especially at low soil N availability (Koide and Kabir 2001). The ECM fungus *Pisolithus tinctorius* (Pers) Coker & Couch was not able

to overcome that reduction, but both the saprotrophic microorganisms and *P. tinctorius* increased *P. resinosa* seedling P content (Koide and Kabir 2001). Addition of forest soil to enhance ECM colonization of *Pseudotsuga menziesii* (Mirb.) Franco var. *glauca* (Beissn.) seedlings caused a reduction in their growth especially at intermediate levels of N fertilization (Kazantseva et al. 2009). The saprotrophic microorganisms present in the fresh forest soil that Kazantseva et al. (2009) added might have immobilized mineral nutrients. In my experiment, however, neither seedling N nor P content differed among seedlings in intact pots (Table 4.3). Moreover, N:P means were similar among seedlings in intact pots (Table 4.2), which suggests no difference in potential seedling N limitation (Güsewell et al. 2003; Koerselman and Meuleman 1996). Although saprotrophic microorganisms seem not to have immobilized N or P in my experiment, I cannot exclude that they immobilized Mg and Ca, and possibly Zn (Fig. 4.6).

It is possible that ECM fungi, which colonized a mean 30.2 % of pine seedling root tips in the presence of fresh root inocula, retained some mineral nutrients. When colonized by several different ECM fungus species, *Pinus sylvestris* L. seedlings had reduced N concentrations (Colpaert et al. 1992), although in my experiment similar N:P means among intact pot treatments that differed in mean percentage ECM root tips (Fig. 4.2b) suggest that N was not retained. ECM fungi might use  $\text{Ca}^{2+}$  to stabilize polyphosphates (Orlovich et al. 1989), thus possibly immobilizing Ca, but the role of Ca in polyphosphate stabilization is controversial (Bucking and Heyser 2000; Orlovich and Ashford 1993).

Growth reduction of seedlings inoculated with fresh roots versus those given autoclaved roots might reflect a carbon cost of mycorrhizal fungi that seedlings cannot satisfy because they have limited photosynthetic capacity. Growth reduction of pine

seedlings by ECM fungi have been observed by other authors (Colpaert et al. 1992; Conjeaud et al. 1996). In those studies, growth depression could be attributed definitively to ECM fungi because pure-culture inocula were used, thereby excluding saprotrophic microorganisms. Others (Corrêa et al. 2008), however, have suggested that growth depression of ECM plants is not a direct consequence of excessive carbon cost of mycorrhizal fungi but instead reflects retention of N by ECM fungi. As already described, N retention in my experiment is unlikely.

Finally, fresh root inocula might have transmitted a parasite or pathogen. Although pines with autoclaved roots received microbial filtrate intended to establish a similar microbial population to that of pines given fresh root inocula, the microbial filtrate passed a small pore-size filter (11  $\mu\text{m}$ ) that might have removed some detrimental fungi or nematodes. I occasionally observed a few nematodes on seedlings' roots, but did not quantify them. I did not see apparent fungal parasites or pathogens in cleared, stained pine seedling roots.

Introduction of parasites or pathogens and excessive carbon cost of ECM do not seem likely to explain the growth reduction associated with the addition of fresh root inocula to intact pots, although I cannot conclusively exclude these phenomena. Fertilization by addition of autoclaved roots, mineral nutrient immobilization by saprotrophic microorganisms, and mineral nutrient retention by ECM fungi are complementary phenomena that might explain the seedling growth reduction. Saprotrophs, however, are most likely to immobilize N or P, which is not consistent with my results. The most probable effect of the addition of autoclaved roots is that they elevated Ca availability sufficiently to increase seedling growth. Field-collected pine roots have two times the Ca, seven times the Fe, and 75 times the Zn of field-collected *S.*

*repens* roots, but have similar concentrations of other macronutrients. Thus, pine roots or their ECM seem to substantially accumulate these elements. The concentrations of Ca, Fe, and Zn in field-collected pine roots are 19, 74, and 213 times their concentrations in flatwoods soil. In my experiment, however, neither Fe nor Zn content differed significantly among intact pot treatments (Table 4.3), and Zn differed only when the extreme intact pot treatments were compared (Fig. 4.6 versus Table 4.3), so supply of Ca by autoclaved root inocula and retention of Ca by ECM fungi are the most plausible phenomena to explain the growth difference that I found for intact pots given all autoclaved versus fresh AM and ECM roots.

#### *Aboveground responses in slotted versus intact pots*

Rotation of in-growth cores inserted in a large volume of soil is a powerful tool for mycorrhiza research (Rillig 2004). For example, rotation of in-growth cores inserted into a grassland reduced AM formation, shoot biomass, N content, and P content of the herb, *Trifolium repens* L. (Johnson et al. 2001), and rotation of in-growth cores in a Lychee grove reduced the dry weight, N, P, and Cu concentrations of foliage of AM *Sapindus saponaria* L. seedlings (D. P. Janos, unpublished data). In my *P. elliotii* var. *densa* experiment, weekly rotation of seedlings did not significantly affect ECM colonization (Fig. 4.2b) or chlorophyll concentration (Fig. 4.4), but it reduced needle dry weight by 33 % (Fig. 4.3). Rotation also significantly diminished total foliar Mn, K, P, N, and Zn (Fig. 4.5) in proportions similar to the reduction of growth, implying that those mineral nutrients in 220 mL of flatwoods soil within a pot were insufficient to maximize pine seedling growth. This illustrates the importance of an extensive extraradical mycelium for ectomycorrhizal pine seedlings as for AM plants.

Nitrogen-to-phosphorus ratio can indicate potential N versus P limitation of plant growth (Koerselman and Meuleman 1996). In my experiment, the mean N:P of pines in all treatments were below 14, which suggests that pine seedlings grown in autoclaved flatwoods soil might have been N-limited. Seedlings in intact pots, with mean N:P below 9, likely were more N-limited than seedlings in slotted pots that had mean N:P just below 14, regardless of rotation. The greater percentage of ECM root tips in slotted than in intact pots seemed to enhance seedling N nutrition more than P uptake, although in intact pots N:P means were not detectably affected by differences in percentage ECM root tips (Table 4.2). The extraradical mycelium beyond slotted pots increased N and P concentrations in similar proportions, and so, rotation did not alter N:P.

Chlorophyll concentration often reflects a plant's mineral nutrient status, and in my experiment total chlorophyll concentration was most strongly correlated with foliar N concentration (Fig. 4.7). Total chlorophyll concentration also was positively correlated with foliar concentrations of Zn, K, Mn, and P, and was negatively correlated with B. Elevated chlorophyll concentrations associated with enhanced mineral nutrition of pines (Fife and Nambiar 1997) can improve photosynthesis capacity (Tissue et al. 1993; Wang and Kellomäki 1997). Phosphorus fertilization enhanced the photosynthetic capacity of *Pinus pinaster* Ait. seedlings (Loustau et al. 1999). The significant positive correlations between total chlorophyll concentration and mineral nutrient concentrations that I found suggest similar effects of mineral nutrients, especially N, for seedling *P. elliotii* var. *densa*.

Although pine seedlings in slotted, static pots had a higher mean total dry weight than seedlings in slotted, rotated pots (Fig. 4.3), they did not differ in mean total chlorophyll concentration. Additionally, although seedlings in slotted, rotated pots did

not differ in mean total dry weight from those in intact pots with autoclaved or fresh ECM root inoculum, they did differ significantly in mean total chlorophyll concentration. This suggests that seedling growth was limited directly by mineral nutrients and not by total chlorophyll concentration and presumed photosynthetic capacity.

Percentage ECM root tips (Fig. 4.2b) and concentrations of N, K, and Zn were higher in seedlings in slotted, rotated pots than in all intact pots (Table 4.2), but dry weight was significantly increased only versus seedlings in intact pots given fresh AM and ECM root inocula. The similarity of growth among these treatments (other than that provided fresh root inocula) suggests that seedlings may be capable of ‘luxury accumulation’ of mineral nutrients such as N, K, and Zn. The accumulation of N, K, and Zn was not a luxury for total chlorophyll concentration, however, because of the positive correlations between these elements and chlorophyll concentration. So called ‘luxury accumulation’ of mineral nutrients by plants might have important ecological consequences in maintaining co-existence among species in nutrient-poor ecosystems (van Wijk et al. 2003).

Even though needle dry weight was positively correlated with concentrations of N and Zn, the luxury accumulation of those elements suggests that they were not likely to have limited seedling growth. Needle dry weight also was positively correlated with P concentration, and no luxury accumulation of P was observed because P concentration did not differ among treatments (Table 4.2). Therefore, even though the mean N:P of seedlings in slotted pots was close to 14, P might be the growth-limiting mineral nutrient of *P. elliotii* var. *densa* seedlings in Florida flatwoods soil. ECM likely enhanced P uptake as suggested by a significant, positive correlation between percent ECM root tips and foliar P concentration ( $r_s = 0.27$ ,  $P_{Bc} = 0.0297$ ), and by high mean P content when

ECM extraradical mycelium had unbroken access to soil beyond slotted, static pots (Fig. 4.5).

I observed a ‘dilution effect’ of plant size on concentrations of Mg and Ca, which were lower in seedlings in slotted, static pots than in intact pots with autoclaved roots (Table 4.2). Such dilution effects are well known for AM fungi that enhance plant N and P nutrition and growth (e.g., Azcon and Barea 1992; Azcon et al. 1991; Pacovsky 1986) without increasing the uptake of Fe, Mn, Ca, and Mg. Thereby, these elements are ‘diluted’ throughout an increased amount of plant tissue, diminishing their foliar concentrations.

ECM fungi might retain Ca and thereby partially compete with their hosts for Ca. The ECM fungus *Suillus bovinus* (L. ex. Fr.) reduced *Pinus sylvestris* shoot Ca concentration under low fertilization (Bucking and Heyser 2000). Even in calcium-rich soil, ECM improved the growth of *Pinus nigra* Arn. ssp. *nigrans* seedlings by lowering foliar Ca concentration and thereby alleviating iron chlorosis (Clement et al. 1977). Flatwoods soil is acidic, and contains a relatively small amount of Ca (125 mg/kg), and the overall mean foliar concentration of Ca in my experiment (0.39 %) was somewhat low compared to that reported for other pine species. *Pinus halepensis* Miller in Greece and Spain had 0.77 % and 0.51 % Ca, respectively (Michopoulos et al. 2007), and *Pinus nigra* ssp. *nigrans* ranged from as much as 1.37 % to 0.16 % Ca depending on the presence or absence of CaCO<sub>3</sub> in the growth medium (Clement et al. 1977). In a low-nutrient solution, however, *Pinus sylvestris* had a foliar Ca concentration of only 0.14 % (Ingestad 1979). Nevertheless, my large, *P. elliotii* var. *densa* seedlings in slotted, static pots had the highest mean Ca content of all treatments (Table 4.3), and so the dilution effect for Ca most likely was a consequence of plant size. Seedlings given fresh

mycorrhizal root inocula in intact pots, however, had the lowest mean Ca content of all treatments in spite of having a mean Ca concentration (Table 4.2) identical to the grand mean Ca concentration. Together, these observations suggest that Ca might have limited the growth of seedlings in intact pots.

#### *Belowground responses*

Pine seedling root dry weight was affected by fresh versus autoclaved root inocula and by pot type in a manner similar to shoot dry weight (Fig. 4.3). Seedlings in intact pots that received autoclaved roots had higher total root dry weight than seedlings given fresh AM and ECM root inocula. Because all intact pot seedlings had statistically indistinguishable root/shoot ratios (Table 4.1), diminished root dry weight probably reflects reduced carbon allocation to roots because of diminished shoot dry weight. Reduced root length (Fig. 4.8a), however, is not solely attributable to reduced root dry weight. It also was influenced by root morphology, as indicated by higher mean specific root length of seedlings given autoclaved roots than those given fresh AM and ECM root inocula (Fig. 4.8b). Seedlings given autoclaved roots produced long, fine roots that compensated for the lack of ECM. In pots inoculated with fresh mycorrhizal roots, long, fine, non-mycorrhizal roots were supplanted by ECM short roots which diminished specific root length. Dosskey et al. (1992) found a similar reduction in specific root length of ECM *Pseudotsuga menziesii*.

Seedlings in slotted, static pots had a significantly lower mean root/shoot ratio than all other treatments. With a root/shoot ratio of 0.39, slotted, static seedlings produced higher mean aboveground dry weight than all other seedlings in the experiment in spite of having predominantly statistically indistinguishable total root dry weight.



Clearly, the high ectomycorrhizal colonization and persistent connection to extraradical hyphae beyond the pots of pine seedlings in slotted, static pots conferred the highest mineral nutrient uptake and produced the greatest shoot dry weight with the lowest allocation to root dry weight (Fig. 4.3).

#### *Implications for flatwoods ecosystems*

In my experiment, the least growth by seedlings in intact pots given fresh mycorrhizal root inocula is most likely to have been a consequence of a pulse of Ca released from autoclaved roots, and Ca retention by ECM fungi. Physical restriction of the ECM mycelium to the limited (220 mL) soil volume of intact pots probably was critical to constraining mineral nutrient accessibility and producing the differences in seedling performance among intact pot treatments. Although ECM fungi can suppress litter decomposition in the field (Gadgil and Gadgil 1975; Högberg and Högberg 2002), they are not likely to have this effect if solution-phase nitrogen does not limit the growth of decomposer microorganisms (Harmer and Alexander 1985). In the field, ECM hyphae can extend far from their host (Cline et al. 2005) making unlikely in flatwoods the suppressive effects of ECM and organic matter that I observed in intact pots.

If autoclaved roots added to intact pots had a fertilization effect by releasing a pulse of mineral nutrients, it suggests the potential importance of the frequent wildfires in flatwoods to establishing *P. elliotii* var. *densa* seedlings. Some mineral nutrients such as K, Ca, and Mg, are released from plant litter by fires, and thereby might favor the growth of surviving pine seedlings. Fires are essential for the maintenance of central Florida ecosystems such as flatwoods and sandhills (Abrahamson 1984). In sandhills, even though a fire (maximum temperature: 732°C) did not affect mineral nutrients in the soil,

N and P concentrations in surviving plants were more than 3 times higher than in unburned sites one-and-a-half months after the fire (Anderson and Menges 1997). In central Florida ecosystems, soil K, Mg, and Ca concentrations can show a short-lived increase after fire (Abrahamson 1984). In my experiment, I found that pine seedlings given autoclaved roots had higher contents of Ca and Mg (Table 4.3) and greater dry weights (Fig. 4.3) than pines given fresh AM and ECM root inocula suggesting that, even if short-lived, increases in Ca and Mg in flatwoods soil after a non-lethal fire might favor pine seedling establishment.

In my microcosm experiment, the treatment that most closely matched field conditions was slotted, static pots because it allowed extraradical hyphae to forage for mineral nutrients from a large volume of soil in spite of the presence of the AM host *T. indica*. In flatwoods, however, the roots of *S. repens* colonize soil far more densely (T. Wyss, personal observation) than *T. indica* roots colonized my microcosms, so *S. repens* might limit the soil volume accessible to ECM fungi. Although ECM mycelia can be disturbed by foraging soil micro- and mesofauna in the field (Ek et al. 1994), such disruptions are not likely to approach the severity of weekly breaking connections to ECM hyphae external to slotted, rotated pots. So, root restriction of the soil volume that can be colonized by ECM fungi in the field probably has a greater effect on pine seedling mineral nutrition than faunal disruption of ECM fungus mycelia.

Survival through the seedling stage is critical to the maintenance of plant populations. Flatwoods ecosystems with their nutrient-poor soils, November to April dry season, and frequent disturbance by wildfires (Abrahamson 1984) may be a very challenging environment for seedling establishment. The results of my microcosm experiment suggest that growth of *P. elliotii* var. *densa* seedlings in flatwoods

ecosystems is favored by extensive ECM extraradical mycelium that improves seedling mineral nutrition, chlorophyll concentration, dry weight, and presumptively, competitive ability. Because ECM fungi often colonize several plants in the field, thereby forming common mycorrhizal networks (Simard et al. 1997b), the survival of *Pinus elliottii* var. *densa* seedlings connected to extensive mycorrhizal networks might be highest when the seedlings are interspersed among adult pines (Booth and Hoeksema 2010). Even if ECM fungus inocula are ubiquitous in flatwoods soil, disturbances that alter mineral nutrient cycling, the incidence of co-occurring, competing plant species, or the extent and functioning of the ECM extraradical mycelium could substantially influence whether pine populations decline.

**Table 4-1** Aboveground and belowground responses of five groups of *Pinus elliotii* var. *densa* seedlings grown in microcosms with autoclaved or fresh ECM and/or AM inoculum, in intact or slotted (rotated or static) pots. Values are means  $\pm$  SE. Significant differences among groups were determined by Kruskal-Wallis analyses of variance followed by all pairwise comparisons post-hoc tests. Within a row, values followed by the same letter are not statistically different at  $P < 0.05$ . Probabilities in bold indicate significance after sequential Bonferroni correction (Rice 1990)

Response variable	Intact pots, autoclaved roots	Intact pots, fresh ECM roots	Intact pots, fresh AM and ECM roots	Slotted, rotated pots	Slotted, static pots	Kruskal-Wallis statistic/ <i>P</i>
Needle dry weight (g)	1.15 $\pm$ 0.05 b	1.07 $\pm$ 0.04 bc	0.89 $\pm$ 0.03 c	1.30 $\pm$ 0.07 b	1.90 $\pm$ 0.14 a	53.91 <b>&lt;0.0001</b>
Stem dry weight (g)	0.28 $\pm$ 0.01 a	0.24 $\pm$ 0.01 a	0.23 $\pm$ 0.008 a	0.22 $\pm$ 0.01 a	0.32 $\pm$ 0.02 a	11.84 0.0186
Chlorophyll <i>a</i> ( $\mu$ g/g)	145.43 $\pm$ 11.54 b	125.68 $\pm$ 8.14 b	130.95 $\pm$ 5.23 b	338.89 $\pm$ 19.16 a	404.12 $\pm$ 17.39 a	125.74 <b>&lt;0.0001</b>
Chlorophyll <i>b</i> ( $\mu$ g/g)	39.69 $\pm$ 4.19 b	33.07 $\pm$ 3.15 b	37.29 $\pm$ 1.86 b	90.69 $\pm$ 6.70 a	105.18 $\pm$ 5.61 a	114.41 <b>&lt;0.0001</b>
Chlorophyll <i>a/b</i> ratio	3.86 $\pm$ 0.14 a	3.85 $\pm$ 0.12 a	3.64 $\pm$ 0.09 a	3.91 $\pm$ 0.09 a	3.96 $\pm$ 0.10 a	6.60 0.1585
Primary radicle dry weight (g)	0.10 $\pm$ 0.01 a	0.10 $\pm$ 0.007 ab	0.08 $\pm$ 0.005 b	0.06 $\pm$ 0.004 a	0.08 $\pm$ 0.008 a	28.45 <b>&lt;0.0001</b>
Fine root dry weight (g)	0.62 $\pm$ 0.02 a	0.55 $\pm$ 0.02 a	0.51 $\pm$ 0.02 ab	0.66 $\pm$ 0.02 b	0.68 $\pm$ 0.03 ab	19.17 <b>0.0007</b>
Root /shoot ratio	0.51 $\pm$ 0.02 a	0.50 $\pm$ 0.02 a	0.53 $\pm$ 0.01 a	0.50 $\pm$ 0.01 a	0.39 $\pm$ 0.02 b	34.46 <b>&lt;0.0001</b>

**Table 4-2** Mineral nutrient concentrations in needles of *Pinus elliotii* var. *densa* seedlings grown in microcosms with autoclaved or fresh ECM and/or AM inoculum, in intact or slotted (rotated or static) pots. Values are means  $\pm$  SE. Significant differences among groups were determined by Kruskal-Wallis analyses of variance followed by all pairwise comparisons post-hoc tests. Within a row, values followed by the same letter are not statistically different at  $P < 0.05$ . Probabilities in bold indicate significance after sequential Bonferroni correction (Rice 1990)

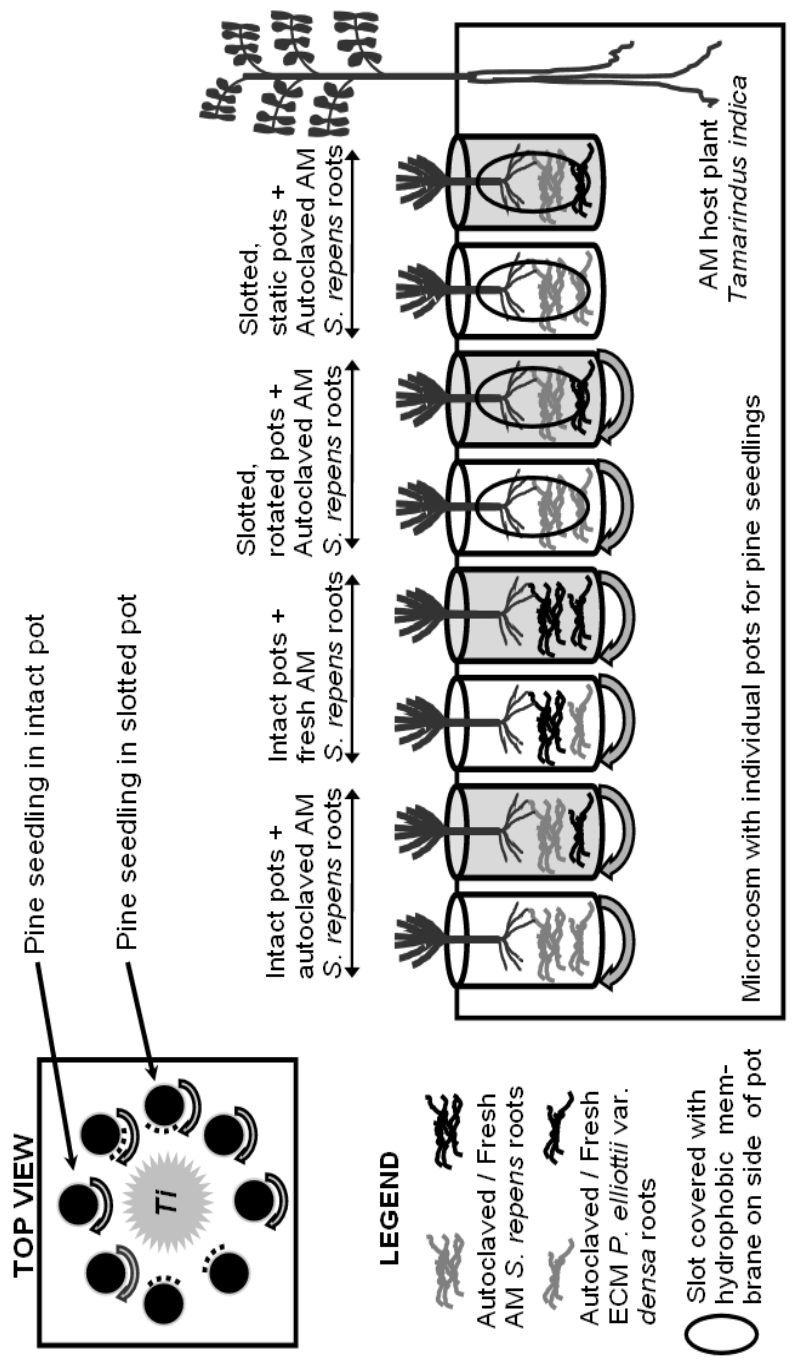
Element or ratio	Intact pots, autoclaved roots	Intact pots, fresh ECM roots	Intact pots, fresh AM and ECM roots	Slotted, rotated pots	Slotted, static pots	Kruskal-Wallis statistic/ $P$
N (% dry wt)	0.64 $\pm$ 0.04 b	0.61 $\pm$ 0.04 b	0.62 $\pm$ 0.04 b	0.96 $\pm$ 0.05 a	1.08 $\pm$ 0.06 a	65.24 <b>&lt;0.0001</b>
P (% dry wt)	0.07 $\pm$ 0.004 a	0.08 $\pm$ 0.004 a	0.08 $\pm$ 0.003 a	0.07 $\pm$ 0.004 a	0.08 $\pm$ 0.004 a	8.36 0.0793
N:P	8.99 $\pm$ 0.61 b	7.48 $\pm$ 0.49 b	8.26 $\pm$ 0.51 b	13.97 $\pm$ 0.67 a	13.46 $\pm$ 0.64 a	67.62 <b>&lt;0.0001</b>
K (% dry wt)	0.61 $\pm$ 0.03 b	0.66 $\pm$ 0.04 b	0.74 $\pm$ 0.02 b	0.94 $\pm$ 0.03 a	1.04 $\pm$ 0.03 a	77.00 <b>&lt;0.0001</b>
N:K	1.08 $\pm$ 0.07 a	1.02 $\pm$ 0.09 a	0.87 $\pm$ 0.05 a	1.05 $\pm$ 0.06 a	1.06 $\pm$ 0.06 a	9.47 0.0505
K:P	8.67 $\pm$ 0.51 b	7.93 $\pm$ 0.39 b	9.76 $\pm$ 0.36 b	14.32 $\pm$ 0.85 a	13.74 $\pm$ 0.77 a	60.93 <b>&lt;0.0001</b>
Mg (% dry wt)	0.20 $\pm$ 0.01 a	0.18 $\pm$ 0.01 ab	0.18 $\pm$ 0.005 ab	0.18 $\pm$ 0.01 b	0.17 $\pm$ 0.01 b	14.61 0.0056
Ca (% dry wt)	0.43 $\pm$ 0.01 a	0.39 $\pm$ 0.03 ab	0.39 $\pm$ 0.01 ab	0.38 $\pm$ 0.01 ab	0.36 $\pm$ 0.01 b	13.83 0.0079
Fe (mg/kg)	178.68 $\pm$ 48.57 a	161.79 $\pm$ 35.61 a	118.38 $\pm$ 13.16 a	159.24 $\pm$ 23.75 a	117.39 $\pm$ 21.58 a	5.60 0.2311
Mn (mg/kg)	147.36 $\pm$ 8.56 c	156.58 $\pm$ 11.78 <sub>bc</sub>	173.27 $\pm$ 8.27 bc	186.55 $\pm$ 7.44 ab	228.05 $\pm$ 10.45 a	37.42 <b>&lt;0.0001</b>

Table 4-2 (Continued)

Element or ratio	Intact pots, autoclaved roots	Intact pots, fresh ECM roots	Intact pots, fresh AM and ECM roots	Slotted, rotated pots	Slotted, static pots	Kruskal-Wallis statistic/ <i>P</i>
B (mg/kg)	30.27 ± 2.40 a	24.26 ± 3.36 ab	28.51 ± 2.02 ab	24.29 ± 1.93 ab	22.17 ± 2.65 b	11.53 0.0212
Cu (mg/kg)	6.77 ± 1.52 a	6.42 ± 1.03 a	7.43 ± 1.13 a	8.26 ± 1.67 a	7.24 ± 1.07 a	0.24 0.9932
Zn (mg/kg)	37.05 ± 1.67 b	34.05 ± 2.25 b	36.35 ± 1.98 b	57.55 ± 2.59 a	61.63 ± 2.94 a	68.28 <b>&lt;0.0001</b>

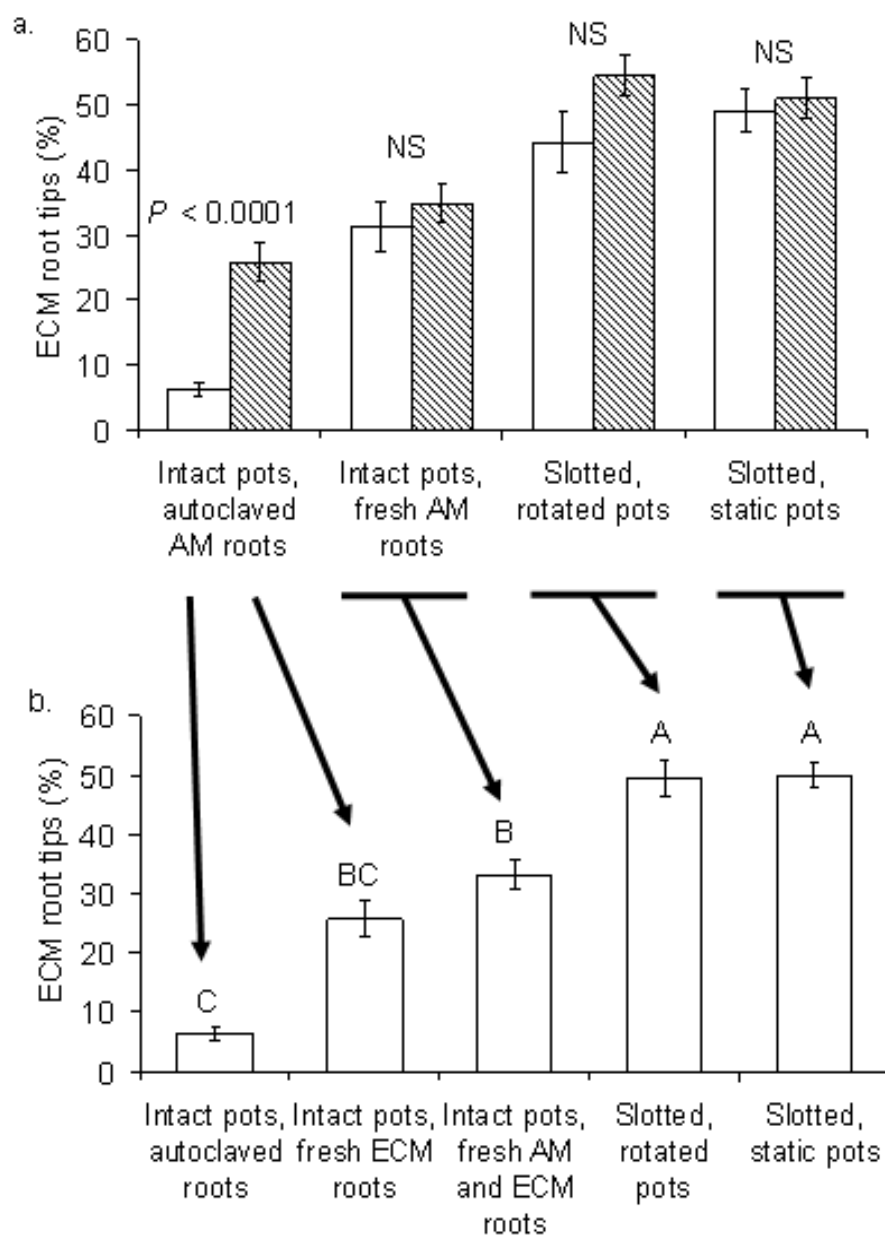
**Table 4-3** Mineral nutrient contents in needles of *Pinus elliotii* var. *densa* seedlings grown in microcosms with autoclaved or fresh ECM and/or AM inoculum, in intact or slotted (rotated or static) pots. Values are means  $\pm$  SE. Significant differences among groups were determined by Kruskal-Wallis analyses of variance followed by all pairwise comparisons post-hoc tests. Within a row, values followed by the same letter are not statistically different at  $P < 0.05$ . Probabilities in bold indicate significance after sequential Bonferroni correction (Rice 1990)

Element	Intact pots, autoclaved roots	Intact pots, fresh ECM roots	Intact pots, fresh AM and ECM roots	Slotted, rotated pots	Slotted, static pots	Kruskal-Wallis statistic/ $P$
N (mg)	7.52 $\pm$ 0.50 bc	6.64 $\pm$ 0.47 c	6.00 $\pm$ 0.40 c	13.16 $\pm$ 1.11 ab	22.05 $\pm$ 2.21 a	68.21 <b>&lt;0.0001</b>
P (mg)	0.86 $\pm$ 0.05 b	0.89 $\pm$ 0.04 ab	0.73 $\pm$ 0.03 b	1.01 $\pm$ 0.09 b	1.72 $\pm$ 0.16 a	29.25 <b>&lt;0.0001</b>
K (mg)	7.04 $\pm$ 0.25 b	6.89 $\pm$ 0.31 b	6.88 $\pm$ 0.23 b	12.21 $\pm$ 0.64 a	20.33 $\pm$ 1.49 a	92.51 <b>&lt;0.0001</b>
Mg (mg)	2.36 $\pm$ 0.11 ab	1.92 $\pm$ 0.12 bc	1.72 $\pm$ 0.07 c	2.35 $\pm$ 0.16 ab	3.35 $\pm$ 0.28 a	36.43 <b>&lt;0.0001</b>
Ca (mg)	5.06 $\pm$ 0.23 ab	4.09 $\pm$ 0.24 bc	3.74 $\pm$ 0.19 c	5.07 $\pm$ 0.31 ab	7.03 $\pm$ 0.56 a	37.69 <b>&lt;0.0001</b>
Fe ( $\mu$ g)	215.35 $\pm$ 68.79 a	186.01 $\pm$ 48.57 a	111.50 $\pm$ 12.20 a	228.10 $\pm$ 41.60 a	241.86 $\pm$ 51.56 a	7.92 0.0944
Mn ( $\mu$ g)	167.02 $\pm$ 6.32 b	163.37 $\pm$ 8.23 b	160.13 $\pm$ 6.33 b	232.87 $\pm$ 9.00 a	447.16 $\pm$ 35.80 a	82.54 <b>&lt;0.0001</b>
B ( $\mu$ g)	34.46 $\pm$ 2.56 a	25.08 $\pm$ 3.10 a	26.56 $\pm$ 1.84 a	30.29 $\pm$ 2.61 a	38.43 $\pm$ 3.66 a	10.22 0.0370
Cu ( $\mu$ g)	7.91 $\pm$ 1.66 a	7.35 $\pm$ 1.30 a	7.30 $\pm$ 1.12 a	11.22 $\pm$ 2.34 a	14.40 $\pm$ 2.59 a	6.30 0.1781
Zn ( $\mu$ g)	43.34 $\pm$ 2.43 bc	36.29 $\pm$ 2.58 c	34.69 $\pm$ 2.26 c	78.73 $\pm$ 6.29 ab	124.41 $\pm$ 10.69 a	73.70 <b>&lt;0.0001</b>

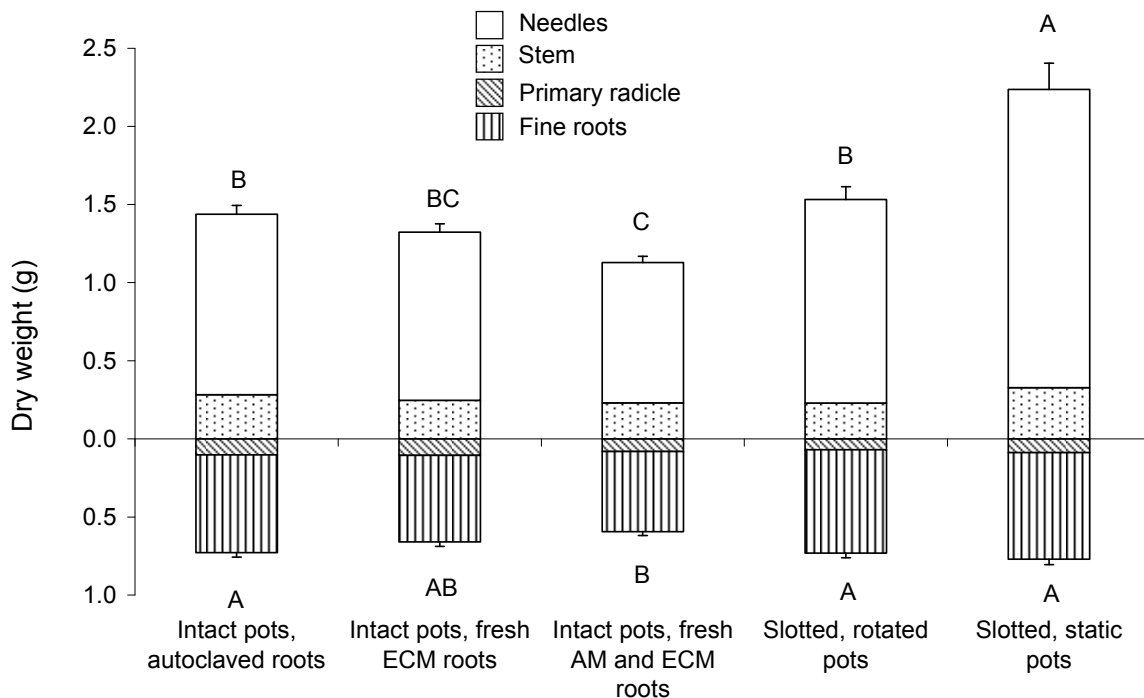


**Figure 4-1** Fully factorial, two-factor microcosm experiment designed to investigate the effect of ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi on performance of *Pinus elliotii* var. *densa* seedlings in flatwoods soil. The AM-pot factor consisted of four levels (intact pot with autoclaved AM roots [*Serenoa repens* roots], intact pot with fresh AM roots, slotted rotated pot [arrows], slotted static pot), and the ECM factor consisted of two levels (autoclaved or fresh ECM root inoculum). The 8 treatment combinations were randomly inserted at equal distances around a central AM *Tamarindus indica* nurse plant in a microcosm filled with fresh flatwoods soil. To control for the effect of rotation of open pots, all pots, except the open static pots, were rotated weekly. The AM and ECM root inocula, represented schematically, were deposited at the bottom of the planting hole at the time pine seedlings were transplanted. The insert on the top left indicates how, in a top view, pine seedlings were arranged at equal distance around a central tamarind in the microcosms. *Ti* = *Tamarindus indica*

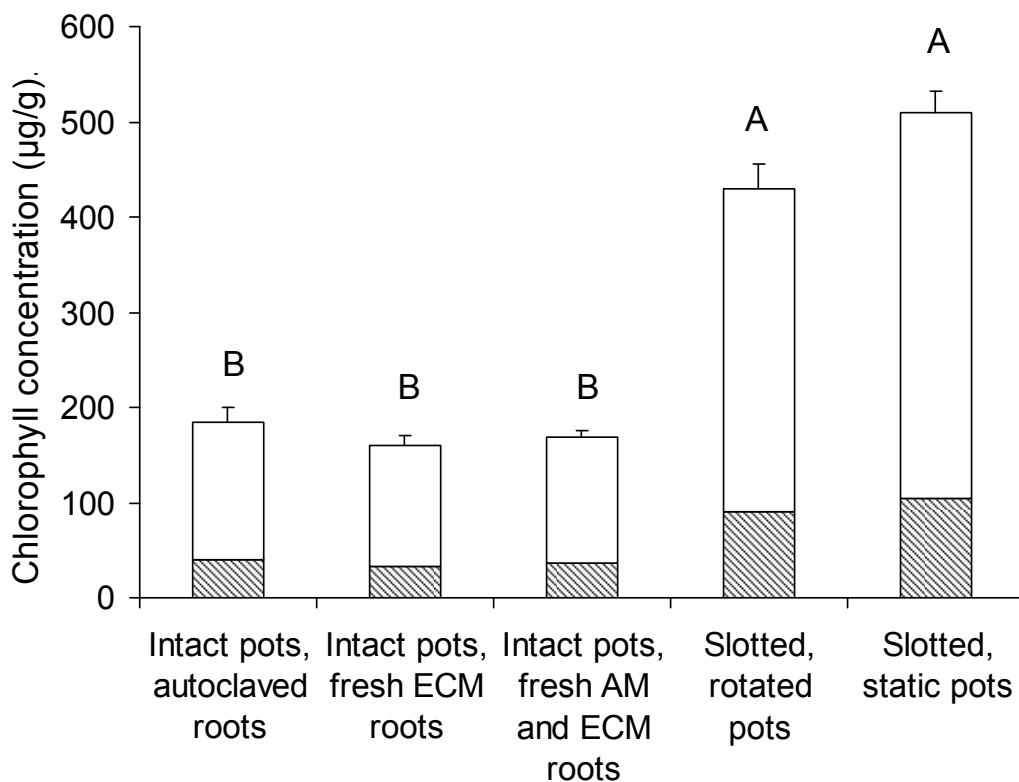




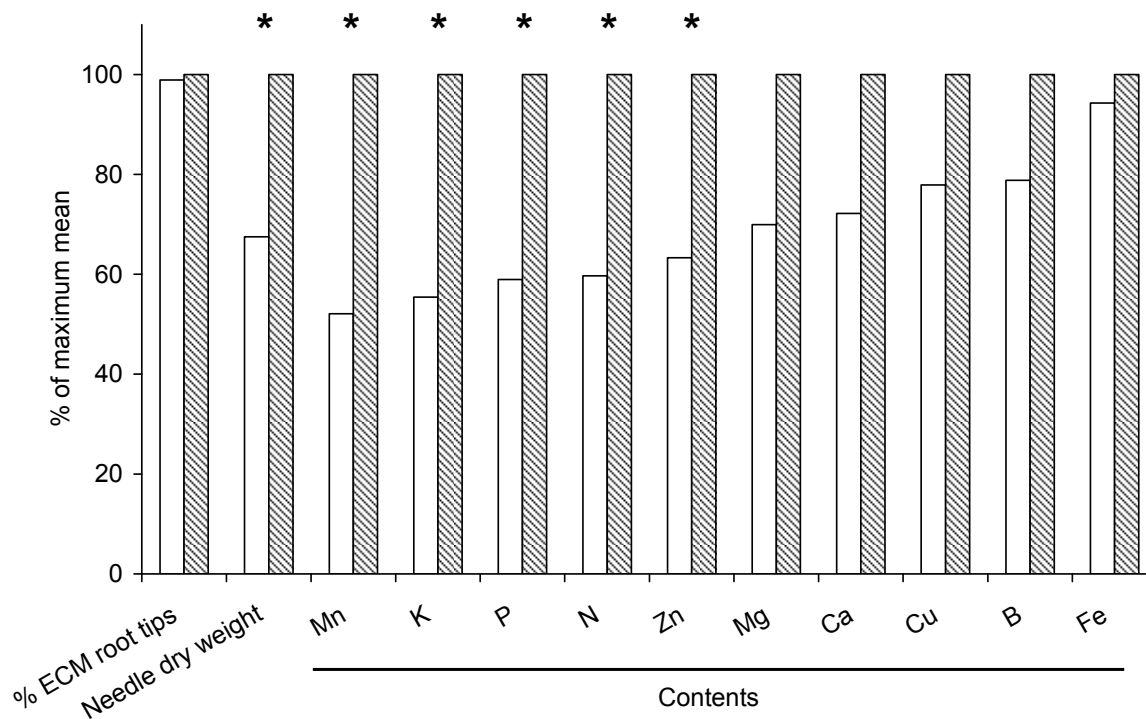
**Figure 4-2** Percentage ECM root tips (mean  $\pm$  1 SE) of *Pinus elliottii* var. *densa* seedlings grown in intact or slotted (static or rotated) pots with fresh or autoclaved AM and/or ECM root inoculum. (a) Open bars: addition of autoclaved ECM roots; hatched bars: addition of fresh ECM roots. NS indicates a non-significant difference in percentage ECM root tips within an AM-pot treatment by separate Kruskal-Wallis tests. (b) Five new treatment groups. As indicated by arrows, seedlings in intact pots that received autoclaved AM roots were maintained as separate groups, while those in other AM-pot treatments were combined for subsequent analyses. Bars topped with the same letter are not significantly different at  $P < 0.05$



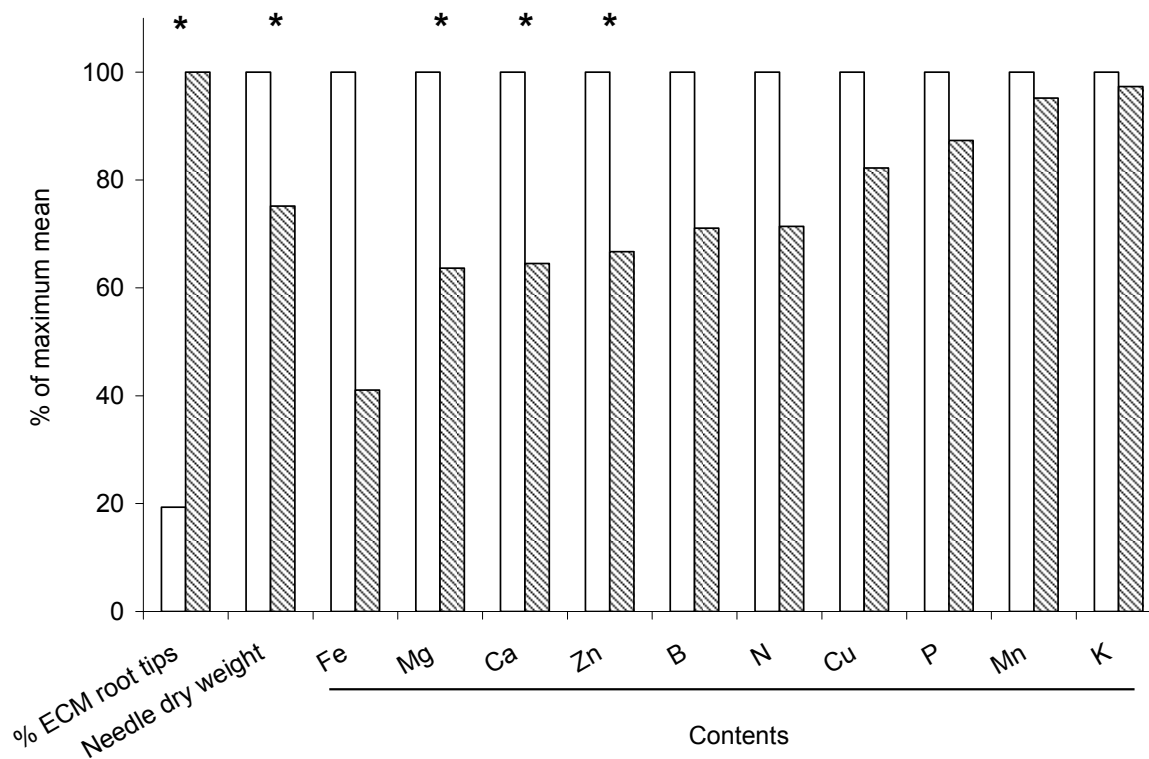
**Figure 4-3** Needle (open bars), stem (dotted bars), primary radicle (diagonal hatched bars) and fine roots (vertical hatched bars) mean dry weights (root weights are shown as positive values below the x-axis) of *Pinus elliotii* var. *densa* seedlings after 9 months in microcosms in intact pots with autoclaved or fresh ECM and/or AM inocula, or in slotted pots (static or rotated). Error bars are SE of total shoot dry weight (needle + stem) and total root dry weight (primary radicle + fine roots). Kruskal-Wallis all pairwise comparison post-hoc tests are shown for total shoot and total root dry weights. Bars topped with the same letter are not significantly different at  $P < 0.05$



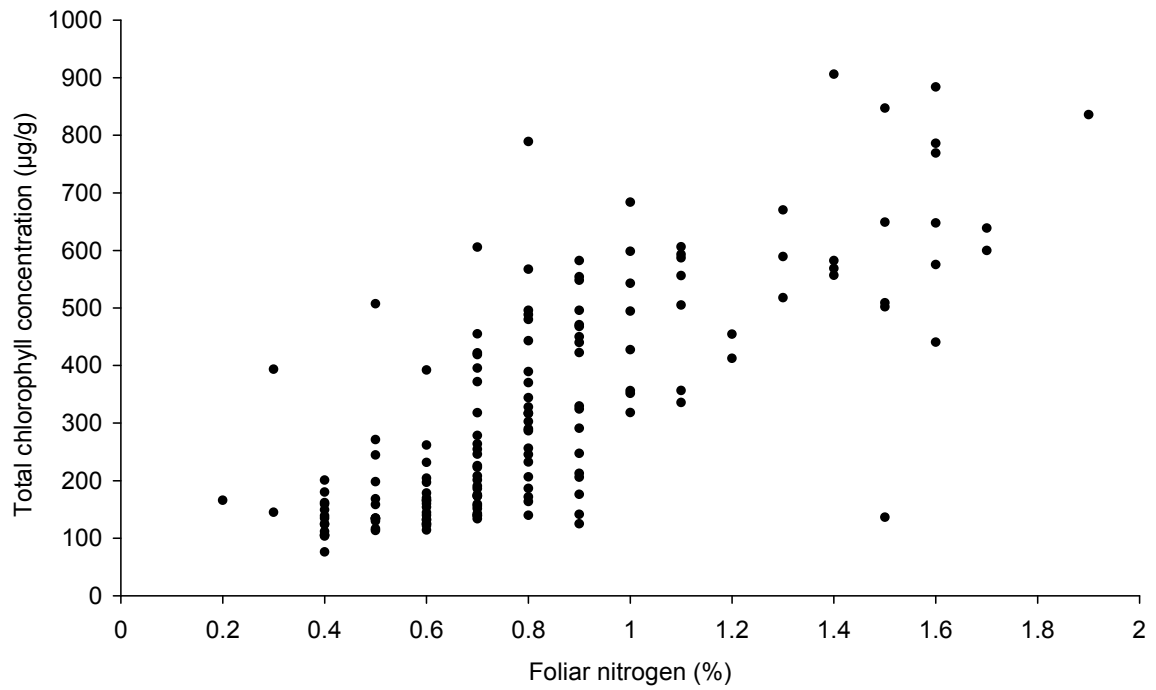
**Figure 4-4** Mean chlorophyll *a* (open bars) and chlorophyll *b* concentrations (hatched bars) of *Pinus elliottii* var. *densa* seedlings after 9 months in microcosms in intact pots with autoclaved or fresh ECM and/or AM inocula, or in slotted pots (static or rotated). Error bars are SE of total chlorophyll concentration. Kruskal-Wallis all pairwise comparison pot-hoc tests are shown for total chlorophyll concentration. Bars topped with the same letter are not significantly different at  $P < 0.05$



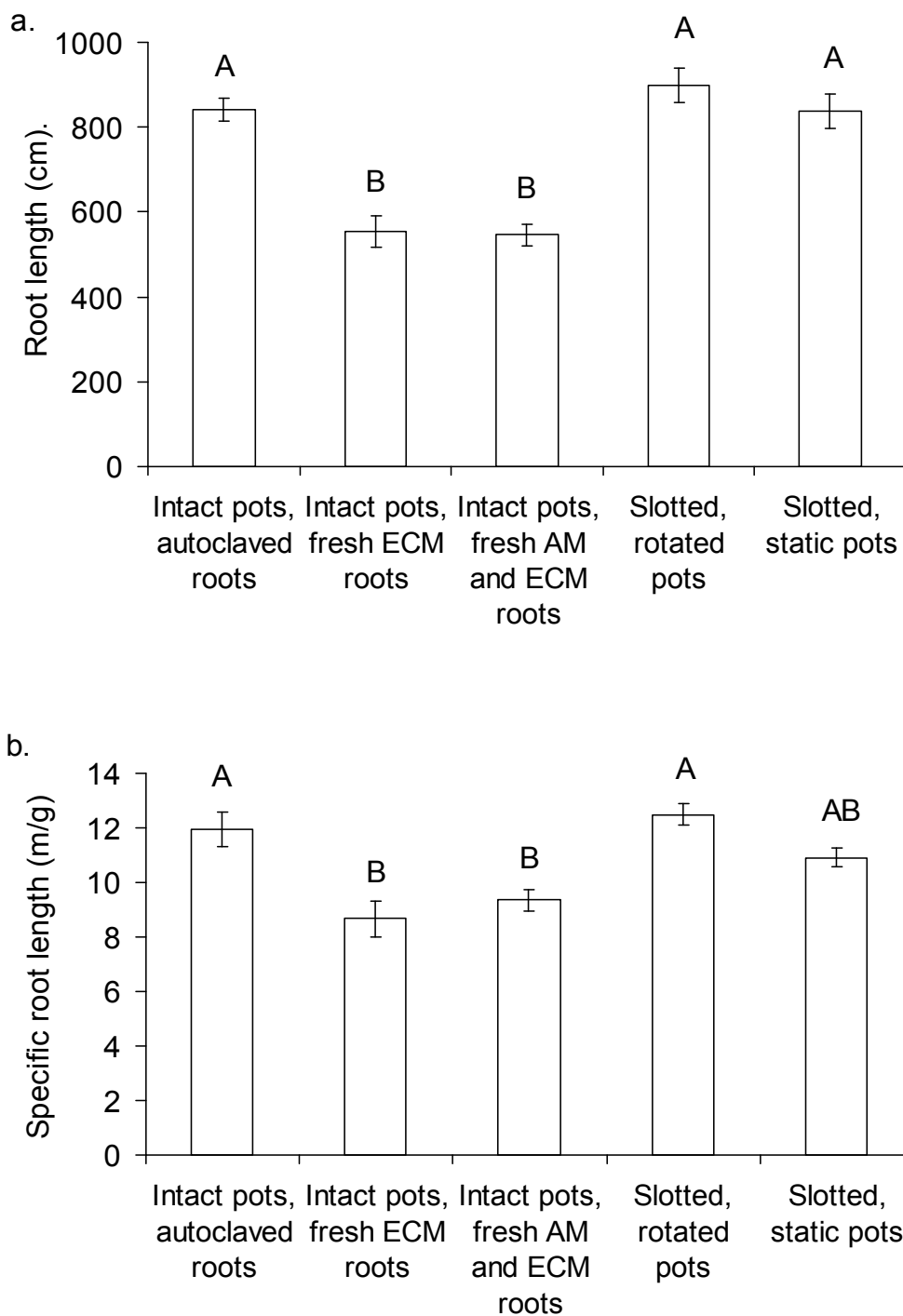
**Figure 4-5** Percent of maximum mean percentage ECM root tips, dry needle biomass, and foliar mineral nutrient contents of *Pinus elliotii* var. *densa* seedlings grown in slotted pots that were rotated weekly (open bars) or remained static (hatched bars). For each response variable, significant differences ( $P \leq 0.005$  after Bonferroni correction) between groups detected with individual Kruskal-Wallis one-way analyses of variance, are indicated by stars above the paired bars



**Figure 4-6** Percent of maximum mean percentage ECM root tips, dry needle biomass, and foliar mineral nutrient contents of *Pinus elliotii* var. *densa* seedlings grown in intact pots either with autoclaved root inocula (open bars) or with fresh AM and ECM root inocula (hatched bars). For each response variable, significant differences ( $P \leq 0.005$  after Bonferroni correction) between groups detected with individual Kruskal-Wallis one-way analyses of variance, are indicated by stars above bars



**Figure 4-7** Mean total chlorophyll (chlorophyll *a+b*) concentration versus nitrogen concentration in needles of *Pinus elliottii* var. *densa* seedlings grown in microcosms. Spearman's rank correlation = 0.79,  $P < 0.001$



**Figure 4-8** (a) Mean root length. (b) Mean specific root length of *Pinus elliottii* var. *densa* seedlings after 9 months in microcosms in intact pots with autoclaved or fresh ECM and/or AM root inocula, or in slotted pots (static or rotated). Error bars are  $\pm 1$  SE. Bars topped with the same letter are not significantly different at  $P < 0.05$

## Chapter 5

### Conclusions

The main objectives of my Ph. D. research were to investigate the time-course of ectomycorrhizal (ECM) colonization of *Pinus elliottii* var *densa* Little & Dorman seedlings in flatwoods in central Florida (Chapter 2), and how the ECM colonization and consequent seedling performance were affected by the density or by the absence versus the presence of adult pines in flatwoods (Chapter 3). Because beneficial effects of ectomycorrhizas in the field were small, I investigated how ECM fungi, and the volume of soil to which extraradical mycelium had access, affected seedling mineral nutrition and growth under controlled greenhouse conditions (Chapter 4). Because fire is a common disturbance in flatwoods, in this concluding chapter, I first describe how fire might affect the performance of *Pinus elliottii* var. *densa* with regard to my findings; I then compare seedling performance among my three experiments. Finally, I describe the availability of ECM inocula in flatwoods at Archbold and the relationship between ectomycorrhizal fungi and pine seedling performance.

#### *Consequences of fire for pine seedling performance*

In addition to its direct effects on plant mortality (Bond and Keeley 2005; Bowman et al. 2009), fire, by volatilizing some nutrients and depositing others in ash, affects mineral nutrient availability in the soil (e.g. Janos 1980; Tuininga and Dighton 2004). Nitrogen, C, S, and to some extent P, can be volatilized from organic matter because of their low volatilization temperatures, while other mineral nutrients with high



volatilization temperatures, such as K, Ca and heavy metals, might be input to the soil in ash (e.g. Johnson et al. 2008; Etiegni and Campbell 1991). Some nutrients from ash, such as K, are easily soluble in water, while others, such as Ca, Mg and Fe are less soluble, and P from ashes is highly insoluble (Khanna et al. 1994). Depending upon fire frequency and season of burning, soil pH can increase and organic horizon thickness can decrease after fire, with enhanced seed germination after diminution of the organic horizon (Neill et al. 2007).

Frequent fires in flatwoods might affect the performance of recruiting pine seedlings through modified mineral nutrient availability. As indicated by foliage N:P ratios lower than 14 in both my field and greenhouse experiments, low N availability in flatwoods soil could limit pine seedling growth. On the other hand, because P and Ca also seemed to limit growth in my greenhouse experiment (Chapter 4), some of the P and Ca deposited as ash could favor seedling growth after a fire. In addition, because foliar Mn concentrations in pine seedlings in the field reflected soil Mn concentration (Chapter 3), and because Mn addition increased the productivity of *Pinus elliottii* var. *elliottii* plantations (Jokela et al. 1991), Mn in ash (Pereira and Ubeda 2010) might enhance pine performance, in particular if undisturbed ECM extraradical mycelium enhances the uptake of Mn (Chapter 4).

By using dendroecological techniques, Higuera and Menges (unpublished results) determined whether post-fire conditions resulted in increased tree ring growth in adult *Pinus elliottii* var. *densa*, related to a pulse of nutrients after fire. Unexpectedly, damage from fires resulted in growth depressions during the years following fires. In their study, Higuera and Menges (unpublished results) found that tree ring growth was affected more by climatic conditions than by fire. In another study, Anderson and Menges (1997) found

that plants in sandhills showed a short-lived increase in foliar concentrations of N, P, K, Mg and Fe after a burn. Even though adult pine growth was not detectably enhanced (Higuera and Menges, unpublished results), an establishing pine seedling having potentially large tissue stores of mineral nutrients, might benefit from a pulse of nutrient release by fire.

In my greenhouse experiment, I found that addition of fresh root inoculum reduced the uptake of Mg, Ca and Zn by seedlings, possibly because of nutrient immobilization by saprotrophic microorganisms degrading fresh root inocula. In southeastern Australian forest soils, ash addition stimulated the respiration of carbon derived from organic matter already present in soils (Khanna et al. 1994). Increased activity of microorganisms in the soil after ash addition resulted in increased N mineralization and elevated soil nitrate concentration, which subsequently was lost through leaching and denitrification (Khanna et al. 1994). In flatwoods, ash deposition after fire might have similar effects on saprotrophic microorganisms. Increased nutrient immobilization through high microbial activity and loss of N through denitrification following N mineralization might exacerbate N limitation of pine seedling growth in flatwoods after a fire, especially because nitrate might not be an appropriate nitrogen source for *P. elliotii* seedlings (L. Bui and DP. Janos, unpublished results). Bui and Janos found that non-mycorrhizal *P. elliotii* var. *elliotii* seedlings in a hydroponic system required ammonium as their nitrogen source.

Altered fire frequency or fire suppression in fire-maintained ecosystems might alter plant community structure and cover (e.g. Menges et al. 1993; Maliakal et al. 2000; Abrahamson 1984). In flatwoods, the frequency of the grass species *Aristida beyrichiana* Trin. & Rupr. was highest shortly after a fire and decreased with time-since-fire (Maliakal

et al. 2000). In contrast, cover by *Serenoa repens* increased with time-since-fire (Maliakal et al, 2000). In a study of vegetation changes after fire suppression in five ecosystems in central Florida, Menges et al. (1993) found that the tree species *Persea borbonia* (L.) Spreng. gained in dominance with time-since-fire, while other species such as *Ilex cassine* L. declined. *Quercus geminata* Small also gained in dominance with time since fire (Menges et al. 1993). Relatively frequent fires might favor pine recruitment by depositing some nutrients in ash, and by preventing overgrowth by shrubs, thereby reducing competition with co-occurring plants that negatively affect the survival and performance of pine seedlings.

#### *Pine seedling chlorophyll and mineral nutrient concentrations*

In my field bioassay (Chapter 2), the mid-point chlorophyll concentration of seedlings among pine stands ranged from 455 to 580  $\mu\text{g/g}$ , but did not change through time, indicating that while the pine seedlings grew, they maintained a constant chlorophyll concentration over their first 6 months of age. In the field experiment (Chapter 3), chlorophyll concentration did not differ among treatments, with an overall average of 298  $\mu\text{g/g}$ , lower than in the field bioassay. In an unpublished greenhouse experiment conducted by a University of Miami undergraduate student, Gabriela Toledo (personal communication), non-mycorrhizal *P. elliotii* var. *densa* seedlings grown in flatwoods soil produced about 320  $\mu\text{g/g}$  chlorophyll, while in my greenhouse experiment (Chapter 4), chlorophyll concentration ranged from 180  $\mu\text{g/g}$  in intact pots to 500  $\mu\text{g/g}$  in slotted pots. Because chlorophyll concentration is tightly related to foliar mineral nutrient concentration, in particular those of N and P, these differences among studies might have been caused by differences in soil nutrient availability. Nitrogen concentration was not

determined for the soils of my field and greenhouse experiments, but P concentration was slightly higher in the soils of the field bioassay than in the field experiment (Chapter 3) or in the greenhouse experiments (bioassay: from 3.25 to 6.75 mg/kg P (weak Bray 1); field experiment: 2 mg/kg P; greenhouse experiments: 1-2.5 mg/kg P), which might have contributed to the high chlorophyll concentrations of seedlings in the field bioassay. Interestingly, the mean chlorophyll concentration of seedlings in the field bioassay was in the same range as the concentration of seedlings in slotted pots in the greenhouse experiment, indicating that the opportunity for ECM fungi to explore a large volume of soil can provide high nutrient uptake and chlorophyll concentration, regardless of seedling age (4.5 versus 9 months, respectively).

In the field bioassay, chlorophyll concentrations of seedlings in no shade was 338  $\mu\text{g/g}$ . This value is close to the 298  $\mu\text{g/g}$  of seedlings in the field experiment, possibly indicating that pine seedlings in the field experiment were not limited by low light. Seedlings might have experienced different light intensities among the three experiments, however, because experiments were performed at different times of year. Although light is less intense during the dry, winter months than during the wet, summer months, winter has fewer cloudy days than summer. My greenhouse experiment ran from October 2008 to June 2009, thus mostly during dry winter months. The duration of my bioassay and field experiments slightly overlapped (field bioassay: from July to December 2009; field experiment: from March to October 2009), so day light intensity was the same for some time during seedling growth in both field experiments.

In my greenhouse experiment (Chapter 4), I observed a “luxury” consumption of some nutrients, such as N, because pine seedlings in slotted rotated pots had a higher foliar N concentration than seedlings in intact pots, but their dry weights did not differ.

Likewise, in the field experiment, foliar Mn concentration was higher in seedlings growing in soil collected within pine stands than beyond pine stands, without resulting in different growth or chlorophyll production (Chapter 3). Although a high N concentration seemed luxury for growth, high foliar N concentration could have contributed to high chlorophyll production by pine seedlings in the greenhouse experiment because of the strong relationship between chlorophyll concentration and foliar nutrient concentrations such as N and P, observed in both my field and greenhouse experiments. High concentrations of mineral nutrients can have important ecological consequences, such as limiting access to nutrients by co-occurring plants of slow-growing plant species (van Wijk et al. 2003) or improving resistance to stressful conditions (Cuesta et al. 2010). Thus, “luxury” consumption of nutrients by pine seedlings might be beneficial under stressful conditions found in flatwoods.

Nutrient limitations can constrain the performance, in particular growth and chlorophyll production, of *Pinus elliottii* var. *densa* seedlings. When grown in the field in containers filled with south Florida hardwood hammock soil, a calcareous iron-poor soil, non-mycorrhizal pine seedlings had increased dry weight and chlorophyll concentration in response to iron fertilization while ECM pine seedlings had only an increased chlorophyll concentration (DP Janos, unpublished results). Chlorophyll production also increased in response to iron fertilization when non-mycorrhizal pine seedlings were grown in flatwoods soil under greenhouse conditions (G. Toledo, unpublished results). In Toledo’s experiment, however, iron fertilization in combination with NPK fertilization had a negative effect on chlorophyll concentration and dry weight of pine seedlings, which most likely was attributable to phosphorus immobilization by added iron. More important than iron, NPK fertilization of seedlings in flatwoods soil resulted in a high

mean chlorophyll concentration and dry weight. Seedlings without added NPK produced a mean 320  $\mu\text{g/g}$  chlorophyll, while NPK-fertilized seedlings produced 680  $\mu\text{g/g}$  chlorophyll (G. Toledo, unpublished results), a much higher concentration than observed in any of my three experiments.

In the experiments reported in this dissertation, seedlings benefited in several different ways from increased mineral nutrient availability: seedling dry weight in intact pots with limited soil volume was enhanced by nutrients released by autoclaved roots (Chapter 4), foliar contents of Mn, K, P, N, and Zn were enhanced by an undisturbed ECM mycelium (Chapter 4), root/shoot ratio was lowered by apparently higher organic matter and estimated nitrogen release in soil collected beyond pine stands than in soil collected within pine stands (Chapter 3), and foliar manganese concentration reflected Mn availability in the soil (Chapter 3).

While limited water availability may restrict mineral nutrient uptake through low diffusion of nutrients during drought (Sardans et al. 2008), too much water during the wet season might limit the functioning of roots in hypoxic soil conditions. Escamilla and Comerford (1998) found that the uptake of K by *Pinus elliotii* var. *elliotii* roots collected in the field was completely inhibited by hypoxic conditions, while P uptake was not affected, indicating that the limitation of some nutrients in flatwoods might be exacerbated during high water table or flooded conditions. Clearly, foliar nutrient concentrations and contents, chlorophyll production, and growth of *Pinus elliotii* var. *densa* seedlings are sensitive to mineral nutrient availability and uptake. Thus, natural or man-made disturbances that affect nutrient cycling and/or the uptake ability of nutrients by ectomycorrhizal fungi in Florida native ecosystems are highly likely to alter pine seedling performance.

*Ectomycorrhizal inocula in flatwoods and seedling performance*

In the field bioassay described in Chapter 2, I found that pine seedlings transplanted to pine stands with different adult densities became colonized by ECM fungi within two weeks of transplant, and that ECM colonization of seedlings was not related to adult pine density. Across all sites, on average, seedlings had 28.1 % of root tips colonized by ECM fungi 4.5 months after transplant. This rapid colonization of seedlings by ECM fungi might be critical for their persistence, and may allow them to establish connections with adults via an extensive, common ectomycorrhizal network. In the field experiment described in Chapter 3, I found that even in the local absence of adult pines, pine seedlings formed up to 49.5 % ECM root tips after 7.5 months in the field. Seedlings in contact with non-autoclaved flatwoods soil collected in the absence of adult pines formed ectomycorrhizas on nearly 50 % of their root tips after 9 months in greenhouse conditions (Chapter 4), similar to ECM colonization of pine seedlings in the absence of adult pines in the field. All three experiments suggest that ECM fungi probably do not limit the recruitment of *Pinus elliottii* var. *densa* in flatwoods at Archbold Biological Station. In the absence of pines, ECM fungi either were present as wind-blown spores or were supported by oaks and possibly plants belonging to the Ericaceae.

Interestingly, although seedling dry weight was positively correlated with percentage ECM root tips in both field studies, percentage ECM root tips usually accounted for a very little variance in pine seedling growth. In the field bioassay (Chapter 2), only 7 % of the variance in total dry weight was explained by percentage ECM root tips, while approximately 10 % was explained in the field experiment (Chapter 3). Although those low values indicate that many other factors, including light, water and

nutrient availability, contribute to pine seedling performance, dry weight of pine seedlings nevertheless is improved by high ECM colonization. Thereby, pine seedlings with immediate access to high levels of ECM inocula in the soil, for example by recruiting within pine stands (Chapter 3), might have improved performance in flatwoods ecosystems.

Not only the amount of ECM colonization on roots, but also the extent of the extraradical mycelium improves conifer seedling performance. In my greenhouse experiment (Chapter 4), I showed that extraradical mycelium contributed to the uptake of Mn, K, P, N and Zn by *P. elliotii* var. *densa* seedlings as well as to growth. The hyphal front of ECM fungi can extend quickly, perhaps as much as 4 mm/day depending on fungus species and temperature conditions (Brownlee et al. 1983; Read et al. 1985; cited in Smith and Read 2008). In pot experiments, the hyphal length of ECM fungi colonizing pine seedlings reached 2000-8000 meters per meter of colonized root in organic soil (Read and Boyd 1986; cited in Smith and Read 2008), showing how ECM fungi can thoroughly exploit the volume of soil from which mycorrhizal plants acquire mineral nutrients (Smith and Read 2008). In consequence, any disturbance of the extraradical mycelium is likely to be detrimental to host plant mineral nutrition.

Except for disturbance of the extraradical mycelium by soil microfauna (Ek et al. 1994), very little research has been conducted on how disturbance modifies the extent of ECM fungus extraradical mycelium in the soil. Fires, if they are intense enough to kill hyphae, may disrupt extraradical mycelia just as they alter ECM fungus communities or mycorrhizal root biomass in soil (Hart et al. 2005; Bastias et al. 2006). Human disturbances such as logging with heavy machinery and/or stump removal also may disrupt ECM mycelia. Teste et al. (2010) studied the transfer of carbon between field-



transplanted ectomycorrhizal seedlings and how that transfer was affected by soil disturbance, *i.e.* by scrapping off the organic layer and top 3 cm of mineral soil. Such soil disturbance did not affect the colonization of seedlings by ECM fungi, but decreased the availability of N and P to *Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco seedlings and altered the bidirectional transfer of carbon between small and large *P. menziesii* var. *glauca* seedlings (Teste et al. 2010). Net transfer of C was greater from large to small seedlings in disturbed soil than in undisturbed soil, suggesting that soil disturbance created a source-sink gradient for transfer of C between seedlings, whereas undisturbed soil did not.

In both my field (Chapter 3) and greenhouse (Chapter 4) experiments, ECM hyphae had the possibility to extend beyond growth containers (either pipes or pots) and improve the mineral nutrition of pine seedlings. Extension of extraradical mycelium benefited seedling performance in the greenhouse, but in the field experiment, in the absence of adult pines the benefit of the extraradical mycelium was not apparent because of root competition in pipes. Within pine stands, however, high ECM colonization, possibly combined with extraradical mycelium extension beyond the pipes and mycorrhizal connections to adult pines compensated for root competition.

In order to definitively determine whether or not ectomycorrhizas improved the mineral nutrition and growth of pine seedlings in flatwoods soil, I conducted a controlled greenhouse experiment (Chapter 4). Very unexpectedly, pine seedlings with about 30 % ECM root tips had a poorer performance than seedlings with only about 6 % ECM root tips, a phenomenon I attributed to the growth conditions in microcosms. The limited volume of soil in pots seemed to change the interaction between pine seedlings and their mycorrhizal fungi, perhaps by restricting the availability of nutrients. In the field

experiment (Chapter 3), the pipes in which I planted seedlings contained 900 mL of soil, which seemed sufficient to avoid a negative effect of addition of fresh versus autoclaved root inoculum and to sustain the mineral nutrition of both the seedlings and their ECM fungus associates, while the 220 mL of pots used in the greenhouse experiment was insufficient.

Besides providing a seed source for pine population regeneration (Teague 2003), adult pine stands supported the highest ectomycorrhizal colonization (Chapter 3) and high ECM fungus diversity on pine seedlings (Chapter 2). Pine stands could moderate air temperature during the summer months and might moderate soil moisture by reducing evaporation from the soil surface during the dry winter months in Florida, but they also might reduce seedlings' resource allocation to growth because of increased allocation to chlorophyll production by shaded seedlings (Chapter 2). Adult pines might enhance seedling water status through hydraulic lift from deep water sources, but adults also can retain rainfall in the canopy if the stand is dense (Liu 1998). In addition, adult pines may have a high demand for water and mineral nutrients, and thus might compete with establishing seedlings. But adults also might compete with understory vegetation, thereby reducing the density and competitive abilities of co-occurring plants (Pecot et al. 2007).

Dickie et al. (2005) constructed a model to assess how tree-seedling interactions change with adult oak tree density, combining facilitation by increased ECM inoculum, and competition by shading (these authors did not include root competition in their model) based on a field experiment with *Quercus macrocarpa* Michx. seedlings. Benefits from high ECM inoculum decreased with diminished adult density, while benefits of light availability increased with diminished adult density, resulting in net benefits for seedlings

at an adult density of 50-60 trees per hectare. In my field bioassay (Chapter 2), the low adult pine density site had 100 trees/ha, the medium density site had 225 trees/ha and the high density site had 475 trees/ha. This suggests that even my low adult pine density site had a higher density of adults than the density at which oak seedling growth was maximal according to Dickie et al. (2005). So the limited growth of pine seedlings in my high pine density site (Chapter 2) was likely to have been a consequence of above- and belowground competition with overstory pines. According to the Dickie et al. (2005) model, ECM colonization of oak seedlings increased up to 100 trees/ha, at which density the benefits of ECM root inocula in the soil reached a plateau. In my field experiment (Chapter 3), ECM colonization of seedlings in flatwoods was lower in the absence than in the presence of adult pines, suggesting that, as in the model of Dickie et al. (2005), the mycorrhizal colonization and performance of pine seedlings in flatwoods might gradually increase with adult pine density up to a plateau of colonization and optimum for performance. At the highest adult pine densities, performance might decrease.

In both my field bioassay (Chapter 2) and field experiment (Chapter 3), I found that co-occurring grasses and shrubs reduced the survival and growth of *Pinus elliotii* var. *densa* seedlings. Both experiments provided clear evidence that pine seedlings suffer from co-occurring vegetation not only through aboveground competition for light, but also, and most importantly, through belowground competition. Neither chlorophyll concentration (reflecting nutrient status) nor foliar nutrient concentrations were affected by competing vegetation in either study, suggesting that competition most likely was for water. Competition might reduce allocation to ECM fungi, thereby reducing root colonization. In young seedlings (4.5 months) in the field bioassay, competition with grass tended to reduce the percentage ECM root tips, but the difference was not

statistically significant (Table 2-5). Low grass density combined with high soil P concentration in the low adult pine density site, however, increased seedling performance, thereby enhancing the seedlings' ability to support high ECM colonization at that site (Chapter 2). For seven-and-a-half month old seedlings in the field experiment, poor seedling performance caused by belowground competition in drilled pipes clearly limited ability to sustain ECM fungi (Fig. 3-7b).

My Ph. D. research shed light on the importance of ectomycorrhizal fungi, of extraradical mycelium, and of competition with co-occurring plants for the survival and performance of *Pinus elliottii* var. *densa* seedlings in flatwoods. Ectomycorrhizas benefited pine seedlings' performance, while performance also determined the level of ECM colonization that seedlings could sustain. Although ECM inocula were widespread in flatwoods, ECM colonization and ECM fungus richness were highest within dense adult pine stands. High ECM colonization in combination with potential connections to common mycorrhizal networks seemed to enhance the competitive ability of pine seedlings within pine stands. Disturbance of the spread of ectomycorrhizal extraradical mycelium was a detriment to pine seedling performance in flatwoods soil. The major detriment to *Pinus elliottii* var. *densa* seedlings, however, was belowground competition with roots of co-occurring species, which reduced seedling performance and ability to sustain ectomycorrhizal fungi.

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