Physiological and genetic changes in poplar during mycorrhizal colonization under phosphorus limitation

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Physiological and genetic changes in poplar during mycorrhizal colonization under phosphorus limitation

Shalaka Desai

Doctoral Dissertation

Submitted in partial fulfillment for the requirements of the degree of Doctor of Philosophy in the Department of Biology at West Virginia University

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2012
ABSTRACT

Physiological and genetic changes in poplar during mycorrhizal colonization under phosphorus limitation

Shalaka Desai

Forest ecosystems are major components of the biosphere and contribute extensive ecosystem services to human society. Trees form a significant carbon storage sink in the global carbon cycle, moderate climate, interact with hydrologic cycles at all scales, and provide food, fuel, and fiber to society. The provision of these benefits depends upon the supply of resources (carbon dioxide, water, nutrients, and light) to the tree. Primary productivity and growth are often limited by nutrient availability, which, in turn, affects the ecological and economical roles of trees. Of the major mineral nutrients, phosphorus (P) availability is often limited in soils due to its high affinity for aluminum and calcium in soil minerals. Typically, only 0.5% of total soil P is available to trees and soil P availability limits the productivity of forest globally (Fig. A).

Since forest trees have evolved in P limited environments, they have developed various mechanisms to overcome this stress. These adaptations include processes that increase the acquisition and uptake of P, such as exudation of compounds into the rhizosphere, and metabolic adjustments that increase P efficiency within metabolic pathways, including the expression of phosphatases that facilitate P turnover within cells (Plassard and Bell 2010, Plaxton and Tran 2011). The exudation of organic carbon by roots facilitates the dissolution of insoluble P-containing minerals and the release of P to the root for absorption. This exudation may involve small organic molecules, such as the organic acids malate, citrate, and malonate, as well as more complex molecules, such as flavonoids.
One significant ecological adaptation to P limitation is the formation of the symbiotic mycorrhizal association between plant roots and soil mycorrhizal fungi. There are several different types of mycorrhizal fungi. Ectomycorrhizal fungi form associations with major forest species, including the spruces, pines, oaks, beeches, birches, and poplars. Arbuscular mycorrhizal fungi form associations with most herbaceous species as well as many tree species. In both mycorrhizal associations, the volume of soil exploited by the root system for mineral absorption is increased by the mycorrhizal hyphae that proliferate through the soil from the plant root surface. In return for access to soil nutrients, the symbiotic mycorrhizal fungi benefit by receiving fixed carbon from the plant host (Balestrini and Lanfranco 2006, Gianinazzi-Pearson et al. 2007, Krajinski and Frenzel 2007, Requena et al. 2007, Séjalon-Delmas et al. 2007, Schaeffer et al. 1995). In addition to increasing the soil volume exploited for mineral acquisition, mycorrhizal fungi may increase P uptake through the activity of higher affinity Pi transport systems (Smith et al. 2011). Mycorrhizal fungi may also increase the amount or variety of root exudates that flow to the rhizosphere, which may play a role in changing nutrient availability. Through these mechanisms, symbiotic associations enhance P uptake in forest trees (Cumming and Weinstein 1990, Klugh and Cumming 2009, Naik et al. 2010).

Poplar species and hybrids are intensively cultivated as renewable sources of biomass because of their rapid growth, extensive genetic variation and ease of propagation. However, the physiological mechanisms underlying soil stress resistance within these poplar species and hybrids are not yet understood. With the recent sequencing of the poplar genome, the use of this species in physiological stress resistance studies may help elucidate the underlying basis of stress resistance in a woody species. Further, although symbiotic mycorrhizal associations have far-reaching ecological significance in forest ecosystems, our understanding of the influences of the association on the genetic basis of environmental stress resistance of trees, including poplar, is limited.

In this dissertation research, physiological, ecophysiological, proteomic and plant biochemistry approaches were used to understand the role of the plant-mycorrhizal symbiotic association under P limitation. By using advanced technology, such as proteomics including two-
My objective for the first chapter was to test if the association of the ectomycorrhizal fungus *Laccaria bicolor* (Marie) with aspen (*Populus tremuloides* Michx.) would alter the plant response to environmental phosphate (Pi) limitation. To test this, we studied different stress related oxidative enzymes, such as catalase and superoxide dismutase, carbon allocation of above and below ground biomass, tissue P accumulation, and root exudation of organic acids. The symbiotic association improved P acquisition in aspen and reduced the oxidative stress responses as well as the exudation responses generated by P limitation. The results confirmed that the acclimation responses of aspen in association with *Laccaria bicolor* under Pi limitation were mediated through enhanced Pi uptake resulting from the symbiotic association.

In the second chapter, I assessed a broader set of physiological changes in aspen under P limitation due to the different ectomycorrhizal species, *Laccaria bicolor* and *Paxillus involutus*. I hypothesized that aspen would have more carbon demand belowground due to mycorrhizal colonization and, to meet this demand, carbon assimilation would increase. Secondary metabolites are also indicators of stress and glycosides in aspen are well defined (Chen et al. 2009). Hence I assayed the secondary metabolites in different plant tissues. As the two fungal species differ in their occurrence, we expected their behavior to differ for the ecophysiological and secondary metabolite parameters. In this study, I found that P limitation significantly limits photosynthetic gas exchange as well as the biochemistry of photosynthesis, but primarily in non-mycorrhizal aspen. These changes were correlated with reductions in tissue P levels that were pronounced in nonmycorrhizal plants.

Finally, to gain a better understanding of the molecular mechanisms of the mycorrhizal symbiotic association, I used proteomic approaches to evaluate the proteins involved in this interaction. The analysis of the proteins that were differentially displayed during ectomycorrhizal and arbuscular mycorrhizal association visualized by two-dimensional electrophoresis (2-DE) of the total protein obtained from poplar roots non-inoculated and associated with two
ectomycorrhizal fungi *L. bicolor* and *P. involutus* and one arbuscular mycorrhizal fungus *Glomus intrradices*, were analyzed. I found significant changes in protein abundances in many metabolic pathways, including those associated with energy, general metabolism, and stress response induced by the arbuscular and ectomycorrhizal association. Similarities and differences between the two groups of fungi suggest that there may be both conserved and unique plant responses to mycorrhizal colonization.

By using the different tools of physiology, ecophysiology, plant biochemistry and proteomics, I attempted to build a broad understanding about the symbiotic association. This research will help to accelerate our understanding of the plant-soil interface and tree stress physiology, which in turn can aid in our understanding of forest ecosystem productivity and response to future changes in the environment.

Figure A. World Map showing soil phosphorus availability (Lynch et al. 2011, Jaramillo-Velastagui, 2011)
ACKNOWLEDGEMENTS

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them” by William Lawrence Bragg.

This would not have been possible without support and encouragement from my family, advisor and all my friends. I owe my deepest gratitude to you all.
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1 The influence of phosphorus availability and *Laccaria bicolor* symbiosis on nutrient acquisition, rhizospheric carbon flux, and reactive oxygen species in *Populus tremuloides* (Aspen)

1.1 Introduction

Tree growth and forest productivity are functions of resource supply and acquisition. Soil nutrient limitations, especially nitrogen (N) and phosphorus (P), constrain the growth of natural and planted forests worldwide (LeBauer and Treseder 2008; St. Clair et al. 2008). Phosphorus biogeochemistry and demand both function to limit phosphate (H$_2$PO$_4^-$) availability at the soil-plant root interface (Fitter and Hay 1993; Vance 2003; Plassard and Bell 2010). On highly weathered, acidic soils, interactions between both inorganic phosphate (Pi) and organic phosphorus (Po) sources with soil aluminum (Al) and iron (Fe) hydroxides lead to precipitation of P from the soil solution (Von Uexku and Mutert 1995; Batjes 1997). On calcareous soils, Pi-Ca precipitation interactions also limit Pi availability at the root-soil interface (Batjes 1997; Vance 2003). Hence, the soluble P accessible at the soil-plant root interface is often in the sub-micromolar concentration range (Plassard and Bell 2010). In agricultural systems, this limitation is overcome by fertilization to maintain plant growth. However, such management practices are not suitable for forest tree production systems. Studies indicate that P limitation plays a major role in forest ecosystems (Elser et al. 2007), and may limit forest tree growth in many forest ecosystems throughout the world (Schutt and Cowling 1985; Gradowski and Thomas 2006; Paoli and Curran 2007; Quesnel and Cote 2009).

Phosphorus starvation leads to a variety of metabolic perturbations that ultimately reduce carbon (C) fixation and plant growth (Raghothama 1999; Kochian et al. 2004). Phosphorus deficiency limits the biochemistry of photosynthesis and increases sink demand for photosynthate for root growth and exudation, and such changes will reduce C fixation and alter patterns of growth (Loustau et al. 1999; Pieters et al. 2001; Gradowski and Thomas et al. 2006). Insufficient P additionally leads to increased hydrogen peroxide production, lipid peroxidation,
and increased activities of antioxidative enzymes as P deficiency disrupts cellular homeostasis (Raghothama 1999). In young leaves of *Morus alba* plants, for example, lipid peroxidation increased and the activity of superoxide dismutase, ascorbate peroxidase, and glutathione reductase also increased in P deficient plants (Tewari et al. 2007).

Plants have developed complex physical, physiological, and symbiotic adaptations to overcome P limitation (Vance 2003, Plassard and Bell 2010; Plaxton and Tran 2011). Changes in carbon allocation, such as changes in the root to shoot ratio, greater number of axial roots, and other root architectural changes, increase the exploration of the soil for this limiting nutrient (Lynch 2011). Physiological changes, such as increases in root exudation, changes in Pi transporter expression, and other metabolic adjustments, function to increase P acquisition and P use efficiency (Neuman and Romheld 1999; Vance 2002; Plassard and Bell 2010). In addition, more than 90% of plants form mycorrhizal associations and rely on these symbiotic fungi to meet their nutritional demands (Tuomi et al. 2001; Plassard and Bell 2010).

The exudation of organic acids (OAs) is one potential mechanism plants utilize to extract P from the rhizosphere. Organic acids may reduce rhizosphere pH and may additionally chelate Ca, Al, and Fe ions in the soil, which will increase the availability of Pi and Po in the soil solution (Dakora and Phillips 2002; Vance 2002; Plassard and Bell 2010). Increased internal OA concentrations within roots (Hoffland et al. 1989; Pellet et al. 1996; Johnson et al. 1996; Neumann et al. 1999) may lead to enhanced OA efflux and Pi scavenging in the rhizosphere (Liao et al. 2006; Shabbaz et al. 2006; Oburger et al. 2009). To support exudation, the activity of enzymes related to OA synthesis increases (Aono et al. 2001; Ryan et al. 2001). For instance, OA release from the cluster roots of *Lupinus albus* and other species has been associated with enhanced activities of phosphophenolpyruvate carboxylase (PEPCase), malate dehydrogenase (MDH), and citrate synthase (CS) (Johnson et al. 1996; Watt and Evans 1999; Ryan et al. 2001).

Ectomycorrhizal (ECM) fungi are important for the survival of many forest tree species under nutrient limiting conditions (Plassard and Bell 2010). The development of mycorrhizal associations is enhanced in response to low soil P availability (Jones et al. 1990, Koide 1991, Ekblad et al. 1995), which can be attributed to increased allocation of photosynthate to roots.
(Thomson et al. 1986, Peng et al. 1993, Graham et al. 1997). ECM fungi live in symbiosis with trees and colonize the soil forming an external mycelium that contributes to the absorbing surface over which mineral nutrients can be absorbed by roots (Schack-Kirchner et al. 2000). In addition to increasing soil exploration, mycorrhizal hyphae improve P acquisition by increasing Pi uptake rates (Cumming 1996) and the extraction of P from Pi complexes or Po sources by releasing organic acids, other metal-chelating compounds, and acid phosphatases (Kroehler et al. 1988, Cumming and Weinstein 1990, Leyval and Berthelin 1993, Wallander 2000, Casarin et al. 2004, Liu et al. 2005). Thus, colonization increases the ecological breadth of mycorrhizal plants by quantitatively and qualitatively increasing the capacity of the plant for nutrient acquisition.

*Populus* is a model tree system that is being utilized extensively in ecological, physiological, and molecular studies worldwide (Jansson and Douglas 2007). Poplar is a preferred plantation species because of its fast growth rate and clonal production ability and there is expectation that species within this genus may help offset the increasing worldwide demand for wood, pulp, and biofuels (Yang et al. 2009). Most industrial plantations are monocultures and the sustainability of production will vary by site fertility and with various management practices (Heilman and Norby 1998, Sayyad et al. 2006). Phosphorous limitation is likely to be very important for future productivity and is considered a limiting factor for growth of poplar species in North American cultivation sites (Liang and Chang 2004). Quaking aspen (*Populus tremuloides* L.) is widely distributed across northern North America (Burns and Honkala 1990). In the eastern United States, aspen is a pioneer species on many disturbed and marginal sites, including abandoned coalmines and areas affected by smelting, where soil factors limit the growth of other native species (Bramble and Ashley 1955; Cumming personal observation). The roots of quaking aspen predominantly associate with ectomycorrhizal fungi (Cripps and Miller 1993, Malloch et al. 1980) and this association may play an integral role in its colonization and survival on these sites. Thus, understanding the role of the poplar-mycorrhizal symbiosis in ameliorating P limitation will help guide sustainable management practices for poplar production and may aid in restoration programs on disturbed sites.
The aim of this study was to assess the influence of *Laccaria bicolor* (Marie) colonization on the phosphate limitation physiology of quaking aspen. We compared the responses of nonmycorrhizal and mycorrhizal *P. tremuloides* plants grown on a range of Pi concentrations (0 to 100 µM) in sand culture for 46 d. Measurements of growth, root and leaf P concentration, the activities of antioxidative enzymes, and exudation of OAs and total organic carbon (TOC) were undertaken. I hypothesized that ectomycorrhizal aspen would be more resistant to P limitation due to increased C allocation to roots and enhanced Pi acquisition capacity. These differences would be expressed as superior growth under P limitation, greater tissue P concentrations, lesser antioxidative enzyme activities, and reduced flux of C to the rhizosphere.
1.2 Materials and methods

1.2.1 Fungal inoculation and ectomycorrhizal formation

*Laccaria bicolor* (Marie) S238N (Institut National de la Recherche Agronomique, Nancy, France) was chosen for its ability to form ectomycorrhizas with aspen. This ECM fungus has been comprehensively characterized at the physiological and molecular levels (Di Battista et al. 1996). The strain was maintained on a modification of modified Melin Norkrans (MMN) agar medium (Cumming et al. 2001). For inoculum production, cultures were grown aseptically in liquid MMN medium for 3 weeks at 25°C in the dark in static culture (Molina and Palmer 1982). Cultures were blended briefly (three times for 3 s) to produce a fungal mycelial slurry to serve as inoculum.

The experimental system for the production of mycorrhizal aspen (*Populus tremuloides* Michx.) seedlings consisted of 6-cm diameter × 25-cm deep pots (Cone-tainers™, Stuewe and Sons, Corvallis, OR, USA) containing acid-washed sand (coarse and fine sand mixture ratio 2:1). For establishing mycorrhizal (Myc) aspen seedlings, a band of fungal slurry was added ~2 cm below the surface of the sand. This inoculation method was found to be successful for generating ECM colonization with *P. trichocarpa* (Baum et al. 2002). For nonmycorrhizal (NM) plants, only MMN liquid medium was added instead of fungal slurry.

Seeds of *P. tremuloides* L. (Sheffield’s Seed Co., Lockey, NY, USA) were planted ~0.5 cm deep in the pots and were kept moist by watering three-times daily with deionized water. After 7 days, seedlings of similar size were thinned to leave a single plant in each pot for the experiment. Following selection, the seedlings were watered three-times daily to field capacity (60 ml pot⁻¹ d⁻¹) with a nutrient solution containing 1.0 mM NO₃, 0.4 mM NH₄, 0.5 mM K, 0.2 mM Ca, 0.1 mM Mg, and SO₄, 50.5 µM Cl, 20 µM Fe, 20 µM B, 2 µM Mn, and Zn, and 0.5 µM Cu, Na, Co, and Mo. Solution pH was adjusted to 5.6 with 0.1 N NaOH. After 2 weeks of seedling growth and ectomycorrhiza formation, Pi in the form of KH₂PO₄ was added to nutrient solution to deliver treatment solutions containing 0, 1, 5, 10, 25, 50, and 100 µM P. Seedlings
were maintained in a climate controlled greenhouse with the shade cloth in place with supplemental lighting (metal halide sources) providing a 14-h photoperiod (272 ± 31 µmol m² s⁻¹) and day/night temperatures of 24/19 ± 3°C; relative humidity fluctuated between 60 and 80% with temperature and time of day. Seedlings were grown for 46 days after treatments (DAT) began.

1.2.2. Growth parameters and ECM colonization

At 46 DAT, seedlings were harvested and shoots were separated from roots and plants were processed as noted below. Leaves (leaf plastochron index 4 and 5) and 4-5 cm of lateral roots were collected, weighed fresh, and flash-frozen in liquid N₂ immediately after harvesting and stored at −80°C until later extraction (below). Similar tissue samples were taken and weighed fresh, dried at 60°C for 48 h, and then weighed to obtain a fresh weight-dry weight ratio to calculate dry mass of all tissues. Remaining tissues were dried at 60°C for 48 h and weighed to obtain plant mass. The mean dry weight:fresh weight ratio (DW/FW) for leaves was 0.23, was not affected by mycorrhizal treatment, and declined from 0.29 at 100 µM Pi to 0.14 at 1 µM Pi. The mean root DW/FW was 0.087 for NM plants and 0.084 for Myc plants, and was not affected by Pi treatment. Dried roots and leaves were processed for P concentrations as described below.

Ectomycorrhizal colonization was quantified by the gridline intersect method (Giovannetti and Mosse 1980). Root samples from each treatment were stored in sterile deionized water at 4°C until staining. These root samples were then processed by staining with 4% (v/v) chlorazol black E (Sigma-Aldrich, St. Louis, MO, USA) overnight (Brundrett et al. 1984, Warwick et al. 1999) and 100 root tips from each sample were viewed for colonization.

1.2.3. Antioxidant enzyme (CAT, APX, SOD, Cu/Zn-SOD and GDP) activity and PEPCase enzyme activity

During enzyme extraction, 0.3–0.4 g fresh mass of leaf or root tissue was ground in liquid N₂ in a pre-cooled mortar and pestle and subsequently homogenized in 2 ml of ice-cold 0.05 M Tris buffer at pH 7.5 containing 4% (w/v) PVP, 1% (w/v) BSA, and 1% (v/v) β-mercaptoethanol (Ranade and Feierabend 1991). The homogenate was centrifuged for 10 min at 4°C at 8000 g
and the supernatant was used for enzyme assays. Protein was determined by the Bradford procedure using BSA (Sigma-Aldrich, St. Louis, MO, USA) as the standard (Bradford 1976) and all enzyme activities were expressed as µkat g⁻¹ protein.

Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H₂O₂ spectrophotometrically at 240 nm for 3 min using a reaction mixture containing 2.975 ml of 100 mM phosphate buffer at pH 7.0, 24 µl of 30% H₂O₂, and 5 µl of supernatant as an enzyme source (Volk and Feierabend 1989).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by measuring the decrease in absorbance due to the conversion of ascorbate to dehydroascorbate. This assay was measured spectrophotometrically as described by De Leonardis et al. (2000) at 290 nm for 3 min using a reaction mixture containing 150 µl of 100 mM phosphate buffer at pH 7.0, 350 µl of 500 µM ascorbic acid, 2.065 ml of sterile water, 300 µl of 1 mM EDTA, 55 µl of 30% H₂O₂, and 80 µl of supernatant.

Guaiacol dependent peroxidase (GDP, EC 1.11.1.7) activity was measured by measuring the increase in absorbance due to the formation of tetra-guaiacol from guaiacol in the presence of H₂O₂ spectrophotometrically at 470 nm (Ranade and Feierabend 1991). The assay was run for 3 min using a reaction mixture containing 2.48 ml of 100 mM phosphate buffer at pH 7.0, 500 µl of 30 mM guaiacol, 4.8 µl of 30% H₂O₂, and 80 µl of supernatant (Volk and Feierabend 1989).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the nitroblue tetrazolium (NBT) reduction method (Beauchamp and Fridovich 1971). The assay medium contained 1.5 ml of 50 mM phosphate buffer (pH 7.8), 600 µl of 13 mM methionine, 300 µl of 75 µM NBT, 300 µl of 2 µM riboflavin, and 70 µl of enzyme extract. The reaction mixture was illuminated at 80 to 90 µmol m⁻² s⁻¹ for 5 min. The reaction mixture without illumination served as the control. The mixture without enzyme developed maximum color and represented the maximum reduction of NBT. Potassium cyanide (5 mM final concentration) was used to distinguish between the cyanide sensitive isoenzyme Cu/Zn-SOD and the cyanide resistant Mn-SOD. The Cu/Zn-SOD was obtained as total activity minus activity in the presence of 5 mM
potassium cyanide.

Phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31) activity was measured according to the method of Jagtap et al. (1998). A ratio of 1.5 ml of homogenizing buffer for 1 g of roots was used to keep the initial volume low. The homogenate was centrifuged at 8000 g at 4°C for 15 min and the supernatant was used for PEPCase activity assays directly. Protein was determined by the Bradford procedure using BSA as the standard. PEPCase was determined by coupling its activity to malate dehydrogenase-catalyzed NADH oxidation in 1.5 ml final volume of a reaction buffer containing 100 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 2.5 mM PEP, 200 µM NADH, 10 mM NaHCO₃, and 15 µg ml⁻¹ malate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA). NADH oxidation was determined at 340 nm at 25°C. Assays were initiated by adding 80 µl aliquots of supernatant.

1.2.4. Determination of organic acids (OAs) and total organic carbon (TOC) in the rhizosphere

Following the separation of shoots from roots, the contents of each pot (roots of one plant and sand substrate) were placed in a beaker. Twenty-five ml of deionized water was added to the root-sand sample and allowed to stand for 5 min (Naik et al. 2009). This root zone extract was collected using a pipette and passed through the root zone sample twice again, removed, and filtered (0.45 µm) immediately. To prepare root zone extracts for organic acid analysis, 800 µl of 10 mM Na₂-EDTA and one drop of 1 M NaOH were added to 10 ml samples of each aqueous root zone extract. This procedure promoted chelation of mineral elements in the solution and prevented suppression of organic acid detection by mineral elements (Cumming et al. 2001). Samples were roto-evaporated and stored at −20°C until analyzed. Residual salt pellets were dissolved in 1 ml of sterile deionized water and the concentrations of organic acids were measured by ion chromatography using a conductivity detector (Dionex ICS-1500, Sunnyvale, CA, USA). For separation of organic acids, a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm ID) was employed with minor modifications of Qiu and Jin (2002) and Cumming et al. (2001). The eluent was 2.3 mM heptafluorobutyric acid at a flow rate of 0.6 ml min⁻¹, the suppressant solution was 5 mM tetrabutylammonium hydroxide at a flow rate of 2.0 ml min⁻¹,
and the analysis time was 20 min. Calibration equations based on peak heights for each organic acid were obtained based on standard organic acid solutions with varying concentrations and their corresponding peaks. Oxalate concentrations were determined spectrophotometrically using an oxalate diagnostic kit (Trinity Biotech, St. Louis, MO, USA). Oxalate standards were prepared from oxalic acid dihydrate (Sigma-Aldrich, St. Louis, MO, USA) as recommended by the manufacturer. Sample organic acid concentrations were adjusted by the dilution factor from the extraction to normalize the values to those existing at field capacity in pots. Concentrations of organic acids in pots without plants were subtracted from values measured for each pot with a plant within the same Myc treatments. Organic acid exudation rates (µmol OA g\(^{-1}\) FW day\(^{-1}\)) were calculated based on daily solution flow through the root zone, measured OA concentrations in the root zone, and root mass.

The total dissolved organic carbon (TOC) was analyzed using a TOC analyzer (Shimadzu TOC-V-CPH, Columbia, MD, USA). Concentrations of TOC in pots without plants were subtracted from values measured for each pot with a plant within the same Myc treatments. Total C exudation rates (mmol C g\(^{-1}\) FW day\(^{-1}\)) were calculated based on daily solution flow through the root zone, measured TOC concentrations in the root zone, and root mass.

1.2.5. Tissue P analysis

Dried leaves and roots were ground to pass a 20-mesh sieve. Samples of ~100 mg were pre-digested overnight with 4 ml of a 1:1 (v/v) mixture of 30% H\(_2\)O\(_2\) and HNO\(_3\) in 75 ml acid washed glass digestion tubes and digested in a block digester for 6 h at 135°C (Jones and Case 1990). Following digestion, the final volume of each sample digest was brought up to a 75 ml with deionized water and these solutions were filtered through No. 44 Whatman filter paper. The P concentrations of the digests were measured spectrophotometrically using the ammonium molybdate assay (Taussky and Shorr 1953).

1.2.6. Statistical analysis

The experiment was established as a randomized blocked, two-way factorial design (6 blocks, 7 Pi concentrations × 2 mycorrhizal treatments) with 10 replicates (\(n = 10, N = 140\)). Blocks
accounted for potential environmental gradients within the greenhouse. Data were log-transformed wherever necessary in order to achieve homogeneity of variance. The effect of Pi on colonization was assessed by regression analysis for Myc plants only. The effect of inoculation with *L. bicolor* on aspen response to nutrient solution Pi concentration was assessed by regression analysis for plant height, length of the longest root, shoot and root biomass, and root:shoot ratio. Effect of mycorrhizal colonization on the response to Pi concentration for antioxidant enzyme activities, rhizosphere organic acids, TOC, and plant P concentrations were also analyzed using regression analysis. Two-way analyses of variance (ANOVA) followed by Tukey’s HSD was used to highlight Pi response means that differed between NM and Myc treatments. Relationships between measured variables and leaf or root P concentrations were assessed using analysis of covariance (ANCOVA). Nonlinear regression using the Michaelis-Menten equation was used to assess differences in the accumulation of Pi by NM and Myc plants:

\[
C_P = \frac{C_{\text{max}} [Pi]}{k_m + [Pi]}
\]

where \(C_P\) is the tissue P concentration, \(C_{\text{max}}\) is the maximal P concentration of that tissue type, \([Pi]\) is the treatment solution Pi concentration, and \(k_m\) is the solution Pi concentration where the tissue P concentration is one-half \(C_{\text{max}}\). \(C_{\text{max}}\) provides an estimate of the asymptote, equivalent to the maximum capacity for P accumulation and \(k_m\) describes the rate at which \(C_P\) changes with treatment Pi, effectively the affinity for Pi accumulation. Differences in the fitted parameters from the model between nonmycorrhizal and mycorrhizal plants were evaluated by using t-tests. Statistical analyses were carried out using SAS JMP 7.0 (SAS Institute, Cary, NC, USA).
1.3 Results

1.3.1. Mycorrhizal colonization, growth, and biomass partitioning

Colonization of aspen roots by *L. bicolor* significantly increased with decreasing Pi concentration in sand culture (Table 1.1). At 100 µM Pi, root colonization was 71% and the colonization increased to 82% at 0 µM Pi (data not presented). NM plants did not show any sign of colonization or short root formation.

Patterns of biomass allocation of NM and Myc aspen seedlings differed slightly in response to Pi availability (Table 1.1). Heights of Myc plants were 17% greater than those of NM across all Pi treatments, with differences between NM and Myc treatments becoming greater at and above 25 µM Pi (*P* = 0.003 for the Pi × Myc interaction). The length of the longest root was 11% greater in Myc than NM plants across Pi treatments (Table 1.1), with differences between mycorrhizal treatments becoming greater as Pi became limiting (*P* < 0.001 for the Pi × Myc interaction). Shoot biomass increased more than 100-fold over the Pi treatment gradient (Fig. 1.1A). Differences between NM and Myc shoot biomass response to Pi were evident, with NM shoot biomass being greater than Myc shoot biomass at and above 25 µM Pi (*P* = 0.040 for the Pi × Myc interaction) (Table 1.1). Root biomass of Myc plants was 16% greater than the root biomass of NM plants across all Pi treatments and this difference was consistent across Pi treatments (Fig. 1B; Table 1.1). The root:shoot ratio was 14% greater in Myc compared to NM aspen seedlings and this pattern was consistent across all Pi treatments (Fig. 1.1C; Table 1.1).

1.3.2. Tissue P concentrations

Differential effects of colonization of aspen by *L. bicolor* on tissue P concentrations were prominent across the Pi gradient (Fig. 1.2). Leaves and roots of Myc seedlings had 259% and 79%, higher P concentrations, respectively, than NM seedlings across all Pi treatments, with differences in tissue P concentrations increasing as Pi availability increased (Fig. 1.2). Analysis using the Michaelis-Menten model indicated that Myc seedlings exhibited significantly greater maximum P concentrations than NM seedlings for both leaves and roots across the Pi treatment
gradient (Table 1.2). In addition, the half-maximal concentration for P accumulation was significantly lower in Myc than NM seedlings for both leaves and roots (Table 1.2), reflecting higher affinity for Pi uptake in plants colonized by *L. bicolor*.

### 1.3.3. Antioxidant enzyme (CAT, APX, GDP, SOD and Zn, Cu-SOD) activity and PEPC activity

The activities of antioxidant enzymes were measured in leaf and root tissues of NM and Myc seedlings as indicators of oxidative stress under Pi limitation. Across all enzymes assessed, the mean activity of antioxidant enzymes across all Pi treatments for NM seedlings was generally greater than the activity of Myc seedlings (Table 1.3, “mean” column). This elevated mean was primarily due to the greater activity of antioxidant enzymes in NM seedling tissues at lower Pi concentrations. This response is reported in Table 1.3, “slope” column: the more negative the slope, the greater the stimulation of enzyme activity by Pi limitation. In the case of CAT, enzyme activity in leaves was consistently higher and, while the slope responses between NM and Myc seedlings were similar (Table 1.3), the overall change was greater in NM plants (Fig. 1.3A). In roots, CAT activity followed a similar pattern of increasing to a greater extent in NM than Myc seedlings as Pi concentration declined (Fig. 1.3B; Table 1.3). APX activity increased significantly in NM seedling leaves and roots with decreasing Pi availability, whereas activity in Myc leaves and roots was significantly less responsive to Pi availability (Table 1.3, “slope” column). Similarly, GDP activity in leaves and roots increased significantly in NM plants below 5 and 10 µM Pi, respectively, yet was non-responsive in Myc seedlings (Table 1.3). Total SOD activity and Zn, Cu-SOD activity were stimulated at low Pi treatments in leaf and root tissues of both NM and M plants, and the increases in activity in NM seedling roots were more pronounced than in Myc seedlings (Table 1.3).

Significant differences in PEPCase activity were also observed between NM and Myc seedlings in response to Pi limitation (Fig. 1.4). In NM seedlings, leaf PEPCase activity exhibited a 7.5-fold increase and root activity a 5.7-fold increase between 100 and 0 µM Pi (Fig. 1.4), whereas the activity of PEPCase in Myc seedlings was not responsive to Pi limitation (Fig. 1.4).
1.3.4. **Organic acid (OA) and total organic carbon exudation**

Citrate, malonate, malate, lactate, and oxalate were the major organic anions released by aspen seedling roots and were detected in declining concentrations in this order. Concentrations of OAs in the rhizospheres of aspen seedlings were extremely low when seedlings were exposed to sufficient Pi supply, especially above 25 µM. Organic acid exudation increased significantly as Pi concentration declined and the extent of stimulation was greater in NM than in Myc seedlings (Table 1.4). These physiological responses are reflected in Table 1.4, “slope” column: the more negative the slope, the greater the stimulation of OA exudation by Pi limitation. For example, citrate was not detectable in root zone samples of seedlings grown at 100 µM Pi, but increased and was measured at concentrations of 119 µM and 68 µM in the rhizospheres of NM and in Myc seedlings grown with 0 µM Pi, respectively (Fig. 1.5A). On a production rate basis, citrate exudation in NM and Myc roots increased below 25 µM Pi and exudation by NM roots exceeded that of Myc roots below 10 µM Pi (Fig. 1.5B). These patterns were similar for all organic acids detected (Table 1.4), with the extent of divergent NM/Myc responses being greater for all organic acids measured (Table 1.4, “slope” column). When integrated together, these consistent differences in the production of OAs between NM and Myc contributed to substantial divergence in C flow to the rhizosphere (Fig. 1.6).

Across all Pi treatments, the concentration of TOC in the rhizosphere of NM plants was 345% greater than that in Myc plants. However, this difference between NM and Myc treatments was driven by Pi limitation, which increased TOC concentrations more substantially in NM plants as Pi concentrations declined (P < 0.001 for the Pi × Myc interaction) (Fig. 1.7A). TOC production in response to changes in Pi in Myc plants was substantially greater than in NM plants, and increased rapidly as Pi became limiting (P = 0.003 for the Pi × Myc interaction) (Fig. 1.7B). Based on measured TOC in the rhizosphere and leaching from pots during the watering cycle, the net dissolved organic C produced by aspen roots ranged from 0.10 to 1.83 mmol C plant\(^{-1}\) d\(^{-1}\) in NM plants to 0.16 to 0.46 mmol C plant\(^{-1}\) d\(^{-1}\) in Myc plants, with greatest production by NM and Myc aspen plants at the lowest Pi supply (Fig. 1.7B).
1.3.5. **Relationships between tissue P, tissue enzyme activity, and exudation**

Analyses of covariance (ANCOVA) were undertaken to assess the impacts of colonization of aspen seedlings by *L. bicolor* on relationships between measures physiological variables. While many stress and acclimation responses to P deficiency differed between NM and Myc seedlings, many of these differences may have been due to the observed divergence of tissue P concentrations in NM and Myc plants (Fig. 1.2).

Leaf CAT activity response appeared to be a consistent function of leaf P concentration across NM and Myc plants (*P* = 0.057 for the Pi × Myc interaction) (Fig. 1.8A) whereas root CAT activity differed between NM and Myc seedlings (*P* < 0.001 for the Pi × Myc interaction) (Fig. 1.8B). The activities of APX and GDP were similarly elevated and increased extensively as tissue P declined in NM plants in comparison to Myc tissues (*P* < 0.001 for the Pi × Myc interactions) (data not presented). Leaf and root SOD and leaf Zn, Cu-SOD were elevated in Myc tissues across the range of tissue P concentrations, although NM activities increased more substantially as tissue P declined (*P* < 0.001 for the Pi × Myc interactions) (data not presented).

Root PEPCase (Fig. 1.9A), citrate exudation (Fig. 1.9B), and TOC exudation (Fig. 1.9C) also showed significant correlations with root tissue P concentration. In each case, the extent of increase in response to declining root P concentration was limited in Myc roots and these lesser responses in PEPCase, citrate exudation, and TOC production compared to NM roots occurred at higher root P concentrations (*P* < 0.001 for the Pi × Myc interactions) (Fig. 1.9).

Organic acids, such as malate and citrate, are byproducts of the tricarboxylic acid (TCA) cycle and PEPCase activity supplies carbon chains for TCA cycle utilization. The exudation of both malate (Fig. 1.10A) and citrate (Fig. 1.10B) increased with increasing root PEPCase activity, with exudation increasing substantially in NM plants as PEPCase activity increased. In Myc plants, exudation and PEPCase activity were limited and did not exhibit any clear associations (Fig. 1.10).
1.4 Discussion

Forest productivity is often limited by nutrients, especially P, yet the physiological changes in forest trees during P limitation and role of the ectomycorrhizal colonization in ameliorating P deficiency are not well defined (Plaxton and Bell 2011). In the current experiment, I hypothesized that mycorrhizal colonization of aspen roots by *L. bicolor* would increase Pi acquisition. This increase would reduce the stress responses, such as oxidative stress, associated with P starvation, reduce root exudation that serves as a P scavenging strategy, and, as a result, lead to increased growth in mycorrhizal plants grown under Pi limitation.

Plants, including woody tree species, exhibit morphological, physiological, and ecological acclimation responses to P limitation that are well documented (Raghothama 1999, Vance et al. 2003, Lynch and Ho 2005; Raghothama and Karthikeyan 2005; Plassard and Bell 2010; Plaxton et al. 2011). These responses include increased root length (Akhtar and Siddiqui 2009, Bakker et al. 2009; Pang et al. 2010), higher root:shoot ratio (Lynch and Brown 2001, Williamson et al. 2001), increased root exudation of protons and carboxylates (Dakora & Phillips 2002; Casarin et al. 2004; Akhtar and Siddiqui 2009), altered Pi uptake rates (Cumming 1996; Colpaert et al. 1999), and altered P metabolism (Rychter and Randall, 1994, Lin et al. 2010) that function to increase Pi acquisition and P use efficiency. Ecologically, plants form symbiotic mycorrhizal associations that lead to morphological and physiological changes in roots that may aid P acquisition by the host plant (Tuomi et al. 2001, Plassard and Bell 2010; Smith et al. 2011).

The association of *L. bicolor* with aspen altered plant response to Pi limitation. Although differences in growth were small, significant differences due to mycorrhizal colonization were evident for shoot and root growth and root length (Table 1.1). Shoot mass of NM plants was greater than Myc plants when Pi was readily available (Fig. 1.1A), whereas root mass of Myc plants was consistently greater than NM plants across all Pi treatments (Fig. 1.1B). Together, these changes reflect a shift in C allocation belowground in Myc plants (Fig. 1.1B), which was further enhanced as Pi became limiting (Fig. 1.1C). Ectomycorrhizal fungi play important roles in enhancing plant growth under adverse conditions, especially nutrient deficiency (Rousseau and Reid 1991, Cumming 1996, MacFall et al. 2002, Wang and Liang 2010, Zhang 2010).
Although P deficiency reduces plant growth, particularly shoot biomass (Ciereszko et al. 1996, Tewari et al. 2007), root growth is often less affected (Benbrahim et al. 1996, Aono et al. 2001) as C is allocated to roots to further explore soil nutrient reserves (Gaume and Machler et al. 2001, Torres et al. 2008, Lopez-Bucio et al. 2003). Our findings are consistent with these reports and the root: shoot ratio of mycorrhizal aspen was 15% greater than that of NM plants, with allocation to roots being more pronounced as Pi became limiting (Fig. 1.1C). Such shifts in the root: shoot ratio reflect changes in C allocation associated with increased sink strength of mycorrhizal roots (Nehls and Hampp 2002; Hobbie 2006) and changes in allocation within the plant association with nutrient scavenging (Lynch and Ho 2005; Raghothama and Karthikeyan 2005).

Although Myc and NM aspen seedlings did not show vast differences in biomass in the current study, they did exhibit significant differences in tissue P concentrations (Fig. 1.2). Modeling of the tissue P patterns indicated that Myc roots had higher affinity for Pi uptake than NM roots (lower $K_m$ values, Table 1.2), which allows the effective acquisition of Pi at lower Pi availabilities (Fig. 1.2). The greater $C_{max}$ values for Myc plants compared to NM plants for both tissues (Table 1.2) indicates a greater capacity for P acquisition in mycorrhizal roots when Pi is not limiting. These differences in P accumulation suggest that *L. bicolor* aids P nutrition of aspen by possessing both higher affinity uptake systems and providing greater capacity for Pi uptake by roots and transfer to the host plant (Cumming 1996, Tarja and Janusz 2011, Cairney and Alexander 1992).

The sustained accumulation of P facilitated by *L. bicolor* in aspen led to significant reductions in oxidative enzyme activities under Pi limitation (Table 1.3, Fig. 1.8). Antioxidative enzymes play important roles in detoxifying ROS in plants under nutrient stress (Kandlbinder et al. 2004). While Ott et al. (2002) and Garfur et al. (2007) indicated the strong involvement of antioxidative enzymes system in response to metal-, drought-, and salinity-induced oxidative stress, little information is available on modulation of oxidative stress by ectomycorrhizal fungi during nutrient deficiency, particularly low P availability. A clear difference in ROS metabolism in response to P availability was observed between NM and Myc plants investigated in the
current study (Fig. 1.3, Table 1.3, Fig. 1.8). In general, mycorrhizal plants were under less oxidative stress than NM plants, as measured by the activity of these antioxidant enzymes, and this difference became more pronounced as P limitation intensified. These differences may result from the maintenance Pi uptake and P homeostasis (Raghothama and Karthikeyan 2005) in mycorrhizal plants (Fig. 1.8). Similarly, Langenfeld-Heyser et al. (2007) found that antioxidant activity of roots of *Populus canescens* under non-stressed conditions was less when colonized by *P. involutus*, although rates declined in both NM and Myc treatments when plants were exposed to NaCl. In contrast, Alvarez et al. (2009) noted elevated antioxidant enzyme activities in roots of *Nothofagus dombeyi* colonized by two ECM fungi under drought stress. These different patterns may reflect differences in the generation of ROS under these different environmental stresses.

In trees, root exudation is responsive to environmental conditions (Klugh and Cumming 2007; Johansson et al. 2009; Naik et al. 2009) and this contribution to rhizodeposition alters nutrient availability in the rhizosphere (Cumming and Weinstein 1990; Wallander 2000; van Hees et al. 2006). A wide range of compounds has been identified in root exudates, with the dominant classes being low molecular weight OAs, amino acids, and sugars (Nguyen et al. 2003; Klugh and Cumming 2007; Johansson et al. 2009). In contrast to annual plants, reports on the rates of exudation from tree roots remain limited and few studies have attempted to investigate exudation patterns of mycorrhizal trees (Ratnayake et al. 1978, Van Hees et al. 2006, Klugh and Cumming 2007; Johansson et al. 2009; Naik et al. 2009). In aspen, the exudation of OAs increased in response to Pi limitation and these increases were more pronounced in NM plants in comparison to plants colonized by *L. bicolor* (Fig. 1.6). The differences between NM and Myc exudation were consistent across all of the organic acids detected (Fig. 1.6) and may have been due to the maintenance of Pi uptake by mycorrhizal roots (Fig. 1.2), which would moderate the threshold inducing a P starvation signal that may initiate exudation (Rouached et al. 2010).

PEPCase activity increases in roots (Pilbeam et al. 1993) and leaves (Hof et al. 1992) in many plant species during P limitation. PEPCase is involved in non-photosynthetic carbon assimilation and catalyzes the processing of phosphoenolpyruvate (PEP) to oxalacetic acid
(OAA), which is a precursor of the TCA cycle. The exudation of malate and citrate, which are products of the TCA cycle, often increases during P limitation (Johnson et al. 1996, Watt and Evans 1999, Ryan et al. 2001). In aspen, NM plants subjected to Pi limitation exhibited significantly higher PEPCase activity in both leaves and roots, with root activity being greater than leaf activity (Fig. 1.4). The high correlation between PEPCase activity and malate and citrate exudation in NM plants (Fig. 1.10), and the observed exudation of malonate, lactate, and oxalate as well, indicates that this C fixation pathway may operate in NM aspen to supply fixed C for exudation in response to Pi limitation. Similar patterns have been noted at the enzyme (Aono et al. 2001) and gene (Penaloza et al. 2005) levels, indicating that PEPcase may function as a Pi-limitation stress acclimation strategy in aspen. In aspen colonized by L. bicolor, however, the limited association between malate and citrate exudation and PEPCase activity (Fig. 1.10) and lesser exudation of all organic acids (Fig. 1.6) suggest that the mycorrhizal association dampens these physiological stress responses. This is most likely due to the maintenance of P acquisition across the full range of Pi treatments (Fig. 1.2), although a fundamental shift in root C processing when L. bicolor colonizes roots cannot be excluded.

Mirroring the increased flux of organic acids to the rhizosphere, TOC exudation also increased substantially with P limitation, with significant differences between NM and Myc aspen as Pi became limiting (Fig. 1.7). This other pool of exuded C may be sugars and amino acids, the production of which increases under P limitation (Ratnayake et al. 1978), and the observed dampening of TOC exudation by the mycorrhizal association may reflect the maintenance of root Pi uptake and homeostasis facilitated by the mycorrhizal association. However, due to lack of ability to separate plant and fungal OA and TOC production in roots colonized by L. bicolor, it is not possible to delineate the contribution of individual symbiotic partner.

Root exudation varies by plant species, edaphic conditions, and with differing mycorrhizal symbionts (Uselman et al. 2000; Klugh and Cumming 2007; Johansson et al. 2009; Naik et al. 2009). These patterns are also evident in the current work. While the release of a variety of C-containing compounds into the rhizosphere under P limitation may reflect the
activity of P-starvation acclimation physiology (Raghothama and Karthikeyan 2005; Plaxton et al. 2011), such losses may also represent a sizable C cost to the plant and may contribute to growth reductions under Pi limitation. In the current study, C production ranged from 1.2 to 20.9 mg C plant\(^{-1}\) d\(^{-1}\) (0.3 to 615.2 mg g\(^{-1}\) DW d\(^{-1}\)) in NM aspen and 0.8 to 5.5 mg C plant\(^{-1}\) d\(^{-1}\) (0.2 to 45.3 mg g\(^{-1}\) DW d\(^{-1}\)) in plants colonized by \(L.\) bicolor. The range of exudation in NM plants closely aligns with reports for exudation in \(P.\) tremuloides and \(P.\) trichocarpa under exposure to Al (Naik et al. 2009), with the highest rates in that study occurring under exposure to Al and in the current study under Pi limitation. Interestingly, these rates of exudation in plants under stress are substantially greater than reports of root exudation in tree species not exposed to edaphic stress (Uselman et al. 2000; Phillips et al. 2009).

The maintenance of Pi uptake and translocation in aspen colonized by \(L.\) bicolor (Fig. 1.2) may underlie much of the observed reductions in stress responses as Pi became limiting. However, analyses of covariance suggest that Myc and NM plants behave differently at the same level of tissue P, suggesting that there may be other moderators of plant response to P limitation. For example, increases in root CAT activity (Fig. 1.8B) and citrate and TOC exudation (Fig. 1.9B and 1.9C) increase in Myc plants at higher root P concentrations than corresponding responses in NM aspen. These moderators of plant response to P limitation in mycorrhizal plants may function through signaling pathways for maintaining P balance (Rouached et al. 2010), result from differential levels of photoassimilates and phytohormones (Cho & Cosgrove, 2002), or through differential action of transcription factors, such as PHRI (Rubio et al. 2001) or WRKY75 (Devaiah et al. 2007) that differ between NM and Myc plants.

Plant response during P limitation is a complicated process involving signaling pathways (Rouached et al. 2010) and metabolic adjustments (Plaxton and Tran 2011) that lead to changes in plant-soil interactions and changes in P acquisition. These changes are moderated through root growth, phosphate transporters, and exudation, all of which may be modified by the mycorrhizal symbiosis. In the current study, we observed significant effects of Pi availability on aspen stress physiology. The activity of antioxidant enzymes increased, suggesting plants were experiencing loss of homeostasis and generation of free radicals as Pi became limiting. PEPCase activity
increased markedly, indicative of a demand for C-containing compounds for exudation, which also increased significantly as Pi concentrations declined. Colonization by the ECM fungus *L. bicolor* modulated these responses, primarily by increasing the acquisition of Pi from the rhizosphere. The maintenance of P uptake and translocation were the result of more effective Pi uptake by mycorrhizal roots and mycorrhizal aspen plants benefited through reduced oxidative stress and lower flow of C to the rhizosphere, both of which represent significant C savings to mycorrhizal aspen. While aspen is considered a stress-resistant, early colonizing species, the current study indicates that aspen’s ability to acclimate to stressful edaphic environments is, in part, due to the formation of mycorrhizal associations that increase its stress resistance and ecological breadth.
Figure 1-1. Effect of phosphorus concentration in nutrient solution on shoot (A) and root (B) biomass and root: shoot ratio (C) of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor.*
Figure 1-2. Effect of phosphorus concentration in nutrient solution on P concentration in leaf (A) and root (B) tissues of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*
Figure 1-3. Effect of phosphorus concentration in nutrient solution on specific activities of catalase (CAT) in aspen seedling leaf (A) and root (B) tissues of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*. Y-axis break in (B) occurs at 0.3 to 0.45.
Figure 1-4. Effect of phosphorus concentration in nutrient solution on specific activities of phosphoenol pyruvate carboxylase (PEPCase) in leaf (A) and root (B) tissues of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*.
Figure 1-5. Effect of phosphorus concentration in nutrient solution on citrate concentrations in the root zone (A) and citrate exudation by roots (B) of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*. Y-axis break in (B) occurs at 0.004 to 0.009.
Figure 1-6. Effect of phosphorus concentration in nutrient solution on cumulative organic acid concentrations in the root zone (A) and organic acid exudation by roots (B) of nonmycorrhizal (NM) and mycorrhizal (Myc) aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Each color bar indicates specific organic acid as indicted.
Figure 1-7. Effect of phosphorus concentration in nutrient solution on TOC concentrations in the root zone (A) and TOC production by roots (B) of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*. Y-axis break in (B) occurs at 0.1 to 1.0.
Figure 1-8. Relationships between catalase (CAT) antioxidant enzyme activity and P concentration in leaves (A) and roots (B) of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*. 
Figure 1-9. Relationships between root phosphoenol pyruvate carboxylase (PEPCase) activity (A), citrate exudation (B), and TOC production (C) and root P concentration in nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor*. Y-axis break in (C) occurs at 3 to 8.
Figure 1-10. Relationships between malate exudation (A) and citrate exudation (B) and root phosphoenol carboxylase (PEPCase) activity in nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi for 46 d. Open symbols indicate nonmycorrhizal treatment and closed symbols indicate aspen colonized with *L. bicolor.*
Table 1-1 Summary statistics from analysis of regression for growth measurements of nonmycorrhizal and mycorrhizal (with *L. bicolor*) aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi in sand culture for 46 d.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mycorrhiza</th>
<th>Pi</th>
<th>Mycorrhiza × Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>% Colonization</td>
<td>nd</td>
<td>nd</td>
<td>31.3</td>
</tr>
<tr>
<td>Plant height</td>
<td>21.5</td>
<td>***</td>
<td>361.6</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>33.5</td>
<td>***</td>
<td>1947</td>
</tr>
<tr>
<td>Shoot biomass (g)</td>
<td>0.09</td>
<td>ns</td>
<td>2470</td>
</tr>
<tr>
<td>Root biomass (g)</td>
<td>10.3</td>
<td>**</td>
<td>1875</td>
</tr>
<tr>
<td>Root:shoot ratio</td>
<td>27.9</td>
<td>***</td>
<td>61.3</td>
</tr>
</tbody>
</table>

*, P < 0.05; **, P < 0.01; ***, P < 0.001; nd, not determined; ns, not significant.
Table 1-2 Estimated P accumulation parameters derived from the Michaelis-Menten equation for leaf and root tissue of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi in sand culture for 46 d. NM = nonmycorrhizal, Myc = mycorrhizal with *L. bicolor*.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mycorrhiza</th>
<th>$C_{max}$ (mg g$^{-1}$ DW)</th>
<th>$k_m$ (µM Pi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>NM</td>
<td>1.69</td>
<td>73.6</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>2.99</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$P^*$ &lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Root</td>
<td>NM</td>
<td>1.84</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>2.58</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$P$ &lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*P = significance of the mycorrhizal treatment effect.*
Table 1-3 Regression parameters describing the activity of antioxidant enzymes in leaves and roots of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi in sand culture for 46 d. NM = nonmycorrhizal, Myc = mycorrhizal with *L. bicolor*.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme</th>
<th>Mycorrhiza</th>
<th>Leaf</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>1.64</td>
<td>-0.0168</td>
<td>0.157</td>
</tr>
<tr>
<td>Myc</td>
<td>0.53</td>
<td>-0.0167</td>
<td>0.082</td>
</tr>
<tr>
<td></td>
<td>$P^3$</td>
<td>&lt;0.001</td>
<td>ns</td>
</tr>
<tr>
<td>APX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>0.401</td>
<td>-0.0214</td>
<td>0.224</td>
</tr>
<tr>
<td>Myc</td>
<td>0.158</td>
<td>0.0001</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GDP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>0.0400</td>
<td>-0.0195</td>
<td>0.0247</td>
</tr>
<tr>
<td>Myc</td>
<td>0.0169</td>
<td>0.0023</td>
<td>0.0159</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>11.0</td>
<td>-0.0184</td>
<td>15.1</td>
</tr>
<tr>
<td>Myc</td>
<td>13.8</td>
<td>-0.0084</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.003</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zn,Cu-SOD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>7.96</td>
<td>-0.0234</td>
<td>12.5</td>
</tr>
<tr>
<td>Myc</td>
<td>5.26</td>
<td>-0.0158</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.005</td>
<td>0.050</td>
</tr>
</tbody>
</table>

1Least square means from the regression $\mu M = e^{\text{slope(Pi)-intercept}}$. \( \mu \text{kat mg}^{-1} \text{ protein min}^{-1} \).
2\( \mu \text{kat mg}^{-1} \text{ protein min}^{-1} \) (µM Pi)$^{-1}$.
3$P$ = significance of the mycorrhizal treatment effect.
4ns = not significant.
Table 1-4 Regression parameters describing the exudation of organic acids by roots of nonmycorrhizal and mycorrhizal aspen seedlings grown with 0, 1, 5, 10, 25, 50, and 100 µM Pi in sand culture for 46 d. NM = nonmycorrhizal, Myc = mycorrhizal with *L. bicolor*.

<table>
<thead>
<tr>
<th>Organic Acid</th>
<th>Mycorrhiza</th>
<th>Response Mean</th>
<th>Response Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>NM</td>
<td>1.014</td>
<td>-0.0752</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>0.503</td>
<td>-0.0630</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>^3&lt;0.001</td>
<td>0.010</td>
</tr>
<tr>
<td>Malonate</td>
<td>NM</td>
<td>1.146</td>
<td>-0.0566</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>0.538</td>
<td>-0.0454</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>&lt;0.001</td>
<td>0.021</td>
</tr>
<tr>
<td>Malate</td>
<td>NM</td>
<td>0.533</td>
<td>-0.0564</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>0.260</td>
<td>-0.0400</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>&lt;0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>Lactate</td>
<td>NM</td>
<td>0.796</td>
<td>-0.0705</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>0.415</td>
<td>-0.0545</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>0.007</td>
<td>0.029</td>
</tr>
<tr>
<td>Oxalate</td>
<td>NM</td>
<td>0.272</td>
<td>-0.0646</td>
</tr>
<tr>
<td></td>
<td>Myc</td>
<td>0.032</td>
<td>-0.0416</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

^1Least square mean from the regression = $e^{\text{slope}(\text{Pi}) - \text{intercept}}$, µmol g$^{-1}$ FW d$^{-1}$.

^2µmol g$^{-1}$ FW d$^{-1}$ (µM Pi)$^{-1}$.

^3*P* = significance of the mycorrhizal treatment effect.
1.5 References:


2 Carbon fixation, carbon allocation, and growth of ectomycorrhizal *Populus tremuloides*

2.1 Introduction

Forest ecosystem productivity is often limited by mineral nutrient availability (LeBauer & Treseder 2008; St. Clair *et al.* 2008; Watt *et al.* 2008). Phosphorus (P) is one of the major mineral nutrients required by forest trees in significant amounts (Elser *et al.* 2007), yet P availability is limited in soils by strong biological demand, sequestration in organic forms, and the chemical binding of inorganic phosphorus (Pi) and organic phosphorus (Po) to aluminum (Al), iron (Fe) or calcium (Ca) ions/compounds prevalent in many natural soil systems (Von Uexkull & Mutert 1995; Batjes 1997). While P limitation may be overcome in agricultural systems by the application of P-containing fertilizers, this management approach is neither sustainable nor practical for large-scale silvicultural production, especially in developing countries.

To overcome P limitation, trees and other plants have developed a suite of physiological and ecological strategies that maintain the balance of internal P homeostasis and maximize P acquisition from the environment (Raghothama 1999; Vance 2003, Plassard & Bell 2010; Plaxton & Tran 2011). Plant-specific responses, termed phosphate starvation responses (PSR), acclimate plants to low P environments (Doerner 2008; Fang *et al.* 2009; Plaxton & Tran 2011). These changes include increases in the root-to-shoot ratio and changes in root architecture that increase soil exploration and acquisition of P (Lynch 2011). Root physiological changes, including root exudation, increased phosphate uptake capacity, and biochemical bypasses, increase P acquisition from soils and P use efficiency within the plant (Dakora & Phillips 2002; Vance 2002; Raghothama & Karthikeyan 2005; Plaxton & Tran 2011). In addition to these changes within the plant, one common strategy for enhancing P acquisition from soil is the formation of the plant-mycorrhizal symbiosis (Plassard & Bell 2010; Smith *et al.* 2011).

The interaction between trees and symbiotic mycorrhizal fungi in soils improves P acquisition under P limitation and benefits tree productivity (Plassard & Bell 2010). The
colonization of roots by ectomycorrhizal (ECM) fungi leads to changes in root architecture (Felton et al. 2009) and ECM fungal mycelia may greatly increase the volume of soil exploited for mineral uptake (Schack-Kirchner et al. 2000, Agerer 2001, Hagerberg et al. 2003). Increased carbon (C) flow to the soil in the form of exudates and enzymes alters the chemistry of the rhizosphere and the availability of P from a broad spectrum of P-containing compounds for ECM and root uptake (Cumming & Weinstein 1990, Leyval & Berthelin 1993, Wallander 2000, Casarin et al. 2004, Liu et al. 2005, Courty et al. 2010).

Changes in C allocation resulting from plant-specific PSR systems or mycorrhizal colonization may alter photosynthesis, the activity of biochemical pathways, and the composition of plant tissues (Ericsson et al. 1999; Lekberg et al. 2010; Plaxton & Carswell 1999). Phosphorus limitation often limits photosynthesis due to inhibition in enzymes involved in Calvin cycle (Tissue et al. 2010). In contrast, colonization by ECM fungi may increase net photosynthesis due to the increased C demands of the fungal partner (Kaschuk et al. 2009) and the provision of P that is frequently limiting in forest ecosystems (Wardle et al. 2004; Wullaert et al. 2010).

According to the Carbon-Nutrient Balance Theory (Bryant et al. 1983), the production of carbon (C) rich secondary metabolites in plants depends on the balance between C and nutrient availability. Under nutrient limitation, photosynthesis continues even though growth is reduced and plants allocate more C to C-based secondary metabolites (Lindroth et al. 1999; Haiko et al. 2007). For example, the concentrations of tannins and salicylates in leaves of Populus alba × P. grandidentata were higher when exposed to lower nitrogen (N) availability (Kleiner 1998). Many plant species have been shown to accumulate anthocyanin during nutrient limiting conditions and the biosynthesis and accumulation of phenolic compounds in plants species under stress also shows that secondary C-containing compounds contribute to the acclimation of plants to stressful environments (Bergmann 1992; Dixon & Paiva 1995; Ismail & Mohamed 2010).

Forest trees are often challenged by biotic and abiotic stresses, including pathogens (Brasier 2009) and nutrient limitation (Biglew & Canham 2007). Although the responses of secondary metabolites are well known in plants under biotic and abiotic stresses, considerably...
less is known about changes in secondary C metabolism in plants in the ectomycorrhizal (ECM) association. In the *Larix deciduas-Suillus tridentinus* symbiosis, for example, higher levels of major secondary C-containing compounds, such as catechin and epicatechin, and minor compounds, such as 4-hydroxybenzoate and 4-o-β glycoside, were observed in the root apices of young mycorrhizas (Weiss *et al.* 1997). No reports concerning interactions between Pi limitation and ECM on the accumulation of secondary compounds in plants could be located.

Trees in the genus *Populus* are widely distributed in the temperate and boreal forest regions. Poplars may be early colonizers as well as old-growth species in various ecosystems (Timoney & Robinson 1996). Species within the genus have economic importance in the pulp and wood industries and plantations of poplar are planted broadly around the world (Cheng & Tuskan 2009). The rapid growth, stress resistance, and genetic variation of many poplars have led to significant efforts to identify species and genotypes for biofuel and phytoremediation applications (Cheng & Tuskan 2009). To support these efforts, poplars have received substantial research attention as a model tree species, leading to the sequencing of the genome of *P. trichocarpa* (Tuskan *et al.* 2006).

In the current study, we assessed the influences of ectomycorrhizal colonization on phosphate limitation responses of *P. tremuloides* Michx. (trembling aspen), specifically investigating photosynthesis and the allocation of carbon between carbohydrates and secondary metabolites (condensed tannins, flavanols, phenolics acids, glycosides and salicylates). I hypothesized the following: (1) that photosynthesis will be reduced due to Pi limitation and increased in ectomycorrhizal plants in response to stimulated C demand in the root system by the fungal partner; (2) that nonmycorrhizal aspen plants are hypothesized to allocate more carbon to secondary compounds under Pi limitation rather than to the production of plant biomass and, (3) that changes in photosynthesis, allocation, and growth expected to be more pronounced in nonmycorrhizal plants than in plants colonized by the ectomycorrhizal symbionts *Laccaria bicolor* and *Paxillus involutus*, due to enhanced Pi acquisition by these symbionts.
2.2 Material and Methods:

2.2.1. Fungal inoculation and plant culture

The *P. involutus* (Batsch) Fr. fungal strain ATCC 200175 (American Type Culture Collection) and *L. bicolor* (Marie) P.D. Orton fungal strain S238N (Institut National de la Recherche Agronomique, Nancy, France) were used as symbionts to colonize seedlings of *P. tremuloides* Michx. (trembling aspen). Fungal cultures were maintained on a modification of modified Melin Norkrans (MMN) agar medium (Cumming et al. 2001). For inoculum production, cultures were grown aseptically in liquid MMN medium for 3 weeks at 25°C in the dark in static culture (Molina and Palmer 1982). Cultures were blended briefly (three pulses for 3 s each) to produce fungal mycelial slurries to serve as inoculum.

The experimental system for the production of mycorrhizal aspen consisted of CP512 treepots (Stuewe and Sons, Corvallis, OR, USA) each containing ~5 L of a mixture (2:1) of sterile acid-washed sand and vermiculite. For establishing mycorrhizal seedlings, a band of fungal slurry was added ~2 cm below the surface of the sand. This inoculation method was found to be successful for generating ECM colonization with *P. trichocarpa* (Baum et al. 2002) and *P. tremuloides* (Desai et al. unpublished). For nonmycorrhizal plants, only MMN liquid medium was added instead of fungal slurry.

Seeds of *P. tremuloides* (Canadian Natural Resources, National Tree Seed Center, Fredericton NB, Canada) were planted ~0.5 cm deep in the pots and were kept moist by watering four-times daily with deionized water. Seedlings were thinned to one seedling per pot 10 days after germination. Thereafter, seedlings were watered four-times daily with 40 ml per pot of a nutrient solution containing 1.0 mM NO₃, 0.4 mM NH₄, 0.5 mM K, 0.2 mM Ca, 0.1 mM Mg and SO₄, 50.5 µM Cl, 20 µM Fe-EDTA, 20 µM B, 2 µM Mn and Zn, and 0.5 µM Cu, Na, Co and Mo. Solution pH was adjusted to 5.6 with 0.1 N NaOH. After an additional 2 weeks, phosphate (Pi) treatments in the form of KH₂PO₄ were added to the baseline nutrient solution to deliver treatment solutions containing 5 or 100 µM Pi. Ten replicate seedlings per Pi/mycorrhizal treatment combination were established. Seedlings were maintained in a climate-controlled
greenhouse with supplemental lighting (mixed metal halide sources) providing a 14-h photoperiod and day/night temperatures of 24/19 ± 3 °C. Seedlings were grown for 46 day after Pi treatments (DAT).

2.2.2. Photosynthesis measurements

Net photosynthesis was measured on 15, 35, and 45 DAT using a Li-COR 6400 portable photosynthesis system with a 6-cm² chamber with red/blue LED light sources (Li-COR Biosciences, Lincoln, NE, USA). Photosynthetic measurements were made between 0900 h to 1600 h daily for the most fully expanded leaf of each plant (4ᵗʰ-5ᵗʰ leaf from the top). A-Cᵢ curves were generated between 50 and 1500 µmol mol⁻¹ CO₂ on three replicate plants per treatment. A-Cᵢ curves were generated for a sequence of 380, 50, 100, 150, 250, 580, 800, 1000, 1200 and 1500 µmol mol⁻¹ CO₂ mole fraction. Vₗₘₜₐₓ was measured on each plant at 380 µmol mol⁻¹ CO₂. Measurements were made at saturating light intensity of 1500 µmol m⁻² s⁻¹. Net photosynthetic rate (A), stomatal conductance (gₛ), transpiration rate (E), and intercellular CO₂ concentration (Cᵢ) were derived.

Chlorophyll florescence was measured using a pulse amplitude modulation (PAM) fluorometer (Walz, Effeltrich, Germany). Dark adaption time of 30 minutes was used prior to taking chlorophyll florescence measurements (Myers, Thomas, & DeLucia 1999). Three replicates per treatment were used for each photosynthetic measurement.

2.2.3. Growth measurements, leaf P concentration, and mycorrhizal colonization

Plant height and number of leaves were measured at 0, 15, 35, and 45 DAT. Leaf phosphorus (P) concentration was measured using four leaf discs sampled from the leaves used during photosynthetic measurement. Leaf punches (4) where flash frozen in liquid nitrogen and stored at -20 °C. Leaf discs were ashed at 475°C for 1 hour, dissolved in one ml 50% HCl, and crucibles rinsed with 1 ml dH₂O. The resulting 2 ml digests were vortexed until clear (~5 min) and inorganic P was detected using the malachite green method (Martin, 1999).

Plants were harvested after 46 days. Five out of ten replicates were used for biomass and
colonization assessment. Plants were separated into roots and shoots and roots were washed thoroughly with dH₂O to removed adhering substrate. Root samples (~1 g) from the center of root systems were stored in sterile distilled water at 4°C. Percent ECM colonization was quantified using the gridline intercept method (Giovanetti and Mosse 1980). The remaining shoot and root tissues were dried at 60°C for 48 h for dry weight.

2.2.4. Tissue C compounds

Tissue samples for secondary metabolite assays were flash frozen in liquid nitrogen and stored at -20 °C. Secondary metabolites were analyzed for three plant tissues: the four youngest leaves (YL) from top, mature leaves (ML), which were the remaining fully expanded leaves below YL, and root tissue (RT), representing pooled roots with diameter < 1 mm.

Plant tissue samples (~1 g) were crushed in liquid nitrogen using a mortar and pestle and ground in the dark in 10 ml of 80% ice-cold methanol. Ground samples were sonicated for 15 min in an ice bath and centrifuged at 4000 rpm for 5 min. Sample extracts were stored at -20 °C. Secondary metabolites were separated by HPLC (Varian, Walnut Creek, CA, USA) using a Luna 5μm C18 column (250 × 4.60 mm) (Phenomenex 519880-40, Torrance, CA, USA) and detection at 254 nm. The mobile phase consisted of a solvent gradient of solvent A (0.5% MeOH in phosphoric acid) and solvent B (100% acetonitrile) that increased stepwise over 45 min. The elution steps were (%B) 0:30:50:65:80 each step for 5 min, followed by column cleaning with 100% MeOH for 15 min and re-equilibration for 5 min with 0.5% MeOH in phosphoric acid while maintaining a constant flow rate of 1.0 ml min⁻¹ (Mellway et al. 2009). HPLC standards for secondary metabolites used were (+) catechin, chlorogenic acid, p-OH-cinnamic acid, myricetin, quercetin, and kamferol, obtained from Sigma Chemical Company (St. Louis, MO, USA). Isohamnetin, salicin, salicortin, and tremulacin standards were obtained from Dr. Richard Lindroth (University of Wisconsin, Madison, WI, USA).
2.2.5. **Statistical analysis**

The experiment was randomized two-way factorial design, with two Pi treatments (5 µM and 100 µM Pi) and three fungal treatments (nonmycorrhizal (NM) control, *L. bicolor* (Lb), *P. involutus* (Pax) and 10 replicates each combination. Samples taken during the 15, 30, and 45 DAT during the experiment (height, number of leaves, \(n = 5\); photosynthesis, and leaf P concentration, \(n = 3\)) were analyzed using repeated measures analysis of variance (ANOVA) with transformations (log) used as needed to meet the assumptions of ANOVA. Lack of statistical significance of interaction factors containing DAT led to presentation of main effects (Pi, mycorrhizae, and their interaction) below. Tukey-Kramer’s HSD tests were utilized to identify significant differences between treatment means.

The effects of Pi concentration and ECM colonization on plant variables at the end of the experiment (biomass, \(n = 5\); tissue secondary metabolites, \(n = 3\)) were analyzed using two-way ANOVA (Pi-by-mycorrhizae). Data were transformed as needed. When ANOVA test indicated asignificant treatment effect, Tukey-Kramer’s HSD tests were utilized to identify significant differences among treatment means. Analysis of covariance (ANCOVA) was used to investigate relationships among measured variables. Statistical analysis was carried out using SAS JMP 7.0 (SAS Institute, Cary, North Carolina, USA).
2.3 Results

2.3.1. Photochemical parameters

There were few statistically significant interactions (two- or three-way) between Pi or mycorrhizal treatments and DAT for photosynthetic parameters measured. Therefore, DAT was treated as a blocking factor and photosynthetic parameters are presented as marginal means across DAT. Photosynthetic CO$_2$ uptake ($A$) was dependent on Pi and mycorrhizal treatments ($P < 0.001$ for the Pi × interaction) (Fig. 2.1A). Photosynthetic CO$_2$ uptake was reduced by 63% in NM poplar, whereas uptake was not significantly affected by Pi limitation in plants colonized by *L. bicolor* or *P. involutus* (Fig. 2.1A). There was no significant effect of DAT on $A$ (data not presented). No significant changes in intercellular CO$_2$ concentration ($C_i$) were observed due to Pi limitation or mycorrhizal colonization (Fig. 2.1B). $C_i$ declined over the course of the treatment period from 282 to 171 µmol mol$^{-1}$. Across Pi treatments, stomatal conductance was reduced by 27% at low Pi ($P = 0.006$ for the Pi effect) (Fig. 2.1C). While this effect was consistent across mycorrhizal treatments, colonization by *L. bicolor* and *P. involutus* differentially affected $g_s$ ($P = 0.020$) in aspen plants. The greatest reduction due to Pi limitation was in nonmycorrhizal plants (Fig. 2.2C), where stomatal conductance was reduced by 44% compared to plants grown at 100 µM Pi. Stomatal conductance declined significantly over DAT from 0.368 mol m$^{-2}$ s$^{-1}$ at DAT 15 to 0.163 mol m$^{-2}$ s$^{-1}$ at DAT 45 (data not presented). The transpiration rate ($E$) of aspen grown at 5µM Pi was 25% less than that of plants grown at 100 µM ($P = 0.004$) (Fig. 2.1D) and this reduction in $E$ by low Pi was consistent across all mycorrhizal treatments. DAT had no significant effect on transpiration.

The light saturated rate of electron transport ($J_{max}$) varied with Pi availability and mycorrhizal colonization ($P < 0.001$ for the Pi × interaction) (Fig. 2.1E). NM aspen exhibited the greatest $J_{max}$ at 100 µM Pi and the greatest inhibition at 5 µM Pi (59% reduction), whereas $J_{max}$ was unaffected by Pi treatment in plants colonized by *L. bicolor* or *P. involutus* (Fig. 2.1E). $J_{max}$ reduced with DAT ($P < 0.001$). In *P. tremuloides*, Pi limitation also limited $V_{cmax}$, the maximum rate of carboxylation of RuBP, and this reduction was dependent on mycorrhizal treatment ($P <$
0.001 for the Pi × interaction) (Fig. 2.1F). NM exhibited the highest $V_{\text{cmax}}$ at 100 µM Pi and showed the greatest (50%) reduction at 5 µM Pi; $V_{\text{cmax}}$ in aspen colonized with Lb and Pax was unaffected by Pi limitation (Fig. 2.1F). $V_{\text{cmax}}$ varied between 50.0 and 62.1 µmol m$^{-2}$ s$^{-1}$ with DAT ($P < 0.001$) (data not presented). The ratio of $J_{\text{max}}$:$V_{\text{cmax}}$, which reflects the relative limitations to photosynthesis between RuBP regeneration and carboxylation, followed similar patterns as $J_{\text{max}}$ and $V_{\text{cmax}}$. In NM aspen, $J_{\text{max}}$/$V_{\text{cmax}}$ declined significantly from 1.31 to 1.10, but was unaffected by Pi in plants colonized by L. bicolor (mean = 1.26) and P. involutus (mean = 1.24) indicating that Pi limitation is affecting RuBP regeneration processes.

The efficiency of photosystem II (Fv/Fm) in aspen plants was significantly reduced by Pi limitation in NM plants and plants colonized by L. bicolor but not P. involutus ($P < 0.036$ for the Pi × interaction) (Table 2.1). Pi-limitation induced reductions of 19 and 17% in Fv/Fm were noted for NM plants and plants colonized by L. bicolor, whereas changes were not evident in plants colonized by P. involutus (Table 2.1). Pi limitation and mycorrhizal colonization also had significant effects on quantum yield ($P < 0.001$ for the Pi × interaction) (Table 2.1). At 100 µM Pi, quantum yield of all mycorrhizal treatments were equal. At 5 µM Pi, quantum yield of aspen colonized by P. involutus was unaffected by Pi limitation. However, quantum yield in NM plants and plants colonized by L. bicolor declined by 26 and 15% at 5µM Pi, respectively (Table 2.1). Photochemical quenching $q_P$ was reduced by Pi limitation, and this effect was ameliorated by colonization by P. involutus, but not L. bicolor ($P = 0.001$ for the Pi × interaction) (Table 2.1). Non-photochemical ($q_N$) did not vary with Pi limitation or mycorrhizal colonization (Table 2.1).

### 2.3.2. Growth parameters

Height growth rate and leaf addition rate of aspen plants depended on both Pi concentration and mycorrhizal status ($P < 0.001$ for the Pi × interactions in each case) (Table 2.2). Height growth among the three mycorrhizal treatments at 100 µM Pi did not differ. At 5 µM Pi, height growth rates were reduced by 66, 28, and 19% in NM plants and plants colonized by L. bicolor and P. involutus, respectively (Table 2.2). Leaf addition rates exhibited similar relative patterns among Pi and mycorrhizal treatments, with plants colonized by the two mycorrhizal symbionts exhibiting lesser reductions in growth than NM plants at 5 µM Pi (Table 2.2). Colonization of
aspen roots was slightly greater in plants colonized by *P. involutus* compared to *L. bicolor* (*P* = 0.003 for the mycorrhizal effect) and increased slightly and consistently for each fungal treatment under Pi limitation (*P* = 0.005 for the Pi effect) (Table 2.2).

Following 45 days of treatment with 5 or 100 µM Pi, aspen plant biomass was significantly reduced by Pi limitation and reductions in shoot and root growth were differentially modulated by *L. bicolor* and *P. involutus* (Fig. 2.2). Shoot growth of aspen did not differ among mycorrhizal treatments at 100 µM Pi. However, reductions in shoot biomass in NM plants (76%) were substantially greater than reduction in plants colonized by either ectomycorrhizal fungi (46%) (*P* < 0.001 for the Pi × interaction) (Fig. 2.2A). Patterns of root response to Pi limitation and among mycorrhizal treatments were more complex. At 100 µM Pi, plants colonized by *P. involutus* allocated ~50% less C to roots than NM plants or plants colonized by *L. bicolor* (Fig. 2.2B). While Pi limitation reduced root biomass in all mycorrhizal treatments, these reductions varied by mycorrhizal treatment (Fig. 2.2B). Root growth was reduced by 91% in NM plants, whereas reductions in plants colonized by *L. bicolor* (36%) and *P. involutus* (28%) were not statistically different from corresponding mycorrhizal treatments at 100 µM Pi (Fig. 2B).

The root: shoot ratios of aspen differed among mycorrhizal treatments and responded differently to Pi limitation (*P* < 0.006 for the Pi × interaction). In NM plants, the root-shoot ratio declined from 6.71 at 100 µM Pi to 2.75 at 5 µM Pi. In contrast, root-shoot ratios were 5.58 and 6.51 at 100 and 5 µM Pi, respectively in aspen colonized by *L. bicolor* and 2.89 and 3.79 at 100 and 5 µM Pi, respectively in aspen colonized by *P. involutus*. These changes in mycorrhizal plants were not statistically significant (data not presented).

The concentration of P in leaf tissue was significantly reduced by Pi limitation, and this effect was dependent on mycorrhizal status (*P* < 0.001 for the Pi × interaction) (Fig. 2.3). NM leaf Pi concentration was 45.5% less than mycorrhizal leaf Pi concentration at 100 µM Pi. NM plants grown at 5 µM Pi had the lowest leaf P concentrations. Although reductions in plants colonized by *P. involutus* were significant, these concentrations were still as high as NM plants grown at 100 µM Pi (Fig. 2.3). The concentration of P in leaves of aspen colonized by *L. bicolor*
was unaffected by P limitation (Fig. 2.3). Leaf Pi concentration did not change with DAT \( (P = 0.378) \).

### 2.3.3. Biochemical parameters

Plant tissue biochemical profiles were measured in young leaves, mature leaves, and roots of aspen at 45 DAT. Pi availability had few significant effects on protein concentrations in aspen mature leaves and roots, except in young leaves where it reduced protein concentrations 16% across all mycorrhizal treatments as Pi became limiting \( (P < 0.020 \text{ for the Pi effect}) \) (Fig. 2.4). Protein concentrations in aspen young leaves and roots colonized by *L. bicolor* and *P. involutus* were 193 and 206% and 297% and 190% greater, respectively, than concentrations in NM plants \( (P < 0.001 \text{ in each case}) \) (Fig. 2.4). Protein concentrations among mature leaves did not exhibit any consistent patterns (Fig. 2.4).

Young and mature leaf starch concentrations declined by 44.7 and 48.4%, respectively, with Pi limitation across mycorrhizal treatment \( (P < 0.001 \text{ for the Pi effect}) \) (Fig. 2.5) but were generally higher in plants colonized by *L. bicolor* and *P. involutus* \( (P < 0.001 \text{ for the mycorrhizal effect in each case}) \) (Fig. 2.5). In roots, starch concentrations were differentially affected by Pi and mycorrhizal treatments, with significant increases occurring in NM plants with Pi limitation whereas concentrations were unchanged in plants colonized by mycorrhizal fungi \( (P < 0.001 \text{ for the Pi \times mycorrhizal interaction}) \).

Soluble sugar concentrations, sucrose, fructose, and glucose, in young and mature leaves exhibited similar patterns in response to Pi limitation and mycorrhizal colonization (Fig. 2.5). For example, concentrations of glucose in young leaves declined by 42.7, 8.2, and 35% in NM plants and plants colonized by *L. bicolor* and *P. involutus*, respectively, at 5 \( \mu \text{M} \) compared to 100 \( \mu \text{M} \) Pi (Fig. 2.5). However, young leaves of plants colonized by *P. involutus* contained 39.8 and 20.7% more glucose than NM plants or plants colonized by *L. bicolor* \( (P = 0.002 \text{ for the Pi \times mycorrhizal interaction}) \) (Fig. 2.5). Across all leaf soluble carbohydrates, plants colonized by *L. bicolor* tended to respond more similarly to NM plants than to plants colonized by *P. involutus* (Fig. 2.5). In aspen roots, the concentrations of glucose and fructose were significantly higher in
NM plants compared to mycorrhizal plants and increased significantly in NM plants under Pi limitation ($P < 0.001$ for the Pi × mycorrhizal interaction in each case) (Fig. 2.5). Concentrations were unaffected by Pi limitation in plants colonized by *L. bicolor* or *P. involutus*, although concentrations of glucose and fructose were elevated by in aspen roots colonized by *L. bicolor* compared to *P. involutus* (Fig. 2.5). In contrast, root sucrose concentrations were greatest in roots colonized by *P. involutus* and increased in all mycorrhizal treatments under Pi limitation ($P < 0.001$ for the Pi × mycorrhizal interaction) (Fig. 2.5).

Tissue condensed tannin and phenol concentrations are considered indicators of stress in many plants. In the case of aspen, accumulation of tannins in young leaves was stimulated by Pi limitation across all mycorrhizal treatments ($P = 0.005$ for the Pi effect), although concentrations in leaves from plants colonized by the two ectomycorrhizal fungi were significantly lower than NM plants ($P < 0.001$ for the mycorrhizal effect) (Fig. 2.6). The accumulation of tannin was 66.9% greater in Pi-starved mature leaves of NM plants compared to NM plants receiving 100 µM Pi; mature leaf tannin concentration was not affected by Pi limitation in plants colonized by *L. bicolor* or *P. involutus* ($P = 0.038$ for the Pi × mycorrhizal interaction). Root tannin concentrations increased in NM plants but not mycorrhizal treatments under Pi limitation ($P < 0.034$ for the Pi × mycorrhizal interaction) (Fig. 2.6). Leaf phenol concentrations were variable and not significantly affected by Pi or mycorrhizal treatments, whereas root phenol concentrations increased slightly yet significantly in NM roots but not mycorrhizal roots ($P < 0.028$ for the Pi × mycorrhizal interaction) (Fig. 2.6).

The accumulation of flavonoid compounds in plant tissues often occurs in response to environmental stress. In aspen, proanthocyanidin production increased by 62.5% due to Pi limitation across all mycorrhizal treatments ($P < 0.014$ for the Pi effect) and accumulation was lower in plants colonized by both symbionts ($P < 0.001$ for the mycorrhizal effect) (Fig. 2.7). Accumulation of proanthocyanidin in mature leaves and roots was also less in mycorrhizal plants ($P < 0.014$ for the mycorrhizal effect). Proanthocyanidin concentrations in roots increased under Pi limitation in NM plants but not mycorrhizal plants ($P < 0.001$ for the Pi × mycorrhizal interaction) (Fig. 2.7). Myrcitin concentrations in young leaves, but not mature leaves, increased
by 218% across all mycorrhizal treatments under Pi limitation, although these increases were more pronounced in NM plants ($P = 0.015$ for the Pi effect and $P < 0.001$ for the mycorrhizal effect). In roots, myrcitin concentrations in NM plants increased 5-fold under Pi limitation, whereas concentrations in roots colonized by *L. bicolor* or *P. involutus* did not change ($P < 0.0001$ for the Pi × mycorrhizal interaction) (Fig. 2.7). As with proanthocyanidin and myrcitin, catachin in young leaves was significantly affected by Pi and mycorrhizal treatments ($P < 0.001$ for the Pi effect and $P < 0.001$ for the mycorrhizal effect) (Fig. 2.7). Under Pi limitation concentrations of catechin doubled across mycorrhizal treatments, with concentrations being 2-fold greater in NM plants than plants colonized by either mycorrhizal fungus (Fig. 2.7). Catechin concentrations in mature leaves and roots were not influenced by Pi or mycorrhizal treatments. Tissue kaempferol concentrations did not show significant effects due to Pi limitation or mycorrhizal treatment (Fig. 2.7).

Out of four well-characterized glycosides in aspen, three were studied in this experiment, *i.e.*, salicin, tremulacin, and salicortin (Fig. 2.8). Concentrations of all three glycosides in young leaves of aspen were higher in NM plants and remained higher under Pi limitation, whereas concentrations in young leaves of aspen plants colonized by *L. bicolor* and *P. involutus* increased under Pi limitation (Fig. 2.8). In mature leaves and roots, the concentrations of glycosides tended to increase with Pi limitation and to a greater extent in NM plants, although these differences were not significant except for tremulacin (Fig. 2.8).

### 2.3.4. Relationships between photosynthesis and biomass and leaf Pi concentration

Net CO$_2$ uptake rate ($A$) measured at each DAT point was positively correlated with leaf P concentration in NM plants ($R^2 = 0.507$) but not in plants colonized by *L. bicolor* ($R^2 = 0.010$) or *P. involutus* ($R^2 = 0.009$) (Fig. 2.9A). These patterns were established by lower leaf P concentrations in NM plants, especially plants grown at 5 µM Pi where leaf concentrations were below ~1.5 mg P g$^{-1}$ DW (Fig. 2.3). Leaf Pi concentrations in mycorrhizal aspen plants were maintained above 1.5 mg P g$^{-1}$ DW under Pi limitation (Fig. 2.3) and mycorrhizal plants were able to sustain net assimilation rate even under Pi limitation (Fig. 2.9A).
A similar pattern was observed between total biomass and leaf Pi concentration in aspen harvested at 45 DAT (Fig. 2.9B). In NM plants, growth was linearly related to leaf P concentration ($R^2 = 0.938$), whereas these associations were not evident in plants colonized by *L. bicolor* ($R^2 = 0.057$) or *P. involutus* ($R^2 = 0.314$) (Fig. 2.9B). Also evident in Fig. 2.9B is the C cost of the mycorrhizal association. At 100 µM Pi, growth of NM aspen was 14.9 and 86.7% greater than plants colonized by *L. bicolor* and *P. involutus*, respectively. However, the mycorrhizal benefit is pronounced among plants grown at 5 µM Pi, where total biomass of NM plants was 19.8 and 29.2% of plants colonized by *L. bicolor* and *P. involutus*, respectively (Fig. 2.9B).
2.4 Discussion

Environmental factors play important roles in forest ecosystem growth and development (Kirkman 2001; Aronsson 1980). Soil nutrient limitation, especially N and P, limit the productivity of many natural and planted forests (Kimmins 1995). Under such conditions, forest trees depend on nutrient acquisition by symbiotic mycorrhizal fungi to meet their nutritional demands. Species within the genus *Populus* are globally distributed, play important ecological roles in natural and managed forest ecosystems, and are economically important through their production of biomass for fuel and fiber (Cheng & Tuskan 2009). These roles have established *Populus* as a model tree genus for understanding the ecological, physiological, metabolic, and genomic characteristics of a major tree genus to support forest management activities (Jansson & Douglas 2007). The current study contributes to this area by investigating the influence of the mycorrhizal association to the response of *P. tremuloides* under Pi limitation. Trembling aspen is a temperate and boreal tree species that often grows on marginal soils that are nutrient limited. Success of aspen on such marginal soils may be possible due to colonization by symbiotic ectomycorrhizal fungi.

2.4.1. Pi limitation reduces C fixation in aspen

We hypothesized that photosynthesis would be reduced due to Pi limitation and increased in ectomycorrhizal plants in response to stimulated C demand in the root system by the fungal partner. Further, colonization of aspen by ECM symbionts would mitigate the effects of Pi limitation through enhanced Pi acquisition. Phosphorus limitation often reduces the rate of photosynthesis (Qin *et al.* 2010; Jacob & Lawlor 1992; Fredeen *et al.* 1989). Photosynthesis is integral to plant growth and limiting photosynthetic C fixation through nutrient limitation will lower productivity (Binkley *et al.* 2010; Gleeson *et al.* 2010; Lazaro-Zermenoto *et al.* 2011). In the current study, photosynthetic CO$_2$ uptake was reduced in NM aspen plants due to Pi limitation, whereas it remained constant in mycorrhizal aspen (Fig. 2.1A). This maintenance of photosynthetic parameters was related to the maintenance of leaf P concentrations above ~1.5 mg g$^{-1}$ in the mycorrhizal treatments (Fig. 2.3 and Fig. 2.9A). The driver for the observed reductions in CO$_2$ uptake could be either changes in sink strength resulting from Pi limitation or
changes in the reactions of photosynthesis. In *Nicotiana tabacum* during Pi limitation, photosynthesis was regulated by sink strength. As growth declined under Pi limitation, sink strength also declined, which reduced CO₂ fixation (Pieters *et al.* 2001). In the current study, this pattern held for NM plants, although reductions in mycorrhizal treatment biomass were not accompanied by down-regulation of CO₂ fixation (Fig. 2.1A and Fig. 2.9A). These data suggest that the increase sink demand from the fungal partner as well as maintenance of Pi uptake may both contribute to higher A rates observed in plants colonized by either *L. bicolor* and *P. involutus*. Sink stimulation of photosynthesis by mycorrhizal fungi is common and reflects increased C allocation to hyphae, fungal partner respiration, and other fungal metabolic demands (Lekberg *et al.* 2010; Hawkes *et al.* 2008; Kaschuk *et al.* 2009).

Stomatal conductance ($g_s$) may be one of the major constraints to rate of photosynthesis as it controls the CO₂ uptake (Walker 1985). Stomatal conductance is dependent on several internal and environmental factors, including ABA signals (Alfredo *et al.* 2004), water (Castell & Terradas 1994), and nutrient limitation (Horacio *et al.* 2009; Howard & Donovan 2010). In the current study, $g_s$ was reduced in NM aspen, but not mycorrhizal aspen, due to Pi limitation (Fig. 2.1C). This reduction may have contributed to observed reductions in A. Although $g_s$ and CO₂ uptake were limited in NM aspen, $C_i$ remained constant in all mycorrhizal treatments (Fig. 2.1B), suggesting that $C_i$ may be regulated within narrow limits in trembling aspen, reductions in A led to reductions in $g_s$, and that the biochemistry of photosynthesis was affected in NM plants by reduced Pi concentration, subsequently limiting CO₂ assimilation within the leaf (Fig. 2.9A).

Photosynthetic electron flux $J_{\text{max}}$ and Rubisco carboxylation $V_{\text{cmax}}$ usually decline with the nutrient stress (Bown *et al.* 2007). In the current study, $J_{\text{max}}$ and $V_{\text{cmax}}$ of NM plants declined significantly whereas these measures were unaffected by Pi limitation in plants colonized by *L. bicolor* and *P. involutus* (Fig. 2.1E and Fig. 2.1F). Leaf Pi influences RuBP regeneration (Plesnicar *et al.* 1994), and patterns of $V_{\text{cmax}}$ exhibited by aspen were similarly dependent on Pi concentration in leaves (Fig. 2.1F and Fig. 2.3). These findings for aspen suggest that the maintenance of tissue Pi concentration above the critical threshold ~1.5 mg Pi g⁻¹ by colonization by *L. bicolor* and *P. involutus* may help sustain photosynthetic biochemical reactions within the
leaf (Fig. 2.3 and Fig. 2.9A). In *Eucalyptus globulus* seedlings, biochemical reactions of photosynthesis were also highly related with the leaf Pi concentration (Turnbull *et al.* 2007; Rao & Terry 1995).

Chlorophyll florescence is often affected by Pi limitation and implies reduced efficiency of PSII photochemical reactions as leaf P declines (Wu *et al.* 2006; Lima *et al.* 2006; Plesniar *et al.* 1994). In the current study, NM plants and plants colonized *L. bicolor* displayed significant reductions in $F_{v}/F_{m}$ at low Pi, whereas a lesser reduction was observed in aspen colonized by *P. involutus* under low Pi (Table 2.1). Quantum yield ($\Phi$) is a measure of the effective use of light by chloroplast in photosynthesis. Reductions in $\Phi$ reflect decrease in efficiency of light harvesting complex and may lead to energy losses through fluorescence (Scholes *et al.* 2011, Kolber *et al.* 1993). Due to Pi limitation in aspen, $\Phi$ declined in NM plants and, to a lesser extent, in aspen associated with *L. bicolor* (Table 2.1). The effect of Pi limitation on $\Phi$ of aspen colonized by *P. involutus* $\Phi$ was negligible (Table 2.1). A similar pattern was observed for qP, which reflects the utilization of light energy in photosynthesis (Table 2.1), which suggests that photosynthetic machinery was negatively affected by Pi limitation in nonmycorrhizal aspen, to a lesser extent in aspen colonized by *L. bicolor*, and not in aspen colonized by *P. involutus*. Although plants colonized with *L. bicolor* maintained leaf Pi concentration during Pi limitation, $F_{v}/F_{m}$, $\Phi$, and qP still declined and these reductions may reflect differences in other non-measured variables, such as other micro- and macronutrients.

Together, these reductions in CO$_2$ fixation and changes in the biochemistry of photosynthesis led to significant reductions in aspen growth and final biomass that were more pronounced in NM plants compared to plants colonized by *L. bicolor* or *P involutus* (Table 2.2, Fig. 2.2, Fig. 2.9A, and Fig. 2.9B). Mycorrhizal colonization moderated plant growth due to increased Pi uptake under Pi limitation, although the two ECM symbionts influenced host plant C allocation and growth-leaf P concentration relationships differently (Fig. 2.3 and Fig. 2.9B). Overall root-shoot ratio in mycorrhizal plants was higher in aspen colonized by *L. bicolor* than aspen colonized by *P. involutus* and these were greater than for NM plants. Pi limitation typically reduces shoot biomass due to reductions in both the number of leaves that are initiated.
and area of individual leaves (Kirschbaum et al. 1992; Rodríguez et al. 1998; Mason et al. 2000; Chiera et al. 2002), which was noted for aspen in the current study (data not presented). Pi limitation often results in increased C allocation to roots, as plants allocate more C belowground to exploit soil Pi (Doerner 2008; Fang et al. 2009; Plaxton & Tran 2011). This has been demonstrated for *P. deltoides*, where root dry mass allocation (%) increased as P supply is reduced (Lewis et al. 2010; Qifu 2009; Walk 2006).

In addition to the mycorrhizal benefits to carbon fixation and growth under Pi limitation noted above for aspen in the current study, there was evidence of the C cost of the mycorrhizal association in plants grown at 100 µM Pi. While not statistically significant, A was slightly greater in plants colonized by both *L. bicolor* and *P. involutus* (Fig. 2.1A) and total plant mass of NM aspen was 14.9 and 86.7% greater than plants colonized by *L. bicolor* and *P. involutus* when grown at 100 µM Pi (Fig. 2.2). This maintenance of A with lower plant biomass is suggestive of elevated maintenance costs, hyphal growth and respiration, associated with the mycorrhizal symbiosis (Lekberg et al. 2010; Hawkes et al. 2008; Kaschuk et al. 2009).

The influence of mycorrhizal colonization by *L. bicolor* and *P. involutus* on growth of aspen under Pi limitation and specifically C allocation to roots was prominent under Pi limitation in the current study (Fig. 2.2). In Chapter 1, colonization by *L. bicolor* increased the root-shoot ratio to a greater extent under Pi limitation than in NM plants, although those changes were relatively small. Differences in the growth patterns between Chapter 1 and the current study may be a result of greater pot size and different growth media used (Haase et al. 2006; Broschat et al. 2010; Mathers et al. 2007; Jackson et al. 2009).

### 2.4.2. Pi limitation changes tissue chemistry and C allocation in aspen

Plant carbon allocation changes under nutrient stress (Fredeen et al. 1989; Wesintein et al. 1991; Cakmak et al. 1994; Laurence et al. 1994; Yen et al. 2009) and this allocation may be modified during mycorrhizal symbiosis (Plassard et al. 2002; Schlieman et al. 2008; Lekberg et al. 2010). In addition, plants accumulate secondary metabolites under a variety of environmental stresses (Bryant et al. 1983; Lees et al. 1994), including nutrient stress (Kleiner 1998). I hypothesized
that Pi limitation would lead to changes in aspen tissue biochemistry reflecting both changes in source-sink relationships and stresses associated with nutrient deficiency. Specifically, I hypothesized that nonmycorrhizal aspen plants would allocate more carbon to secondary compounds under Pi limitation rather than to the production of plant biomass and that the maintenance of Pi uptake through the ECM associations would reduce these changes in tissue biochemistry.

Young leaf and root protein concentrations were substantially lower in NM aspen plants compared to plants colonized by either ECM symbionts (Fig. 2.4). While a significant body of work has developed assessing transcriptional and translational responses of various plant hosts to ECM colonization (e.g., Johansson et al. 2004), little work on general biochemical responses related to protein levels is available. Burgess et al. (1996) noted that colonization of roots of Eucalyptus grandis by Pisolithus tinctorius led to dramatic reductions in the expression of root proteins. These findings are opposite those for aspen roots and young leaves in the present experiment. It may be that increased levels of soluble proteins in the actively growing shoots and roots in aspen result from increased P acquisition (Fig. 2.3) and subsequent stimulation of protein synthesis in these tissues.

The concentrations of starch and soluble carbohydrates in plant tissues reflect the balance of CO₂ fixation, respiration, and the assimilation of carbon into biomass. Assimilation of CO₂ in leaves is influenced by Pi content in the leaves, as Pi is required for C export from chloroplasts and continued fixation (Walker & Sivak, 1986, Pieters et al. 2001). Under Pi limitation, starch accumulates in chloroplasts and starch may accumulate in other tissues from altered C demand within Pi limited tissues. Starch and soluble carbohydrates may also decline under severe P limitation, as the reactions of photosynthesis are limited (Gonzalez-Meler et al. 2001; Groot et al., 2001). In the current experiment, starch and soluble sugar concentrations declined in young and mature leaves of aspen at 5 µM compared to 100 µM Pi (Fig. 2.5). However, plants colonized by P. involutus exhibited elevated starch and sugar concentrations at both Pi concentrations while plants colonized by L. bicolor exhibited carbohydrate profiles more similar to NM plants. In contrast, starch and soluble carbohydrates increased in NM aspen roots under Pi
limitation, but remained unchanged in roots colonized by *L. bicolor* or *P. involutus* (Fig. 2.5). Glucose and fructose concentrations were substantially greater in NM roots compared to the two ECM treatments, whereas sucrose concentrations were greater in mycorrhizal roots (Fig. 2.5). These patterns of elevated starch and sucrose and lower monosaccharide concentrations in mycorrhizal roots may reflect lower demand (Fig. 2.2) for carbohydrates relative to assimilation (Fig. 2.1) and the preferential use of glucose and fructose by the symbionts (Hughes & Mitchell 1995)

Changes in tissue secondary C compounds have been reported during mycorrhizal colonization and reductions in these may reflect changes in host physiology to facilitate colonization or changes in nutrient acquisition and C assimilation (Blilou et al. 1999; Medina et al. 2003; García-Garrido and Ocampo 2002; Baum et al. 2009). In aspen, the accumulation of total tannins increased under Pi limitation in young and mature leaves of NM plants, while no changes were noted in mycorrhizal aspen (Fig. 2.6). This pattern may reflect changes in host physiology resulting from mycorrhizal colonization or improved Pi acquisition, which functions to reduce stress and maintain C allocation within the plant (Keski-Saari & Julkunen-Tiito 2003). There were no consistent patterns among treatments or tissues in total phenol concentration (Fig. 2.6).

Specific flavonoids in leaves and roots of aspen were altered by Pi limitation and colonization by ECM fungi (Fig. 2.7). Proanthocyanidin and myrcitin were elevated in young leaves and roots of NM aspen and increased under Pi limitation (Fig. 2.7). Catechin in young leaves also increased under Pi limitation. These changes were mitigated by colonization by either ECM symbiont. These changes in C allocation, and the lack of change in mycorrhizal plants, suggests that Pi limitation alters the allocation of fixed C in NM plants and the maintenance of Pi uptake in ECM plants negates these responses. The concentrations of glycosides in aspen and their responses to Pi limitation and mycorrhizal colonization were slightly different from the flavonoids. Young leaves of NM plants exhibited elevated salicin, tremulacin, and, to a lesser extent, salicortin concentrations compared to leaves of mycorrhizal plants, regardless of Pi
availability (Fig. 2.8). Under Pi limitation, concentrations of these compounds increased in mycorrhizal plants, but did not attain levels exhibited in NM plants (Fig. 2.8).

The accumulation of these compounds is in accord with the CNB hypothesis and is supported by recent systems biology approaches (reviewed by Amtmann et al. 2005). Under Pi limitation, NM aspen plants contained higher C based secondary metabolite concentrations and this accumulation was mitigated by colonization by *L. bicolor* or *P. involutus*. This may be a result of greater sink strength in mycorrhizal aspen than NM aspen or due to the lower Pi acquisition by NM aspen that limits sink strength by limiting building blocks necessary for growth. Previous studies have shown that, in *P. tremuloides*, the predictions of CNB theory hold under low nutrient availability (Donaldson et al. 2006).

**2.4.3. Colonization by ECM maintains Pi acquisition and alleviates stress responses in aspen**

To maintain Pi acquisition and P homeostasis in P-limiting environments, plants may change C allocation to roots and form symbiotic associations with mycorrhizal fungal partners belowground to increase the capacity to access Pi from soils. One of the most notable benefits of the mycorrhizal association is increased acquisition of P through greater exploration by fungal hyphae, increased Pi uptake rates, or other systems that function to scavenge P from soils (Smith and Read 2011). We hypothesized that the effects of low Pi availability on aspen would be reduced in plants colonized by *L. bicolor* and *P. involutus* due to enhanced acquisition of Pi from the root zone. Leaf Pi concentrations in aspen were sustained at higher concentrations in mycorrhizal plants under Pi limitation compared to NM plants (Fig. 2.3). However, there were significant differences in the patterns of leaf Pi concentrations indicating that differences exist among fungal partners in maintaining Pi uptake under Pi limitation. Aspen colonized by *L. bicolor* maintained constant leaf Pi concentrations across both Pi treatments (Fig. 2.3). In contrast, tissue concentrations in NM plants and plants colonized by *P. involutus* declined with Pi limitation, although concentrations in plants colonized by *P. involutus* were elevated relative to NM plants (Fig. 2.3). Differences among ectomycorrhizal species in Pi acquisition have been
reported in *Pinus rigidia* and *Pinus sylvestris* that reflected differences in Pi uptake rates under Pi limiting conditions (Cumming 1996; Colpaert et al. 1999).

The maintenance of Pi acquisition by ECM in aspen under stress sustained photosynthetic CO₂ fixation by aspen, which contributed to the maintenance of growth under Pi limitation (Fig. 2.1 and Fig. 2.9). The alleviation of stress responses by *L. bicolor* and *P. involutus*, including the accumulation of a variety of secondary C-containing compounds, indicates that the ECM association plays a critical role in maintaining nutrient homeostasis under Pi limitation in aspen. However, changes in C allocation among root growth, starch, and soluble carbohydrate pools indicate that ECM symbionts do not behave uniformly in acquiring Pi and translating uptake to plant physiological responses. Ectomycorrhizal species-specific changes in host physiology are thus important when addressing plant-mycorrhizal-soil interactions (Cumming 1996; Trocha et al. 2010; Ostonen et al. 2009).
2.5 Conclusions

Phosphorus availability is a major constraint to forest tree productivity in natural ecosystems. To overcome Pi limitation, forest trees may express a variety of physiological acclimation systems that increase P uptake and P use efficiency and also form symbiotic interactions with ectomycorrhizal fungi to increase the acquisition of Pi from soils. In the current study, aspen seedlings colonized by *L. bicolor* and *P. involutus* exhibited increased Pi acquisition under Pi limitation. As predicted by the CNB hypothesis, aspen responded to Pi limitation with reductions in photosynthesis and growth, reductions in carbohydrates in leaves and increases in roots, and increases in secondary metabolite production that reflect altered C allocation in aspen. These changes were significantly less or not evident in aspen colonized with *L. bicolor* and *P. involutus*. The ECM symbiosis, therefore, changes the environmental thresholds where the CNB hypothesis applies for aspen under Pi limitation. Finally, differences in Pi acquisition and C allocation were also evident between aspen colonized with *L. bicolor* and *P. involutus*, indicating that ECM species-specific variation may influence host plant response to Pi limitation.
Figure 2-1. (A) Photosynthesis \( A \) (µmol m\(^{-2}\) s\(^{-1}\)), (B) intercellular CO\(_2\) concentration \( C_i \), (C) stomatal conductance \( g_s \), (D) transpiration \( E \), (E) Light saturated rate of electron transport \( J_{\text{max}} \), and
(F) maximum carboxylation capacity ($V_{cmax}$) of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (n = 3 plants at 3 repeated measurement times, 15, 30, and 45 DAT). Different letters indicate that means are significantly different ($P \leq 0.05$ by Tukey HSD).
Figure 2-2. (A) Shoot dry weight and (B) root dry weight of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (n = 5). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-3. Leaf Pi concentrations of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (*n* = 3). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-4. Protein concentrations of young leaves, mature leaves, and roots of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of least square means (n = 3). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-5. Starch, glucose, fructose, and sucrose concentrations of young leaves, mature leaves, and roots of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT.
Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (n = 5). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-6. Tannin and phenol concentrations of young leaves, mature leaves, and roots of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (n = 5). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-7. Proanthocyanidin, myrcitin, catechin, and kamferol concentrations of young leaves, mature leaves, and roots of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi.
for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of the least square means (n = 3). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-8. Salicin, tremulacin, and salicortin concentration of young leaves, mature leaves, and roots of *P. tremuloides* plants grown under low (5 µM) and high (100 µM) Pi for 45 DAT. Plants were nonmycorrhizal (NM) or colonized with *L. bicolor* (Lb) or *P. involutus* (Pax). Error bars are standard errors of least square means (n = 3). Different letters indicate that means are significantly different (*P* ≤ 0.05 by Tukey HSD).
Figure 2-9. (A) Photosynthesis ($A$) and leaf P measured on ($n = 3$) of 15, 30, and 45 DAT. (B) Total plant biomass and leaf P were measured on ($n = 5$) plants of 45 DAT. Lines are least squares regression for each mycorrhizal treatment.
Table 2-1 Chlorophyll florescence\(^1\) measurements of nonmycorrhizal (NM) and mycorrhizal *P. tremuloides* with *L. bicolor* (Lb) and *P. involutus* (Pax) plants as a function of delivered Pi

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5 µM Pi</th>
<th>100 µM Pi</th>
<th>(P_{\text{Pi*Myc}})^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F_v/F_m)</td>
<td>NM</td>
<td>0.60 ± 0.03b</td>
<td>0.73 ± 0.03a</td>
</tr>
<tr>
<td></td>
<td>Lb</td>
<td>0.62 ± 0.03b</td>
<td>0.75 ± 0.03a</td>
</tr>
<tr>
<td></td>
<td>Pax</td>
<td>0.78 ± 0.03a</td>
<td>0.81 ± 0.03a</td>
</tr>
<tr>
<td>Quantum yield ((\Phi))</td>
<td>NM</td>
<td>0.57 ± 0.02c</td>
<td>0.77 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Lb</td>
<td>0.66 ± 0.02b</td>
<td>0.76 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Pax</td>
<td>0.75 ± 0.02a</td>
<td>0.78 ± 0.02a</td>
</tr>
<tr>
<td>Photochemical quenching (qP)</td>
<td>NM</td>
<td>0.52 ± 0.02c</td>
<td>0.96 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Lb</td>
<td>0.66 ± 0.02b</td>
<td>0.99 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Pax</td>
<td>0.96 ± 0.02a</td>
<td>0.96 ± 0.02a</td>
</tr>
<tr>
<td>Non-photochemical quenching (qN)</td>
<td>NM</td>
<td>0.33 ± 0.08a</td>
<td>0.35 ± 0.08a</td>
</tr>
<tr>
<td></td>
<td>Lb</td>
<td>0.29 ± 0.08a</td>
<td>0.40 ± 0.08a</td>
</tr>
<tr>
<td></td>
<td>Pax</td>
<td>0.32 ± 0.08a</td>
<td>0.33 ± 0.08a</td>
</tr>
</tbody>
</table>

\(^1\) Means and standard errors of the least square means (n = 3)

\(^2\) Probability of the Pi*Mycorrhizal interaction, ns = not significant.
Table 2-2 Growth measurements\(^1\) of nonmycorrhizal (NM) and mycorrhizal *P. tremuloides* with *L. bicolor* (Lb) and *P. involutus* (Pax) plants as a function of delivered Pi.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5 µM Pi</th>
<th>100 µMPi</th>
<th>(P_{\text{Pi*Myc}}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height growth rate (cm week(^{-1}))</td>
<td>NM  4.06 ± 0.05 d</td>
<td>9.19 ± 0.05 a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Lb  6.68 ± 0.53 c</td>
<td>9.23 ± 0.05 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pax  7.00 ± 0.05 bc</td>
<td>8.64 ± 0.05 ab</td>
<td></td>
</tr>
<tr>
<td>Leaf addition rate (leaf number week(^{-1}))</td>
<td>NM  1.96 ± 0.05 d</td>
<td>3.88 ± 0.05 ab</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Lb  3.13 ± 0.5 bc</td>
<td>4.01 ± 0.05 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pax  2.94 ± 0.05 c</td>
<td>3.60 ± 0.05 abc</td>
<td></td>
</tr>
<tr>
<td>Colonization (%)</td>
<td>NM  -</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lb  76.9 ± 1.4 ab</td>
<td>71.9 ± 1.4 b</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Pax  81.5 ± 1.4 a</td>
<td>77.0 ± 1.5 ab</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Means and standard errors of the least square means (n = 5)

\(^2\) Probability of the Pi*Mycorrhizal interaction, ns = not significant
2.6 References


Kleiner, K.W., 1998. Effect of nitrogen availability on the growth and phytochemistry of hybrid poplar and the efficacy of the *Bacillus thuringiensis cry1 A (a) d-endotoxin* on gypsy moth. Canadian journal of forest research, 28(7) 1055-1067.


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3 Differentially expressed protein profiles in *Populus tremuloides* in symbiotic association with the ectomyccorhizal and arbuscular mycorrhizal fungi

3.1 Introduction

Forest ecosystems comprise approximately 30% of terrestrial biomes and are represented by a variety of forest types (Schmitt et al. 2009). Forests play significant roles in modulating biogeochemical cycles and provide extensive ecosystem services and economic benefits to human society (Costanza et al. 1997, Krieger 2001, Patterson and Coelho 2009, Schmitt et al. 2009, Lindner et al. 2010). Primary productivity of terrestrial ecosystems, including forests, is often limited by nutrient availability (St. Clair et al. 2008, Quesnel and Côté 2009, Vitousek et al. 2010), which, in turn, affects the ecological roles and economic benefits provided by trees. As a response to variation and limitation in soil nutrient availability, forest trees, and most other forms of vegetation, have evolved symbiotic associations with soil mycorrhizal fungi (Brundrett 2009). In these symbioses, morphological and physiological changes and specializations of the root system increase access to soil nutrients that are often limited in supply or present in forms or compartments that are unavailable to the host plant (Smith and Read 1997).

The two major types of mycorrhizal association are formed by the ectomycorrhizal (ECM) fungi and the vesicular-arbuscular mycorrhizal (AM) fungi. These two groups play similar functional roles in plant-soil interactions, but have very different patterns of colonization and influences on root structure (Smith and Read 1997). In the ECM association, fungal hyphae grow within the root among host plant root cortical cells without penetrating them. Fungal hyphae form a network throughout the cortex, termed the Hartig net, a hyphal mantle that covers the root surface, and colonization frequently leads to the formation of characteristic “short roots” within the host plant root system (Smith and Read 1997). Extramatrical hyphae may extend from the mantle into the soil and these provide effective access to the soil for nutrient exploration. In the AM association, hyphae penetrate the cell walls of the host plant and form arbuscules, clusters of finely divided hyphae enveloped by host cell membrane, in host plant root cortical cells. As with the ECM, AM hyphae extend from the surface of the root and explore the soil for
nutrient acquisition (Harrison 2005). In these two types of mycorrhizal association, the Hartig net in ECM and arbuscules in AM are the sites where carbon and mineral nutrients are exchanged between the host plant root and mycorrhizal symbionts (Harrison 2005, Nehls et al. 2006).

In the ECM association, common in many forest tree species, and AM association, common in crop plants as well as tree species, both the partners mutually benefit. Mycorrhizal fungi receive fixed carbon from the host tree (Jones and Last 1991) and, in turn, nutrients are transferred to the host plant by the associated fungi (Smith and Read, 2008). The volume of soil exploited by the root system for mineral absorption is increased by the mycorrhizal hyphae that proliferate through the soil from the tree root surface (Schützendübel and Polle 2002, Sikes et al. 2009). This increased soil exploration and the addition of new metabolic systems, such as high affinity nutrient transporters and nutrient scavenging systems, increase the uptake of N, P, Zn, and Cu from the soil solution and from complex sources within the soil matrix (Cumming and Weinstein 1990, Trocha et al. 2010, Ostonen et al. 2009, Farzaneh et al. 2011, Zaefarian et al. 2011).

The two mycorrhizal associations are unique in that ECM root structure, colonized short roots, and AM arbuscules display similar morphological characteristics across hundreds of host species, indicating that these mycorrhizal associations fundamentally impact host root development (Bruns et al. 1995, Horan et al. 1988, Malajczuk et al. 1990, Smith and Read 1997, Tagu et al. 2002). Therefore, there may be common metabolic pathways involved in the mycorrhizal root that are activated in the colonization process. To understand the mechanisms underlying changes in roots resulting from the mycorrhizal association, researchers have used a variety of physiological techniques to compare mycorrhizal and nonmycorrhizal plants (Sikes et al. 2009, Fransson and Johansson 2009, Ramos et al. 2009, Lehto and Zwiazek 2011, Hobbie et al. 2009). Such approaches, however, have certain limitations. Physiological studies are restricted only to the specific parameter being examined and may overlook other critical plant responses to the mycorrhizal symbiosis. More recently, researchers have used genomic approaches to assess metabolic changes in the root-mycorrhizal association (Martin and Nehls...
2009, Wright et al. 2005, Heller et al. 2008, Balaji et al. 2009). However, microarray and similar transcriptome studies provide information of transcripts expressed and may not reflect those plant responses that result when transcripts are translated into proteins (Twyman 2004).

Recently, proteomic tools have become more refined, and analysis of differentially expressed proteins allows researchers to elucidate of plant response to changing environmental and ecological conditions (Chaves et al. 2003, Kieley et al. 2006, Muneer et al. 2012). Unlike physiological assessments, which may be limited in scope by technical capacity, and genomic analyses, which capture changes in gene expression but not translation, protein evaluation provides a global and integrated view of the metabolic status of an organism (Zivy et al. 2000, Muller 2009, Bickel et al. 2009). Proteomic approaches have been successfully used to identify both host and symbiont proteins expressed in the mycorrhizal association (Natera et al. 2000, Bestel-Corre et al. 2002, Rolfe et al. 2003, Liang et al. 2007, Nagendran et al. 2009). For example, mycorrhizal roots of _Medicago truncatula_ with _Glomus mosseae_ showed up/down regulation of numerous proteins, including glutathione-S-transferase, fucosidase, myosin-like protein, serine hydroxymethyl transferase, and cytochrome-c oxidase (Bestel-Corre et al. 2002). In the _Eucalyptus-Pisolithus_ association, mycorrhizal short root development was associated with the production of numerous fungal proteins, which came to dominate the protein profile (Burgess and Dell 1996). Such responses refine the understanding of host response to the mycorrhizal symbiosis.

Poplar species and hybrids are intensively cultivated as renewable sources of biomass because of their rapid growth, extensive genetic variation, and ease of propagation (Dillen et al. 2010). With the recent sequencing of the poplar genome (Tuskan et al. 2006), the use of species within the genus _Populus_ in plant-mycorrhizal interaction studies has the potential to clarify the underlying basis of the role of mycorrhizal associations in the physiology of a woody species. Trees in the genus _Populus_ form associations with both the ECM and AM fungi (Dhillion 1994, Obase et al. 2008). Colonization by AM fungi is considered to occur early in the tree’s lifespan, while colonization by ECM fungi occurs later as trees mature (Smith and Read 1997). This dual pattern may reflect ecological succession and plant access to soil nutrient pools that change as
ecosystems age (Selosse et al. 2006). This pattern of dual colonization presents a unique opportunity to investigate differences in tree function in response to these divergent symbionts.

In order to gain better insight into the biochemical molecular mechanisms affecting roots during symbiotic interactions, the present study used the analysis of proteins that were differentially displayed during mycorrhizal association using two-dimensional electrophoresis (2-DE). Total protein patterns were obtained from *P. tremuloides* Michx. roots that were nonmycorrhizal and roots associated with the ECM fungi *Laccaria bicolor* Marie and *Paxillus involutus* (Batsch) Fr. and the AM fungus *Glomus intraradices* Schenck & Smith. Separation of peptides by LC-ESI MS and peptide sequencing were used in combination with bioinformatics to determine putative identity of many of the individual proteins present. These patterns are discussed in light of the differences in colonization between ECM and AM fungi and potential changes in the trajectory of host root metabolism resulting from colonization.
3.2 Materials and Methods

3.2.1. Fungal Inoculation and Plant Culture

The *Laccaria bicolor* (Marie) fungal strain S238N (Institut National de la Recherche Agronomique, Nancy, France) and *Paxillus involutus* (ATCC 200175 Batsch Fries) were chosen for their ability to form ectomycorrhizas with aspen. The strains were maintained on modified (MMN) agar medium (Molina and Palmer 1982). For inoculum production, cultures were grown aseptically in liquid MMN medium for 3 weeks at 25°C in the dark. Cultures were blended briefly (three 3-s pulses) to produce a fungal slurry inoculum for plant production.

The AM inoculum consisted of *Glomus intraradices* Schenck & Smith (mixed isolates IA509, BU105, and FL737) (INVAM 234-2) (INVAM-International Culture Collection of Vesicular Arbuscular Mycorrhizal Collection, West Virginia University, Morgantown, WV, USA). Fungi were generated on roots of sudangrass in a greenhouse. Root systems and adhering pot soil were chopped, wet-sieved (500 µm and 38 µm mesh sizes) to remove most sudangrass roots, sand, and soil before adding the resulting concentrated inoculum, containing mostly spores collected on the 38 µm sieve, to acid-washed sand. Final concentration of inoculum was one part initial inoculum soil to ten parts acid washed sand.

The experimental plant growth system consisted of 4-cm diameter × 18-cm deep pots (“Cone-tainers™, Stuewe and Sons, Corvallis, OR, USA) containing acid-washed sand (coarse and fine sand mixture ratio 2:1). For establishing ECM seedlings, the fungal slurry was added as a band at ~2 cm depth below the surface of the sand. The AM inoculum-sand mix was added to the surface 10 cm of pots to produce AM seedlings. For NM plants, only MMN liquid medium was added to the sand.

Seeds of *P. tremuloides* (Sheffield’s Seed Co., Lockey, NY, USA) were planted ~0.5 cm deep in pots and were kept moist by watering three-times daily with deionized water. After 7 d, seedlings were thinned to obtain single plants of similar size in each pot for the experiment.
Following selection, the seedlings were watered three-times daily to field capacity (40 ml per pot) with a modified Johnson’s nutrient solution (Naik et al. 2009). Solution pH was adjusted to 5.6 with 0.1 $N$ NaOH. Seedlings were maintained in a climate-controlled greenhouse with supplemental lighting (mixed metal halide sources) providing a 14-h photoperiod and day/night temperatures of 24/19 ± 3°C. Relative humidity fluctuated with temperature and day. Seedlings were grown for 46 days. At harvest, 5 seedlings were combined to form one biological replicate to have sufficient biomass for protein extraction. For each treatment, 3 biological replicates were used for extraction and separation of root proteins as noted below.

### 3.2.2. Protein Extraction

At harvest, plant roots were washed with distilled water and fresh tissues were immediately weighed, frozen in liquid nitrogen, and subsequently stored at -80°C. Samples were crushed to a fine powder using liquid nitrogen in a pre-cooled mortar and pestle and stored at -80°C until extraction. Total protein was extracted from root samples according to Yang et al. (2009), with slight modification of the solubilization buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% C7BzO, 20 mM dithiothreitol (DTT), 5 mM tributylphosphine (TBP), 2% IPG buffer (pH 3-10)).

### 3.2.3. Protein Separation and Analysis

Protein quantification was conducted using the 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA). Proteins were separated by 2-D electrophoresis. In the first dimension, 0.25 mg of protein in solubilization buffer was used to rehydrate the non-linear IPG strips (11 cm, pH 3-10)(Amersham Biosciences) for 12 hr at room temperature according to the manufacturer’s instructions. Isoelectric focusing was carried out on a Bio-Rad IEF system (PROTEAN i12™ IEF system 164-6000) as described by the manufacturer (Bio-Rad, Hercules, CA, USA). The IPG strips were stored at -80°C and then the second dimensional SDS-PAGE was performed using precast gradient acrylamide gels (8-16% Tris-HCl, 8–16% polyacrylamide
gel) (Bio-Rad Criterion Precast Gel #345-0105) at 100 V. The SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250.

The 2-D gel image analysis was performed using Delta2D software (Decodon GmbH, Gerifswald, Germany). Spot intensities were normalized for each gel and a consensus spot pattern was created for which to compare all gels. A total of 1,124 consistently reproducible spots were detected on the gels of NM (control), Lb (aspen roots colonized with \textit{L. bicolor}), Pi (aspen roots colonized with \textit{P. involutus}) and Gi (aspen roots colonized with \textit{G. intraradices}). Spots having at least a 2-fold expression difference (P < 0.001 by Student’s t-test) between at least one of the mycorrhizal and NM treatments were selected for further analysis. Differentially-expressed protein spots were excised from the gels. Protein digestion with trypsin was performed according to Powell et al. (2005). After extraction from the gel, peptides were separated using reverse phase nanospray capillary LC columns that were coupled directly to an LTQ-FT mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA). Capillary columns were fabricated in-house; each consisted of 75 µm inner diameter packed with approximately 8 cm of C18 silica beads (5 µm bead diameter and 200 Å pores) (Bruker-Michrom Inc., Auburn, CA). The composition of liquid chromatography buffer (LCB) was 0.1% acetic acid, 70% acetonitrile, 0.5% heptafluorobutyric acid. The percentage of LCB against 100% acetonitrile throughout the gradient was: 0% for 1 min, 0-15% from 1 to 3 min, 15-62% from 3 to 22 min, 62-100% from 22 to 25 min, 100% from 25 to 28 min, 100 to 0% from 28 to 29 min, 0% from 29 min to 35 min. MS acquisition settings included full scan mode with the FT mass analyzer followed by 3 data-dependent CID scans of the 3 most intense ions that occurred in the LTQ ion trap and spray voltages of 2.5-3 kV using the Sequest searching algorithm (Thermo Fisher Scientific, Inc. Waltham, MA). CID mass spectra were searched against the \textit{Populus} database (downloaded January 2011 from the Joint Genome Institute) and a contaminants database (The common Repository of Adventitious Proteins from The Global Proteome Machine downloaded January 2011). A decoy database search was used, reverse of the \textit{Populus} and contaminants database, to determine that the false discovery rate for the database searching was 0%. All spectra were further searched against \textit{L. bicolor} and \textit{P. involutus} databases (Joint Genome Institute, downloaded January 2012). The fungi contributed negligibly to the protein content of spots as
only one *Paxillus involutus* protein was detected from all analyzed spots in the *Paxillus involutus* treatment, and two *Laccaria bicolor* proteins were detected from all spots in the *Laccaria bicolor* treatment. An acceptable Sequest peptide had a minimum Cn score of 0.1. Tryptic peptides with a +1 charge state were further considered if they had a cross-correlation (Xcorr) of at least 1.5 for charge state +1, 2 for charge state of +2, and 2.5 for charge state of +3. Only proteins with at least two sequenced peptides were further considered. In cases where multiple proteins were identified per gel spot, the protein abundance index (PAI) was determined for each protein. For a given protein, PAI is the ratio of the number of detected tryptic peptides and the number of observable tryptic peptides (Rappsilber et al., 2002). The protein with the greatest PAI was chosen as the representative for a given gel spot.
3.2.4. Data Analysis

The experiment was established as a completely random design, with four mycorrhizal treatments (NM, *L. bicolor*, *P. involutus*, and *G. intraradices*) with 15 replicate plants per treatment. Biomass was measured on each plant. Root tissue from five plants was combined for protein extractions to give three biological replicates per mycorrhizal treatment for proteomic analyses. Growth data were analyzed using one-way analysis of variance using JMP software (SAS Institute, Cary, North Carolina, USA). Delta2D software version 4.0 (Decodon) was used to identify differentially expressed proteins as noted above.

Poplar gene identifications were annotated to the corresponding *Arabidopsis* gene identifications and gene functions using BLAST searches and the *Arabidopsis* database. The false discovery rate was 0% and was determined by searching all CID spectra against a decoy database consisting of the *Populus* database in reverse. All proteins identified in the current study were of plant origin and are presented in Appendix 1.
3.3 Results

3.3.1. Growth

*Populus tremuloides* colonized by the mycorrhizal fungi *L. bicolor*, *P. involutus*, and *G. intraradices* displayed significantly greater biomass than NM seedlings (Fig. 3.1). For the ECM fungi, shoot and root mass of plants colonized by *L. bicolor* were 27 and 44% greater than NM plants, whereas shoots and roots were 83 and 68% greater in plants colonized by *P. involutus* (Fig. 3.1). In plants colonized by the AM fungus *G. intraradices*, shoot biomass was 54% greater and root biomass 69% greater than NM aspen (Fig. 3.1). Colonization by *L. bicolor* and *G. intraradices* led to greater allocation of biomass to roots, as measured by root-shoot ratios, compared to NM plants, whereas plants colonized by *P. involutus* allocated the least to roots relative to shoots (Fig. 3.1).

3.3.2. Effects of Mycorrhization of Roots on Protein Profiles

Across all mycorrhizal treatments, 1,124 protein spots were matched that were present in all mycorrhizal treatments. A comparison of global protein expression based on mean volumes elucidated broad differences in protein profiles between NM plants and plants colonized by either ECM or AM fungi. A heat map was generated using the protein expression data and a cluster analysis was used to compare NM and the three mycorrhizal treatments (Fig. 3.2). Based on the expression levels, AM formed a distinct branch from NM and both of the ECM fungi. Patterns of protein expression in roots colonized by *P. involutus* next separated NM and *L. bicolor* colonized roots. More broadly, aspen colonized by *P. involutus* and *G. intraradices* exhibited unique patterns of up/down-regulated proteins compared to NM plants, whereas plants colonized by *L. bicolor* exhibited patterns of protein expression more aligned with NM plants (Fig. 3.2).

Proteins identified were involved in a variety of physiological processes, such as carbon metabolism, energy production, cell metabolism, rescue and defense, and protein synthesis.
(Appendix 1). Of the identified proteins, those exhibiting at least 2-fold up- or down-regulation relative to the NM controls were grouped to physiological function by mycorrhizal fungus to assess symbiont-specific changes in protein expression. Patterns of up-regulated proteins (Fig. 3.3) broadly agreed with the global expression profile (Fig. 3.2). Roots colonized by *P. involutus* and *G. intraradices* exhibited a high number of up-regulation proteins compared to NM roots of aspen (Fig. 3.3). In contrast, roots colonized by *L. bicolor* had relatively few distinctly up-regulated proteins compared to NM roots (Fig. 3.3). Patterns of down-regulated proteins were also in agreement with the global profile (Fig. 3.4). Plants colonized by *L. bicolor* exhibited the fewest differences compared to NM roots, those colonized by *P. involutus* were intermediate, and those colonized by *G. intraradices* exhibited the greatest differences (Fig. 3.4).

Expression of proteins involved in carbon metabolism, such as cinnamyl-alcohol dehydrogenase, caffeoyl-CoA-methyltransferase, and tryptophan synthase, were up-regulated, whereas expression of carbonate dehydratase, acetyl-CoA C-acetyltransferase, and chalcone isomerase were down-regulated significantly due to symbiotic association with both ECM and AM fungi. Glycine dehydrogenase was up-regulated due to the *P. involutus* symbiotic association, but had no change due to the *L. bicolor* and *G. intraradices* symbiotic associations. Similarly, 3-methylcrotonly-CoA carboxylase was up-regulated in the *L. bicolor* treatment, down-regulated in *G. intraradices* treatment, and exhibited no change due to *P. involutus*. Other proteins involved in carbon metabolism, such as ubiquitin ligase, were up-regulated in aspen roots due to *P. involutus* and *G. intraradices*, but had no change due to the *L. bicolor* symbiotic association. Overall, the percentages of up/down-regulated proteins and the specific proteins involved in carbon metabolism that were differentially expressed were distinct among all mycorrhizal treatments (Fig. 3.3 and Fig. 3.4).

Proteins involved in energy production were more up-regulated than down-regulated in plants colonized by all three symbionts, although fungal specific patterns were evident (Fig. 3.3 and Fig. 3.4). Up-regulated proteins included 2, 3-bisphosphoglycerate-independent phosphoglycerate mutase, citrate hydrolase, malate dehydrogenase, and fructose-bisphosphate aldolase. In contrast, tropinone reductase/dehydrogenase, ATP-synthase, and triose phosphate
isomerase were down-regulated during colonization by *L. bicolor*, *P. involutus*, and *G. intraradices*. Some proteins involved in energy production, such as pyruvate dehydrogenase, were up-regulated in aspen due to *P. involutus* and *G. intraradices* colonization, but did not change due to the *L. bicolor* symbiotic association. In contrast, malate dehydrogenase was highly up-regulated in *L. bicolor* in comparison to *P. involutus* and *G. intraradices* treatments.

Proportionally, more proteins involved in metabolism (assimilation, nutrition, and water relations) were up-regulated by mycorrhizal colonization compared to other physiological groups (Fig. 3.3 and Fig. 3.4). Uniformly up-regulated proteins included methionine adenosyltransferase, nitrite reductase, and glutamine synthase in mycorrhizal roots. Down-regulated metabolic proteins included UDP-xylose, vacuolar ATP-synthase subunit E1, and 2-oxoacid dehydrogenase family protein.

Between 4 and 24 proteins related to stress and defense were up-regulated in response to the ECM and AM symbioses, with a greater number of stress-related proteins were affected by colonization by *P. involutus* and *G. intraradices* than by *L. bicolor* (Fig. 3.3). DREPP plasma membrane polypeptide family protein and heat shock proteins were highly expressed in *P. tremuloides* roots due to mycorrhizal colonization with *L. bicolor*, *P. involutus*, and *G. intraradices*. The reactive oxygen scavenging enzyme L-ascorbate peroxidase was up-regulated by colonization with *P. involutus* and *G. intraradices*, whereas catalase was down-regulated in aspen roots due *L. bicolor* and *G. intraradices* colonization and was unchanged due to the *P. involutus* symbiotic association.

The greatest extent of change in protein expression was in the areas of energy production and the TCA cycle (31% of the identified proteins) (Fig. 3.5). Among these proteins, biotin carboxylase (5-fold), fructose-bisphosphate (5-fold), and glyceraldehydes-3-phosphate dehydrogenase (2-fold) were notably up-regulated due to mycorrhizal colonization of aspen by *L. bicolor*. Pyruvate dehydrogenase (5-fold), aldehyde dehydrogenase (5-fold), and aconitate hydratase (2-fold) were up-regulated due to mycorrhizal colonized by *P. involutus*. Malate dehydrogenase (more than 5-fold), cinnamyl-alcohol dehydrogenase (more than 5-fold), and NADH-ubiquinone oxidoreductase (5-fold) proteins were up-regulated due to mycorrhizal
colonization of aspen by \textit{G. intraradices}. Down-regulated proteins in this group were aminomethyltransferase in \textit{L. bicolor}, ATP synthase in \textit{P. involutus}, and caffeoyl-CoA-3-methyltransferase in \textit{G. intraradices} (Fig. 3.5).

Stress related proteins commonly up-regulated due all three mycorrhizal fungi were proline rich-family protein, oxidoreductase, chitinase, and low expression of osmotically responsive protein (Fig. 3.6). Peroxidase was unchanged in aspen due to mycorrhizal colonization with \textit{L. bicolor}, whereas it was highly up-regulated by colonization with \textit{P. involutus} and \textit{G. intraradices} (Fig. 3.6).
3.4 Discussion

*Populus* has become a model woody plant system for investigating the ecological, physiological, and molecular basis of traits such as growth rate, wood production, and abiotic and biotic stress resistance (Tuskan et al. 2006). These traits and others are influenced by the formation of symbioses between the host plant and soil mycorrhizal fungi. *Populus* forms mycorrhizal association with ECM fungi as well as AM fungi in nature (Dhillion 1994, Obase et al. 2008), and these associations are known to play significant roles in the growth, development, and physiology of woody plants (Molina 1994, Dell 2002, Siddiqui and Pichtel 2008). The current work investigated protein identification and expression changes in aspen roots in association with the ECM fungi *L. bicolor* and *P. involutus* and the AM fungus *G. intraradices*.

3.4.1. Changes in proteins associated with plant nutrition

Mycorrhizal fungi may alter a wide variety of host plant physiological processes, including mineral uptake (Clark and Zeto 2000, Chalot et al. 2006, Alberton and Kuyper 2009) and water relation (Lehto et al. 2011). In previous work (Chapter 2), the association of the ECM fungi *L. bicolor* and *P. involutus* with *P. tremuloides* seedlings increased leaf tissue P concentrations by 43% and 83%, respectively. Such changes in Pi acquisition may result from or be involved in altered protein expression in the roots of host plants. Mycorrhizal fungi also change micronutrient uptake, such as Cu or Zn (Clark and Zeto 2000, Alberton and Kuyper 2009, Helmisaari et al. 2009), and these modifications in host plant nutrition due to mycorrhizal colonization may have induced some of the changes in protein expression observed in aspen.

In the current study, few proteins were identified that are involved directly in nutrient uptake or assimilation, which may reflect the limitations of the extraction method that most likely identifies the most abundantly expressed proteins and may not effectively extract the low levels of plasma membrane transporters in roots (Rockstroh et al. 2011). Other metabolic changes observed as changes in protein expression may reflect differences in the assimilation of nutrients related to different rates of uptake or preferential nutrient source uptake by fungi and transfer to host roots. For example, proteins participating in nitrogen assimilation, such as
glutamine synthase and glutamate-ammonia ligase, were up-regulated in the current study in mycorrhizal aspen roots. Similar patterns were found for *Zea mays* colonized by *Glomus fasciculatum* (Cliquet and Stewart 1993) and *Fagus sylvatica* with *Lactarius* sp. and *Russula* sp. (Martin et al. 1986).

### 3.4.2. Changes in proteins associated with carbon processing

It has been estimated that 10-20% of plant photoassimilates are transferred from the host plant to mycorrhizal fungal symbionts (Rygiewicz and Andersen 1994, Hogberg et al. 2001), which may lead to increases in photosynthesis during mycorrhizal colonization (Kaschuk 2009). In addition, this increased sink strength induced by mycorrhizal colonization may alter C allocation in the host plant root with concomitant changes in protein expression. The root cortical cell is the point where both symbiotic partners are in close proximity and is the active site of nutrient and C exchange between partners (Harrison 2005, Kalliokoski et al. 2010).

Proteins corresponding to C metabolism were up-regulated in mycorrhizal aspen roots colonized with *L. bicolor*, *P. involutus*, and *G. intraradices*. The most commonly expressed proteins in aspen mycorrhizal roots colonized by *L. bicolor*, *P. involutus*, and *G. intraradices* were malate dehydrogenase, 2,3-bisphosphoglycerate, fructose-bisphosphate aldolase, and pyruvate dehydrogenase. In addition, isocitrate dehydrogenase/NADP$^+$ was highly expressed in *L. bicolor* and succinate dehydrogenase aconitase, pyruvate decarboxylase, and citrate hydrolase were additionally highly expressed in *P. involutus*. Malate dehydrogenase, NADH-cytochrome reductase, and NADH-ubiquinone oxidoreductase were commonly expressed in the *G. intraradices* association. The common up-regulation of enolase, acetyl-CoA C-acetyltransferase, NADH-isocitrate dehydrogenase, peptidyl-prolyl cis-trans isomerase, and cyclophilin (CYP2) across the three symbionts may reflect increased C demand and exchange in the mycorrhizal association and is in agreement with the results of other studies on mycorrhizal associations (Duplessis et al. 2005, Tagu et al. 1996, Nehls et al. 2001).

In the current study, proteins involved in carbon metabolism, energy production, and the TCA cycle were shown to accumulate, which is in agreement with many gene expression studies.
(Tagu et al. 1996, Carneiro et al. 1996, Kim et al. 1998, Nehls et al. 1998, Nehls et al. 2001, Wright et al. 2000, Sundaram et al. 2001, Johansson et al. 2004, Duplessis et al. 2005). For example, gene expression studies on *Eucalyptus globulus* with *Pisolithus microcarpus* during mycorrhizal developmental stages demonstrated consistent up-regulation of specific classes of functional genes, including those involved in the TCA cycle and glycolysis, reflecting the ongoing C demand of the symbiosis (Duplessis et al. 2005). In the current study, similar proteins were up-regulated in *P. tremuloides* due to mycorrhizal colonization with *L. bicolor*, *P. involutus*, and *G. intraradices*. These findings suggest that the fungal partner stimulates the accumulation of proteins involved in carbon metabolism or the TCA cycle to satisfy C demand of the symbiosis or other C-consuming processes, such as exudation, functioning in the root.

### 3.4.3. Changes in proteins due to stress and defense

Mycorrhizal colonization reduces some environmental stresses, for example low phosphate availability (Chapters 1 and 2), or may conversely prime host plant metabolism to cope with environmental stress (Garcia-Garido and Ocampo 2002). Such changes the expression of stress related genes and enzymes would be expected to alter plant-environment interactions (Hohnjec et al. 2005). Conversely, the host root-mycorrhizal association may induce host plant stress systems reflecting the quasi-pathogenic nature of the colonization process (Ruiz-Lozano et al. 1996, Niemi et al. 2007, Seddas et al. 2008). Accumulation of proteins associated with cell defense is well documented in the AM symbiosis (Harrison and Dixon 1993). Recently, similar patterns have been observed for the ECM association, which is in accordance with the results of the present study (Appendix 1). For example, studies on *Betula pendula* associated with different isolates of *P. involutus* and *Hebeloma cylindrosporum* found accumulation of proteins related to cell defense, including pathogenesis-related protein (PR-proteins) and phenylalanine-ammonia-lyase (Feugey et al. 1999).

In the current study, colonization of *P. tremuloides* with ECM and AM fungi generally increased expression of stress-related proteins. Up-regulated proteins included 20S proteasome
beta subunit, DNA repair protein, heat shock proteins 70S and 60S, low expression of osmotically-responsive protein, oxidoreductase, peptidyl-prolyl cis-trans isomerase, and DREPP plasma membrane polypeptide family proteins (Fig. 3.6). Similarly, colonization of *Medicago truncatula* by *Glomus mosseae* increased the expression of SOD (Bestel-Corre et al. 2002) and roots of *Nothofagus dombeyi* colonized by *Descolea antarctica* or *Pisolithus tinctorius* exhibited elevated activities of catalase, ascorbate peroxidase, and glutathione reductase (Alvarez et al. 2009), indicating that the mycorrhizal association induces a wide range of plant stress systems (Garcia-Garido and Ocampo 2002). Such broad changes in the expression of stress resistance-related proteins would be expected to have implications for host plant responses to environmental stress. It is interesting to note, however, that aspen colonized by *L. bicolor* exhibited much less responsive activities of antioxidant systems (Chapter 1). In this latter case, increased Pi acquisition by ECM roots obviated the metabolic perturbations induced by environmental P limitation and subverted the expression of ROS scavenging enzymes.
3.5 Conclusion

In nature, a single tree is colonized with different ectomycorrhizae and, in some cases, by vesicular arbuscular mycorrhizae as well (Chen et al. 2000). These associations may also vary with variable environmental conditions (Mason et al. 1983, Jasper et al. 1991, Andrade et al. 1997, Wallander 2000, Landeweert et al. 2001). Such variation may reflect the ability of different fungi to help the plant host to adapt to variable environmental conditions. Ectomycorrhizal fungi and arbuscular mycorrhizal fungi increase in mineral and water uptake and increase host resistance to abiotic and biotic stresses (Balestrini and Lanfranco 2006, Gianinazzi-Pearson et al. 2007, Krajinski and Frenzel 2007, Requena et al. 2007, Séjalon-Delmas et al. 2007, Schaeffer et al. 1995). In the current study, functional characterization of mycorrhizal aspen proteins was achieved via proteomics analysis to better understand differences in the physiological processes underlying the interaction between aspen and the ECM species *L. bicolor* and *P. involutus* and the AM species *G. intraradices*.

There were significant differences in protein profiles from roots colonized by the ECM fungi and the AM fungus in comparison to NM roots as well as between the two ECM symbionts. The greatest differences in protein profiles existed between NM roots and roots colonized by *G. intraradices* (Fig. 3.2). Of the two ECM symbionts, protein expression profiles of roots colonized by *L. bicolor* more closely aligned to NM roots than roots colonized by *P. involutus*. These patterns may reflect fungal-specific ecological niche differentiation: the establishment of aspen on mineral soils may be aided first by AM fungi and early successional ECM fungi, such as *P. involutus*. In contrast, *L. bicolor* is a later successional species and these differences may translate to fungal-specific changes in root physiology associated with resource availability in early versus late successional soils (Cox et al. 2010). Conversely, these differences may reflect more complex temporal and spatial variation in resource utilization by different mycorrhizal symbionts (van der Heijden et al. 1999).
Proteins involved in carbon processing, metabolism, and cell defense were both up-regulated and down-regulated due to the mycorrhizal associations, and specific changes in protein expression in these functional categories differed between the mycorrhizal treatments (Fig. 3.2 and Fig. 3.3). Such differences may reflect underlying differences in niche separation noted above related to differential nutrient uptake capacities/preferences and specific differences in C demand between the different mycorrhizal symbionts. In the case of stress related systems, differences between ECM and AM fungi and may also correspond to the differences in their processes of colonization by ascomycetes and basidiomycetes. Such broad changes in protein expression induced by *L. bicolor*, *P. involutus*, and *G. intraradices* reflect the significant changes in host physiology brought about by mycorrhizal colonization and such changes induced by mycorrhizae would be expected to modulate host plant physiology and interaction with the environment (Morgan et al. 2005), as noted in earlier chapters.
Figure 3-1. A, Shoot and B, root biomass (g FW) of nonmycorrhizal (NM) *P. tremuloides* plants and plants colonized with *L. bicolor* (Lb), *P. involutus* (Pi) and *G. intraradices* (Gi) after 46 days.
Figure 3-2. Hierarchical clustering of changes in protein expression during the symbiotic association in aspen. Protein expression profiles were determined from non-inoculated (NM) *P. tremuloides* roots and roots in symbiotic association with *L. bicolor* (Lb), *P. involutus* (Pi), and *G. intraradices* (Gi) and after 46 days. The color code for relative expression levels is shown on the side.
Figure 3-3. Distribution of up-regulated proteins in *P. tremuloides* roots colonized by two ectomycorrhizal fungi (*L. bicolor* and *P. involutus*) and one arbuscular mycorrhizal fungi (*G. intraradices*) compared to non-mycorrhizal plants.
Figure 3-4. Distribution of down-regulated proteins in *P. tremuloides* roots colonized by two ectomycorrhizal fungi (*L. bicolor* and *P. involutus*) and one arbuscular mycorrhizal fungi *G. intraradices* compared to non-mycorrhizal plants.
Figure 3-5. Quantitative response of selected proteins involved in carbon and energy metabolism in *P. tremuloides* roots due to colonization with two ectomycorrhizal fungi (*L. bicolor* (Lb) & *P. involutus* (Pi)) and one arbuscular fungi (*G. intraradices* (Gi)) after 46 days. Aspen growth without mycorrhizal colonization is the nonmycorrhizal treatment (NM).
Figure 3-6. Quantitative response of selected stress proteins in *P. tremuloides* roots due to colonization with two ectomycorrhizal fungi (*L. bicolor* (Lb) & *P. involutus* (Pi)) and one arbuscular fungi (*G. intraradices* (Gi)) after 46 days. Aspen grown without mycorrhizal colonization is the nonmycorrhizal treatment (NM).
3.6 References:


4 General Conclusion and Evaluation of Hypotheses

Phosphorus (P) is one of the major mineral nutrients required by forest trees that, is limited in availability in forest soils due to a suite of biogeochemical factors. This limitation in natural ecosystems may limit forest productivity and the provision of ecosystem services, including wood and fiber production and modulation of the carbon and hydrologic cycles. In response to P limitation, forest trees have developed adaptations that increase P acquisition from soils and increase the efficiency of P utilization within the plant. These adaptations include enhanced rates of inorganic P (Pi) uptake, increased exudation of organic compounds that increase P solubility in soils, and the formation of symbiotic associations with soil mycorrhizal fungi. Mycorrhizal fungi, both ectomycorrhizal (ECM) fungi and arbuscular mycorrhizal (AM) fungi, modify the interface between soil and plant roots and play critical roles in P acquisition.

*Populus* is a model tree system that is being utilized extensively in ecological, physiological, and molecular studies worldwide. Species within this genus are preferred plantation species because of their fast growth rates and clonal production abilities and there is expectation that species within this genus may help offset the increasing worldwide demand for wood, pulp, and biofuels. In nature, many mycorrhizal fungal partners colonize single trees, and species within the genus *Populus* may form associations with both ECM and AM fungi. Mycorrhizal colonization increases mineral, especially Pi, uptake, water acquisition, and increases host resistance to different environmental stress (Andrade et al. 1997, Jasper et al. 1991, Mason et al. 1983, Landeweert et al. 2001, Wallander 2000, Jentschke & Goldbold, 2000, Bellion et al. 2006). Given these important roles, the aim of this dissertation was to understand the physiology of *Populus*, specifically *P. tremuloides*, under Pi limitation and the influence of mycorrhizal colonization on tree responses.
4.1 Evaluation of Chapter 1 Hypotheses

In first chapter, nonmycorrhizal trembling aspen (*P. tremuloides*) seedlings colonized by *Laccaria bicolor* were grown at different levels of Pi concentrations. A suite of physiological measures was assessed and the following general hypothesis was evaluated:

1. ECM aspen would be more resistant to P limitation due to increased Pi acquisition capacity and this difference would be expressed as superior growth under Pi limitation, greater tissue P concentrations, lesser antioxidative enzyme activities, and reduced flux of C to the rhizosphere.

**Confirmed, except for growth responses.** I observed increased Pi uptake in *P. tremuloides* due to colonization by *L. bicolor*. This increase in P nutrition reduced the stress responses that were evidenced in nonmycorrhizal (NM) seedlings under Pi limitation. Significant reductions in physiological stress responses included reduced antioxidative enzyme activity in leaves and roots, lower PEPCase activity associated with processing carbon (C) for the TCA cycle, and a reduction in organic acid exudation and total organic carbon exudation from mycorrhizal root systems compared to NM roots.

Although colonization by *L. bicolor* led to substantially higher tissue P concentrations that those in NM seedlings as Pi availability declined, there was no observed growth benefit to the seedling. The lack of growth differential may reflect the C cost of the ECM association. In this case, the benefit of the ECM association was not realized, although elevated Pi uptake, reduced oxidative stress, and reduced C exudation observed in aspen colonized by *L. bicolor* are clearly beneficial to seedling health. The lack of growth differential may also reflect the short duration of the experiment.

4.2 Evaluation of Chapter 2 Hypotheses

The aim of the second chapter was to understand the functioning of different ECM species colonizing aspen under Pi limitation. In the second chapter, aspen were grown under Pi-sufficient and Pi-limiting conditions and I studied changes in ecophysiological parameters and
tissue biochemistry of NM *P. tremuloides* seedlings and seedlings in symbiotic association with *L. bicolor* and *P. involutus* and the following hypotheses were assessed:

1. Photosynthesis will be reduced due to Pi limitation in NM plants and increased in ECM plants in response to stimulated C demand in the root system by the fungal partners.

*Partially confirmed.* Pi limitation significantly reduced photosynthetic CO$_2$ fixation in NM plants. Photosynthesis of plants colonized by *L. bicolor* or *P. involutus* and grown at 5 μM Pi was comparable to Pi-sufficient seedlings; these rates were not statistically greater than rates of NM seedlings under P-sufficient conditions. These reductions in photosynthesis in NM plants appear to be due to P-limitation effects on the photobiochemistry in aspen. Allocation (growth) of ECM roots was less than NM roots at 100 μM Pi and substantially greater than NM plants at 5 μM Pi, reflecting differences in C allocation priorities in mycorrhizal plants and efficiency of Pi acquisition in the ECM association.

2. Nonmycorrhizal aspen plants will allocate more carbon to secondary compounds under Pi limitation rather than to the production of plant biomass.

*Confirmed.* Under Pi limitation, there were reductions in leaf starch and soluble sugar concentrations in NM leaves and increases in NM roots. At the same time, there were significant increases in the concentrations of flavonoids in young leaves on NM plant. Concentrations of glycosides in young leaves on NM plants were unaffected by Pi limitation, but were consistently elevated in NM compared to ECM plants.

3. Changes in photosynthesis, allocation, and growth would be more pronounced in NM plants than plants colonized by the ECM symbionts *L. bicolor* and *P. involutus* due to enhanced Pi acquisition by these symbionts.

*Confirmed.* In this experiment, plants colonized by *L. bicolor* and *P. involutus* performed better than NM aspen. Perturbations to photosynthesis, growth, and tissue chemistry were nonexistent or greatly reduced by the two ECM symbionts. This effect was evidently due to the maintenance of Pi acquisition under Pi-limiting conditions. However, I also conclude that the increase in Pi
uptake is not the only factor responsible for the performance of plants colonized with ECM during Pi limitation. Pi uptake was greater in mycorrhizal aspen colonized with *L. bicolor*, but aspen colonized with *P. involutus* performed better under P stress for the ecophysiological parameters assessed.

### 4.3 Evaluation of Chapter 3 Hypotheses

In the last chapter, the advanced tools of proteomics were used to analyze protein expression in mycorrhizal roots of aspen colonized with two ECM species, *L. bicolor* and *P. involutus*, and one AM fungus, *G. intraradices*. While no specific hypotheses were developed for this discovery-based research, the following general hypotheses could be evaluated:

1. Proteins involved in energy and C metabolism would be differentially expressed during mycorrhizal colonization, reflecting the fixed C cost of the symbiosis.

   **Confirmed.** Proteins involved in energy production and C metabolism were up-regulated due to mycorrhizal colonization. These may reflect the costs of sustaining the mycorrhizal symbionts as well as potential changes in C processing resulting from the mycorrhizal symbioses.

2. Proteins involved in defense and general metabolism would be differentially expressed, reflecting aspen response to the colonization process by ECM and AM fungi.

   **Confirmed.** There were similarly significant changes in the expression of proteins involved in defense and metabolism. The former may result from the stresses of fungal colonization in the root, even though these are symbiotic associations where each symbiont benefits from the association, and reflect fundamental plant response to pathogen invasion. Changes in metabolism, and the levels of proteins, may result from changes in ion uptake, the induction of specific transporters involved resource exchange in the symbiotic association, and/or the activation of metabolic pathways involved in providing and/or assimilating resources in the symbiotic association.
3. ECM and AM fungi would elicit different responses in aspen, reflecting differences in the colonization and C costs of the two mycorrhizal associations.

Differences in protein profiles could be used to separate ECM from AM fungi. However, the number of differentially expressed proteins in aspen due to mycorrhizal colonization was significantly greater in *P. involutus* and *G. intraradices* than in roots colonized by *L. bicolor*. As noted in Chapter 2, the physiological differences between NM plants and aspen colonized by *L. bicolor* were significantly less than the differences between NM plants and plants colonized by *P. involutus*, especially under Pi limitation. These physiological differences may reflect fundamental changes that were noted in protein expression in Chapter 3.

### 4.4 General Conclusion

*Populus* is a valuable model tree system for assessing the fundamental genetic and physiological adaptation to environmental stress, such as nutrient limitation and biotic interactions. Trees within the genus *Populus*, including aspen, form mycorrhizal associations with both ECM and AM fungi, making this genus amenable to investigating fundamental differences in tree response to these two different symbiotic groups. According to my results, aspen physiology is broadly affected by Pi limitation, leading to low tissue P concentrations, increases in oxidative stress, lower photosynthesis, reductions in carbohydrate production, increases in secondary metabolites, and, ultimately, reductions in growth (Fig. 4.1). Mycorrhizal colonization ameliorates these Pi limitation stress responses in aspen due to the maintenance of Pi acquisition under low Pi environments. Alteration in the host plant biochemistry and physiology also depends on species and type of mycorrhizal symbionts, suggesting that mycorrhizal biodiversity plays a role in plant response to the environment. These results contribute to the base understanding of tree behavior in natural ecosystems where P deficiency is a major worldwide problem and mycorrhizal associations are dominant ecological factors modifying plant-soil interactions.
Figure 4-1. Illustration of general conclusion of plant nutrient limitation affecting plant proteins, physiology and carbon partitioning, which in turn affects the forest ecosystem.
## Appendix-1

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Appendix. 1. Identification of proteins expressed that were > 2-fold up or down regulated in aspen root colonized by *L. bicolor, P. involutus, or G. intraradices*. 