ROLE OF MYCORRHIZAL NETWORKS IN DRY DOUGLAS-FIR FORESTS

by

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Abstract

Mycorrhizal networks (MNs) are fungal hyphae that connect the roots of at least two plants, potentially providing a conduit for interplant resource transfer. Interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco) is an obligate ectomycorrhizal (EM) tree species that has high potential to form MNs with neighboring trees because of its receptivity to a diverse community of EM fungi. This MN potential is expected to be greatest among conspecific trees. In this thesis, I determined the influence of MNs formed by residual Douglas-fir trees on interplant carbon transfer and survival, growth, physiology, and EM status of neighboring naturally regenerated and planted Douglas-fir seedlings. To do this, I used MN-restricting treatments and isotope gas-labeling techniques on sites harvested with variable tree retention to investigate how varying: i) proximity to conspecific trees affects EM colonization and performance of planted seedlings; ii) ‘donor’ tree size affects seedling establishment and carbon or nitrogen transfer, and; iii) soil disturbance stress affects net carbon transfer between established seedlings. Because I used physical barriers (i.e., mesh bags) to control for the presence and characteristics of the MN, I also verified the effectiveness of different-sized mesh pores at reducing hyphal connections between plants in the greenhouse. In my experiments, I found that MN-mediated colonization was not the dominant mechanism responsible for EM colonization of planted seedlings; other sources of inoculum (e.g., spores, sclerotia, hyphal fragments) were more important. I found that mature trees not only competed for resources with seedlings but offered some facilitative effects at intermediate distances within their rooting zones. My key finding was that access to a MN with residual trees benefited seedling survival and that this corresponded with increased carbon and nitrogen transfer to seedlings. In addition, I found that there was consistently a net gain in carbon by one seedling in a MN and this net transfer increased with relative growth rate of the receiver seedling. These results indicate that MNs can facilitate interplant carbon transfer and be important in regeneration dynamics in dry Douglas-fir forests.
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Co-Authorship Statement

Chapter 2 was co-authored with Justine Karst, Drs. Melanie Jones, Suzanne Simard and Daniel Durall. Justine Karst and I are equal contributors, with authorship ranking based on time spent on the laboratory and data analysis work. She and I identified, designed and conducted the research, including data collection, analysis and manuscript preparation. Melanie Jones, Suzanne Simard and Daniel Durall assisted with manuscript revision. Suzanne Simard also assisted with data analysis and Daniel Durall was responsible for the molecular analysis of fungal samples.

Chapter 3 was co-authored with Drs. Suzanne Simard and Daniel Durall. I conducted the research, including data collection, analysis and manuscript preparation. Suzanne Simard, Daniel Durall, and I designed the experiment and assisted with manuscript revision. Daniel Durall facilitated the molecular analyses of the fungal samples.

Chapter 4 was co-authored with Dr. Suzanne Simard. I conducted the research, including data collection, analysis and manuscript preparation. Suzanne Simard and I designed the experiment and assisted with manuscript revision.

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1 INTRODUCTION

1.1 Context

Interior Douglas-fir (Pseudotsuga menziesii var. glauca (Beissn.) Franco) is an obligate ectomycorrhizal (EM) tree species with broad fungal receptivity. Under natural conditions, I expect that Douglas-fir seedlings are colonized with native EM by linking into a mycorrhizal network (MN) with neighboring trees. Poor regeneration of planted Douglas-fir seedlings on logged sites in the Interior Douglas-fir (IDF) biogeoclimatic zone of British Columbia (Newsome et al., 1990; Heineman et al., 2003) may be partly due to poor early EM formation. It may particularly limit conifer regeneration in the very dry and dry IDF subzones, or at the IDF ecotone with the Ponderosa Pine or Bunchgrass zones, where Douglas-fir seedlings are more limited by soil water availability than in wetter forest types. In the dry IDF forests, Douglas-fir seedlings may require linkage into a MN to survive, and their growth and competitiveness with other plants may also benefit from the association. Some MN research has been done in the Interior Cedar Hemlock (ICH) zone, where the carbon benefits of shared mycelia between Douglas-fir and paper birch were examined (Simard et al., 1997a). Simard et al. (1997a) observed a 3-10% net carbon transfer from paper birch to Douglas-fir, with more transfer where receiver Douglas-fir was shaded. Based on this research, it has been suggested that MNs have the potential to affect the distribution of resources within the plant and fungal community, and hence interplant competition (Read, 1997).

Following this work, Robinson & Fitter (1999) have argued that unequivocal evidence for direct movement of carbon via fungal hyphae from one plant to another (i.e., a MN-only pathway) in the field is still lacking. Although their research suggested that most transfer occurred through a MN pathway, Simard et al. (1997a) also found that 18% of total carbon transfer occurred to unlinked arbuscular mycorrhizal (AM) western red cedar (Thuja plicata), suggesting that both direct mycelial and indirect soil pathways were important. Whether greater amounts of carbon are transferred through a MN or soil will affect conservation and management practices, and these and other studies suggest that further investigation of MNs is merited (Selosse et al., 2006, Whitfield, 2007). Robinson & Fitter (1998) suggest that there is a need to conduct more field experiments.
with better controls, such as physical barriers capable of restricting the formation of hyphal links, as well as improved isotope labeling methodology and clear demonstration of the existence of MNs. If strong evidence for the ecological importance of MNs accumulates, then silvicultural strategies that promote the conservation of existing MNs may help achieve sustainable forest management objectives, especially for tree species that are difficult to regenerate in stressful environments.

1.2 Objectives and Hypotheses

The general objectives of this thesis are:

i) To determine whether there is potential for MNs to form among conspecific trees in dry Douglas-fir forests.

ii) To determine the influence of MNs with residual trees on interplant carbon transfer and survival, growth, physiology, and EM status of naturally regenerated and planted Douglas-fir seedlings.

iii) To establish effects of donor size, distance from donor, or soil disturbance on the extent of interplant carbon transfer or seedling performance.

The main objectives and hypotheses of this thesis are:

a) To determine the effectiveness of physical or chemical methods at preventing the formation of ectomycorrhizas on seedlings. I hypothesized that decreasing mesh pore size of physical barriers, or increasing the rate of fungicide exposure, would proportionally decrease the occurrence of hyphal links between seedlings.

b) To determine the effects of MN access and tree proximity to conspecific trees on EM colonization of planted seedlings. I hypothesized that: (i) there is potential for MNs to form, (ii) MN-mediated colonization is the dominant mechanism promoting the continuity of EM communities from trees to seedlings, and (iii) there is a critical distance at which the EM community of planted seedlings is most similar to trees.
c) To determine the effects of MN access and tree proximity on seedling performance. I hypothesized that seedling performance would increase with increasing access to the MN and proximity to mature trees resulting from greater facilitation by MNs than competition for resources.

d) To determine the effects of MN access and ‘donor’ tree size on seedling establishment and carbon or nitrogen transferred. I hypothesized that seedling survival, growth, and physiological responses, as well as carbon and nitrogen transfer, would increase with greater access to a MN. Secondly, I hypothesized that the extent of carbon transfer would increase with ‘donor’ tree size.

e) To determine whether net carbon transfer occurs between seedlings, MNs are the dominant transfer pathway, and transfer is enhanced by stress imposed by soil disturbance. I hypothesized that net carbon transfer occurs between conspecific seedlings along a source-sink gradient, and that net transfer increases with access to a MN or soil disturbance.

1.3 Literature Review

1.3.1 Facilitation

Competition is important, but only one of several ecological interactions regulating plant establishment and community structure, dynamics, productivity, and diversity (Bruno et al., 2003). Facilitation among plants is also considered of primary importance (Callaway, 1995). While Frederic Clements’ super-organism theory of plant communities fostered intense research on competition in most of the 20th century (Tilman, 1988), some plant ecologists were also observing that positive interactions may be important and a general phenomena. Phillips (1909) was one of the first to publish a report suggesting facilitation was a determinant of community structure and diversity. He found that seedlings of pinyon pine (Pinus edulis) occurred mostly under the shade of older trees, not in the open. Since then, many studies, mostly in dry ecosystems, have documented spatial patterns that are structured by positive interactions (Bertness & Callaway, 1994; Callaway, 1995; Callaway et al., 2002; Bruno et al., 2003).
Facilitation among plants can occur through various mechanisms. A large plant can shade and protect seedlings from extremes in temperature and light intensity, which can reduce water loss and photoinhibition during stomatal closure (Callaway, 1995). Many soil scientists have also measured higher levels of nutrients in soils directly beneath the canopy of trees. Through the pumping of water and nutrients from deep in the mineral soil, trees may enrich the soil via hydraulic redistribution and litterfall, thereby increasing availability of water and limiting nutrients to seedlings and shallow-rooted plants. Other biotic mechanisms, such as substrate modification, increased protection from herbivores (plant defense guilds), increased pollination, and sharing of beneficial soil bacteria and mycorrhizas, may also result in facilitation among plants. As the long-running debate about controls over structure, dynamics, and diversity in plant communities continues, there is a need to improve our understanding about mutualistic relationships, which could be as influential on plant communities as antagonistic relationships (Bruno et al., 2003).

1.3.2 Mycorrhizas

Mycorrhizas are ubiquitous symbioses that form between plant roots and fungi. Mycorrhizal fungi increase plant uptake of soil nutrients (nitrogen and phosphorus) and water (Smith & Read, 1997) and in return, the host plant provides carbon to the fungal partner. After several decades of research, mycorrhizas are commonly referred to as the main nutrient absorbing organ of the host plant. Most mycorrhizal research has attributed plant growth responses to nutrient relations, but there is increasing evidence for other mechanisms as well. For instance mycorrhizal fungi can protect host plant roots against soil pathogens (Kropp & Langlois, 1990; Morin et al., 1999) or heavy metal toxicity (Perry et al., 1987; Jones et al., 1987). Some species of mycorrhizal fungi can improve soil aggregate stability (Wright & Upadhyaya, 1998) or increase weathering of soil minerals (Jongmans et al., 1997; Van Breemen et al., 2000).

There are seven classes of mycorrhizal fungi, of which the two most ubiquitous are ectomycorrhizal fungi (EMF) or arbuscular mycorrhizal fungi (AMF) (Peterson et al., 2004). Mycorrhizal fungi that form: i) a mantle enveloping the root, ii) a Hartig net (hyphae around the root epidermal and cortical cells), and iii) extraradical hyphae or
rhizomorphs, are EMF. Ectomycorrhizal fungi are mutualistic partners with the majority of North American commercial tree species (Molina & Trappe, 1982). The majority of EMF are Basidiomycetes and Ascomycetes, and are mostly found in boreal and temperate forest ecosystems (Allen, 1991) where low availability of nitrogen is characteristic (Fisher & Binkley, 2000).

Establishment of conifer tree seedlings on certain reforestation sites depends on EM development to capture scarce site resources (Perry et al., 1987). Ectomycorrhizal fungi can aid seedlings in overcoming nutrient and moisture limitations, and can decrease transplant shock (Marx, 1991), especially on degraded sites. Inoculation trials have not consistently improved survival and growth of commercial stock (Jones et al., 2003), and contributing factors include poor colonization and persistence of inoculated fungal species as well as high native inoculum potential of reforestation sites (Teste et al., 2004). Unless sites have very low inoculum potential, management efforts are best focused on conserving the native EM community for satisfactory seedling colonization and performance.

1.3.3 Mycorrhizal networks

Tree seedlings with broad fungal receptivity (i.e., high number and diversity of EMF species) can easily associate with several different EMF species simultaneously. The number and diversity of EMF typically increases with host size and age (Jones et al., 2003). To some degree, EMF species show little host specificity, and they can therefore associate with multiple hosts (Molina et al., 1992). Trees of the same or different species can be compatible with the same species of EMF and be connected to one another by a network of hyphae (Newman, 1988). Mycorrhizal networks (MN) linking plants can function as conduits for resource (carbon, nutrients and water) sharing and serve as fungal inoculum sources for new seedlings (Newman, 1988; Selosse et al., 2006).

Björkman (1960) was the first to provide evidence for carbon transfer via mycorrhizal links. He injected $^{14}$C-glucose into stems of spruce and pine, and later detected it in nearby achlorophyllous Monotropa hypopitys. The possibility that carbon could be transferred between EM plants via a MN was first investigated by Reid & Woods (1969), but they were unable to provide conclusive evidence for $^{14}$C transfer.
between pines via *Thelephora terrestris*. In the 1980’s, the first studies clearly demonstrating carbon transfer between plants via a MN were published. Much of the research on carbon transfer was done by D.J. Read and co-workers (Francis & Read, 1984; Read *et al*., 1985; Finlay & Read, 1986; Grime *et al*., 1987) in the laboratory using seedlings grown in microcosms, $^{14}$C and autoradiography.

By the 1990’s, there was evidence that carbon could be transferred belowground between two plants via a MN and that a net carbon gain could be measured (Simard *et al*., 1997a), with potentially far-reaching ecological implications (e.g., increased coexistence and biodiversity) for plant communities (Read, 1997; Whitfield, 2007). The evidence that MNs played an important role in interplant carbon transfer, however, was criticized (Robinson & Fitter, 1998; Fitter *et al*., 1999; Fitter & Robinson, 2000; Fitter, 2001). Much of the controversy has centered around MNs formed by arbuscular mycorrhizal (AM) plants, where transferred carbon appears to remain in the fungal tissue (Pfeffer *et al*., 2004). One exception was a study by Lerat *et al*. (2002), who clearly showed that carbon was transferred from AM trout lily (*Erythronium americanum*) to AM sugar maple (*Acer saccharum*) seedlings in the spring, and in the reverse direction in the fall, suggesting that the direction of transfer was governed by source-sink relationships shifting with plant phenology. In both seasons, the transferred carbon was found in both receiver roots and shoots, which the authors suggested was available for receiver plant growth. In contrast to AM systems, studies with EM plants have consistently shown that MNs facilitate interplant carbon transfer (Reid & Woods, 1969; Brownlee *et al*., 1983; Read *et al*., 1985; Finlay & Read, 1986; McKendrick *et al*., 2000). The strongest evidence exists for mycoheterotrophic plants, which have been shown to receive significant amounts of carbon from surrounding trees through MNs formed by EMF (Bidartondo, 2005; Selosse *et al*., 2006). For MNs formed between chlorophyllous EM plants, studies have shown that unlabeled plants (receiver plants) receiving carbon isotopes from labeled plants (donor plants) usually had lower concentrations of carbon isotope in shoots compared to roots (Read *et al*., 1985; Simard *et al*., 1997a,b) or no carbon isotope in the shoots at all (Wu *et al*., 2001). Whether the receiver plant directly benefits from carbon transfer still remains unclear, but some authors have argued that
even transferred carbon that remains in fungal tissue can serve as a subsidy to the receiver plant’s EM system (Perry, 1998; Perry, 1999; Simard & Durall, 2004).

Source-sink relationships that form between donor and receiver plants appear to regulate the magnitude and direction of interplant carbon transfer (Simard et al., 2002, Lerat et al., 2002). For example, shading one of two plants in a network can result in an interplant source-sink carbon gradient by altering relative photosynthetic rates, and has resulted in carbon transfer from illuminated to shaded plants (Finlay & Read, 1986; Simard et al., 1997a). Here, the shaded plant is the ‘receiver’ plant (i.e., carbon sink) and the plant in full sun is the ‘donor’ plant (i.e., carbon source). Most MN-transfer studies have focused on altering sink strength by shading a plant whereas few studies have altered source strength. One exception was a study by Fitter et al. (1998), who altered source strength by elevating CO₂ levels of donor plants and increasing available carbon in their roots. However, this had no effect on the growth of the donor plant or carbon transfer to receiver plants.

In the EM symbiosis, carbon typically moves from plant to fungus in carbohydrates via membrane transport processes (Smith & Read, 1997). The mechanism for the opposite movement of carbon in MNs, that is from fungus to plant, is unknown (Fitter & Robinson, 2000), but certainly exists in mycoheterotrophic plants (Smith & Read, 1997). Since amino acids or low-weight nitrogenous compounds can move from fungal to plant tissue (Näsholm et al., 1998), it is conceivable that MN-transferred carbon is converted into nitrogenous compounds before moving into ‘receiver’ plants (Simard & Durall, 2004). Even so, it is still possible that fungal sugars or hyphal collapse at the fungal-plant interface are two other pathways for carbon transfer from fungal to plant tissue as proposed for mycoheterotrophic plants (Bidartondo, 2005).

In addition to carbon transfer, other direct and indirect effects of the MN on receiver seedlings have been examined. For example, interplant nitrogen and phosphorus transfer has been demonstrated in laboratory and field experiments (Simard et al., 2002; Selosse et al., 2006, and references therein). Intraspecific water transfer has also been measured in the laboratory (Brownlee et al., 1983) and field (Querejeta et al., 2003; Egerton-Warburton et al., 2007). Other inferred MN effects include enhanced seedling establishment (Horton et al., 1999), increased mycorrhization (Simard et al., 1997b), and
increased seedling survival (Onguene & Kuyper, 2002; McGuire, 2007) and growth (McKendrick et al., 2000; Booth, 2004). Also, since MNs appear to facilitate transfer of carbon, nutrients, and water between trees, seedlings capable of connecting to a MN may have a competitive advantage over seedlings that are not connected (Dickie et al., 2002). Other researchers have also suggested that MNs can benefit the mycorrhizal community by providing continuity through time and space (Perry et al., 1989; Jonsson et al., 1999; Cline et al., 2005). It is noteworthy that such benefits have not always been measured in MNs. Various plant assemblages have been examined for their potential to form a MN (Kennedy et al., 2003; Dickie et al., 2004), but these studies have not determined whether carbon, nutrients, or water transfer occurs.

In summary, up to now, research has provided evidence for the existence of MNs (Finlay & Read, 1986; Wu et al., 2001; Kennedy et al., 2003; Lian et al., 2006), transfer of resources between plants (Simard et al., 1997a; Lerat et al., 2002, Egerton-Warburton et al., 2007), and the effects of MNs on seedling survival or growth (McKendrick et al., 2000; Booth, 2004; McGuire, 2007). While many reviews have suggested the potential important role of MNs in ecosystem functioning (Newman, 1988; Wilkinson, 1998; Simard et al., 2002; Leake et al., 2004; Selosse et al., 2006), no study has yet simultaneously investigated whether MNs can facilitate plant establishment and whether such facilitation occurs through interplant resource transfer in the field.

1.4 Overview of Thesis

In Chapter 2, I re-examined the effectiveness and usefulness of the most frequently used methods of controlling EM colonization and MN formation. To do so, I tested the effectiveness of chemical and physical methods at controlling formation of EM on Douglas-fir seedlings. Specifically, I demonstrated the efficacy of the fungicides, Topas® and Senator®, at various concentrations and application frequencies at reducing EM colonization; I also noted any fungicide effects on community composition. In addition, I tested the ability of nylon mesh of various pore sizes to prevent hyphal penetration of several EM fungi, and in doing so, alter the EM community composition of neighboring seedlings.
In Chapter 3, I determined whether MN-mediated colonization could promote the continuity of EM communities from residual trees to seedlings. I also determined the critical proximity at which the EM community of seedlings was most similar to that of residual trees, and whether seedlings and residual trees shared the same EM taxa and genet. For this study, I constructed mesh bags using similar pore sizes tested in Chapter 2 to restrict access to the MN.

In Chapter 4, I examined how competitive and facilitative interactions between residual trees and seedlings varied with seedling-tree proximity and access to a MN. The research was conducted on the same field sites using the same mesh bags as in Chapter 3.

In Chapter 5, I determined whether access to a MN or ‘donor’ tree size affected seedling survival, growth, and carbon or nitrogen transfer in the field using advance regeneration trees of different sizes. I employ the same mesh bags used in Chapters 3 and 4 and custom-built large air-tight gas-labeling bags for carbon isotope pulse-labeling.

In Chapter 6, I determine whether net carbon transfer occurred between Douglas-fir seedlings, and whether it was influenced by access to a MN or soil disturbance.

Finally, in Chapter 7, I bring the thesis to closure with general conclusions and the strengths and weaknesses of my research. I also propose alternative approaches to the research methods and suggest future research needs.
1.5 References


2 METHODS TO CONTROL ECTOMYCORRHIZAL COLONIZATION: EFFECTIVENESS OF CHEMICAL AND PHYSICAL BARRIERS

2.1 Introduction

In mycorrhizal research, evaluation of mycorrhizal effects on plant performance often requires comparisons between mycorrhizal and non-mycorrhizal plants. Creating effective, yet feasible methods to control mycorrhizal colonization in the field has become of utmost importance as there has been a recent demand to increase the ecological relevance of mycorrhizal research (Read, 2002). This requires moving away from laboratory-based work to experiments conducted in natural environments.

Currently, most studies have obtained non-mycorrhizal plants by employing one of three methods: substrate sterilization (via autoclaving, steam sterilization or gamma irradiation), the creation of mutant plants unable to form mycorrhizas, or the use of fungicides applied to soil around plant roots. Sterilizing soil can result in substantial changes in its chemical and physical properties (Lenis et al., 1991; Chambers & Attiwill, 1994; Sheremata et al., 1997; Shaw et al., 1999); moreover, its application in the field is futile because contamination is certain. The development of defective plants that lack the ability to form mycorrhizas has been limited to a few plant species associating with arbuscular mycorrhizal fungi (AMF) (Marsh & Schultze, 2001). More research is also required to determine whether the functioning of mutants is otherwise identical to non-mutant plants (Kahiluoto et al., 2000). Of the fungicides, benomyl has been effectively used to reduce arbuscular mycorrhizal colonization of plants in the field by as much as 80% (Hartnett & Wilson, 1999; Wilson et al., 2001; Callaway et al., 2004; Dhillion & Gardsjord, 2004). Benomyl, no longer licensed for use in some countries and relatively ineffective against basidiomycetes, is however, not an option to control ectomycorrhizal (EM) fungi. Fungicides have generally not been employed in EM systems (but see Page-Dumroese et al., 1996; Manninen et al., 1998).

1 A version of this chapter has been published as: Teste FP, Karst J, Jones MD, Simard SW, Durall DM. 2006. Methods to control ectomycorrhizal colonization: effectiveness of chemical and physical barriers. Mycorrhiza 17: 51-65 (including 6 figures) and has been reproduced here with kind permission of Springer Science+Business Media. All articles published in Mycorrhiza are protected by copyright, which covers the exclusive rights to reproduce and distribute the article (e.g., as offprints), as well as all translation rights. No material published in Mycorrhiza may be reproduced photographically or stored on microfilm, in electronic data bases, video disks, etc., without first obtaining written permission from the publisher.
Ectomycorrhizal fungal communities are more taxonomically diverse than AMF communities, thus requiring a broad spectrum fungicide to adequately decrease EM colonization. Of the three methods currently employed to control mycorrhization, the use of fungicides appears the most feasible for field research in EM systems.

Two fungicides, Topas® and Senator®, have been suggested by greenhouse managers for control of EM hyphal growth. Propiconazole, the active ingredient in Topas® (25% a.i.), interferes with ergosterol biosynthesis, which is critical to the formation of fungal cell membranes (Kendrick, 2000). The lack of normal sterol production slows or stops the growth of the fungus, effectively preventing further infection and/or invasion of host tissues (Kendrick, 2000). Propiconazole incorporated into agar media at 1 ppm or higher inhibited growth of many EM fungal strains (Zambonelli & Iotti, 2001; Laatikainen & Heinonen-Tanski, 2002). Colonization of *Pinus sylvestris* roots by EM fungi decreased by approximately 20%, with some morphotypes affected more than others, when propiconazole was applied for two consecutive years in the field at a rate of 250g l⁻¹ every two weeks (Manninen *et al*., 1998). Thiophanate-methyl, the active ingredient in Senator® (70% a.i.), interferes with the functioning of microtubules, so that treated cells cannot divide. Thiophanate-methyl targets the cells of ascomycetes (Kendrick, 2000), but to our knowledge has not been used to control EM fungi.

Studies of mycorrhizal networks (MNs) in plant communities form a unique subset of studies on mycorrhizal effectiveness (Simard & Durall, 2004). They require comparisons between plants that are linked with those that are not linked by a MN (Simard *et al*., 1997; Booth, 2004). In these studies, control plants may be mycorrhizal, but hyphal linkages between plants must be absent. While non-mycorrhizal or non-linked controls are easily established in the laboratory using substrate sterilization techniques, this is more problematic in the field where seedlings are grown in native soils. Mesh barriers constructed of either steel or nylon have been used to prevent formation of ectomycorrhizal connections between plants (e.g., Francis & Read, 1984; Schüepp *et al*., 1992; Booth, 2004; Kranabetter, 2005), or provide root-free compartments where mycorrhizal hyphae can explore and grow. To allow movement of soil solution while restricting penetration of roots and hyphae, mesh with pores 1 μm or
smaller has been used (Robinson & Fitter, 1999; Johnson et al., 2001; Zabinski et al., 2002; Cardoso et al., 2004), even though it is unknown whether it prevents formation of mycorrhizal connections between plants. Furthermore, given that hyphal width varies (from 1.5 to 9 μm), a mesh with pore sizes larger than 1 μm may restrict penetration of some mycorrhizal fungal species but not others. Consequently, the mesh pore size could alter the EM fungal community composition. Ectomycorrhizal fungi vary in their ability to absorb and transport nutrients and water (Simard & Durall, 2004); therefore, any alteration of the community may affect transport within the MN.

The objective of this study was to examine the effectiveness of chemical and physical methods at controlling formation of ectomycorrhizas on Douglas-fir seedlings. We tested the effectiveness of the fungicides, Topas® and Senator®, at various concentrations and application frequencies. We predicted that both fungicides would reduce EM colonization, however, we expected that colonization of ascomycete fungi would be particularly reduced with the application of Senator®. Thus, the composition of the EM fungal community would be altered compared to untreated controls. In addition, we tested the effectiveness of nylon mesh with various pore sizes at preventing hyphal penetration, and its effects on EM community composition of neighboring seedlings. We predicted that percent colonization and similarity of EM communities between seedlings on opposite sides of the mesh barrier would decrease with decreasing mesh pore size.

2.2 Materials and methods

2.2.1 Field soil collection

On August 27-28 of 2003, we collected 600 l of soil from the Black Pines variable retention cut (also known as a green-tree retention cut where some trees are not harvested) and adjacent forest approximately 50 km northwest of Kamloops, British Columbia (120°26’W, 50°42’N). The Black Pines variable retention cut occurs in the dry cool subzone of the Interior Douglas-fir (IDFdk) biogeoclimatic zone (Meidinger & Pojar, 1991). It has an elevation of 1180 m asl and loamy Gray Luvisolic soil (Krizic et al., 2004). The plant community is dominated by residual Douglas-fir (Pseudotsuga menziesii var. glauca (Beissn.) Franco) and subalpine fir (Abies lasiocarpa (Hook.) Nutt.)
trees and advanced regeneration (saplings), with shrub and herbaceous layers dominated by soopolallie (*Sherpherdia canadensis* (L.) Nutt.) and pinegrass (*Calamagrostis rubescens* Buckley), respectively.

We collected forest floor (30 cm x 30 cm) together with mineral soil (to 40 cm depth) from 15 random locations in 1 ha of the Black Pines forest. This soil was used for both experiments. The fifteen samples were combined and thoroughly mixed, then stored at room temperature until needed (see below).

2.2.2 Plant material

Interior Douglas-fir seedlings (seedlot #48520, British Columbia Ministry of Forest Tree Seed Center, Surrey, British Columbia, Canada) were grown at the University of British Columbia (Vancouver, Canada) greenhouse (temperature minimum 20 °C, temperature maximum 24 °C, humidity average 60 %). Seeds were moist-stratified at 4°C for 21 days. Seeds were then sterilized in constantly mixed 3% H₂O₂ for two hours. Styroblock™ 512B trays (Beaver Plastics Ltd., Edmonton, Alberta, Canada) were cut in half horizontally and filled with autoclaved peat and sawdust (3:1, v:v). Three seeds were sown in each cavity and 4 weeks later were thinned to one seedling per cavity. The trays were placed under a mist tent for 12 days and then moved to a greenhouse bench for the remaining time. To improve seedling vigor and discourage mycorrhizal colonization, we applied 1.9 g l⁻¹ water soluble Rose Plant Food (Miracle-Gro, Scotts Canada Ltd., Mississauga, Ontario, Canada) (18:24:16 N:P:K) once per week for 4 weeks following germination. Afterwards, we fertilized with 4 ml l⁻¹ Peter’s solution (Plant-Prod®, Plant Products Co. Ltd., Brampton, Ontario, Canada) (20:20:20 N:P:K) once per week until the seedlings were transplanted into the treatment pots. For the duration of the two concurrent experiments (five months), natural daylight in the greenhouse was supplemented by 400 W high pressure sodium lamps to maintain an 18 hour photoperiod.

2.2.3 Fungicide experiment

*Experimental design and treatments*

On September 16, 2003, 14-week-old seedlings were transplanted into 3.2 l pots (175 mm x 180 mm) (Listo Products Ltd., Surrey, British Columbia, Canada) with
drainage holes. The pots contained field soil mixed with perlite (3:1, v:v). A 3x3x3 factorial set of treatments with a separate control group was replicated 10 times in a completely randomized design, where the factors were fungicide type, rate of application, and frequency of application (270 seedlings + 10 controls = 280 total). The three fungicide types were Senator®, Topas®, and a combination of the two fungicides (both from Engage Agro Corporation, Guelph, Ontario, Canada). The three rates of application were: 0.5, 1 or 1.5 ml l⁻¹ of Senator®; and 0.5, 1 or 1.5 g l⁻¹ of Topas®. Recommended concentrations of Senator® and Topas® are 0.5 ml l⁻¹ and 0.5 g l⁻¹, respectively. To our knowledge this is the only study assessing the effect of these fungicides on EM fungi thus, we decided as a starting point to use the above rates. The fungicide was mixed with water and added at a constant volume of 600 ml pot⁻¹; therefore, seedlings that were treated with Senator® and Topas® in combination received 300 ml of each fungicide-water mixture. The three frequencies of application were: once at the beginning of the experiment, every two months (three applications total), or every month (five applications in total). For each fungicide application, we drenched the soil around the seedlings, avoiding contact with foliage. Additionally, ten control seedlings were grown in pots to which only water was applied. On September 30, 2003, initial height was recorded for all seedlings. The seedlings were watered as necessary and their locations re-randomized monthly.

Seedling measurements

On February 10, 2004, the height of all surviving seedlings was measured. Shoots were removed, dried at 65°C for 48 hours and weighed. The roots and intact soil of up to seven replicates were stored at 4°C for 45 days before processing. Each root system was soaked in tap water, rinsed clean of soil, and cut into 1 cm fragments. The sample was then divided approximately in half, and one half was dried and weighed. We used this measurement to estimate dry weight of the remaining roots, which were weighed wet, and then cleared and stained following the methodology of Phillips & Hayman (1970) to assess percent EM colonization. For a given seedling, percent EM colonization was calculated as:

\[
\text{Percent EM Colonization} = \frac{\text{Active EM root tips}}{\text{Active EM root tips} + \text{Active nonEM root tips}} \times 100
\]
A root tip surrounded by a mantle was classified as mycorrhizal.

In addition to assessing percent colonization, we recorded the abundance and richness of EM morphotypes in each of the treatments. Root systems of the remaining three replicates from each of the ten treatments were carefully washed under running tap water and then cut into approximately 1 cm pieces. All root fragments were placed in a baking dish containing water and thoroughly mixed. We randomly subsampled and counted up to 100 EM, or 100 non-EM root tips, whichever came first. Generally, EM tips were turgid and smooth, had emanating hyphae or rhizomorphs (Harvey et al., 1976), and had a Hartig net. A root tip that was dark and wrinkled, or was somewhat hollow and fragmented under minimal pressure was classified as ‘dead’. Gross morphology of EM roots and rhizomorphs were described using a stereomicroscope, while the mantle, cystidia, and emanating hyphae were described using a compound microscope under 400x or 1000x magnification. When possible, mantles were peeled by separating the fungal tissue from the root with forceps and micro-scalpels, and then described. Morphological descriptions were made with reference to Agerer (1985–1998), Ingleby et al. (1990), Goodman et al. (1996), and Hagerman et al. (2001). Morphotyped roots were then dried and weighed.

2.2.4 Mesh barrier experiment

Experimental design and treatments

To test the effect of pore size on penetration by EM fungi, we grew seedlings in 3.2 l pots divided vertically by nylon mesh barriers with different pore sizes. The pore sizes of the four meshes were: 0.2 μm (catalogue number 25007, polyamide type 250 membrane, Sartorius AG, Goettingen, Germany), 1 μm (catalogue number 03-1/1 Nitex, Sefar America Inc., Depew, NY, USA), 20 μm (catalogue number 03-20/14 Nitex), and 500 μm (catalogue number 06-500/47 Nitex). Control pots were divided by an impermeable acetate sheet to test for EM contamination through insufficient sterilization, or water and airborne EM propagules. Each of the five barrier treatments was replicated 12 times in a completely randomized design. The pots were first sterilized in a 20% bleach solution for at least one hour, cut in half vertically, and then reassembled using non-toxic adhesive silicone sealant (catalogue number 3145-Grey-RTV; mil-A-46146,
Dow Corning Midland, MI, USA) to attach the mesh and hold the two halves of the pot together. Each pot had two compartments. On August 30, 2003 one compartment was filled with field soil mixed with perlite (3:1, v:v), watered, and planted with 14-week-old seedlings (see Plant Material for growth conditions). Three weeks after the seedlings were transplanted into the unsterilized soil, the second compartments were filled with sterilized field soil. Uncolonized 17-week-old seedlings were then transplanted into the sterilized soil and watered. The purpose of transplanting seedlings into the unsterilized field soil 3 weeks prior to the introduction of seedlings into the other half of the pot was to insure that the seedlings were already colonized by EM fungi when the experiment was started. We refer to the initially transplanted seedlings as “source seedlings”. If hyphae from the source seedlings were able to penetrate a mesh of a given pore size, we expected to see mycorrhizal root tips on “recipient” seedlings grown in sterilized field soil (Fig. 1).

Once all source and recipient seedlings had been transplanted into the pots, the seedlings were watered as necessary. Just prior to transplanting, we destructively subsampled fifteen source seedlings to quantify EM colonization following the methodology of Phillips and Hayman (1970). Afterwards, pot location on the greenhouse bench was re-randomized monthly. Initial shoot height was measured shortly after transplanting, on September 30, 2003.

Seedling measurements

At harvest, January 11, 2004, shoot height and biomass (dried at 65°C for 48 hours) were measured. During the harvest, we also inspected mesh barriers for signs of hyphal penetration using a stereomicroscope. We chose to randomly select ten replicates per mesh barrier treatment for morphotyping using similar methods outlined above (5 treatments x 2 seedlings per pot x 10 replicates = 100 seedlings). Three replicate sets of one root tip per morphotype from different seedlings were lyophilized prior to storage for subsequent molecular analysis. On average, 3% of the total root tips per morphotype examined were sent for molecular analysis. The remainder of the morphotyped roots were dried and weighed with the remainder of the root sample.
2.2.5 Molecular confirmation of EM fungal species identification

Total genomic DNA was extracted from single EM tips by pulverizing them for 45 seconds at a speed of 5.0 units using a Bio101 Systems Fast Prep FP120 high frequency shaker (Q-biogene, Carlsbad, CA, USA). DNA was isolated using the procedure of Baldwin & Egger (1996). The final DNA pellet was dried using a speed vacuum concentrator and then re-suspended in 50 μl EDTA-TE buffer.

Following DNA extraction and isolation, the internal transcribed spacer (ITS) region of the fungal nuclear rDNA was specifically amplified by the primers NS11 and NLC2 (Martin & Rygiewicz, 2005). PCR reactions typically included 1 μL template DNA, 18.6 μL sterile purified water (Barnested Nanopure Diamond water purifier), 0.2 mM deoxyribonucleotides (dNTPs), 2.5 μl 10x PCR buffer, 1.5 mM MgCl2, 0.48 mM each primer, 1.6 mg ml⁻¹ bovine serum albumin (BSA), and 0.25 U μl⁻¹ AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA). Samples were amplified using a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA). A 10 min hot start was followed by PCR cycling as follows: 45 s at 94°C followed by 34 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, ramping 72°C for 1 min with a 1s extension after each cycle, and extension at 72°C for 10 min, and then the temperature was held at 4°C. The PCR products were visualized on 1.5% agarose gels using a Gel Logics 440 (Kodak Instruments, Rochester, NY, USA). The PCR product was cleaned using the QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, USA). Prior to sequencing, the large ITS fragment produced above, was re-amplified in a nested PCR reaction using the primers ITS 1 and ITS 4 (White et al., 1990). PCR products were quantified and then sequenced using a 3730 DNA Capillary Sequencer (Applied Biosystems) at the University of British Columbia Nucleic Acid and Protein Services Unit. All unique morphotypes were sequenced and then aligned using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). Taxonomic matches were based on BLAST results with >98% sequence similarity.

2.2.6 Statistical analysis

The fungicide experiment examined a 3x3x3 factorial set with a separate control group of treatments (i.e., separate from the factorial but combined in the layout) in a
completely randomized design (Bergerud, 1989). We used percent colonization data obtained from the cleared and stained roots and normalized the data with a square root transformation for analysis of variance (ANOVA). We analyzed EM community data (richness and diversity, relative abundance of morphotypes with >5% of EM root tips), seedling growth, and square root of percent colonization, first by using the GLM procedure in SAS (SAS Institute Inc., 1999). We then ran a second GLM procedure with a contrast statement to compare the control treatment against all other treatment combinations. Analyses on data collected from cleared and stained roots and morphotyped root tips were done separately, and consequently graphed separately. ANOVA tables were constructed manually to obtain the proper experimental error terms and degrees of freedom. When significant main treatment effects occurred, we separated means using the Bonferroni multiple comparison test.

For the mesh barrier experiment, the percent colonization and EM richness for both seedlings per pot were used to calculate the Steinhaus index of EM community similarity (Legendre & Legendre, 1998) and to calculate the difference in morphotype richness (integral of the number of morphotypes on the donor root system minus the number on the receiver root system). The effects of mesh pore size on EM community data (richness difference and Steinhaus index of similarity), percent EM colonization and seedling growth (shoot height, biomass and root biomass) were detected with a one-way ANOVA using the GLM procedure in SAS (SAS Institute Inc., 1999). For both percent EM colonization and seedling growth, the difference in the response variable between source and recipient seedlings within a pot was calculated and used in the analysis. Differences were considered significant at $\alpha=0.05$. Where significant mesh barrier treatment effects occurred, we separated means using the Bonferroni multiple comparison test. Effects of sterilization on seedling growth and total percent EM colonization were analyzed using the TTEST procedure for each mesh size (SAS Institute Inc., 1999).

2.3 Results

2.3.1 Fungicide treatments

Approximately 30% of the roots of control seedlings (i.e., seedlings receiving only water) were colonized after 21 weeks in the treatment pots. Application of
fungicide reduced EM colonization by up to 50%, depending on fungicide type ($P<0.01$) but not application concentration ($P=0.85$) (Table 2.1). The most effective treatment regime was Topas® applied alone or in combination with Senator® (Fig. 2.2a). Senator® alone was less effective at decreasing EM colonization, with only a 36% reduction compared with 56% reduction using Topas®. There were no differences associated with different application frequencies (Fig. 2.2b) and there were no significant interactions among any combination of the three treatment factors ($P>0.05$, Table 2.1). None of the fungicides applied at any concentration or application frequency affected seedling height or shoot or root biomass (Table 2.1).

A total of eight morphotypes were identified and described (Table 2.2). Two had ≥ 98% sequence matches of their ITS sequences to *Wilcoxina rehmi* and *Thelephora terrestris* accessions in Genbank. DNA from the other six morphotypes either did not amplify or had less than 98% sequence homology with genotypes in Genbank. One morphotype was not identifiable and was classified as undifferentiated. Only the Rhizopogon/Suillus-type formed rhizomorphs; the remainder had relatively smooth mantles (Table 2.2).

On average we observed more morphotypes on seedlings that were subject to fungicides than those that were not (Fig. 2.3). However, neither EM community richness ($P=0.21$) nor diversity ($P=0.31$) was significantly affected by the fungicides. The relative abundance of *Wilcoxina rehmi* mycorrhizas (the most common ectomycorrhiza) as a percentage of all root tips examined was reduced by Topas® applied alone or in combination with Senator®, when compared to Senator® alone or the control (Fig. 2.4). The abundance of *Cenococcum geophilum*, the other dominant ascomycetous mycorrhiza, was not affected by application of fungicides ($P=0.58$, data not shown). Similarly, the abundances of Rhizopogon/Suillus- and Tomentella-type mycorrhizas, the most abundant basidiomycetes, were also not affected by fungicide treatment ($P=0.66$, $P=0.79$, respectively, data not shown).

### 2.3.2 Mesh barrier treatments

Source seedlings had greater shoot height, shoot biomass, root biomass, and EM colonization than recipient seedlings across all mesh treatments except the 20 µm pore
size (Table 2.3), and mesh size did not affect the magnitude of these differences (Table 2.4). Across all mesh sizes, on average, 50 and 21% of roots of source and recipient seedlings were colonized by EM fungi, respectively. These colonization levels contrast early measurements where colonization of source seedlings was less than 1%.

We found six distinct morphotypes on source seedlings, (Table 2.2). Most of the six morphotypes were represented in all mesh treatments (Fig. 2.5). *Wilcoxina rehmii* ectomycorrhizas comprised > 85% of the community on source and recipient seedlings separated with mesh barriers of 1 µm or larger (> 80%). By contrast, both the 0.2 µm and 1 µm pore-sized meshes blocked the formation of *Rhizopogon/Suillus*-type mycorrhizas on recipient seedlings (Fig. 2.5). This type formed approximately 5% of the mycorrhizas on source seedlings. MRA-type morphotypes were found on all source seedlings, but were absent from recipient seedlings of all mesh treatments. *Thelephora terrestris* ectomycorrhizas formed an increasingly high proportion of the community on recipient seedlings as mesh size decreased, whereas they were not found on source seedlings. The abundance of *Cenococcum geophilum* mycorrhizas was too low to be useful in detecting mesh effects.

Ectomycorrhizal community similarity, which takes into account richness and relative abundance, between recipient seedlings versus source seedlings increased with mesh pore sizes greater than 0.2 µm (*P*<0.01) (Fig. 2.6a). The EM communities separated by the full barrier (control) or by mesh of pore size 0.2 µm were significantly dissimilar from those separated by mesh with pore sizes 1 µm and larger (Fig. 2.6a). The difference in morphotype richness between source and recipient seedlings was large in the full barrier treatment and generally decreased as mesh size increased (*P*=0.09) (Fig. 2.6b). When examined under the microscope, we observed hyphae penetrating pore sizes of 1 µm and larger, and roots penetrating only 500 µm pores. Three of the mesh barriers were torn in pots of the 0.2 µm mesh treatment; these replicates were omitted from the analyses.
2.4 Discussion

2.4.1 Fungicide effects on EM colonization

This study suggests that fungicides can be used to significantly reduce EM colonization in controlled experiments. Topas® was more effective than Senator® at reducing EM colonization levels. The manufacturer’s recommended concentration was effective in reducing colonization, and there was no advantage to applying Topas® repeatedly during the course of the experiment. In our study, EM colonization decreased by as much as 56% compared with the control. Douglas-fir control seedlings in this experiment had relatively low levels of colonization (approximately 30%) but these levels are typical for greenhouse-grown interior Douglas-fir (5-42%) (Hagermann & Durall 2004; Teste et al., 2004). Our results are consistent with another study using propiconazole. Manninen et al. (1998) found that 0.15 g of propiconazole applied to seedlings in the field (versus 9.6 g at the highest application frequency in our study) caused a decrease in EM colonization of almost 33% (from 67 to 45% colonization) two years after 2 year-old nursery grown Pinus sylvestris seedlings were outplanted.

Although the fungicides did not eliminate EM colonization altogether, we propose that Topas® reduces colonization to an extent to be useful for field studies. Similar decreases in arbuscular mycorrhizal colonization following benomyl application have resulted in substantial changes in structure of the plant community. For example, reductions in arbuscular mycorrhizal colonization of 60% have changed plant nitrogen and phosphorus concentrations and aboveground community productivity in boreal grassland communities (Dhillion & Gardsjord, 2004). Hartnett & Wilson (1999) found that a 75% decrease in arbuscular mycorrhizal colonization coincided with biomass decreases of dominant C4 grasses. Callaway et al. (2004) reported that interactions between native grassland species and the invasive Centaurea maculosa were substantially altered when experimental plots were treated with benomyl; the fungicide decreased arbuscular mycorrhizal colonization by >80%, resulting in a C. maculosa biomass decrease when mixed with Koeleria cristata or Festuca idahoensis. Assuming reductions in arbuscular and EM colonization result in similar functional responses in plant communities, we expect that Topas® applied at the recommended rate once every
five months will reduce EM colonization sufficiently to affect seedling performance in
the field.

The specificity of the fungicides for ascomycetes and basidomycetes differed
from that expected. Senator® is reported to be more effective against ascomycetes than
basidiomycetes, and yet it appeared to have no effect on Wilcoxina rehmii, a dominant
ascomycete in this study. Manninen et al. (1998) reported that propiconzaole was also
more effective at inhibiting ascomycete than basidiomycete symbionts and this was
confirmed by Laatikainen & Heinonen-Tanski (2002). The latter found that low
concentrations of propiconazole (0.1 ppm) increased growth of Suillus bovinus and S.
variegatus strains grown in vitro, and that these fungi were tolerant of concentrations up
to 1 ppm. In our study, the effectiveness of propiconazole (Topas®) could not be
predicted strictly by taxonomic status. For example, it caused a substantial reduction in
colonization by Wilcoxina rehmii, but not by Cenococcum geophilum, another important
ascomycete. Colonization by the basidiomycetes forming Thelephora terrestris,
Tomentella-type, and Rhizopogon/Suillus-type mycorrhizas either increased or was not
affected by either fungicide, however. In our study, Topas® targeted the most abundant
EM fungi, Wilcoxina rehmii, so that the additional application of Senator® provided no
further advantage.

Other fungicides have had variable effects on EM colonization. O’Neill &
Mitchell (2000) applied benomyl to Picea sitchensis seedlings and found that
colonization was reduced from 60% to 20%; however, only one morphotype, Wilcoxina
mikolae, was observed on the nursery grown seedlings. In another study, the percent of
roots colonized by Thelephora terrestris or Laccaria laccata decreased when 0.3%
Dithane M-45 was applied to Pinus patula seedlings grown in pouches, and similar
reductions in hyphal dry weight occurred when the fungicide was applied to in vitro
cultures (Reddy & Natarajan, 1995). A wide range of responses was exhibited by 64
strains of EM fungi grown in vitro and exposed to relatively low concentrations (<10
ppm) of five fungicides (benomyl, chorothalonil, copper oxychloride, mane and
propiconazole) (Laatikainen & Heinonen-Tanski, 2002). Conversely, in some other
laboratory studies, fungicides have increased EM colonization (Pawuk et al., 1980; Marx
& Rowan, 1981; de la Bastide & Kendrick, 1990). This effect is likely due to the
selective inhibition of fungi that are competitive towards EM fungi (Summerbell, 1988). In our study, interactions among EM fungi could have resulted in the increase in basidomycetes observed. *Wilcoxina rehmii*, a rapid colonizer of nursery seedlings (Mikola, 1988) was suppressed by the application of Topas®. Removal of this rapid colonizer could have allowed other EM fungi to colonize seedling root tips. Surveys of the entire fungal community on a large number of replicate seedlings are required to investigate this possibility.

Our results suggest that Topas® should be effective at reducing morphotypes commonly found in greenhouse bioassays of field soils, but there are two caveats. First, we could not assess the effects of fungicides on rare EM fungal species or those that do not colonize seedlings in greenhouses. Second, Topas® may affect seedling physiology and/or other soil biota. These impacts are more difficult to identify and quantify by short term experiments in a greenhouse setting. Propiconazole has been shown to have growth-regulator effects on plants in the Solanaceae family (Kendrick, 2000), and it has also been shown to affect soil fauna, such as flagellates (Ekelund *et al.*, 2000), as well as soil respiration (Elmholt, 1992). Topas® is recommended for prevention of a variety of foliar fungal diseases, and its mode of action by preventing ergosterol synthesis makes it likely to also affect non-target saprotrophic and parasitic soil fungi. A change in this community would alter potential food substrates of soil fauna. In experiments where EM reduction is of primary concern, and side-effects on the soil biota is unimportant, then applications of Topas® can be an effective treatment regime. Given that the active ingredient in Topas® is fungistatic, repeated applications may be required where there is high hyphal turnover, as would happen over a temperate growing season, or where there is high fungal propagule pressure; both of these conditions occur in field situations.

2.4.2 Mesh barrier effects on hyphal penetration

Our study indicates that mesh with pore size 0.2 µm is effective at reducing hyphal penetration and mycorrhizal colonization of neighboring seedlings. However we conclude that the threshold for restricting EM hyphal penetration lies between 0.2 and 1 µm. Ectomycorrhizal richness tended to increase in sterilized compartments where mesh size equaled or exceeded 1 µm, suggesting hyphae from the source seedlings
compartment penetrated the mesh and colonized the recipient seedlings growing in the sterilized compartment. Of even greater significance, EM community similarity between source and recipient seedlings greatly increased in meshes \( \geq 1 \mu m \). If the recipient seedlings were mycorrhiza-free, differences in richness alone should have indicated mesh effectiveness at restricting hyphal penetration, regardless of abundance, but the small number of morphotypes may have rendered richness as a measure with little resolving power.

The EM community observed in our study was typical for interior Douglas-fir seedlings inoculated with field soil and grown in the greenhouse (Jones et al., 1997; Simard et al., 1997; Hagerman & Durall, 2004; Teste et al., 2004). The six morphotypes formed on the source seedlings also represented a broad range of mantle types (texture and thickness), width of emanating hyphal forms (width and extension 3 to 7 \( \mu m \)), as well as the presence or absence of rhizomorphs. They allowed us to test the effectiveness of the pore sizes at preventing hyphal penetration by different EM fungi. We might predict, for example, that a mesh with a smaller pore size would be required to prevent penetration of single hyphae, compared to the size required to stop penetration of rhizomorphs. Our findings support this prediction since we found that the rhizomorph-forming *Rhizopogon/Suillus*-type morphotype was restricted by a mesh size between 1 to 20 \( \mu m \). We propose that meshes with pore sizes smaller than 1 \( \mu m \) would be adequate in field situations.

Although mesh with 0.2 \( \mu m \) pores was the most effective at reducing hyphal penetration, it was very fragile. This characteristic of nylon mesh with pore sizes smaller than 1 \( \mu m \) has been noted previously (Tarafdar & Marschner, 1994). Our results suggest that field experiments requiring fine mesh (0.2 \( \mu m \)) should use more durable nylon (i.e. mesh thickness > 115 \( \mu m \)) or metal-based mesh.

Our finding that mesh with pore sizes between 0.2 \( \mu m \) and 1 \( \mu m \) are most effective at inhibiting EM colonization must be interpreted cautiously because some EM were found in sterilized soils with a 0.2 \( \mu m \) mesh barrier. Within the sterilized compartment of these pots, the EM community was reduced but not eliminated. For example, *Wilcoxina rehmii* was on the recipient seedlings, regardless of the mesh barrier type, but was not observed in control pots, suggesting that hyphal penetration or spore
dispersal may have occurred. We are uncertain why *Wilcoxina rehmii* was not found in the sterilized compartment of the control pots. Further research is warranted on *Wilcoxina rehmii* propagating strategies in nurseries (e.g., hyphal and spore) and morphological plasticity. We also found that *Thelephora terrestris* had colonized root tips in one seedling of the control treatment (i.e., sterilized soil with a full barrier), confirming previous studies that it is a common greenhouse contaminant. Statistical analyses were run without *Thelephora terrestris* (data not shown); however, results were similar, and did not change our conclusions about the hyphal restriction properties of the mesh treatments. MRA-type mycorrhizas were also only observed on source seedlings across all mesh treatments, suggesting that chemical changes induced by autoclaving may have inhibited this particular EM fungus. Rhizomorphs were completely excluded from sterilized compartments separated by 1 or 0.2 µm mesh.

The use of mesh barriers versus fungicides for controlling EM colonization depends on the ecological processes that must be maintained and those that can be compromised in the experiment. Future MN research can benefit from the use of mesh barriers. Mesh barriers with a gradient of pore sizes have the potential to tease out carbon and nutrient pathways (soil-only, hyphal-only, rhizomorph-only, etc.) in resource sharing MN studies. However, installing mesh barriers will disrupt soil structure and potentially reduce water flow through small pore sizes. If the purpose of mesh is to exclude mycorrhizal hyphae, and maintain non-mycorrhizal status of the enclosed host, the soil contained in the mesh barrier compartment will require sterilization. Mesh with pore sizes < 1 µm appear to reduce hyphal penetration, however care will be required to exclude fungal propagules arriving via air or water pathways. We suggest that mesh barriers, apart from their disruptive installment, are a more promising method than fungicides to completely exclude fungi.

**Acknowledgements**

We are grateful to Graeme Hope and Shannon Berch for their aid at the early stages of the field work. We thank Peter McAuliffe, Jon Millar, Dave Enns, and Mike Carlson for their valuable insight at the beginning of the greenhouse work. We also thank Candis Staley, Amanda Schoonmaker, and Lenka Kudrna, for assistance with applying the fungicide treatments, morphotyping, and the molecular analysis,
respectively. Tony Kozak and Wendy Bergerud provided useful insights on the data analysis. Funding was provided by a Forest Science Program of Forest Investment Innovation of British Columbia grant to S. Simard, a Fonds Québécois de la Recherche sur la Nature et les Technologies scholarship to J. Karst, Natural Sciences and Engineering Research Council of Canada Discovery Grant to M. Jones, and Canadian Foundation for Innovation grants to S. Simard and D. Durall.
Table 2.1  Analysis of variance for effect of fungicide type (F), concentration (C), and application frequency (A) on square root percent ectomycorrhizal colonization $\sqrt{\text{PEC}}$ and size of Douglas-fir (*Pseudotsuga menziesii* var. *glauc*a) seedlings after 5 months.

| Source of variation          | df | $\sqrt{\text{PEC}}$ MS | $\sqrt{\text{PEC}}$ F | $\sqrt{\text{PEC}}$ P | Height MS | Height F | Height P | Shoot biomass MS | Shoot biomass F | Shoot biomass P | Root biomass MS | Root biomass F | Root biomass P |
|------------------------------|----|-------------------------|------------------------|------------------------|------------|----------|----------|------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Control vs. all others       | 1  | 13.55                   | 9.67                   | <.0001                 | 0.03       | 0.00     | 0.9756   | 0.01             | 0.01           | 0.9038         | 0.03           | 0.32           | 0.5700         |
| Fungicide type               | 2  | 9.70                    | 6.92                   | <.0001                 | 69.40      | 2.06     | 0.1316   | 0.29             | 0.60           | 0.5487         | 0.08           | 1.01           | 0.3670         |
| Concentration                | 2  | 0.23                    | 0.16                   | 0.8500                 | 16.10      | 0.48     | 0.6313   | 0.62             | 1.29           | 0.2786         | 0.04           | 0.48           | 0.6210         |
| Application frequency        | 2  | 22.81                   | 16.28                  | <.0001                 | 46.00      | 1.37     | 0.2587   | 0.74             | 1.55           | 0.2163         | 0.11           | 1.47           | 0.2346         |
| FxC                          | 4  | 1.56                    | 1.11                   | 0.3526                 | 22.90      | 0.68     | 0.6068   | 0.36             | 0.75           | 0.5625         | 0.06           | 0.77           | 0.5455         |
| FxA                          | 4  | 1.99                    | 1.42                   | 0.2304                 | 47.90      | 1.42     | 0.2302   | 0.15             | 0.32           | 0.8660         | 0.05           | 0.68           | 0.6059         |
| CxA                          | 4  | 0.25                    | 0.18                   | 0.9480                 | 8.32       | 0.25     | 0.9111   | 0.73             | 1.52           | 0.1997         | 0.16           | 2.11           | 0.0835         |
| FxCxA                        | 8  | 1.44                    | 1.03                   | 0.4181                 | 17.90      | 0.53     | 0.8306   | 0.43             | 0.90           | 0.5208         | 0.02           | 0.29           | 0.9677         |
| Error                        | 135| 1.40                    |                        |                        | 33.70      | 0.48     | 0.48     | 0.08             |                |                |                |                |                |

*PEC*: percent ectomycorrhizal colonization.
Table 2.2 Description of morphological characteristics of ectomycorrhizas observed on Douglas-fir (*Pseudotsuga menziesii* var. *glauc*a) grown in the fungicide (F) and mesh (M) study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Morphotype and Blast match</th>
<th>Macroscopic description</th>
<th>Mante type(s)</th>
<th>Emanating hyphae</th>
<th>Rhizomorphs</th>
<th>Cystidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>F and M</td>
<td><em>Rhzopogon/ Suillus</em>-type (R/S)</td>
<td>Unbranched to subterete silvery white mycorrhiza with rough texture</td>
<td>Outer: felt prosenchyma, hyphae 3-4 µm smooth, and thick-walled; inner: net synenchyma, thin, hyphae 2 µm</td>
<td>5 µm wide; no clamps, crystalline ornamentation, and elbow-like bends</td>
<td>Compact brown with crystalline ornamentation and elbow-like bends</td>
<td>Absent</td>
</tr>
<tr>
<td>F and M</td>
<td><em>Thelephora</em>-type (T) Blasted to <em>Thelephora terrestris</em>, Accession No. U83486, 619/627 base pairs = 99%</td>
<td>Unbranched or irregular bright orange to brown (sometimes whitish) mycorrhiza with smooth reflective texture</td>
<td>Outer: net synenchyma, hyphae 3 µm wide; inner: incomplete interlocking irregular synenchyma, hyphae 4-5 µm wide</td>
<td>Rare, 3 µm wide; clamps, smooth with occasional enlarged hyphal junctions</td>
<td>Absent</td>
<td>Common, 40-50 µm long and 3 µm wide with basal clamp</td>
</tr>
<tr>
<td>F and M</td>
<td><em>Cenococcum geophilum</em> (Cg)</td>
<td>Unbranched, black mycorrhiza with rough hairy texture</td>
<td>Outer: net synenchyma in a stellate pattern, hyphae 6 µm wide; inner: net synenchyma</td>
<td>5-6 µm wide black, straight</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>F and M</td>
<td><em>Wilcoxina</em>-type (W) Blasted to <em>Wilcoxina rubra</em>, Accession No. DQ069001, 510/519 base pairs = 98%</td>
<td>Irregular dark brown to orangish mycorrhiza, often wrinkled, also called E-strain</td>
<td>Outer: not seen; inner: patchy and incomplete net prosenchyma, hyphae 2 µm wide</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>F and M</td>
<td><em>Mycelium radicis atrovirens</em>-type (MRA)</td>
<td>Unbranched black to brown mycorrhiza with curled hairy or very rough texture</td>
<td>Outer: felt prosenchyma, hyphae 3 µm wide; inner: net synenchyma, hyphae 2-3 µm wide</td>
<td>Rare, 5-7 µm wide, no clamps, smooth but becoming progressively more verrucose away from the mantle</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>F and M</td>
<td>Undifferentiated (Undif)</td>
<td>Young orange mycorrhiza with no distinct characters</td>
<td>Barely visible net synenchyma readily turning into Hartig net</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>F</td>
<td><em>Tomentella</em>-type (Tom)</td>
<td>Swollen dark-brown sandy textured mycorrhiza</td>
<td>Outer: squarish incomplete interlocking irregular synenchyma with thick-walled hyphae; inner: net synenchyma</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>F</td>
<td><em>Piloderma</em>-type (P)</td>
<td>Yellow coarsely felt mycorrhiza with abundant rhizomorphs</td>
<td>Not determined</td>
<td>Absent</td>
<td>Finely verrucose, septa common, not clamped, approximately 3 µm wide</td>
<td>Absent</td>
</tr>
</tbody>
</table>
Table 2.3 Effect of sterilization on growth and EM colonization of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings.

<table>
<thead>
<tr>
<th>Mesh (µm)</th>
<th>Soil</th>
<th>Height increment (cm)</th>
<th>SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>10</td>
<td>15.2</td>
<td>2.6</td>
</tr>
<tr>
<td>S</td>
<td>12</td>
<td>9.7</td>
<td>2.6</td>
</tr>
<tr>
<td>U</td>
<td>12</td>
<td>13.9</td>
<td>0.8</td>
</tr>
<tr>
<td>S</td>
<td>12</td>
<td>9.0</td>
<td>0.8</td>
</tr>
<tr>
<td>U</td>
<td>11</td>
<td>20.2</td>
<td>2.4</td>
</tr>
<tr>
<td>S</td>
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<td>14.5</td>
<td>2.4</td>
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<tr>
<td>U</td>
<td>12</td>
<td>17.8</td>
<td>3.6</td>
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<tr>
<td>S</td>
<td>8</td>
<td>12.5</td>
<td>3.6</td>
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<tr>
<td>U</td>
<td>12</td>
<td>20.6</td>
<td>4.1</td>
</tr>
<tr>
<td>S</td>
<td>9</td>
<td>10.8</td>
<td>4.1</td>
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<tr>
<th></th>
<th>n</th>
<th>Shoot gain (g) SEM</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
<td>0.906</td>
<td>0.137</td>
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<tr>
<td></td>
<td>12</td>
<td>0.420</td>
<td>0.137</td>
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<tr>
<td></td>
<td>12</td>
<td>0.967</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
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<td>0.341</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.555</td>
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<tr>
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<td>0.858</td>
<td>0.379</td>
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<tr>
<td></td>
<td>12</td>
<td>1.511</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.606</td>
<td>0.319</td>
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<th></th>
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<th>Root gain (g) SEM</th>
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<td>0.316</td>
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<td>0.064</td>
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<td>0.123</td>
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<tr>
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<td>0.347</td>
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<table>
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<tr>
<th></th>
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<td>9</td>
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<td>0.155</td>
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<td>0.440</td>
<td>0.056</td>
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<tr>
<td></td>
<td>11</td>
<td>0.557</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.492</td>
<td>0.145</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.423</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.347</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.566</td>
<td>0.083</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Percent EM colonization (%) SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>43</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>57</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>33</td>
<td>8</td>
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<tr>
<td></td>
<td>7</td>
<td>57</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

A series of t-tests were used to determine differences between grown in unsterilized (U) and sterilized (S) soils for each mesh barrier treatment. Seedling growth is expressed as height and biomass measured after 5 months. SEM: standard error of the mean.
Table 2.4  Effect of mesh treatment on growth and ectomycorrhizal colonization of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings.

<table>
<thead>
<tr>
<th>Mesh (µm)</th>
<th>Height increment difference (cm)</th>
<th>SEM</th>
<th>n</th>
<th>Shoot gain difference (g)</th>
<th>SEM</th>
<th>n</th>
<th>Root gain difference (g)</th>
<th>SEM</th>
<th>n</th>
<th>Root:Shoot gain ratio difference</th>
<th>SEM</th>
<th>n</th>
<th>EM colonization difference (%)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>10</td>
<td>5.7</td>
<td>a</td>
<td>± 2.6</td>
<td></td>
<td>10</td>
<td>0.486</td>
<td>a</td>
<td>± 0.220</td>
<td>0.025</td>
<td>a</td>
<td>± 0.088</td>
<td>-0.550</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>12</td>
<td>a</td>
<td>± 2.4</td>
<td></td>
<td>12</td>
<td>0.627</td>
<td>a</td>
<td>± 0.201</td>
<td>0.231</td>
<td>a</td>
<td>± 0.084</td>
<td>-0.117</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>a</td>
<td>± 2.6</td>
<td></td>
<td>10</td>
<td>0.900</td>
<td>a</td>
<td>± 0.220</td>
<td>0.330</td>
<td>a</td>
<td>± 0.100</td>
<td>0.080</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8</td>
<td>a</td>
<td>± 2.9</td>
<td></td>
<td>8</td>
<td>0.313</td>
<td>a</td>
<td>± 0.247</td>
<td>0.157</td>
<td>a</td>
<td>± 0.118</td>
<td>0.052</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>9</td>
<td>a</td>
<td>± 2.7</td>
<td></td>
<td>9</td>
<td>0.818</td>
<td>a</td>
<td>± 0.232</td>
<td>0.181</td>
<td>a</td>
<td>± 0.088</td>
<td>-0.205</td>
<td>ab</td>
</tr>
</tbody>
</table>

Response differences between source and recipient seedlings were calculated for each pot. This single number was used in the ANOVA for each response variable. Statistically significant mesh treatment effects detected by a Bonferroni multiple comparison test are designated by different letters ($P<0.05$).

*SEM*: standard error of the mean.
Figure 2.1  Schematic diagram of the pot design used to test hyphal penetration of mesh barriers.
Figure 2.2 Effect of a) fungicide type and b) application frequency on percent ectomycorrhizal colonization (determined by clearing and staining root tips) of Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings. Fungicide abbreviations: S= Senator® and T=Topas®. Frequency abbreviations: A= once upon commencement of the experiment, B=every two months, and C= once a month. Statistically significant fungicide treatment effects detected by a Bonferroni multiple comparison test are designated by different letters (*P*<0.05). Error bars are one standard error of the mean.
Figure 2.3 Relative abundance of morphotypes (*Tomentella*-type (Tom) *Thelephora terrestris* (T); *Mycelium radicis atrovirens*-type (MRA); *Wilcoxina rehmii* (W); *Cenococcum geophilum* (Cg); *Rhizopogon/Suillus*-type (R/S); *Piloderma*-type (P) and Undifferentiated (Undif) found on morphotyped Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) root systems grown in soil treated with different a) fungicide types and b) application frequency. Fungicide abbreviations: S= Senator® and T=Topas®. Frequency abbreviations: A= once upon commencement of the experiment, B=every two months, and C= once a month.
Figure 2.4  Relative abundance of Wilcoxina rehmii ectomycorrhizas, as a percentage of all root tips examined on Douglas-fir (Pseudotsuga menziesii var. glauca) grown in soil treated with fungicides. Fungicide abbreviations: S= Senator® and T=Topas®. Statistically significant fungicide type treatment effects detected by a Bonferroni multiple comparison test are designated by different letters ($P<0.05$). Error bars are one standard error of the mean.
Figure 2.5  Relative abundance of morphotypes *(Thelephora terrestris* *(T)*; *Mycelium radicis atrovirens*-type (MRA); *Wilcoxina rehmii* *(W)*; *Cenococcum geophilum* *(Cg)*; *Rhizopogon/Suillus*-type *(R/S)*; and Undifferentiated *(Undif)*, as a percentage of all root tips examined on source *(S)* and recipient *(R)* Douglas-fir *(Pseudotsuga menziesii* var. *glauca)* separated by a mesh barrier.
Figure 2.6 Ectomycorrhizal community differences. 

**a)** Steinhaus similarity index for ectomycorrhizal communities observed on source and recipient seedlings separated by a mesh barrier. 

**b)** Richness difference = number of morphotypes observed on source Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) root systems minus morphotypes present on recipient Douglas-fir separated by a mesh barrier. Statistically significant mesh treatment effects detected by a Bonferroni multiple comparison test are designated by different letters (*P*<0.05). Error bars are one standard error of the mean.
2.5 References


**Chambers DP, and Attiwill PM. 1994.** The ash-bed effect in *Eucalyptus regnans* forest - chemical, physical and microbiological changes in soil after heating or partial sterilization. *Australian Journal of Botany 42:* 739-749.


3 ROLE OF MYCORRHIZAL NETWORKS AND TREE PROXIMITY IN ECTOMYCORRHIZAL COLONIZATION OF PLANTED SEEDLINGS

3.1 Introduction

The importance of mycorrhizal networks (MNs) to plant and fungal ecology, and their role in ecosystem recovery following disturbance, is gaining increasing attention (Taylor, 2006). The likelihood that mycorrhizal networks (MN) form in communities dominated by ectomycorrhizal (EM) plants appears high given low host specificity of most EM fungal species (Molina et al., 1992). Although there have been numerous studies of EM fungal specificity and function, controversy still remains over whether MNs can physically connect tree roots of the same or different species (Selosse et al., 2006). This is likely due to the artificial conditions of laboratory studies (Brownlee et al., 1983; Read et al., 1985; Finlay & Read, 1986a,b; Wu et al., 2001) or to a lack of direct observations of MNs in the field (Simard & Durall, 2004; Booth, 2004; McGuire, 2007). However, recent studies using molecular techniques (Kennedy et al., 2003; Lian et al., 2006) are providing strong evidence that MNs occur in the field.

Establishing seedlings are colonized by propagules of EM fungi (spores, sclerotia, hyphae of excised EM root tips) or by roots contacting mycelium of an established plant (i.e., MN-mediated). Established trees facilitate seedling EM colonization indirectly by harboring a high soil inoculum potential or perhaps directly by a MN (Borchers & Perry, 1990; Berman & Bledsoe, 1998; Dickie et al., 2002). Mycorrhizal network-mediated colonization of seedlings by established vegetation was first demonstrated in the field by Fleming (1983; 1984), and has been suggested as the most important method of colonization in forests (Robertson, 1954; Newton, 1992). This is supported by studies showing that seedlings planted near mature trees usually have higher EM fungal species richness and diversity than those in the open (Kranabetter & Wylie, 1998; Durall et al., 1999; Cline et al., 2005). Distinct shifts in EM community composition (i.e., species occurrence and abundance) have also occurred when seedlings regenerated near trees compared to far away (Dickie et al., 2002; Outerbridge & Trofymow, 2004; Dickie & Reich, 2005).

A version of this chapter has been submitted and is now in revision: Teste, FP, Simard, SW, and Durall, DM. The role of mycorrhizal networks and tree proximity in ectomycorrhizal colonization of planted seedlings.
None of these field studies clearly show, however, that MN-mediated colonization is the dominant mechanism for EM fungal spread. Newman (1988) suggested that MN-mediated colonization of seedlings would be faster, greater, and more species rich than seedling colonization in the absence of a MN. However, these hypotheses have yet to be carefully tested. Separating colonization by MNs from that by EM soil inoculum or wind-dispersed spores is needed to determine the ecological importance of MN-mediated colonization.

We define mycorrhizal network potential as a measure of the likelihood that a MN occurs between two plants. If two plants growing in close proximity share many of the same EM taxa or genets, then there is a strong likelihood that a MN exists between the two plants, and hence have a high MN potential. In this study, we used morphological and molecular techniques to compare EM fungi on residual trees and seedlings in order to determine the MN potential between these plants in the field.

The objectives of this study were firstly, to determine the potential for a MN to form between Douglas-fir trees and seedlings in the field, secondly, to determine the relative importance of MN-mediated colonization of seedlings compared to colonization from all other sources, and thirdly, to determine how restricting the inoculum source and varying the proximity to conspecific residual trees affect the EM colonization of planted seedlings. The following hypotheses were tested: i) MN-mediated colonization is the dominant mechanism promoting the similarity of EM communities between residual trees to seedlings; ii) there is a distance at which the EM community of planted seedlings is most similar to residual trees; (iii) there is high potential for seedlings and conspecific trees to share the same EM taxa and genets, and hence potential to form a MN; and iv) EM status (colonization, richness, and diversity levels) of seedlings is greatest where there is access to all inoculum sources.

3.2 Material and methods

3.2.1 Site description

The study sites were located within the Thompson Dry, Cool Interior Douglas-fir (IDF) biogeoclimatic variant (IDFdk2) 25 to 50 km north of Kamloops, British Columbia, Canada (spanning 50°51’N, 120°25’W to 50°56’N, 120°18’W). This area is
near the northern-most limit of interior Douglas-fir in North America, where precipitation ranges from 300 to 750 mm annually (Meidinger & Pojar, 1991). The six study sites have loamy Gray Luvisolic soils (Soil Classification Working Group, 1998) and tree canopies dominated by interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco). Understory vegetation was dominated by arbuscular mycorrhizal (AM) pinegrass (*Calamagrostis rubescens* Buckl.), nodding onion (*Allium cernuum* Roth), white hawkweed (*Hieracium albiflorum* Hook.), and several minor EM shrubs and herbs (Hagerman *et al.*, 2001).

3.2.2 Experimental design

Six study sites were selected in spring 2004 (Table 3.1). All sites had been logged 1-11 years previously using variable retention harvesting, where a low density of pole-sized Douglas-fir trees were retained throughout. On each site, residual Douglas-fir trees at least 30 m apart were numbered and then four were randomly selected and assigned to one of four mesh treatments, where seedlings were planted into bags with 0.5 µm, 35 µm or 250 µm pore sizes, or directly into soil (see Table 3.1 for tree characteristics). Douglas-fir seedlings were then planted at four randomly selected distances (0.5 m, 1.0 m, 2.5 m, 5.0 m) from the residual tree either directly into the soil or into a mesh-bag (15 cm diameter and 35 cm deep) containing carefully excavated soil. We minimized disturbance to the MN by carefully excavating the soil into three distinct soil layers (intact forest floor, A, and some of the B horizon) then placed these layers back in the same order. Prior to planting the seedlings in the ground of the no mesh treatment, the surrounding soil was disturbed to ensure initial soil disturbance was consistent across mesh treatments. The four distance locations were offset from each other to avoid spatial interdependence. These treatments were organized in a split plot design with six replicate sites, where the whole plot effect was mesh pore size and the split plot effect was distance from the residual tree. The interior Douglas-fir seedlings (Seedlot # 42309, British Columbia Ministry of Forest and Range Tree Seed Center, Surrey, BC, Canada) that were planted had been commercially grown in a nursery for one year in 512A styroblocks™ (Beaver Plastics, Edmonton, AB, Canada).

The purpose of the mesh treatments was to assess the capacity for residual trees to...
actively colonize nearby seedlings via a MN, thus determining the importance of MN-mediated colonization. Seedlings planted in a 0.5 µm mesh bag could be colonized by wind- or soil-borne propagules, but not by the tree’s MN because the pores were too small for hyphal penetration, preventing the formation of mycorrhizal hyphal connections with other generalist EM plants (Teste et al., 2006). Seedlings planted in 35 and 250 µm mesh bags allowed us to distinguish MN colonization by individual hyphae from that by rhizomorphs. Field and lab observations showed that intact rhizomorphs did not penetrate the 35 µm mesh. However, we did notice that rhizomorphs were capable of breaking down into an unstructured form (loose hyphae) at the surface of the 35 µm mesh, which allowed penetration, but these occurrences were rare. All mesh bags were made out of sturdy plain-weave nylon (Plastok Ltd., Birkenhead, UK), had a volume of 6185 cm³, and the 0.5, 35, and 250 µm mesh had an estimated percent open pore space of 13.3%, 34.0%, and 54.1%, respectively. Because of widespread belief that fine mesh would impede water flow, we conducted a small pot experiment, which clearly showed that soil water moved freely across the fine 0.5 µm mesh within minutes. Seedlings planted directly into soil could potentially be colonized by all inoculum sources, including short-distance hyphal connections with ‘contact’ exploration type EM (Agerer, 2001). The occurrence of short-distance MN connections was considerably reduced in the 250 µm mesh treatment because the thickness (~0.32 mm) of the nylon mesh was greater than the typical length (0 - 0.25 mm) of the extraradical hyphae or cystidia of EM ‘contact’ exploration types found in this study. The purpose of the four distance treatments was to assess the spatial influence of residual trees on EM communities of planted seedlings.

A wedge-shaped area (35 m²) was trenched around each tree in a southern direction to a soil depth of 50 - 70 cm to exclude the influence of surrounding tree roots. Trenches were lined with heavy polyethylene and refilled with excavated soil. All trees within a 2 m radius of the sides of the wedge-shaped areas and North side of residual trees were felled. All vegetation in the wedge-shaped area was clipped regularly throughout the first growing season and at the beginning of the second growing season to minimize shading effects on seedlings. Douglas-fir seedlings were grown in containers for one year prior to planting, were non-mycorrhizal (based on a random subsample of 10
and determined using the methods described below) at the time of planting, and ranged in height from 13 to 30 cm.

3.2.3 Sampling of residual tree ectomycorrhizas

From August 26 to 30, 2005, soil cores (10 cm diameter, 20 cm deep) were collected in four cardinal directions (North, South, West, and East) approximately 2 cm from the outer rim of the mesh bags. For seedlings planted directly into the soil (i.e., without mesh bags), soil cores were taken from the same distances away from each seedling only after the seedling had first been carefully excavated. Samples were placed in plastic bags and stored at 4 °C until further processing. All samples were processed within 4 months after field sampling.

3.2.4 Sampling of seedling ectomycorrhizas

Seedlings were harvested immediately after soils were sampled for residual tree EM (14 months after planting). After clipping off the shoots, root systems with some surrounding soil were placed in plastic bags and stored at 4 °C until further processing. All samples were processed within 6 months after field sampling.

3.2.5 Morphotyping and molecular identification of ectomycorrhizas

Roots of residual trees and seedlings were carefully washed under running tap water and then cut into approximately 2 cm pieces. All root fragments were placed in a baking dish containing distilled water and thoroughly mixed. We randomly subsampled and counted up to 200 residual tree and 300 seedling EM root tips. Gross morphology of EM roots and rhizomorphs was described using a stereomicroscope, while the mantle, cystidia, emanating hyphae, and Hartig net were described using a compound microscope under 400 or 1 000 X magnification. Morphological descriptions were made with reference primarily to Goodman et al. (1996) and Hagerman et al. (2001) and to a minor extent to Ingleby et al. (1990) and Agerer (1985–1998). After morphotyping, root systems were dried and weighed.

DNA extraction and PCR amplification of the ITS region of fungal nuclear rDNA was conducted on one subsample (typically 5 EM tips) per morphotype per host type. Extraction and isolation of DNA, sequencing and nucleotide Basic Local Alignment
Search Tool (BLAST) searches followed methods outlined in Twieg et al. (2007). Primer pairs used in PCR amplifications were: ITS1-F and ITS4; NSI1 and NLC2 (Martin & Rygiewicz, 2005). Samples that were not successfully amplified or sequenced were given an EM taxon name based on morphotyping data. Out of the 154 root tips extracted, 89% yielded DNA for BLAST searches.

3.2.6 Microsatellite analyses of *Rhizopogon vinicolor* ectomycorrhizas

Whenever *Rhizopogon*-type ectomycorrhizas were encountered on residual tree or seedling root systems, at least one tip was randomly selected for fragment analysis. These tips were placed in distilled water and frozen at -80 °C until DNA was extracted. We extracted, isolated, and sequenced *Rhizopogon*-type DNA following the methods outlined above. Prior to fragment analysis, we confirmed using an NCBI BLAST search that *Rhizopogon*-type samples corresponded to *Rhizopogon vinicolor* Smith (Kretzer et al., 2003a). To identify the *Rhizopogon vinicolor* genotypes, four separate PCR amplification reactions using primer sets Rv 53, Rve 1.34, Rve 2.77, and Rve 3.21 developed by Kretzer et al. (2003b) were used. We then analysed the fragment sizes using GeneMapper software (Applied BioSystems, Foster City, CA, USA, version 4.2).

3.2.7 Statistical analyses

All statistical analyses were carried out using the R statistical environment for statistical computing and graphics (R Development Core Team, 2006). Multivariate community data, univariate analyses, and linear mixed-effects model fitting were performed in R with the vegan (Oksanen et al., 2007), stats (R Development Core Team, 2006), and nlme (Pinheiro et al., 2007) packages, respectively. The split plot design was analysed using a linear mixed-effects model (Pinheiro & Bates, 2000), where sites were used as blocks and set as the random factor. The whole plot and split plot fixed factors were the mesh and distance treatments, respectively. Planted seedlings representing the mesh and distance treatment combinations on each site were the experimental units (n=6). For the residual tree root analyses, the experimental units were the sum of the four core subsamples.

*Community composition, structure, and similarity analyses*
Relative abundance (RA) of EM taxa was calculated as the abundance of a species divided by the sum of abundances of all EM taxa in an experimental unit (McCune et al., 2002). EM taxa accumulation curves and rank-abundance curves (i.e., Whittaker curves) were fit using the functions, specaccum (with method “Coleman”) and radfit. Mantel tests and Analysis of Similarity (ANOSIM) were carried out with the functions, mantel and anosim, respectively. Nonmetric Multidimensional Scaling (NMS) ordinations were performed using the metaMDS function to visualize the similarity between residual tree and seedling EM communities at the whole plot level.

Analysis of treatment effects

To test for treatment effects with the multivariate community data, we carried out Blocked Multi-Response Permutation Procedures (MRBP) following the guidance of McCune et al. (2002) with the function, mrpp (with blocks set as the strata). Percent EM colonization was calculated following Teste et al. (2006). Ectomycorrhizal richness on residual trees was relativized based on total number of EM root tips at each distance since it is known that richness decreases with fewer root tips sampled (Taylor, 2002). We calculated EM taxa richness and Shannon diversity index (H’) using functions, specnumber and diversity, respectively. The H’ emphasizes species richness because it is weighted towards rare species (Magurran, 2004). Contingency tables were constructed for presence and absence of EM taxa grouped by mesh treatments and analyzed with chisq.test (Pearson’s Chi-square test) and Monte Carlo simulated P-values. The lme function (method set to restricted maximum likelihood) was used to fit the linear mixed-effects models. Variance partitioning and significance of the fixed effects were determined with the functions, anova and summary of the fitted lme objects. When a significant (P < 0.05) fixed effect was found, pairwise mean difference comparisons using 95% simultaneous confidence intervals (SCI) with the Bonferroni method were employed. The plotted SCI concurrently assessed i) which means were significantly different, ii) the effect size, iii) precision, and iv) the range of plausible values for the population (Gardner & Altman, 2000).
3.3 Results

3.3.1 Ectomycorrhizal community composition and structure

A total of 36 EM taxa were found on either residual tree or seedling root systems (Table 3.2). Residual trees hosted 32 EM taxa and seedlings hosted 26 EM taxa, with 22 taxa in common. More unique EM taxa were found on residual trees than on seedlings (Table 3.2). \textit{Wilcoxina rehmii} dominated EM communities on both trees and seedlings; mycorrhizas formed by \textit{Rhizopogon rudus}, \textit{Cenococcum geophilum}, \textit{Rhizopogon vinicolor}, and \textit{Amphinema byssoides} were also abundant (Fig. 3.1).

A total of 30 228 residual tree- and 23 387 seedling-EM root tips were observed and counted. Both EM communities were sufficiently sampled to determine that the residual trees had significantly higher EM taxa richness than the seedlings (Fig. 3.2). Rank-abundance curves for both communities were best described (best fit and lowest AIC value) by the log-normal model, signifying moderately high evenness (Magurran 2004) (Fig. 3.3). The residual tree EM community curve had a shallower slope indicating that it was more even than the seedling EM community (Fig. 3.3).

3.3.2 Ectomycorrhizal community similarity

Trees and seedlings shared 61% of all EM taxa found in this study (Table 3.2) and 83% of those taxa had a relative abundance greater than 5% (Fig. 3.1). The NMS ordination method showed a high degree of overlap between the residual tree and seedling EM communities (Fig. 3.4). Community analyses based on abundance and presence-absence of EM taxa also showed high similarity between the residual trees and seedlings (Table 3.3).

3.3.3 \textit{Rhizopogon vinicolor} genet analysis

We successfully sequenced 24 \textit{Rhizopogon vinicolor} samples, of which 16 produced clear fragments for four different loci (Table 3.4). Five of the \textit{R. vinicolor} samples allowed us to determine if residual trees (five \textit{Rhizopogon vinicolor} samples from various distances) and associated seedlings (two \textit{Rhizopogon vinicolor} samples at two distances) were harboring the same fungal genet (i.e., same individual fungus). At Big Pines, three \textit{R. vinicolor} samples found on a tree shared all the same fragment sizes at
the four loci with one *Rhizopogon vinicolor* sample from a seedling planted in 35 µm mesh at 0.5 m from that tree. This analysis provides evidence that both plants were associated with the same fungal genet and, hence, that a MN had formed between the Douglas-fir seedling and a mature tree in the field.

3.3.4 Mesh effects on seedling ectomycorrhizal community

At the community level, mesh treatment had no effect on the EM seedling community (Table 3.3). Similarly, seedling EM colonization, richness and diversity were unaffected by the mesh treatments (data not shown). The interaction between mesh and distance was tested as a fixed effect but was not significant for the univariate responses presented above. Given the complexity of the design and its lack of balance (uneven number of replicates per treatment combination), we were not able to directly test for mesh and distance interactions at the community level (multivariate responses). However, following the advice of McCune et al. (2002), we visualized interactions using joint plot overlays on an ordination diagram. In all cases, treatments were patterned as perpendicular vectors indicating independence and non-interaction. Only 50% of all EM taxa encountered were found on seedlings in the 0.5 µm mesh as opposed to an average of 77% on seedlings having partial or full access (35, 250 µm, and no mesh) to a MN (Fig. 3.3), but this trend was statistically weak ($X^2 = 6.5, P = 0.09$). Furthermore, four EM taxa (*Inocybe aurea*, *Lactarius deterrimus*, *Russula brevipes*, and *Tuber* sp.) were uniquely excluded by the 0.5 µm mesh (i.e., absent from the 0.5 µm mesh but present in at least one of the other mesh treatments).

3.3.5 Distance effects on the residual tree and seedling ectomycorrhizal communities

Distance treatments influenced the composition of both the tree and seedling EM communities (Table 3.3). Seedlings furthest from residual trees had a simpler EM community composition, mainly dominated by *Wilcoxina rehmii*, compared to seedlings in closer proximity (Fig. 3.6). Ectomycorrhizal colonization of root samples from residual trees was high but not affected by distance from the bole (Fig. 3.7). However, both EM richness and diversity in samples from residual trees decreased with distance (Fig. 3.7). Similar to residual trees, EM colonization of planted seedlings was unaffected
by distance from residual trees (Fig. 3.8). However, seedling EM richness and diversity were greatest at 0.5 m and decreased with distance (Fig. 3.8).

3.4 Discussion

3.4.1 MN-mediated colonization

Our first hypothesis, that MN-mediated colonization is the dominant mechanism promoting continuity of the EM community (Newton, 1992), was rejected. Even though there was high potential for MNs to form on our sites (see below), we found that wind- and soil-borne EM inoculum already present in mesh bags (could include chlamydospores, sclerotia, hyphal fragments, severed tree EM tips, and disrupted network hyphae) was the most important inoculum source for newly colonized seedlings. This is similar to McGuire’s (2007) observations in an EM monodominant rain forest. However, we did find that restricting access to a MN reduced the occurrence of some EM taxa. Furthermore, four EM species were absent from seedlings with restricted access to the MN of our residual trees, implying that specific groups of EM fungi depended on MN-mediated colonization for spread. This result needs to be interpreted cautiously, however, because this outcome may have occurred by chance. The EM fungi that were absent had smooth mantles with limited extraradical hyphae, representing contact (Lactarius deterrimus and Russula brevipes) or short-distance (Inocybe aurea and Tuber sp.) exploration morphology types (Agerer, 2001). Therefore MNs may still be considered important for colonization of EM species with poor-exploration strategies.

3.4.2 Lateral extent of EM communities

Seedling EM richness and diversity increased with proximity to trees, suggesting that nearby residual trees acted as EM refugia and facilitated the spread of EM fungi to establishing seedlings. Our second hypothesis, that there is a distance where the EM community of seedlings is most similar to trees, was supported. Indeed, seedlings closest to the residual trees had the most similar EM communities compared to those at further distances. The proximity pattern observed in this study was also noted in other studies assessing the effect of distance from live trees on seedling EM status (Dickie et al., 2002; Outerbridge & Trofymow, 2004). In contrast to Dickie et al. (2005) and Hagerman et al.
(1999), we found no clear relationship between percent EM colonization in planted seedlings and distance to the closest live tree. We suspect that resilient soil propagules and wind-dispersed spores of rapidly growing EM fungal species, such as *Wilcoxina rehnii*, *Rhizopogon vinicolor*, and *Thelephora terrestris*, accounted for the high EM colonization levels on our most distant seedlings.

EM taxa richness and diversity of the residual trees also significantly decreased with distance, corresponding with our estimated decline in root density. Patterns of declining residual tree root and EM root tip densities with distance (data not shown) are consistent with estimates based on coarse-root growth rates of interior Douglas-fir in the IDF biogeoclimatic zone (Richardson, 2000). As expected, this general pattern has also been found in other Douglas-fir forests (Outerbridge & Trofymow, 2004; Cline et al., 2005; Luoma et al., 2006) as well as other coniferous and hardwood forests (Durall et al., 1999; Hagerman et al., 1999; Kranabetter et al., 1999; Dickie et al., 2002; Dickie & Reich, 2005).

The AM herbaceous plants that dominated the understory vegetation on our sites may also have negatively affected the EM fungal communities on seedlings furthest away from mature trees, where MN potential and EM status were lowest and AM plant abundance highest. Invasion or increases of AM plants have been shown to reduce EM fungal abundance and change EM species composition in the dry pinyon pine forests of the USA (Haskins & Gehring, 2004). Dominance of early post-disturbance plant communities by AM herbes is common in IDF forests such as ours (Stark et al., 2006). We suspect that below-ground competition with AM plants is an important factor reducing EM seedling recruitment and establishment in our dry forest ecosystems (McHugh & Gehring, 2006).

### 3.4.3 MN formation potential

The Douglas-fir trees and seedlings in this study shared most of the observed EM taxa, and community analyses supported our third hypothesis that the potential for MNs to form in these dry forests is high. These results agree with other studies demonstrating the potential for an MN to exist between mature trees and surrounding seedlings (Jonssson et al., 1999; Matsuda & Hijii, 2004; Cline et al., 2005) and between different
species of woody plants (Horton & Bruns, 1998; Horton et al., 1999; Simard et al., 1997; Kennedy et al., 2003; Dickie et al., 2004).

Molina et al. (1992) offered convincing arguments for high potential of MN formation in the field between EM hosts of the same or different species. Their discussion involved EM species specificity patterns and multiple-host fungal ranges as the norm rather than the exception. Their argument relied on the assumption that plant hosts sharing the same EM fungal species would be connected via a MN. We agree with Kennedy et al. (2003), however, that their concept would be strengthened by identification of the same EM fungal genet on multiple host plants; the availability of more advanced molecular techniques allows us to provide more convincing evidence of the occurrence of MN connections in the field. Our microsatellite analysis of *Rhizopogon vinicolor* genets indeed showed that a residual tree and nearby seedling shared the same EM fungal genet. Lian et al. (2006) also found the same *Tricholoma matsutake* genets connecting *Pinus densiflora* trees in a field study in Japan.

3.4.4 EM inoculum potential and community composition

Seedlings with access to all sources of inoculum, including MNs, soil propagules and spores, and that were also in close proximity to trees, tended to have the greatest EM colonization, richness, diversity, and abundance; thus, we could not reject our fourth hypothesis that EM status is greatest where there is global access to all inoculum sources. Of these inoculum sources, wind-dispersed EM fungal spores may not have been the dominant colonization vectors because epigeous and hypogeous sporocarps were infrequent on the six sites during the two-year study period, which is consistent with previous studies showing a paucity of fruitbodies in recent clearcuts (Durall et al., 2006). Since *Rhizopogon* species were abundant on root tips, especially on seedlings that could not form MNs, *Rhizopogon* may have colonized seedlings from a viable and tenacious spore bank and/or from disrupted network hyphae in the soil (Kjøller & Bruns, 2003). The most abundant EM taxon, *Wilcoxina rehmii*, likely colonized seedlings via soil propagules (chlamydospores, sclerotia, hyphal fragments, severed tree EM tips, and disrupted network hyphae) and to a lesser extent via MN’s (Mikola, 1988).
We were surprised that Wilcoxina rehmii was the most abundant EM taxon on both residual trees and seedlings. Typically, Wilcoxina occurs as a greenhouse bioassay contaminant and is completely absent from mature trees (Cline et al., 2005). Yet, Mikola (1988) noted that this species can be a rapid colonizer of new root tips under field conditions. It is possible that the high abundance of Wilcoxina was an artifact of the small soil disturbances caused during mesh bag insertions. However, Wilcoxina has been noted as an abundant member of the woody understory plant community in similar Interior Douglas-fir forests (Hagerman et al., 2001). Thus, it is likely that Wilcoxina rehmii mycelia are shared among multiple herbaceous and woody plant species, and is an aggressive colonizer of interior Douglas-fir roots. Given that planted seedlings were non-mycorrhizal at the time of planting, we conclude that the ectendomycorrhizal Wilcoxina rehmii is a naturally occurring and abundant member of these dry coniferous forests.

Some of the most abundant EM taxa, including Rhizopogon rudus, Cenococcum geophilum, Rhizopogon vinicolor, and Amphinema byssoides were present on both the residual trees and seedlings. Thelephora terrestris, by contrast, was abundant only on seedlings furthest away from residual trees, a pattern also observed by Kranabetter & Wylie (1998), Durall et al. (1999), and Cline et al. (2005). The rank-abundance curves for both communities were best described by a log-normal model, similar to patterns in other regenerating EM forests (Visser, 1995; Jonsson et al., 1999; Cline et al., 2005). The spatial structure of the EM communities we observed may reflect on the different abilities of EM fungal species to compete for tree or seedling roots in these forests.

3.4.5 Conclusions

We present evidence that MN-mediated colonization is not the dominant mechanism promoting continuity of the EM community from mature trees to nearby establishing seedlings. Our results instead suggest that soil- or wind-borne EM inoculum (e.g., chlamydomycetes, sclerotia, hyphal fragments, severed tree EM tips, and disrupted network hyphae) were the main vectors responsible for EM colonization of seedlings. Regardless, we show there is high potential for MNs to form between established trees and seedlings in these forests, and MNs may be important for colonization of certain EM species on establishing seedlings. At forest harvest, retaining residual trees at sufficient
spatial distribution to maintain a diverse EM community appears important for recovery of the EM fungal community following disturbance. This may be especially important where EM hosts are surrounded by AM vegetation, as was the case in our study. Mature tree root zones extend several meters beyond the tree crown, ensuring seedling colonization and continuity of the EM fungal community both spatially and through time.

Acknowledgements

We acknowledge David Huggard and Shannon Berch for their thoughtful comments at the early stages of the study. We are very grateful to Amanda Schoonmaker for her help during the field work. We are indebted to Gordon Leschyson for renting his CanDig® (Kamloops, BC, Canada) mini-excavator and Rocky Hudson at High Country Cold Storage Ltd. for the nursery-grown seedlings. We thank Bill Clarke, Lenka Kudrna, and MaryAnn for help with the molecular work in the laboratory. We also thank Brendan Twieg for his insightful advice during the morphotyping, molecular and community data analyses. Tony Kozak and Val LeMay provided advice on data analysis. Funding was provided by a Forest Science Program of Forest Investment Innovation of British Columbia grant, a Canadian Foundation for Innovation grant, and a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant (DG) to SWS, an NSERC PGS scholarship to FPT, and an NSERC DG to DMD. We declare that the experiments comply with the current laws of the country in which they were performed.
Table 3.1  Site and residual interior Douglas-fir (\textit{Pseudotsuga menziesii} var. \textit{glauca}) tree characteristics. Values for tree characteristics are means with one standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Big pines</th>
<th>Columbine pines</th>
<th>Dogwood pines</th>
<th>Douglas pines</th>
<th>Fairy pines</th>
<th>Robin Hood pines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevation (m)</td>
<td>1100</td>
<td>1180</td>
<td>1140</td>
<td>1210</td>
<td>1120</td>
<td>1130</td>
</tr>
<tr>
<td>Aspect</td>
<td>Southeast</td>
<td>South</td>
<td>Southeast</td>
<td>North</td>
<td>Southeast</td>
<td>Southwest</td>
</tr>
<tr>
<td>Mean slope (%)</td>
<td>25</td>
<td>15</td>
<td>5</td>
<td>5</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Selected tree characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>102 (11)</td>
<td>91 (13)</td>
<td>102 (29)</td>
<td>123 (1)</td>
<td>83 (24)</td>
<td>50 (7)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>11.7 (4.2)</td>
<td>11.3 (1.5)</td>
<td>11.1 (3.7)</td>
<td>11.4 (2.0)</td>
<td>9.4 (2.2)</td>
<td>5.6 (1.3)</td>
</tr>
<tr>
<td>DBH (cm)</td>
<td>11.4 (3.1)</td>
<td>13.8 (1.7)</td>
<td>12.1 (2.8)</td>
<td>11.6 (1.9)</td>
<td>12.1 (0.6)</td>
<td>8 (1.5)</td>
</tr>
</tbody>
</table>
Table 3.2  List of observed ECM taxa on Interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) trees and seedlings on six variable retention sites in the IDF biogeoclimatic zone of BC, Canada, in August 2005.

<table>
<thead>
<tr>
<th>Morphotypesa</th>
<th>Closest BLAST match</th>
<th>Database Accession number</th>
<th>Total base pairs aligned</th>
<th>NCBI % Similarity or UNITE score</th>
<th>Consensus ECM taxa</th>
<th>Host</th>
<th>Presence of rhizomorphs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphinema</td>
<td>Amphinema byssoides</td>
<td>NCBI AY838271</td>
<td>524</td>
<td>99</td>
<td>Amphinema byssoides</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>InoB</td>
<td>Tomentella botryoides</td>
<td>UNITE UNITE UNITE</td>
<td>NA</td>
<td>835</td>
<td>Tomentella botryoides</td>
<td>Seedling</td>
<td>No</td>
</tr>
<tr>
<td>Buffy</td>
<td>Sebacina epigaea</td>
<td>NCBI AF490397</td>
<td>689</td>
<td>93</td>
<td>Sebacina epigaea</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Caramel, BYO</td>
<td>Tuber sp.</td>
<td>NCBI AY634113</td>
<td>682</td>
<td>98</td>
<td>Tuber sp.</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>Cenococcum</td>
<td>Cenococcum geophilum</td>
<td>NCBI AY394919</td>
<td>586</td>
<td>98</td>
<td>Cenococcum geophilum</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>Cortinarius</td>
<td>Cortinarius erythrinus</td>
<td>AY696900</td>
<td>540</td>
<td>96</td>
<td>Cortinarius erythrinus</td>
<td>Tree</td>
<td>Yes</td>
</tr>
<tr>
<td>Fuzzy white, Heb</td>
<td>Suillus lakei</td>
<td>NCBI DQ367917</td>
<td>711</td>
<td>99</td>
<td>Suillus lakei</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>RussG</td>
<td>Lactarius rubracteus</td>
<td>NCBI DQ97882</td>
<td>594</td>
<td>98</td>
<td>Lactarius rubracteus</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>HB, HGLB, HBG</td>
<td>Inocybe sp.1</td>
<td>NCBI A893286</td>
<td>565</td>
<td>97</td>
<td>Inocybe sp.1</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>HT, SHT</td>
<td>Sebacina sp.</td>
<td>NCBI AF44048</td>
<td>748</td>
<td>97</td>
<td>Sebacina sp.</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>HW, HWil(T), Tom3</td>
<td>Pseudotomentella tristis</td>
<td>NCBI AY702789</td>
<td>630</td>
<td>99</td>
<td>Pseudotomentella tristis</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>HY, LacC, HW2</td>
<td>Inocybe dulcamara</td>
<td>UNITE UDB001196</td>
<td>NA</td>
<td>1015</td>
<td>Inocybe dulcamara</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>Irregular Hairy Yellow</td>
<td>Russula fragilis</td>
<td>NCBI DQ367914</td>
<td>778</td>
<td>99</td>
<td>Russula fragilis</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Lac(T)</td>
<td>Tricholoma sp.</td>
<td>NCBI DQ47754</td>
<td>609</td>
<td>98</td>
<td>Tricholoma sp.</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Lr, Lact</td>
<td>Lactarius deterrimus</td>
<td>NCBI AF249286</td>
<td>735</td>
<td>97</td>
<td>Lactarius deterrimus</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>LBO</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>LBO</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>O, OB, HO</td>
<td>Inocybe pudica</td>
<td>NCBI AY228341</td>
<td>287</td>
<td>95</td>
<td>Inocybe pudica</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>PinkRuss, InoP</td>
<td>Inocybe sp.2</td>
<td>NCBI AY751558</td>
<td>394</td>
<td>94</td>
<td>Inocybe sp.2</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>BRuss, PRuss(T), Russ1</td>
<td>Russula brevipes</td>
<td>NCBI AEF39714</td>
<td>593</td>
<td>99</td>
<td>Russula brevipes</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>PDB(S)</td>
<td>Russula brevampelina</td>
<td>NCBI AY061734</td>
<td>679</td>
<td>99</td>
<td>Russula brevampelina</td>
<td>Seedling</td>
<td>No</td>
</tr>
<tr>
<td>PDB(T)</td>
<td>Tricholoma moseri</td>
<td>NCBI AEF377211</td>
<td>694</td>
<td>96</td>
<td>Tricholoma moseri</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Piloderma</td>
<td>Piloderma fallax</td>
<td>NCBI AY010280</td>
<td>571</td>
<td>98</td>
<td>Piloderma fallax</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>R(T)</td>
<td>Inocybe aurea</td>
<td>UNITE UDB000612</td>
<td>363</td>
<td>Inocybe aurea</td>
<td>Tree</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Rhizopogon</td>
<td>Rhizopogon vinicolor</td>
<td>NCBI AEF263931</td>
<td>700</td>
<td>100</td>
<td>Rhizopogon vinicolor</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>Suil, Suil2, R2(S)</td>
<td>Rhizopogon rudus</td>
<td>NCBI AEF377107</td>
<td>598</td>
<td>98</td>
<td>Rhizopogon rudus</td>
<td>Both</td>
<td>Yes</td>
</tr>
<tr>
<td>Rough brown</td>
<td>Tomentella bryophila</td>
<td>UNITE UDB000035</td>
<td>1133</td>
<td>Tomentella bryophila</td>
<td>Tree</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Rn, Russ2(T), Russ3(T), RussD(T), RussD, YTRuss(T)</td>
<td>Russula adusta</td>
<td>NCBI AY061652</td>
<td>438</td>
<td>97</td>
<td>Russula adusta</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>Silv, SBFT, PRuss(S), EmRuss</td>
<td>Russula nigricans</td>
<td>NCBI DQ367915</td>
<td>770</td>
<td>100</td>
<td>Russula nigricans</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>T1, T2, Lac(S)</td>
<td>Thelephora terrestris</td>
<td>NCBI DQ068970</td>
<td>636</td>
<td>99</td>
<td>Thelephora terrestris</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Tom1, Tom4, UT1, UT2, HWil(S)</td>
<td>Tomentella subclaveligera</td>
<td>NCBI AY748886</td>
<td>619</td>
<td>98</td>
<td>Tomentella subclaveligera</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>Tom2</td>
<td>Thelephoraceae</td>
<td>NCBI U38467</td>
<td>612</td>
<td>99</td>
<td>Thelephoraceae</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>Tub</td>
<td>Tomentella subtestacea</td>
<td>UNITE UDB000304</td>
<td>NA</td>
<td>1068</td>
<td>Tomentella subtestacea</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>UHT</td>
<td>Rhizopogon rogeri</td>
<td>NCBI AEF071497</td>
<td>497</td>
<td>97</td>
<td>Rhizopogon rogeri</td>
<td>Seedling</td>
<td>Yes</td>
</tr>
<tr>
<td>Will, Wil2, MRA, Undif</td>
<td>Wilcoxina rehmii</td>
<td>NCBI AEF266708</td>
<td>571</td>
<td>98</td>
<td>Wilcoxina rehmii</td>
<td>Both</td>
<td>No</td>
</tr>
<tr>
<td>YRuss</td>
<td>Laccaria bicolor</td>
<td>NCBI DQ367906</td>
<td>749</td>
<td>100</td>
<td>Laccaria bicolor</td>
<td>Tree</td>
<td>No</td>
</tr>
<tr>
<td>YTRuss(S)</td>
<td>Sebacina inocrustans</td>
<td>NCBI AY143340</td>
<td>791</td>
<td>97</td>
<td>Sebacina inocrustans</td>
<td>Seedling</td>
<td>No</td>
</tr>
</tbody>
</table>

aFor photographs and concise morphotype descriptions, please contact the corresponding author. bNCBI is the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). cUNITE is a molecular database for the identification of ectomycorrhizal fungi (Köljalg et al., 2005).
Table 3.3 Summary of multivariate community analyses showing degree of similarity between residual interior Douglas-fir \textit{(Pseudotsuga menziesii var. glauca)} tree and seedling EM communities and treatment effect differences.

<table>
<thead>
<tr>
<th>Community analysis method</th>
<th>Grouping entity</th>
<th>Data matrix type</th>
<th>Similarity measure</th>
<th>Multiple comparisons</th>
<th>Statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mantel test</td>
<td>tree vs. seedling</td>
<td>Presence-absence</td>
<td>Chao</td>
<td>-</td>
<td>r = 0.629</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abundance</td>
<td>Chao</td>
<td>-</td>
<td>r = 0.305</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ANOSIM</td>
<td>tree vs. seedling</td>
<td>Presence-absence</td>
<td>Sørensen</td>
<td>-</td>
<td>R = 0.156</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abundance</td>
<td>Sørensen</td>
<td>-</td>
<td>R = 0.244</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRBP</td>
<td>mesh treatment levels</td>
<td>Presence-absence</td>
<td>Chao</td>
<td>-</td>
<td>A = 0.133</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abundance</td>
<td>Chao</td>
<td>-</td>
<td>A = 0.021</td>
<td>0.003</td>
</tr>
<tr>
<td>MRBP</td>
<td>distance treatment levels</td>
<td>Presence-absence</td>
<td>Chao</td>
<td>-</td>
<td>A = 0.100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence-absence</td>
<td>Chao</td>
<td>0.5 m vs 2.5 m</td>
<td>A = 0.062</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence-absence</td>
<td>Chao</td>
<td>0.5 m vs 5.0 m</td>
<td>A = 0.162</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence-absence</td>
<td>Chao</td>
<td>1.0 m vs 5.0 m</td>
<td>A = 0.115</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abundance</td>
<td>Chao</td>
<td>-</td>
<td>A = 0.035</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The Mantel r and ANOSIM R statistics ranges from -1 to +1 where: a value of -1 indicates complete dissimilarity; a value of 0 indicates a completely random grouping; and +1 indicates a completely similar grouping. The Mantel r and ANOSIM R associated p-values evaluates how likely is an observed dissimilarity or similarity due to chance. The MRBP chance-corrected within-group agreement (A) is a statistic used to test the hypothesis of no difference between two or more groups and represents the effect size (ranges from 0 to 1 where a value of 1 indicates that two or more groups are completely different). The associated p-value evaluates how likely is an observed difference due to chance (McCune & Grace, 2002). Only statistics with a value > 0.1 and p<0.05, were considered for multiple comparisons. Non-significant (p<0.05) multiple comparison tests are not included.
Table 3.4 Summary of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) tree and seedling *Rhizopogon vinicolor* microsatellite marker analysis. Genets were identified only when four loci were successfully analysed.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Rv53</th>
<th>Rve1.34</th>
<th>Rve2.77</th>
<th>Rve3.21</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D^I$</td>
<td>0.67</td>
<td>0.37</td>
<td>0.42</td>
<td>0.43</td>
</tr>
<tr>
<td>$F_{IS}^1$</td>
<td>0.02</td>
<td>0.15</td>
<td>-0.03</td>
<td>-0.01</td>
</tr>
</tbody>
</table>

Trees (n = 9, g = 6)
- Number of alleles: 4, 5, 5, 6
- Number of homozygotes: 5, 7, 3, 2
- Number of heterozygotes: 4, 2, 6, 7
- Heterozygocity (%): 44%, 22%, 67%, 78%
- Fragment sizes (bp) of shared genet: 251, 254, 196, 196, 220, 222, 213, 213

Seedlings (n = 7, g = 6)
- Number of alleles: 4, 2, 4, 5
- Number of homozygotes: 6, 7, 2, 5
- Number of heterozygotes: 1, 0, 5, 2
- Heterozygocity (%): 14%, 0%, 71%, 29%
- Fragment sizes (bp) of shared genet: 251, 254, 196, 196, 220, 222, 213, 213

1Gene diversity ($D$) and $F_{IS}$ values were taken from Kretzer *et al.* (2003).
2Number of samples (n) analyzed and genets (g) found.
Data presented are for the four successfully analysed loci.
Figure 3.1 Relative abundance of residual tree and seedling EM taxa.
Figure 3.2 Residual tree and seedling EM taxa accumulation curves with confidence intervals.
Figure 3.3 Residual tree and seedling EM taxa rank abundance plots (i.e., Whittaker plots). The well-known log-normal species abundance model had the best fit.
Figure 3.4  NMS ordination of residual tree and seedling EM fungal communities. Sorensen distance measure was used; $R^2 = 0.66$. 
Figure 3.5  Relative abundance of EM taxa found on seedlings having access to the MN (35 µm, 250 µm, and no mesh) or where it was restricted (0.5 µm mesh). * denotes relative abundance = 0 for MN restricted.
Figure 3.6  Relative abundance of common (>1%) EM taxa found on *Pseudotsuga menziesii* var. *glauc*a seedlings growing at the four distances from *Pseudotsuga menziesii* var. *glauc*a residual trees.
Figure 3.7 Distance effects on EM status (colonization, richness and diversity) of *Pseudotsuga menziesii* var. *glauca* residual trees. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top right of each graph are the four distance treatment means.
Figure 3.8  Distance effects on EM status (colonization, richness and diversity) of *Pseudotsuga menziesii* var. *glauca* seedlings. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top right of each graph are the four distance treatment means.
3.5 References


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4 MYCORRHIZAL NETWORKS AND DISTANCE FROM MATURE TREES ALTER PATTERNS OF COMPETITION AND FACILITATION IN DRY DOUGLAS-FIR FORESTS

4.1 Introduction

Competition is widely accepted as the principal factor structuring plant communities (Tilman, 1982), whereas facilitation has been considered of secondary importance in most ecosystems (Bruno et al., 2003). Plants usually compete with other plants or soil microbes for resources, but their establishment can be facilitated by a broad suite of organisms, from pollinators to animals to soil organisms (Molles & Cahill, 2007). Although facilitation has been examined previously (Phillips, 1909), most studies predated the common use of experimental design in the field and thus received little critical testing (Bertness & Callaway, 1994). More comprehensive experiments that include multiple interactions are required to inform ecological theory for a more accurate and inclusive understanding of processes that regulate patterns in natural plant communities (Bruno et al., 2003).

In mature temperate forests, where canopy and subcanopy trees interfere with many neighboring trees and plants, belowground interactions are considered as important as aboveground interactions (Casper & Jackson, 1997; Canham et al., 2006). When seedlings preferentially establish near mature trees, a so-called “nurse-protégé”, or commensalism interaction is thought to occur, where seedlings benefit from the trees but the trees are unaffected. This type of facilitation typically occurs because a neighboring tree modifies the physical and/or biotic conditions either above- or belowground, leading to positive effects on seedlings (Bertness & Callaway, 1994). Potential benefits to seedlings of establishing near a mature tree include; i) buffered air or soil temperatures; ii) greater water availability; iii) greater soil nutrient availability (“island of fertility” phenomenon (Flores & Jurado, 2003)); iv) better soil aeration; v) protection from herbivores; vi) reduced soil erosion (Flores & Jurado, 2003), and vi) greater access to beneficial root symbionts (Kranabetter, 1999; Dickie et al., 2002).

1 A version of this chapter has been submitted to Oecologia and is now in revision: Teste, FP and Simard, SW. Mycorrhizal networks and distance from mature trees alter patterns of competition and facilitation in dry Douglas-fir forests.
Most research examining the effects of established plants on seedlings have decoupled competitive (Tilman, 1982; Packer & Clay, 2003) from facilitative (Callaway, 1995) interactions, but only a few studies have determined their net effect under natural or experimental conditions (Callaway & Walker, 1997; Dickie et al., 2005; Kennedy & Sousa, 2006). Generally these studies demonstrate that facilitation is important to seedling establishment in arid and semi-arid ecosystems, but this may be a publication bias since most facilitation studies have been conducted in these ecosystems (Flores & Jurado, 2003). Facilitation may also occur in more productive ecosystems, but to our knowledge this research has been limited, possibly because facilitative effects are more transient, dynamic or difficult to detect (Simard et al., 2006). Nevertheless, there is increasing evidence that mature trees in northern forests can facilitate seedling survival (Perry et al., 1989; Dawson, 1993; Klinka et al., 2004). Moreover, facilitative interactions are expected to become increasingly important to seedling recruitment in forest ecosystems of North America over the next century as they are subject to warming and drying conditions associated with climate change (Gomez-Aparicio et al., 2004; Hamann & Wang, 2006).

Clearcutting is a common harvesting practice in the dry Douglas-fir forests of North America, but mature trees are sometimes retained for their biodiversity values (Newsome et al., 1991). Little attention has been paid, however, to the spatial influences of the mature trees on regenerating seedlings (Scholes & Archer, 1997). Previous studies on spatial patterns of competition (Tilman, 1988) and facilitation (Callaway, 1995) have either dichotomously compared “near” and “far away” distance effects or have established transects on a distance scale (Dickie et al., 2005) that extends far beyond the belowground influence of trees. There is a need for more focused studies that identify the presence of a critical proximity where there are net positive or negative effects within the rooting zone of mature trees.

Ectomycorrhizas (EM) are integral with a tree’s life history and they function as the tree’s primary nutrient absorbing organ (Smith & Read, 1997). Ectomycorrhizal fungi can determine the structure and dynamics of plant communities and are major players of belowground plant interactions (Fitter, 2001). For example, establishment of southern beech seedlings occurred only within the rooting zone of adult southern beech
trees in New Zealand (Baylis, 1980). Baylis (1980) noted that EM fungi played an important role in the expansion of southern beech forests since the fungi also were restricted to the rooting zone of adult southern beech trees. A mycorrhizal network (MN) is comprised of fungal hyphae that connect the roots of two or more plant roots, and can involve one or more fungal species (Simard & Durall, 2004). There is increasing evidence that MNs can facilitate seedling establishment near trees directly by promoting resource sharing (e.g., carbon (C), nutrient, and water transfer) or indirectly by mediating seedling colonization by later successional EM species (Newman, 1988; Selosse et al., 2006; Twieg et al., 2007); however, supportive field studies are scarce (Simard & Durall, 2004). To our knowledge, no study has attempted to determine the relative contributions of mature tree competition and EM facilitation on net seedling performance.

Our objective was to examine how competitive and facilitative interactions between mature trees and seedlings vary with seedling-tree proximity and access to a MN. The following hypotheses were tested: i) seedling productivity will increase with greater access to a MN; ii) seedling productivity will increase with proximity to mature trees; iii) the increased productivity will result from greater facilitation by MNs than competition for resources; iv) seedling nutrient uptake will increase with proximity to mature trees as predicted by the “island of fertility” phenomenon (Flores and Jurado 2003). We expect there will be a localized zone of net benefit to seedlings immediately outside the crown of the mature tree as a result of greater facilitation than competition. Under the crown, the competitive effects of mature tree roots will outweigh their beneficial MN effects.

4.2 Methods

4.2.1 Site description and experimental design

The study sites were located within the Dry, Cool Interior Douglas-fir (IDF) biogeoclimatic subzone (IDFdk) north of Kamloops, British Columbia, Canada. This area is near the northern-most limit of interior Douglas-fir in North America (Hosie, 1990), where precipitation ranges from 300 to 500 mm annually, of which less than half falls during the growing season (Lloyd et al., 1990). On our study sites, tree canopies were dominated by interior Douglas-fir (Pseudotsuga menziessi var. glauca (Beissn.)
Franco) and understories were dominated by arbuscular mycorrhizal (AM) pinegrass (Calamagrostis rubescens Buckl.), nodding onion (Allium cernuum Roth) and white hawkweed (Hieracium albiflorum Hook.).

Six study sites were selected in spring 2004 and are characterized in detail in Teste et al. (in review; Chapter 3). In brief, the sites had a mean elevation of 1147 m, southerly aspect, and 14% slope. All sites had been logged 1-11 years previously using variable retention harvesting, where a low density of suppressed but mature Douglas-fir trees (average 10 m tall, 93-yrs-old) were retained throughout. The sites were separated by 1-25 kms. On each site, residual Douglas-fir trees were numbered and then four were randomly selected and assigned to one of five mesh treatments (mesh bags (15 cm diameter; 35 cm depth) with 0 µm (impermeable), 0.5 µm, 35 µm or 250 µm pore sizes; or no mesh bag) (see Table 4.1 for tree characteristics). Douglas-fir seedlings were then planted at four randomly selected distances (0.5 m, 1.0 m, 2.5 m, 5.0 m) from the residual tree into one of these mesh treatments containing carefully excavated soil. The four distance locations were offset from one another to avoid spatial interdependence. These treatments were organized in a split plot design with six replications, where the whole plot factor was mesh pore size and the split plot factor was distance from the residual tree. Planted interior Douglas-fir seedlings (Seedlot # 42309, British Columbia Ministry of Forests and Range, Tree Seed Center, Surrey, BC, Canada) had been commercially grown in a nursery for one year in 512A styrobloks™ (Beaver Plastics, Edmonton, AB, Canada).

The purpose of the mesh treatments was to restrict seedling access to an MN. Seedlings planted in a 0.5 µm mesh bag could not be colonized by the tree’s MN, nor could hyphal anastomosis occur between the tree and seedling because the pores were too small for hyphal penetration (Teste et al., 2006). Seedlings planted in 35 and 250 µm mesh bags could form an MN by single hyphae or rhizomorphs plus hyphae, respectively. Seedlings planted directly into soil (without mesh bags) could form hyphal MNs, rhizomorph MNs, or short-distance hyphal connections between ‘contact’ exploration type EM (Agerer, 2001). The occurrence of short-distance MN connections were considerably reduced in the 250 µm mesh treatment because of the thickness (~0.32 mm) of the nylon mesh was greater than the typical length (0 - 0.25 mm) of the extraradical
hyphae or cystidia of EM ‘contact’ exploration types found in this study. Seedlings planted into impermeable bags grew in isolation and served as indicators of maximum growth potential without interference from mature tree roots. Since we observed no occurrence of root grafting, comparisons between the no mesh and 0.5 µm mesh treatments indicated the effect of the MN on seedlings performance. In a previous study, we determined that the potential for MNs to occur between residual trees and seedlings was very high on these sites (Teste et al., in review; Chapter 3). The purpose of the four distance treatments was to assess the spatial influence of resource competition and facilitation by residual trees on planted seedling performance.

A wedge-shaped area (35 m²) was trenched around each tree in a southerly direction to a soil depth of 50 - 70 cm in order to exclude the influence of surrounding tree roots. Trenches were lined with heavy polyethylene and refilled with excavated soil. All vegetation in the wedge-shaped area was clipped regularly throughout the first growing season and at the beginning of the second growing season to minimize shading effects on seedlings. Where necessary, nearby trees were felled to eliminate any additional root or light competition. Douglas-fir seedlings were grown in containers for one year prior to planting; they were non-mycorrhizal at the time of planting and ranged in height from 13 to 30 cm.

4.2.2 Survival, growth, and physiology

Survival, height, and root-collar diameter were assessed for all seedlings at the end of August in 2004 and 2005. All seedlings were destructively sampled between August 26-30, 2005, their shoots severed from roots (see below), and both tissues oven-dried at 70°C for 48 hours prior to weighing.

Gas exchange measurements were conducted with a CIRAS-1 portable photosynthesis system (PP Systems Inc., Amesbury, MA, USA) attached to a clear cylindrical conifer leaf cuvette (PLC5 (A), PP Systems Inc., Amesbury, MA, USA). Measurements were conducted on all trees and seedlings at all sites in June, July, and August of 2005 from 10:00 to 15:00 on days with a photosynthetically active radiation (PAR) greater than 1000 µmol m⁻² s⁻¹. Ambient air temperature typically ranged from 20 to 30 ºC. During measurements, carbon dioxide (CO₂) concentration of air entering the
cuvette was kept constant at 400 μmol mol$^{-1}$ with a fixed air-flow rate of 400 μmol s$^{-1}$. Temperature inside the cuvette generally ranged from 25 to 35 ºC. For seedlings, measurements were made on attached lateral shoots (current-year needles) when possible, and the needles were arranged in the cuvette such that self-shading was minimized. Gas exchange measurements were recorded only when photosynthesis and transpiration rates inside the cuvette were steady for at least 90 s. For trees, we measured gas exchange rates using a cut-branch technique (Watts & Neilson, 1978; Dang et al., 1997), where south-facing branches in full sun were detached with extendable pruners. The stem of a falling branch was immediately placed under water. The branch stem was then re-cut under water and remained in the vial of water until gas exchange measurements were completed (typically within 15 minutes). Needles inside the cuvette were harvested in August and kept frozen until further processing. One-sided leaf area was measured with a LI-3100 leaf area meter (LICOR Inc., Lincoln, NE, USA). Intrinsic water-use efficiency (WUE) was calculated as the ratio of CO₂ assimilation to H₂O transpiration.  

Chlorophyll fluorescence was measured with a hand-held chlorophyll fluorometer OS-30p (Opti-Sciences Inc., Tyngsboro, MA, USA) on the same seedlings and during the same days as the gas exchange measurements. Two attached needles were placed together under special leaf clips and dark-adapted for 30 minutes; they were then flashed with a high intensity actinic light (1000 μE) for 2 s with a modulation intensity of 2. The OS-30p fluorometer automatically measured minimum fluorescence yield ($F_o$), maximum fluorescence yield ($F_m$), and calculated the ratio of variable to maximum fluorescence ($F_m - F_o/F_m$), also known as the quantum efficiency of open photosystem II centers. The ratio of variable to maximum fluorescence is highly correlated with the rate of carbon assimilation and can serve as an indication of the plant’s overall photosynthetic performance (Maxwell & Johnson, 2000). 

Predawn (3:30 – 6:00) and midday (11:00 – 14:00) seedling twig water potential ($ψ_{seedling}$) were measured with a pressure chamber (Plant Water Status Console Model 3005, Soil Moisture Equipment Corporation, Santa Barbara, CA, USA). A small lateral branch was clipped from the seedling and immediately inserted into the pressure chamber. Pressure was slowly increased then recorded when the first sap droplet
appeared from the severed xylem tracheids. Measurements were taken twice on all Douglas-fir seedlings: August 4-6 and August 20-21, 2005.

4.2.3 Soil and root sampling

From August 26 to 30, 2005, soil cores (10 cm diameter, 20 cm deep) were sampled approximately 2 cm from the outer rim of the mesh bags in the four cardinal directions (North, South, West, and East). For seedlings planted directly into the soil (i.e., without mesh bags), soil cores were taken only after the seedlings were first carefully excavated. Seedling roots had grown approximately 8 cm radially and to a depth of 30-40 cm. All soil core and seedling root samples were placed in plastic bags and stored at 4°C until further processing. For the soil cores, soil was sieved with 1 and 4 mm sieves and the roots plucked out. All roots were oven-dried at 70°C for 48 hours then weighed. Tree root density (g cm⁻³) around each seedling was expressed as root mass divided by the volume of soil from which the roots originated.

Mineral soil samples were air dried and sieved (2 mm). Soil analyses were carried out by the Analytical Laboratory in Victoria, BC using the methods outlined in Tiessen & Moir (1993) for total C, McGill & Figueiredo (1993) for total N, Kalra & Maynard (1991) for total P, and soil pH in H₂O.

4.2.4 Foliar nutrient analysis

All needles from were removed from shoots of all seedlings, dried overnight at 70°C, weighed, and then sent to the Analytical Laboratory in Victoria, British Columbia (B.C. Ministry of Forests and Range, Research Branch) for analysis of total N, P, S, Ca, Fe, B, K, Mg, Mn, Zn, Cu, and C (Kalra & Maynard, 1991).

4.2.5 Carbon isotope analysis

With the aid of a dissecting microscope, sturdy forceps, and fine-textured sandpaper, dry wood (early and late wood) was removed from the 2005 growth-ring on all trees and seedlings. For the trees, the 2005 early- and late-wood sections were removed from cores that had previously been sampled in the field, then manually ground. For the seedlings, the first 10 cm above the root-collar was sectioned and then manually ground. After homogenizing the powder, we sampled 1.5-2 mg of wood tissue for C isotopic
composition. The samples were combusted, CO$_2$ was liberated, and this was analyzed for the ratio $^{13}$C/$^{12}$C with a continuous flow Europa Hydra 20/20 isotope ratio mass spectrometer at the UC Davis Stable Isotope Laboratory (Davis, CA, USA). The C isotope ratio ($\delta^{13}$C) was calculated as:

$$\delta^{13}C = \left( \frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{standard}} - 1 \right) \times 1000$$

in ‰ units, where the sample ratio is relative to the Vienna-PeeDee Belemnite (V-PDB) standard ($1.1237 \times 10^{-2}$). The resulting $\delta^{13}$C values were used as a relative index of WUE (Pate, 2001) for the 2005 growing season.

4.2.6 Statistical analyses

All statistical analyses were carried out using the R statistical environment for statistical computing and graphics (R Development Core Team, 2006). Univariate analyses and linear mixed-effects model fitting were performed in R with the stats (R Development Core Team, 2006), nlme (Jose Pinheiro et al., 2007), and lme4 (Bates, 2007) packages. The survival data was analyzed with a mixed effect logistic regression using the lme4 function (family set to binomial) of the lme4 package (Bates, 2007). The split plot design was analyzed using a linear mixed-effects model (Pinheiro & Bates, 2000), where sites were used as blocks (n=6) and set as the random factor. The whole plot and split plot fixed factors were the mesh and distance treatments, respectively.

Planted seedlings representing the mesh and distance treatment combinations were the experimental units. Statistical analyses examining the distance effect on net productivity considered only seedlings with some access to the MN (i.e., all seedlings except the ones growing in the 0.5 μm mesh bag); here, we were interested in defining the zone of net benefit, requiring that seedlings were affected by both competition and facilitation. Performance of isolated seedlings (impermeable bags) was compared to that of all other treatment seedlings combined using t-tests. Tree root density was compared among distances using a randomized complete block design, where distance was the fixed factor and block was the random factor. In this case, the experimental units were the sum of the four soil core subsamples taken in proximity to each seedling. Since we incorporated time as a factor in the seedling physiological measurements, the data was considered a
repeated measures design. Given that our data were unbalanced because of seedling mortality, we used a mixed-effects model approach (Pinheiro & Bates, 2000), while inducing the best fit serial correlation structure for the model (Schabenberger & Pierce, 2002). Here, the whole plot, split plot, and split-split plot factors were mesh, distance, and time treatments, respectively, with blocks as the random factor (i.e., split-split plot layout). The lme function (method set to restricted maximum likelihood) was used to fit the linear mixed-effects models. Variance partitioning and the significance of the fixed effects were determined with functions, anova and summary of the fitted lme objects. Where a significant ($P \leq 0.05$) fixed effect was found, we used the Bonferroni method for pairwise mean difference comparisons using 95% simultaneous confidence intervals (SCI). The plotted SCI concurrently assessed: i) which means were significantly different, ii) the effect size, iii) precision, and iv) the range of plausible values for the population (Gardner & Altman, 2000).

4.3 Results

4.3.1 Survival, growth, and nutrient uptake

Mesh and distance interacted significantly to affect seedling survival ($P = 0.03$). Logical mean comparisons (elicited by an interaction plot, not shown) indicate that seedlings growing in the 0.5 µm mesh at 5.0 m had significantly lower survival than seedlings having full access to the MN (no mesh) at the same distance (Fig. 4.1). Furthermore, when averaged across distance treatments seedling survival increased from 81% in the 0.5 and 35 µm mesh treatments to >95% in the no mesh or 250 µm treatments; this increase was large but not significant ($P = 0.20$) (Fig. 4.1). Seedlings growing at 2.5 m from mature trees tended to have the highest survival (96%) on average ($P = 0.38$).

Mesh treatment had no effect on two-year seedling growth, shoot or root biomass, or foliar nutrient contents, but distance did significantly affect these variables (Table 4.2). Seedling height (Fig. 4.2), shoot biomass (Fig. 4.3), root biomass (Fig. 4.3) and needle biomass (Fig. 4.2) were lowest at 0.5 m (optimum at 2.5 m and 5.0 m) distance from mature trees. As a group, seedlings that were interacting with mature trees had greater total heights (t-value = 4.72, $P < 0.01$) and diameters (t-value = 22.88, $P = < 0.01$) than
seedlings growing in isolation (impermeable bags). Similarly, total foliar N, P (Fig. 4.5), S, Ca, Fe, B, K, Mg, Mn, Zn, Cu, and C (data not shown) content of seedlings were greatest at distances of 2.5 from mature trees. This result suggests that nutrient uptake was greater at the intermediate distances and correlated with the associated height, shoot and needle biomass peaks at the same distance. Root density of mature trees decreased with distance from the bole (Fig. 4.4).

4.3.2 Physiological responses

Predawn twig water potential tended to increase slightly with mesh size (Table 4.3). Seedlings with full access to the MN had a mean \( \psi_{\text{seedling}} \) of -0.45 MPa (CI 0.17 MPa) compared to -0.67 MPa (CI 0.17 MPa) for seedlings in 0.5 \( \mu \)m mesh bags, suggesting that MNs helped seedlings recover overnight from day-time water stress. None of the other physiological responses were affected by the mesh treatments (Table 4.3). Time of measurement significantly affected all of the physiological responses, where \( \text{CO}_2 \) assimilation and transpiration rates were highest in July and instant WUE, midday water potential, and chlorophyll fluorescence were highest in August. The interaction between mesh treatment and time was diagnosed with an interaction plot and was ignored because it was visually weak. Seedlings growing closest to mature trees had significantly lower \( \text{CO}_2 \) assimilation rates and seedling wood \( \delta^{13}\text{C} \) (hence, lower seasonal WUE) compared to seedlings growing at further distances (Fig. 4.6).

4.3.3 Soil moisture and soil nutrients

There was no difference in soil moisture content between the inside and outside of the mesh bags (Table 4.4). These results agree with a small pot experiment, which clearly showed that soil water moved freely across the fine 0.5 \( \mu \)m mesh within minutes. These results indicated that the fine mesh bags allowed unrestricted movement of soil water. Soil carbon and nitrogen concentration tended to be greater inside the fine than coarser mesh bags (Table 4.4).

Soil moisture content inside the mesh bags increased with distance from mature trees (Table 4.5) and was significantly lower at 0.5 m and 1.0 m than at 5.0 m (Fig. 4.7). Soil carbon, nitrogen, phosphorus, and pH did not vary with distance (Table 4.5).
4.4 Discussion

4.4.1 Mycorrhizal network effects

Linking into an MN with large trees tended to improve water relations, but the mesh treatment effects were weak. Mycorrhizal networks improved survival, but only at the farthest distance from the tree. Supporting evidence that MNs played a role in mediating tree-seedling interactions comes from molecular analysis of the shared EM community and the sympatric distributions of the tree and seedling roots, which together indicate high potential for MNs to exist on these sites (Teste et al., in review; Chapter 3). Notably, survival increased from 60% to 100% at 5 m where seedlings had full access to the MN, whereas survival generally remained below 83% where MN access was restricted (across all distances). This suggests that mature trees were highly competitive under their crowns, but beyond the dripline, access to the MN resulted in a net positive benefit to survival. The significant interaction between MN and distance also indicated that full access to MNs increased seedling survival 5.0 m from mature trees; at this furthest distance from the tree, we expect that soil resource competition from understory plants was most suppressive to the establishing seedlings (Delong et al., 2005). These results demonstrate that MN effects in dry forests are spatially complex, just as are the distributions of EM fungal species (Erland & Taylor, 2003).

Recently, using a similar approach to our study, McGuire (2007) found that restricting MN access with mesh pots reduced seedling performance in a tropical EM monodominant rain forest. She germinated seeds directly in mesh pots, in contrast with our study, where we planted large, commercially grown, one year-old seedlings. Our large caliber seedlings are less prone than germinants to nutrient and water stress during their first year in the field (Newsome et al., 1991), which is when the MN effect is likely most important in alleviating drought-caused mortality (Simard & Durall, 2004; Gomez-Aparicio et al., 2004). We suggest that germinating seeds are more affected by MNs than nursery-grown seedlings planted in the field (McGuire, 2007).

Nara (2006a) also assessed the facilitative effects of MNs associated with established plants on nearby seedling establishment. Whereas Nara (2006a) avoided de novo formation of mycorrhizas from spores to ensure that seedlings established within the
existing MN, we physically restricted access to the MN using mesh bags. We suggest that Nara (2006a) provided evidence for a “MN-inoculation effect” (or indirect MN effect), rather than a direct “MN effect” that could include nutrient transfers (Taylor, 2006), because the MN of the established plants served to inoculate nearby seedlings. The resulting mycorrhization likely accounted for the increase they observed in seedling performance. Seedlings in our study could form mycorrhizas from spores, sclerotia, hyphal fragments, and severed tree EM tips from the soil in which they were planted, and hence did not rely on the MN for colonization. Under these conditions, the increase in seedling performance could only be attributable to a direct MN effect.

The increase in soil carbon and nitrogen concentration that resulted from decomposition of the severed roots inside the fine mesh bags (0.5 µm) may have affected seedling performance (McPhee & Aarssen, 2001). However, seedling survival and water relations were suppressed in the fine mesh relative to the other treatments, suggesting there was no beneficial effect of the greater nutrient availability.

4.4.2 Proximity to mature trees

Our second hypothesis, that seedling productivity increases with proximity to mature trees, was rejected because seedling survival, height, shoot and needle biomass were greatest at the intermediate and larger distances. Seedling root biomass, by contrast, was greatest at the furthest distance from the mature trees, corresponding with declining mature root densities. The growth peak at 2.5 and 5.0 m was outside the enriched drip line of the tree canopies (approx. 1 m from the bole) (Scholes & Archer, 1997); at those distances, there was an increase in total available resources due to the absence of the focal tree canopy interception of water as well as declining mature root competition for soil water and nutrients. Notably, focal tree light interception was not a factor in this study because seedlings were planted in a southerly direction on generally south-facing slopes, where, at this latitude, shade rarely occurred. Dickie et al. (2005) also found maximal seedling growth at intermediate distances from the edge of an oak forest. Intraspecific EM studies generally show that seedlings have greater survival (McGuire, 2007), growth (Nara & Hogetsu, 2004; Nara, 2006b), and EM colonization (Borchers & Perry, 1990; Dickie et al., 2002; Nara & Hogetsu, 2004) when establishing near adult trees compared
to seedlings removed from any direct effect of the focal tree. Seedlings that were grown in isolation in this study were significantly less productive, suggesting that proximity to mature trees facilitated their establishment.

Close proximity to adult trees has been shown to reduce the survival of conspecific seedlings (Packer & Clay, 2003), but it has proven beneficial under certain circumstances (Newbery et al., 2000). Facilitation of conspecific seedling establishment by MNs could result in kin-selection, where seedlings benefit from connection to the larger fungal network of neighbouring parent trees (Sellosse et al., 2006). This may be particularly relevant in old forests characterized by closed canopies and small gap disturbances, or in old forests subject to frequent, mixed disturbances where establishment conditions are uncertain. The latter condition characterizes our monospecific Douglas-fir forests.

Typically, increased nutrient levels (“island of fertility” phenomenon) are found in proximity to “nurse-plants” and can be an important mechanism underlying increased seedling establishment (Flores & Jurado, 2003; Dickie et al., 2007). In our study, seedling nutrient uptake was greatest at intermediate distances from mature trees, refuting our fourth hypothesis that nutrient uptake increased with proximity to mature trees. It is possible in our forests that the acidic litter that accumulates under the tree crown reduced nutrient availability (Prescott et al., 2004). A similar spatial pattern of foliar N and P has also been found in oak forests (Dickie et al., 2005). By contrast, most studies have found greater levels of foliar N and P in EM seedlings growing adjacent to mature EM trees (Baylis, 1980; Borchers & Perry, 1990; Dickie et al., 2002; Nara & Hogetsu, 2004; Nara, 2006a,b).

Close proximity to mature trees affected seedling physiology throughout the growing season. Photosynthetic rates and seasonal WUE were greater among seedlings furthest away from mature trees compared with those that were closest. Our results contrast with Dickie et al. (2007), who found that oak seedlings growing in soil collected 1 m away from adult oak trees had higher photosynthetic rates than seedlings in control soil. In our study, soil moisture content decreased with proximity to mature trees and this was coupled with the lowest photosynthetic rates and seasonal WUE, suggesting that resource competition for soil N was highest adjacent to the trees. Given that MN
potential was also high near the mature trees, it is possible that either water or carbon transfer (i.e., resource sharing) occurred from the mature trees to seedlings, partly compensating the lowered CO$_2$ assimilation and transpiration (Simard et al., 1997; Querejeta et al., 2003). These spatial patterns probably change over time as seedlings develop, highlighting the importance of quantifying signals like wood $\delta^{13}$C to provide a historical record of plant stress during a growing season or over the full seedling establishment period (Pate, 2001).

4.4.3 Facilitation and competition dynamics

Douglas-fir seedlings growing within the rooting zone of mature Douglas-fir trees experienced facilitative and competitive effects attributable to belowground processes. Facilitation was likely the result of an increase in EM status, MN potential (Teste et al., in review; Chapter 3), and perhaps resource sharing. At the same time, competition outside the crown dripline was for soil nutrients and soil water, but not for light, since all other vegetation was removed; this belowground competition caused a decrease in growth and physiological activity of nearby seedlings. Inside the dripline, seedlings were deprived of soil resources because of crown interception and competition. At intermediate distances, seedling growth and nutrient uptake peaked, indicating there existed a zone of net benefit, where facilitation benefits outweighed competition costs (Fig. 8). This is illustrated in the graphical model show in Fig. 8, which assumes that: i) facilitation and competition decrease exponentially with distance from trees (McGuire, 2007; Packer & Clay, 2003), ii) competitive effects outweigh facilitative effects at close distances in these arid ecosystems (Callaway et al., 2002), iii) competition intensity is a function of root density, such that root competition doesn’t exist where root density declines to zero (Schenk, 2006), and iv) facilitative effects decline less dramatically with distance than competitive effects, and can extend even further away due to the extended mycelial frontier (Smith et al., 2003; Taylor, 2006). Hence, competition and facilitation are spatially disjunct (Dickie et al., 2005). A similar graphic model was proposed by Dickie et al. (2002), and later validated by Dickie et al. (2005), but it focused on variation in facilitation and competition interactions with tree density rather than with proximity.
Douglas-fir forest encroachment into grasslands is driven partly by facilitation (Kennedy & Sousa, 2006), but also partly by changing climate and disturbance patterns (Arsenault & Klenner, 2004). Our model shows how competition and facilitation vary spatially and where seedling establishment is most likely to succeed. It could be used to inform larger, spatially explicit models predicting the dynamics of forest encroachment into grasslands or predicting forest recovery following disturbance (Canham et al., 2004; Hamann & Wang, 2006).

4.4.4 Conclusions

This study provides support for the existence of a zone of net benefit for seedling establishment in the proximity of mature trees in dry forest ecosystems. Facilitation and competition simultaneously affected Douglas-fir seedlings growing at various distances from within the rooting system of mature Douglas-fir trees. Our results suggest that competition for soil resources was more intense than facilitation at close proximity, but this was relaxed at intermediate distances. We present evidence for beneficial MN effects on seedling survival and water status, however further research is needed to verify these results. The role of conspecific trees indirectly facilitating seedling establishment via an increase in EM colonization has been investigated previously (Dickie et al., 2002; Dickie et al., 2005), but we extend these to include the important role of MNs on EM seedling regeneration within AM-dominated communities. Our results have important implications for tree establishment in harvested sites dominated by native AM herbaceous plants, grasslands encroaching on bordering forests with climatic or disturbance regime shifts, or degraded sites invaded by exotic AM weeds. Retaining conspecific mature trees following disturbance at a sufficient spatial distribution for regeneration within zones of net benefit in these naturally patchy forests may become increasingly important with changing climatic conditions.

Acknowledgements

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process. We are grateful to Dr. Robert Guy for his valuable advice on the physiological measurements and the carbon isotope analyses. Tony Kozak and Val LeMay provided advice on data analysis. Funding was provided by a Forest Sciences Program of Forest Investment Innovation of British Columbia grant, a Canadian Foundation for Innovation grant, a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant to SWS, and an NSERC PGS scholarship to FPT. We declare that the experiments comply with the current laws of the country in which they were performed.
Table 4.1 Mature interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) tree characteristics. Values for tree characteristics are means and one standard deviation in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Mean values for 2005</th>
<th>June</th>
<th>July</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>93 (27)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height (m)</td>
<td>10.1 (3.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DBH (cm)</td>
<td>11.5 (2.6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Assimilation (μmol CO₂ m⁻² s⁻¹)</td>
<td>4.58 (3.09)</td>
<td>4.34 (2.12)</td>
<td>3.19 (2.13)</td>
<td>6.33 (3.95)</td>
</tr>
<tr>
<td>Transpiration (mmol H₂O m⁻² s⁻¹)</td>
<td>0.97 (0.58)</td>
<td>1.01 (0.57)</td>
<td>0.89 (0.62)</td>
<td>1.00 (0.56)</td>
</tr>
<tr>
<td>Intrinsic WUE (μmol CO₂ mol⁻¹ H₂O)</td>
<td>5.50 (3.83)</td>
<td>4.48 (2.14)</td>
<td>4.24 (2.14)</td>
<td>7.91 (5.63)</td>
</tr>
<tr>
<td>δ¹³C (%)</td>
<td>-24.50 (0.90)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

WUE = water use efficiency, DBH = diameter at breast height.
Table 4.2  Analysis of variance table for fixed effects of mesh size, distance from mature tree and mesh x distance interaction on productivity of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings after two years in the field.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Height increment</th>
<th>Diameter increment</th>
<th>Needle biomass</th>
<th>Shoot biomass</th>
<th>Root biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>F</em></td>
<td><em>P</em></td>
<td><em>F</em></td>
<td><em>P</em></td>
<td><em>F</em></td>
</tr>
<tr>
<td>Mesh (M)</td>
<td>3</td>
<td>0.90</td>
<td>0.4669</td>
<td>1.44</td>
<td>0.2774</td>
<td>1.56</td>
</tr>
<tr>
<td>Distance (D)</td>
<td>3</td>
<td>5.41</td>
<td>0.0028</td>
<td>10.66</td>
<td>&lt;0.0001</td>
<td>8.19</td>
</tr>
<tr>
<td>M x D</td>
<td>9</td>
<td>0.62</td>
<td>0.7742</td>
<td>0.34</td>
<td>0.9557</td>
<td>1.12</td>
</tr>
</tbody>
</table>
Table 4.3  Analysis of variance table for fixed effects of mesh size, distance from mature tree, time, and interactions on physiological responses of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) seedlings after two years in the field.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>CO₂ assimilation</th>
<th>Transpiration</th>
<th>Chlorophyll fluorescence</th>
<th>Instant WUE</th>
<th>δ¹³C</th>
<th>Predawn water potential</th>
<th>Midday water potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Mesh (M) 3</td>
<td>2.07</td>
<td>0.1470</td>
<td>1.31</td>
<td>0.3066</td>
<td>0.15</td>
<td>0.9276</td>
<td>1.15</td>
</tr>
<tr>
<td>Distance (D) 3</td>
<td>5.89</td>
<td>0.0017</td>
<td>2.22</td>
<td>0.0976</td>
<td>1.31</td>
<td>0.2818</td>
<td>0.47</td>
</tr>
<tr>
<td>Time (T) 2</td>
<td>10.85</td>
<td>&lt;0.0001</td>
<td>33.72</td>
<td>&lt;0.0001</td>
<td>46.15</td>
<td>&lt;0.0001</td>
<td>20.74</td>
</tr>
<tr>
<td>M x D 9</td>
<td>1.32</td>
<td>0.2502</td>
<td>0.77</td>
<td>0.6480</td>
<td>0.89</td>
<td>0.5408</td>
<td>0.36</td>
</tr>
<tr>
<td>M x T 6</td>
<td>2.40</td>
<td>0.0309</td>
<td>1.01</td>
<td>0.4207</td>
<td>0.44</td>
<td>0.8474</td>
<td>1.69</td>
</tr>
<tr>
<td>D x T 6</td>
<td>2.08</td>
<td>0.0596</td>
<td>1.08</td>
<td>0.3758</td>
<td>1.14</td>
<td>0.3441</td>
<td>0.95</td>
</tr>
<tr>
<td>M x D x T 18</td>
<td>0.62</td>
<td>0.8807</td>
<td>0.56</td>
<td>0.9247</td>
<td>0.58</td>
<td>0.9060</td>
<td>0.42</td>
</tr>
</tbody>
</table>

105
Table 4.4  Effect of mesh treatment on the mean difference between inside and outside mesh bag soil moisture content, soil total carbon (C), soil total nitrogen (N), soil available phosphorus (P), and soil pH. The mesh fixed effect P-values generated from the mixed-effect model fits are shown.

<table>
<thead>
<tr>
<th>Mesh (μm)</th>
<th>Soil moisture difference (g kg(^{-1})) 95% CI</th>
<th>Soil C difference (g kg(^{-1})) 95% CI</th>
<th>Soil N difference (g kg(^{-1})) 95% CI</th>
<th>Soil P difference (mg kg(^{-1})) 95% CI</th>
<th>Soil pH difference 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.9 -2.0 to 3.9</td>
<td>481 207 to 756</td>
<td>16.9 7.1 to 26.7</td>
<td>-7 -55 to 40</td>
<td>-0.24 -0.62 to 0.13</td>
</tr>
<tr>
<td>35</td>
<td>0.3 -2.7 to 3.3</td>
<td>149 -111 to 408</td>
<td>6.1 -3.2 to 15.3</td>
<td>-44 -89 to 1</td>
<td>0.03 -0.33 to 0.38</td>
</tr>
<tr>
<td>250</td>
<td>-0.7 -3.5 to 2.0</td>
<td>109 -126 to 345</td>
<td>4.9 -3.5 to 13.3</td>
<td>-38 -79 to 3</td>
<td>-0.10 -0.42 to 0.22</td>
</tr>
</tbody>
</table>

\(^{a}\)kg of H\(_2\)O per kg of soil

\(^{b}\)95% confidence interval (CI); when zero is not included in the 95% CI then the mesh treatment effect is considered statistically significant.
Table 4.5  Analysis of variance table for fixed effects of mesh size, distance from mature tree, and the interaction between mesh and distance on soil moisture, soil total carbon (C), soil total nitrogen (N), soil available phosphorus (P), and soil pH measured inside the mesh bags. Analysis of variance assumptions were met after a logarithmic transformation of total C, total N, and available P.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Moisture df</th>
<th>F</th>
<th>P</th>
<th>Total C df</th>
<th>F</th>
<th>P</th>
<th>Total N df</th>
<th>F</th>
<th>P</th>
<th>Available P df</th>
<th>F</th>
<th>P</th>
<th>pH df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesh (M)</td>
<td>2</td>
<td>0.03</td>
<td>0.9674</td>
<td>3</td>
<td>0.95</td>
<td>0.4427</td>
<td>0.42</td>
<td>0.7414</td>
<td>0.44</td>
<td>0.7309</td>
<td>2.00</td>
<td>0.1611</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance (D)</td>
<td>3</td>
<td>5.73</td>
<td>0.0035</td>
<td>3</td>
<td>1.16</td>
<td>0.3349</td>
<td>0.65</td>
<td>0.5880</td>
<td>0.45</td>
<td>0.7156</td>
<td>1.95</td>
<td>0.1349</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M x D</td>
<td>6</td>
<td>0.52</td>
<td>0.7867</td>
<td>9</td>
<td>0.79</td>
<td>0.6234</td>
<td>0.60</td>
<td>0.7893</td>
<td>1.17</td>
<td>0.3355</td>
<td>0.99</td>
<td>0.4619</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1 Mesh and distance treatment effects on *Pseudotsuga menziesii* var. *glauca* seedling survival after two growing seasons. Error bars are one standard error of the mean.
Figure 4.2 Distance effects on height and needle biomass of *Pseudotsuga menziesii* var. *glauca* seedlings. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.3 Distance effects on shoot and root biomass of *Pseudotsuga menziesii* var. *glauca* seedlings. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.4 Distance effects on root density of *Pseudotsuga menziesii* var. *glauca* mature trees. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.5 Distance effects on foliar N and P of *Pseudotsuga menziesii* var. *glauca* seedlings. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.6  Distance effects on photosynthesis and wood $\delta^{13}C$ of *Pseudotsuga menziesii* var. *glauca* seedlings. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.7 Distance effects on soil moisture inside mesh bags. Plots show pairwise mean difference comparisons with 95% simultaneous confidence intervals (SCI) using the Bonferroni method. Numbers at the top of each graph are the four distance treatment means in order (from left to right) of distance (0.5, 1.0, 2.5, 5.0 m).
Figure 4.8  Graphical model highlighting the simultaneous influences of facilitation and competition on seedlings. A zone of net benefit occurs at intermediate distances where facilitation overwhelms rapidly diminishing competitive effects.
4.5 References


**Bates D. 2007.** *lme4: Linear mixed-effects models using S4 classes.* R package version 0.99875-7.


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5 CARBON AND NITROGEN TRANSFER VIA MYCORRHIZAL NETWORKS MAY FACILITATE SEEDLING SURVIVAL

5.1 Introduction

Mycorrhizal networks are fungal hyphae that directly connect roots of two or more plants. There is evidence that MN’s exist (Finlay & Read, 1986; Lian et al., 2006), mediate interplant resource transfer (Lerat et al., 2002; Simard et al., 1997), influence plant competition (Kytöviita et al., 2003), and play a role in ecosystem functioning (Leake et al., 2004; Simard & Durall, 2004; Selosse et al., 2006). The role of MNs in interplant carbon transfer has been controversial (Robinson & Fitter, 1998; Fitter et al., 1999; Perry, 1999; Wilkinson, 1999; Fitter & Robinson, 2000; Whitfield, 2007). Much of the controversy surrounds MNs formed by arbuscular mycorrhizal (AM) plants, where transferred carbon appears to remain in the fungal tissue (Pfeffer et al., 2004). However, studies on plants associated with ectomycorrhizal (EM) fungi have consistently shown that MNs facilitate interplant carbon transfer (Reid & Woods, 1969; Brownlee et al., 1983; Read et al., 1985; Finlay & Read, 1986; McKendrick et al., 2000). The most unequivocal evidence comes from mycoheterotrophic plants, which have been shown to receive ecologically significant amounts of carbon transferred from surrounding trees through MNs (Bidartondo, 2005; Selosse et al., 2006).

If MNs provide an extra source of carbon to carbon-limited receiving EM plants, then an increase in plant fitness may be expected. This extra carbon may be critically important for survival of suppressed EM seedlings in the understory of forests (Read et al., 1985). Evidence for the effects of MNs on seedling establishment (Nara, 2005), survival (McGuire, 2007) and growth (Booth, 2004) suggests they can be beneficial or without consequence (Kranabetter, 2005), but it remains unclear whether carbon transfer is responsible for MN effects on seedling performance.

There is evidence that interplant carbon transfer through MNs is regulated by source-sink relationships between plants (Francis & Read, 1984; Read et al., 1985; Finlay & Read, 1986; Grime et al., 1987; Simard et al., 1997).

¹A version of this chapter will be submitted for publication: Teste, FP, Simard, SW, Durall, DM, Guy, RD, Jones, MD, and Schoonmaker, AL. Carbon and nitrogen transfer via mycorrhizal networks facilitate seedlings survival.
For example, increasing the sink strength (e.g., by shading, clipping or natural senescence) of ‘receiver’ plants has resulted in increased carbon transferred from ‘donor’ plants (Simard & Durall, 2004). It has also been hypothesized that carbon movement in MNs is regulated by the source strength of donor plants (Grime et al., 1987; Pietikäinen & Kytöviita, 2007). Maple seedlings transferred more carbon to trout lilies when their leaves were fully expanded in summer compared to spring, but this also corresponded with increased sink-strength of developing trout lily corms (Lerat et al., 2002). Where increasing source strength of ‘donor’ plants has been manipulated in isolation of sink strength using elevated CO₂, the extent of carbon transfer has not increased (Fitter et al., 1998). Thus, there is a need for more research to clarify the implications of altering source strength on interplant carbon transfer.

Interplant nitrogen transfer can also occur (Selosse et al., 2006 and references therein) but evidence about the relative importance of MNs compared to indirect transfer pathways is lacking (He et al., 2003). The extent of nitrogen transferred also appears to be regulated by source-sink gradients between plants (Simard et al., 2002; He et al., 2004), but the degree of mycorrhization (van der Heijden, 2002) and levels of other macronutrients in the soil (Ekblad & Huss-Danell, 1995) are also important factors. Recently, nitrogen transfer has been observed between non-nitrogen fixing EM and AM plants (He et al., 2006), suggesting that MNs are not always necessary, since hyphal fusion between Glomeromycete and Basidiomycete/Ascomycete fungi is unlikely. These studies do not rule out a role for MNs, but rather, demonstrate that indirect pathways exist. More research separating the contribution of MN and indirect transfer pathways to nitrogen transfer is needed.

The main objective of this study was to determine whether access to a MN enhances seedling establishment and carbon or nitrogen transferred between EM plants, and whether this varies by donor tree size. The following hypotheses were tested: greater access to a MN results in greater i) seedling survival, ii) seedling growth and physiological responses, iii) carbon and nitrogen transfer from donor to receiver, and iv) the magnitude of carbon transferred is positively correlated with donor tree size.
5.2 Material and methods

5.2.1 Site description and experimental design

The full description of the study site can be found in Teste et al. (2006). The study site was selected in spring 2004. The site had been logged one year previously using variable retention harvesting, where advanced regeneration interior Douglas-fir (Pseudotsuga menziesii var. glauca) trees (referred to as ‘donor’ trees hereafter) were retained in low densities throughout (see Table 5.1 for tree characteristics). Over 400 donor trees with heights spanning 0.2 to 2 m tall were identified and numbered, of which 160 were randomly selected and assigned to one of four mesh treatments. In these four treatments, seeds were sown or seedlings were planted into mesh bags with 0.5 µm, 35 µm or 250 µm pore sizes, or directly into soil (no mesh) on May 22, 2004. All were 0.5 m away from the selected donor tree in a direction randomly selected from the four cardinal points (N, S, E, or W).

For 80 of the donor trees, 25 interior Douglas-fir seeds (referred to as ‘in situ seedlings’ hereafter) were manually sown into the surface soil of the randomly assigned mesh bag treatment. Seed predation was excluded by mosquito netting, which was either glued onto the rim of the mesh bags or buried into the surrounding soil for the no mesh treatment. The mosquito netting was removed after germination. For the remaining 80 donor trees, interior Douglas-fir seedlings (referred to as ‘planted seedlings’ hereafter) were planted into the assigned mesh bag treatment. When inserting the mesh bags (15 cm diameter and 35 cm deep), we minimized soil disturbance by carefully excavating the soil into three distinct soil layers (intact forest floor, A horizon, and some of the B horizon) and replacing these layers into the bags in the same order. Prior to placing the seed or planted seedlings in the ground of the no mesh treatment, the surrounding soil was disturbed to ensure initial soil disturbance was consistent across treatments. The seed and seedlings were considered separate experiments, and the four mesh treatments in each were organized in a completely randomized design with 20 replicates.

Seeds (seedlot #48520) were moist-stratified at 4°C for 21 days prior to sowing in the field. Planted seedlings (seedlot #48520, British Columbia Ministry of Forest and Range Tree Seed Center, Surrey, BC, Canada) were grown at the University of British
Columbia (Vancouver, Canada) greenhouse for 6 months in 512B styroblocks™ (Beaver Plastics, Edmonton, AB, Canada), and were non-mycorrhizal and ranged in height from 5 to 19 cm at time of planting.

The purpose of the mesh treatments was to control seedling access to a MN (Robinson & Fitter, 1999). The mesh was also used to assess the capacity for donor trees to actively colonize nearby seedlings via a MN, thus determining the importance of MN-mediated colonization. Seedlings planted in 0.5 µm mesh bags could be colonized by wind- or soil-borne propagules, but not by the donor tree’s MN, nor could hyphal anastomosis occur between fungi extending from donor roots and seedling roots, because the pores were too small for hyphal penetration (Teste et al., 2006). Seedlings planted in 250 µm mesh bags could form a MN by single hyphae or rhizomorphs. In our previous field and lab studies (Teste et al., in review; Chapter 3), we never observed intact rhizomorphs penetrating the 35 µm mesh. We did notice, however, that rhizomorphs were capable of breaking down into an unstructured form at the surface of the 35 µm mesh, thus allowing penetration by small groups of hyphae, but these occurrences were rare. Hence, MN’s formed by seedlings in the 35 µm mesh bags would primarily occur by individual hyphae. Seedlings planted directly into soil (no mesh) could form hyphal and rhizomorph MNs, and their roots were free to intermingle with tree roots, thus permitting short-distance hyphal connections between ‘contact’ exploration type EM (Agerer, 2001). The occurrence of short-distance MN connections was considerably reduced in the 250 µm mesh treatment because the thickness (~0.32 mm) of the nylon mesh was greater than the typical length (0 - 0.25 mm) of the extraradical hyphae or cystidia of EM ‘contact’ exploration types found in this study. Since we observed no occurrence of root grafting, comparisons between the no mesh and 0.5 µm mesh treatments indicated the full potential effect of the MN on seedling performance, resource transfer, and EM colonization.

All mesh bags were made out of sturdy plain-weave nylon (Plastok Ltd., Birkenhead, UK), had a volume of 6185 cm³, and the 0.5, 35, and 250 µm mesh had an estimated percent open pore space of 13.3%, 34.0%, and 54.1%, respectively. Because of concern that fine mesh might impede water flow, we conducted a small pot experiment to test for differences in water flow across the mesh. We found that soil water moved
freely across the 0.5µm mesh within minutes.

A square area (3 m x 3 m) was trenched around each donor tree and seedling pair to a soil depth of 50 - 60 cm to exclude the influence of surrounding tree roots. Trenches were lined with heavy polyethylene and refilled with excavated soil. All vegetation in the square area was clipped throughout the first growing season and then sprayed twice (2005 and 2006) with glyphosate (donor trees and seedlings were covered) to eliminate interspecific plant interactions.

5.2.2 Survival, growth, physiology, and foliar nitrogen

Seedling survival, height, and root-collar diameter were assessed at the end of August in 2004, in May and August in 2005, and in May and July in 2006. Survival of in situ seedlings was determined based on i) the proportion of live to total seedlings sown per mesh bag, or ii) the frequency of mesh bags with live seedlings. Survival ‘i)’ relativized for the total number of germinating seeds per mesh bag, thereby not confounding microsite effects on survival, while ‘ii)’ was simply based on presence or absence of a live seedling per mesh bag. All whole seedlings were destructively sampled between July 21 and August 1, 2006, their shoots severed from roots (see below), and both tissues oven-dried at 70 °C for 48 hours, then weighed. We noted at the time of harvest that root extent appeared similar among all treatments, suggesting that lateral root extention was not confounding MN treatment effects; thus, treatments differed primarily according to the presence or pore-size of the mesh bags.

Gas exchange measurements were conducted with a LI-6400 portable photosynthesis system (LICOR Inc., Lincoln, NE, USA) attached to a LI-6400-05 conifer leaf chamber (LICOR Inc., Lincoln, NE, USA). Measurements were conducted on all trees and seedlings in July and August of 2005 and in July of 2006 from 10:00 to 15:00 on days with a photosynthetically active radiation (PAR) greater than 1000 μmol m⁻² s⁻¹. Ambient air temperature typically ranged from 20 to 30 °C. During measurements, carbon dioxide (CO₂) concentration of air entering the chamber was kept constant at 400 μmol mol⁻¹ with a fixed molar flow rate of 400 μmol s⁻¹ and temperature inside the chamber less than 25 °C. Measurements were made on attached lateral shoots, and the needles were arranged in the chamber such that self-shading was minimized. Gas
exchange measurements were recorded only when photosynthesis and transpiration rates inside the chamber were steady for at least 90 s. Needles inside the chamber were harvested in August and kept frozen until further processing. One-sided leaf area was measured with a LI-3100 leaf area meter (LICOR Inc., Lincoln, NE, USA) and ImageJ (v. 1.36b) computer software (Rasband, 1997-2007). Intrinsic water-use efficiency (WUE) was calculated as the ratio of CO\textsubscript{2} assimilation to H\textsubscript{2}O transpiration.

Chlorophyll fluorescence was measured with a hand-held chlorophyll fluorometer OS-30p (Opti-Sciences Inc., Tyngsboro, MA, USA) following the method outlined in Teste & Simard (in review; Chapter 4) during the same days as the gas exchange measurements. A subsample (0.5 g) of of current-year needles was removed from shoots of donor trees and planted seedlings, dried overnight at 70 °C, weighed, and then sent to the Analytical Laboratory in Victoria, British Columbia (B.C. Ministry of Forests and Range, Research Branch) for analysis of total N (Kalra & Maynard, 1991).

5.2.3 Carbon and nitrogen isotope labeling

By May 2006, replication in the 0.5 µm mesh treatment was very low due to poor survival; therefore, for C transfer measures only, we transplanted 2 year-old naturally regenerated seedlings (dug up from a nearby skid trail) into five of the 0.5 µm mesh bags to increase replication. We categorized donor trees into three broad size classes (small, medium, and large) based on height and canopy diameter. Large gas labeling bags (30, 60, and 100 l) were custom-made with 5-ply transparent gas-tight polyethylene/nylon (FoodSaver®, Jarden Corp., Rye, NY, USA). Our calculations (based on previous research and experience) indicated that small, medium, and large donor trees should be pulsed-labeled with 900, 1300, and 3200 ml of $^{13}$CO\textsubscript{2}, respectively, in order to potentially detect δ$^{13}$C values 10 ‰ above background in receiver seedlings. Prior to isotope gas labeling, 1 ml gas-tight syringes (Hamilton Co., Reno, NV, USA) and a LI-6251 CO\textsubscript{2} analyzer (LICOR Inc., Lincoln, NE, USA) were used to determine the amount of time needed for donor trees to reach the compensation point inside the gas labeling bag after injecting regular CO\textsubscript{2} gas. This preliminary data determined the ideal duration of the pulse period for complete assimilation of the $^{13}$CO\textsubscript{2}.
From July 14 to 17, 2006, donor trees were pulse-labeled with CO$_2$ (99% $^{13}$C, Cambridge Isotope Laboratories, Inc.) using a 0.5 l gas-tight super syringe (Hamilton Co., Reno, NV, USA). After three hours, receiver seedling shoots were carefully covered with thick plastic bags to prevent accidental aerial enrichment when donor tree gas-labeling bags were removed and flushed. After a few minutes, the thick plastic bags were removed from the receivers. After all gas-labeling bags were removed and flushed, potted interior Douglas-fir seedlings (referred to as ‘aerial control’ seedlings hereafter) were placed in between the trees and seedlings to estimate the amount of re-fixed carbon via plant or soil respiration. After a seven day chase period, we harvested all donor trees and seedlings (shoots and roots). All plant material was immediately placed into air-tight plastic bags and surrounded with dry ice during transport to the lab, where they were kept frozen at -20º C until they were oven dried.

To examine interplant nitrogen transfer, one medium-sized donor tree was selected in May 2005 and isolated in a square area (~16 m$^2$) by trenching (50 cm deep). In June 2005, a two year-old naturally regenerating interior Douglas-fir seedling (collected on-site from a skid trail) was planted in each of the four cardinal and four intercardinal directions (North, South, West, East, N-E, N-W, S-E, S-W) at 25-100 cm distance from the donor tree. In spring 2006, we used a fine spade to insert 0.5 µm mesh into the soil (30 cm deep), individually surrounding three of the eight seedlings. Nitrogen labeling of the donor tree began on August 10, 2006 and finished approximately 6 weeks later. We used a similar approach to He et al. (2006), where centrifuge tubes (15 ml) were attached to branch ends of the donor tree. Branch ends were fitted to the tubes by removing some needles furthest away from the terminal bud but leaving at least 5 cm of intact needles inside the tube. After the tubes were attached, we injected 10 ml of NH$_4$NO$_3$ solution (90 mM, 98%+, $^{15}$N$_2$, Cambridge Isotope Laboratories, Inc.) into each one. The tubes were then covered with plasteline (Le Beau Touché, Chavant, Inc.) to reduce evaporation and keep them firmly attached to the tree branch. Altogether, 12 tubes were attached to donor branches with a total of 120 ml isotope solution. At the time of harvest, 76 ml of solution had been taken up by the tree. All tubes were intact and did not show any signs of leaks or spills.
5.2.4 Carbon and nitrogen isotope analysis

Donor tree and receiver seedling tissue (shoots & roots) was oven-dried at 70°C for 48 hours and then weighed. Tissue was first roughly ground to 0.25 mm with a SM 2000 heavy-duty cutting mill (Retsch® Newtown, PA, USA) and then thoroughly mixed. A MM 200 ball mill (Retsch® Newtown, PA, USA) was used to turn a 100 mg subsample of the rough-ground tissue into a 0.01 mm fine powder. After homogenizing the fine powder, we sampled 1 mg of shoot and root tissue for C and N isotopic composition. The samples were combusted and the liberated CO₂ and N₂ was analyzed for the ratio $^{13}$C/$^{12}$C and $^{15}$N/$^{14}$N with a continuous flow Europa Hydra 20/20 and Europa Integra (enriched samples) isotope ratio mass spectrometer at the UC Davis Stable Isotope Laboratory (Davis, CA, USA). The C or N isotope ratio ($\delta^{13}$C or $\delta^{15}$N) was calculated as:

$$\delta^{13}$C or $\delta^{15}$N = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

in ‰ units, where $R = ^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N. The sample ratio is relative to the Vienna-PeeDee Belemnite (V-PDB) standard ($1.1237 \times 10^{-2}$) for C and N₂-atmospheric gas ($3.677 \times 10^{-3}$) for N. The resulting $\delta^{13}$C or $\delta^{15}$N values were used to determine whether seedlings had an excess of $^{13}$C or $^{15}$N above natural abundance levels.

5.2.5 Carbon and nitrogen excess calculations

To convert $\delta^{13}$C or $\delta^{15}$N into mg of $^{12}$C- or $^{14}$N- equivalent excess in seedlings, we followed a modified version of the procedure outlined in Boutton (1991). Sample tissue (shoots and roots separately) $\delta^{13}$C values were converted to the absolute isotope ratio (R):

$$R_{\text{sample}} = \left[ \left( \frac{\delta^{13}C}{1000} \right) + 1 \right] \times R_{\text{standard}} \quad (1)$$

The molar fractional abundance (F) was calculated as:

$$F = \frac{R_{\text{sample}}}{R_{\text{sample}} + 1} \quad (2)$$

The mass-based fractional abundance (MF) was then calculated as:

$$MF = \frac{F \times 13}{((F \times 13) + (1 - F) \times 12)} \quad (3)$$
The background (natural abundance) MF values (on a size-tissue specific basis with average \( n = 4 \)) were subtracted from the sample MF values to yield a change in MF (\( \Delta MF \)). Excess sample tissue \(^{13}\)C (excess \(^{13}\)C) was calculated as:

\[
\text{excess } ^{13}\text{C (mg)} = \text{Mean tissue C content } \times \text{tissue mass (mg)} \times \DeltaMF
\] (4)

Finally, excess \(^{13}\)C was converted to excess sample tissue \(^{12}\)C-equivalent (excess \(^{12}\)C-equivalent) by the equation:

\[
\text{excess } ^{12}\text{C-equivalent} = \text{excess } ^{13}\text{C} \times \left( \frac{12}{13} \right) \times Df
\] (5)

where \( Df \) is a dilution factor that corrects for the slight dilution of \(^{13}\)CO\(_2\) by the \(^{12}\)CO\(_2\) initially present in the gas-labeling bag. The same procedure for converting \( \delta^{15}\)N to excess values was used but we replaced molecular weights 13 and 12 with 15 and 14, respectively, in equations (3) and (5); and there was no \( Df \).

5.2.6 Sampling of donor tree and receiver seedling ectomycorrhizas

Concurrently with the donor tree harvest in summer 2006, we randomly selected lateral donor root sections from around each of the receiver seedlings. For seedlings planted directly into soil (i.e., without mesh bags), donor lateral roots were taken only after the seedlings were first carefully excavated. Donor tree root samples and the root systems of receiver planted seedlings were placed in plastic bags with loose soil and stored at 3 °C until further processing. All samples were processed within 2 months after field sampling.

In early May of 2005 and 2006, three \textit{in situ} seedlings were harvested from each mesh treatment, with each from a different donor tree, for EM colonization assessment. The rest of the seedlings were harvested immediately after sampling the donor tree roots (26 months after planting). After clipping off the shoots, root systems with surrounding soil were placed in plastic bags and stored at 3 °C until further processing. All samples were processed within 3 months after field sampling.

5.2.7 Morphotyping and molecular identification of ectomycorrhizas

Roots of donor trees and seedlings were carefully washed under running tap water and then cut into approximately 2 cm pieces. All root fragments were placed in a tray containing distilled water and thoroughly mixed. We randomly subsampled and counted
200 EM root tips from each donor tree and seedling. Morphological descriptions were made with reference primarily to Goodman et al. (1996) and Hagerman et al. (2001) and to a minor extent to Ingleby et al. (1990) and Agerer (1985–1998). Roots were then dried and weighed.

DNA extraction and PCR amplification of the ITS region of nuclear rDNA was conducted on two subsamples per morphotype per host type. Extraction and isolation of DNA, sequencing and nucleotide Basic Local Alignment Search Tool (BLAST) searches followed methods outlined in Twieg et al. (2007). Primer pairs used in PCR amplifications were: ITS1-F and ITS4; NS11 and NLC2 (Martin & Rygiewicz, 2005). Samples that were not successfully amplified or sequenced were given an EM taxon name based on morphotyping data. Out of the 62 root tips extracted, 90% yielded DNA for BLAST searches.

5.2.8 Statistical analyses

All statistical analyses were carried out using the R (R Development Core Team, 2006) statistical environment for statistical computing and graphics. A binomial regression model with a logit link function (logistic regression) and a quasi-binomial generalized linear model (GLM) were used to analyze the seedling survival data, implemented with the glm function (family set to binomial or quasi-binomial). The significance of the regression and treatment levels were determined with a deviance-based test (Faraway, 2006) and Wald test (Hosmer & Lemeshow, 2000), respectively. Percent EM colonization was calculated as the number of EM root tips divided by the total number of root tips (EM and nonEM) multiplied by 100. We calculated EM taxa richness and Shannon diversity index (H’) using functions specnumber and diversity, respectively. The H’ emphasizes species richness because it is weighted towards rare species (Magurran, 2004). The Morisita-Horn similarity index (CMH) (Magurran, 2004) was calculated following Schoonmaker et al. (2007). Contingency tables were constructed with function chisq.test (Pearson’s Chi-square test) to test for mesh treatment effects on the occurrence of $^{13}$C-enriched seedlings and on the presence and absence of EM taxa. The effects of the mesh treatments on seedling growth, physiology, excess $^{12}$C- and $^{14}$N-equivalent, and EM status (colonization,
richness, diversity) were detected with a one-way analysis of variance using function `aov`. Physiological responses of donor trees were compared to that of receiver seedlings using t-tests. To assess the influence of donor size (height, diameter, stem volume, age, and biomass) on seedling excess $^{12}$C-equivalent, we performed simple linear regression with function `lm`. Statistical differences were considered significant at $P \leq 0.05$.

5.3 Results

5.3.1 Survival, growth, and physiological responses

Survival of *in situ* seedlings increased with mesh size (Fig. 5.1). *In situ* seedlings growing in no mesh (full access to the MN) had significantly greater survival (for both types of survival) than *in situ* seedlings growing in the 0.5 $\mu$m mesh ($P = 0.04$). The no mesh treatment was a more realistic representation of full access to a MN compared to the 250 $\mu$m mesh treatment because short-distance hyphal connections could form between EM taxa (e.g., *Lactarius*, *Russula*, *Tomentella*, and *Tuber* spp.) with a ‘contact’ exploration type (Agerer, 2001). Survival of planted seedlings was not affected by the mesh treatments ($P = 0.17$).

Mesh treatment had no effect on *in situ* or planted seedling growth responses (height, root-collar diameter, shoot biomass and root biomass). In 2006, *in situ* seedlings growing in no mesh tended to have the greatest WUE ($P = 0.07$). None of the other physiological responses were affected by the mesh treatments for either the *in situ* or planted seedlings.

Donor trees had greater CO$_2$ assimilation rates ($P < 0.01$), ratio of variable to maximum fluorescence ($F_m - F_o/F_m$) ($P = 0.05$), and foliar nitrogen content ($P < 0.01$) compared to planted seedlings in 2006 (Table 5.1).

5.3.2 Carbon and nitrogen transfer

Carbon isotope transferred from donors was detected in the shoots and roots of *in situ* receiver seedlings (Fig. 5.2). There was a greater frequency of $^{13}$C-enriched *in situ* seedlings (shoots + roots) growing in no mesh compared to the other mesh treatments (except 0.5 $\mu$m, $P = 0.19$) (Fig. 5.2) ($P < 0.01$). Average amount of excess $^{12}$C-
equivalent transferred also tended to be greatest in \textit{in situ} seedlings (shoots + roots) growing in the no mesh treatment (Fig. 5.2; \( P = 0.07 \)).

Carbon was also transferred to the shoots and roots of most of the planted seedlings (Fig. 5.3). However, mesh treatment had no effect on the number of seedlings that were \( ^{13} \text{C} \)-enriched \(( P = 0.70)\) or the average amount of excess \( ^{12} \text{C} \)-equivalent transferred \( ( P = 0.91)\). Out of 36 aerial control seedlings, two were marginally enriched with \( ^{13} \text{C} \) (Excess \( ^{12} \text{C} \)-equivalent = 0.064 and 0.059 mg), suggesting that very small amounts of \( ^{13} \text{C} \) were respired and taken up in receiver shoots compared with amounts that were transferred belowground.

Transferred nitrogen was detected in the shoots and roots of three of the five \textit{in situ} seedlings growing in soil (i.e., no mesh) (Fig. 5.4). None of the three \textit{in situ} seedlings growing in the 0.5 \( \mu \text{m} \) mesh showed evidence of \( ^{15} \text{N} \) transfer (Fig. 5.4). \textit{In situ} seedlings growing in no mesh tended to have greater amounts of excess \( ^{14} \text{N} \)-equivalent in shoots \( ( P = 0.08) \) and roots \( ( P = 0.13) \) than in 0.5 \( \mu \text{m} \) mesh seedlings.

5.3.3 Effect of donor size on carbon transfer

The amount of carbon transferred to \textit{in situ} seedlings decreased with increasing donor size (Fig. 5.5) and age \(( R^2 = 0.49, P < 0.01 \)). The strongest relationship was found between \textit{in situ} seedling root excess \( ^{12} \text{C} \)-equivalent and donor tree diameter (Fig. 5.5). Regression analysis indicated no relationship \(( R^2 = 0.0003, P = 0.94) \) between \( \delta^{13} \text{C} \) of donor shoot and donor stem diameter, suggesting that labeling efficiency was uniform across all donor sizes. Allocation of \( ^{13} \text{C} \) (i.e., \( ^{12} \text{C} \)-equivalent) to donor roots decreased with decreasing donor size (Fig. 5.6). Roots sampled from large donor trees tended to have a richer (5.2 vs. 4.1 EM taxa, \( P = 0.07 \)) and more diverse (1.3 vs. 0.9, \( P = 0.01 \)) EM community than those from small donor trees.

5.3.4 Potential for mycorrhizal networks to form

A total of 32 EM taxa were found on both donor tree and seedling (\textit{in situ} and planted) root systems (Table 5.2). Donor trees and seedlings hosted 31 and 20 EM taxa, respectively, with 19 EM taxa in common. More unique EM taxa were found on donor trees than on seedlings (Table 5.2). The EM community was made up of many rare and a few abundant EM taxa (Fig. 5.7). The five most abundant EM taxa found on donor trees
and seedlings were *Wilcoxina rehmii*, *Rhizopogon vinicolor*, *Russula brevipes*, *Cenococcum geophilum*, and *Amphinema byssoides* (Fig. 5.7). Seedling (*in situ* and planted) EM communities were similar (colonization, richness, and diversity did not differ among seedling types, with $C_{MH}$ similarity index = 93%) so we merged the two data sets for comparisons with the donor tree EM communities. Donor trees and seedlings (*in situ* and planted) shared 59% of all EM taxa found in this study (Table 5.2) and 80% of those taxa had a relative abundance on root tips greater than 5% (Fig. 5.7). The $C_{MH}$ similarity index indicated there was 84% similarity between the donor tree and receiver seedling EM communities.

5.3.5 Mesh effects on ectomycorrhizal communities

After one growing season, *in situ* seedling EM colonization tended to be greatest (99%) in no mesh ($P = 0.06$) but this effect disappeared in the following growing season. *In situ* seedling EM richness and diversity were unaffected by the mesh treatments (data not shown). After three growing seasons, planted seedling EM diversity was greater in no mesh than the 0.5 µm mesh treatment ($P = 0.05$), whereas colonization and richness were unaffected (Fig. 5.8).

5.4 Discussion

5.4.1 Mycorrhizal network effects on seedling performance

Full access to a MN improved survival of seedlings grown from seed (*in situ* seedlings) in the field but this was not the case for planted seedlings initially grown in a greenhouse. These results suggest MNs are more beneficial for newly germinated seedlings struggling to establish under the harsh conditions found in these dry forests (Vyse *et al.*, 2006). Therefore, we do not reject our first hypothesis that seedling survival increases with greater access to a MN where seedlings are grown from seed in the field, but we do reject it where seedlings are first grown to a fair size in the greenhouse and then planted in the field (*planted seedlings*). Movement of resources via a MN has been found to occur along source-sink gradients (Francis & Read, 1984; Finlay & Read, 1986) and the struggling *in situ* seedlings likely acted as stronger sinks for resources than planted seedlings. Resource-sharing promoted by donor trees may have been relatively
more beneficial for *in situ* seedlings than planted seedlings because they were significantly smaller (i.e., lower surface areas for photosynthesis and nutrient uptake) and less physiologically active (i.e., lower photosynthetic rates and WUE). Even though their absolute physiological activities tended to be lower, however, the relative demand for nitrogen in the foliage of *in situ* seedlings was likely relatively greater than planted seedlings, which had older needles and the capacity to recycle foliar nitrogen (Lambers *et al.*, 1998). These mechanisms could explain why *in situ* seedlings appeared to benefit more from resource sharing via MNs.

The magnitude of the MN effect is ecologically significant because full access to the network increased survival by 26.1% (occurrence basis) compared to no access to the network. Since seedling survival in these dry forests is low, and regeneration failure is commonplace (Newsome *et al.*, 1991; Simard *et al.*, 2003), we hypothesize that small amounts of resource gains provided by a MN could make the crucial difference for successful forest establishment. Our results agree with McGuire (2007), who found increased survival of seedlings where they had access to a MN in a dense monodominant EM tropical forest. The beneficial MN effects observed in our study came from one small tree versus several large trees in the study of McGuire, suggesting MNs with several mature ‘edge’ trees may be even more beneficial in regeneration of small canopy gaps that are created by small scale disturbances in dry forests (Vyse *et al.*, 2006).

The mesh treatments did not affect seedling growth or physiology, leading us to reject our second hypothesis that access to a MN will increase seedling growth and physiological responses. Seedling growth and physiology may have not been affected by access to a MN because all seedlings were growing in full sunlight conditions. Other studies suggest that MN effects on growth or physiology are more pronounced for receiver seedlings growing in shade, where they are stronger sinks for transferred resources (Francis & Read, 1984; Simard *et al.*, 1997). It is also possible that we missed the critical period for capturing physiological response differences among mesh treatments because we were unable to measure the germinating seedlings in the first year due to their small size.
5.4.2 Carbon and nitrogen transfer

Interplant carbon and nitrogen transfer from donor trees was facilitated by access to MNs for seedlings grown from seed in the field (*in situ* seedlings). The average amounts of carbon and nitrogen transferred tended to be highest where *in situ* seedlings had full access to a MN, thus supporting our third hypothesis. That transfer occurred in all mesh sizes suggests that both hyphae and rhizomorphs were important in nutrient transfer, supporting earlier research involving transfer through different EM taxa (Brownlee *et al.*, 1983; Simard *et al.*, 1997). The absolute amounts quantified after a 7-day chase period in mid-summer were low, but other studies examining transfer over several periods during the growing season suggest that transferred amounts peak in the fall when root and mycorrhizal development is greatest (Lerat *et al.*, 2002; Philip, 2006). At our harsh sites where carbon fixation is limited by low soil water availability (Fleming *et al.*, 1998), carbon and nutrient transfer accumulated over the whole growing season or at critically important periods may be significant to survival of establishing seedlings.

Interplant carbon transfer also occurred to a lesser extent in the fine mesh treatment, indicating resources also moved through soil or a discontinuous MN pathway (Simard & Durall, 2004). We think that a discontinuous MN pathway (analogous to synaptic clefts and gap junctions in neural networks) can still transfer a considerable amount of carbon between plants. Since root tips of *in situ* seedlings in the fine mesh treatment were heavily colonized by EM fungi and were not in close proximity to the mesh material, we suspect that carbon moved predominantly via a discontinuous MN pathway. This pathway implies that small gaps (produced by the mesh barrier) were present between the extraradical mycelium of two mycorrhizal plants (i.e., donor tree and *in situ* seedlings). In nature, small gaps in the MN are likely to occur because of incompatibility of some of the involved fungal taxa (Webster & Weber, 2007), mycelium fragmentation by soil fauna (Tuffen *et al.*, 2002; Johnson *et al.*, 2005) or hyphal degradation.

Carbon transfer between donor trees and planted seedlings was variable, agreeing with most other carbon transfer studies (Read *et al.*, 1985, Simard *et al.*, 1997, Marler *et al.*, 1998). Increasing complexity of the MN pathway, however, from hyphae to hyphae plus rhizomorphs, had no effect on the extent of carbon transferred. Roots and EM
mycelium of planted seedlings were well developed, often observed growing along the surface of the mesh material. Under these optimal conditions, all MN pathways appeared equally important in interplant carbon transfer.

The movement of carbon and nitrogen transferred between donor trees and both types of seedlings may have been regulated by a foliar nitrogen source-sink gradient as indicated by the significantly greater foliar nitrogen content in needles of donor trees. Carbon and nitrogen could move as free amino acids across the Hartig net of seedlings (Simard & Durall, 2004). It is also possible that transfer between donor trees and seedlings was regulated by a carbon source-sink gradient, or by both carbon and nitrogen gradients, since donor trees had greater CO₂ assimilation rates and and tolerance to environmental stress than planted seedlings.

5.4.3 Influence of source strength on carbon transfer

We expected carbon transfer to occur from donor trees to regenerating seedlings along a physiological source-sink gradient, where donor trees were photosynthesizing at higher rates than the seedlings. Earlier work in AM communities, for example, suggested that increasing dominance of canopy source plants promoted carbon transfer to understory sink seedlings (Grime et al., 1987), particularly where they were highly dependent on mycorrhizas (van der Heijden, 2002). We found, however, that larger Douglas-fir donor trees did not transfer more carbon than small donors to receiver seedlings, thus refuting our fourth hypothesis that donor tree size is an important regulator of the magnitude of transfer. In fact we found the inverse relationship, where increasingly larger donor trees transferred even less carbon to seedlings. This trend could not be explained by carbon allocation patterns to donor tree roots since the percentage of carbon allocated to donor tree belowground parts (roots and EM) increased with donor tree size. However, our results could be explained by the relative sink strength of in situ seedlings. In situ seedlings would have had relatively greater sink strength when connected to smaller than larger donor trees. It could also be explained by the considerably older age of larger understory (donor) trees that had existed for many decades in the shade of these forests (Heyerdahl et al., 2007); their greater age may have
been associated with higher root maintenance respiration costs and fewer exudates available for transfer than in smaller donors.

Carbon allocation patterns to MNs could also influence carbon transfer patterns, and they may be governed less by donor tree size than canopy light conditions, spatial orientation of the extraradical mycelia, or composition of EM community. Larger donors had more diverse EM communities than smaller donors because of a greater number of non-networking taxa, which could have been competing sinks for donor carbon. Allocation of carbon to extraradical mycelia forming a MN is likely patchy and may be related more to light patterns and EM community composition than whether the MN is intact, discontinuous or disrupted (Nakano-Hylander & Olsson, 2007).

Our results agree with research by Fitter et al. (1998) on AM plants that increasing the source strength does not affect the amount of carbon transfer. Our results also share similarities with Pietikäinen & Kytöviita (2007), who found that benefits of MNs were lowest when seedlings were grown in the vicinity of non-defoliated adult plants but greater with increasing defoliation of adult plants (Pietikäinen & Kytöviita, 2007). As in our study, they found that donor trees with greater capacity to fix atmospheric carbon (i.e., ability to provide more carbon to the MN) did not translate into greater benefits to seedlings. As explained above, the large donor trees in our study were older than the smaller trees and likely had considerably greater maintenance cost to support old tissue (Lambers et al., 1998). This could explain why they transferred less carbon to the MN.

5.4.4 Mycorrhizal network formation potential

The potential for MNs to form was high because the donor tree and seedling EM community compositions were similar. The majority of the shared EM taxa were also the most abundant members of these communities. Our other studies in similar forests show that older, larger Douglas-fir trees share most of the same EM fungal taxa, as well as the same Rhizophogon vinicolor genet, with planted seedlings (Teste et al., in review; Chapter 3). Our findings corroborate other studies showing high potential for MNs to form with conspecific (Jonsson et al., 1999; Matsuda & Hijii, 2004; Haskins & Gehring, 2005;
Cline et al., 2005) as well as different tree species (Kennedy et al., 2003; Twieg et al., 2007).

In this study, larger donor trees had a more diverse EM community than smaller ones, with many rare EM taxa not shared with the in situ seedlings. With greater abundance of competing rare EM sinks in large donors, shared taxa would have been allocated less carbon than in small donors. This, combined with the lower MN potential, could explain why larger donor trees transferred less carbon to neighboring seedlings than smaller donors.

5.4.5 Mycorrhizal network-mediated colonization

Mycorrhizal network-mediated colonization did not play an important role in transmitting EM fungi from donor trees to seedlings. This is because the EM inoculum (spores, sclerotia, severed tree EM tips and network hyphae) potential of the site was high, enabling rapid colonization of the planted seedlings’ extensive root systems from soil inside the mesh bags. Ectomycorrhizal inoculum is often maintained at high enough levels following clearcutting that colonization of planted seedlings is not hindered (Jones et al., 2003). Our results do not disprove that MN-mediated colonization of planted seedlings occurred; it is possible that MNs formed after initial colonization and subsequent anastomosis (Glass et al., 2004; Jakobsen, 2004).

5.4.6 Conclusions

This study presents evidence that seedlings with access to a MN have reduced risk of mortality and can receive more carbon and nitrogen from ‘donor’ trees than seedlings with no access to a MN. Seedlings grown from seed in the field benefited more from the MN than planted seedlings, possibly because their relative sink strength was greater. Increasing size of ‘donor’ trees was associated with declining carbon transfer, which may be related to their more advanced age of suppression, or greater degree of root colonization by non-networking fungi. It appears that donor plant source strength is a less likely explanation for variation in extent of carbon transfer than receiver plant sink strength. Mycorrhizal networks appear to be ecologically important for seedlings establishing near conspecific trees in dry forests, and they may become increasingly
important following disturbance, site degradation, exotic AM weed invasion, or under increasing climatic stress.

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Table 5.1 Advanced regeneration interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) tree and seedling characteristics in 2006. Values for characteristics are means with one standard deviation in parentheses. *WUE* Water use efficiency.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Donor trees mean values</th>
<th>Donor trees</th>
<th>Receiver seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (years)</td>
<td>39 (16)</td>
<td>26 (11)</td>
</tr>
<tr>
<td></td>
<td>Height (cm)</td>
<td>128 (49)</td>
<td>76.4 (33.1)</td>
</tr>
<tr>
<td></td>
<td>Root-collar diameter (cm)</td>
<td>1.93 (1.06)</td>
<td>0.83 (0.51)</td>
</tr>
<tr>
<td></td>
<td>Stem volume (cm³)</td>
<td>584 (922)</td>
<td>67 (93)</td>
</tr>
<tr>
<td></td>
<td>Assimilation (µmol CO₂ m⁻² s⁻¹)</td>
<td>5.98 (1.97) a</td>
<td>5.75 (2.47)</td>
</tr>
<tr>
<td></td>
<td>Transpiration (mmol H₂O m⁻² s⁻¹)</td>
<td>2.02 (0.68) a</td>
<td>1.98 (0.61)</td>
</tr>
<tr>
<td></td>
<td>Intrinsic WUE (µmol CO₂ mol⁻¹ H₂O)</td>
<td>3.31 (1.48) a</td>
<td>3.19 (1.42)</td>
</tr>
<tr>
<td></td>
<td>Chlorophyll fluorescence (<em>Fₘ - Fₜ/Fₘ</em>)</td>
<td>0.75 (0.09) a</td>
<td>0.79 (0.03)</td>
</tr>
<tr>
<td></td>
<td>Foliar nitrogen (mg cm⁻²)</td>
<td>91.9 (25.2) a</td>
<td>87.1 (28.6)</td>
</tr>
</tbody>
</table>

Statistically different tree and seedling physiological variables were detected with *t*-tests (*P* = 0.05) and are designated by different letters. *nd* not determined.
Table 5.2  List of observed EM taxa on Interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) trees and seedlings (in situ and planted) on the Black Pines variable retention site in the IDF biogeoclimatic zone of BC, Canada, in August 2006.

<table>
<thead>
<tr>
<th>Morphototype^a</th>
<th>Closest BLAST match</th>
<th>Database Accession number</th>
<th>Total base pairs aligned</th>
<th>NCBI % similarity</th>
<th>Consensus EM taxa</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphinema</td>
<td><em>Amphinema byssoides</em></td>
<td>NCBI AY838271</td>
<td>557</td>
<td>98</td>
<td><em>Amphinema byssoides</em></td>
<td>Both</td>
</tr>
<tr>
<td>Bol, Heb</td>
<td><em>Cortinarius erythrinus</em></td>
<td>NCBI AY669690</td>
<td>485</td>
<td>96</td>
<td><em>Cortinarius erythrinus</em></td>
<td>Both</td>
</tr>
<tr>
<td>Cenococcum</td>
<td><em>Cenococcum geophilum</em></td>
<td>NCBI AY394919</td>
<td>645</td>
<td>99</td>
<td><em>Cenococcum geophilum</em></td>
<td>Both</td>
</tr>
<tr>
<td>Piloderma, Cort</td>
<td><em>Piloderma fallax</em></td>
<td>NCBI DQ179125</td>
<td>473</td>
<td>99</td>
<td><em>Piloderma fallax</em></td>
<td>Both</td>
</tr>
<tr>
<td>Ino (brown red)</td>
<td>Mycorrhizal fungi</td>
<td>NCBI AY330697</td>
<td>129</td>
<td>86</td>
<td>Brown red</td>
<td>Tree</td>
</tr>
<tr>
<td>Ino/Lac</td>
<td><em>Lactarius rufus</em></td>
<td>NCBI DQ97868</td>
<td>726</td>
<td>100</td>
<td><em>Lactarius rufus</em></td>
<td>Both</td>
</tr>
<tr>
<td>Lac (fuzzy yellow)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Fuzzy yellow</td>
<td>Tree</td>
</tr>
<tr>
<td>Lac (green)</td>
<td><em>Lactarius rubrilacteus</em></td>
<td>NCBI DQ97882</td>
<td>414</td>
<td>98</td>
<td><em>Lactarius rubrilacteus</em></td>
<td>Both</td>
</tr>
<tr>
<td>Lac/Russ (smooth orange)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Smooth orange</td>
<td>Tree</td>
</tr>
<tr>
<td>Wilcoxina</td>
<td><em>Wilcoxina rehmii</em></td>
<td>NCBI AF266708</td>
<td>610</td>
<td>99</td>
<td><em>Wilcoxina rehmii</em></td>
<td>Both</td>
</tr>
<tr>
<td>Rhizopogon</td>
<td><em>Rhizopogon vinicolor</em></td>
<td>NCBI AY63930</td>
<td>419</td>
<td>99</td>
<td><em>Rhizopogon vinicolor</em></td>
<td>Both</td>
</tr>
<tr>
<td>Russ (long em hyphae)</td>
<td></td>
<td>NCBI DQ377383</td>
<td>464</td>
<td>99</td>
<td><em>Cortinariaceae</em></td>
<td>Both</td>
</tr>
<tr>
<td>Russ (pink orange)</td>
<td></td>
<td><em>Russula fragilis</em></td>
<td>NCBI DQ367914</td>
<td>493</td>
<td><em>Russula fragilis</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Russ (silver-brown)</td>
<td></td>
<td><em>Inocybe sp.</em></td>
<td>NCBI DQ822816</td>
<td>291</td>
<td><em>Inocybe sp.</em></td>
<td>Both</td>
</tr>
<tr>
<td>Russ (yellow tip)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Yellow tip</td>
<td>Both</td>
</tr>
<tr>
<td>Russula</td>
<td><em>Russula brevipes</em></td>
<td>NCBI AF349714</td>
<td>535</td>
<td>99</td>
<td><em>Russula brevipes</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Suillus</td>
<td><em>Suillus lakei</em></td>
<td>NCBI DQ367917</td>
<td>523</td>
<td>98</td>
<td><em>Suillus lakei</em></td>
<td>Both</td>
</tr>
<tr>
<td>Russ b, Thel</td>
<td><em>Laccaria bicolor</em></td>
<td>NCBI DQ148850</td>
<td>542</td>
<td>99</td>
<td><em>Laccaria bicolor</em></td>
<td>Both</td>
</tr>
<tr>
<td>Thelephora</td>
<td><em>Thelephoraceae</em></td>
<td>NCBI AJ93344</td>
<td>707</td>
<td>99</td>
<td><em>Thelephoraceae</em></td>
<td>Seedling</td>
</tr>
<tr>
<td>Tom, Tom (hairy)</td>
<td></td>
<td><em>Pseudotomentella tristis</em></td>
<td>NCBI AJ899688</td>
<td>493</td>
<td><em>Pseudotomentella tristis</em></td>
<td>Both</td>
</tr>
<tr>
<td>Tomentella (thick-wall cystidia)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Tomentella1</td>
<td>Both</td>
</tr>
<tr>
<td>Tomentella (smooth)</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Tomentella2</td>
<td>Both</td>
</tr>
<tr>
<td>Truncocolumella</td>
<td>Mycorrhizal fungi</td>
<td>NCBI EF026606</td>
<td>446</td>
<td>94</td>
<td><em>Truncocolumella</em></td>
<td>Both</td>
</tr>
<tr>
<td>Pale brown/white silver</td>
<td></td>
<td><em>Pseudotomentella mucidula</em></td>
<td>NCBI AF274768</td>
<td>502</td>
<td><em>Pseudotomentella mucidula</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Pink em hyphae</td>
<td><em>Suillus caeruleusens</em></td>
<td>NCBI L54096</td>
<td>538</td>
<td>99</td>
<td><em>Suillus caeruleusens</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Fuzzy silver</td>
<td><em>Sebacina</em></td>
<td>NCBI D440851</td>
<td>698</td>
<td>98</td>
<td><em>Sebacina</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Skinny shiny faded pink</td>
<td></td>
<td><em>Tylospora sp.</em></td>
<td>NCBI DQ482022</td>
<td>433</td>
<td><em>Tylospora sp.</em></td>
<td>Both</td>
</tr>
<tr>
<td>Pyramidal felty</td>
<td><em>Ecectomycorrhiza</em></td>
<td>NCBI AY310838</td>
<td>215</td>
<td>96</td>
<td><em>Pyramid felty</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Wine smooth shiny jigsaw</td>
<td></td>
<td><em>Rhizoscyphus ericae</em></td>
<td>NCBI AY762620</td>
<td>503</td>
<td><em>Wine SS jigsaw</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Pale yellow jigsaw</td>
<td></td>
<td><em>Russula turci</em></td>
<td>NCBI AY061720</td>
<td>421</td>
<td><em>Russula turci</em></td>
<td>Tree</td>
</tr>
<tr>
<td>Gray cluster</td>
<td></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>Gray cluster</td>
<td>Both</td>
</tr>
<tr>
<td>Caramel</td>
<td><em>Russula cernua</em></td>
<td>NCBI AY061730</td>
<td>544</td>
<td>98</td>
<td><em>Russula cernua</em></td>
<td>Tree</td>
</tr>
</tbody>
</table>

^aFor photographs and concise morphotype descriptions, please contact the corresponding author. ^NCBI is the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). nd = not determined.
Figure 5.1 Survival of *in situ* seedlings (grown from seed germinating in the field) after two years (May 2006) in the field. (A) survival calculated as a proportion of live seedlings to total number of seed that germinated per mesh bag (i.e., count basis). (B) survival based on presence or absence of live seedlings per mesh bag (i.e., occurrence basis). The mesh treatment was a significant predictor of survival for both type of survival responses (logistic regression, \( P = 0.011 \); quasi-binomial GLM, \( P = 0.036 \)). Values are means ± 1 standard error of the mean. Bars with different letters indicate significant differences between means across mesh treatments.
Figure 5.2  Shoot and root $\delta^{13}$C and excess $^{12}$C-equivalent of *in situ* seedlings grown in mesh treatments at 0.5 m away from labeled donor trees in the field. The $\delta^{13}$C background mean (solid line) and 99 % confidence intervals (dotted line) appear as horizontal lines on the left portion of the figure. Values above the 99 % confidence interval are considered enriched and are marked as asterisks on the right portion of the figure.
Figure 5.3  Shoot and root $\delta^{13}C$ and excess $^{12}C$-equivalent of planted seedlings grown in mesh treatments at 0.5 m away from labeled donor trees in the field. The $\delta^{13}C$ background mean (solid line) and 99 % confidence intervals (dotted line) appear as horizontal lines on the left portion of the figure. Values above the 99 % confidence interval are considered enriched and are marked as asterisks on the right portion of the figure.
Figure 5.4 Shoot and root $\delta^{15}$N and excess $^{14}$N-equivalent of *in situ* seedlings grown with (0.5µm) or without mesh bags at 0.5 m away from one labeled donor tree in the field. The $\delta^{15}$N background mean (solid line) and 99 % confidence intervals (dotted line) appear as horizontal lines on the left portion of the figure. Values above the 99 % confidence interval are considered enriched and are marked as asterisks on the right portion of the figure.
Figure 5.5  The relationships between (A) donor tree diameter, (B) donor tree stem volume, (C) donor tree dry biomass and enriched *in situ* seedling root excess $^{12}$C-equivalent.
Figure 5.6 Allocation of $^{12}$C-equivalent to labeled donor root systems. Values are means with 95% confidence intervals. Statistically significant donor size class means determined by Tukey’s test are designated by different letters ($P < 0.05$).
Figure 5.7 Relative abundance of donor trees and seedlings (\textit{in situ} and planted) EM taxa.
Figure 5.8  Mesh effects on EM diversity planted of *Pseudotsuga menziesii* var. *glauca* seedlings. Values are means with 95% confidence intervals. Statistically significant mesh treatment effects determined by Tukey’s test are designated by different letters ($P < 0.05$).
5.5 References


He XH, Critchley C, Ng H, and Bledsoe C. 2004. Reciprocal N (15NH4+ or 15NO3–) transfer between nonN2-fixing *Eucalyptus maculata* and N2-fixing *Casuarina cunninghamiana* linked by the ectomycorrhizal fungus *Pisolithus* sp. *New Phytologist* **163**: 629-640.


6 NET CARBON TRANSFER BETWEEN CONSPECIFIC SEEDLINGS IN THE FIELD

6.1 Introduction

Interplant carbon transfer facilitated by a mycorrhizal network (MN) is an ecological phenomenon with potentially far-reaching consequences for plant communities (Newman, 1988; Read, 1997; Wilkinson, 1998). A MN is comprised of fungal hyphae connecting two or more plant roots of the same or different species, and it is apparent that most plants establish these structures in nature (Fitter, 2001). A MN can transfer carbon, nutrients, or water between plants (Selosse et al., 2006; Egerton-Warburton et al., 2007; Meding & Zasoski, 2008) and these functions may benefit seedling establishment and survival under harsh (e.g., nutrient poor or droughty) conditions or in light-limiting environments (Simard et al., 2002; Selosse et al., 2006).

Carbon has been shown to transfer belowground in both directions between two plants, and net transfer in one direction has been shown to occur where source-sink gradients exist between the connected plants (Simard et al., 1997a). Here, a so-called ‘donor’ source plant transfers more carbon than it gains from a ‘receiver’ sink plant, and the magnitude of net transfer from the source has been shown to increase with receiver sink strength (Simard et al., 1997a). The evidence for net carbon transfer through MNs continues to be hotly debated in the literature, however, and the role of MNs in ecosystem dynamics is still sought-after (Whitfield, 2007).

Some of the controversy about the role of MNs in interplant carbon transfer also involves arbuscular mycorrhizal (AM) plants, where transferred carbon appears to remain in the fungal tissue rather than being transferred to associated plant tissue (Pfeffer et al., 2004). Some scientists suggest that without transfer to plant tissues, MN-mediated transfer is insignificant to plant ecology (Robinson & Fitter, 1999), whereas others have argued that any carbon subsidy to a plant’s mycorrhizas can benefit the plant itself (Perry, 1999; Simard & Durall, 2004). Regardless, most studies examining MNs in ectomycorrhizal (EM) plants find that carbon is transferred to the tissue of connected plants (Leake et al., 2004), with the odd exception (Wu et al., 2001).

1 A version of this chapter will be submitted for publication: Teste, FP, Simard, SW, Durall, DM, Guy, RD, and Berch, SM. Net carbon transfer between conspecific seedlings in the field.
Since amino acids or low-weight nitrogenous compounds can move from fungal to plant tissue (Näsholm et al., 1998), it is conceivable that MN-transferred carbon is converted into nitrogenous compounds before moving into ‘receiver’ plants (Simard & Durall, 2004). Even so, it is still possible that fungal sugars or hyphal collapse at the fungal-plant interface are two other pathways for carbon transfer from fungi to plant tissue as proposed for mycoheterotrophic plants (Bidartondo, 2005). To move the debate on MN-mediated interplant carbon transfer, there is a need to confirm that carbon transferred through MNs moves into the shoots of ‘receiver’ plants (Robinson & Fitter 1999).

If MNs can facilitate interplant carbon transfer along source-sink gradients, then plants growing under harsh conditions that limit photosynthetic activity may benefit from this process more so than plants growing in fertile conditions (Callaway et al., 2002). The stress-gradient hypothesis, that facilitation is more important to plants growing under harsh conditions (Bertness & Callaway, 1994), has been challenged by studies showing the opposite effect (Maestre et al., 2005; Riginos et al., 2005; Maestre et al., 2006), but more field studies are required (Lortie & Callaway, 2006). Soil disturbances associated with forest harvesting can accentuate the limiting effects of stressful environments on seedling establishment (Fleming et al., 1998), but the ameliorating effects of MN-facilitation under such conditions have not been studied.

The morphology of EM fungi forming a MN may affect the magnitude of carbon transfer. For instance, MNs comprised of EM fungi with prolific rhizomorphs may transfer more water between plants than MNs comprised of simple hyphae (Brownlee et al., 1983; Egerton-Warburton et al., 2007). Based on close examination of rhizomorph structure and development, it has been hypothesized that rhizomorphs are the most important fungal structures involved in interplant resource transfer (Read et al., 1985; Cairney, 1992; Cairney & Smith, 1992). It remains to be seen whether carbon transfer is comparably greater in rhizomorph- than hyphae-dominated networks.

The main objectives of this study were to determine whether net carbon transfer occurs between conspecific EM seedlings, transfer occurs through MNs, and transfer is enhanced by stress imposed by soil disturbance. The following hypotheses were tested: i) net carbon transfer occurs between conspecific seedlings along a source-sink gradient, ii) net transfer increases with access to a MN, iii) net carbon transfer increases with soil
disturbance, iv) carbon is transferred to both shoots and roots of ‘receiver’ seedlings; and v) net carbon transfer is greater in root tips colonized by EM taxa that form rhizomorphs.

Much of the skepticism on interplant carbon transfer via MNs lies in the methodologies and experimental designs used in previous studies (Bergelson & Crawley, 1988; Robinson & Fitter, 1999; Fitter & Robinson, 2000; Fitter, 2001). Our study addresses several limitations noted in previous work on interplant carbon transfer via MNs (Robinson & Fitter, 1999), where we: i) use mesh barriers to prevent hyphal connections; ii) fully reciprocate all dual-labeling deliveries; iii) use controls to detect possible re-fixation of respired labeled carbon tracers (from ‘donor plants and soil) by the shoots of ‘receiver’ plants (Leake et al., 2004), iv) use ‘donor’ specific activities for net carbon transfer calculations; and v) examine plant regulatory factors, such as relative growth rate, that may generate a sink for carbon transfer.

6.2 Methods

6.2.1 Site description and experimental design

Characteristics of the three study sites (Dairy Creek, O’Connor Lake, Black Pines) are described in Table 6.1 and Hope (2006). The three sites were established between 1997 and 2001 for the Long-Term Soil Productivity (LTSP) project (Powers, 2006). For our study, an untreated patch was randomly located at each LTSP site, in which two 5 x 25 m experimental plots were established (10 m between plots, 50 m from nearest LTSP plot). On one plot, the forest floor and top 3 cm mineral soil layer were scraped off (referred to as ‘FFMIN’ soil treatment hereafter), whereas the other plot was left undisturbed (referred to as ‘NO’ soil treatment hereafter). Twenty-four independent subplots were then created in each soil treatment plot using a small Bobcat® excavator; each square (~9 m²) subplot was trenched to a soil depth of 50 cm to exclude the influence of surrounding roots and to contain the carbon radioisotope.

In June 2004 and 2005, naturally regenerated interior Douglas-fir seedlings (referred to as ‘natural seedlings’ hereafter; see Table 6.1 for characteristics) were carefully excavated from surrounding roadcuts (on average 1 km away) and transplanted in the middle of each subplot. Each subplot was then numbered and randomly assigned to one of four mesh treatments (mesh bags with 0.5 µm, 35 µm or 250 µm pore sizes; or
A few days later, nursery-grown interior Douglas-fir seedlings (referred to as ‘planted seedlings’ hereafter; see Table 6.1 for characteristics) were planted into the mesh bags 0.5 m away from the natural seedling. These treatments were organized in a split plot design with three replications, where the whole plot effect was the soil disturbance type and the split plot effect was the mesh pore size.

Planted seedlings (seedlot #48520, British Columbia Ministry of Forests and Range Tree Seed Center, Surrey, BC, Canada) were grown at the University of British Columbia (Vancouver, Canada) greenhouse for 6 months in 512B styroblocks™ (Beaver Plastics, Edmonton, AB, Canada); they were non-mycorrhizal and ranged in height from 5 to 19 cm at time of planting. Due to poor survival of the planted seedlings (mainly at O’Connor Lake and Black Pines), we replanted some of the subplots with non-mycorrhizal commercial nursery grown seedlings in May 2005 (Seedlot # 42309, British Columbia Ministry of Forests and Range, Tree Seed Center, Surrey, BC, Canada).

All vegetation in each square subplot was clipped throughout the first growing season and then sprayed twice (2005 and 2006) with glyphosate to eliminate interspecific plant interactions. When inserting the mesh bags (15 cm diameter and 35 cm deep), we minimized soil disturbance by carefully excavating the soil into three distinct soil layers (intact forest floor, A horizon, and some of the B horizon) and replacing these layers into the bags in the same order. Prior to planting the seedlings in the ground of the no mesh treatment, the surrounding soil was disturbed to ensure initial soil disturbance was consistent across mesh treatments. The characteristics of the mesh bags are outlined in Teste et al., (in review; Chapter 3).

The purpose of the mesh treatments was to restrict seedling access to a MN (Robinson & Fitter, 1999). Seedlings planted in a 0.5 µm mesh bag could be colonized by wind- or soil-borne propagules, but not by fungi associated with nearby natural seedlings, nor could hyphae of natural seedlings anastomose with those of planted seedlings once colonized because the pores were too small for hyphal penetration (Teste et al., 2006). Seedlings planted in 35 and 250 µm mesh bags could form a MN by single hyphae or rhizomorphs plus hyphae, respectively. Field and lab observations demonstrated intact rhizomorphs did not penetrate the 35 µm mesh. However, we did notice that rhizomorphs were capable of breaking down into an unstructured form (loose
hyphae) at the surface of the 35 µm mesh, thus allowing penetration, but these occurrences were rare. Seedlings planted directly into soil (no mesh) could form hyphal and rhizomorph MNs, and their roots were free to intermingle with other roots. Root intermingling between natural and planted seedlings was not observed, however (the closest distance separating root tips was 5 cm in the no mesh treatment treatment and 30 cm for the other mesh treatments), thus providing a unique opportunity to compare carbon transfer through the implausible soil pathway (0.5 µm mesh) versus the MN pathway (no mesh) (Simard & Durall, 2004). Differences in transfer between the 250 µm mesh and no-mesh treatments, on the other hand, would indicate the combined effects of mesh presence and degree of proximity of natural and planted seedling roots.

6.2.2 Carbon isotope labeling

Gas labeling bags (10 l) were custom-made with 5-ply transparent gas-tight polyethylene/nylon (FoodSaver®, Jarden Corp., Rye, NY, USA). Prior to isotope gas labeling, 1 ml gas-tight syringes (Hamilton Co., Reno, NV, USA) and a LI-6251 CO$_2$ analyzer (LICOR Inc., Lincoln, NE, USA) were used to determine the amount of time needed for ‘donor’ seedlings to reach the compensation point inside the gas labeling bag after injecting regular CO$_2$ gas. This preliminary data determined the ideal duration of the pulse period for complete assimilation of the $^{13}$CO$_2$.

From June 26 to July 1, 2006, seedlings were pulse-labeled twice (between 8:00 and 9:30, then again between 11:00 and 12:30) with 200 mL of $^{13}$CO$_2$ (99% $^{13}$C, Cambridge Isotope Laboratories, Inc.) using a 0.5 l gas-tight super syringe (Hamilton Co., Reno, NV, USA) or with 4.44 MBq gaseous $^{14}$CO$_2$ (released from 0.25 ml of Na$_2^{14}$CO$_3$ with lactic acid). Seedlings labeled with $^{14}$C were also injected twice with 200 ml of regular CO$_2$ gas ($^{12}$CO$_2$) immediately after release of $^{14}$CO$_2$ in order to match the CO$_2$ concentration inside the gas labeling bags of seedlings labeled with $^{13}$C. Both seedlings (natural and planted) in a subplot were labeled with $^{13}$CO$_2$ or $^{14}$CO$_2$ (total of 90 seedlings labeled).

After six hours, all seedling shoots were carefully covered with thick plastic bags to prevent accidental aerial enrichment when gas-labeling bags were removed and flushed. After a few minutes, the thick plastic bags were removed from the natural and
planted seedlings. After all gas-labeling bags were removed and flushed, potted interior Douglas-fir seedlings (referred to as ‘aerial control’ seedlings hereafter) were placed in between the natural and planted seedlings to estimate the amount of re-fixed carbon via plant or soil respiration. After a 7 day chase period, we harvested shoots and roots of all seedlings. We carefully excavated root systems and only removed clumps of soil when encountered. All plant material was immediately placed into air-tight plastic bags and surrounded with dry ice during transport to the lab, where they were kept frozen at -20 °C until they were oven dried. In the lab, remaining soil was carefully washed off root systems with running tap water in a series of tubs.

6.2.3 Carbon isotope analysis

Natural and planted seedling tissue was oven-dried at 70°C for 48 hours and then weighed. Tissue was first roughly ground to 0.5 mm with a digital ED-5 mid-sized mill (Thomas Scientific©, Swedesboro, NJ, USA) and then thoroughly mixed. A MM 200 ball mill (Retsch® Newtown, PA, USA) was used to turn a 100 mg subsample of the rough-ground tissue into a 0.01mm fine powder. After homogenizing the fine powder, we sampled 1 mg of shoot and root tissue for C isotopic composition. The samples were combusted and the CO₂ liberated was analyzed for the ratio \(^{13}C/^{12}C\) with a continuous flow Europa Hydra 20/20 and Europa Integra (enriched samples) isotope ratio mass spectrometer at the UC Davis Stable Isotope Laboratory (Davis, CA, USA). The amount of \(^{14}C\) (dpm) of each sample was simultaneously measured by subsampling liberated CO₂ (during combustion) with a gas-tight syringe and analyzing with a liquid scintillation counter. The C isotope ratio (\(\delta^{13}C\)) was calculated as:

\[
\delta^{13}C = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]

in ‰ units, where \(R = ^{13}C/^{12}C\). The sample ratio is relative to the Vienna-PeeDee Belemnite (V-PDB) standard (1.1237 x \(10^{-2}\)) for C. The resulting \(\delta^{13}C\) and \(^{14}C\) values were used to determine if seedlings had an excess of \(^{13}C\) and/or \(^{14}C\) above natural abundance levels.
6.2.4 Carbon excess, bidirectional and net transfer calculations

To convert $\delta^{13}$C into mg of $^{12}$C-equivalent excess in seedlings we followed a modified version of the procedure of Boutton (1991) outlined in Teste et al. (in review; Chapter 5). To convert ‘ppm’ $^{14}$C into mg $^{12}$C-equivalent excess in seedlings we first calculated the radioactivity of the sample (sample $^{14}$C):

$$sample\ ^{14}C\ (Bq) = \frac{sample\ radioactivity\ (dpm) - background\ radioactivity\ (dpm)}{60} \quad (1)$$

Tissue (shoots or roots) radioactivity (tissue $^{14}$C) was calculated as:

$$tissue\ ^{14}C\ (Bq) = \left( \frac{tissue\ weight\ (mg)}{sample\ weight\ (mg)} \right) \times sample\ ^{14}C \quad (2)$$

The tissue excess in radioactivity (excess tissue $^{14}$C) was then calculated as:

$$excess\ tissue\ ^{14}C\ (mmol) = \frac{tissue\ ^{14}C}{specific\ activity\ (Bq/mmol)} \quad (3)$$

Finally, excess tissue $^{14}$C was converted to excess tissue $^{12}$C-equivalent (excess tissue $^{12}$C-equivalent) by the equation:

$$excess\ tissue\ ^{12}C\ -\ equivalent\ (mg) = excess\ tissue\ ^{14}C \times 12 \quad (4)$$

Excess plant $^{12}$C-equivalent was calculated as the sum of excess shoot and root $^{12}$C-equivalent.

Bidirectional transfer was the sum of excess $^{12}$C-equivalent received by both the natural and planted seedlings in a given subplot (i.e., in a seedling group). Net transfer was based on excess plant $^{12}$C-equivalent that was received from partner ‘donor’ seedling. Net transfer was the difference between excess $^{12}$C-equivalent received by the natural seedling and that received by the planted seedling. Positive net transfer meant that the natural seedling received more carbon (a net gain) than the planted seedling, and a negative net transfer indicated the opposite. For example, if net transfer = 0.7 mg ($^{12}$C-equivalent) then we say that natural seedlings received a net carbon gain of 0.7 mg; if net transfer = - 0.4 mg ($^{12}$C-equivalent) then we say that planted seedlings received a net carbon gain of 0.4 mg. Only values above a 99 % confidence interval (based on seedling background levels) for both $\delta^{13}$C and $^{14}$C dpm were considered enriched (i.e., net transfer greater than zero).
6.2.5 Sampling, morphotyping, and molecular analyses of seedling ectomycorrhizas

From July 8 to 13, 2006, non-labeled natural and planted seedlings were destructively harvested for estimating background carbon isotope ($\delta^{13}$C and $^{14}$C) levels of plant tissue and for EM morphotyping. Natural and planted seedling root systems were severed from shoots and placed in plastic bags with loose soil and stored at 3 °C until further processing. All samples were processed within 3 months after field sampling. Details on root sample preparation, morphotyping, and molecular analyses (PCR, sequencing, BLAST search, and microsatellite analyses of *Rhizopogon vinicolor* EM) can be found in Teste et al. (in review; Chapter 3). Finally, we randomly sampled 20 EM tips from each of the three most abundant EM taxa on all labeled and non-labeled seedlings for isotope analysis. We then processed these tips as indicated above for seedling tissues and calculated carbon transfer in the same manner.

6.2.6 Statistical analyses

All statistical analyses were carried out using the R (R Development Core Team, 2006) statistical environment for statistical computing and graphics. The split plot design was analyzed using a linear mixed-effects model (Pinheiro & Bates, 2000) using function `lme` (package `nlme`, Jose Pinheiro et al., 2007) where sites were used as blocks (n=3) and set as the random factor. The whole plot and split plot fixed factors were the soil disturbance and mesh size treatments, respectively. We performed t-tests with function `t.test` to determine if seedling shoots accumulated more carbon isotope than roots. The effect of mesh treatment on net carbon transfer to the three most abundant EM taxa was detected with one-way analysis of variance using function `aov`. To assess the influence of seedling size and growth on seedling net carbon transfer, we performed simple linear regression with function `lm`. Percent EM colonization was calculated following Teste et al. (2006). The Morisita-Horn similarity index ($C_{MH}$) (Magurran, 2004) was calculated following Schoonmaker et al. (2007). Statistical differences were considered significant at $P \leq 0.05$. 

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6.3 Results

6.3.1 Soil disturbance and MN effects on net carbon transfer

There was a net transfer of carbon between natural and planted seedlings (Fig. 6.1). The amount of net carbon transferred was unaffected by the mesh or soil treatments (Fig. 6.1). We found significantly greater gross and net carbon transfer to shoots compared to roots (Table 6.2). The amounts of labeled carbon found in the shoots of aerial control seedlings were trivial and not significantly greater than zero (Table 6.2).

Greater net carbon transfer occurred to *Rhizopogon vinicolor* tips than the other two abundant EM taxa (Fig. 6.2). Host carbon was not preferentially allocated to ‘donor’ seedling *R. vinicolor* tips compared to other EM tips based on excess $^{13}$C ($P = 0.58$) or excess $^{14}$C ($P = 0.98$). Therefore, it was unlikely that this trend in Fig. 6.2 was confounded by host carbon allocation differences between the EM taxa.

6.3.2 Seedling relationships with net carbon transfer

Net carbon transfer occurred to 21 natural (51.2%) and 20 planted (48.8%) seedlings. This result indicates that natural and planted seedlings did not consistently act as net ‘donors’ or net ‘receivers’, respectively. Instead, the magnitude of net carbon transferred was regulated by ‘receiver’ seedling size, where it increased with seedling stem volume and shoot biomass, whether seedlings were naturals or planted (Fig. 6.3). Net carbon transfer also increased with seedling relative height growth rate (Fig. 6.4).

6.3.3 Natural and planted seedling ectomycorrhizal communities

A total of 5 EM taxa were found on both natural and planted seedling root systems (Table 6.3). Natural and planted seedlings hosted 5 and 4 EM taxa, respectively, with 4 EM taxa in common (Table 6.3). Natural seedlings had higher levels of EM colonization (96%) than planted seedlings (79%) ($P < 0.01$). The two most abundant EM taxa found on natural and planted seedlings were *Wilcoxina rehmii* and *Rhizopogon vinicolor* (Fig. 6.5). Natural and planted seedlings shared 80% of all EM taxa found in this study and 100% of those taxa had a relative abundance on root tips greater than 5% (Fig. 6.6). The $C_{MH}$ similarity index indicated there was 94% similarity between the natural and planted seedling EM communities.
We successfully amplified DNA from 52 *Rhizopogon vinicolor* samples, of which 19 produced clear fragments for four different microsatellite loci. Eight of the *R. vinicolor* samples allowed us to determine if natural (4 samples) and associated planted seedlings (4 samples) were harboring the same fungal genet (i.e., same individual fungus). Out of these four seedling groups, we found one seedling group (comprised of a natural seedling and planted seedling in a 35μm mesh bag at the DA site in the forest floor and mineral soil removal treatment) shared the same *Rhizopogon vinicolor* genet. These results provide evidence for the formation of MN between Douglas-fir seedlings in the field.

### 6.4 Discussion

#### 6.4.1 Seedling relationships with net carbon transfer

Net carbon transfer occurred between conspecific seedlings, and it appeared to occur along an interplant source-sink gradient. Seedlings with the greatest relative growth rates received the greatest net carbon transfer, agreeing with our first hypothesis that the magnitude of net carbon transfer is positively correlated with sink strength. Rapidly growing ‘receiver’ seedlings likely acted as stronger sinks for carbon than ‘receiver’ seedlings with moderate or low growth rates. It is also possible that transfer was regulated by EM fungal factors, such as degree of colonization, density of rhizomorphs, abundance of rhizomorph-forming EM taxa, and richness and diversity of MN-forming EM taxa (van der Heijden, 2002). Unfortunately we were not able to examine this because of the safety and logistic issues associated with molecular analyses of 14C-enriched EM root tips.

#### 6.4.2 Soil disturbance and MN effects on net carbon transfer

Net transfer of carbon between conspecific seedlings tended to increase with access to a continuous MN, but this effect was not significant, suggesting that other pathways were more important. Almost all natural and planted seedling root tips were colonized by EM, implying that carbon transferred predominantly via a discontinuous MN pathway, where carbon entered the soil matrix from ‘donor’ EM mycelia and was then rapidly picked up by ‘receiver’ EM mycelia. The MN connections have traditionally
been thought of as intact continuous hyphal links but the mycorrhizal root systems can also include discontinuous hyphal pathways (Simard & Durall, 2004) involving the same or different species. The presence of a MN, whether or not it includes continuous or a mix of continuous with discontinuous links, is what matters most to seedlings receiving resources from these networks. The possibility for transfer through a soil pathway was minimized by the absence of root intermingling (gaps of 5 to 30cm separated interplant root tips). With such a large spatial gap between donor and receiver EM root tips and such a short labeling chase period, any carbon leaked into the soil would have been immediately immobilized by competing soil microbes rather than moving through soil pores via mass flow (Högberg & Read, 2006; Högberg et al., 2008).

Net carbon transfer was also not affected by soil disturbance, disagreeing with Callaway et al. (2002) that biotic facilitation is more important under harsh conditions. It is possible, however, that our soil treatments did not impose a large enough stress on receiver seedlings to affect transfer. Nevertheless, we reject our second and third hypotheses that net carbon transfer to conspecific seedlings increases with access to a continuous MN and with soil disturbance.

Greater amounts of carbon were transferred to shoots than roots of seedlings, and we determined that this was not due to re-fixation of respired CO₂ (from soil, hyphae, or ‘donor’ seedling) in the shoots. These results support our fourth hypothesis that net carbon transfer occurs along MN pathways to both shoots and roots of ‘receiver’ seedlings. We provide evidence opposing the findings of Wu et al. (2001), who suggest that carbon is not transferred to shoots of EM plants, but agrees with other studies demonstrating that carbon is transferred to both ‘receiver’ shoots and roots, albeit at lower amounts in shoots (Read et al., 1985, Simard et al., 1997a, Simard et al., 1997b). In Wu et al. (2001), the shoots of the ‘receiver’ seedlings were covered in aluminum foil, thus inhibiting photosynthetic activity and reducing their sink strength. In our study, ‘receiver’ seedlings were growing in full sunlight and at a time of year (early July) where carbon and nitrogen demands in the shoots are high. Under these conditions, shoots may have been stronger sinks than roots for MN-transferred carbon.

_Rhizopogon vinicolor_ EM root tips received greater amounts of transferred carbon than the other two abundant EM taxa found on ‘receiver’ seedlings. This finding agrees
with our fifth hypothesis that net carbon transfer is greater to root tips colonized by EM taxa forming rhizomorphs. Using time-course autoradiography, Wu et al. (2001) observed higher radioactivity in rhizomorphs compared to the mycelium of *Pisolithus tinctorius* forming an MN between *Pinus densiflora* seedlings. Rhizomorphs potentially provide the most important pathway for carbon transfer between plants forming a MN (Brownlee et al., 1983; Read et al., 1985), but rhizomorph connections are more variable and may be less frequent than simple hyphal connections (Finlay & Read, 1986). Our results agree with the suggestion that rhizomorphs may facilitate interplant carbon transfer over greater distances than the extending mycelia (Finlay & Read, 1986).

### 6.4.3 Potential for mycorrhizal networks to form

The potential for MNs to form between natural and planted seedlings was high because their EM community compositions were very similar and because abundance of the most common taxa was identical. This is also supported by fragment analysis (microsatellite marker analysis) showing the same *Rhizopogon vinicolor* genet on the natural and planted seedling in one of the seedling pairs. Natural seedlings were excavated kms away and must have harbored different *Rhizopogon vinicolor* genets than the genets present as resilient soil propagules (spores, sclerotia, or hyphal fragments) in the subplots; this is because the largest distance found between different genets of this species has been only 2 m (Kretzer et al., 2003). Given that planted seedlings were non-mycorrhizal at time of planting, we suggest that the occurrence of the same *Rhizopogon vinicolor* genet on natural and planted seedlings arose through colonization by extraradical hyphae or rhizomorphs from a MN. These results corroborate our associated studies of Douglas-fir (Teste et al., in review, Chapter 3; Teste et al., in review, Chapter 5), as well as other studies showing high potential for MNs to establish among conspecific tree species (Jonsson et al., 1999; Matsuda & Hijii, 2004; Haskins & Gehring, 2005; Cline et al., 2005).

### 6.4.4 Conclusion

This study presents evidence that net carbon transfer occurs between conspecific seedlings through a MN comprised of continuous and discontinuous hyphal pathways. The harsh conditions we created by soil disturbance did not affect net carbon transfer, but
it is possible that our treatments were insufficient to affect factors most limiting to seedling establishment. Contrary to previous studies, more carbon was gained by receiver shoots than roots, possibly because sink strength in the shoots was greater than the roots due to time of year and full sunlight conditions. Net carbon transfer increased with receiver seedling relative growth rate and was significantly greater to *Rhizopogon vinicolor* than other abundant EM taxa. Mycorrhizal networks appear to facilitate net transfer of carbon between seedlings in dry forests under full sun conditions, and its correlation with sink-strength suggests this may be even more important for performance of seedlings rapidly regenerating in forest canopy gaps.

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Table 6.1 Site and interior Douglas-fir (*Pseudotsuga menziesii* var. *glauc*a) seedling characteristics in 2006. Values for characteristics are means with one standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Site characteristics</th>
<th>Dairy creek (DA)</th>
<th>O'Connor lake (OC)</th>
<th>Black pines (BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude; Longitude (ū)</td>
<td>50.51; 120.25</td>
<td>50.53; 120.21</td>
<td>50.56; 120.17</td>
</tr>
<tr>
<td>Elevation (m)</td>
<td>1150</td>
<td>1180</td>
<td>1075</td>
</tr>
<tr>
<td>Slope (%)</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Aspect</td>
<td>North</td>
<td>South west</td>
<td>South</td>
</tr>
<tr>
<td>Soil type</td>
<td>Brunisolic Gray Luvisol</td>
<td>Brunisolic Gray Luvisol</td>
<td>Brunisolic Gray Luvisol</td>
</tr>
<tr>
<td>Soil texture</td>
<td>Silt loam</td>
<td>Silt loam</td>
<td>Silt loam</td>
</tr>
<tr>
<td>Humus form</td>
<td>Hemimor</td>
<td>Hemimor</td>
<td>Hemimor (Mormoder)</td>
</tr>
<tr>
<td>Seedling characteristics</td>
<td>Natural regeneration</td>
<td>Planted</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>4 to 6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>30.6 (9.1)</td>
<td>26.4 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Root collar diameter (cm)</td>
<td>0.77 (0.21)</td>
<td>0.60 (0.13)</td>
<td></td>
</tr>
<tr>
<td>Stem volume (cm³)</td>
<td>17.3 (14.6)</td>
<td>8.2 (4.7)</td>
<td></td>
</tr>
<tr>
<td>Shoot dry biomass (g)</td>
<td>16.9 (10.7)</td>
<td>9.3 (5.6)</td>
<td></td>
</tr>
<tr>
<td>Root dry biomass (g)</td>
<td>5.4 (2.8)</td>
<td>3.7 (1.7)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.2  Average carbon transfer between natural regeneration interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) and planted seedlings, and carbon assimilation by aerial control seedlings in 2006.

<table>
<thead>
<tr>
<th></th>
<th>Shoot</th>
<th>Root</th>
<th>Lower</th>
<th>Upper</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excess $^{12}$C-equivalent (mg) (receiving $^{14}$C)</td>
<td>0.106</td>
<td>0.025</td>
<td>-0.011</td>
<td>0.172</td>
<td>1.762</td>
<td>0.083</td>
</tr>
<tr>
<td>Excess $^{12}$C-equivalent (mg) (receiving $^{13}$C)</td>
<td>0.131</td>
<td>0.025</td>
<td>0.038</td>
<td>0.174</td>
<td>3.144</td>
<td>0.002</td>
</tr>
<tr>
<td>Aerial control excess $^{12}$C-equivalent (mg) (receiving $^{14}$C)</td>
<td>0.0006</td>
<td>-</td>
<td>-0.0012</td>
<td>0.0023</td>
<td>0.627</td>
<td>0.534**</td>
</tr>
<tr>
<td>Gross transfer (mg $^{12}$C-equivalent)</td>
<td>0.237</td>
<td>0.05</td>
<td>0.05</td>
<td>0.323</td>
<td>2.754</td>
<td>0.008</td>
</tr>
<tr>
<td>Net transfer (mg $^{12}$C-equivalent)</td>
<td>0.133</td>
<td>0.041</td>
<td>0.017</td>
<td>0.167</td>
<td>2.455</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*95% confidence intervals for the differences between the shoot and root means.

**Based on a simple t-test which was used to determine if aerial control seedlings had significant amounts of labeled carbon.
Table 6.3 List of observed EM taxa on Interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) natural regeneration (Natural), or planted (Planted) seedlings, or both hosts on the three LTSPs (Dairy Creek, O'Connor Lake, and Black Pines) sites in the IDF biogeoclimatic zone of BC, Canada, in July 2006.

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>Closest BLAST match</th>
<th>Database Accession number</th>
<th>Total base pairs aligned</th>
<th>NCBI % similarity</th>
<th>Consensus EM taxa</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphinema</td>
<td><em>Amphinema byssoides</em></td>
<td>NCBI</td>
<td>613</td>
<td>98</td>
<td><em>Amphinema byssoides</em></td>
<td>Natural</td>
</tr>
<tr>
<td>Cenococcum</td>
<td><em>Cenococcum geophilum</em></td>
<td>NCBI</td>
<td>599</td>
<td>99</td>
<td><em>Cenococcum geophilum</em></td>
<td>Both</td>
</tr>
<tr>
<td>Rhizopogon</td>
<td><em>Rhizopogon vinicolor</em></td>
<td>NCBI</td>
<td>544</td>
<td>99</td>
<td><em>Rhizopogon vinicolor</em></td>
<td>Both</td>
</tr>
<tr>
<td>Suillus</td>
<td><em>Suillus lakei</em></td>
<td>NCBI</td>
<td>588</td>
<td>99</td>
<td><em>Suillus lakei</em></td>
<td>Both</td>
</tr>
<tr>
<td>Wilcoxina</td>
<td><em>Wilcoxina rehmii</em></td>
<td>NCBI</td>
<td>397</td>
<td>97</td>
<td><em>Wilcoxina rehmii</em></td>
<td>Both</td>
</tr>
</tbody>
</table>

*aFor photographs and concise morphotype descriptions, please contact the corresponding author. bNCBI is the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).
Figure 6.1  Net carbon transfer to Douglas-fir seedlings (natural or planted) grown in the soil disturbance and mesh treatments in the field. Mean background $\delta^{13}$C and $^{14}$C dpm were used to calculate 99 % confidence intervals (CI). Only values above the 99 % CI for both $\delta^{13}$C and $^{14}$C dpm are considered enriched and are marked as asterisks on the figure. Analysis of variance indicated that the soil disturbance ($P = 0.53$) and mesh ($P = 0.30$) treatments had no effect on net carbon transfer.
Figure 6.2  Net carbon transfer to the three most abundant EM taxa (Cg: *Cenococcum geophilum*; Rv: *Rhizopogon vinicolor*; Wr: *Wilcoxina rehmi*) found on Douglas-fir seedlings (natural or planted). Values are means with 95% confidence intervals.
Figure 6.3 The relationships between seedling (natural or planted) (A) stem volume or (B) shoot dry biomass and net carbon transfer to enriched receiver seedlings (natural or planted).
Figure 6.4 Relationship between enriched receiver seedling (natural or planted) relative growth rate and net carbon transfer.
Figure 6.5 Relative abundance of natural and planted seedlings EM taxa.
6.5 References


7 CONCLUSIONS

The role of MNs in plant ecology appears complex, dynamic, and potentially important for ecosystem functioning, but current research approaches represent a gross simplification of real-world processes (Whitfield, 2007). Current studies on MNs have provided evidence for their existence (Finlay & Read, 1986; Wu et al., 2001; Kennedy et al., 2003; Lian et al., 2006), their role in interplant resource transfer (Simard et al., 1997; Lerat et al., 2002, Egerton-Warburton et al., 2007), and their indirect benefits to seedlings (McKendrick et al., 2000; Booth, 2004; McGuire, 2007), and many reviews have suggested they play an ecologically relevant role in plant communities (Newman, 1988; Wilkinson, 1998; Simard et al., 2002; Leake et al., 2004; Selosse et al., 2006). In this thesis, I investigated whether MNs simultaneously increase plant survival and facilitate interplant resource transfer in the field. My general objectives were:

1. To determine whether there is potential for MNs to form among conspecific trees in dry Douglas-fir forests.
2. To determine the influence of MNs with residual trees on interplant carbon transfer and survival, growth, physiology, and EM status of naturally regenerated and planted Douglas-fir seedlings.
3. To examine effects of donor size, distance from donor, or soil disturbance on the extent of interplant carbon transfer or seedling performance.

7.1 Main research findings

The over-arching research question of my thesis was: Provided that MNs exist in dry Douglas-fir forests, are they important for seedling establishment and growth, and can they facilitate interplant carbon transfer? I was able to answer many other questions as well, but here are my most pertinent conclusions:

7.1.1 The potential for mycorrhizal networks to form in the field is high

The results from Chapters 3, 5 and 6 supported my hypothesis that the potential for MNs to form in these dry Douglas-fir forests is high; in these three studies, trees and seedlings shared 83%, 80%, and 100%, respectively, of their most abundant EM taxa. The
potential for MNs to form tended to decrease with distance from trees (Chapter 3) and with size (and age) of established trees (Chapter 5). The genet analyses of *Rhizopogon vinicolor* showed that a tree (Chapter 3) or naturally regenerated seedling (Chapter 6) shared the same fungal individual with neighboring seedlings. These studies provide evidence that MNs, and specifically networks formed by *Rhizopogon vinicolor*, exist in the field.

**7.1.2 Mycorrhizal network-mediated colonization is not always the dominant mechanism promoting ectomycorrhizas to establishing seedlings**

My hypothesis, that MN-mediated colonization is the dominant mechanism promoting continuity of the EM community, was rejected (Chapter 3 and Chapter 5). I found instead that soil- or wind-borne EM inoculum (e.g., spores, hyphal fragments, and severed tree EM tips) were the main vectors responsible for EM colonization of seedlings. These are important findings because they indicate that MN-mediated colonization is not always, as once thought (Fleming, 1983; Newman, 1988), the dominant mechanism promoting continuity of the EM community from trees to nearby seedlings. Nevertheless, my studies do not discount that MN-mediate colonization does occur simultaneously with the other mechanisms or is important under different environmental conditions.

**7.1.3 Mycorrhizal networks increase survival of naturally regenerated seedlings**

My key hypothesis, that linkage into a MN can increase seedling survival in the field, was supported for seedlings grown from seed but rejected for seedlings transplanted to the field from a greenhouse. Full access to a MN resulted in the greatest survival of germinating seedlings (Chapter 5), suggesting that MNs are important for germinating seedlings struggling to establish under the harsh conditions found in these dry forests (Vyse et al., 2006). Many growth and physiological responses were unaffected by MNs, however, indicating that the effects are subtle and difficult to detect under full sunlight conditions. The survival increases we observed appeared associated with carbon transfer from donor seedlings through the MN. These findings are impressive and fill an important gap in MN research because they demonstrate that the effects of carbon transfer through a MN is ecologically relevant to forest dynamics. The importance of
MNs may even be greater in the forest understory or in forest gaps, where mature ‘donor’ trees are numerous and sink strength of establishing seedlings is high.

7.1.4 Mycorrhizal networks facilitate carbon transfer to seedling shoots and roots

I found that interplant carbon transfer was facilitated by increased access to a MN when the ‘receiver’ seedlings were germinated from seed in the field (Chapter 5) but not when they were first grown and then transplanted from the greenhouse into the field, or transplanted from roadcuts into soil disturbance plots (Chapter 5 and 6). In Chapter 6, I provide evidence for interplant net carbon transfer through a MN comprised primarily discontinuous rather than continuous pathways; the possibility for transfer through a soil pathway was minimized by the absence of root intermingling (the closest distance separating root tips was 5 cm in the no mesh treatment and 30 cm for the other mesh treatments). A key finding was that greater amounts of carbon was transferred to shoots than roots of seedlings, and this was not due to re-fixation of respired CO₂ (from soil, hyphae, or ‘donor’ seedling) in the shoots. My findings address some of the major concerns outlined by Robinson & Fitter (1999) and have the potential to remove some of the controversy surrounding the role of MNs in ecosystem functioning (Whitfield, 2007).

7.2 Strengths of the thesis

An obvious strength of this thesis is that all experiments were conducted in the field under natural conditions (except Chapter 2). Also, two out of four field studies occurred over 3 growing seasons, which is considerably longer than most other field research on MNs. All of the ‘donor’ plants were already established, relatively old, and covered an extensive area, increasing the realism of my research. The ‘receiver’ seedlings were all non-mycorrhizal at time of planting thus eliminating any confounding EM inoculation effects.

The use of mesh bags, without sterilizing the excavated soil to control for the MN, was an important strength of my thesis. Mesh bags restricted access to the MN and provided evidence for a direct “MN effect” (Taylor, 2006). Nara (2006) also assessed the facilitative effects of MNs but avoided de novo formation of mycorrhizas from spores to ensure that seedlings established within the existing MN. I suggest that Nara (2006) provided evidence for a “MN-inoculation effect” (indirect “MN effect”), rather than a
direct “MN effect” that could include nutrient transfers, because the MN of the established plants served to inoculate nearby seedlings. The resulting mycorrhization likely accounted for the increase in observed seedling performance. Seedlings in my thesis could form mycorrhizas from spores, sclerotia, hyphal fragments, and severed tree EM tips from the soil in which they were planted, and hence did not rely on the MN for inoculation. Under these conditions, the increase in seedling performance could only be attributable to a direct “MN effect”.

In Chapter 5, I present results showing that access to a MN can increase seedling survival and that this was associated with carbon and nitrogen transfer. The novelty of these results lies in their demonstration, for the first time, that nutrient transfers via MNs are significant to plant community dynamics. This was achieved by successfully germinating seed in situ and using custom-built large gas-labeling bags for the delivery of $^{13}$CO$_2$.

In Chapter 6, I provide evidence for interplant net carbon transfer through an MN; the possibility for transfer through a soil pathway was minimized by the absence of root intermingling (the closest distance separating root tips was 5 cm in the no mesh treatment and 30 cm for the other mesh treatments). With such a large spatial gap between donor and receiver EM root tips and such a short labeling chase period, any carbon leaked into the soil would have been immediately immobilized by competing soil microbes rather than moving through soil pores via mass flow (Högberg & Read, 2006; Högberg et al., 2008). In this study, I also addressed many of the concerns of Robinson & Fitter (1999). For example, the microsatellite marker analyses (fragment analyses) of Rhizopogon vinicolor genets indicated that hyphal connections and MNs occurred between seedlings. The use of physical barriers and fully reciprocated labeling also address their concerns that net transfer occurred through MNs. This study is unique because ‘donor’ seedlings were excavated several kms away and therefore must have harbored different Rhizopogon vinicolor genets than the genets present as resilient soil propagules (e.g., hyphal fragments or severed EM root tips) in the subplots. Kretzer et al. (2003) had previously shown that the largest inter-genet distance for Rhizopogon vinicolor was only 2 m; hence, the non-mycorrhizal ‘receiver’ seedlings must have been colonized via a MN comprised of the same Rhizopogon vinicolor genet on conspecific ‘donor’ seedlings.

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7.3 Weaknesses of the thesis and alternatives

In Chapter 2, I achieved low EM colonization levels, shedding some doubt on the biological significance of using fungicides as controls for EM colonization. Douglas-fir seedlings are slow to colonize in the greenhouse; hence, future greenhouse bioassays using Douglas-fir should be conducted over a period of at least 12 months. If this is not possible, then using a readily colonized donor plant, such as a *Pinus* sp., could also be an alternative. The presence of EM greenhouse contaminants made the interpretation of the results difficult and the findings less robust. An alternative approach for reducing EM contamination could have been to conduct this study in a highly efficient, particulate air-filtering chamber with filtered water.

Typically, the success rate of molecular analyses of EM root tips increases with the number of samples processed. Therefore, in Chapter 3, analysis of more EM root tips could have increased the chances of successfully identifying genets during fragment analysis. More analyses could have also helped determine the influence of the various mesh treatments on *Rhizopogon vinicolor* hyphal connections in the field.

In Chapter 4, I could have sown seed, as in Chapter 5, instead of using commercially-grown nursery seedlings. The interpretation of water-use efficiency via δ¹³C is not straightforward and could have been improved by including some δ¹⁸O data (Barbour, 2007). For Chapter 3 and 4, I could have included a fifth distance treatment (e.g., 20 m away) where the influence of the residual tree was negligible, allowing for a more accurate estimate of the competitive and facilitative effects of the residual trees on the seedlings.

In Chapter 5, growing a greater number of replicates from seed would have allowed me to estimate foliar nitrogen of germinants in the field. I also should have included more background (natural abundance) samples for the aerial control seedlings. The δ¹³C values were variable, and because I included only 4 background samples for the aerial control seedlings, some enrichment of two seedlings may have occurred via re-fixation of respired carbon.

In Chapter 6, the soil disturbance treatment was not harsh enough to physiologically stress the seedlings; this may explain why the soil disturbance treatments had no effect on net carbon transfer or seedling responses. Also, I intended to use the
naturally regenerated seedlings as ‘donor’ source plants, but mortality and slow growth rendered them physiologically similar to the planted seedlings.

Finally in Chapters 5 and 6, I did not collect “time-zero” pulse-labelled samples because of low numbers of replicates. Therefore, I needed to rely on previous research to estimate carbon loss via respiration before calculating percent carbon transfer values.

7.4 Potential applications of the research

This research has some direct and indirect applications for applying variable retention silvicultural systems (Kohm & Franklin, 1997) for the natural regeneration of Douglas-fir seedlings in the interior dry-belt forests of B.C. Retaining live mature trees at sufficient spatial distribution after harvesting can help maintain a diverse EM community. This may be most important in dry forests where EM hosts are surrounded by AM vegetation.

The influence of mature tree root zones can extend several meters beyond the tree crown, ensuring seedling colonization, continuity of the EM fungal community, and increase the potential for MNs to form both spatially and through time. Within this extensive rooting zone, we found that residual trees, MNs and carbon transfer had a net facilitative effect on establishment and survival of seedlings at intermediate distances. Retaining conspecific mature and advanced regeneration trees following disturbance at a sufficient spatial distribution for regeneration within zones of net benefit in these naturally patchy forests may become increasingly important with increasing climatic stress. Planting seedlings around residual EM host trees may also increase seedling performance on sites that are degraded or invaded by exotic AM weeds.

7.5 Future research directions

Here, I present a few avenues for future research on MNs in forest ecosystems. Large and growth-affecting amounts of C and N may be transferred to understory plants by canopy dominant trees via MNs. Future research could focus on plants in forest understories or canopy gaps, where MNs may be critical to the survival and growth of plants struggling in low or variable light conditions. Of considerable interest is whether established plants and their MNs facilitate the establishment of target species on severely disturbed sites or species migrating with climate change. Future work could also
determine whether MNs and resource transfer are important regulators of understory mycoheterotrophic and mixotrophic plant community composition and diversity. Determining the degree of mycoheterotrophy and the importance of MNs to chlorophyllous species in families such as Burmanniaceae and Gentianaceae would also be an interesting research area (Leake, 2004). The use of natural abundance isotopic signatures of C and N could be used for this purpose instead of tracers (Staddon, 2004).

Identifying the compounds involved in interplant carbon transfer via MNs would be useful. For instance, it would be interesting to determine whether carbon is moving as amino acids, carbohydrates, or both. If carbohydrates move from fungi to plants (reverse of the normal flow of carbon), then it is important to discover whether there is a new cellular pathway or whether carbon is simply moving through a pathway already known (Smith & Read, 1997).

To assess whether mature trees are transferring carbon to seedlings, a novel stem injection technique using $^{13}$C-labeled sugars coupled with a continuous pulse-labeling approach needs to be developed. The use of $^{14}$C is theoretically possible but not pragmatic in the field when dealing with large trees. Finally, further evidence that MNs comprised of additional or multiple species needs to be explored using autoradiography techniques and microsatellite markers. These approaches used together could potentially provide the unequivocal evidence needed to convince even the heartfelt skeptic.
7.6 References


