THE ROLE OF ECTOMYCORRHIZAL FUNGI IN CARBON TRANSFER WITHIN COMMON MYCORRHIZAL NETWORKS

by

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ABSTRACT

Ectomycorrhizae, one of the largest groups of mycorrhizal fungi, have low host specificity, colonizing mainly tree species in temperate forest systems worldwide. Because these fungi readily colonize many trees, hyphae form belowground connections between root systems of the same or different tree species whereby carbon and nutrients flow directly between rhizospheres. This biological phenomenon has been described as a consequence of common mycorrhizal networks and has been suggested as one potential factor in regulating composition and structure of plant communities. Ectomycorrhizae and belowground carbon transfer between paper birch (*Betula papyrifera* Marsh.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) was studied in three laboratory studies and one field study to determine the role ectomycorrhizal fungi play in belowground carbon transfer between neighbouring mycorrhizal plants. Pot systems, root chambers, and pulse labeling were used in the laboratory to examine: 1) minimum detection levels required to detect belowground carbon transfer, 2) the magnitude and direction of carbon transfer through hyphal pathways compared to alternate soil pathways, and 3) the effects of fungal species on the magnitude of carbon transfer. Results from the laboratory show that >16 ml $^{13}CO_2$ and at least 10 μCi $^{14}CO_2$ pulse-labels are required to detect carbon transfer and is size dependent, that more net carbon transfer occurs when hyphal connections are left intact than not, and that fungal specificity tends to affect carbon transfer. Reciprocal isotopic labeling of paper birch and Douglas-fir in spring, summer and fall was used to determine phenological effects on the magnitude and direction of transfer. Carbon moved from paper birch to Douglas-fir in summer and in the opposite direction in spring and fall representing 4-34% net transfer (proportion of the total isotope assimilated in the system) through hyphal pathways. Phenology also affected carbon transfer through soil pathways. These laboratory and field findings support and extend previous studies in belowground carbon transfer, providing strong evidence for the role ectomycorrhizal fungi play in belowground transfer. Further studies are required to

determine if transfer amounts observed in these experiments are of a sufficient magnitude to influence plant biodiversity, productivity, and fitness.

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DEDICATION

"There must be a beginning of any great matter, but the continuing unto the end until it be thoroughly finished yields the true glory." Francis Drake 1587

For everyone who pleaded with me to quit and for all who encouraged me to continue along this path to the end; for my Dad who is still here to see me achieve my goal; for my Uncle who is no longer with me but always encouraged my dreams.

CHAPTER ONE: Overview and Summary

Introduction

Belowground carbon transfer and common mycorrhizal networks (CMNs) have been discussed extensively in the literature (Wilkinson, 1998; Perry, 1999; Simard *et al.*, 2002; Bever, 2003; Booth, 2004; Giovannetti *et al.*, 2004; Simard & Durall, 2004). Mycorrhizal fungi form symbiotic associations with herbaceous plant species and temperate coniferous and deciduous tree and shrub species world-wide (Allen *et al.*, 1995; Horton *et al.*, 1999). Both ectomycorrhizal and, to a greater degree, arbuscular mycorrhizal fungi have low host specificity (Allen, 1991; Molina *et al.*, 1992; Fitter *et al.*, 2001). As a result, hyphal connections can develop between root systems of the same or different plant species, providing biological conduits for carbon moving between plants (Jones *et al.*, 1997; Simard *et al.*, 1997b).

Hyphal connections have previously been identified in the laboratory using autoradiography (Francis & Read, 1984; Finlay & Read, 1986a, b; Duddridge *et al.*, 1988; Wu *et al.*, 2001) and direct observation (Newman *et al.*, 1994), despite difficulties visualizing the fragile, microscopic structures. Autoradiography techniques have been helpful in following carbon movement between mycorrhizal plants but are technologically limited by specific radioisotope detection levels and unsuitability to field situations (Wu *et al.*, 2001). Newman *et al.* (1994) successfully observed hyphal connections between grasses, legumes and arbuscular mycorrhizal woody plants, but to date ectomycorrhizal hyphal connections have not been visually identified in the field. Neither autoradiography nor visual observations have been used to estimate the number or functional activity of hyphal connections between mycorrhizal plants in the field.

Other studies have used carbon isotopes to demonstrate one-way, bi-directional, and net carbon transfer between mycorrhizal plants (Watkins *et al.*, 1996; Graves *et al.*, 1997; Simard *et*

al., 1997a, c, d; Fitter *et al.*, 1998; Lerat *et al.*, 2002; Pfeffer *et al.*, 2004). Isotopes have also been used to examine interplant nutrient (nitrogen and phosphorus) transfer through common mycorrhizal networks (Finlay & Read, 1986b; Francis *et al.*, 1986; Newman & Ritz, 1986; Bethlenfalvay *et al.*, 1991; He *et al.*, 2003) and how transfer is affected by soil microbes (Bonkowski *et al.*, 2001; Klironomos & Hart, 2001; Perez-Moreno *et al.*, 2001; Tuffen *et al.*, 2002). Despite the outcomes from these studies, the identity of the primary carbon transfer pathway (Fitter *et al.*, 1999) and the role and biological importance of ectomycorrhizal fungi in belowground carbon transfer remain unclear (Perry, 1999; Robinson & Fitter, 1999; Fitter *et al.*, 2001; Simard & Durall, 2004).

Belowground carbon fluxes between root systems may be best understood by depicting the major carbon transfer pathways functioning between plants (Newman & Ritz, 1986, Martins, 1993). Newman & Ritz (1986) previously described two transfer pathways to explain phosphorus movement between vesicular-arbuscular mycorrhizal plants, where: 1) phosphorus transfers from one root system to another through hyphal connections (the direct transfer pathway); and 2) phosphorus flows from donor roots to receiver uncolonized or mycorrhizal roots through the soil (the soil pathway). In the soil pathway, nutrients transfer in soil solution through diffusion and mass flow. A third carbon transfer pathway intermediate between these two is where carbon flows partially in hyphae and partially in the soil (the discontinuous soil-hyphal pathway) (Simard *et al.*, 2002; Simard & Durall, 2004). Carbon transfer between individual plants of the same species may also occur through root grafts (Fraser *et al.* 2005).

Carbon transfer through hyphal pathways has been documented using arbuscular mycorrhizal and ectomycorrhizal plants grown in the laboratory (Francis & Read, 1984; Fitter *et al.*, 1998; Watkins *et al.*, 1996; Simard *et al.*, 1997c, d; Wu *et al.*, 2001; Pfeffer *et al.*, 2004) and in the field (Simard *et al.*, 1997a; Lerat *et al.*, 2002). In the laboratory, Pfeffer *et al.* (2004) investigated carbon movement from fungus to plant, and from one root system to another, using

^{13}C and ^{14}C labeled substrates supplied to transformed carrot (*Daucus carota*) roots and the arbuscular mycorrhizal fungus, *Glomus intraradices*. In this case, carbon flowed in one direction, from donor to receiver roots, but transferred carbon remained in receiver fungal tissues. Fitter *et al.* (1998), Watkins *et al.* (1996), Graves *et al.* (1997), and Wu *et al.* (2001) also found that carbon moved between mycorrhizal plants, but that labeled carbon was not transferred out of the fungus to receiver plant tissues. In contrast, Simard *et al.* (1997a, d) and Lerat *et al.* (2002), who examined ectomycorrhizal networks among arbuscular and ectomycorrhizal tree species in the field, found that transferred carbon moved into receiver plant tissues.

Source-sink gradients between plants have been demonstrated in the laboratory and field to influence the amount and direction of CMN-mediated carbon transfer (Simard *et al.* 2002, and references therein). Using reciprocal labeling with stable and radioactive carbon isotopes in the field, for example, Simard *et al.* (1997a) found that carbon moved bi-directionally between ectomycorrhizal paper birch and Douglas-fir, with a 3% net gain by Douglas-fir in full sun compared with 10% in deep shade. Simard's work corroborated earlier laboratory studies by Read *et al.* (1985), who found that ^{14}C transfer between *Pinus sylvestris* increased with shading of the receiver plant. In another field study by Lerat *et al.* (2002), radioactive carbon isotope moved one way between arbuscular mycorrhizal trout lily (*Erythronium americanum*) and sugar maple (*Acer saccharum*), with the direction of transfer changing between spring and fall, depending on foliation and photosynthetic status of the two plant species.

In spite of this progress, identity of the dominant transfer pathways and dynamic nature of carbon transfer over a growing season continue to hinder our understanding of the mechanism and significance of interplant carbon transfer. In both the field studies of Simard *et al.* (1997a) and Lerat *et al.* (2002), for example, there was evidence for transfer simultaneously through common mycorrhizal networks and soil pathways. Simard *et al.* (1997a) found that 1-18% of the total amount of carbon transferred between ectomycorrhizal paper birch and Douglas-fir was

transferred through the soil to arbuscular mycorrhizal western red cedar (*Thuja plicata*). Lerat *et al.* (2002) similarly found that labeled carbon was transferred through soil pathways from arbuscular mycorrhizal plants to ectomycorrhizal yellow birch (*Betula alleghaniensis*). In a laboratory study, Simard *et al.* (1997d) found that bi-directional transfer between paper birch and Douglas-fir was of the same magnitude whether through intact or severed hyphal pathways.

Bi-directional and net carbon transfer, either through common mycorrhizal networks or alternate soil pathways, could be influenced by the biology of the fungal species (Fitter, 2001). Arbuscular mycorrhizal fungi, in the phylum Glomeromycetes (Schüßler *et al.*, 2001), often associate with herbaceous plant hosts and increase phosphorus acquisition, while ectomycorrhizal fungi, in the phyla Ascomycetes and Basidiomycetes (Smith & Read, 1997), usually colonize woody plant hosts and enhance nitrogen nutrition (Allen, 1991). Both mycorrhizal types produce extensive networks of extraradical mycelia, composed of many individual fungal species. The wide range of anatomical (hyphae, fruiting bodies, rhizomorphs), physiological (nutrient acquisition, metabolite transfer) and ecological (environmental tolerances) differences (Finlay & Read, 1986a, b) have yet to be considered in laboratory and field studies of carbon transfer.

Rationale

This thesis addresses several of the information gaps illustrated above, including: 1) whether transfer is more important through hyphal than soil pathways, 2) whether there is one-way, bi-directional, or net transfer between plants, 3) whether transferred compounds remain in fungal tissues or are transferred to plant tissues, and 4) the identity of biological and ecological factors that regulate the direction and magnitude of transfer.

Objectives

The overall objectives of this thesis build on the work of Simard *et al.* (1997a, b, c, d) in determining the role and significance of common mycorrhizal networks in belowground interplant carbon transfer. Three laboratory and one field study were designed to determine:

1. The minimum ^{13}C and ^{14}C pulse levels required to detect belowground carbon transfer (CHAPTER TWO),
2. The relative importance of hyphal transfer compared to soil transfer pathways (CHAPTER THREE),
3. The role of host plant phenology in the magnitude and direction of carbon transfer (CHAPTER FOUR),
4. The role of fungal species in the magnitude of carbon transfer (CHAPTER FIVE).

In CHAPTER TWO, seedlings grown in pots were labeled with stable and radioactive carbon isotopes to determine the minimum pulse level required to detect carbon flow between plants, and to determine whether carbon allocation patterns changed with increasing carbon pulse concentration. In CHAPTER THREE, laboratory root chambers were used to control for the presence of the hyphal pathway, and dual isotope labeling used to compare the relative magnitude of carbon transferred through hyphal and soil pathways. CHAPTER FOUR used reciprocal isotope labeling at three times in the growing season to determine the effect of host plant phenology on the magnitude and direction of carbon transfer between mycorrhizal plants. CHAPTER FIVE used stable isotope labeling and root chambers to examine whether fungal host specificity affected the magnitude of one-way carbon transfer, and tested the relative importance of hyphal, soil, and discontinuous soil-hyphal pathways in facilitating transfer. CHAPTER SIX,

a summary and synthesis of CHAPTERS ONE-FIVE, discusses future directions for research on CMN's and plant-soil ecology.

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*CHAPTER TWO: Minimum pulses of stable and radioactive carbon isotopes to detect belowground carbon transfer between plants

Introduction

Studies examining carbon dioxide effects on ecophysiology, carbon sequestration and carbon dynamics in terrestrial ecosystems have become increasingly important to plant and soil biologists (Schimel, 1995; Paterson *et al.*, 1996; Gavito *et al.*, 2000; Kellomaki & Wang, 2001; Niklaus *et al.*, 2001; Rønn *et al.*, 2002). Elevated levels of atmospheric carbon compounds, leaching of soil-mobile organic nutrients, storage of soil carbon in the short and long-term, and microbial transformations of carbon in the rhizosphere are recognized as key components of the carbon cycle (Fitter & Garbaye, 1994; Bonkowski *et al.*, 2001). Soil carbon, once an overlooked and underestimated factor in carbon cycling, is now recognized as an important component of the total carbon pool (Schimel, 1995). As a result, novel experimental approaches using stable and radioactive isotopes are being developed to learn more about carbon movement in plant-soil systems (Simard *et al.*, 1997a; He *et al.*, 2003).

Carbon translocation in plant-soil systems can occur entirely in the soil or can be mediated by soil organisms (Newman & Ritz, 1986; Newman 1988; Simard & Durall, 2004). In the rhizosphere soil, carbon can move by mass flow or diffusion, both processes that are influenced by soil structure, porosity, organic matter content and soil type (Rillig *et al.*, 1999; de Kroon & Visser, 2003). Of the soil organisms that mediate transfer, mycorrhizas are commonly the most important for translocating carbon from plants to soil, or between plants (Molina *et al.*, 1992; Onguene & Kuyper, 2002; Bever, 2003; Pfeffer *et al.*, 2004; Simard & Durall, 2004). Soil carbon could move between plants either entirely within mycorrhizal hyphae, or partially within hyphae and partially within soil (Newman, 1988).

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* A version of this chapter will be submitted for publication.
Philip LJ, Simard SW, Jones MD. Minimum pulses of stable and radioactive carbon isotopes to detect belowground carbon transfer between plants.

Carbon allocation, translocation within the rhizosphere, and transfer among plants are often studied using stable and radioactive carbon isotope labeling techniques (Francis & Read, 1984; Finlay & Read, 1986a; Simard *et al.*, 1997a, c, d; Haile-Mariam *et al.*, 2000; Bromand *et al.*, 2001; Bucking & Heyser, 2001; Saggar & Hedley, 2001; Wu *et al.*, 2001; Johnson *et al.*, 2002; Pfeffer *et al.*, 2004). While pulse labeling methods are not novel, applying these techniques to study belowground carbon movement between trees is quite innovative. Pulse labeling with $^{13}CO_{2\ (gas)}$ and/or $^{14}CO_{2\ (gas)}$ has been used, for example, to examine: 1) root carbon flow and carbon allocation patterns (Simard *et al.*, 1997a, c, d; Niklaus *et al.*, 2001; Saggar & Hedley, 2001; Grayston *et al.*, 2004); 2) carbon flow among plants through soil and mycorrhizal pathways (Watkins *et al.*, 1996; Graves *et al.*, 1997; Simard *et al.*, 1997a, c, d; Niklaus *et al.*, 2001; Wu *et al.*, 2001; Pfeffer *et al.*, 2004); 3) microbial respiration (Zak *et al.*, 1989; Strain *et al.*, 1992); 4) litter decomposition (Bromand *et al.* 2001); and 5) microbial activity using Stable Isotope Probing (Grayston *et al.*, 2004).

Isotope levels that have been used to experimentally pulse-label plant tissues with ^{13}C range from 50 ml to 200 ml of 99 atom% $^{13}CO_{2\ (gas)}$, (Newman, 1988; Watkins *et* al, 1996; Graves *et* al., 1997; Simard *et al.*, 1997a, c, d; Fitter *et al.*, 1998; Pfeffer *et al.*, 2004), and with ^{14}C from 185kBq to 7.4 MBq (Johnson *et al.*, 2002; Simard *et al.*, 1997a, d; Lerat *et al.*, 2002; Wu *et al.*, 2001). While isotopic labeling has demonstrated the presence of a variety of belowground carbon transfer pathways (Simard *et al.*, 1997a), the application of carbon isotopes at elevated CO_2 levels may overestimate the quantity of carbon moving within a plant-soil system through changes in photosynthetic rates, internal plant carbon gradients, or mycorrhizal associations (Robinson & Fitter, 1999). Errors in estimating carbon fluxes, such as those within and between plants, need to be addressed to improve our understanding of the biological relevance and regulation of carbon allocation and carbon transfer patterns. A more accurate prediction of the amount of carbon moving in a plant-soil system is also important for

understanding the positive and negative feedback mechanisms that regulate interspecific interactions in the rhizosphere and between plants (Bever, 2003).

In this laboratory study, I examined the effects of carbon isotope pulse labeling levels on carbon allocation and carbon transfer patterns in ectomycorrhizal paper birch (*Betula papyrifera* Marsh.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). The two specific objectives were: (1) to determine the minimum pulse of $^{13}CO_2$ or $^{14}CO_2$ for detecting interplant carbon movement between plants along belowground pathways, and (2) to determine whether carbon allocation patterns within plants change with low levels of elevated carbon dioxide. My experimental system included mixtures of Douglas-fir and paper birch seedlings grown in pots in a greenhouse for ten months.

Materials and Methods

Experimental design

Belowground one-way carbon transfer was studied in two similar but separate laboratory experiments in which paper birch and Douglas-fir were grown together in pots. In the first experiment, the effect of isotope levels on carbon transfer between paper birch and Douglas-fir was tested within three pulse levels of 99% $^{13}CO_2$: 4, 8, and 16 ml. In the second experiment, paper birch and Douglas-fir were pulsed within three levels of $^{14}CO_2$: 5, 10, and 15 μCi. In each experiment, two species were labeled: (1) paper birch donor seedlings, with neighbouring unlabeled Douglas-fir seedlings serving as receiver plants (abbreviated 13PB-DF or 14PB-DF) and (2) Douglas-fir donor seedlings, with unlabeled paper birch serving as receiver seedlings (13DF-PB or 14DF-PB). Each detection experiment used a 2x3 factorial design where one factor was either donor species, or donor versus receiver plant of the same species, and the second factor was pulse treatment level. By labeling the two species in separate treatments, isotopic discrimination differences between the two tree species and their mycorrhizae can be

quantified. This design was replicated four times for the $^{13}CO_2$ detection experiment and three times for the $^{14}CO_2$ detection experiment, each in a completely randomized design.

Soil and mycorrhizal inoculum

Soil samples were randomly collected at depths of 30cm from a reforested clear-cut site in the southern interior of British Columbia in the summer of 2001. The site occurred at 50°24.14N, 118°57.61W and an elevation of 1015 m. The original stand of mature paper birch and Douglas-fir was clear-cut logged in 1998. The collected samples were pooled, sieved to 2 mm, and stored for five days at 4 °C. The pooled sample was mixed with Perlite® in a ratio of three parts field soil to one part Perlite® (by volume), and the soil mixture used to fill 10 cm x 10 cm x 20 cm pots. The soil served as the ectomycorrhizal inoculum source for seedlings that were transplanted into the pots.

Preparation of the transplanted plant material

Paper birch seeds were sterilized in 10% H_2O_2 and rinsed in sterile distilled water, while Douglas-fir seeds were scarified by shaking in 35% H_2O_2 for 15 minutes followed by surface sterilization in 3% H_2O_2 for approximately five hours (Jones *et al.*, 1997). Following this treatment, three seeds per species were sown on the soil surface in each pot. Seeds were also sown on Leach Tubes® (Ray Leach 'Container' Single Cell System, Stuewe & Sons, Corvallis, USA) filled with autoclaved field soil to detect: 1) greenhouse fungal contaminants (such as the common ectomycorhizal fungus, *Thelephora* spp. (Jones *et al.*, 1997; Simard *et al.*, 1997b), and dark septate fungal endophytes such as *Phialocephala fortinii*,(Jumpponen, 2001; Jumpponen *et al*, 1998) ; 2) natural abundance (unlabeled/control) levels of carbon isotopes; and 3) possible aerial contamination of isotope following pulses in the laboratory. Sterilized sand was applied

over the seeds in a 1 mm layer to reduce moisture loss, seed contamination by fungal contaminants, as well as algal and moss growth. Leach Tubes® and pots were completely randomized on a single bench in the greenhouse at the University of British Columbia-Okanagan (formerly, Okanagan University College), Kelowna, British Columbia, Canada. Seedlings were grown for ten months using natural irradiance, temperatures of 18-24 °C, photoperiod extension to 14-hours, daily misting, and monthly applications of Ingestad's Nutrient Solution (Mason, 1980). Following seed germination, pots were thinned to one seedling per species and were re-randomized monthly on the greenhouse bench.

Pulse-chase regimes

Pulse labeling in each detection experiment was applied using a modification of the procedure developed by Simard *et al.* (1997a, c, d). Prior to labeling, the shoot of a donor seedling was sealed inside a 10 L, airtight, gas-sampling bag fitted with a septum for gas injections with a hypodermic needle. The root systems were also sealed from shoots using medium weight polyethylene-plastic and aluminum foil covering the soil surface. The sealed pots were then placed inside a fumehood modified with grow lights (PAR [photosynthetically active radiation] of 300 μmols $m^{-2}s^{-1}$) and timers. Seedlings grown in Leach Tubes® were placed adjacent to pots in the fumehood.

Preliminary trials, conducted to determine CO_2 utilization over time by paper birch and Douglas-fir, were used to determine the pulse period for donor plants. Seedlings were pulsed for five (paper birch) or eight (Douglas-fir) hours by injecting either i) 4, 8 or 16 ml $^{13}CO_2$, or ii) 85% lactic acid into one 1.5 ml microcentrifuge tube containing $Na_2{}^{14}CO_3$ to release 5, 10, or 15 μCi (0.19, 0.37, 0.56 MBq) $^{14}CO_2$. Additionally, seedlings pulsed with $^{14}CO_2$ were injected with 4, 8 or 16 ml $^{12}CO_2$ (containing 1.11% $^{13}CO_2$) to achieve equivalent total carbon dioxide concentrations between ^{13}C labeling bags in the first experiment and ^{14}C labeling bags in the

second experiment. The total CO_2 concentration ($^{12+13}CO_2$ or $^{12+13+14}CO_2$) inside the labeling bags was measured using a portable gas analyzer (LI-6251 CO_2 Gas Analyzer, Li-COR, Lincoln, Nebraska, USA) that measured 1 ml samples at the end of the pulse period to approximate the end concentration of total CO_2 in the labeling bag. A CO_2 calibration curve generated from gas standards ranging from 0-4 times ambient CO_2 concentration (measured concentration = 0.96+237.31*gas standard; r^2=0.97; $p<0.0001$) was used to approximate total CO_2 concentration.

The gas-sampling bags were removed at the end of the pulse period to release residual $^{13}CO_2$ and $^{14}CO_2$ gases into the fumehood. Seedlings remained in the fumehood for the six-day chase under a 14-hour photoperiod. A 20 cc syringe fitted with an 18-gauge hypodermic needle was used to water seedlings in the fumehood three times during the chase period.

Photosynthetic rate

Leaf net photosynthetic rates of three paper birch and three Douglas-fir seedlings were measured under laboratory conditions (24°C; ambient CO_2 conditions; PAR 300 μmols $m^{-2}s^{-1}$) using a portable photosynthesis unit (LCi portable photosynthesis system, model LCi-001, ADC Bioscientific Ltd., Hoddesdon, Herts, England, EN 11 ODB). The measured seedlings were grown under the same conditions and were comparable in size and appearance to the pulsed seedlings.

Tissue sampling

After the chase period, seedlings in pots and Leach Tubes® were harvested, tissues separated into shoot and root tissue samples, and roots washed. All sampled tissues were oven-dried for 48 hours at 55-60 °C and ground using a Wiley® Mill with 20 and 40 μm metal mesh screens. One 1 mg or 2 mg sub-sample per tissue was removed and combusted for %C and analyzed for ^{13}C or ^{14}C content at the stable isotope facilities of the University of Alaska-Fairbanks and the

University of California-Davis. Sub-samples of each tissue type (roots and shoots) were combusted to determine %C and analyzed for ^{13}C (δ in ‰) or ^{14}C (dpm) content. These values were used to estimate excess ^{13}C or ^{14}C in plant tissues and whole seedlings (see below) (Appendices I, II, III). Stable isotope data at the University of Alaska-Fairbanks were obtained using continuous-flow isotope ratio mass spectrometery (CFIRMS). Instrumentation was either a ThermoFinnigan Delta+ with a Carlo Erba NC2500 elemental analyzer or a Delta+XL with a Costech ESC 4010 elemental analyzer. Stable isotope ratios were reported in δ notation as parts per thousand (‰) deviations from the international standard PDB (carbon).

Mycorrhizae

Root systems of non-labeled plants grown in pots were examined for percent colonization and richness of ectomycorrhizal morphotypes. Roots were separated from shoots, washed and cut into 3 cm segments. Percent colonization per seedling was estimated by examining randomly-selected root segments under dissecting and compound microscopes, and counting the number of ectomycorrhizae occurring over a maximum of 200 root tips. Morphotype richness was determined by summing the number of morphotypes found per seedling. Fungal morphological characteristics (root-tip branching, colour, texture and hyphal patterns) were described and compared with previously published ectomycorrhizal descriptions (Ingleby *et al.*, 1990; Goodman *et al.* 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b) to identify morphotype categories.

Statistical analyses

The carbon isotope content in each tissue type was calculated from excess $\delta^{13}C$ (‰) and total carbon values for ^{13}C (Appendix I), and from dpm values and total carbon values for ^{14}C (Appendix II) (Simard *et al.*, 1997 a, d). Seedling biomass (g), whole seedling donor and receiver isotope contents (‰ or μg), tissue isotope contents (‰ or μg), isotope composition $\delta^{13}C$

(‰), and root:shoot ratios were compared between labeled tree species and pulse treatments using two-way ANOVA (n=4 for ^{13}C; n=3 for ^{14}C) for a 2x3 factorial design (SPSS software version 10.0). Polynomial contrasts were used to test linear and quadratic trends among treatment means. Means were separated among pulse treatments using Tukey's tests. For each pulse treatment, one-way transfer amounts (i.e. the amount of isotope received) were compared with zero using t-tests. Mean values for seedling characteristics, isotope contents, and transfer quantities were reported with corresponding standard error (SE) values.

Results

Seedling characteristics

Paper birch seedlings were larger than Douglas-fir in both experiments ($p<0.001$ for ^{13}C and ^{14}C). Neither root nor shoot biomass of either paper birch or Douglas-fir varied with pulse level or tree species in the ^{13}C or ^{14}C experiments ($p>0.05$) (Table 2.1). In the ^{13}C experiment, paper birch shoot (1.59 g ± SE 0.16) and root (1.47 g ± SE 0.22) biomass were similar among ^{13}C pulses and did not differ whether they served as donors or receivers ($p>0.05$ for both tissues). Douglas-fir tissue biomass (0.12 g ± SE 0.14 in shoots, 0.12 g ± SE 0.15 in roots) in the ^{13}C experiment also did not differ between pulse levels ($p=0.075$ for shoots, $p=0.133$ for roots) or between donors and receivers ($p>0.05$ for both tissues). Paper birch pulsed with ^{14}C had comparable shoot (2.90 g ± SE 0.31) ($p=0.805$) and root (3.28 g ± SE 0.33) ($p=0.902$) biomass among pulses and between donors and receivers ($p>0.05$ for both tissues). Douglas-fir tissue biomass also did not vary among ^{14}C pulses for shoots (0.27 g ± SE 0.04) or roots (0.31 g ± SE 0.04) nor did donors differ from receivers ($p=0.083$ for shoots, $p=0.088$ for roots).

Root:shoot ratios of paper birch and Douglas-fir did not vary with pulse level in either the ^{13}C or ^{14}C experiments ($p>0.05$, Table 2.2). Paper birch (0.90 ± SE 0.03) and Douglas-fir (1.35 ± SE 0.20) had similar root: shoot ratios regardless of whether they were donors or receivers in

the ^{13}C experiment (p=0.155 for paper birch, p=0.403 for Douglas-fir). Ratios between tissues in the ^{14}C experiment did not vary with pulse level (1.15 ± SE0.06, p=0.085 for paper birch; 1.42 ± SE 0.32, p=0.557 for Douglas-fir). In the ^{14}C experiment, paper birch donors had greater root:shoot ratios (1.28 ± SE 0.08) than receivers (1.01 ± SE 0.08) (p=0.020).

Paper birch net leaf photosynthetic rate (4.97 μmols $m^{-2}s^{-1}$ ± SE 0.65) was three times greater than that of Douglas-fir (1.54 μmols $m^{-2}s^{-1}$± SE 0.14) (p<0.0001).

Mycorrhizal status

Paper birch seedlings were colonized by six ectomycorrhizal morphotypes while Douglas-fir seedlings were colonized by four (Ingleby *et al.*, 1990; Goodman *et al.*, 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b) (Table 2.3). Three morphotypes, excluding the incomplete morphotype, were common to paper birch and Douglas-fir, colonizing 38% of root tips in paper birch and 52% in Douglas-fir. The shared morphotypes were E-strain 1, E-strain 2 and MRA (*Mycelium radicis atrovirens*). Incomplete morphotypes were found on both tree species.

CO_2 concentration in sampling bags

At the end of the pulse period, CO_2 concentration remaining in the sampling bags differed significantly between donor species (p<0.0001 for both experiments). In the ^{13}C experiment, sampling bags with donor seedlings had mean total CO_2 concentration ranging from 4.3 x 10^2 ppm ± SE 1.3 to 5.23 x 10^2 ppm ± SE 1.5. In the ^{14}C experiment, donor seedlings had mean total CO_2 concentrations ranging from 4.8 x 10^2 ± SE 2.7 ppm to 8.73 x 10^2 ± SE 4 ppm CO_2 remaining. Pulse level effects on residual CO_2 concentration were not significant in the ^{13}C (1.62 x 10^2 ppm) (p=0.526) or ^{14}C (2.35 x 10^2 ppm) experiments (p=0.281) and are represented here as the means of the pulse levels in each experiment.

Isotope content of paper birch and Douglas fir seedlings

Aerial Isotope Contamination

Control seedlings grown to monitor possible aerial isotope contamination had $\delta\ ^{13}C$ values (‰) of -31.62 and -30.67 for paper birch shoots, and -31.04 and -30.60 for roots. Douglas-fir levels (dpm) were -29.14 and -29.97 for shoots and -28.95 and -28.43 for roots.

Isotope contamination levels in the ^{14}C experiment were 102.25 dpm and 112.51 for paper birch shoots and roots while Douglas-fir shoots and roots had levels of 103.42 and 102.78.

Whole seedling ^{13}C and ^{14}C enrichment

Paper birch whole seedlings were more enriched in ^{13}C than Douglas-fir regardless of whether they were donors or receivers ($p<0.0001$). In the ^{13}C experiment, paper birch seedlings tended to be most enriched in the 16 ml treatment ($p=0.085$) regardless of whether they were donors or receivers ($p=0.847$) (Table 2.4). Planned linear contrasts showed that mean ^{13}C enrichment of donor and receiver paper birch seedlings increased with pulse level ($p=0.033$). Douglas-fir donors were more enriched with ^{13}C than receivers ($p=0.001$), with similar enrichment among ^{13}C pulse levels ($p=0.470$).

Paper birch whole seedling donors were more enriched in ^{14}C than Douglas-fir donors ($p<0.0001$) (Figure 2.1a). Donor seedling enrichment was also affected by pulse level ($p=0.023$). Planned linear contrasts showed ^{14}C donor seedling enrichment (mean of paper birch and Douglas-fir) was significantly affected by pulse level ($p=0.007$), with both paper birch and Douglas-fir donors most enriched by 15 μCi. For both donor species, isotope content was more enriched in the 15 μCi treatment compared to the 5 μCi treatment ($p=0.018$), but neither of these levels differed from the 10 μCi treatment ($p>0.05$) as separated by post hoc Tukey tests. The isotope content in whole receiver seedlings also depended on receiver species, with paper birch

more enriched than Douglas-fir ($p<0.0001$) (Figure 2.1b). Pulse level effects on receiver ^{14}C enrichment were not significant ($p=0.349$).

^{13}C allocation in tissues

When expressed as $\delta^{13}C$ (‰), isotope composition of paper birch shoots and roots was greatest in the 16 ml pulse ($p=0.001$ for shoots; $p=0.012$ for roots) and was greater in donor than receiver seedlings ($p=0.001$ for shoots; $p=0.009$ for roots) (Table 2.4). Douglas-fir ^{13}C enrichment (‰) was similar among pulse levels for shoots ($p=0.590$) and roots ($p=0.463$), but was greater in donor than receiver seedlings ($p<0.001$ for shoots, $p=0.004$ for roots) (Table 2.4).

^{14}C allocation in tissues

Donor shoot and root tissues were significantly more enriched in ^{14}C than receiver shoot and tissues for both paper birch and Douglas-fir ($p<0.0001$ for both tissue types and species) (Tables 2.5 and 2.6). However, pulse level had no effect on root or shoot tissue ^{14}C content of either paper birch or Douglas-fir donors or receivers ($p>0.05$) (Tables 2.5 and 2.6). In donors, more ^{14}C was allocated to shoots than roots for either paper birch (61% in shoots and 39% in roots) or Douglas-fir (66% and 34%). For receivers, the opposite was true. On average, 65% and 72% of received isotope remained in roots of paper birch and Douglas-fir, respectively (Table 2.6).

One-way transfer

Natural abundance levels (‰) in the ^{13}C experiment control seedlings were -30.79, -30.49, and -32.49 for paper birch shoots, and -29.95, -29.35, and -30.39 for paper birch roots. Comparably, Douglas-fir control levels were -28.59 and -28.38 for shoots, and -28.14, -26.85, and -28.43 for roots.

In the ^{13}C experiment, $\delta\ ^{13}C$ delta values were higher in Douglas-fir receiver roots than paper birch receiver roots ($p<0.0001$) (Table 2.4). Del values for Douglas-fir receiver roots were higher in the 4 ml than 8 or 16 ml treatments but those for paper birch did not vary by pulse ($p>0.05$).

In the ^{14}C experiment, isotope enrichment of receiver paper birch or Douglas-fir was not affected by pulse level ($p=0.349$); however, isotope enrichment was greater in Douglas-fir receivers than paper birch receivers ($p<0.0001$) (Figure 2.1a, b).

Discussion

Greater ^{13}C and ^{14}C isotope content in receiver roots of Douglas-fir than paper birch suggest that carbon transfer occurred from Douglas-fir to paper birch in the 16 ml ^{13}C pulse level, and all ^{14}C pulse levels, but there was no evidence of transfer of either isotope from paper birch to Douglas-fir. Our results contrast with previous studies showing net transfer from paper birch to Douglas-fir (Simard *et al.* 1997), suggesting that isotope levels were either too low in donor roots, or that source-sink gradients between the two species differed in our system. The occurrence of isotope in receiver roots of Douglas-fir at all tested ^{14}C pulse levels, as well as one ^{13}C level, suggests that 5-15 μCi ^{14}C and 16 ml ^{13}C are useful for detecting one-way carbon transfer from Douglas-fir to paper birch in seedlings of this size. Lack of isotope in Douglas-fir receivers may be related to the greater proportion of label remaining in Douglas-fir than paper birch receiver roots, much lower shoot and root biomass in Douglas-fir than paper birch receivers, and lower leaf photosynthetic rates in Douglas-fir than paper birch. This suggests that Douglas-fir receivers had lower carbon demands compared to the much larger paper birch, which had larger shoots and roots, and hence greater root and hyphal turnover rates as well as mycorhizosphere exudation and sloughing (Finlay & Read 1986a; Duddridge *et* al., 1988; Finlay, 1989b; Rygiewicz *et al*, 1997; Simard & Durall, 2004).

Another factor influencing interspecific source-sink patterns is mycorrhizal colonization pattern. Paper birch was colonized by more ectomycorrhizal fungi than Douglas-fir, probably with larger amounts of extraradical mycelium. While both tree species were colonized by ectomycorrhizae, three species were common between paper birch and Douglas-fir, one of which was a dark septate fungal endophyte. Fungi in this group can be described as mutualistic or parasitic. A previously established parasitic relationship between this fungus and Douglas-fir suggests that two common fungi rather than three were influential in carbon flow.

Isotope contents in donor root and shoot tissues suggest pulse levels of either ^{13}C or ^{14}C had little influence on isotope allocation patterns in either paper birch or Douglas-fir. The highest pulse of ^{13}C resulted in greater allocation to shoots than roots of donor paper birch, but this elevated level should not result in greater transfer from roots. Root:shoot ratios tended to be lower for paper birch (<1) than Douglas-fir (>1) in the ^{13}C experiment, as did atom% ^{13}C, which may partly explain why more isotope was transferred from fir to birch than vise versa. Nevertheless, root and shoot biomass, as well as absolute isotope contents were much lower in Douglas-fir than birch, implying that paper birch roots have greater demand as well as greater potential to exploit soil resources than Douglas-fir roots (Waring, 1985; Finlay & Soderstrrom, 1992. Higher photosynthetic rates and root area in paper birch compared to those in Douglas-fir suggest paper birch assimilated and translocated carbon more readily in our experiment.

Carbon allocation results from this study contrast with previous studies, possibly because lower CO_2 levels that were close to ambient conditions were used here. Shifts in carbon allocation patterns have been attributed to unrealistically high total carbon pulse concentrations in earlier studies (Paterson *et al.*, 1996; Simard *et al.*, 1997a, c, d; Robinson & Fitter, 1999; Kellomaki & Wang, 2001; Niklaus *et al.*, 2001). For example, Paterson *et al.* (1996), using $^{14}CO_2$ pulse labeling and two CO_2 concentrations, found increasing root:shoot ratios for wheat (*Triticum aestivum*) and decreasing root:shoot ratios for rye grass (*Lolium perenne*) with

increased CO_2, but found no effect on carbon allocation in Bermuda grass (*Cynodon dactylon*). Allocation of ^{14}C to rye grass and wheat rhizospheres increased with elevated CO_2 concentrations, while the rhizosphere microbial fraction was less than the soil fraction under the highest CO_2 concentration. A different study by Kellomaki & Wang (2001) tested the effects of elevated CO_2 on resource allocation patterns of birch seedlings (*Betula pendula* Roth.). Elevated CO_2 did not alter root:shoot ratios during early birch seedling growth but it resulted in increased root:shoot ratios during later growth stages.

During this study, we monitored the pulse period in preliminary trials to ensure that the seedlings were not exposed to unrealistically high levels of isotope during the pulse for longer than they were capable of using the isotope. By using low pulse levels, appropriate pulse periods and large gas-sampling bags, total CO_2 concentrations at the end of the pulse period were close to ambient levels. Increasing the gas-sampling bag volume would have slowed the rate of isotope uptake by possibly diluting the ratio of labeled to unlabeled molecules, allowing for longer isotope pulses and more "continuous" labeling. Bromand *et al.* (2001) demonstrated the importance of carbon pulse technique on carbon allocation patterns. Using wheat plants (*Triticum aestivum* L. cv. 'Katepwa'), ^{13}C was applied at regular intervals (directly proportional to the rate of photosynthesis) weekly for six hours in a closed chamber. After 13 weeks, plants had consistent ^{13}C abundance in all plant fractions. By contrast, plants pulsed only during the early stages of growth had greater differences in isotope abundance among plant parts.

Carbon fluxes within and between ectomycorrhizal plants may be related to differences in fungal or plant-regulated carbon gradients. Simard & Durall (2004) have suggested that physiological differences, such as the ability of a fungus to drain carbon from a plant, or plant characteristics such as root biomass or age (Rygiewicz *et al.*, 1997), may influence the direction and magnitude of carbon transfer between plants. Carbon gradients can also occur in concert with rhizosphere nitrogen gradients that are influenced by microbial transformations or

fertilization (Bethlenfalvay *et al.*, 1991; Bradley & Fyles, 1995; Wang *et* al, 1995; Bonkowski *et al.*, 2001; Klironomos & Hart, 2001; Perez-Moreno & Read, 2001; DeLong *et al.*, 2002; Rönn *et al*, 2002). This study showed there were tree species differences in carbon assimilation rates, with paper birch photosynthesizing at nearly four times the rate of Douglas-fir. Donor seedlings with higher labile carbon concentrations would likely transfer more carbon to sinks in their own rhizosphere, or in the rhizosphere or fungi colonizing nearby receiver seedlings. Multiple fungal species could then compete as carbon sinks. The presence of many carbon sinks may be one explanation for the small and variable amount of carbon received by the two species. Because carbon can be drained by many sources and because ^{13}C detection levels are so high, detection of carbon transfer using ^{13}C may require larger or longer pulses of stable isotope (Simard *et al.*, 1997c, d). Detection of transfer using radioisotopes, however, may be less affected by multiple carbon sinks, since ^{14}C can be detected at much lower levels.

In our experiment, we found three ectomycorrhizal morphotypes shared in common between paper birch and Douglas-fir root systems, suggesting there was the potential for hyphae to connect root systems of the two tree species. Past laboratory and field studies have also established that paper birch and Douglas-fir are frequently colonized by generalist mycorrhizae (Jones *et al.*, 1997; Simard *et al.*, 1997b; Philip *et al.*, submitted 2004). However, because we used microscopy techniques instead of molecular analysis, we were unable to determine whether the ectomycorrhizae on each tree species were genetically identical and whether hyphal linkages actually formed. Even genetically dissimilar but compatible fungi could have anastomosed (Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004) and formed linkages. We could not determine this without somatic compatibility tests. Autoradiography would have been useful in confirming the presence of linkages (Wu *et al.*, 2001), however the minimum detection level for autoradiography may have been too high for the ^{14}C pulse levels used in this study.

Even though paper birch and Douglas-fir shared ectomycorrhizal morphotypes in common, carbon transfer could have occurred through disconnected hyphal linkages or entirely through soil pathways via diffusion or mass flow (Simard *et al.*, 1997a, d; Simard & Durall, 2004). No attempt was made in this experiment to separate carbon transfer among these pathways. In the discontinuous pathway, carbon moves from one colonized root-tip into the soil and is then taken up by either an uncolonized or colonized root-tip of a second seedling. This type of transfer could have occurred in our pot systems. Paper birch roots grew quickly and dominated the pot area. Douglas-fir root systems were noticeably smaller and were quickly displaced by the larger paper birch root systems. Because the two species' root systems were growing in close proximity, carbon could have transferred by diffusion or mass flow. Diffusion usually occurs over shorter distances in the rhizosphere than mass flow, but it is plausible that soluble organic carbon compounds moved by both processes in our experiments.

In summary, the low ^{13}C and ^{14}C pulse levels used in this study had no effect on carbon allocation patterns in donor seedlings, eliminating the possibility that pulse level would result in an over-estimation of carbon transfer. However, there was little evidence for transfer of ^{13}C to either birch or fir suggesting much higher ^{13}C pulse levels are required for transfer detection. By contrast, all pulse levels of ^{14}C resulted in transfer from fir to birch, but not in the opposite direction, possibly because of lower sink strength of the considerably smaller Douglas-fir than paper birch. Further studies are needed to test higher levels of ^{13}C and ^{14}C pulse levels on carbon allocation and carbon transfer using plants of different source and sink strengths.

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Table 2.1 Seedling tissue biomass (g) in 10-month old paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* seedlings pulsed with three isotope levels in two experiments.

		Biomass		*p* values	
Tissue	Experiment	Paper Birch	Douglas-fir	Paper Birch	Douglas-fir
Shoots	^{13}C experiment			0.699	0.075
	4 ml	1.53 ± 0.15*	0.10 ± 0.02		
	8 ml	1.76 ± 0.15	0.14 ± 0.03		
	16 ml	1.72 ± 0.09	0.12 ± 0.01		
	^{14}C experiment			0.805	0.985
	5 μCi	3.17 ± 5.64	0.26 ± 0.05		
	10 μCi	2.83 ± 6.54	0.26 ± 0.07		
	15 μCi	2.71 ± 4.72	0.30 ± 0.09		
Roots	^{13}C experiment			0.766	0.133
	4 ml	1.31 ± 0.16	0.13 ± 0.02		
	8 ml	1.55 ± 0.18	0.14 ± 0.02		
	16 ml	1.47 ± 0.10	0.13 ± 0.02		
	^{14}C experiment			0.902	0.692
	5 μCi	3.52 ± 6.58	0.26 ± 0.05		
	10 μCi	3.22 ± 5.75	0.28 ± 0.07		
	15 μCi	3.11 ± 5.98	0.39 ± 0.10		

*Means represent ± 1 standard error. Differences between pulses were not significant (α=0.05).

Table 2.2 Seedling root:shoot ratios in 10-month old paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* seedlings pulsed with three isotope levels in two experiments.

	Ratio		*p* values	
Experiment	Paper Birch	Douglas-fir	Paper Birch	Douglas-fir
^{13}C experiment			0.063	0.923
4 ml	0.85 ± 0.06*	1.47 ± 0.34		
8 ml	0.88 ± 0.06	1.13 ± 0.11		
16 ml	0.82 ± 0.07	1.44 ± 0.49		
^{14}C experiment			0.845	0.316
5 μCi	1.12 ± 0.12	1.04 ± 0.08		
10 μCi	1.19 ± 0.14	1.17 ± 0.18		
15 μCi	1.14 ± 0.09	2.04 ± 0.93		

*Means represent ± 1 standard error. Differences between pulse levels were not significant (α=0.05).

Table 2.3 Morphological characteristics and abundance (number of observed root tips per seedling expressed as a percentage (%) of the total number of root tips examined) of ectomycorrhizae on paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) grown together in pots for ten months.

Morphotype	Morphological Characteristic	Paper Birch	Douglas-fir
Non-mycorrhizal	root hairs visible; Hartig net and mantle absent	68.2	68.0
Incomplete	immature mantle cells	43.8	29.8
E-strain I	root tips brown/black; large oblique mantle cells; verrucose emanating hyphae	125.0	41.0
E-strain II	similar to E-strain I; emanating hyphae absent	74.3	58.8
Hebeloma-*like*	root tips cream/brown; copious emanating, clamped hyphae	127.0	0.0
Laccaria-*like*	root tips white; infrequent emanating hyphae; thick mantle; mantle cells narrow	114.0	0.0
MRA (Mycelium radicis atrovirens)	root tips black, hairy to smooth; narrow, angular mantle cells; emanating hyphae thick-walled, black	14.0	7.0

Table 2.4 Mean seedling isotope composition (δ^{13}(‰)) in 10-month old paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* pulsed with three $^{13}CO_2$ levels. Values are means ± 1 standard error.

	Paper Birch		Douglas-fir	
Treatments	Donors		Donors	
	Shoot	Root	Shoot	Root
16 ml	143.51±18.28*	44.52±15.00	256.99±105.62	68.64±75.02
8 ml	94.03±19.22	28.65±9.59	380.47±34.57	126.14±39.03
4 ml	27.08± 13.33	-8.98±7.80	367.46±113.16	28.98±34.63
Treatments	Receivers		Receivers	
	Shoot	Root	Shoot	Root
16 ml	-31.01±0.61	-33.75±2.18	-30.57±0.27	-29.19±0.59
8 ml	-31.32±0.37	-30.08±0.27	-31.00±0.47	-27.93±0.81
4 ml	-31.05±0.27	-29.83±0.28	-31.30±0.41	-24.60±5.98

Differences between pulse levels were significant for donor paper birch shoots and roots but not for Douglas-fir shoots or roots. Paper birch receivers were similar between pulses while pulse differences for Douglas-fir receiver shoots and roots were significant (α=0.05).

Table 2.5 Mean donor seedling isotope contents in tissues of 10-month old paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* pulsed with three isotope levels in the ^{14}C experiment.

Donor Tissue	Paper Birch	Douglas-fir
Shoot	1962.68 ± 1.29*	851.74 ± 5.12
Root	1277.12 ± 5.16	438.43 ± 3.93

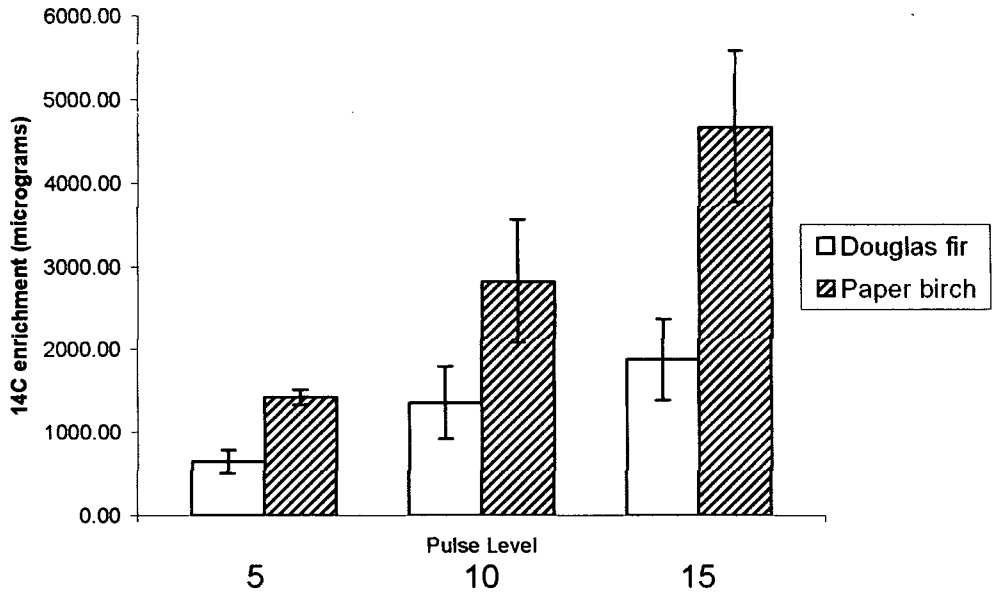
*Means represent tissue enrichment quantities in μg ± 1 standard error. Differences between pulse levels were not significant (α=0.05).

Table 2.6 Mean receiver seedling isotope contents (mg) in 10-month old paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* pulsed with three isotope levels in the ^{14}C experiment.

	Paper Birch		Douglas-fir	
Experiment	Shoot	Root	Shoot	Root
5 μCi	18.06 ± 1.11*	20.59 ± 1.36	0.81 ± 1.06	1.95 ± 1.16
10 μCi	19.29 ± 1.37	31.56 ± 2.08	1.35 ± 1.10	6.88 ± 3.93
15 μCi	5.22 ± 0.54	21.84 ± 1.94	3.34 ± 5.53	5.07 ± 6.82

*Means represent tissue enrichment quantities in μg ± 1 standard error. Differences between pulse levels were not significant (α=0.05).

a)



b)

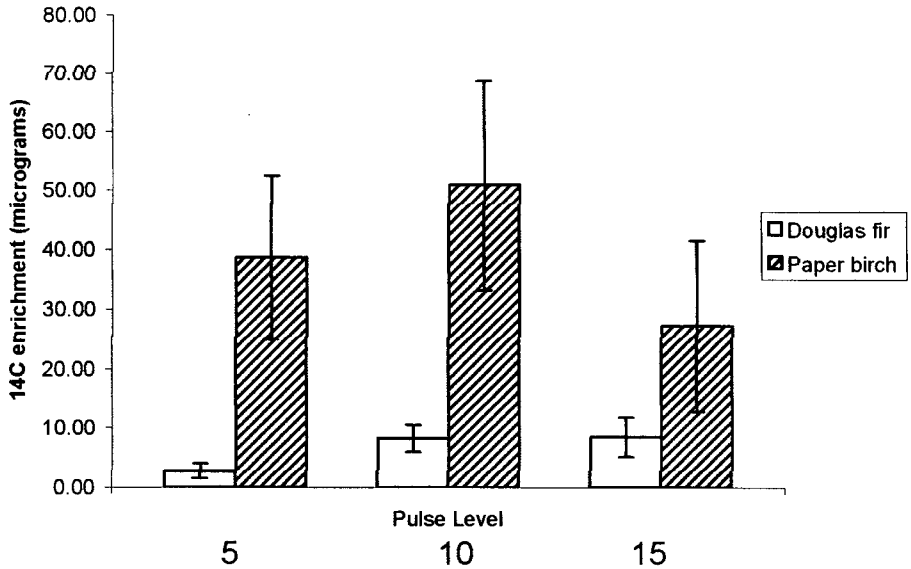


Figure 2.1 Whole seedling enrichment of (a) donors and (b) receivers following pulse labeling with three levels (15, 10, 5 μCi) of $^{14}CO_2$.

*CHAPTER THREE: Common mycorrhizal networks play a significant role in belowground carbon transfer between paper birch (*Betula papyrifera)* and Douglas-fir (*Pseudotsuga menziesii)* seedlings in laboratory root chambers

Introduction

Ectomycorrhizal fungi can colonize and potentially form a common mycorrhizal network (CMN) between deciduous and coniferous tree species (Molina *et al.*, 1992; Jones *et al.*, 1997; Wallenda & Read, 1999; Booth, 2004), forming a direct conduit for interplant transfer of carbon or nutrients (Newman, 1988). Hyphal linkages have previously been confirmed using visual observation (Newman *et al.*, 1994) or ^{14}C labeling coupled with autoradiography (Francis & Read, 1984; Finlay & Read, 1986; Wu *et al.*, 2001). In other studies, one-way, bi-directional or net transfer of carbon has been demonstrated between different tree species using carbon isotopes (Simard *et al.*, 1997a; Lerat *et al.*, 2002), but the identity of the primary transfer pathway has remained under debate (Fitter *et al.*, 1999). Understanding the role CMNs play in interplant transfer will improve our understanding of plant interactions and also is a necessary first step in understanding factors that influence transfer between plants.

Several contentious issues remain central to the debate on whether CMN-mediated carbon transfer is an ecologically significant process in plant communities (Perry, 1999; Fitter *et al.*, 2001; Simard & Durall, 2004). These include: (1) whether the CMN pathway is more important than soil or other pathways (Newman *et al.*, 1994; Watkins *et al.*, 1996; Wilkinson, 1998); (2) whether there is one-way, two-way or net transfer of carbon; (3) the identity of the transferred carbon compounds and the mechanisms by which they move; (4) whether transferred compounds remain in fungal tissues or move to plant tissues; (5) identity of the ecological

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*A version of this chapter will be submitted for publication.
Philip LJ, Simard SW, Jones MD. The ectomycorrhizal hyphal pathway plays a significant role in belowground carbon transfer between paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) seedlings in laboratory root chambers.

factors that regulate the direction and magnitude of transfer, and (6) whether net transfer is of sufficient magnitude to affect plant productivity, fitness or interactions with other organisms.

There are four possible pathways through which carbon can transfer between plants (Newman & Ritz, 1986, Martins, 1993). These include (1) carbon transfers from one root system to another through mycorrhizal hyphal connections (the direct transfer pathway) (Newman & Ritz, 1986); 2) carbon flows from donor roots to receiver uncolonized or mycorrhizal roots through the soil (the soil pathway) in solution by diffusion and mass flow (Newman & Ritz, 1986); 3) carbon flows partially in hyphae and partially in the soil (the discontinuous soil-hyphal pathway) (Simard *et* al, 2002; Simard & Durall, 2004); or 4) carbon flows between plants of the same species through root grafts (Fraser *et al.*, 2005).

In a laboratory study, Simard *et al.* (1997d) compared carbon transfer between hyphal and soil pathways using *Betula papyrifera* Marsh (paper birch) and *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) seedlings grown side-by-side in root pouches. In one treatment, the CMN that had developed between seedlings was severed once prior to labeling, and in the other treatment it was left intact. The paired seedlings were reciprocally labeled for one hour with carbon isotopes to quantify bi-directional and net carbon transfer. While carbon moved in two directions, they found there was no significant net gain in carbon by either species as well as no difference in transfer between the CMN and soil pathway treatments. This contrasted with the field study of Simard *et al.* (1997a), where net transfer occurred from paper birch to Douglas-fir, and little transfer occurred through soil pathways to arbuscular mycorrhizal cedar seedlings. Factors that appeared important in the lack of net transfer in the laboratory study included potential hyphal reconnection following the single severing, and low replication of treatments.

In this study, we improved upon the root-box study of Simard *et al.* (1997d) by using enhanced reciprocal carbon isotope labeling techniques, repeated CMN severing, and increased treatment replication. The objectives of our study were to: 1) determine whether carbon transfer

between the two species is bi-directional, 2) determine if there is a net gain in carbon by either species; and 3) compare the magnitude and direction of carbon transfer between the CMN and soil transfer pathways. We hypothesize that transfer is bidirectional, but with net transfer from paper birch to Douglas-fir, and that more carbon is transferred through the CMN than soil pathway.

Materials and Methods

Experimental design

Carbon transfer was studied in a laboratory experiment in which paper birch and Douglas-fir were grown in separate but adjacent root-restrictive mesh pouches. The effects of hyphal connections and labeling schemes on transfer were tested in a 2x2 factorial structure of treatments that were replicated six times in a completely randomized design. The two hyphal treatments were: (1) hyphae left intact, and (2) hyphae severed. In the severed treatment, hyphal connections were cut with a long handled instrument with attached razor blade inserted between root pouches immediately prior to the labeling period and daily during the chase period. In the intact treatment, hyphal connections were left undisturbed. The two labeling scheme levels, designed to account for potential species and isotope effects on transfer, were: (1) paper birch seedlings pulse labeled with $^{13}CO_2$ and Douglas-fir seedlings pulsed with $^{14}CO_2$ (abbreviated 13PB-14DF), and (2) Douglas-fir seedlings pulse labeled with $^{13}CO_2$ and paper birch seedlings pulsed with $^{14}CO_2$ (13DF-14PB). Each seedling was pulsed with either 15 ml 99% $^{13}CO_2$ (Cambridge Isotope Laboratories) or 15 μCi (0.60 MBq) $^{14}CO_2$ (Amersham Ltd.).

Soil and mycorrhizal inoculum

For growing seedlings in the root chambers, six soil samples were randomly collected to depths of 30 cm during the spring of 2000 from a reforested clear-cut site in the southern interior of

British Columbia, Canada (1015 m elevation at 50°24.14N, 118°57.61W). The original stand of 120 year-old paper birch and Douglas fir was clear-cut logged in 1998.

The soil samples were pooled, sieved to 2 mm, and stored for seven days at 4 °C prior to root chamber assembly and seedling establishment. The soil was mixed with Perlite® in a ratio of three parts field soil to one part Perlite® (by volume) and then used to fill 10 cm x 20 cm root-restrictive pouches comprised of 28 μm Nitex® nylon mesh fabric. The chosen mesh size was shown by Fitter *et al.* (1998) to exclude roots but permit hyphal penetration. Two pouches were placed side-by-side between two 20 cm x 20 cm plastic plates secured with foam spacers and metal clips. The two root pouches were pressed against each other to minimize any air gaps that may be created by severing hyphae, thereby minimizing potential disruption of the soil pathway. The root chambers were covered in aluminum foil to minimize evaporation and reduce algal and moss growth during the experiment. The pooled field soil served as the ectomycorrhizal inoculum source for the seedlings. Assembled root chambers were randomly placed on a greenhouse bench at the University of British Columbia-Okanagan (formerly, Okanagan University College), Kelowna, British Columbia, Canada.

Preparation of the plant material

Paper birch seeds were surface-sterilized in 10% H_2O_2 and rinsed in sterile distilled water, while Douglas-fir seeds were scarified by shaking in 35% H_2O_2 for 15 minutes, followed by surface sterilization in 3% H_2O_2 for approximately five hours (Jones *et al.*, 1997). Following this treatment, three seeds per species were sown on the soil surface of root pouches and covered with a 1 mm layer of sterilized sand to reduce moisture loss, seed contamination by fungal contaminants, as well as algal and moss growth. Seeds were also sown on Leach Tubes® (Ray Leach 'Conetainer' Single Cell System, Stuewe & Sons, Corvallis, USA) filled with autoclaved

field soil for use in: (1) detecting possible greenhouse fungal contaminants (such as the common ectomycorrhizal fungus, *Thelephora* spp. (Jones *et al.*, 1997; Simard *et al.*, 1997b), and dark septate fungal endophytes such as *Phialocephala fortinii* (Jumpponen, 2001; Jumpponen *et al.*, 1998) , (2) detecting potential aerial isotope transfer during pulse labeling in the laboratory, and (3) determining natural (unlabeled) isotope abundance levels. Control seedlings grown in Leach Tubes® were preferable to non-mycorrhizal controls grown in root pouches because of the likelihood there would be size differences and therefore physiological differences between non-mycorrhizal and mycorrhizal paper birch and Douglas-fir. Root pouch controls would potentially influence the magnitude and direction of transfer and hinder interpretation. Seedlings were grown for eight months using natural irradiance, temperatures of 18-24 °C, photoperiod extension to 14 hours by high-pressure sodium lights, daily misting, and monthly applications of Ingestad's Nutrient Solution (Mason, 1980). Following seed germination, pouches were thinned to one seedling per pouch.

Pulse-chase regimes

Pulse labeling was applied using modifications of the procedure developed by Simard *et al.* (1997a, c, d). Immediately prior to labeling, hyphal connections were cut or left intact. Seedling shoots were then sealed inside air-tight gas sampling bags (10 L volume) fitted with a septum for injections with a hypodermic needle. The root systems were also sealed from shoots using medium weight polyethylene-plastic covering the soil surface and aluminum foil protecting the soil surface and root chamber surfaces. The sealed root chambers were then placed inside a fumehood modified with grow lights (photosynthetically active radiation [PAR] = 300 μmols m^{-2} s^{-1}). The seedlings in Leach Tubes®, grown for detecting aerial isotope contamination during labeling, were placed adjacent to the root chambers in the fumehood.

Seedlings were pulsed for eight hours (pulse period determined in preliminary trials) by injecting either 15 ml of $^{13}CO_2$ gas or injecting 85% lactic acid to release 15 μCi, (0.60 MBq) $^{14}CO_2$ from $Na_2{}^{14}CO_3$ that was frozen, then thawed, in one 1.5 ml microcentrifuge tube and taped to the inside of the bag. Additionally, bags containing seedlings pulsed with $^{14}CO_2$ were injected with 15 ml 99%+1% $^{12+13}CO_2$ to achieve equivalent total carbon dioxide concentrations in both sets of labeling bags. The total concentration ($^{12+13}CO_2$ or $^{12+13+14}CO_2$) in 1 ml gas samples removed from the labeling bags at the end of the pulse period was monitored using a portable gas analyzer (LI-6251 CO_2 Analyzer, Li-COR, Lincoln, Nebraska, USA). Using a CO_2 calibration curve generated from gas standards ranging from zero to four times ambient CO_2 concentration (y[mV] = b + ax.standard (mV/ppm); measured concentration in 1 ml sample (y[mV]) = 0.96 + 237.31 * gas standard (mV/ppm); r^2=0.97; $p<0.0001$), the total CO_2 concentration relative to ambient CO_2 levels was estimated at the end of the pulse period.

After eight hours, the gas-sampling bags were removed to release residual $^{12}CO_2$, $^{13}CO_2$ and $^{14}CO_2$ gases into the fumehood. Seedlings in two root chambers were harvested immediately following the pulse period. Remaining seedlings were left in the fumehood for a six-day chase period under a 14-hour photoperiod. Seedlings were watered three times during the chase period using a syringe fitted with a hypodermic needle. Hyphal connections between root pouches were re-cut daily to reduce hyphal anastomoses between root pouches.

Tissue sampling

After the chase period, remaining seedlings in root chambers were harvested, shoot and root tissues separated, and roots washed. The same was done for control and natural abundance seedlings grown in Leach Tubes®. All tissues were oven-dried for 48 hours at 55-60°C, and ground using a Wiley® Mill with 20 μm and 40 μm metal mesh screens. One 2 mg sub-sample of each tissue type (shoots, roots) was combusted to determine %C and analyzed for ^{13}C or ^{14}C

content at the Stable Isotope Facility, Department of Agronomy, University of California-Davis. These values were used to estimate excess mg ^{13}C or ^{14}C in plant tissues. Instrumentation was either a ThermoFinnigan Delta+ with a Carlo Erba NC2500 elemental analyzer or a Delta+XL with a Costech ESC 4010 elemental analyzer. Stable isotope ratios were reported in δ notation as parts per thousand (‰) deviations from the international standard PDB (carbon).

Mycorrhizae

Root systems of additional, non-labeled plants were examined for percent colonization and richness of ectomycorrhizal morphotypes. To do this, roots were removed from seedlings grown in unpulsed root chambers, washed and cut into 3 cm segments, and root tips examined to a maximum number of two-hundred root tips. Percent colonization per seedling was estimated by examining randomly-selected segments under dissecting and compound microscopes, and counting the number of ectomycorrhizae in the first two-hundred root-tips. Morphotype richness was determined by summing the number of morphotypes found per sample. Fungal morphological characteristics (root-tip branching, colour, texture and hyphal patterns) were described and compared with previously published ectomycorrhizal descriptions to identify morphotype categories (Ingleby *et al.*, 1990; Goodman *et al.*, 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b).

Statistical analyses

The ^{13}C isotope excess (mg) in each tissue type was calculated from $\delta^{13}C$ and percent carbon values per sample (μg), and the ^{14}C isotope excess (mg) was similarly calculated from dpm values and percent carbon values (μg) per sample. These values were used to calculate carbon isotope content of donor and receiver tissues as well as whole seedlings (Simard *et al.*, 1997a, d) (Appendices I, II). Means were calculated from experimental units (i.e., each replicate of the

hyphal treatments and labeling schemes) to compare hyphal treatment effects (e.g. severed versus intact hyphae) as well as species and isotope effects (e.g. paper birch in 13PB-14DF versus Douglas-fir in 13DF-14PB). Seedling biomass (g), and donor and receiver isotope contents (mg), were compared between treatments and labeling schemes using two-way ANOVA (n=6) for a 2x2 factorial design (SPSS software version 10.0).

Whole seedling isotope contents were used to compare bi-directional and net carbon transfer between labeling schemes and treatments using two-factor ANOVA (n=6) (Appendix III). Where significant differences occurred between the two labeling schemes (e.g. transfer of ^{13}C from birch to fir in the labeling scheme 13PB-14DF compared to ^{14}C transfer from birch to fir in the labeling scheme 13DF-14PB), a correction factor was applied to the ^{14}C isotope contents in order to equalize the two labeling schemes; correction factors were calculated on a treatment-species-tissue-specific basis (n=6) (Simard *et al.*, 1997a, d). For example, excess mg^{13}C received by Douglas-fir roots measured in the labeling scheme 13PB-14DF, was divided by excess mg^{14}C received by Douglas-fir roots in the reciprocal labeling scheme 14PB-13DF, of the same severing treatment. Mean correction factors (n=6) were applied to mg-^{14}C values to obtain analogous mg-^{13}C-equivalent values for each tissue. Corrected tissue values were summed to obtain donor and receiver whole plant corrected ^{14}C values (i.e., mg-^{13}C-equivalent) and ^{13}C (mg) values. Bi-directional transfer was calculated as the sum of isotope received by whole paper birch and Douglas-fir seedlings, and net transfer was the difference in isotope received by Douglas-fir from that received by paper birch (where positive values indicate net transfer to Douglas-fir and negative values indicate net transfer to paper birch) (Appendix III). Bi-directional and net transfer were compared between treatments using t-tests (n=24). For each treatment, net transfer was also compared with zero using t-tests. Net transfer was compared to total isotope assimilated by both species and expressed as a proportion of total isotope fixed (%) per root chamber.

Results

Seedling characteristics

Paper birch seedlings (0.4769 g ± 0.0091) were 1.3 times larger than Douglas-fir seedlings (0.3724 g ± 0.0139) when labeling was conducted. Biomass of Douglas-fir roots did not differ between labeling schemes (0.2267 g ± 0.0157) (p=0.423) but did differ between hyphal connection treatments (0.1949 g ± 0.0198 in the severed treatment compared to 0.2585 g ± 0.0213 in the intact treatment) (p=0.046). By contrast, Douglas-fir shoots (0.1458 g ± 0.0140) (p=0.667; p=0.812), paper birch roots (0.2984 g ± 0.0163) (p=0.765; p=0.982), and paper birch shoots (0.1786 g ± 0.0090) (p=0.988; p=0.791) did not differ between labeling schemes or hyphal treatments, respectively.

Mycorrhizae

A total of nine ectomycorrhizal morphotypes occurred on non-labeled paper birch and Douglas-fir seedlings (Table 3.1). Paper birch and Douglas-fir each averaged four morphotypes per seedling. One to three morphotypes (E-strain 1, E-strain 2, *Mycelium radicis atrovirens* [MRA]) were shared in common between the two seedlings in each root chamber, colonizing on average 48% and 62% of paper birch and Douglas-fir root tips, respectively.

Total CO_2 concentration in sampling bags

At the end of the pulse period (immediately before the bags were removed), mean CO_2 concentration in bags pulsed with $^{13}CO_2$ averaged 0.0323 x 10 000 ppm ± 0.0033, while that in bags pulsed with $^{14}CO_2$ averaged 0.0515 x 10 000 ppm ± 0.0077. Ambient CO_2 concentration in the lab was 0.0360 x 10 000 ppm. For bags pulsed with $^{13}CO_2$, CO_2 concentration at the end of the pulse did not differ between species (0.0384 x 10 000 ppm ± 0.0040 for Douglas-fir and

0.0285 x 10 000 ppm ± 0.0051 for paper birch; p=0.338) or hyphal treatments (p=0.990). In $^{14}CO_2$-labeled bags, by contrast, species effects were significant (0.0651 x 10 000 ppm ± 0.0106 for Douglas-fir in 13PB-14DF compared to 0.0310 x 10 000 ppm ± 0.0060 for paper birch in 13DF-14PB) (p=0.043) while treatment effects were not (0.0515 x 10 000 ppm ± 0.0077) (p=0.866). The species difference most likely resulted from the fact that Douglas-fir seedlings were smaller than paper birch (particularly the shoots) and had lower leaf photosynthetic rates (see Chapter IV), and therefore had lower potential for assimilating carbon than paper birch.

Isotope content and distribution

Seedlings monitoring potential aerial isotope contamination had whole seedling and tissue isotope contents that did not differ significantly from ^{13}C natural abundance levels (p>0.05) and ^{14}C control levels (p>0.05). Mean natural abundance levels were -30.98 ‰ and -30.18 ‰ for paper birch and -29.32 ‰ and -28.05 ‰ for Douglas-fir shoots and roots, respectively. Control ^{14}C levels were 79.83 dpm and 76.40 dpm for paper birch and 80.20 dpm and 79.03 dpm for Douglas-fir shoots and roots, respectively.

Donors

At the beginning of the chase, initial C isotope content (excess mg ^{13}C or ^{14}C per seedling) of paper birch in the labeling scheme, 13PB-14DF, was 1.0464 mg ^{13}C and 0.0857 mg ^{14}C in shoot tissue, and 1.0493 mg ^{13}C and 0.1673 mg ^{14}C in root tissue (n=6). Douglas-fir in 13DF-14PB had initial C isotope contents of 1.2809 mg ^{13}C and 0.0741 mg ^{14}C in shoots, and 0.3886 mg ^{13}C and 0.1736 mg ^{14}C in roots (n=6). Initial isotope contents of Douglas-fir in 13PB-14DF and of paper birch in 13DF-14PB were not obtained due to sample loss during analysis at the University of California-Davis Isotope Facility. Based on the available initial data, root boxes where paper birch donors were pulsed with ^{13}C contained 1.26 times as much ^{13}C as Douglas-fir ^{13}C donors (2.0957 excess mg^{13}C versus 1.6696 excess mg^{13}C). Comparably, paper birch ^{14}C receivers

initially contained 0.2530 excess mg^{14}C whereas Douglas-fir ^{14}C receivers contained 0.2477 excess mg^{14}C.

At the end of the chase period, whole seedling excess ^{13}C content of ^{13}C-donor paper birch and Douglas-fir seedlings (in 13PB-14DF and 13DF-14PB, respectively) averaged less in the severed treatment (0.1718 mg ^{13}C ± 0.1251) than the intact treatment (0.9649 mg ^{13}C ± 0.2111) ($p<0.0001$) (Table 3.2, Figure 3.1a). Whole seedling excess ^{13}C content of ^{13}C-donors did not differ between species (0.5684 mg ^{13}C ± 0.1457) ($p=0.054$). On a tissue basis, mean isotope content of donor shoots pulse labeled with ^{13}C did not differ between severing treatments ($p=0.146$) (Table 3.2). However, ^{13}C-donor Douglas-fir shoots were more enriched in ^{13}C than birch shoots averaged over both hyphal treatments ($p=0.006$) (Table 3.2), representing 68.14% of the whole seedling excess isotope content in fir and 3.27% in birch shoots, respectively. Paper birch ^{13}C-donor roots (13PB-14DF) contained similar amounts of ^{13}C as Douglas-fir ^{13}C-donor roots (13DF-14PB) averaged over both hyphal severing treatments (0.3332 mg ^{13}C ± 0.0881) ($p=0.445$). ^{13}C-donor roots contained less excess ^{13}C in the severed than intact treatment, representing 56.45% and 73.78% of total seedling isotope content, respectively ($p=0.001$) (Table 3.2).

Whole seedling ^{14}C content of donor seedlings pulsed with $^{14}CO_2$ did not differ between severing treatments ($p=0.168$) (Figure 3.1a), but was greater in Douglas-fir (13PB-14DF) than paper birch donors (13DF-14PB) ($p<0.0001$) (Table 3.2). Root and shoot tissue excess ^{14}C contents of ^{14}C-donor paper birch and Douglas-fir were also similar between hyphal treatments ($p=0.068$ for shoots; $p=0.598$ for roots) (Table 3.2). Paper birch and Douglas-fir donor roots contained similar amounts of ^{14}C ($p=0.230$), but fir donor shoots contained more isotope than birch donor shoots ($p=0.042$) (Table 3.2).

Receivers

On average, paper birch and Douglas-fir whole seedlings received significantly more ^{13}C in the intact treatment than the severed treatment (p=0.001) (Figure 3.1b). By contrast, the amount of excess ^{13}C received on average by paper birch did not differ from that received by Douglas-fir (1.2318 mg $^{13}C \pm 0.1773$) (p=0.871). Roots of paper birch received similar amounts of ^{13}C as roots of Douglas-fir (p=0.093) (Table 3.2). Most of the received isotope remained in roots (94.08 % of whole seedling isotope content in the severed treatment; 78.96 % in the intact treatment), but receiver roots in the severed treatment were significantly less enriched than roots in the intact hyphal treatment (p<0.001) (Table 3.2). Shoots receiving ^{13}C were also more enriched in the intact treatment than the severed treatment (p=0.007), but only clearly for Douglas-fir (Table 3.2). ^{13}C content of receiver shoots was significantly greater in Douglas-fir than birch (p=0.002). Receiver shoot enrichment with ^{13}C represented on average 11.85% of total seedling ^{13}C content per root box in the severed treatment and 42.09% in the intact treatment.

As with ^{13}C, there was a tendency for paper birch and Douglas-fir seedlings to receive more ^{14}C in the intact than the severed treatment (p=0.107) (Figure 3.1b). The amount of ^{14}C received by whole seedlings was on average greater for Douglas-fir than paper birch (p=0.001) (Table 3.2). Douglas-fir receiver roots and shoots were more enriched in ^{14}C than birch roots or shoots (p=0.003 for roots; p=0.006 for shoots). The ^{14}C content in receiver roots was greater in the intact than severed treatment (p=0.015), whereas severing had little effect on the amount of ^{14}C received by shoots (p=0.109).

Carbon transfer

Both Douglas-fir and paper birch seedlings received significant amounts of isotope from their neighbour when compared with zero transfer (p=0.001 for bi-directional transfer and p=0.048 for net transfer), indicating that carbon transfer was bi-directional. Bi-directional transfer (Appendix

III, sum of mg ^{13}C and mg ^{13}C equivalent received by both seedlings in a root chamber) was greater in the intact hyphal treatment than the severed treatment (p=0.002) (Figure 3.2). Net transfer was positive on average, indicating net transfer from paper birch to Douglas-fir. It was significantly greater in the intact than the severed treatment (p=0.011) (Figure 3.2). The amount of isotope received by Douglas-fir represented 2% of total carbon fixation (expressed as a proportion of the total amount of excess isotope fixed by both species in a root chamber) in the severed treatment and 9% of total carbon fixation in the intact hyphal treatment.

Discussion

Carbon moved in two directions between paper birch and Douglas-fir seedlings in the root chambers, with a net gain by Douglas-fir that was equivalent to 2-9% of that fixed by both tree species in the root chamber. This finding confirms the first part of our hypothesis, that transfer is bidirectional, but with net transfer from paper birch to Douglas-fir. We believe this is the first laboratory study showing significant net carbon transfer between different plant species via CMNs as a result of experimental hyphal manipulation. The magnitude of net transfer in the intact treatment is similar to that observed in the field study reported in Chapter Four and that of Simard *et al.* (1997a).

Although both ^{13}C and ^{14}C were used to measure bidirectional and net carbon transfer in root chambers, the uptake, storage and transfer of the two isotopes differed between Douglas-fir and paper birch, such that Douglas-fir donors (shoots and roots) were more enriched than paper birch donors in both isotopes, particularly ^{14}C. Donor isotope content also differed between hyphae treatments, with less donor ^{13}C enrichment where hyphae were cut than where they were left intact. There are several possible explanations for greater isotope enrichment in donor Douglas-fir compared to paper birch and for differences between donor isotope content between hyphal treatments. First, Douglas-fir donors had higher specific activity (greater ratio of labeled

versus unlabeled carbon) than paper birch donors, probably because the isotope was less diluted in the smaller biomass of Douglas-fir than paper birch. The ^{14}C content was particularly higher in Douglas-fir, and this may be attributed to the slower gas diffusion rates of the heavier ^{14}C isotope around the smaller seedlings, so that the small Douglas-fir needles would have had greater exposure per unit leaf area than the paper birch leaves. The higher ^{14}C content of Douglas-fir than paper birch donor roots may be attributed to the greater proportion of coarse roots in Douglas-fir, which may have turned over and sloughed isotope at a slower rate than the finer paper birch roots (with higher surface area to volume ratios for root and fungal turnover processes). The Douglas-fir roots were also 1.3 times more colonized by ectomycorrhizae than paper birch, which may have increased the carbon isotope drain from shoots to roots (and also increased the possibility that fungi were storing labeled carbon more on Douglas-fir than paper birch roots.

The lower donor isotope content in the severed than intact treatment may have resulted from lowered fungal respiration, carbon demand, and isotope re-uptake following severing of the hyphae, whereas leaving fungi intact allowed them to continue forming extraradical mycelia thus increasing relative carbon demand. Severing of the dark septate fungal endophyte shared by Douglas-fir and paper birch would particularly enhance these treatment differences: dark septates are sometimes referred to as pseudomycorrhizal as they can act parasitically or mutualistically, and severing would have reduced their potential for demanding and storing more labeled carbon. Differences in mycorhizosphere composition between severing treatments may have also contributed to the isotope differences of donors, whereby microbial demand and transformation may have been reduced in severed the mycorhizospheres. Finally, differences in donor isotope contents between species and hyphal treatments may have resulted from contamination during sample preparation and analysis (e.g., unrealistically high percent carbon values per sub-sample may have skewed the data calculations).

A challenge with dual-labeled experiments is in comparing ^{14}C with ^{13}C isotope values. Simard *et al.* (1997a, d) used correction factors to account for isotopic differences between labeling schemes and plant species. I used the same method to calculate mg^{13}C equivalent values from the ^{14}C data, which presented some problems in interpreting higher corrected isotope values. One possible explanation for the large difference between equivalent and ^{13}C values may be an anomalous data point or outlier which skewed the corrected values. One possible solution to simplify this corrected data may be to analyze ^{14}C equivalents and ^{13}C together and separately analyze uncorrected ^{14}C data and ^{13}C.

The second part of our hypothesis, that more carbon is transferred through the CMN than soil pathways, was also confirmed by this experiment. We found that approximately three times more isotope was transferred bidirectionally through an intact than severed CMN. This question has remained under lively debate in the literature. Carbon transfer through hyphal pathways has previously been studied in the laboratory in ectomycorrhizal (Wu *et al.*, 2001, Simard *et al.*, 1997d) and vesicular-arbuscular mycorrhizal systems (Fitter *et al.*, 1998, Watkins *et al.*, 1996). Wu *et al.* (2001) used autoradiography to follow the path of labelled carbon transferring one-way from donor to receiver pine seedlings. While autoradiography successfully showed active hyphal linkages, transferred carbon could not be detected in receiver plant tissues. The results of Wu *et al.* (2001) contrast with Simard *et al.* (1997d), who studied two-way interspecific carbon transfer using laboratory microcosms, dual isotope labelling techniques, and the same plant species as in this study. Simard *et al.* (1997d) found that carbon moved in two directions to root and shoot receiver tissues, but that severing of hyphae did not significantly influence the magnitude of either bi-directional or net transfer. In this study, however, our ability to detect net transfer was likely improved by the daily hyphal severing procedures that prevented hyphal re-connection, better treatment replication, and the longer isotope pulse period. Our observation that transferred carbon moved into shoots and roots of receiver plants concurs with Simard *et al.*

(1997a, d) and Lerat *et al.* (2002), but differs from Wu *et al.* (2001) and Fitter *et al.* (1998). Fitter *et al.* (1998) found that carbon transferred between interconnected vesicular-arbuscular plants, *Plantago lanceolata* and *Cynodon dactylon*, but found no evidence that carbon moved from fungal tissues into receiver plant tissues. The contrasting results of these studies may be attributed to the differing mycorrhizal fungal groups (arbuscular mycorrhizal fungi versus ectomycorrhizal fungi), where a greater amount of transferred isotope may have been stored in arbuscular fungal storage structures.

Further evidence that carbon was transferred through a common mycorrhizal network was that paper birch and Douglas-fir shared in common one to three ectomycorrhizal morphotypes over approximately half of their root tips. Emanating hyphae from these common fungi would have linked the two root systems, and this was supported by our visual observations of hyphae bridging the adjacent root pouches immediately prior to labelling. However, because we used microscopy and morphotyping rather than molecular analysis for identification of ectomycorrhizae, we were unable to unequivocally determine whether the seedlings were colonized by genetically identical fungi. Autoradiography would have been useful in confirming the presence of linkages (Wu *et al.*, 2001), and in quantifying the number and/or activity of the hyphal connections; however, the minimum detection level for autoradiography may be inadequate to do this. Furthermore, autoradiography works well only in a two-dimensional root system, which is more artificial than our system. Autoradiography, in combination with hyphal sampling and analysis by liquid scintillation, may increase our ability to identify functional linkages and detect transfer to receiver root systems.

Severing hyphal linkages between seedlings every day of the chase period may have improved our ability, in comparison to Simard *et al.* (1997d), to detect differences in net and bi-directional transfer between hyphal severing treatments. Given the rapid rates at which mycorrhizal hyphae can regenerate (Brandes *et al.*, 1998) and anastomose (hyphal fusion)

(Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004), it is possible that Simard *et al.* (1997d) could not detect a severing effect on net transfer because hyphal linkages re-formed during the six-day chase period after a single severing treatment. In our study, we severed hyphae between root pouches multiple times to reduce the possibility of re-anastomosis prior to the end of the chase period.

Our detection of significant net transfer through intact hyphal connections may also have been influenced by the longer isotope pulse period than in Simard *et al.* (1997d). We pulsed paper birch and Douglas-fir for eight hours, and maintained equitable CO_2 levels in each sampling bag with supplementary $^{12+13}CO_2$ injections, representing a substantial improvement over the one-hour pulse technique of Simard *et al.* (1997d). Extending the pulse period may have resulted in greater isotope labeling and allocation to donor roots.

Our detection of net carbon transfer to Douglas-fir in this study may be attributable to interspecific differences in carbon allocation patterns in pulsed donor seedlings immediately following the pulse and after six days. Even though pulse labeling with either ^{13}C or ^{14}C resulted in greater amounts of total isotope in donor Douglas-fir roots than paper birch roots, there was net transfer from paper birch to Douglas-fir. It is possible that the larger, finer and more diffuse root system of paper birch resulted in a more even distribution of isotope pulsed through the CMN for greater uptake by Douglas-fir receiver roots. Larger root to shoot ratios in birch suggests birch roots and mycorrhizas have the potential to explore and exploit more of the soil matrix than Douglas-fir, and form more abundant and species-rich associations with ectomycorrhizal fungi and bacteria that may facilitate carbon transfer. In our experimental systems, paper birch seedlings also had higher net photosynthetic rates and foliar nitrogen concentrations than Douglas-fir (Chapter 4), representing an interspecific carbon and nitrogen source-sink gradient along which carbon transferred from birch to fir. Plant factors previously suggested to regulate carbon transfer through CMNs include interplant differences in net

photosynthetic rate, nutrient status, total biomass, and age (Read *et al.*, 1985; Newman, 1988; Bethlenfalvay *et al.*, 1991; Simard *et al.* 2002).

In this experiment, the surface of the soil in root chambers was covered with polyethylene-plastic to prevent foliar re-assimilation of CO_2 respired by the roots or hyphae. If I had not done that, CO_2 release from roots and severed hyphae, followed by aerial CO_2 transfer, could have reduced differences in C transfer between the severed and intact treatments. While it is difficult to obtain reliable estimates of fungal respiration (Smith *et al.*, 2003), Ek (1997) found 43% to 64% of carbon allocated to ectomycorrhizal mycelium in birch was respired. Even so, we were unable to detected anapleurotic uptake of CO_2 that was respired by roots or hyphae of individual seedlings (data not shown). The amount of C transfer measured in the severed treatment therefore represented C transfer through soil pathways in solution or gas, since aerial C transfer was stopped by the plastic and CMN transfer was prevented by repeatedly severing the hyphae potentially bridging the mesh barriers. Transfer through the soil pathway could be subject to the same regulatory processes as transfer through the hyphal pathway because it is possible for carbon to transfer through hyphal and soil pathways simultaneously. This is possible because not all root tips in a single root system are colonized or functional at the same time (Rygiewicz *et al.*, 1997).

The implications of interplant carbon transfer to plant community dynamics has been discussed in recent reviews (Newman, 1988; Miller & Allen, 1992; Perry *et al.*, 1992, Fitter, 2001; Simard *et al.*, 2002; Bever, 2003; Booth, 2004; Simard & Durall, 2004). Common mycorrhizal networks could, for example, assist in establishment of seedlings, recovery of plant communities following disturbance, and alteration of competitive interactions. In our study, net carbon transfer from paper birch to Douglas-fir may allow Douglas-fir to establish and grow in the understory of early successional paper birch. Paper birch and Douglas-fir naturally co-exist in western North American forest ecosystems (Simard *et al.*, 2004), and the benefits of tree

species mixtures to forest productivity, forest health, and biodiversity are well documented [Juha *et al.*, 2002; DeLong *et al.*, 2002; Hauessler & Bergeron, 2004]. The complex relationship between common mycorrhizal networks and aboveground plant community dynamics appears to involve a tightly linked plant-soil system (Bever 2003).

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Table 3.1. Ectomycorrhizal morphotypes and mean percent colonization of non-labeled paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) seedlings grown together in root chambers for eight months.

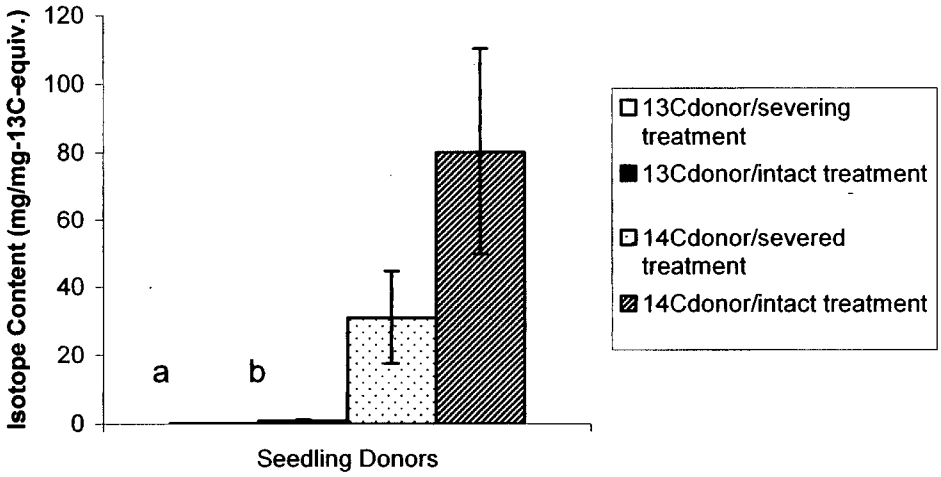
		Percent Colonization per 200 root tips	
Morphotype	Characteristics	Paper Birch	Douglas-fir
Non-mycorrhizal	root hairs visible	8.50±2.81	9.25±4.40
Incomplete	immature mantle	5.80±3.14	43.60±8.74
Cenococcum-like	root tips black, hairy; stellate mantle pattern	2.30±0.01	0
E-strain 1	root tips brown/black; oblique mantle cells; verrucose emanating hyphae	24.00±5.20	22.15±8.69
E-strain 2	similar to E-strain 1; emanating hyphae absent	56.00±8.20	8.10±16.52
Hebeloma 1-like	root tips cream/brown; copious emanating, clamped hyphae	0	19.30±0.01
Hebeloma 2-like	similar to Hebeloma 1; emanating hyphae absent	11.70±3.34	0.50±1.16
Laccaria-like	root tips white; mantle thick, narrow cells; emanating hyphae infrequent	11.20±2.27	16.60±6.90
MRA	root tips black, hairy to smooth; angular mantle cells; emanating hyphae thick-walled, black	47.18±10.12	7.60±1.72
Rhizopogon-like	emanating hyphae brown/yellow with elbow bends	0	14.90±4.52
Tomentella-like	root tips black; emanating hyphae black, clamped	80.00±32.70	1.00±0.07

Table 3.2. Isotope content (mg ^{13}C or mg-^{13}C-equivalent per tissue) of paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) seedlings in the root chamber experiment, where seedlings were reciprocally labelled with ^{13}C or ^{14}C in severed and intact hyphal treatments, and then harvested for isotope analysis after six days.

Species	Tissue	Treatment	Isotope Content of Donors		Isotope Content of Receivers	
			^{13}C(mg)	$^{14}C_{(mg\text{-}^{13}C\text{-equivalent})}$	^{13}C(mg)	$^{14}C_{(mg\text{-}^{13}C\text{-equivalent})}$
Birch	shoot	severed	0.0001±0.0200*	0.0010±0.0001	0.0001±0.0200	0.0001±0.0200
Birch	root	severed	0.0258±0.0630	0.0228±0.0107	0.6471±0.3204	0.0003±0.0002
Fir	shoot	severed	0.2453±0.6006	4.5123±2.5286	0.0726±0.0987	0.0475±0.0293
Fir	root	severed	0.0726±0.1683	57.6252±5.0514	0.5380±0.2763	0.7302±0.4067
Birch	shoot	intact	0.0182±0.0442	0.0111±0.0051	0.0001±0.0200	0.0014±0.0034
Birch	root	intact	0.5338±0.3158	0.6337±0.3068	1.6860±0.5810	0.1218±0.2870
Fir	shoot	intact	0.6776±0.4096	89.9305±9.0473	0.8349±0.6125	0.1777±0.1577
Fir	root	intact	0.7006±0.5651	69.4077±4.1306	1.1486±0.5363	2.2026±1.7834

* Mean ± 1 Standard Error

(a).



(b).

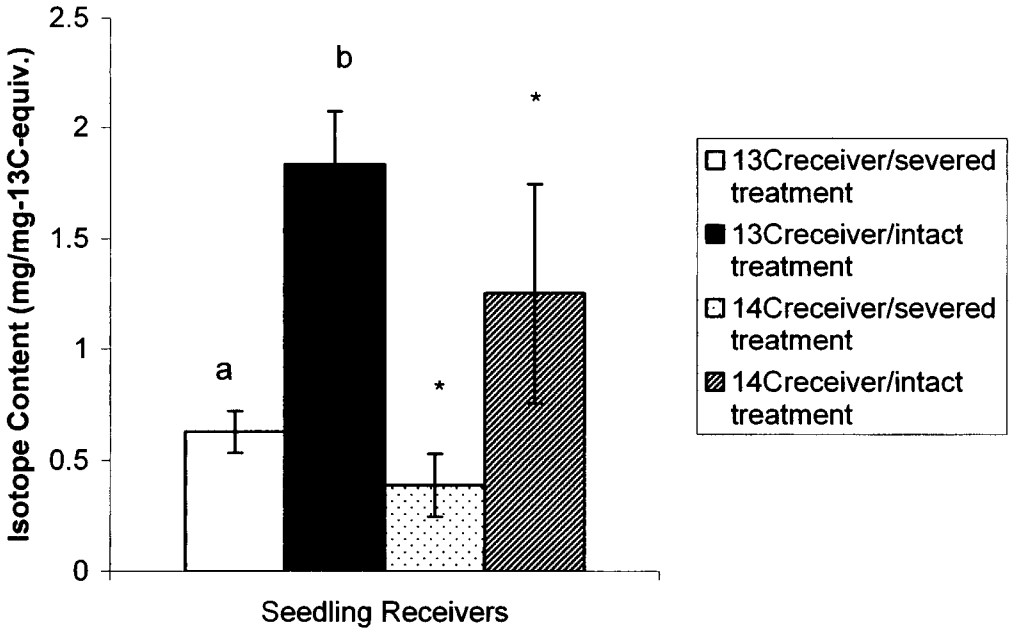


Figure 3.1 Whole seedling isotope content of (a) donor (^{13}C in mg and ^{14}C in mg-^{13}C-equiv.) and (b) receiver (^{13}C in mg and ^{14}C in mg-^{13}C-equiv.) seedlings in the severed and intact

treatments. Histograms represent means of the two species (N = 6). Error bars are one standard error. Different letters or symbols within an isotope indicate significant differences between hyphal severing treatments according to two-factor ANOVA (α=0.05).

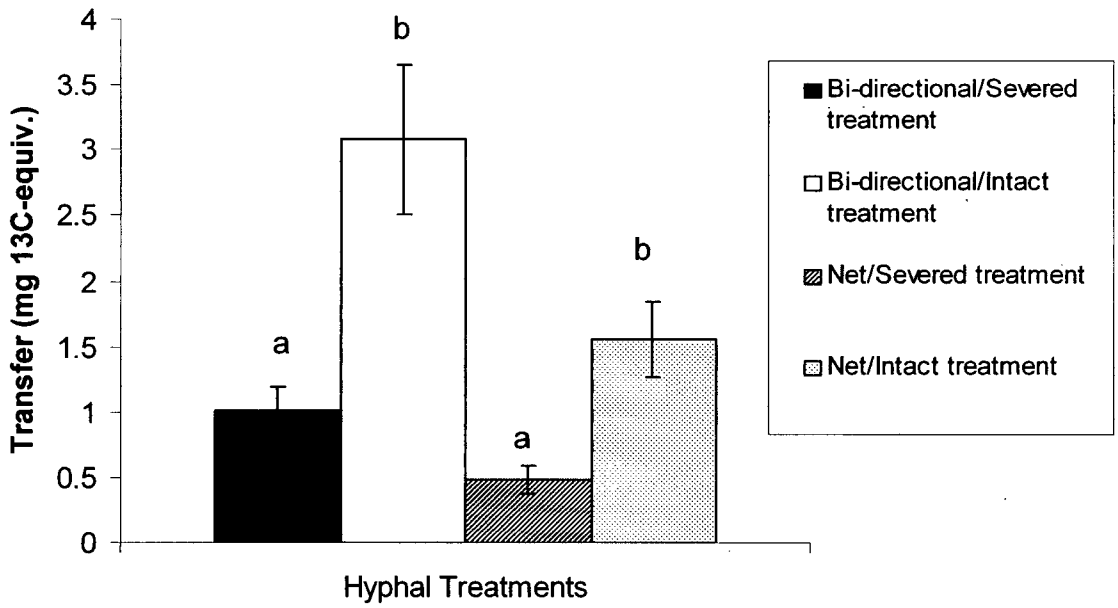


Figure 3.2 Bi-directional and net transfer between paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) where hyphae were left intact or severed. Error bars are one standard error. Positive transfer amounts indicate net transfer from birch to fir. Different letters for bidirectional or net transfer indicate differences between hyphal severing treatments according to two-factor ANOVA (α=0.05).

*CHAPTER FOUR: Phenology affects interplant carbon transfer through mycorrhizal and soil pathways in the field

Introduction

Temperate forest trees depend on mycorrhizal associations for survival, with many fungi forming associations with multiple plant species, potentially forming interplant common mycorrhizal networks (CMN) (Molina *et al.*, 1992; Jones *et al.*, 1997; Simard *et al.*, 1997b; Simard & Durall, 2004). There is increasing evidence that CMNs can facilitate movement of carbon and minerals between plants (Francis & Read, 1984; Finlay & Read, 1986; Newman, 1988; Newman *et al.*, 1994; Simard *et al.*, 1997a, d; Wu *et al.*, 2001; Lerat *et al.*, 2002; Pfeffer *et al.*, 2004). Direct acquisition of carbon or nutrients from the CMN is advantageous compared with root uptake from soil solution because it bypasses less efficient soil transformations. Hyphae are also less costly to generate than roots, have a lower biomass to absorptive surface area ratio, can rapidly colonize sites, and are able to exploit small soil pores inaccessible to roots (Brandes *et al.*, 1998).

While belowground carbon transfer through ectomycorrhizal or arbuscular-mycorrhizal networks has been studied previously in the laboratory (Brownlee *et al.*, 1983; Finlay& Read, 1986; Simard *et al.*, 1997d; Wu *et al.*, 2002; Pfeffer *et al.*, 2004) and field (Simard *et al.*, 1997a, Lerat *et al.*, 2002), the identity of the primary carbon transfer pathways remains contentious (Fitter *et al.*, 1999). Newman and Ritz (1986) suggest that two main carbon transfer pathways exist between mycorrhizal plants. One is a direct transfer pathway, where carbon, phosphorus, or nitrogen moves from one mycorrhizal root system to another entirely through hyphal linkages (i.e., the continuous hyphal pathway). This pathway can be viewed as a mycorrhizal bypass, where carbon movement occurs within hyphae, compartmentalizing carbon away from saprobes or chemical and physical processes that deplete carbon from the soil (Newman & Ritz, 1986).

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* A version of this chapter will be submitted for publication.
Philip LJ, Simard SW, Jones MD. Phenology affects direction of carbon transfer in the field.

The second is an indirect soil pathway, where carbon or nutrients move between non-mycorrhizal roots through the soil (i.e., the continuous soil pathway). Carbon moves along this pathway as soluble organic carbon in the soil solution or through respiratory release of CO_2 followed by re-fixation in anapleurotic reactions in uncolonized and colonized roots (Wingler *et al.*, 1996). Intermediate between these two pathways is the discontinuous-soil pathway, where carbon moves between roots partially within mycorrhizal hyphae and partially within the soil.

Recognition of multiple carbon transfer pathways, functioning alone or simultaneously between plant neighbours, is crucial to our understanding of transfer effects on plant interactions and the factors that influence transfer between plants (Newman, 1988). In addition to this, CMN-mediated transfer remains contentious because it is unknown (1) whether there is one-way, bi-directional or net transfer of carbon; (2) whether transferred compounds remain in fungal tissues or move to plant tissues; (3) what biological factors regulate the direction and magnitude of transfer, and (4) whether net transfer is of sufficient magnitude to affect plant fitness, productivity or interactions with other organisms.

Common mycorrhizal networks and belowground carbon transfer have been studied previously in the field in both ectomycorrhizal (Simard *et al.*, 1997a) and arbuscular mycorrhizal (Lerat *et al.*, 2002) forest plant communities. In an ectomycorrhizal community of paper birch (*Betula papyrifera* Marsh) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings, Simard *et al.* (1997a) quantified the magnitude and direction of belowground carbon transfer using reciprocal labeling with stable and radioactive carbon isotopes. Carbon moved in two directions between paper birch and Douglas-fir, with no net transfer when seedlings were two years old, but increasing to 3-10% net transfer from paper birch to Douglas-fir when they were three years-old, with more in deep shade than full sun. Western redcedar (*Thuja plicata* D. Don), an arbuscular-mycorrhizal species, received <1% of the total amount of carbon transferred between paper birch and Douglas-fir in the first year, increasing to 18% in the second year,

indicating the soil pathway was operative simultaneously with the hyphal pathway. In a second field study in an arbuscular mycorrhizal system, Lerat *et al.* (2002) examined how changes in plant source-sink relationships through the growing season affected the direction of carbon transfer. Using ^{14}C pulse-labeling techniques to examine one-way carbon transfer, Lerat *et al.* (2002) found that fully foliated trout lily (*Erythronium americanum* Ker-Gawl) supplied radioactive carbon to emergent sugar maple (*Acer saccharum* Marsh) seedlings in the spring, while later in the season, foliated maple supplied carbon to senescent trout lily corms.

These studies together suggest that carbon transfer is a dynamic process that varies annually and seasonally, and that a one-time measurement in summer is insufficient to evaluate the ecological importance of CMNs to interspecific interactions between paper birch and Douglas-fir. For instance, coniferous Douglas-fir photosynthesizes earlier and later in the growing season than deciduous paper birch, potentially resulting in net carbon flow from Douglas-fir to paper birch along a labile carbon concentrating gradient in spring and fall. In mid-summer, however, the direction of net carbon transfer may reverse when birch is photosynthesizing at a higher rate than Douglas-fir.

This field study examined bi-directional and net carbon transfer between fully illuminated ectomycorrhizal paper birch and Douglas-fir at three host plant phenological stages during a single growing season. We compared transfer along soil and hyphal transfer pathways by quantifying two-way transfer between ectomycorrhizal paper birch and Douglas-fir, and one-way transfer to arbuscular mycorrhizal western redcedar, bearberry (*Arctostaphylous uva-ursi* (L.) Spreng.) and fireweed (*Epilobium angustifolium* L.), and ectomycorrhizal western larch (*Larix occidentalis* Nutte.). Our specific objectives were to determine (1) the role that CMN and soil pathways play in carbon transfer, and (2) whether the magnitude and direction of transfer vary with host plant phenology.

Methods

Study Design

Carbon transfer was studied in a field experiment in which ectomycorrhizal paper birch and Douglas-fir were grown in seedling groups with western redcedar and western larch. After two years growth, paper birch and Douglas-fir were reciprocally pulse labeled with stable and radioactive carbon isotopes at three phenological stages of host seedlings. At the three stages tested, paper birch seedlings were: (1) beginning to leaf-out in May (spring), (2) completely leafed out in July (summer), and (3) beginning to senesce in October (fall). For Douglas-fir seedlings, this corresponded with: (1) pre-bud burst (spring), (2) after terminal bud-set but during active diameter growth (summer), and (3) after cessation of diameter growth (fall). The two labeling schemes, designed to account for potential species and isotope effects on transfer, were: (1) where paper birch seedlings were pulse labeled with $^{13}CO_2$ and Douglas-fir seedlings with $^{14}CO_2$ (abbreviated 13PB-14DF), and (2) where Douglas-fir seedlings were pulse labeled with $^{13}CO_2$ while paper birch seedlings were pulsed with $^{14}CO_2$ (13DF-14PB). Seedlings were pulsed with either 200 ml 99% $^{13}CO_2$ or 200 μCi (7.4 MBq) $^{14}CO_2$ released from $Na_2{}^{14}CO_3$. Plant phenology and labeling scheme effects were tested in a 3x2 factorial structure of treatments, replicated five times in spring, five times in summer, and four times in fall in a completely randomized design.

Site Description

This study was conducted within two fenced plots, each measuring approximately 50 m^2, on a reforested clear-cut site in the southern interior of British Columbia, Canada. The sites were located at 50°24.14 N, 118°57.61W at elevation 1 015-1 029 m. The original stand of 120 year-old paper birch and Douglas-fir was clear-cut logged in 1998 and replanted with a mixture of Douglas-fir, lodgepole pine (*Pinus contorta* Dougl. ex Loud.) and western larch seedlings in

1999. Herbaceous vegetation that naturally occurred on the site included kinnikinnick (*Arctostaphylous uva-ursi* (L.) Spreng.), Canada goldenrod (*Solidago canadensis* L.), Canada thistle (*Cirsium arvense* (L.) Scop.), falsebox (*Pachistima myrsinites* (Pursh) Raf), fireweed (*Epilobium angustifolium* L.), thimbleberry (*Rubus parviflorus* Nutte), thread-leaved daisy (*Erigeron filifolius* var. *filifolius* (Hook.) Nutt.), and wild strawberry (*Fragaria virginiana* Duchesne ssp. *virginiana*). The study sites were located on mesic sites in the Interior Cedar Hemlock (ICH) biogeoclimatic zone, specifically the Shuswap variant of the moist, warm ICH (ICHmw2) subzone (Lloyd *et al.*, 1990). The ICH zone is characterized by a climate with moderately dry summers and snowy winters. The soil type was Hemo Feric Podzol with 10-40% coarse fragment content, soil texture loam to silt loam and humus layer classified as Mor.

Preparation of seedling groups

Each experimental unit consisted of a five-seedling group comprised of paper birch, Douglas-fir, western redcedar, western larch and an extra Douglas-fir control. Seedlings were planted 50 cm apart, except for the Douglas-fir control, which was planted 15 cm from the centre seedling to monitor possible isotope contamination during labelling (Figure 4.1). Following planting, groups were surrounded by soil trenches, 30 cm in depth and lined with heavy polyethylene plastic. Soil trenches were established in August 2000 in order to (a) entrain roots toward the centre of the group, and (b) minimize potential isotope contamination to adjacent groups. All species except paper birch were grown in 415 PSB styroblocks using standard nursery practices. Paper birch seedlings were naturally regenerated and transplanted from nearby cut-blocks.

Photosynthetic measurement

Leaf net photosynthetic rate of all tree species was measured in each experimental unit immediately prior to pulse labeling in spring, summer and fall at ambient temperature and light

conditions. Measurements were made using a portable photosynthesis unit (LCi portable photosynthesis system, model LCi-001, ADC Bioscientific Ltd., Hoddesdon, Herts, England, EN 11 ODB). Leaf area measurements were not taken.

Pulse-chase regime

Prior to pulse labeling

One day before each pulse labeling, all herbaceous vegetation was clipped at the root collar. Additional unlabeled seedling groups were harvested the day before labeling for analysis of natural isotope abundance levels. Potential hyphal connections between Douglas-fir control seedlings and the remaining seedlings were severed by digging a 20 cm deep trench immediately prior to pulse labeling. Heavy polyethylene plastic was also used to cover the ground surface to prevent refixation of respired label from roots during the pulse and chase periods.

During pulse labeling

Paper birch and Douglas-fir seedlings in an experimental unit were pulse labeled with either ^{14}C or ^{13}C. Seedlings were enclosed in air-tight gas-sampling bags (10 L) fitted with septa for gas injections with a hypodermic needle, and then were pulsed by injecting either 200 ml 99% $^{13}CO_2$ gas (Cambridge Isotope Laboratories) directly into the bag, or by releasing $^{14}CO_2$ gas from 200 μCi (7.4 MBq) $Na_2{}^{14}CO_3$ (Amersham Ltd.) using 85% $C_3H_6O_3$ (lactic acid). For ^{14}C labeling, the gas-sampling bags were fitted with a closed 1.5 ml microcentrifuge tube containing $Na_2{}^{14}CO_2$ (aqueous) stock solution, one injection porthole, two gas exhaust portholes, and one styrofoam spacer to separate the bag walls (Figure 4.2). One gas porthole was connected to an air gas-cylinder using latex tubing, and the second to a CO_2 trap containing soda lime using latex tubing. To prevent escape of labeled CO_2 during the pulse period, duct tape was used to seal gas-sampling bags around seedling root collars and secure them to the plastic ground-sheets. This

ensured a tight seal between the soil and labeled shoots, reducing the potential for any respired gases to diffuse from the soil into the atmosphere. The gas-sampling bag was secured to a wire support placed adjacent to the pulsed seedling using metal clips.

Prior to labeling with ^{14}C, each gas exhaust porthole was closed to maintain an air-tight seal. The $^{14}CO_2$ gas was released when the microcentrifuge tube was opened from the outside of the bag and lactic acid was injected into the microcentrifuge tube through the injection porthole. At the same time, 200 ml 99%+1% $^{12+13}CO_2$ was injected into the gas-sampling bag to ensure the total CO_2 concentration inside the ^{14}C bags ($^{12+13+14}CO_2$) and ^{13}C bags ($^{12+13}CO_2$) was equivalent. After a two-hour pulse period, the microcentrifuge tube was closed and the two gas-exhaust portholes opened in preparation for $^{14}CO_2$ evacuation. The $^{14}CO_2$ was released into one gas exhaust porthole from the compressed air cylinder, forcing the remaining $^{12+13+14}CO_2$ out of the bag and into the soda lime trap. The bags were flushed in this manner for approximately 30 minutes and then removed. For ^{13}C, $^{12+13}CO_2$ gas was injected directly into the gas sampling bags through the septum. The ^{13}C bags were removed at the beginning of the ^{14}C flushing process. The seedling groups and ground sheets were then left undisturbed for a nine-day chase period.

Tissue harvest and analysis

After the chase, shoots were severed from roots using pruning shears and all roots were manually removed from surrounding soil. Shoot and root tissues were placed in plastic bags and stored on dry ice in coolers prior to transport to the laboratory. In the lab, shoot tissues were transferred to paper bags and stored in a –20 °C freezer, while roots were rinsed and then stored.

All sampled tissues were oven-dried for 48 hours at 55-60°C. Coarse tissue fragments were splintered into smaller pieces with pruning shears, and all tissues then ground using a Wiley® Mill with 20 and 40 μm metal mesh screens. One 2 mg sub-sample per tissue was

combusted for %C and analyzed for ^{13}C (δ in ‰) or ^{14}C (dpm) content at the Stable Isotope Facility, Department of Agronomy, University of California-Davis using mass spectrometry and liquid scintillation. These values were used to estimate excess ^{13}C and ^{14}C content (mg) in plant tissues and whole seedlings (Appendices I, II, III). Instrumentation was either a ThermoFinnigan Delta+ with a Carlo Erba NC2500 elemental analyzer or a Delta+XL with Costech ESC 4010 elemental analyzer. Stable isotope ratios were reported in δ notation as parts per thousand (‰) deviations from the international standard PDB (carbon). Instrument precision was <0.2‰.

Mycorrhizas

Root systems of extra non-labeled field-grown seedlings were examined for mycorrhizal status. Ectomycorrhizal species' roots were washed, cut into 3 cm segments, and randomly selected root segments then examined under dissecting and compound microscopes. Fungal morphological characteristics (root-tip branching, colour, texture and hyphal patterns) were compared with previously published ectomycorrhizal descriptions to identify morphotype categories (Ingleby *et al.*, 1990; Goodman *et al.*, 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b). Arbuscular mycorrhizal species' roots were washed, cleared and stained with Chlorazol Black E, and examined for intracellular hyphae, arbuscules, and vesicles (Brundrett *et al.* 1994).

Statistical analysis

Seedling comparisons were based on whether seedlings were considered donors or receivers. For example, paper birch ^{13}C donors could transfer ^{13}C to Douglas-fir receivers that were pulsed with ^{14}C, as well as to non-labeled receiver seedlings. Similarly, Douglas-fir ^{14}C donors could transfer ^{14}C to paper birch receivers pulsed with ^{13}C. Receiver seedlings, including paper birch, Douglas-fir, and non-labeled western redcedar, western larch, fireweed and Douglas-fir controls, could receive ^{13}C and/or ^{14}C from paper birch and Douglas-fir donor seedlings.

Excess ^{13}C isotope (mg) in shoot and root tissues was calculated from $\delta^{13}C$ and total carbon values (mg) (Appendix I), and excess ^{14}C isotope (mg) was similarly calculated from dpm values and total carbon values (mg) (Appendix II). These values were used to calculate carbon isotope content of donor and receiver tissues and whole seedlings (Simard *et al.*, 1997a, d). Species isotope means were used to compare phenology treatments and labeling scheme effects on donor and receiver isotope contents (mg) using a two-way ANOVA for a 3x2 factorial design (SPSS software version 10.0). Seedling biomass (g) and leaf photosynthetic rate (μmols $m^{-2}s^{-1}$) were compared using the same ANOVA model. The sum of isotope transferred one-way from a donor seedling to all receivers was used to calculate the proportion (%) of total isotope transferred within a seedling group (Appendix III).

Whole seedling isotope contents were used to compare bi-directional and net carbon transfer between labeling schemes and phenology treatments using two-factor ANOVA (n=5 spring, and summer; n=4 fall). Where significant differences occurred between the two labeling schemes (e.g., transfer of ^{13}C from paper birch to Douglas-fir in the labeling scheme, 13PB-14DF, compared to transfer of ^{14}C from paper birch to Douglas-fir in the labeling scheme, 13DF-14PB), a correction factor was applied to ^{14}C isotope contents in order to equalize the two labeling schemes; correction factors were calculated on a treatment-species-tissue-specific basis using either ratios (Simard *et al.*, 1997a, d; Chapter Three) or by performing linear or polynomial regression analyses. Mean correction factors were applied to mg-^{14}C values to obtain mg-^{13}C-equivalent values for each tissue. Corrected tissue values were summed to obtain donor and receiver whole plant corrected ^{14}C values (i.e., mg-^{13}C-equivalent) and ^{13}C (mg) values. Using corrected values, bi-directional transfer was calculated as the sum of isotope received by whole paper birch and Douglas-fir seedlings, and net transfer was the difference in isotope received by Douglas-fir from that received by paper birch (where positive values indicate net transfer to Douglas-fir, and negative values indicate net transfer to paper birch). Bi-directional and net

transfer were compared among phenology treatments using one-way ANOVA (n=3), and means were separated using Tukey tests. For each treatment, net transfer was also compared with zero using t-tests. Net transfer was compared to total isotope assimilated by both donor species and expressed as a proportion of total isotope fixed (%).

Results

Seedling biomass

Douglas-fir seedlings were 3-5 times larger than paper birch throughout the experiment ($p<0.05$), and all seedlings were largest in the fall (Table 4.1). Paper birch and Douglas-fir shoots were of similar size in summer and fall ($p=0.291$ for paper birch, $p=0.551$ for Douglas-fir) but were smaller in spring ($p<0.05$ for both species), while roots were similar in spring and summer ($p=0.747$ for paper birch, $p=0.898$ for Douglas-fir) but larger in fall ($p<0.05$ for both species). Redcedar shoots were smaller in spring than in summer ($p=0.005$) or fall ($p=0.022$), but roots in summer were not different from those in spring ($p=0.323$) or fall ($p=0.072$). Larch shoots and roots were smaller in spring than fall ($p=0.006$ for shoots, $p=0.023$ for roots), but shoots were similar between summer and fall ($p=0.974$). Larch root biomass was similar in spring and summer ($p=0.253$) and summer and fall ($p=0.400$).

Photosynthesis

Leaf net photosynthetic rates reflected seedling phenology (Table 4.1). Paper birch leaf assimilation rate increased from spring to fall ($p<0.001$) while Douglas-fir ($p=0.461$), redcedar ($p=0.051$) and larch ($p=0.145$) assimilation rates remained the same throughout the experimental period (Figure 4.3). Assimilation rate of paper birch was greater than that of Douglas-fir in summer ($p<0.001$), but less in spring ($p=0.002$) and similar in fall ($p=0.100$) (Fig. 4.3).

Mycorrhizae

Paper birch, Douglas-fir, and western larch were colonized with ectomycorrhizal fungi and western redcedar seedlings were associated with arbuscular mycorrhizal fungi. Eleven ectomycorrhizal morphotypes were observed on non-labeled seedlings (Table 4.2) (Ingleby *et al.*, 1990; Goodman *et al.*, 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b). Paper birch and Douglas-fir shared seven morphotypes in common (*Mycelium radicis atrovirens* [MRA], *Cenococcum*-like, E-strain 1, E-strain 2, *Tomentella* 1-like, *Hebeloma* 1-like). In comparison, western larch seedlings shared only two morphotypes with Douglas-fir (MRA, E-strain 1) and one with paper birch (E-strain 1). Morphotypes specific to a single tree species were *Lactarius*-like and *Laccaria*-like on paper birch roots, and *Rhizopogon*-like on Douglas-fir. Redcedar was approximately 30% colonized by arbuscular mycorrhizal fungi. No attempt was made to determine fungal genera for arbuscular mycorrhizal samples.

Isotope content of paper birch and Douglas-fir

Donors

Whole seedling excess ^{13}C content was greater for Douglas-fir than paper birch donors pulsed with $^{13}CO_2$ (111.24 mg ± 36.95 versus 25.07 mg ± 7.77) ($p=0.010$), but did not vary with phenology for either species ($p=0.176$) (Figure 4.4). Donor shoot ^{13}C (39.86 mg ± 19.58) also was similar between species ($p=0.185$) and among seasons ($p=0.136$). Polynomial contrasts detected a quadratic trend with phenology, however, with ^{13}C content of donor shoots peaking in summer ($p=0.049$). Roots of donor Douglas-fir contained more ^{13}C than roots of paper birch (44.95 mg ± 13.10 versus 11.64 mg ± 5.32) ($p=0.006$). Donor roots were more enriched in fall (56.17 mg ± 22.24) than spring or summer ($p<0.05$).

For donor seedlings pulsed with ^{14}C, whole seedling isotope content was greater in Douglas-fir than paper birch ($p<0.001$), but did not vary with phenology ($p=0.886$) (average over

all phenologies and species 1.79 x 10^{-5} mg ± 0.31) (Figure 4.5). Donor shoots contained similar amounts of ^{14}C throughout the growing season (p=0.900), with Douglas-fir donor shoots more enriched than paper birch (2.45 x 10^{-5} mg ± 0.38 versus 0.66 x 10^{-5} mg ± 0.15) (p<0.001). Shoots contained 86.37% and 87.85% of whole seedling ^{14}C content for paper birch and Douglas-fir, respectively. Donor root enrichment was similar among phenologies (0.24 x 10^{-5} mg ± 0.06) (p=0.059), but differed between donor species (p=0.001). Polynomial contrasts detected a linear trend in donor root ^{14}C enrichment among phenologies (p=0.020), with the least enrichment in spring (0.15 x 10^{-5} mg ± 0.05), increasing in summer then fall.

Receivers

Douglas-fir whole seedlings received similar amounts of ^{13}C as paper birch whole seedlings (14.91 mg ± 3.44) (p=0.254) (Figure 4.4). Seedlings received the least amount of ^{13}C from donors in summer (8.08 mg ± 2.54), followed by spring (10.51 mg ± 5.07) and then fall (28.95 mg ± 8.24) (p=0.008). Receiver seedlings were similarly enriched in spring and summer (p=0.974), but more so in fall than summer (p=0.020) or spring (p=0.012). Paper birch and Douglas-fir received on average 3-5% of the total amount of ^{13}C in donors in each seedling group, regardless of phenological stage.

Douglas-fir and paper birch shoots received similar amounts of ^{13}C (p=0.703), with the greatest enrichment in summer (5.37 mg ± 1.26) compared to spring or fall (p<0.05). Paper birch shoots contained 22.92% of whole seedling ^{13}C content compared to 8.11% for Douglas-fir shoots. Douglas-fir and paper birch roots also received similar amounts of ^{13}C (p=0.058). ^{13}C enrichment of receiver roots was less in summer (2.71 mg ± 71) than fall (p=0.003), but summer and fall were similar to spring (p=0.518).

As with ^{13}C, the amount of ^{14}C received by whole seedlings did not differ between species (average 0.04 x 10^{-5} mg ± 0.03) (p=0.123). There was a tendency for phenology to affect

the total amount of ^{14}C received by paper birch and Douglas-fir (p=0.058), with the greatest amounts received in fall. Over the three seasons, paper birch and Douglas-fir received on average 31% and 24% of the total amount of ^{14}C fixed by a seedling group, respectively.

Douglas-fir and paper birch shoots received similar amounts of ^{14}C (0.04×10^{-5} mg ± 0.03) (p=0.508), regardless of phenology (0.108). However, polynomial contrasts detected a linear trend in receiver shoot ^{14}C content among phenologies (p=0.063), with peak transfer in fall. Paper birch and Douglas-fir receiver shoots contained 83.09% and 93.90% of whole seedling ^{14}C content, respectively. Paper birch receiver roots received similar amounts of ^{14}C as Douglas-fir (average 0.02 x 10-5 mg ± 0.10) (p=0.063), and there were no differences among phenological stages (p=0.231).

Isotope content of control Douglas-fir

Douglas-fir control seedlings used to detect potential aerial isotope contamination received ^{13}C and ^{14}C from donor seedlings. Whole seedling ^{13}C content of Douglas-fir controls in summer was greater than that in spring (p<0.0001), but similar to fall (p=0.883). The amounts were significantly different from zero in summer (p<0.05). The amount of ^{13}C contamination in control Douglas-fir shoot and root tissues was also affected by phenology (p=0.004 for shoots, p<0.0001 for roots). Shoot enrichment in summer was significantly greater than in spring (p=0.003) but similar to fall (p=0.084). Root isotope content in summer was larger than in spring (p=0.013) and smaller than in fall (p=0.011).

For ^{14}C, Douglas-fir controls were contaminated similarly in spring, summer and fall, with whole seedling content of 0.05×10^{-5} μg ± 0.01 (p=0.095), shoot content of 0.05×10^{-5} μg ± 0.01 (p=0.069) and root content of 0.01×10^{-5} μg ± 0.0005 (p=0.155). Enrichment was significantly greater than zero in spring (p<0.001) and fall (p=0.039) but not summer (p=0.103).

Isotope content of non-pulsed receivers

Western redcedar and western larch both received ^{13}C from donor seedlings. Phenology affected whole seedling ^{13}C content in redcedar ($p<0.001$) and larch ($p=0.019$) (Table 4.3). For redcedar, greater whole seedling enrichment occurred in spring than summer ($p=0.006$) or fall ($p<0.001$). For larch, whole seedling ^{13}C content was greater in summer than spring ($p=0.018$) but was similar to fall ($p=0.119$). Whole seedling enrichment in redcedar and larch was significantly different than zero in spring ($p<0.001$ for redcedar, $p=0.045$ for larch), summer ($p<0.001$ for redcedar, $p<0.001$ for larch), and fall ($p=0.041$ for redcedar, $p=0.001$ for larch). Redcedar shoots were more enriched with ^{13}C in spring than summer or fall ($p<0.001$), while roots were more enriched in summer than spring or fall ($p<0.05$). Isotope received by larch shoots, were similar throughout the experimental period ($p=0.287$), but roots were more enriched in summer and fall ($p=0.478$) than spring ($p<0.001$).

Western redcedar and western larch also picked up ^{14}C from donor seedlings (Table 4.3). Western redcedar received more ^{14}C in fall ($p=0.016$) than spring ($p=0.021$) or summer ($p=0.041$), while western larch received the same amount throughout the experiment ($p=0.436$). Redcedar ^{14}C enrichment was significantly different than zero in spring ($p=0.015$) but not in summer ($p=0.054$) or fall ($p=0.055$). Larch whole seedlings enrichment was significantly different from zero throughout the experiment ($p=0.013$ in spring, $p<0.001$ in summer, and $p=0.034$ in fall). Redcedar shoots received similar amounts of ^{14}C through the experiment ($p=0.450$) while roots were more enriched in fall than spring or summer ($p<0.05$). Larch shoots were more enriched in fall than summer or spring ($p<0.05$), whereas transfer to larch roots was not affected by phenology ($p>0.05$).

Phenology effects on bi-directional and net carbon transfer

Carbon moved in two directions in spring, summer and fall, with a net gain by paper birch in spring and fall, and by Douglas-fir in summer (Figure 4.7). Bi-directional carbon transfer between paper birch and Douglas-fir was affected by phenology ($p=0.038$), with greater transfer in fall than spring.. Bi-directional transfer was significantly greater than zero in spring ($p=0.002$), summer ($p=0.001$), and fall ($p<0.001$). Net carbon transfer also tended to vary with phenology ($p=0.062$), and was greatest in summer, followed by fall and then spring. Net transfer between paper birch and Douglas-fir was significantly greater than zero in spring ($p=0.008$), summer ($p=0.047$), and fall ($p=0.018$). Net transfer in spring, summer, and fall was equivalent to 12.50%, 3.80%, and 34.40% of total isotope fixed by the seedling group, respectively.

Discussion

Phenology affected the magnitude and direction of belowground carbon transfer between ectomycorrhizal and arbuscular-mycorrhizal plants in the field. Belowground transfer was bi-directional throughout the growing season, with a net gain by birch in spring and fall, and by fir in summer. Net transfer represented 4-34% of the total isotope fixed by donor seedlings, with more transfer in fall than spring or summer. Paper birch and Douglas-fir received 16% and 7% of total carbon transferred in fall, respectively, 21% and 27% in summer, and 12% and 10% in spring. The results suggest that carbon transfer is a dynamic process that is affected by shifting source-sink carbon gradients among plants throughout the growing season.

The phenology effects detected agrees with Lerat *et al.* (2002), who found that the direction of carbon flow reversed between spring and summer in concert with shifting carbon allocation gradients among plants. The magnitude and direction of net transfer we measured in summer were also similar to that found by Simard *et al.* (1997a), where 3-10% of total isotope fixed was transferred from paper birch to Douglas-fir in late July. The consistency between my

results and Simard *et al.* (1997a) and Lerat *et al.* (2002) suggests that this experiment provided a realistic assessment of plant phenology effects on belowground carbon transfer in the field.

I found that carbon transferred from Douglas-fir to paper birch in spring, when paper birch leaves were beginning to emerge and Douglas-fir had broken winter dormancy. Net transfer occurred in the reverse direction in summer, however, when paper birch leaves were fully expanded and Douglas-fir actively growing. The direction of carbon movement changed again in fall, when carbon moved from Douglas-fir to senescing paper birch.

In addition to the hyphal pathway, phenology also affected the magnitude of carbon moving through the soil pathway, where arbuscular-mycorrhizal western redcedar received more carbon from pulsed neighbours in fall than spring or summer. The amount of transfer through soil pathways was substantial, representing 30-47% of the total carbon transferred within a seedling group. In summer, I found that 30% of the carbon was transferred one-way to arbuscular mycorrhizal redcedar, compared with 23% two-way transfer between paper birch and Douglas-fir, and 3.8% net transfer from paper birch to Douglas-fir. Soil transfer in this study was considerably larger than the 1-18% soil transfer detected by Simard *et al.* (1997a). The amount of carbon received by western redcedar was nearly twice the amount observed in Simard *et al.* (1997a), and could be due to the larger redcedar biomass in our experiment and the method used to express transfer fractions.

Multiple soil transfer pathways appeared functional during all phenological stages in this study, as suggested by the occurrence of transfer between ectomycorrhizal as well as arbuscular mycorrhizal plants. Bi-directional transfer between paper birch and Douglas-fir was greater than transfer to arbuscular western redcedar, suggesting the more transfer occurred through hyphal pathways, but that a substantial amount also transferred through the soil pathway or discontinuous soil-hyphal pathway. Similar to our study, Lerat *et al.* (2002) used tree species of a different class of mycorrhizae to compare carbon transfer through soil pathways with that

through an arbuscular mycorrhizal pathway. While autoradiography did not detect isotope in ectomycorrhizal yellow birch controls in spring or fall, liquid scintillation analysis detected that yellow birch was enriched with ^{14}C transferred from trout lily in spring. Uptake by yellow birch was attributed to potential passive absorption from the soil, and suggests that carbon was moving in soil pathways at the same time as hyphal pathways. The presence of simultaneous carbon transfer pathways throughout the growing season suggests that the mechansisms and factors controlling carbon transfer patterns are complex.

My results show that carbon moved into root and shoot receiver tissues, contrasting with earlier laboratory observations that transferred carbon remained in receiver fungal tissues (Francis & Read, 1984; Waters *et al.*, 1994; Watkins *et al.*,1996; Fitter *et al.*, 1998; Wu *et al.*, 2001; Pfeffer *et al.*, 2004). For example, Wu *et al.* (2001) used autoradiography in an ectomycorrhizal system to follow the path of labeled carbon transferring one-way from donor to receiver pine seedlings. While autoradiography successfully visualized active hyphal linkages, carbon could not be detected in receiver plant tissues. Similarly, Pfeffer *et al.* (2004) used ^{13}C and ^{14}C labelled substrates to investigate carbon movement between arbuscular-mycorrhizal root systems via a common mycorrhizal network. Labeled glucose moved to receiver roots via mycorrhizal connections but remained in fungal structures. By contrast, in the only two field studies published to date, a substantial amount of transferred carbon clearly moved into root and shoot tissues of receiver plants (Simard *et al.*, 1997b; Lerat *et al.*, 2002). It is possible in the earlier lab studies that sink strength of receiver plants was muted compared to the field studies because of excised shoots (Pfeffer *et al.* 2004), covered shoots (Wu *et al.*, 2001), or lower ambient light conditions (Fitter *et al.* 1998). The condition under which carbon remains in fungal structures or moves into plant roots or shoot tissues is unclear (Simard & Durall, 2004).

In this study, patterns of belowground carbon transfer among the three labeling times may be linked to interspecific physiological differences in either plant or fungal characteristics

(Fitter *et al.*, 1999; Simard & Durall, 2004). This may include interspecific differences in carbon allocation patterns, net photosynthetic rate, nutrient status, total biomass, and age (Read *et al.*, 1985; Newman, 1988; Bethlenfalvay *et al.*, 1991; Simard *et al.*, 1997a). Pulse labeling resulted in greater photosynthate flux between shoots and roots of paper birch relative to Douglas-fir, possibly due to is greater foliar photosynthetic rate and root carbon sink strength. Larger root: shoot ratios in paper birch than Douglas-fir, particularly in spring and fall, also suggests paper birch roots have the potential to exploit more soil resources (Waring, 1985), and form more abundant and species-rich associations with ectomycorrhizal fungi (DeLong *et al.*, 2002; Simard & Durall, 2004). For example, Simard *et al.* (1997c) found that 49% of total photosynthate was allocated to paper birch roots compared with only 41% for Douglas-fir roots, corresponding with 45% of transferred carbon moving into paper birch shoots compared with only 13% into Douglas-fir shoots. Similarly in this study, 23% and 8% of transferred ^{13}C was destined for paper birch and Douglas-fir receiver shoots, respectively.

Interspecific differences in leaf photosynthetic rates at the three phenological stages likely influenced interspecific carbon allocation patterns between shoots and roots, thereby affected carbon transfer patterns. In spring, birch seedlings had a lower carbon assimilation rate than Douglas-fir, and therefore birch may be considered a carbon sink and Douglas-fir a source within the plant community network. In that case, net carbon transfer would occur from source to sink plants species through hyphae. This interspecific source-sink relationship reversed in summer, when fully foliated paper birch had a higher net photosynthetic rate than Douglas-fir, corresponding with a reversal in the direction of net transfer. Proportionately greater summer growth of paper birch roots would favour further ectomycorrhizal colonization (Rygiewicz *et al.*, 1997). At the fall labeling, paper birch leaves were sensecing and re-absorbing organo-nutrients, while Douglas fir maintained its net photosynthetic rate, once again shifting the source-sink pattern between species.

The seasonal pattern of belowground carbon transfer, where the magnitude of bi-directional transfer increased from spring to summer to fall, could be related to seasonal changes in the carbon drain strength of the seedling group. In our study, labeled carbon moved to several plants, including the other labeled seedlings and western larch and western redcedar. Additionally, in spring, carbon could have moved to arbuscular-mycorrhizal fireweed and naturally regenerated ectomycorrhizal paper birch and Douglas-fir. Western larch was large and flushing in spring, and potentially a stronger sink than paper birch for carbon transferred from Douglas-fir through a CMN. In subsequent labelings in spring and fall, these extra neighbouring plants were removed from the seedling group, potentially reducing overall sink strength of the plant community as the growing season progressed. These results suggest that the presence of more than one receiver species, or diverse carbon sinks, may serve to divert some of the carbon that could have transferred directly between paper birch and Douglas-fir. Increasing magnitude of bi-directional transfer through the growing season may also have been related to increasing mycorrhizal colonization, as rooting area, number of root tips and root length would increase with shoot and root carbon sequestration as the season progresses (Rygiewicz *et al.* 1997).

Greater magnitude of carbon transfer between ectomycorrhizal paper birch and Douglas-fir than to arbuscular mycorrhizal western redcedar suggests that a common ectomycorrhizal network facilitated carbon transfer between paper birch and Douglas-fir (Newman, 1988; Molina *et al.*, 1992; Newman *et al.*, 1994). The presence of a CMN linking paper birch and Douglas-fir is suggested by my observation that most root tips were associated with shared ectomycorrhizae. It is also supported by previous morphotyping and molecular studies of these two species (Jones *et al.* 1997; Simard *et al.* 1997b; Sakakibara *et al.* 2002). At the same time, western larch shared several morphotypes in common with paper birch and Douglas-fir, potentially linking into the same CMN. However, because I used microscopy and morphotyping rather than molecular analysis or autoradiography for identification of ectomycorrhizae, we were unable to determine

if the seedlings were colonized by genetically identical fungi. Nor could I estimate the degree of hyphal linkage between seedlings. Carbon transfer that occurred to trenched Douglas-fir control seedlings could have resulted from re-connection with labelled paper birch and Douglas-fir during the chase. Reconnection is possible over nine days given documented rates at which hyphae can regenerate (Brandes *et al.*, 1998) and anastomose (Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004).

The role of mycorrhizal fungi and the implications of interplant carbon transfer to plant community dynamics have been discussed in recent reviews (Newman, 1988; Miller & Allen, 1992; Perry *et al.*, 1992; Fitter, 2001; Simard *et al.*, 2002; Bever, 2003; Booth, 2004; Simard & Durall, 2004). Carbon and nutrient transfer via common mycorrhizal networks could, for example, assist in seedling establishment, alter above- and belowground competitive interactions, and improve recovery of plant communities following disturbances. A similar magnitude of net transfer in our study with that observed from *Eichornia crassipes* clonal parents to offspring ramets suggests that transfer may affect Douglas-fir and paper birch perfomance (Alpert *et al.*, 1991). In this study, net carbon transfer from paper birch to Douglas-fir in summer may allow Douglas-fir to grow and compete in the understorey of early successional paper birch. In contrast, paper birch would benefit from net supplemental carbon gains from Douglas-fir for greater shoot biomass production in spring and root biomass production in fall. Paper birch and Douglas-fir naturally co-exist in North American forests (Simard *et al.*, 2004) and the benefits of tree species mixtures to forest productivity, forest health, and biodiversity are well known (Juha *et al.*, 2002; Hauessler & Bergeron, 2004; Simard *et al.*, 2005).

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Table 4.1 Mean seedling biomass (g) and leaf assimilation rate ($\mu mols/m^{-2}s^{-1}$) measured in spring, summer and fall for Douglas-fir), paper birch, western larch, and western redcedar that had grown in seedling groups for two years in the field.

Tree Species	Phenology	Biomass (g)		Assimilation ($\mu molsm^{-2}s^{-1}$)
		Shoot	Root	
Douglas-fir	spring	18.13±2.31*	10.59±1.41	3.94±0.54
	summer	45.27±6.06	12.03±2.18	3.19±0.34
	fall	54.49±8.21	24.41±3.56	3.60±0.36
Paper birch	spring	4.13±0.62	4.75±0.80	1.71±0.26
	summer	7.56±1.06	3.77±0.44	7.95±0.49
	fall	10.93±2.67	11.80±2.44	5.62±0.87
Larch	spring	46.16±5.93	16.89±2.67	4.92±0.60
	summer	129.31±21.36	25.78±4.16	3.70±0.31
	fall	137.97±30.38	33.40±5.23	5.32±0.78
Redcedar	spring	26.64±4.59	10.87±1.26	2.88±0.21
	summer	71.45±9.01	15.11±1.91	2.11±0.20
	fall	65.88±14.39	24.08±4.49	2.99±0.39

* Mean ± 1 standard error

Table 4.2 Ectomycorrhizal morphotypes of non-labeled paper birch and Douglas-fir seedlings grown in seedling groups with western redcedar , and western larch for two years in the field.

Morphotype	Key Characteristics
Non-mycorrhizal	root hairs visible; no visible Hartig net or mantle cell development
Incomplete	immature mantle and Hartig net; few or absent emanating hyphae
Cenococcum-like	root tips black, hairy; stellate mantle pattern; thick mantle layers
E-strain 1	root tips brown/black; oblique mantle cells; verrucose emanating hyphae
E-strain 2	similar to E-strain 1; emanating hyphae absent
Hebeloma 1-like	root tips cream/brown; copious emanating, clamped hyphae; narrow mantle cells
Laccaria-like	root tips white; mantle thick, narrow cells; emanating hyphae infrequent
Lactarius-like	root tips cream to tan; wide lacticifer cells with inclusions
MRA	root tips black, hairy to smooth; angular mantle cells; emanating hyphae thick-walled, black
Rhizopogon-like	root tips brown to tan; emanating hyphae brown/yellow with elbow bends
Tomentella 1-like	root tips black; emanating hyphae black, clamped

Table 4.3 Mean excess ^{13}C and ^{14}C isotope content measured in spring, summer and fall for non-labeled Douglas-fir aerial isotope control seedlings, western larch , and western redcedar grown in seedling groups for two years in the field.

	^{13}C content (µg)			^{14}C content (µg[x10^{-7}])		
Tree Species	Spring	Summer	Fall	Spring	Summer	Fall
Redcedar						
whole plant	937.28±203.15	254.44±61.87	109.29±63.20	2.50±0.50	4.22±1.45	18.80±7.82
shoot	932.93±204.06	208.86±56.62	102.26±64.48	1.50±0.50	3.00±1.41	5.38±3.82
root	4.35±3.42	45.58±14.33	7.03±6.38	1.00±0.00	1.10±0.10	13.38±4.48
Larch						
whole plant	275.57±198.92	628.76±127.37	225.69±77.30	7.80±4.20	1.00±0.00	25.25±4.25
shoot	259.01±200.41	458.05±111.13	114.61±84.33	6.80±5.90	0.34±0.00	16.50±5.96
root	16.56±9.34	170.72±39.35	111.07±24.85	1.00±0.00	0.66±0.03	8.73±0.56
Douglas-fir						
whole plant	8.13±3.21	181.31±37.57	160.29±43.19	2.30±0.21	5.50±2.48	10.62±3.79
shoot	1.30±11.29	122.29±32.14	44.56±27.01	1.10±0.10	4.50±2.48	9.62±3.79
root	6.84±3.25	59.02±11.19	115.73±20.53	1.20±0.13	1 .00±0.00	1.00±0.00

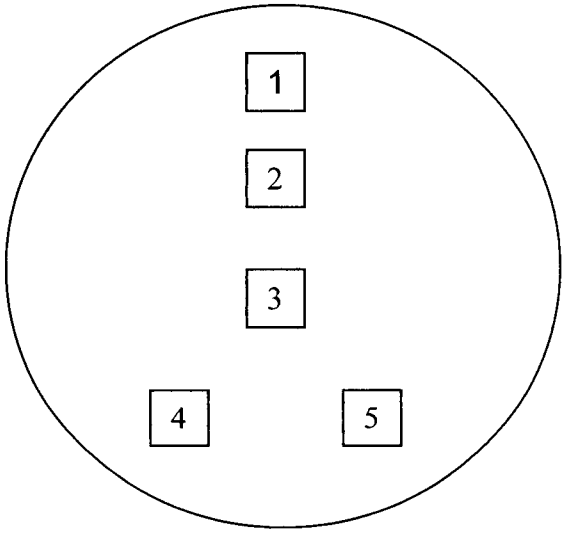


Figure 4.1 Diagram of a seedling group encircled by a 30 cm deep trench. Seedling 1 is a Douglas-fir used for aerial control monitoring, 2 is either a paper birch or a Douglas-fir seedling, 3 is either a paper birch or Douglas-fir seedling, 4 is a western larch seedling and 5 is a western redcedar seedling. Seedling 1 is 15 cm from seedling 2. Seedlings 2-5 are planted equidistantly from each other.

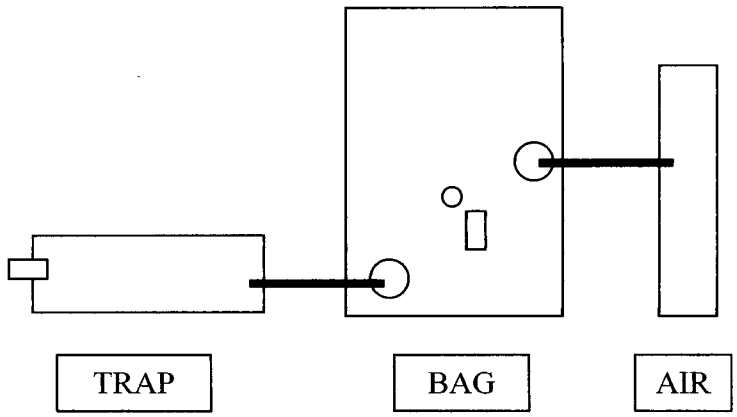


Figure 4.2. Diagram of the gas-sampling bag apparatus, compressed air cylinder, and soda lime trap. Large circles indicate the gas exhaust portholes for bag evacuation. The small circle indicates the injection porthole, which is adjacent to the micro-centrifuge tube taped to the inside of the bag.

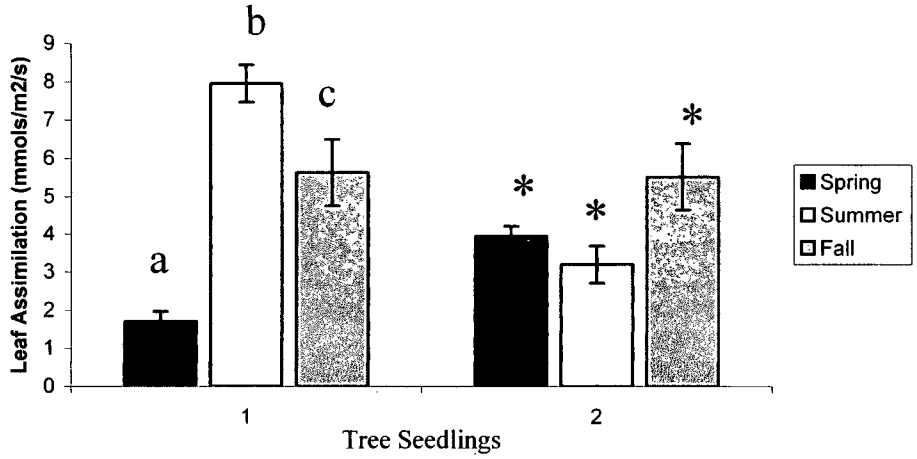


Figure 4.3 Mean leaf assimilation of (1) paper birch and (2) Douglas-fir measured before labeling in spring, summer, and fall in the field.

Means represent standard errors. Means followed by different letters (paper birch) represent significant differences as detected by ANOVA ($p<0.05$). Means followed by the same symbol (Douglas-fir) represent no significant differences as tested by ANOVA ($p>0.05$).

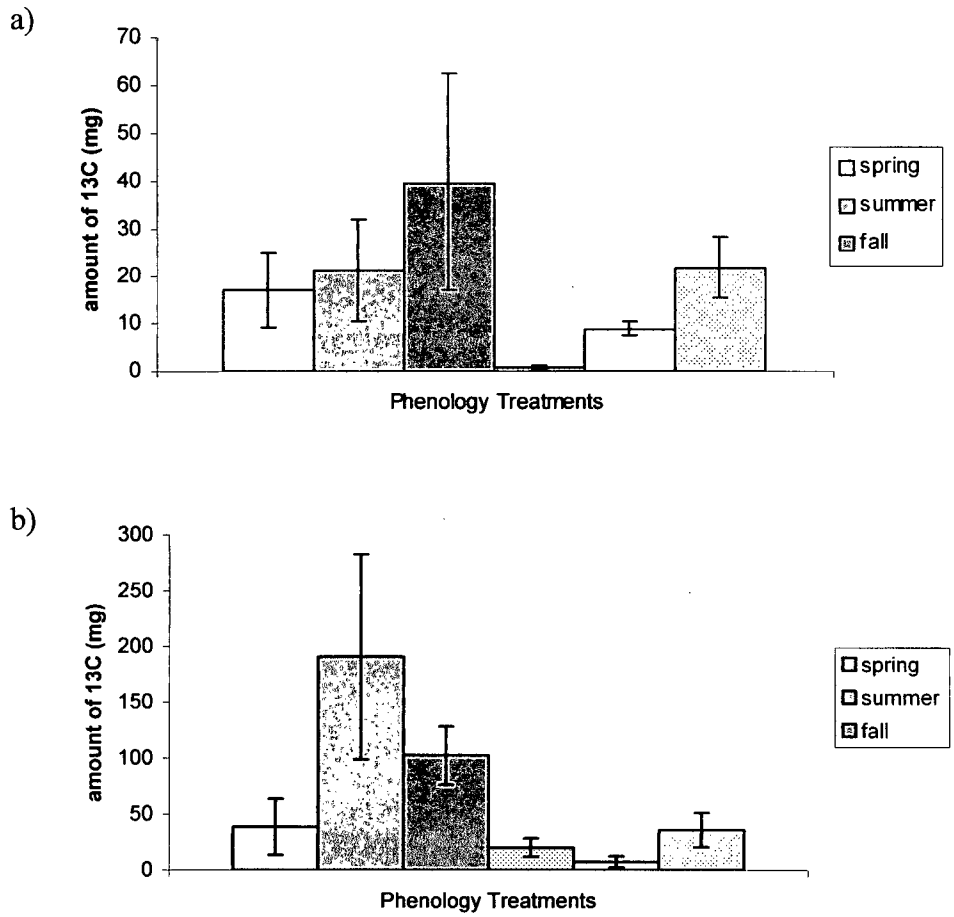
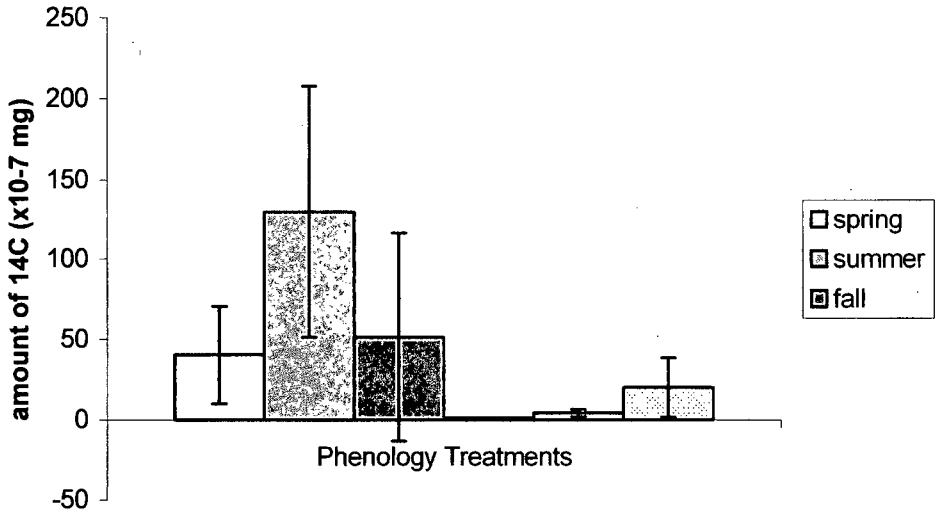


Figure 4.4 Whole seedling ^{13}C content of a) paper birch and b) Douglas-fir ■ donors (solid bars) and ⊡ receivers (stippled) measured in spring, summer, and fall. Error bars are one standard errors. Units abbreviated mg refer to micrograms carbon.

a)



b)

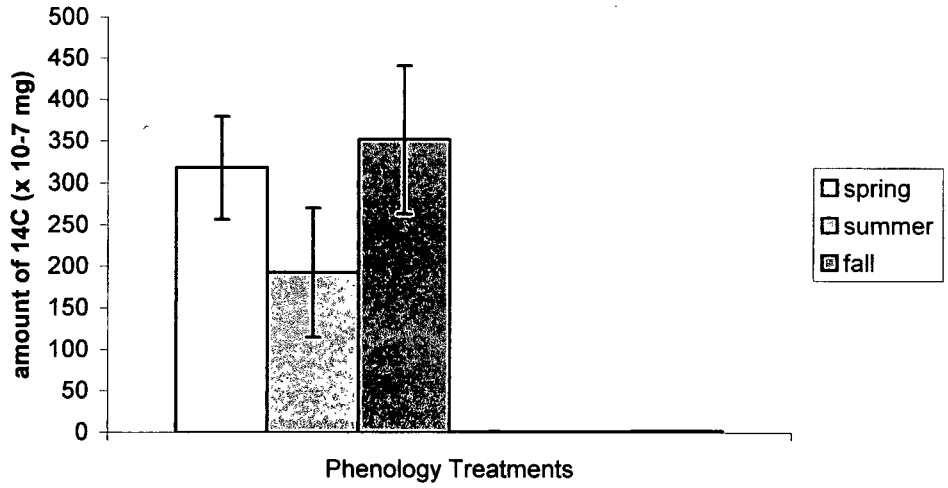


Figure 4.5 Whole seedling ^{14}C content of a) paper birch and b) Douglas-fir ■ donors (solid bars) and ⊡ receivers (stippled) measured in spring, summer, and fall. Error bars are one standard errors. Different letters within a receiver or donor species represent phenology treatment effects according to one-way ANOVA. Units abbreviated mg refer to micrograms C.

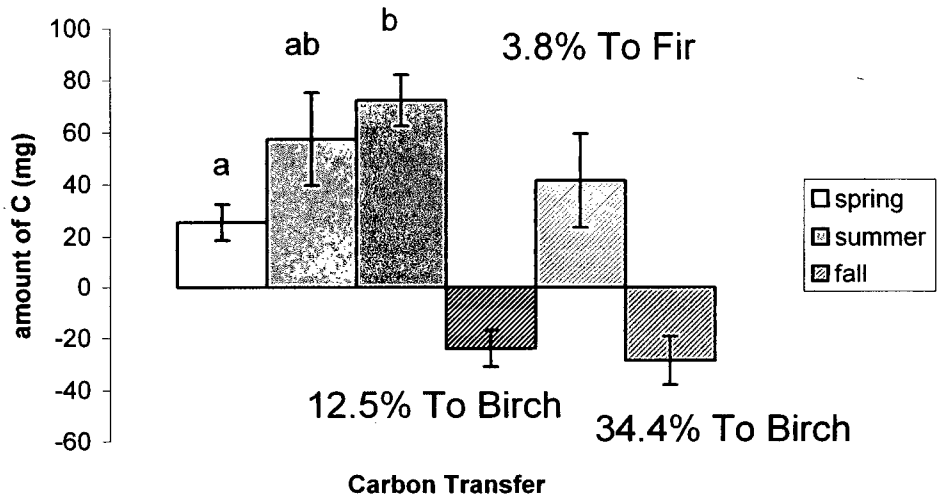


Figure 4.6 Bi-directional■ and net▨ carbon transfer between pulsed paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii)* in spring, summer and fall in the field. Units abbreviated mg refer to micrograms carbon.

*CHAPTER FIVE: Fungal specificity regulates the magnitude of carbon transfer

Introduction

Mycorrhizal fungi are ubiquitous soil microorganisms that form symbiotic associations with plant root tips, acquiring carbon from plant hosts in return for nutrients and water (Allen, 1991; Johnson *et al.*, 1997; Zeide, 1998; Robinson & Fitter, 1999). One group of mycorrhizal fungi, ectomycorrhizae, are considered generalists, associating with root systems of many different plant host species (Molina *et al.*, 1992). Where different plant species or individuals share ectomycorrhizal fungal species in common, their root systems may form hyphal linkages, forming a common mycorrhizal network (CMN). Common mycorrhizal networks (CMN) have been suggested to link some coniferous and deciduous plant species (Jones *et al.*, 1997; Simard *et al.*, 1997b; Horton *et al.*, 1999; Booth, 2004), and facilitate direct interplant transfer of carbon, nutrients, and water (Newman & Ritz, 1986; Newman, 1988). Of the soil organisms that mediate transfer, mycorrhizas are commonly the most important for translocating carbon from plants to soil, or between plants through the mycorrhizal pathway (Newman, 1988; Molina *et al.*, 1992; Onguene & Kuyper, 2002; Bever, 2003; Pfeffer *et al.*, 2004; Simard & Durall, 2004). In addition to the mycorrhizal pathway, however, carbon and nutrients have been shown to transfer through soil and discontinuous soil-hyphal pathways (Simard *et al.*, 1997a; Simard & Durall, 2004). In the rhizosphere soil, carbon can move by mass flow or diffusion, and both processes are influenced by soil structure, porosity, organic matter content and soil types (Rillig *et al.*, 1999; de Kroon & Visser, 2003).

Microscopic hyphal connections have been documented in the laboratory using visual observations (Newman *et al.*, 1994) and autoradiography (Finlay & Read, 1986; Duddridge *et al.*, 1988; Wu *et al.*, 2001), but field identification using these techniques remains difficult. As a

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* A version of this chapter will be submitted for publication.
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result, the presence of hyphal pathways in the field has been indirectly inferred from isotope experiments, where carbon transfer between plants sharing ecto- or arbuscular mycorrhizal fungi is compared with transfer to non-mycorrhizal control plants, or plants with fidelity to a different class of fungi (Simard *et al.* 1997a; Lerat *et al.*, 2002; Chapter Four). Because these field studies provide only indirect evidence for CMN facilitation of carbon transfer, the relative importance of hyphal versus soil pathways remains contentious.

In the discontinuous soil-hyphal pathway, carbon is transferred partially through mycorrhizal fungi and partially through the soil. It occurs where the fine, delicate hyphal network is disrupted by physical processes or by soil organisms, such as arthropods, bacteria, macroinvertrebrates, and protozoans (Duchesne *et al.*, 1987; Fitter & Garbaye, 1994; Nurmiaho-Lassila *et al.*, 1997; Bonkowski *et al.*, 2001; Klironomos & Hart, 2001; Perez-Moreno & Read, 2001; DeLong *et al.*, 2002; Ronn *et al.*, 2002; Tuffen *et al.*, 2002). While the hyphal fragments can re-connect through anasotomosis (Brandes *et al.*, 1998) in arbuscular mycorrhizal networks (Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004), it is unknown how much of this occurs in ectomycorrhizal networks. As a result, the discontinuous hyphal network may be prevalent in ectomycorrhizal systems, where carbon flows from a mycorrhizal root tip into the soil, then moves by mass flow or diffusion to a neighbouring rhizosphere, where it is taken up by either mycorrhizal or uncolonized root tips.

Ectomycorrhizal fungal species in a CMN may vary physiologically, with differential carbon demand from host plants or differential abilities to link plants and transfer carbon. In addition, ectomycorrhizal fungal species can differ in colonization rate (Jones *et al.*, 1997; Simard *et al.*, 1997b; Chapter Two, Three), percent colonization per root system, rate of metabolite transfer to plant hosts (Brandes *et al.*, 1998), substrate utilization (Chalot & Brun, 1998), production of mycelial strands or rhizomorphs, and mycelial growth rate. Roots colonized by fast growing fungi may be able to form hyphal connections to plant hosts at a faster

rate and more extensively compared with slower-growing ectomycorrhizal fungi. The ectomycorrhizal fungal species may also indirectly influence carbon transfer soil pathways by differentially influencing rates of root turnover or root exudation, root death (Rygiewicz *et al.*, 1997) and rhizosphere organism communities (Garbaye & Dupponis, 1992; Garbaye, 1994). For example, *Suillus bovinus* (Fr.) O. Kuntze colonized *Pinus sylvestris* lateral roots to a greater degree and accumulated more ^{14}C-labeled assimilate from adjacent plants than the *Larix eurolepsis* specific fungi, *Suillus grevillei* (Klotzsch) Sing. or *Bolentinus cavipes* (Opat.) Kalchbr. (Finlay, 1989a, b). In contrast, the amount of assimilate accumulated by larch was greater when colonized by *Suillus grevillei* or *Bolentinus cavipes*. In arbuscular mycorrhizal studies, fungal colonization had little effect on the amount or direction of carbon transfer (Watkins *et al.*, 1996; Fitter *et al.*, 1998).

Because ectomycorrhizal fungi differ in their physiologies and plant host specificity, ectomycorrhizal fungal species have the potential to influence the pattern of belowground carbon transfer. While plant-derived characteristics have been shown to influence the direction and magnitude of belowground carbon transfer through common mycorrhizal networks (Read *et al.* 1985; Simard *et al.* 1997a; Lerat *et al.* 2002; Simard & Durall, 2004; Chapter Four), the influence of mycorrhizal fungal species on these factors has not been well studied. Here, I studied the effect of fungal specificity on the magnitude of one-way carbon transfer between paper birch (*Betula papyrifera* Marsh.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings. My specific study objectives were to: 1) determine if fungal host specificity affects the magnitude of one-way carbon transfer, and 2) compare the relative importance of carbon transfer through hyphal pathways compared to alternate soil pathways.

Materials and Methods

Experimental design

The effect of fungal specificity on one-way carbon transfer was studied in a laboratory experiment where paper birch (*Betula papyrifera* Marsh) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings were grown together in root-restrictive fabric pouches. Host-specific fungal inoculations were used to develop fungal specificity treatments for comparing the continuous hyphal, discontinuous soil-hyphal, and soil transfer pathways (Table 5.1). The effects of fungal specificity and donor tree species on one-way transfer were tested in a 2x5 factorial structure of treatments that was replicated three times in a completely randomized design. The five fungal specificity treatments included paper birch and Douglas-fir each inoculated with: 1) non-mycorrhizal sterile agar plugs (soil pathway), 2) the paper birch-specific fungus, *Leccinum scabrum* (discontinuous soil-hyphal pathway), 3) the Douglas-fir-specific fungus, *Rhizopogon vinicolor* (discontinuous soil-hyphal pathway), 4) the paper birch-specific fungus, *Leccinum scabrum* plus the Douglas-fir-specific fungus, *Rhizopogon vinicolor* (discontinuous soil-hyphal pathway), and 5) the generalist fungus, *Cenococcum sp.* (continuous hyphal pathway). The two donor species were: 1) paper birch, with unlabeled Douglas-fir serving as a receiver (abbreviated 13PB-DF) and, 2) Douglas-fir, with unlabeled paper birch serving as a receiver (13DF-PB). Donor seedlings were pulsed over two hours with 30 ml 99% $^{13}CO_{2\ (gas)}$.

Preparation of the plant and fungal material

Paper birch seeds were surface-sterilized in 10% H_2O_2 and rinsed in sterile distilled water, while Douglas-fir seeds were scarified by shaking in 35% H_2O_2 for 15 minutes, followed by surface sterilization in 3% H_2O_2 for approximately five hours (Jones *et al.*, 1997). Following this treatment, two seeds per species were sown on the surface of autoclaved soil filling bleached

Leach Tubes® (Ray Leach 'Conetainer' Single Cell System, Stuewe & Sons, Corvallis, USA). The soil in Leach Tubes was comprised of ProMix® potting soil mix mixed with Perlite® in a ratio of three parts potting soil to one part Perlite® (by volume). Seeds were also sown on Leach Tubes® for use in: (1) detecting possible growth chamber fungal contaminants (such as the common ectomycorrhizal fungus *Thelephora* spp. (Jones *et al.*, 1997; Simard *et al.*, 1997b) and dark septate fungal endophytes such as *Phialocephala fortinii* (Jumpponen *et al.*, 1998; Jumpponen, 2001)), (2) detecting potential aerial isotope transfer during pulse labeling in the growth chamber, and (3) determining natural (unlabeled/control) isotope abundance levels. Seeds were germinated and allowed to grow in the absence of ectomycorrhizal fungi for three to four weeks in a controlled growth chamber with mean temperature of 24 °C, photoperiod extension to 16 hours by high-pressure sodium lights, light intensity of 240-275 μmols $m^{-2}s^{-1}$, daily misting, and monthly applications of modified Ingestad's Nutrient Solution (Mason, 1980). Extra seedlings were removed from Leach Tubes® and roots examined under dissecting and compound microscopes to ensure contaminants were absent prior to inoculation. Seedlings were also transplanted from the Leach Tubes® into the root chambers.

Fungal isolates and inoculation of plant material

The ectomycorrhizal isolates (Table 5.1) were maintained on modified Melin-Norkranns (MMN) agar media (Marx, 1969) in the dark at room temperature (21°C) at the University of British Columbia. Fungi were maintained on MMN agar plates in order to standardize colony size between isolates. After four months, colonies were sub-cultured aseptically in the laboratory. Fungal specificity treatments were established using a modified paper-sandwich technique for rapid synthesis of ectomycorrhizas (Chilvers *et al.*, 1986). Using this technique, three 1 cm plugs of the standardized colonies were aseptically removed from the MMN agar plates, and

used to inoculate each of three sterile absorbent paper discs (1.5 cm in diameter). These were placed 2 cm apart on the surface of sterile MMN agar plates. The paper discs were monitored and, when required, moistened using sterile, dilute MMN liquid media. After approximately four weeks, fungal mycelia covered 25-50% of each paper disc. At this time, inoculated paper discs were aseptically transferred to uncolonized paper birch and Douglas-fir roots removed from Leach Tubes® under sterile conditions. Seedling roots were aseptically placed in root pouches, such that roots were between the inoculated paper discs and 1-2 cm layer of sterile soil. Additionally, three to five 1 cm plugs from standardized colonies were placed adjacent to roots and paper discs. Sterile soil was placed around the inoculated roots, and the seedlings were then placed in soil pouches inside the root chambers (see below). A thin layer of sterilized sand was applied to the inside surface of root pouches to reduce moisture loss, seed contamination by fungal contaminants, as well as algal and moss growth. Inoculated root chambers were returned to the growth chamber for the duration of the experiment.

Preparation of root chambers

For growing seedlings in the root chambers, autoclaved ProMix® potting soil mix was mixed with Perlite® in a ratio of three parts potting soil to one part Perlite® (by volume) and then used to fill 18 cm x 20 cm root-restrictive fabric pouches. One soil pouch was placed between two 20 cm x 20 cm plastic plates, which were then secured with foam spacers and metal clips. The root chambers were covered in aluminum foil to minimize evaporation and reduce algal and moss growth during the experiment. Assembled root chambers were randomly placed in one growth chamber at the University of British Columbia, Vancouver, British Columbia, Canada. The seedlings were grown for nine months, during which time they received daily misting with sterile distilled water, and monthly applications of sterile Ingestad's Nutrient Solution (Mason, 1980).

Pulse-chase regimes

Pulse labeling was applied using modifications of the procedure developed by Simard *et al.* (1997a, c, d) and was similar to the techniques used in Chapter Two and Three. Immediately prior to labeling, donor shoots were sealed inside air-tight gas sampling bags (10 L volume) fitted with a septum for injections with a hypodermic needle, and roots were sealed from shoots using medium weight polyethylene-plastic. Control seedlings for detecting aerial isotope contamination were placed adjacent to the root chambers in the growth chamber.

Seedlings were pulsed for two hours by injecting 30 ml of $^{13}CO_2$ gas into the gas-sampling bag. After two hours, the gas-sampling bags were removed to release residual $^{13}CO_2$ gases outside of the growth chamber. Seedlings were left in the chamber for a five-day chase period under a 16-hour photoperiod. Seedlings were watered two times during the chase period. No attempt was made to monitor the total $^{12+13}CO_2$ concentration inside the labeling bags or assess leaf photosynthetic rates.

Tissue sampling

After the chase period, seedlings in root chambers and Leach Tubes® were harvested, and shoot and root tissues separated. All tissues were oven-dried for 48 hours at 55-60 °C and ground using a Wiley® Mill with 20 and 40 μm metal mesh screens. One 2 mg sub-sample of each tissue type (shoots, roots) was combusted to determine %C and analyzed for excess ^{13}C content at the Stable Isotope Facility, Department of Agronomy, University of California-Davis. Instrumentation was either a ThermoFinnigan Delta+ with a Carlo Erba NC2500 elemental analyzer or a Delta+XL with a Costech ESC 4010 elemental analyzer. Stable isotope ratios were reported in δ notation as parts per thousand (‰) deviations from the international standard PDB (carbon). Instrument precision was <0.2‰. These values were used to estimate excess mg ^{13}C in plant tissues and whole seedlings (Appendix I).

Mycorrhizae

Before oven-drying, root systems of labeled plants were examined for percent colonization of ectomycorrhizal isolates and potential fungal contaminants. Roots were removed from root pouches, separated from shoots, washed, and cut into 3 cm segments. Percent colonization per seedling was estimated by examining randomly-selected segments under dissecting and compound microscopes, and counting the number of ectomycorrhizae in the first one hundred root-tips. Fungal morphological characteristics (root-tip branching, colour, texture and hyphal patterns) were described and compared with previously published ectomycorrhizal descriptions (Ingleby *et al.*, 1990; Goodman *et al.*, 1996; Jones *et al.*, 1997; Simard *et al.*, 1997b).

Statistical analyses

The carbon isotope content in each tissue type was calculated from excess $\delta^{13}C$ (‰) and total carbon values (mg) of the treated sample (Appendix I). These values were used to calculate excess carbon isotope content of donor and receiver tissues as well as whole seedlings (Simard *et al.*, 1997a, d). Seedling biomass (g), and donor and receiver tissue isotope content (mg) were compared between tree species and fungal specificity treatments using two-way ANOVA (n=3) for a 2x5 factorial design (SPSS software version 10.0). Whole seedling isotope content was used to compare one-way carbon transfer between tree species and fungal specificity treatments using two-factor ANOVA (n=3). Where significant differences occurred, Tukey's tests were applied to separate means among fungal inoculation treatments. For each treatment, one-way transfer was compared with zero using t-tests. One-way transfer was expressed as a proportion of total isotope fixed by donor species (%). Mean values for seedling characteristics and excess isotope contents were reported with corresponding standard error (SE) values.

Results

Seedling characteristics

Paper birch donors and receivers were similar in size (1.21 g ± 0.10, p=0.113) and averaged four times larger than Douglas-fir (0.33 g ± 0.03) (p<0.001). Douglas-fir donors (0.37 g ± 0.04) were slightly larger than Douglas-fir receivers (0.29 g ± 0.03) (p=0.040). Paper birch biomass tended to vary among fungal specificity treatments (p=0.063). Planned linear contrasts showed paper birch was largest when inoculated by the generalist fungus, *Cennococcum* (1.54 g ± 0.18) and smallest when non-mycorrhizal (0.87 g ± 0.11) (p=0.025). Douglas-fir biomass was affected by fungal specificity treatment (p=0.001), and was greater when both Douglas-fir and paper birch were inoculated with the Douglas-fir-specific fungus, *Rhizopogon vinicolor* (0.50 g ± 0.04) compared to the remaining treatments (0.33 g ± 0.03) (p<0.001).

Paper birch shoot biomass did not differ between donors and receivers (0.82 g ± 0.07) (p=0.422), but donor roots were larger than receiver roots (0.48 g versus 0.31 g) (p=0.0042). By contrast, neither shoots (0.16 g ± 0.02) nor roots (0.16 g ± 0.01) of Douglas-fir differed between donors and receivers (p=0.178 for shoots, p=0.215 for roots). Paper birch shoot biomass did not vary with fungal specificity treatment (p=0.201), but root biomass was lower in the non-mycorrhizal treatment (0.26 g ± 0.05) than the others (0.39 g ± 0.19) (p=0.029). Douglas-fir shoots (0.09 g ± 0.01) and roots (0.11 g ± 0.02) were smaller when inoculated with *Cenococcum sp.* than in the other treatments (0.17 g ± 0.44 for shoots) (p=0.002 for shoots, p=0.042 for roots).

For both paper birch and Douglas-fir, root:shoot ratios were similar between donors and receivers (p=0.461 for paper birch, p=0.857 for Douglas-fir) and among fungal specificity treatments (p=0.329 for paper birch, p=0.602 for Douglas-fir). Root:shoot ratio averaged 0.53 ± 0.06 for paper birch and 1.31 ± 0.17 for Douglas-fir.

Mycorrhizal status

Percent colonization did not differ between tree species or among fungal specificity treatments, averaging 8.17% ± 2.31 ($p>0.05$). Douglas-fir was more colonized than paper birch in treatment 2, where both seedlings were colonized by the Douglas-fir-specific fungus, *Rhizopogon vinicolor* (13% versus 5%) ($p=0.05$), and in treatment 4, where the Douglas-fir-specific fungus, *Rhizopogon vinicolor,* colonized Douglas-fir and the paper birch-specific fungus, *Leccinum scabrum*, colonized paper birch (18% versus 3%) ($p=0.05$).

Isotope content of paper birch and Douglas-fir seedlings

Natural abundance levels ($\delta^{13}C$ (‰)) for unlabeled paper birch seedlings were -31.50‰ for shoots and -30.19‰ for roots), while those for Douglas-fir were -30.15‰ for shoots and -29.56‰ for roots. Whole seedling enrichment was greater in donors than receivers for paper birch (2.73 mg ± 0.70 for donors versus 0.86 mg ± 0.41 for receivers) ($p=0.008$) and Douglas-fir (0.39 mg ± 0.12 for donors versus 0.03 mg ± 0.02 for receivers) ($p<0.001$). Paper birch donors and receivers were more enriched than Douglas-fir (mean of donors and receivers was 1.80 mg ± 0.41 for paper birch versus 0.21 mg ± 0.07 for Douglas-fir) ($p>0.05$). Analysis of variance showed that fungal specificity had no effect on whole seedling enrichment for either donors or receivers of paper birch or Douglas-fir ($p>0.05$), but linear contrasts showed a tendency for greatest enrichment of paper birch and Douglas-fir donors in treatment 4, where each host species was inoculated with their respective host-specific fungus ($p=0.09$ for paper birch; $p=0.10$ for Douglas-fir).

Shoots of donors were more enriched in ^{13}C than shoots of receivers for both paper birch (2.03 mg ± 0.59 for donors, 0.53 mg ± 0.25 for receivers) and Douglas-fir (0.32 mg ± 0.11 for donors, 0.02 mg ± 0.01 for receivers) ($p=0.039$ for paper birch, $p=0.008$ for Douglas-fir). Linear contrasts showed a there was a tendency for fungal specificity treatment to affect shoot isotope

content in paper birch (1.79 mg ± 0.81; p=0.18) and Douglas-fir (0.35 mg ±0.20, p=0.05) donors and receivers, with the greatest enrichment where paper birch was inoculated with its species-specific fungus and Douglas-fir was inoculated with its species-specific fungus (treatment 4). Root isotope enrichment did not differ between donors and receivers for either paper birch or Douglas-fir (p=0.129 for paper birch, p=0.386 for Douglas-fir). Root isotope content of Douglas-fir donors and receivers tended to be affected by fungal specificity treatment (p=0.07) with the highest root enrichment in treatment 2, where Douglas-fir and paper birch was inoculated with Douglas-fir species-specific fungus (0.19 mg ±0.10). Isotope content of paper birch roots was also affected by specificity treatment (p=0.03), and were most enriched when inoculated with *Leccinum scabrum* (1.31 mg ± 0.56) and least when inoculated with *Cenococcum sp.* (0.57 mg ± 0.17).

One-way carbon transfer

Carbon was transferred between paper birch and Douglas-fir in all fungal treatments. Averaged over all fungal specificity treatments, paper birch received 0.86 mg ± 0.32 from Douglas-fir, and conversely Douglas-fir received 0.03 ± 0.10 from paper birch (Figure 5.1). The greatest one-way transfer occurred when paper birch was inoculated with its species-specific fungus and Douglas-fir was inoculated with its species-specific fungus (treatment 4) (3.10 mg ± 1.16 received by paper birch, 0.35 mg ±0.20 received by Douglas-fir). The second rank-order treatment for amount of isotope received by paper birch (2.14 mg ± 1.21) was treatment 5, where seedlings were inoculated with the generalist fungus, *Cenococcum*. Likewise, the second rank-order treatment for Douglas-fir was treatment 2, where both Douglas-fir and paper birch were inoculated with *Rhizopogon vinicolor*. The amount of carbon transferred did not differ from zero in this treatment (p>0.05).

Discussion

The effect of fungal host specificity on the magnitude of belowground carbon transfer in common mycorrhizal networks remains unclear following this experiment. I found that carbon transfer occurred through three different pathways: the discontinuous soil-hyphal pathway, the soil pathway, and the hyphal pathway. Carbon transfer between paper birch and Douglas-fir tended to be greatest where paper birch was associated with its host-specific fungus, *Leccinum scabrum scabrum*, and where Douglas-fir was colonized with its host-specific fungus, *Rhizopogon vinicolor*, suggesting most carbon transferred through the discontinuous soil-hyphal pathway. The second largest amount of carbon transferred to paper birch occurred where both paper birch and Douglas-fir were colonized by the same generalist fungus, *Cenococcum* sp., suggesting the direct hyphal pathway was also operative. That some carbon was received by both Douglas-fir and paper birch where they were non-mycorrhizal also suggests that some transfer occurred through the soil pathway by mass flow or diffusion.

The low ectomycorrhizal colonization of my seedlings in treatments 1-4 may explain the overall low levels of interplant carbon transfer. I confirmed that only 8% of the root tips of paper birch and Douglas-fir were infected with the inoculated fungi. The resulting low level of transfer agrees with earlier suggestions that transfer is related to the degree of mycorrhizal colonization, mycorrhizal species, and the proportion of active to inactive hyphal connections (Finlay, 1989a, b; Simard & Durall, 2004; Chapter Two, Three). Nevertheless, this study improves upon previous ectomycorrhizal studies, which either did not assess the relative abundance of ectomycorrhizal colonization per seedling (Wu *et al.*, 2002) or used indirect methods to determine the presence of hyphal linkages (Simard *et al.*, 1997a, c, d; Lerat et al. 2002). However, because I used microscopy and morphotyping rather than molecular analysis for identification of ectomycorrhizae, I was unable to unequivocally determine whether the seedlings were colonized by the original fungi.

The largest amount of carbon transferred between paper birch and Douglas-fir through the discontinuous soil-hyphal pathway, suggesting that labeled exudates moved between root systems in the soil solution or through anapleurotic uptake of labeled CO_2 diffusing from rhizosphere to the next plant. Earlier preliminary tests (data not shown) determined, however, that anapleurotic fixation by roots and hyphae did not occur; therefore, I expect that most labeled carbon moved through the soil-hyphal pathway in root and hyphal exudates. The greater amount of carbon transferred through the discontinuous soil-hyphal than the other two pathways suggests that mycorrhizal inoculation alone was the most important factor influencing transfer. Myocrrhizal inoculation resulted in larger seedlings of both paper birch and Douglas-fir, which should exude more carbon into the rhizosphere, and therefore have greater potential for transferring carbon in exudates to neighbouring seedlings.

There was greater transfer where seedlings were colonized by a generalist fungus, *Cenococcum* sp. compared to non-myocrrhizal seedlings. *Cenococcum geophilum* Fr. is a frequent ectomycorrhizal fungus of low specificity that colonizes most temperate coniferous and deciduous tree species, suggesting a high likelihood that it forms hyphal connections between tree species.

These results agree with previous laboratory studies showing that transfer can occur through multiple pathways in ectomycorrhizal (Wu *et al.*, 2001, Simard *et al.*, 1997d) and vesicular-arbuscular mycorrhizal systems (Fitter *et al.*, 1998, Watkins *et al.*, 1996; Pfeffer *et al.*, 2004). Wu *et al.* (2001) used autoradiography to follow the path of labeled carbon transferring one-way from donor to receiver pine (*Pinus densiflora* Sieb. Et Zucc.) seedlings inoculated with an unidentified basidomycete fungal species (T01) isolated from *Pinus densiflora* growing in a nursery. Wu *et al.* (2001) cultured T01 axenically in flasks filled with an autoclaved mixture of peat and vermiculite, transferred sub-samples to inoculation plates containing one-month-old pine seedlings, and after 8 months transferred inoculated seedlings to cloth sheets in labeling

chambers for four to five weeks prior to autoradiography. Given the rapid rates at which mycorrhizal hyphae can regenerate (Brandes *et al.*, 1998; Rygiewicz *et al.*, 1994) and anastomose (Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004), it is possible that hyphal connections then formed between donor and receiver seedlings. However, hyphal links may not have formed between all root tips during the four to five week establishment period, resulting in multiple pathways not easily distinguished using autoradiography alone. Despite this, Wu *et al.* (2001) found that autoradiography successfully visualized active hyphal linkages, but carbon transfer into receiver plant tissues could not be detected. In my study, labeling with ^{14}C rather than ^{13}C, followed by autoradiography and analysis of hyphal and plant tissue by liquid scintillation counting, may have increased our ability to identify functional linkages and detect transfer through hyphal and soil-hyphal pathways.

Only one ectomycorrhizal fungus was used by Wu *et al.* (2001), but a different or greater number of inoculated fungal species may have resulted in greater transfer given the physiologically and functional diversity of mycorrhizal fungi. The study by Wu *et al.* (2001) contrasts with that of Simard *et al.* (1997d), who used field soil from plantations of paper birch and Douglas-fir as an ectomycorrhizal inoculum source for their laboratory root-box study. While ectomycorrhizal status in root-boxes was not assessed, a bioassay showed high species richness and high colonization by common ectomycorrhizal morphotypes between paper birch and Douglas-fir, suggesting a high likelihood that hyphal connections would form between root systems (Jones *et al.*, 1997; Simard *et al.*, 1997b). Simard *et al.* (2002) and Fitter *et al.* (1999) have previously suggested that a diversity of mycorrhizal fungi result in a complex pattern of carbon source and sink strengths, with a higher likelihood for greater or more variable carbon transfer.

Trends observed in this laboratory study strengthen the argument for using new inoculation and pulse labeling methodologies to compare carbon transfer between continuous

hyphal, discontinuous hyphal-soil, and soil pathways.

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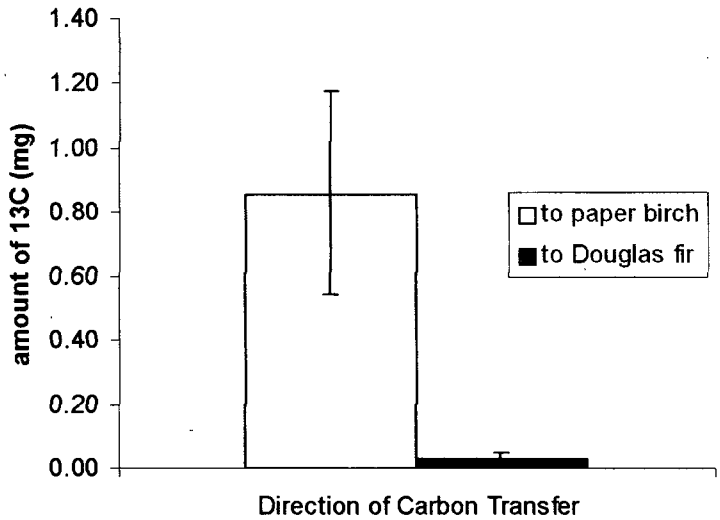
Table 5.1 Ectomycorrhizal isolates used to inoculate paper birch and Douglas-fir for the fungal specificity treatments. Following inoculuation, seedlings were grown in root chambers for nine months.

Treatment	Fungus	Host	Active carbon pathway	Culture Origin
1	Non-mycorrhizal	N/A	soil	UBC
2	*Leccinum scabrum*	Paper birch	discontinuous soil-hyphal	OSU
3	*Rhizopogon vinicolor*	Douglas-fir	discontinuous soil-hyphal	UAFC
4	*Leccinum scabrum* and	Paper birch	discontinuous soil-hyphal	OSU
	Rhizopogon vinicolor	Douglas-fir		UAFC
5	*Cenococcum sp.*	Paper birch	continuous hyphal	OUC
		Douglas-fir		

Cultures were obtained from: University of British Columbia-Okanagan (formerly Okanagan University College), Kelowna, British Columbia (OUC); Oregon State University, Corvallis, Oregon (OSU); University of Alberta Fungal Collection (UAFC); University of British Columbia (UBC), Vancouver, British Columbia.

Figure 5.1 Amount of excess carbon isotope received by whole seedling paper birch (*Betula papyrifera*) and Douglas-fir (*Pseudotsuga menziesii*) grown in root chambers for nine months



CHAPTER SIX: Conclusions and Future Directions

Conclusions

The role of mycorrhizal fungi and common mycorrhizal networks (CMNs) in belowground transfer has been discussed extensively in the literature (Wilkinson, 1998; Perry, 1999; Simard *et al.*, 2002; Bever, 2003; Simard & Durall, 2004). Recent laboratory and field studies in belowground carbon transfer have provided evidence for the widespread incidence of mycorrhizal networks in natural systems (Simard *et al.*, 1997a; McKendrick *et al.*, 2000; Klironomos & Hart, 2001; Perez-Moreno *et al.*, 2001; Zhou *et al.*, 2001; Booth, 2004), variable transfer activity (Watkins *et al.*, 1996; Fitter *et al.*, 1998; Simard *et al.*, 1997a, d; Wu *et al.*, 2001; Pfeffer *et al.*, 2004), plant-driven transfer regulation (Simard *et al.*, 1997a, d; Lerat *et al.*, 2002), and fungal derived processes within CMNs (Giovannetti *et al.*, 2004). Despite recent advances, the biological importance of hyphal compared to soil transfer remains uncertain (Newman, 1988). Additionally, evidence for one-way, bi-directional, or net carbon transfer is still debated along with whether transferred carbon is stored simply in fungal tissues or also in plant tissues, or whether it of a sufficient magnitude to influence plant community dynamics (Simard &Durall, 2004).

Building on the work of Simard *et al.* (1997a, b, c, d) and other recent studies, I demonstrated that carbon moves belowground through both hyphal and soil pathways using stable and radioactive carbon isotopes in the laboratory and the field. Transfer through both pathways was detectable using ^{13}C, ^{14}C, or $^{13}C/^{14}C$ reciprocal labeling techniques. Using low levels of carbon isotopes as well as hyphal manipulation, significantly greater net carbon transfer through the hyphal pathway compared to soil pathways was demonstrated for the first time in the laboratory. In the field, I found that net carbon transfer was affected by source-sink patterns

corresponding to different host plant phenologies, corroborating results from previous studies where interplant photosynthate sinks were experimentally manipulated in the field (Simard *et al.*, 1997a; Lerat *et al.*, 2002). Changes in the magnitude and direction of net transfer were synchronized with important periods of plant development, suggesting common mycorrhizal networks play an important ecological role in plant communities. For example, belowground carbon transfer to paper birch in spring (during early leaf development) and fall (leaf senescence) corresponds to high carbon demands during growth. While examples of plant-driven hyphal and soil transfer seem certain from these studies, support for fungal-driven transfer through hyphal and soil transfer pathways remains uncertain with tendencies for fungal specificity (*Rhizopogon* spp. for Douglas-fir) to increase the magnitude of carbon transfer.

Future directions

I examined belowground carbon transfer using pulse-labeling with stable and radioactive isotopes. One drawback associated with this technique is that carbon transferred to fungal tissues or receiver tissues was not measured directly; by contrast, autoradiography allows visualization of carbon flow from the donor plant to the recipient (Francis & Read, 1984; Finlay & Read, 1986a, b; Duddridge *et al.*, 1988; Wu *et al.*, 2001). Autoradiography techniques themselves are technologically limited, however, by specific radioisotope detection levels and application to field situations (Wu *et al.*, 2001). Additionally, neither, autoradiography or visual observations (Newman *et al.* 1994) have been used to estimate the number or functional activity of hyphal connections between mycorrhizal plants in the field. Future studies should include autoradiography by first addressing the technique's minimum detection levels required for observing carbon transfer in arbuscular and ectomycorrhizal systems. Secondly, new studies should include combinations of pulse labeling, autoradiography and liquid scintillation

techniques in order to distinguish between labeled carbon in fungal structures versus plant tissues. For example, Wu *et al.* (2001) could not detect labeled carbon in receiver plant tissues with autoradiography alone, suggesting the minumum detection level was lower than anticipated in their pine system. Liquid scintillation could be used in combination with autoradiography to improve detection of low levels of labeled carbon. Thirdly, autoradiography should be extended from to include microscopic autoradiographs of plant-fungal interfaces in the laboratory and to larger interplant interactions in the field. These applications may assist in pinpointing the location of labeled carbon in common mycorrhizal networks and determining the extent, regulation and mechanisms of fungal carbon storage. This technique may also help address whether transferred carbon stored in fungal structures is important to plant performance (Simard *et al.* 2002). Factors influencing carbon storage or, mobilization from resistant fungal structures, such as arbuscular mycorrhizal vesicles or ectomycorrhizal sclerotia or rhizomorphs, at present is unknown.

Pulse-labeling with carbon isotopes has raised concerns regarding the total carbon concentration pulsed seedlings are exposed to in laboratory and field experiments (Fitter *et al.* 1998). My laboratory and field experiments, used reciprocal labeling with ^{13}C /^{14}C and additional injections of $^{12+13}CO_2$ in an attempt to equalize total carbon concentrations. Future studies should consider adopting a continuous labeling approach, closely monitoring total carbon concentrations over longer, more sustained pulse periods. The labeling apparatus used in past studies and those used in my experiments, could be modified for continuous labeling and also improved by designing labeling bags that include more permanent bag-supportive materials (plastic tubing instead of sytrofoam spacers), allow for larger gas volumes, incorporate solar-powered fans (circulation of labeled air at regular intervals), and provide protective sleeves for plant stems (important for air-tight seals and to reduce damage to seedlings during bag removal).

The influence of soil microbes on belowground carbon transfer has been studied little, but the studies that do exist have varied in whether microbes enhanced or inhibited transfer through common mycorrhizal networks (Bonkowski *et al.*, 2001; Klironomos & Hart, 2001; Perez-Moreno *et al.*, 2001; Tuffen *et al.*, 2002). None of these studies identified the mechanism by which microbes influenced transfer. New advances in fluorescent dyes and laser scanning confocal microscopy could be used to study the role and mechanisms by which mycorrhizal associated bacteria affect interplant carbon transfer. Autoradiography and labeled carbon substrates could also be used to follow the movement of microbes through hyphal pathways.

Interactions between common mycorrhizal networks, carbon transfer, and other organisms could also be applied to new field studies to determine the role of common mycorrhizal networks in seedling establishment under varied environmental conditions. Implications of the effects of common mycorrhizal networks on seedling establishment have been suggested previously (Eissenstat *et al.*, 1990; Horton *et al.*, 1999; Dickie *et al.*, 2002; Onguene *et al.*, 2002; Simard &Durall, 2004), but longer term studies under more realistic field conditions are still needed to better understand their importance at different temporal and spatial scales. Information is also needed on the role of common mycorrhizal networks on seedling mortality under climatically (e.g., drought) or biologically (e.g., diseased) stressed conditions. These studies could build on my observation that net transfer coincides with biologically significant periods, such as seedling establishment in spring, competition for resources in summer, and belowground carbon allocation in fall.

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APPENDIX 1: Example Stable Isotope Calculations

Stable Carbon Isotope:

Stable carbon isotope calculations were completed using values from treated or pulsed seedlings and untreated, control seedlings at natural abundance.

(1) $\delta^{13}C$ (‰ per mil) values of treated samples were converted to $R_{t\ sample}$, the absolute isotope ratio

$R_{t\ sample} = {}^{13}C/{}^{12}C = [(\delta^{13}C/1000) + 1)] \times R_{standard}$, where $R_{standard} = 0.0112372$ (PDB Standard)

$\delta^{13}C$ (‰ per mil) values of control samples were converted to $R_{c\ sample}$, the absolute isotope ratio

$R_{c\ sample} = {}^{13}C/{}^{12}C = [(\delta^{13}C/1000) + 1)] \times R_{standard}$, where $R_{standard} = 0.0112372$ (PDB Standard)

(2) fractional abundances (A_{ts}) of treated seedlings were related to $R_{t\ sample}$ of the corresponding treated samples

$A_{ts} = {}^{13}C/({}^{13}C + {}^{12}C) = R_{t\ sample}/(R_{t\ sample} + 1)$

fractional abundances (A_{cs}) of control samples were related to $R_{c\ sample}$ of the control samples

$A_{cs} = {}^{13}C/({}^{13}C + {}^{12}C) = R_{c\ sample}/(R_{c\ sample} + 1)$

(3) the quantity of ^{13}C in the treated sample, A_{ts}, was related to the total carbon content (mg) of the treated sample

$A_{ts} \times C_{total\ ts}\ (mg) = mg\,{}^{13}C_{t\ sample}$

the quantity of ^{13}C in the control sample, A_c, was related to the total carbon content (mg) of the control sample

$A_{cs} \times C_{total\ cs}\ (mg) = mg\,{}^{13}C_{c\ sample}$

(4) ^{13}C enrichments, $mg^{13}C_{sample}$, were obtained using ^{13}C contents in treated samples

$$mg^{13}C_{t\ sample} - (A_{cs} \times mg^{12+13}C_{t\ sample}) = \text{excess } mg^{13}C_{sample}$$

(5) tissue enrichments ($mg^{13}C$) in treated seedlings were obtained by relating shoot and root tissue biomass (mg) to excess $mg^{13}C_{t\ sample}$

$$\text{excess } mg^{13}C_{t\ sample} \times \text{tissue biomass (mg)} = \text{excess } mg^{13}C_{tissue}$$

(6) whole plant enrichments ($mg^{13}C$) in treated seedlings were obtained by summing tissue fractions

$$\text{excess } mg^{13}C_{shoot\ tissue} + \text{excess } mg^{13}C_{root\ tissue} = \text{excess } mg^{13}C_{w\ plant}$$

APPENDIX 1I: Example Radioactive Isotope Calculations

Radioactive Carbon Isotope:

Radioactive carbon isotope calculations were completed using dpm values from treated or pulsed seedlings and untreated, control seedlings.

(1) radioactivity values of treated samples ($Bq^{14}C$ or $dpm^{14}C$), were converted to $mg^{14}C$ based on the batch activity (λ) of $Na_2{}^{14}CO_3$, $\lambda = 1.96$ GBq/mmol and was expressed per mg C ($Bq^{14}C_{mgC}$)

$$Bq^{14}C_{mgC} = Bq^{14}C_{t\ sample}/mg\ C_{t\ sample}$$

(2) radioactive units (Bq) were converted to $mols^{14}C$ using λ, and then $mg^{14}C$ using the molecular weight of ^{14}C ($mw^{14}C$)

$$mols^{14}C_{mgC} = Bq^{14}C_{mgC}/\lambda$$

$$mg^{14}C_{mgC} = mols^{14}C_{mgC}/mw^{14}C$$

(3) ^{14}C enrichment in treated samples, excess $mg^{14}C_{mgC}$ was calculated by subtracting control ($Bq^{14}C_{control}$) values from treated sample values

$$mg^{14}C_{mg\ C\ t\ sample} - mg^{14}C_{mg\ C\ control} = \text{excess } mg^{14}C_{mg\ C\ t\ sample}$$

(4) tissue enrichments ($mg^{14}C$) in treated seedlings were obtained by relating shoot and root tissue biomass (mg) to excess $mg^{14}C_{mg\ C\ t\ sample}$

$$\text{excess } mg^{14}C_{mg\ C\ t\ sample} \times \text{tissue biomass (mg)} = \text{excess } mg^{14}C_{tissue}$$

(5) whole plant enrichments ($mg^{14}C$) in treated seedlings were obtained by summing tissue fractions

$$\text{excess } mg^{14}C_{shoot\ tissue} + \text{excess } mg^{14}C_{root\ tissue} = \text{excess } mg^{14}C_{w\ plant}$$

APPENDIX III: Example Carbon Transfer Calculations

Bi-directional (BT) and net (NT) transfer calculations were based on whole plant levels of excess isotope and were calculated for labeling schemes 14PB-13DF and 13PB-14DF. The first labeling scheme represents paper birch seedlings pulsed with ^{14}C simultaneously with Douglas-fir seedlings pulsed with ^{13}C. The second labeling scheme represents the opposite of the above where paper birch is pulsed with ^{13}C and Douglas-fir pulsed with ^{14}C.

(1) Transfer estimated using the labeling scheme 14PB-13DF, was expressed as a proportion of the total ^{13}C or ^{14}C assimilated by PB, DF and PB + DF.

BT = isotope received by both donor and receiver seedlings

BT = (DF excess mg^{14}C received from PB) + (PB excess mg^{13}C received from DF)

(2) Transfer (NT) using the labeling scheme 13PB-14DF, was estimated by calculating the difference between isotope received. Positive net transfer indicates a greater amount of isotope was received by Douglas-fir than paper birch. Negative net transfer indicates the reverse, where paper birch receives more isotope than Douglas-fir.

NT = (DF excess mg^{13}C received from PB) – (PB excess mg^{14}C received from DF)

One-way (OT) transfer calculations were based on whole plant levels of excess isotope and were calculated for labeling schemes 13DF-PB, 13PB-DF, 14DF-PB, 14PB-DF. Briefly, the first labeling scheme 13DF-PB represents Douglas-fir pulsed with ^{13}C while paper birch was not pulsed with any isotope.

(3) Transfer estimated using the labeling scheme 13DF-PB, was expressed as a proportion of the total ^{13}C assimilated by PB, DF and PB + DF.

OT = excess mg^{13}C isotope received by PB from DF