KIN-SELECTED SIGNAL TRANSFER THROUGH MYCORRHIZAL NETWORKS IN DOUGLAS-FIR

by

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Abstract

Mycorrhizal networks create pathways for movement of resources and information molecules belowground. A mycorrhizal network is formed when two or more plants are linked by the same mycorrhizal fungus. Experiments have demonstrated movement of carbon and nitrogen between Douglas-fir and neighboring plants in response to source-sink dynamics, seasonality, and differences in age of linked plants. Furthermore, the network appears to act a conduit for information chemicals, where defense chemicals are transferred in response to herbivory or pathogen attack. Because of recent evidence implying the capacity for Douglas-fir to recognize kin, as well as differential colonization of Douglas-fir by ectomycorrhizas based on tree relatedness, this thesis aimed to determine whether Douglas-fir would preferentially transfer carbon and/or nitrogen through mycorrhizal networks to kin over strangers in response to herbivory treatment. Using seedlings with and without access to a mycorrhizal network (restricted or permitted via mesh pore size), stable isotope probing was used to track carbon and nitrogen in the system. One seedling of a pair was designated as the 'donor' and defoliated immediately prior to photosynthesizing with 99%-^{13}C-CO_2 as well as pulse-labelling with 99%-^{15}N ammonium nitrate. Both a greenhouse and field experiments were performed to corroborate results. Transfer was determined by measuring $\delta^{13}C$ and $\delta^{13}N$ in tissues (needle, stem, root) of kin and stranger seedlings. Data was analyzed using linear mixed effects models. Significantly more carbon was transferred to kin than strangers, and through the mycorrhizal network than when the mycorrhizal network was blocked. Furthermore, herbivory (in the form of western spruce budworm defoliation as well as manual defoliation) induced transfer of carbon to kin over strangers. Douglas-fir families differed in their tendency to transfer carbon and nitrogen to kin. Molecules potentially involved in defense signaling were identified using liquid chromatography coupled with mass spectroscopy. Ectomycorrhizal fungi that can form mycorrhizal networks were found on all seedlings. We conclude that preferential carbon transfer through mycorrhizal networks occurs between kin in Douglas-fir and is amplified by herbivory stress. Herbivory is not necessary for transfer, as some transfer also occurred in the no-herbivory treatment.

Lay Abstract

Mycorrhizal networks, formed by mycorrhizal fungi that connect Douglas-fir trees, allow for the transfer of resources and information chemicals. This thesis tested whether Douglas-fir could transfer defense signals through the mycorrhizal network to its neighbours, whether the identity of neighbors as kin or strangers affected their response to this transfer, and whether herbivory amplified the response. The hypothesis was that Douglas-fir could recognize kin, and preferentially warn their kin about an herbivore predator threat. Transfer of carbon through mycorrhizal networks was detected, was greater to kin than strangers, and was amplified by herbivory. In addition, some families were more inclined to share information with kin than others, demonstrating that not all families are the same.

Preface

A version of Chapter One has been published with myself as first author:
Gorzelak MA, Asay AK, Pickles BJ, Simard SW. 2015. Interplant communication through mycorrhizal networks mediates complex adaptive behaviour in plant systems. *AoB Plants*. 7:plv505; doi:10.1093/aobpla/plv050. Suzanne W. Simard conceived of the idea for this review, Monika Gorzelak wrote the first draft with guidance from SWS, and Amanda K. Asay and Brian John Pickles contributed to subsequent drafts of the paper. This paper and this chapter provide the theoretical framework for this thesis and contextualizes the work within current research.

Chapter Two is based on a greenhouse experiment which was designed, developed, conducted and interpreted by Monika Gorzelak with intellectual guidance and support from Suzanne W. Simard. Amanda K. Asay volunteered significant amounts of time and energy during the extensive set-up of this experiment.

Chapter Three is based on a field experiment which was designed, developed, conducted and interpreted by Monika Gorzelak with intellectual guidance and support from Suzanne W. Simard. Amanda K. Asay and Daniel Malvin were indispensable in executing the field work.

Chapter Four is based a growth chamber experiment as well as samples archived from the greenhouse experiment in Chapter Two. This experiment was designed, developed, conducted and interpreted by Monika Gorzelak.

Chapter Five summarizes Illumina MiSeq fungal root tip sequencing data generated from experiments in Chapter two and three. These experiments were designed, developed, conducted and interpreted by Monika Gorzelak.

All three experiments are supported by and represent a contribution to the Mother Tree Project which strives to understand the impact of mycorrhizal networks on tree regeneration in British Columbia as part of a NSERC Strategic Project Grant awarded to Suzanne W. Simard.

Table of Contents

List of Tables

List of Figures

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Chapter 1. Literature Review

Mycorrhizas

The term mycorrhiza (literally 'fungus-root') was first coined by AB Frank in 1885 (Trappe, 2005). Most terrestrial plants form symbiotic mutualisms with mycorrhizal fungi (Smith & Read, 2008; Brundrett, 2009). The origins of this symbiosis are ancient and this symbiosis may have facilitated the movement of plants onto land approximately 400 Mya (Redecker *et al*., 2000; Humphreys *et al*., 2010), by helping plants to acquire mineral nutrients from soil in exchange for photosynthates. The mycorrhizal symbiosis is a many-to-many relationship: plants tend to form symbioses with a diverse array of fungal species (broad host receptivity) and likewise, fungal species are able to colonize plants of different species (broad host range). While most mycorrhizal fungi are broad host generalists, forming diffuse mutualisms, a few appear to be specialists, occurring exclusively on a single host (Lang *et al.*, 2011).

Plant species tend to display fidelity to specific classes of mycorrhizal fungi, and entire ecosystems are often dominated by one class or the other. By far the most ubiquitous class is arbuscular mycorrhizal fungal (AMF) class, comprised of obligate biotrophs in the Glomeromycota that form arbuscules and sometimes vesicles within the root cells of hosts. AMF symbioses dominate temperate grasslands, tropical forests, and agricultural systems, but also associate with some temperate forest trees, such as members of the Cupressaceae and Aceraceae families. The AMF are microscopic with few morphological features to distinguish between species, and are classified by spore appearance and molecular markers, and continue to stir controversy due to the atypical genetics of these fungi (Koch *et al*., 2004; Ehinger *et al*., 2009).

The other major class of mycorrhizas is the ectomycorrizal fungal (EMF) class. Although fewer plant species have been found to form symbioses with EMF, in comparison to AMF (Brundrett, 2009), the hosts of EMF tend to be widely dispersed, abundant, and dominant members of their assemblages. For example, EMF hosts include most coniferous trees (all of the Pinaceae), the majority of woody shrub species in temperate and boreal forests, and the Dipterocarpaceae, which results in EMF also being common in tropical forests. Root tips harbouring EMF (Massicotte *et al.*, 1987) are distinguishable by macroscopic features (Peterson *et al.*, 2004) including: i) the mantle (a fungal sheath that encases a colonized root tip); ii)

extramatrical mycelium (diffuse hyphae that extend out into the surrounding soil), and iii) the Hartig net (hyphae that surround root cortical cells (Peterson *et al.*, 2004)). Some species of EMF form epigeous mushrooms and others form hypogeous truffles, predominantly from the phyla Basidiomycota and Ascomycota. EMF appear to have evolved separately in association with multiple plant families, with as many as 66 incidences identified thus far from phylogenetic evidence (Tedersoo *et al.*, 2010). Some exceptional plant families and genera are capable of forming viable symbioses with EMF and AMF simultaneously (e.g., Salicaceae, *Eucalyptus*).

A further class of mycorrhizas, that appears to have evolved from the EMF, is the ericoid mycorrhiza fungal (ERMF) class. ERMF originate from several fungal lineages, and form primarily with plants in the family Ericaceae, but have also been shown to occur simultaneously on both ERMF and EMF host plants (Villarreal-Ruiz *et al*., 2004). The ERMF occur throughout the world, but they are most common in boreal forests, alpine tundra, Arctic tundra, and heathland ecosystems. The ERMF can co-occur with EMF on a single site or in a single MN (Gorzelak *et al.*, 2012). Importantly, the dominant class of mycorrhizal association has ecosystem-level consequences for resource availability, as recently demonstrated by lower soil C:N ratio in systems dominated by AMF than those dominated by EMF/ERMF (Averill *et al.*, 2014).

Mycorrhizal networks: structure and function

A mycorrhizal network (MN) is formed when multiple plants are linked belowground by a continuous AMF (Kiers *et al*., 2011), EMF (van der Heijden & Horton, 2009; Beiler *et al.*, 2010) or ERMF (Bidartondo, 2005) genet. Networks can be exclusive to a subset of plants able to form mycorrhizal associations with a particular fungus, as in Beiler *et al.*, (2010), where two fungal species and one tree species were implicated in MN formation. However, MNs can also include multiple generalist fungal species connecting multiple plant species from disparate plant families (Molina & Horton, 2015). In the case of AMF, the network may connect many or most of the plants in a local area, because these fungi tend to be generalists and the host plants tend to have low specificity for the species of AMF fungi, enabling multiple linkages to form with multiple plants (Molina *et al.*, 1992). It is probable that EM, AMF and ERMF networked communities overlap; for example, in western North America, a single mixed forest comprised of approximately ten tree species can include approximately 100 fungal species, with most linked

together in a complex MN that includes EMF, AMF and ERMF networks simultaneously linking some plants capable of forming multiple types of MNs, with other plants linking into only one network, and those plants with specialist fungal partnership requirements linking into a more limited MN, all of which co-exist (Simard *et al.*, 2013). Some tree genera, such as *Populus* and *Salix*, can simultaneously form EMF and AMF networks, and others, such as mycoheterotrophic orchids, can integrate EMF and ERMF networks. Evidence for the occurrence of MNs has been accumulating for half a century (Björkman, 1960; Reid & Woods, 1969; Francis & Read, 1984; Newmann, 1988), and their significance for ecosystems has been studied intensively in the past two decades (Simard *et al*>, 1997c; Fitter>*et al*>, 1998). There is increasing evidence that MNs can influence plant establishment (Dickie *et al.*, 2002; Nara, 2006), survival (Teste *et al.*, 2009; Bingham & Simard, 2011, 2012), physiology (Wu *et al.*, 2001, 2002), growth (Teste *et al.*, 2010) and pathogen and insect defense chemistry (Song *et al.*, 2010, 2014; Babikova *et al.*, 2013). This influence is thought to occur because the MN serves either as a pathway for interplant exchange of resources and information molecules or as a source of fungal inoculum (see reviews by Simard *et al.*, 2012, 2013, 2014). For instance, anastomosis with existing MNs of established plants is considered the most common mechanism for mycorrhizal fungal colonization of regenerating plants *in situ* (van der Heijden & Horton, 2009; Kariman *et al.*, 2012). Colonization of establishing seedlings by MNs enables them to acquire sufficient soil nutrients for root and shoot growth and hence survival (Kariman *et al.*, 2012).

As with mycorrhizal colonization, interplant resource and signal fluxes through MNs have the potential to alter plant behaviour (Gorzelak *et al.*, 2015). The arrival of resources or information via MNs is a source of stimuli that must be integrated by a receiving plant. Fluxes through MNs have been shown to include carbon (Simard *et al*., 1997a,b), water (Egerton-Warburton *et al.*, 2007; Bingham & Simard, 2011), nitrogen (Teste *et al.*, 2009), phosphorus (Eason *et al.*, 1991), micronutrients (Asay, 2013), stress chemicals (Song *et al.*, 2010, 2014; Babikova *et al.*, 2013) and allelochemicals (Barto *et al.*, 2011), and can occur between plants of the same or different species. Understanding the potential effects of these fluxes on plant behaviour, however, first requires an understanding of transfer processes, and the factors that regulate these processes. For instance, interplant resource exchanges are thought to be regulated by source-sink relationships within the MN, where one plant that is rich in nutrients serves as a source (donor) of organic compounds for a neighboring plant that is poor in nutrients, which thus

serves as a sink (receiver) (Simard *et al*., 1997a,c). The long-distance transport of carbon and/or nutrients appears to occur predominantly by advective mass flow driven by the source-sink gradient generated by these interplant nutrient differences (Simard *et al.*, 2012; Heaton *et al.*, 2012a). Mass flow can also be generated by fungal mycelium growth, and diffusion or active transport mechanisms may operate during active fungal cell expansion at growing mycelium fronts (Heaton *et al.*, 2012b).

The primary importance of plant sink strength in governing the magnitude and direction of resource transfer through MNs is illustrated in studies showing transfer of carbon to rapidly growing EMF saplings with high transpiration rates, or to shaded EMF seedlings with high respiration demands for survival and growth (Read, 1985; Simard *et al*., 1997a; Teste *et al*., 2009, 2010). Organic compounds are thought to enter the transpirational stream of the receiver plant via the xylem, and then be actively transported to rapidly expanding biosynthetic tissues. Plant source strength also drives transfer under certain conditions. This was demonstrated in the rapid transfer of labile carbon from the roots of injured EMF seedlings to healthy neighbours (Song *et al.*, 2014), and in the transfer of nitrogen from N_2-fixing or fertilized source plants to non-N_2-fixing sink plants (He *et al.*, 2009). Increasing source strength by CO_2 fertilization of plants in AMF networks, by contrast, has had no effect on carbon transfer (Fitter *et al.*, 1998). Although either source or sink strength may dominate under certain circumstances, it is more likely that the simultaneous behaviour of both source and sink plants (and sources and sinks within the mycelium itself) influences carbon and nutrient transfer through MNs. For instance, the direction and magnitude of carbon transfer changed over a growing season due to simultaneous changes in phenology, and hence source and sink strength, of different plants involved in an EMF network in mixed temperate forest (Philip, 2006) and Low Arctic Tundra (Deslippe & Simard, 2011). Carbon and nitrogen are thought to travel through MNs together in simple amino acids (Simard *et al.*, 2015). These molecules travel through the MN rapidly, moving from donor plants to the fungal mycelium within one or two days (Wu *et al.*, 2002; Heaton *et al.*, 2012a) and to the shoots of neighbouring plants within three days (Wu *et al.*, 2002).

In addition to resources such as carbon and nutrients, studies using experimental designs that prevent the above-ground transfer of volatile organic compounds (VOC's) and control for the presence of an MN, have provided indirect evidence indicating that stress molecules may

also transfer from injured to healthy plants through MNs. It appears that these stress compounds may travel even more rapidly than carbon, nutrients or water. For example, herbivore- and pathogen-induced defense enzymes were up-regulated in undamaged neighbors in as little as 6 hours following insect or fungal infestation of donor plants linked in AMF MNs (Song *et al*., 2010, 2014; Babikova *et al*., 2013). Song *et al.*, (2014) found up-regulation of four defense-related genes in healthy neighbours 6 h after AMF tomato donors were infested with the insect *Spodoptera litura*, likely in response to signaling via the jasmonate pathway through the MN. They showed that production of defense enzymes increased receiver resistance to pests, as indicated by lower weight gain and hence lower fitness of the herbivore. In an earlier study, Song *et al.* (2010) infested AMF tomato plants with the foliar necrotrophic fungus, *Alternaria solani*, and similarly found that six defense genes encoding for defense enzymes were activated after 65 h in the un-infested neighbours. In this study, the use of mutant controls and the genes that were up-regulated suggested that salicylic acid and jasmonic acid pathways were involved in signaling (or in signal detection via "eaves-dropping") through the MN. In a study where Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *glauca* (Mayr)) was defoliated with *Choristoneura occidentalis*, Song *et al*., (2015) reported a physiologically significant transfer of photosynthetic carbon as well as stress response in interlinked ponderosa pine (*Pinus ponderosa* Douglas *ex* C.Lawson) seedlings. The experiment consisted of interior Douglas-fir (grown in pairs with ponderosa pine in a 3 x 3 full factorial design testing 3 mycorrhizal treatments (network with 35µm mesh, no network with 0.5µm mesh, and no mesh bags) and 3 defoliation treatments (western spruce budworm (*Choristoneura occidentalis*), manual defoliation, and control). Donors (Douglas-fir) were subjected to defoliation treatment, pulsed with ^{13}C-CO_2 24 hours later and receiver seedlings (ponderosa pine) harvested 6 days after the pulse-labeling. Song *et al.*, (2015) measured root and shoot excess ^{12}C equivalent (mg), which is the equivalent mass of carbon that would be transferred using ambient ^{12}C-CO_2 (atmospheric CO_2 contains < 1% ^{13}C and is thus lighter than the 99% ^{13}C-CO_2 label). They found a significant increase in transferred carbon in the mycorrhizal treatment in response to manual defoliation, but not spruce budworm defoliation (the spruce budworms did not feed vigorously on the Douglas-fir, which explains the lack of response). Song *et al.*, (2015) also measured enzyme activity (peroxidase, polyphenol oxidase, and superoxide dismutase) in receiver ponderosa pine and found these were increased in these networked seedlings in response to both manual and insect defoliation. This, and the

previous study, show a rapid physiological response of recipient plants to putative stress signals transmitted through MNs. It is important to note that no study has identified a stress or defense chemical moving through a MN. All of the studies described in this section provide indirect and circumstantial evidence for defense molecule transfer.

Douglas-fir forests

Two varieties of Douglas-fir are recognized in western North America: coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*) and interior Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *glauca* (Mayr)), which diverged from each other approximately 2.11 Mya (Gugger *et al.*, 2010). Interior Douglas-fir forest structure, function, and biogeography are detailed in Simard, (2009) and briefly described here for reference. Interior Douglas-fir occupies a distinct range east of the coastal variety (Lavender & Hermann, 2013) and is distinguished via genetic and chemical markers (Gugger *et al.*, 2010). Interior Douglas-fir (hereafter referred to as simply 'Douglas-fir') range from as far south as northern Mexico (Hermann & Lavender, 1999; Lavender & Hermann, 2013) and as far north as 55° (Griesbauer & Green, 2010a) covering a span of 4,500km (Lavender & Hermann, 2013). Douglas-fir is a foundational species, serving as an ecosystem structuring force due to its long-lived nature and extensive distribution (Ellison *et al.*, 2005; Simard, 2009). Douglas-fir's high genetic variability (Gugger *et al.*, 2010) may explain its phenotypic plasticity (St Clair *et al.*, 2005) and in turn, its ability to grow in many forest types.

In warm and dry climates, Douglas-fir tends to be limited by soil moisture and in northern climates tends to be limited by annual temperature (Griesbauer & Green, 2010b). Douglas-fir are most productive in the semi-arid central interior of British Columbia (the interior Douglas-fir biogeoclimatic zone IDF), becoming more patchy in the southern interior and transitioning to Ponderosa pine (*Pinus ponderosa* Laws) and bunchgrass ecosystems in drier climates (Huggard *et al.*, 2005). At higher elevations, IDF borders lodgepole pine forests of the montane spruce (MS) biogeoclimatic zone (Huggard *et al.*, 2005). In wetter climates, Douglas-fir becomes a mid to early seral species in a mixed forest with other broadleaf and conifer species (Meidinger & Pojar, 1991), becoming more rare in wetter climates, where it is replaced by western redcedar (*Thuja plicata* Don.) and helmlock (*Thuja heterophylla* (Raf.) Sarg. as moisture increases (Meidinger & Pojar, 1991).

Importantly, Douglas-fir, and interior Douglas-fir forests have a history of being studied from the perspective of MNs (Simard, 2009; van der Heijden & Horton, 2009). A 20m x 20m MN in a dry Douglas-fir forest has been mapped (Beiler *et al.*, 2010) *in situ*. Transfer of carbon and nitrogen through MNs has been documented in these forests (Simard *et al*>, 1997c; Philip, 2006; Teste>*et al*>, 2009, 2010). With a myriad of studies demonstrating the importance of MN to ecosystem function (Simard *et al.*, 2012) in interior Douglas-fir forests, this system is an ideal candidate for deeper studies of the effects of MNs.

Defense signaling

Western spruce budworm (*Choristoneura occidentalis*) in an important pest threatening Douglas-fir in British Columbia as average summer temperatures increase due to climate change (Woods *et al*., 2010). Western spruce budworms (WSB) are foliage feeding larvae that consume needles and young shoot tips (Alfaro *et al.*, 2014). To reduce damage by WSB, Douglas-fir express secondary metabolites that reduce the feeding efficiency of WSB, including alpha- and beta-pinene, and myrcene (Redak & Cates, 1984). Reduced feeding by WSB is also associated with increased foliar total nitrogen (Redak & Cates, 1984). In order to mount a defense, plants sense the presence of a defoliator, and, at least in *Arabadopsis thaliana*, are able to mount a differential response depending on the manner of defoliation: chewing or sucking (Appel *et al.*, 2014). In response to WSB defoliation in Sitka spruce (*Picea stikensis*), genes involved in plant defense, terpenoid biosynthesis, ethylene signaling, transport, and secondary metabolism are upregulated (Ralph *et al.*, 2006), and those involved in photosynthesis are downregulated (Ralph *et al.*, 2006). Conifer secondary metabolism is able to react and respond to insect herbivory. This response is triggered by methyl jasmonate in Douglas-fir (Huber *et al.*, 2005), which is a defense chemical common in plants (Wasternack, 2007).

Jasmonates are responsible for communication about defoliation within a plant (Wasternack, 2007), but may also be responsible for communicating insect attack between plants through belowground MN pathways (So*ng et al*, 2010, 2015a). Inter-plant communication also occurs via volatile organic compounds (VOCs) through aboveground pathways (Karban *et al.*, 2014). And whilst studies in aboveground communication have been criticized for, among other things, signals not necessarily being targeted or directed at neighbours (Karban *et al.*, 2014), a mycorrhizal belowground pathway could provide a targeted pathway for signal transfer. The MN as an information conduit is more likely to transfer water-soluble molecules such as jasmonates

(which can also be emitted as volatiles) rather than VOCs. Most plants utilize the jasmonate pathway (Wasternack, 2007), which makes jasmonate a universal plant signal and could account for the interspecific communication documented by Song *et al.*, (2015). In Douglas-fir, jasmonates induce terpene synthesis (Huber *et al.*, 2005), and terpenes, in turn, are associated with decreased weight gain by defoliating WSB (Redak & Cates, 1984). Jasmonates are thus likely to be involved in signal molecules transferred through MN in response to WSB herbivory in Douglas-fir.

Kin selection

Kin recognition, the ability for an organism to distinguish between genetic kin and non-kin individuals, was demonstrated in *Cakile edentula* by Dudley & File, (2007). Plants grown with strangers allocated more biomass to their roots as compared to plants grown with kin. Dudley & File, (2007) interpreted this as evidence of reduced competition between kin plants, which is a form of altruism, although they did not test the mechanism. Biedrzycki *et al.*, (2010) showed that kin recognition is mediated through root exudates in *Arabadopsis thaliana*. Also in *A. thaliana,* kin recognition was demonstrated through altruistic leaf orientation when placed next kin and this was shown to be mediated via photoreceptors by testing knock-out plants. It appears that there may be multiple mechanisms of kin recognition. Furthermore, Asay, (2013) and Pickles *et al.*, (2017) demonstrated kin recognition in Douglas-fir through a mycorrhizal network. Kin Douglas-fir seedlings had more colonization than stranger seedlings, and ~ 70% of the ^{13}C in PLFAs (phosphlipid fatty acids) of plant roots, ectomycorrhizal root tips, and soil fungal communities, implicating that mycorrhizas responded to host relatedness. A recent study by File et al (2012) in AM systems also demonstrated a mycorrhizal effect (File *et al*., 2012a). Using *Artemisia ambrosiafolia* L., File et al (2012) found evidence of a larger MN in plants when in the neighbourhood of kin.

In this thesis, “kin” was defined as full-siblings of known parentage. In all experiments described, Douglas-fir seedlings used were sourced from seed orchards where their parentage was controlled. All originated from the same provenance. “Stranger” is defined as known genetic non-relatives. The term “family” refers to a particular line of “kin”. Douglas-fir from the same family are kin (as defined above). Douglas fir from different families are strangers. Multiple

families were tested in this thesis in order to establish the universality of kin selection in Douglas-fir.

Research objective and thesis outline

The objective of this thesis was to determine if defense signals are preferentially transferred to kin over stranger Douglas-fir through MNs in response to herbivory defoliation. In this research, defense signaling is inferred from direct evidence of carbon and nitrogen transfer. All references to defense signal transfer refer to indirect evidence, that is, changes to carbon/nitrogen transfer in response to stimulus.

The specific questions addressed were as follows:

1. Is there a small amount of transfer that occurs through MN regardless of stimulus? (Chapters 2 and 3)
2. All things being equal, would a donor Douglas-fir preferentially transfer a signal to its kin over a stranger Douglas-fir? (Chapter 2)
3. Can transfer be stimulated by applying WSB defoliation (Chapter 2)? And/or can a similar response be induced with manual defoliation (Chapters 2 and 3)?
4. Can signal transfer be localized to the MN, or is signal transferred through soil belowground pathways (Chapter 3) and/or through root interactions (Chapter 2)?
5. Can the signal transferred be identified using non-specific whole metabolome profiling (Chapter 4)?
6. Finally, is there evidence of fungal colonization forming the MN in these Douglas-fir? And are communities of mycorrhizal fungi more alike in kin over strangers, sharing more fungal connections, and accounting for any increases in transfer? (Chapter 5)

Chapter 2 describes a greenhouse study where three Douglas-fir seedlings were grown in a pot (2 were kin, 1 was a stranger), and permitted to form a MN. One of the kin was assigned 'donor' status, and was defoliated and pulse-labelled with stable isotopes. This defoliated and labelled donor could 'choose' whether to send signals to either of the two receivers: a kin or a stranger. Stable isotope content in receiver seedlings was used to determine if preferential transfer to kin occurred in this "choice" experiment.

Chapter 3 describes a field based study where kin and stranger Douglas-fir were planted in pairs with and without MN access. Using similar techniques to Chapter 3, donor seedlings were manually defoliated and pulse labelled with stable isotopes. Stable isotope content in receiver seedlings was used to determine if more labelled was transferred kin over stranger in response to simulated herbivory stress.

Chapter 4 describes whole metabolome profiling of needles harvested in from the greenhouse experiment in Chapter 2 to identify the signal being transferred. Attempts to simplify the system using a single mycorrhizal fungus colonizing two kin or stranger Douglas-fir seedlings grown in a growth chamber are described.

Chapter 5 describes high-throughput sequencing of fungal root tip communities of Douglas-fir seedlings from Chapter 2 and Chapter 3. Comparisons of fungal community structure are used to understand preferential transfer through MN to kin over stranger seedlings.

Chapter 2: Kin recognition and herbivory greenhouse experiment

Introduction

Mycorrhizal networks have the potential to impact the behaviour of plants by facilitating transfer of resources and information chemicals between linked plants (Gorzelak *et al.*, 2015). Plant behaviour can be described as the acquisition and processing of information that results in modification of what the plant does (Trewavas, 2009). Signalling and responding to signalling, with, for example, changes to secondary metabolism are considered plant behavioural responses. Plasticity is a form of plant behaviour, and is advantageous as a definition because it can be measured. Plasticity can be exemplified by positional changes in response to light (Smith, 2000; Gundel *et al.*, 2014). Plants may, in fact, respond to cues that have yet to be identified (Gagliano *et al.*, 2012).

Kin recognition was demonstrated in *Cakile edentula* by Dudley & File, (2007). Plants grown with strangers allocated more biomass to their roots as compared to plants grown with kin. Biedrzycki *et al.*, (2010) showed that kin recognition is mediated through root exudates in *Arabadopsis thaliana* (Biedrzycki *et al.*, 2010). Also in *A. thaliana*, kin recognition was demonstrated through altruistic leaf orientation when placed next to kin and this was shown to be mediated via photoreceptors by testing knock-out plants. It appears that there may be multiple mechanisms of kin recognition. Using *Artemisia ambrosiafolia* L., File *et al.*, (2012b) found evidence of a larger MN as represented by higher hyphal counts in the neighbourhood of kin. Asay (2013) also noted an increase in colonized root tips in kin over stranger in Douglas-fir. The presence of kin may prime the mycorrhizal fungi to more easily form associations with kin over strangers.

Studies probing kin recognition must be replicated among different families to test variation within the species of interest, as in Dudley & File, (2007). Testing kin responses in multiple families allows for the determination of whether responses may be due to intra-specific variation, rather than a kin recognition response. A single family may contain a suite of mutations that respond differently to other families, making the recognition response being measured a bias of that particular family rather than a pan-species ability to respond favourably to kin through recognition.

Recent work has implicated that Douglas-fir may be capable of kin recognition (Asay, 2013; Pickles *et al.*, 2017). Asay (2013) determined that Douglas-fir growing with kin had higher foliar micronutrient content (zinc, boron and iron) than those with strangers; and that kin were more highly colonized by ectomycorrhizal fungi than strangers. Pickles *et al.*, (2017), meanwhile, found in the same experiment that more stable isotope ^{13}C (after pulse labelling with ^{13}C-CO_2) was transferred to kin over Douglas-fir strangers in some families, through MNs. Taken together, these studies suggest that kin recognition may be possible for Douglas-fir.

Western spruce budworm (WSB) is a conifer defoliating insect whose main target in British Columbia is Douglas-fir (Alfaro *et al.*, 2014). WSB elicits defense responses in Douglas-fir, including production of secondary metabolites that reduce the feeding efficiency of WSB (Redak & Cates, 1984). Furthermore, in an indirect demonstration of defense signal transfer through a MN, defoliation of Douglas-fir with WSB elicited defense enzyme production in MN-linked ponderosa pine seedlings (Song *et al*., 2015). Thus Douglas-fir responds to WSB defoliation by producing a defense signal, which appears to be transferred to neighbouring conifers via MN. The defense signal has the potential to travel via MN connections interspecifically and also to conspecifics. In the case of conspecifics, the putative defense signal may be sent preferentially to kin.

Plants were grown three to a pot, with two levels of **relatedness**: two *kin* and one *stranger*. One of the kin was selected to be a 'donor', underwent a **defoliation** treatment (*manual defoliation, western spruce budworm defoliation, or no-herbivory control*), and was labelled with ^{13}C-CO_2 and ^{15}N-ammonium nitrate over 27 hours. Receiver seedlings (the unlabeled kin and stranger in the pot), and the one donor seedling were destructively harvested to determine label content of tissues of all seedlings. Seedlings were grown in one of three **belowground** treatments: *MN* (hyphae only-permitted by growing seedlings in 35µm pore sized mesh bags which block roots and permit hyphae therefore allowing the formation of a *mycorrhizal network*), *F* (both roots and hyphae *free* to interact-no mesh bag), or *B* (*blocked* root and hyphal contact-blocked by growing seedlings in 0.5µm mesh bags which block both roots and hyphae). In experiment 1, the donors were manually defoliated, western spruce budworm defoliated or no-herbivory control. In experiment 2, all donor seedlings underwent a no-herbivory control. In both experiments, the donor was given a "choice" to transfer label to kin and/or strangers, in order to test the following questions. 1) Is there preferential carbon transfer to neighboring kin over

strangers through MNs in response to herbivory stress in Douglas-fir seedlings? 2) If, as previous experiments have shown, a small amount of carbon would transfer regardless of stimulus, is the constitutive transfer to kin greater than strangers? 3) Does the type of defoliation affect whether carbon transfer to kin is preferred? 4) Does transfer occur through the MN, with less transferred when both roots and fungi were free to interact, and none transferred where both roots and fungal hyphae of seedlings were blocked? 5) Do families differ in their responses to these treatments.

Methods

In a first experiment, two families were used in a 3 x 3 x 2 factorial set of treatments, with three **belowground** treatments (*mycorrhizal network* only *MN* with roots blocked by growing seedlings in 35µm mesh bags; soil pathway only with both hyphae and roots *blocked (B)* by growing seedlings in 0.5 µm mesh bags*;* and naturally planted with roots and hyphae *free (F)* to interact), three **defoliation** treatments applied to the donor seedling (*manual defoliation, western spruce budworm defoliation, or no-herbivory control*), and two **relatedness** treatments (*kin* and *stranger*) replicated 10x for families A and B in a completely randomized design. Each greenhouse pot contained three Douglas-fir seedlings: two A's (*kin*) and one B (*stranger*). One A seedling was designated a 'donor' and the other two seedlings (A and B) were kin-receiver and stranger-receiver. Thus seedling A was given a 'choice' to transfer signal either to kin or stranger in response to treatment. This resulted in 90 greenhouse pots containing two comparisons per pot for a total of 180 experimental units.

In a second experiment involving four families (A, B, E, and F), the same 'choice' greenhouse pot set-up as described above, was used. In this case, a 3 x 4 x 2 factorial design was used with three **belowground** treatments (*free (F), MN,* and *blocked (B)*), 4 **families** (*A, B, E, F*) and two **relatedness** treatments (*kin* and *stranger*) were replicated five times in a completely randomized design. In this second experiment there were 30 pots containing two comparisons per pot for a total of 60 experimental units.

Seeds of full-sibling Douglas-fir were obtained from the British Columbia Ministry of Forests, Land, and Natural Resource Operations (MFLNRO) at the Kalamalka Research Forest Station. These seeds were artificial crosses of known parentage. All seed used in this experiment was from the same provenance. Each experimental unit consisted of three Douglas-fir seedlings

(two seedlings of one family and the third seedling from a different family) grown from seed in a 4L pot containing 50% natural forest soil mixed with 50% greenhouse potting mixture consisting of 75% peat and 25% perlite (West Creek Farms, Fort Langley, BC, Canada). For the **belowground** treatments, the three seedlings in each pot were individually grown in either 0.5µm pore-sized mesh (*blocked (B)*), 35µm pore-sized mesh (*MN*) (Plastok Meshes and Filtration Ltd. Birkenhead UK), or with no mesh bags (*free (F)*), with each seedling in its own bag (when applicable). Both mesh sizes prevent root passage, however the 35µm pore-sized mesh allows the passage of ectomycorrhizal hyphae (*MN* treatment), whereas the 0.5µm pore-sized mesh does not (*blocked* treatment), although soil pathway solute transfer is possible through this mesh size (Teste *et al.*, 2006). The no bag treatment is referred to as *free (F)*.

Experimental units were grown for 8 months prior to treatment. Neither fertilizer nor supplementary light were provided to the experimental units. Pots were watered to field capacity twice per week after a daily 2-week watering regiment during germination. A fine gravel layer was applied to soil surfaces to prevent "damping-off" fungus. Herbivory treatments were applied immediately before pulse-labelling donors using ^{13}C-CO_2. Here, one of the two kin seedlings in an experimental unit was selected as a 'donor' to which a herbivory treatment was applied, immediately followed by the pulse-labelling. For the herbivory treatments, donors were either untreated controls, or had their needles cut with scissors (approximately 50% of needles cut in half for a total of 25% defoliation), or were inoculated with western spruce budworm (ten 2nd instar budworms placed on fresh buds per seedling). The 2nd instar western spruce budworms were kindly provided by John Dedes at the Great Lakes Forestry Research Centre, Forestry Canada, Sault Ste. Marie, Canada. It is important to note that, to prevent airborne signal transfer, each individual seedling within a pot was sealed into an airtight plastic bag immediately prior to defoliation of the donor seedling (the donor's bag was sealed immediately after budworm application or manual defoliation).

Pulse-labelling

Pulse-labelling was performed by sealing each seedling in a 50cm X 30cm plastic bag (FoodSaver®) in which an injection valve had been installed. Each bag was filled with ambient air to capacity, sealed completely at the base of the stem using Plastalina modelling clay (Craft Smart®, Irving Texas USA) and Tuck® Contractors sheathing tape (Cantech, Montreal QC, Canada) and tested for air tightness by gently squeezing the bag. Only air-tight bags were used.

Each donor seedling received 100ml of 99 atom% ^{13}C-CO_2 (Cambridge Isotopes Laboratory, Inc. Tewksbury, MA, USA) before being allowed to photosynthesize for 27 hours. Receiver seedlings were also bagged to prevent any transfer of airborne molecules, not only from the donor within their own experimental unit but also from adjacent experimental units within the same greenhouse.

Furthermore, each donor seedling was provided with 1mL of 20mM ^{15}N-ammonium nitrite (Cambridge Isotopes Laboratory, Inc. Tewksbury, MA, USA) in the form of a 1.5mL microcentrifuge tube taped securely to a healthy branch, with needles dipped into the labelling liquid. The ^{15}N label was allowed to absorb for the same 27 hour period as the ^{13}C-CO_2 label. All bags contained water droplets the next day when the experimental units were harvested, indicating active photosynthesis had been occurring. All seedlings were bagged and labelled between 08:00 and 11:00, and harvested the next day began at 11:00. All experimental units were labelled and harvested within a period of 10 days in mid-May, 2016.

Seedling sampling and elemental analysis

After the 27 hour pulse labelling period, seedlings were destructively harvested, with separation of root from aboveground biomass, and separation of stems from needles. Roots were stored at 4°C in coolers until cleaned and processed for mycorrhizal colonization assessment via root tip harvesting (see Chapter 5 for details), over a period of approximately 3 months. A 3g sample of needles was frozen immediately in liquid nitrogen and stored at -80°C for LCMS analysis (see Chapter 4). Remaining needles and stems were dried in a 60°C oven for 3 days (until no change in mass), ground in liquid nitrogen for carbon and nitrogen isotopic content analysis in 3mg samples for low enrichment (receiver seedlings) and 2mg samples for high enrichment (donor seedlings), in tin cups. Carbon and nitrogen isotopic content was determined using combustion analysis with elemental analysis (Elementar, Hanau, Germany), in C, N mode, paired with an isotope ratio mass spectrometer (IRMS, Isoprime, Cheadle, UK). The C or N isotope ratio ($\delta^{13}C$ or $\delta^{15}N$) was calculated as:

(eq. 1) $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} -1)$

Where $R_{standard}$ = Vienna-PeeDee Belemnite (VPDB) standard (1.1237×10^{-2}) for C, and N_2 atmospheric gas (3.677×10^{-3}) for N.

Statistical analysis

All statistical analyses were performed in R 3.3.3 (R core team, 2017). Linear mixed effects (LME) models were used using the nlme (Pinheiro *et al*., 2017) and lme4 (Bates *et al.*, 2015) packages in R. ANOVA results were determined using lmerTest (Kuznetsova *et al.*, 2016), which implements the Satterthwaite (1946) approximation for denominator degrees of freedom, based on the SAS proc mixed theory (SAS, 1978).

The transfer of carbon and nitrogen in the system were analyzed separately. Furthermore, experiment 1 (including **defoliation** treatment) and experiment 2 (*no-herbivory control* **defoliation** only) were also analyzed separately.

In experiment 1, stable carbon and nitrogen isotope values of both donor and receiver needles, stems, and roots were normally distributed. Fixed effects were **relatedness** (*kin* vs *stranger*); **belowground** treatment (*MN, B, F*) and **defoliation** treatment (*western spruce budworm, manual defoliation, no-herbivory control*) (eq 1). The experimental unit, the greenhouse pot, was used as a random effect. Donor needle ^{13}C was used as a covariate to account for variation due to photosynthetic rate of incorporation of the ^{13}C-CO_2 pulse, as per (eq. 1). A similar model was used to test nitrogen stable isotope content (eq. 2).

(eq. 1) *Receiver C ~ Donor Needle C + Donor root C + relatedness + belowground + defoliation +relatedness*defoliation + relatedness*belowground + defoliation*belowground, random =1|experimental unit*

(eq. 2) *Receiver N ~ relatedness + belowground + defoliation +relatedness*defoliation + relatedness*belowground + defoliation*belowground, random =1|experimental unit*

Donor stem ^{13}C, and donor N (all tissues) were tested as potential covariates but did not improve the explanatory power of the model and were removed.

For experiment 2, differences between families were tested (with no-herbivory control defoliation treatment only) was performed as per (eq. 3) for ^{13}C and (eq. 4) for ^{15}N.

(eq. 3) *Receiver C ~ Donor C + relatedness + belowground + family + relatedness*belowground + relatedness*family + belowground*family, random =1|experimental unit*

Stable nitrogen isotope values were log transformed to achieve a normal distribution. Receiver needle nitrogen was square-root transformed. Fixed and random effects were the same as for the ^{13}C models (eq. 4).

(eq. 4) *Receiver N ~ Donor needle N + relatedness + belowground + family + relatedness*belowground + relatedness*family + belowground*family, random =1|experimental unit*

Donor stem, and root ^{15}N did not predict receiver ^{15}N content and were not used in the models. All models used were tested for normality of the resultant residuals to ensure the models were valid (Gurka *et al*., 2006). Model fit was determined using the R package piecewiseSEM (Lefcheck, 2016).

LME models were used to generate standard effect sizes (SES) (Schielzeth, 2010), from the regression coefficients of the response variables (^{13}C and ^{15}N tissue content of kin vs stranger-including roots, stems, and needles). SES was calculated based on standardized coefficients derived from the LMEs. The intercept of a regression coefficient (of the response variable) was removed to allow all levels of the treatments to be compared, and then divided by the standard deviation of the response variable. Dividing by standard deviation sets the variance to 1, thereby standardizing the value. Standard effect size was determined by subtracting the standardized coefficients of stranger from kin, allowing for the determination of size and directionality of the difference (a positive value denotes more ^{13}C or ^{15}N found in kin). A 95%

confidence interval was determined for each comparison by using the pooled standard error for all the terms considered. Thus the SES was interpreted as the additional change in treatment level, in standard deviations, for one standard deviation in the predictor variable (Schielzeth, 2010; Bates *et al.*, 2015). Standardizing in this manner allows for comparing between models, and between experiments. The benefit of presenting the data using standardized coefficient differences as standard effect sizes is that it allows partitioning the variance among the treatments to gain a more nuanced view of the significant effects across those treatments.

SES figures compliment ANOVA tables. ANOVA tables determine whether the means are different between response variables. SES figures, on the other hand, compare regression coefficients associated with response variables. In all SES figures, positive values denote a standardized coefficient that is higher in kin over stranger. The higher the coefficient, the greater the slope of the linear relationship as modelled by the LME. Thus, if kin ^{13}C increases more quickly (larger slope), than stranger ^{13}C (smaller slope), it can be said that there is a larger effect size in kin over stranger with respect the treatment in question. Henceforth, this is referred to as "effect size", which ought not to be confused with the mean of a response variable (as presented in ANOVA tables). ANOVA and effect sizes need not always agree. Using kin as an example, a significant ANOVA and a non-significant effect size indicates kin is greater, but there is no difference in the rate of change between kin and stranger; both increase by approximately the same amount for a given change in the predictor variable. A significant ANOVA and significant effect size indicates kin is greater, and the rate of increase is significantly greater in kin compared to stranger; kin increases at a faster rate than stranger for a given change in the predictor variable. Furthermore, 95% confidence intervals are represented by the error bars, which are used to determine statistical significance in each case. If the error bar crosses zero, the effect is not statistically different (all means were standardized by setting the variances to be equal to 1), that is, the standardized value for stranger is not different to the standardized value for kin within a 95% confidence interval.

Since kin was significantly greater than stranger, it was possible to present the standardized coefficient differences in terms of kin minus stranger, with respect to the belowground treatments and herbivory treatments. The ^{13}C values are presented in Figure 3 (needles), Figure 4 (stems) and Figure 5 (roots); and ^{15}N values are presented in Figure 6 (needles), and Figure 7 (stems). In all SES figures, positive values denote a standardized

coefficient that is higher in kin over stranger. This is a comparison of regression coefficients among treatments (made comparable within and among figures due to standardization—that is, the effect size is comparable between ^{13}C and ^{15}N models, as well as between experiment 1 and experiment 2).

Results

Experiment 1

The relatedness treatment and belowground treatments affected all receiver ^{13}C tissue content, as well as the herbivory x belowground interaction was significant in needles and stems, but not roots. Marginal significance (defined as $p>0.15$ in this thesis) was observed for the relatedness x belowground interaction (Table 1, Figure 1). Relatedness affected ^{15}N in needles and stems, but not roots (

Figure **2.** Box and whisker plots of 15N nitrogen content in receiver tissues, organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle 15N content is shown in the left-most column by a.) relatedness, b.) defoliation treatment, and c.) belowground treatment. Stem 15N content is presented in the same order in the middle column: d.) relatedness, e.) defoliation treatment, f.) belowground treatment; and root 15N is shown in the right-most column, spanning the same set of fixed effects g.) relatedness, h.) defoliation treatment, and i.) belowground treatment.

Table 2, Figure 2). However, the belowground treatment affected ^{15}N roots (

Table 2, Figure 2). Interaction effects in ^{15}N data included defoliation x belowground and belowground x relatedness for needles, and defoliation x belowground for stems (Table 2

Table 2).

In needles, in the free (F) belowground treatment, kin ^{13}C effect size was greater than stranger only with worm defoliation, and not in the no-herbivory and manual defoliation treatment (Figure 3). In the MN and B belowground treatments, all three herbivory treatments resulted in a significant ^{13}C effect size to kin over stranger (Figure 3). In stems, all belowground and herbivory treatments resulted in a significant ^{13}C effect size to kin over stranger (Figure 4). In roots, in the free (F) belowground treatment, a significant ^{13}C effect size was found in kin over stranger in the worm defoliation and manual defoliation herbivory treatments, but not in the no-herbivory control treatment. In the MN (networked) belowground treatment, only the worm defoliation treatment resulted in a significant ^{13}C effect size to kin over stranger, and no significant effect size of ^{13}C was found in any herbivory treatment with blocked (B) belowground treatment (Figure 5). The root ^{13}C results do not correspond to the ANOVA results (Table 1), which may be due to the structure of the data where many values are outliers (Figure 1), and thus are interpreted with caution. LME models of needle, stem and root ^{13}C tissue content explained 57%, 56%, and 61% respectively of the variation in the data and residuals were normally distributed (Table 3).

A significant ^{15}N effect size was found in kin over stranger needles in the belowground treatment (Figure 6), and then only in the no-herbivory and manual defoliation herbivory treatments, but not in the worm defoliation herbivory treatment. In stems, a significant ^{15}N effect size was found in strangers over kin in one treatment combination only: MN belowground and manual defoliation herbivory (Figure 7). In roots, no differences were found between kin and stranger ^{15}N content, even though the kin mean was significantly different in the ANOVA (

Table 2). LME models of needle, stem and root ^{15}N tissue content explained 50%, 62%, and 77% of the variation in needle, stem, and root ^{15}N tissue, respectively, (Table 4). The residuals were not normally distributed, indicating that some observations had a larger effect on the model than others rendering the models suspect. Many data transformations were attempted, these LME models represent the best possible fit.

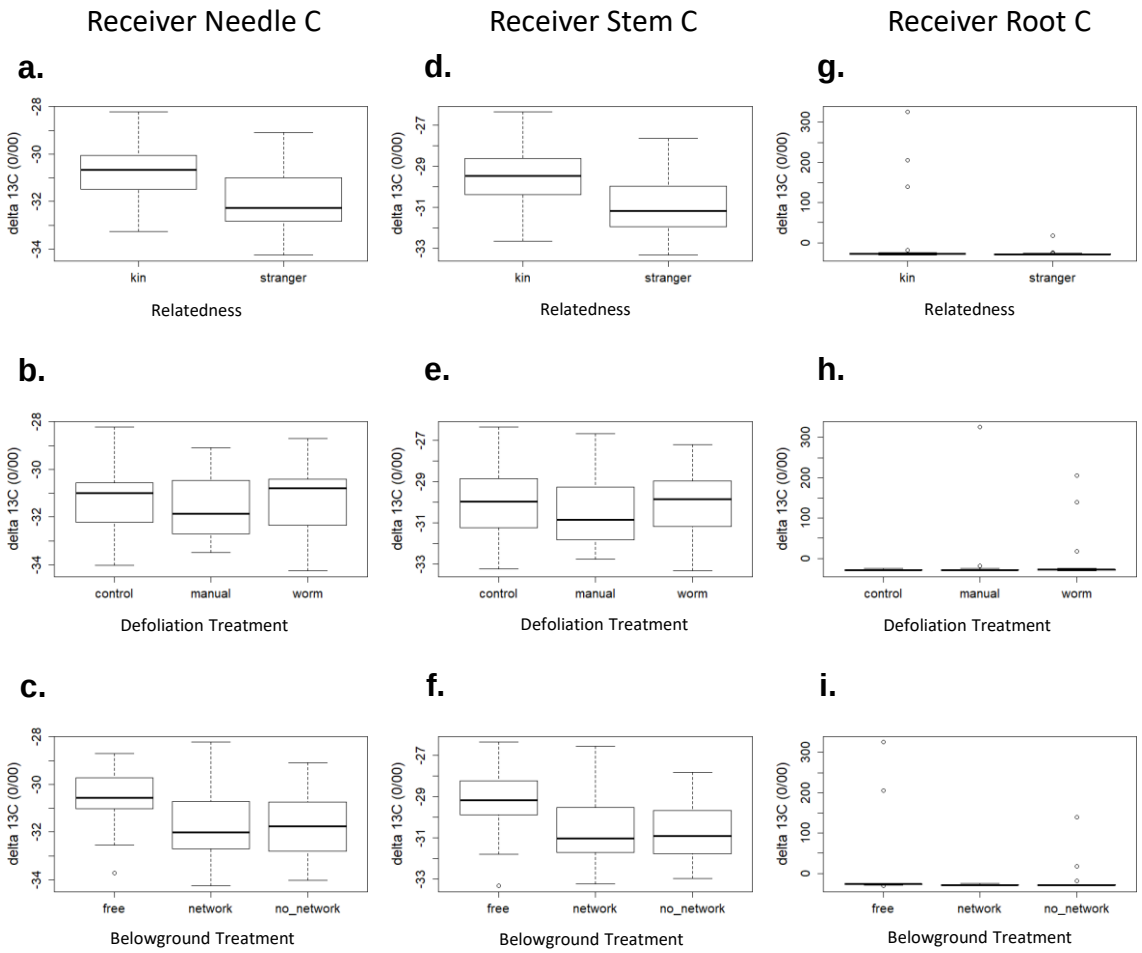

Figure 1. Box and whisker plots of ^{13}C carbon content in receiver tissues, organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle ^{13}C content is shown in the left-most column by a.) relatedness, b.) defoliation treatment, and c.) belowground treatment. Stem ^{13}C content is presented in the same order in the middle column: d.) relatedness, e.) defoliation treatment, f.) belowground treatment; and root ^{13}C is shown in the right-most column, spanning the same set of fixed effects g.) relatedness, h.) defoliation treatment, and i.) belowground treatment.

Table 1. ANOVA table of effect of **relatedness**, **belowground**, and **defoliation** treatments on the movement of pulse-labeled ^{13}C from donor to receiver seedlings grown in pots based on linear mixed-effects modeling of ^{13}C content in roots, stems and needles of receiver seedlings, as determined using the Satterthwaite approximation for degrees of freedom.

Receiver-NeedleC

	df	F-value	p-value
Intercept	1, 84.98	66.432	**0.000**
Covariate-needle	1, 57.38	0.140	0.710
Covariate-root	1, 58.49	3.745	*0.058*
Relatedness	1, 57.38	50.868	**0.000**
Herbivory	2, 57.46	0.674	0.513
Belowground	2, 57.46	22.289	**0.000**
Related x Herb	2, 57.49	1.420	0.250
Related x Below	2, 57.26	2.053	*0.138*
Herb x Below	4, 57.55	2.862	**0.031**

Receiver-StemC

	df	F-value	p-value
Intercept	1, 82.72	50.998	**0.000**
Covariate-needle	1, 58.28	0.437	0.511
Covariate-root	1, 56.72	4.036	**0.049**
Relatedness	1, 57.62	45.907	**0.000**
Herbivory	2, 57.63	0.398	0.674

Belowground	2, 57.87	21.555	**0.000**
Related x Herb	2, 57.85	0.531	0.591
Related x Below	2, 57.35	0.733	0.485
Herb x Below	4, 57.77	3.013	**0.025**

Receiver-RootC

	df	F-value	p-value
Intercept	1, 80.23	107.450	**0.000**
Covariate-needle	1, 55.91	0.016	0.900
Covariate-root	1, 58.40	9.572	**0.003**
Relatedness	1, 56.82	21.844	**0.000**
Herbivory	2, 57.19	0.149	0.862
Belowground	2, 57.16	43.939	**0.000**
Related x Herb	2, 56.87	1.387	0.258
Related x Below	2, 57.05	0.703	0.499
Herb x Below	4, 57.59	1.459	0.227

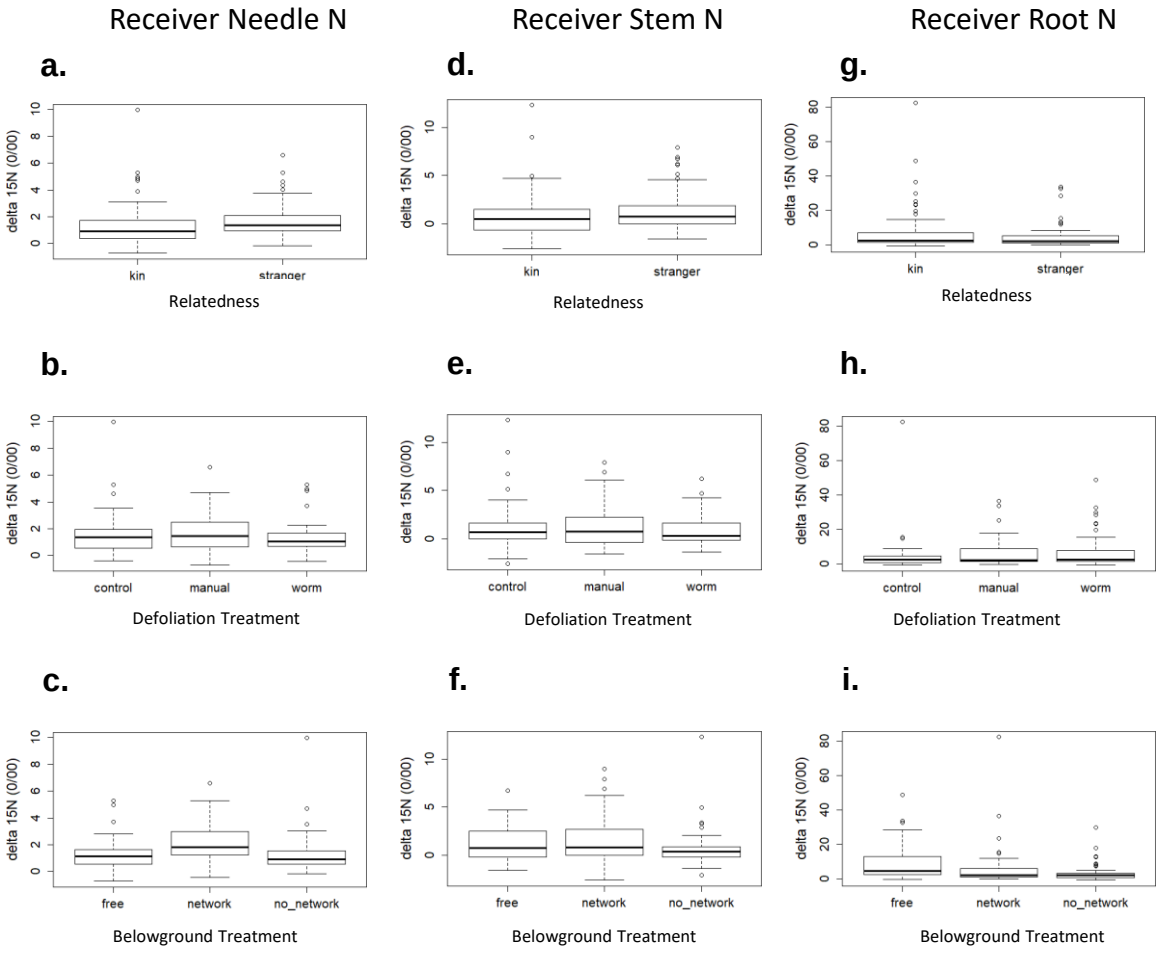

Figure 2. Box and whisker plots of 15N nitrogen content in receiver tissues, organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle 15N content is shown in the left-most column by a.) relatedness, b.) defoliation treatment, and c.) belowground treatment. Stem 15N content is presented in the same order in the middle column: d.) relatedness, e.) defoliation treatment, f.) belowground treatment; and root 15N is shown in the right-most column, spanning the same set of fixed effects g.) relatedness, h.) defoliation treatment, and i.) belowground treatment.

Table 2. ANOVA table of effect of **relatedness**, **belowground**, and **defoliation** treatments on the movement of pulse-labeled ^{15}N from donor to receiver seedlings grown in pots based on linear mixed-effects modeling of ^{15}N content in roots, stems and needles of receiver seedlings, as determined using the Satterthwaite approximation for degrees of freedom.

Receiver-NeedleN

	df	F-value	p-value
Intercept	1, 88.19	3.899	**0.000**
Root Donor N	1, 56.41	20.216	**0.000**
Herbivory Treatment	2, 56.85	0.528	0.592
Belowground	2, 57.05	1.958	*0.151*
Relatedness	1, 56.53	13.156	**0.001**
Herb x Below	4, 56.96	2.918	**0.029**
Herb x Related	2, 56.66	0.446	0.643
Below x Related	2, 56.43	3.410	**0.040**

Receiver-StemN

	df	F-value	p-value
Intercept	1, 72.05	7.004	**0.000**
Root Donor N	1, 69.78	21.976	**0.000**
Herbivory Treatment	2, 53.11	0.050	0.951
Belowground	2, 52.66	0.613	0.545
Relatedness	1, 50.60	6.781	**0.012**
Herb x Below	4, 53.40	2.570	**0.048**

Herb x Related	2, 50.30	2.005	*0.145*
Below x Related	2, 49.90	0.321	0.727

Receiver-RootN

	df	F-value	p-value
Intercept	1, 74.25	3.531	**0.001**
Root Donor N	1, 60.15	51.259	**0.000**
Herbivory Treatment	2, 59.44	0.890	0.416
Belowground	2, 59.17	8.786	**0.000**
Relatedness	1, 58.35	0.527	0.471
Herb x Below	4, 59.36	1.427	0.236
Herb x Related	2, 58.18	1.513	0.229
Below x Related	2, 58.27	0.719	0.491

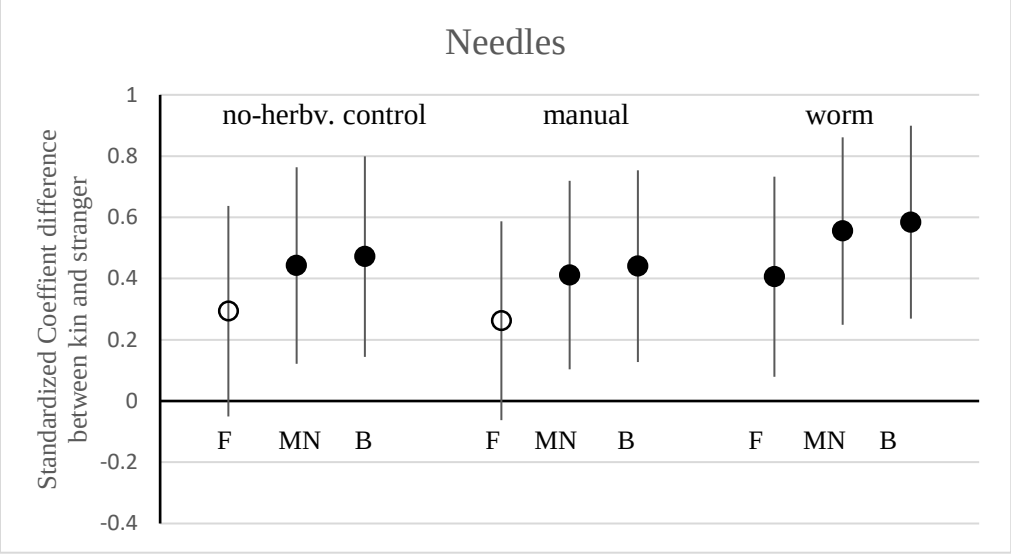

Figure 3. Effect size of seedling relatedness on ^{13}C transfer, as found in needles, between a pulse-labeled donor to a *kin* or *stranger* receiver. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between kin and stranger ^{13}C needle content. Positive values indicate more ^{13}C in kin needles. Error bars indicate the 95% confidence interval. Herbivory treatments are clustered into control, manual, and worm defoliation. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

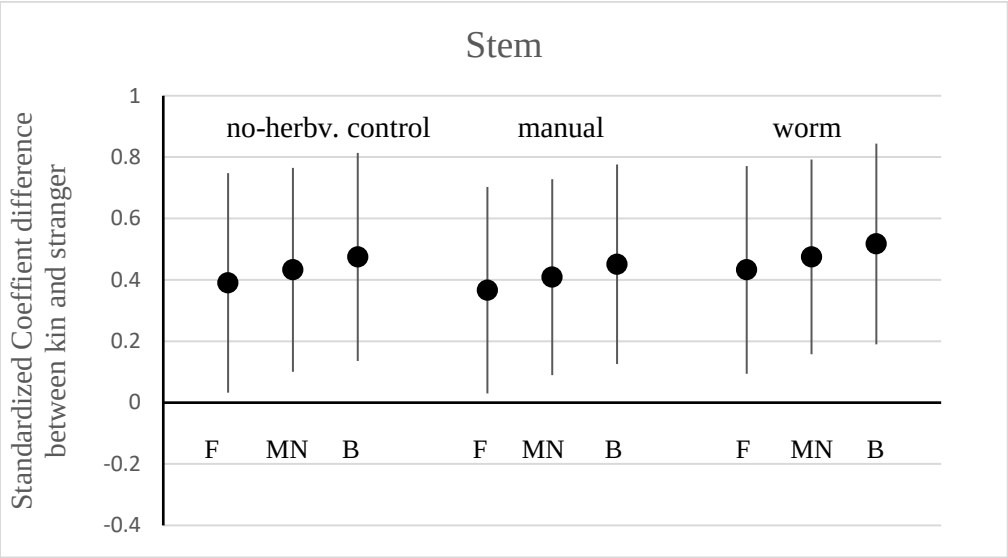

Figure 4. Effect size of seedling relatedness on ^{13}C transfer, as found in stems, between a pulse-labeled donor to a *kin* or *stranger* receiver. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between kin and stranger ^{13}C stem content. Positive values indicate more ^{13}C in kin stems. Error bars indicate the 95% confidence interval. Herbivory treatments are clustered into control, manual, and worm defoliation. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

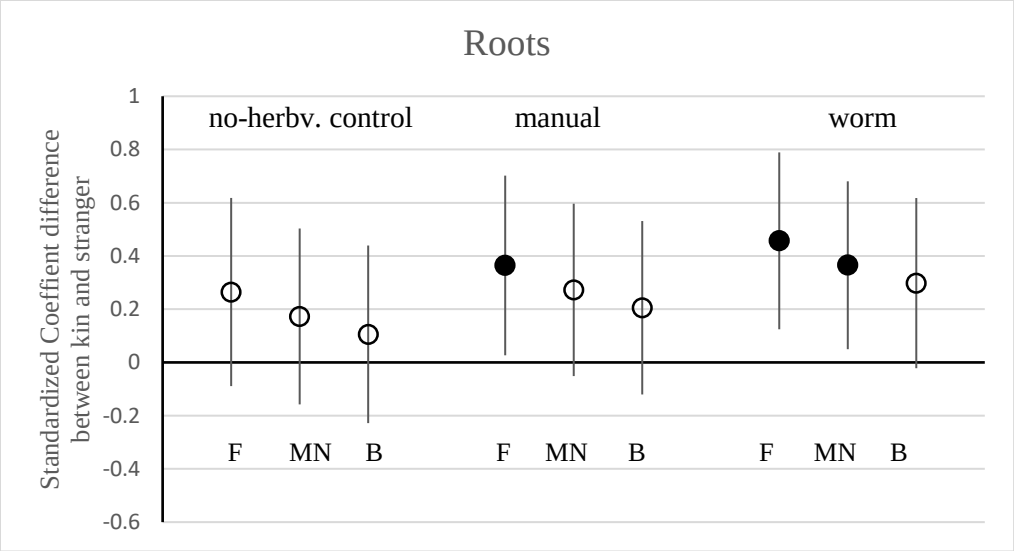

Figure 5. Effect size of seedling relatedness on ^{13}C transfer, as found in roots, between a pulse-labeled donor to a *kin* or *stranger* receiver. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between kin and stranger ^{13}C root content. Positive values indicate more ^{13}C in kin roots. Error bars indicate the 95% confidence interval. Herbivory treatments are clustered into control, manual, and worm defoliation. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

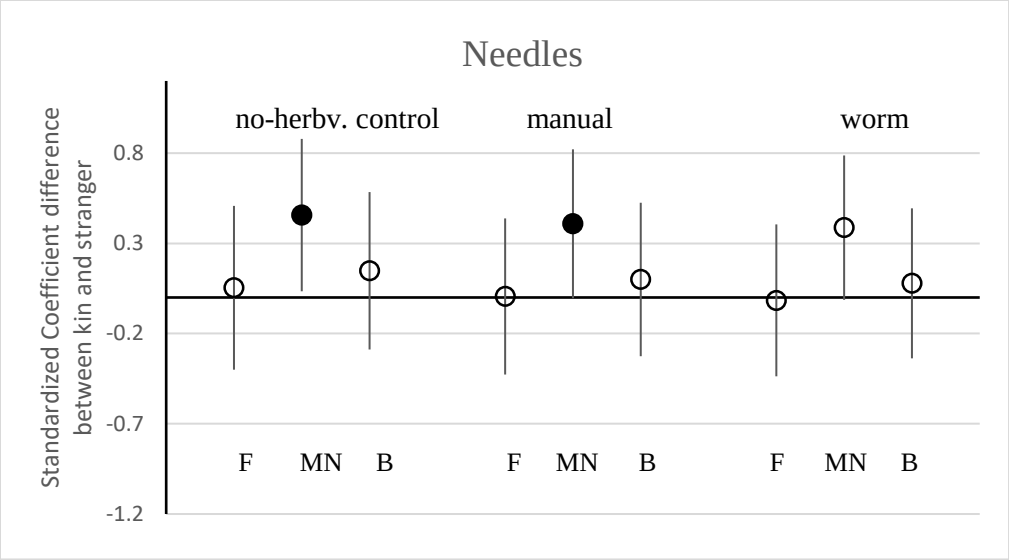

Figure 6. Effect size of seedling relatedness on ^{15}N transfer, as found in needles, between a pulse-labeled donor to a *kin* or *stranger* receiver. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between kin and stranger ^{15}N needle content. Positive values indicate more ^{15}N in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

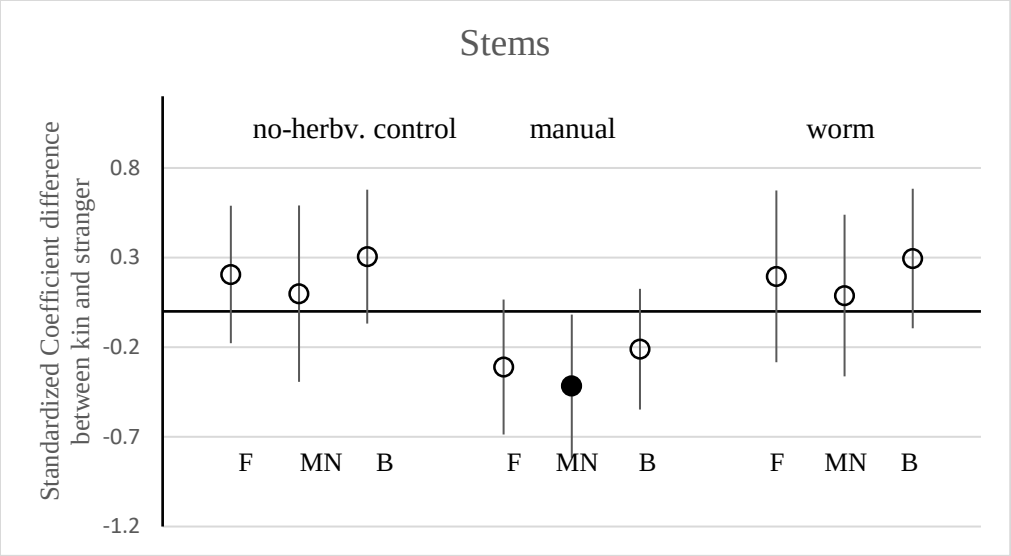

Figure 7. Effect size of seedling relatedness on ^{15}N transfer, as found in stems, between a pulse-labeled donor to a *kin* or *stranger* receiver. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between kin and stranger ^{15}N needle content. Positive values indicate more ^{15}N in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

Table 3. Summary of LME models used to investigate ^{13}C movement from donor to receiver seedlings in response to herbivory treatments when networked, not-networked, and free to interact. Note that the Shapiro-Wilk statistic and associated p-value refer to the test for normality of residuals.

LME model	Marginal R^2	Conditional R^2	Shapiro-Wilk statistic	p-value
Receiver-Needle	0.544	0.568	0.992	0.738
Receiver-Stem	0.516	0.559	0.993	0.791
Receiver-Root	0.560	0.612	0.988	0.396

Table 4. Summary of LME models used to investigate ^{15}N movement from donor to receiver seedlings in response to herbivory treatments when networked, not-networked, and free to interact. Note that the Shapiro-Wilk statistic and associated p-value refer to the test for normality of residuals.

LME model	Marginal R^2	Conditional R^2	Shapiro-Wilk statistic	p-value
Receiver-Needle	0.379	0.500	0.972	0.016
Receiver-Stem	0.321	0.623	0.974	0.028
Receiver-Root	0.514	0.772	0.956	0.001

Experiment 2

Receiver tissue ^{13}C content was significant for relatedness and family, as well as relatedness x family, and belowground x family for both stems and needles. Root ^{13}C content was significant for family and belowground x family, and only marginally significant for relatedness x family (Table 5, Figure 8). The ^{15}N nitrogen data had many outliers (Figure 9), and fewer significant ANOVA results than the ^{13}C carbon data (Figure 8). Family was significant in the ^{15}N needle data, but only marginally significant for stems and roots. Marginal significance was also found for the related x family interaction in both needles and roots, belowground x family in roots, and relatedness in roots only (Table 6). Standardized coefficient differences (*kin* minus *stranger*) of ^{13}C values are presented in Figure 10 (needles), Figure 11 (stems) and Figure 12 (roots); and ^{15}N values are presented in Figure 13 (needles), where significant effect sizes are parsed out between treatment groups.

In needle ^{13}C, significant effect sizes were greater in kin than stranger in all family treatments except family E in the blocked (B) and free (F) belowground treatments (Figure 10). In stems, all belowground treatments resulted in significant effect sizes where kin was greater

than stranger, except in family E in the blocked (B) belowground treatment (Figure 11). In root ^{13}C, only family A resulted in significant effect sizes of kin greater than stranger in the free (F) and MN belowground treatment (Figure 12). LME models of needle, stem and root ^{13}C tissue content explained 80%, 80%, and 65% respectively of the variation in the data (Table 7); residuals were normally distributed.

In needle ^{15}N, significant effect sizes were found to be greater in stranger than kin needles in the MN (mycorrhizal network) belowground treatment in Family A only (Figure 13). Neither stem nor root showed any other significant effect sizes for the treatment combinations, despite some significant interactions in the ANOVA table (Table 5). LME models of needle, stem and root ^{15}N tissue content explained 59%, 66%, and 65% of the variation in needle, stem, and root ^{15}N tissue, respectively, (Table 8). The residuals were normally distributed for roots, but not for needles and stems indicating that for those tissues, some observations had a larger effect on the model than others rendering the models suspect. Many data transformations were attempted, these LME models represent the best possible fit.

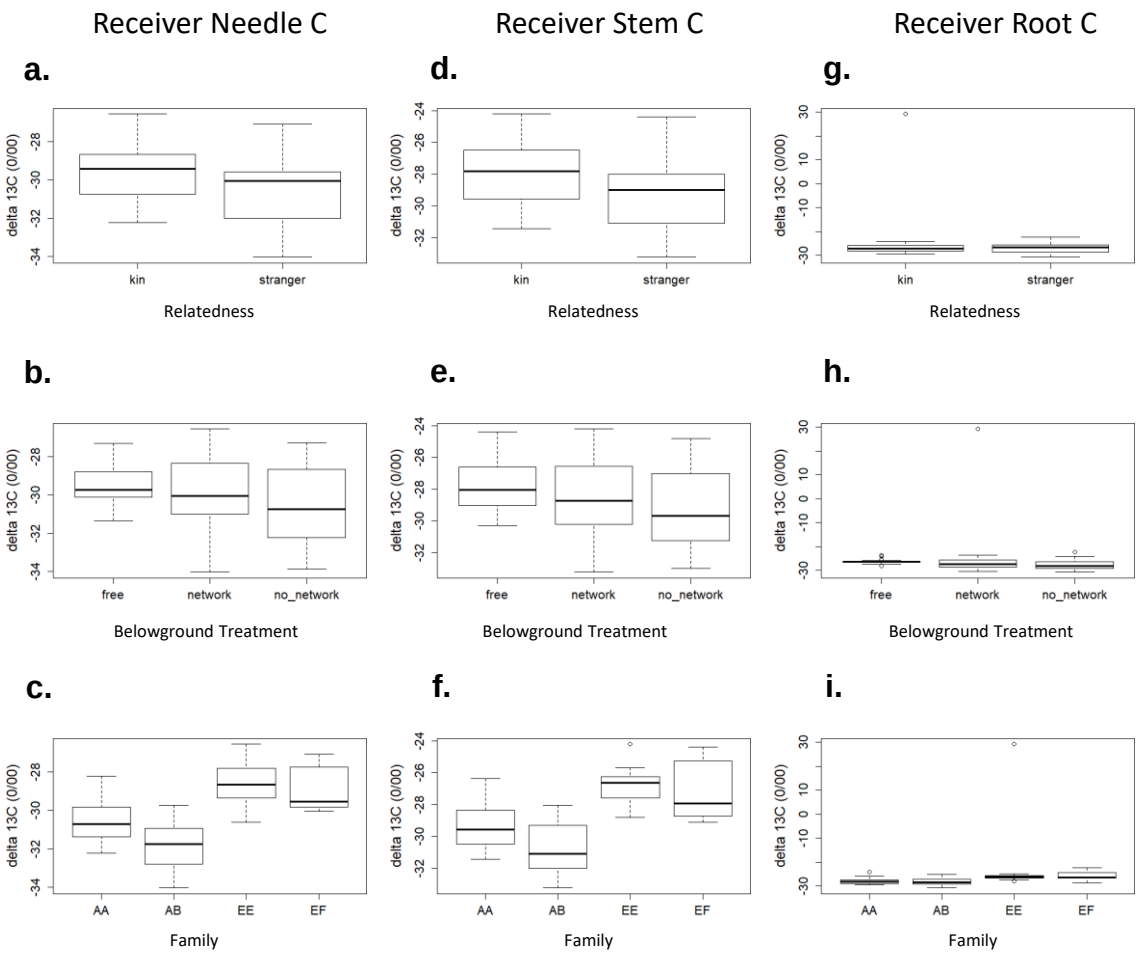

Figure 8. Box and whisker plots of ^{13}C carbon content in receiver tissues in Experiment 2 with no defoliation treatments applied (all experimental units equivalent to herbivory-control), organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle ^{13}C content is shown in the left-most column by a.) relatedness, b.) belowground treatment, and c.) family. Stem ^{13}C content is presented in the same order in the middle column: d.) relatedness, e.) belowground treatment, f.) family; and root ^{13}C is shown in the right-most column, spanning the same set of fixed effects g.) relatedness, h.) belowground treatment, and i.) family.

Table 5. ANOVA table of effect of **relatedness**, **belowground**, and **family** on the movement of pulse-labeled ^{13}C from donor to receiver seedlings grown in pots based on linear mixed-effects modeling of ^{13}C content in roots, stems and needles of receiver seedlings, as determined using the Satterthwaite approximation for degrees of freedom.

Receiver-NeedleC

	df	F-value	p-value
Intercept	1, 37.88	48.646	**0.000**
Donor needle C	1, 28.91	0.209	0.651
Relatedness	1, 28.74	13.237	**0.001**
Belowground	2, 29.13	0.368	0.695
Family	1, 29.10	39.149	**0.000**
Related x Below	2, 28.78	0.727	0.492
Related x Family	1, 28.70	4.194	**0.050**
Below x Family	2, 29.12	4.029	**0.029**

Receiver-StemC

	df	F-value	p-value
Intercept	1, 38.48	36.934	**0.000**
Donor needle C	1, 30.35	0.469	0.499
Relatedness	1, 29.17	13.974	**0.001**
Belowground	2, 29.84	0.358	0.702
Family	1, 30.03	38.629	**0.000**
Related x Below	2, 29.23	1.871	0.172
Related x Family	1, 29.07	3.172	**0.085**
Below x Family	2, 29.88	5.687	**0.008**

Receiver-RootC

	df	F-value	p-value
Intercept	1, 42.35	39.433	**0.000**
Donor needle C	1, 27.53	0.380	0.543
Relatedness	1, 27.62	0.005	0.943
Belowground	2, 27.92	1.646	0.211
Family	1, 27.44	23.631	**0.000**
Related x Below	2, 27.49	2.910	*0.071*
Related x Family	1, 27.71	3.380	*0.077*
Below x Family	2, 27.95	7.367	**0.003**

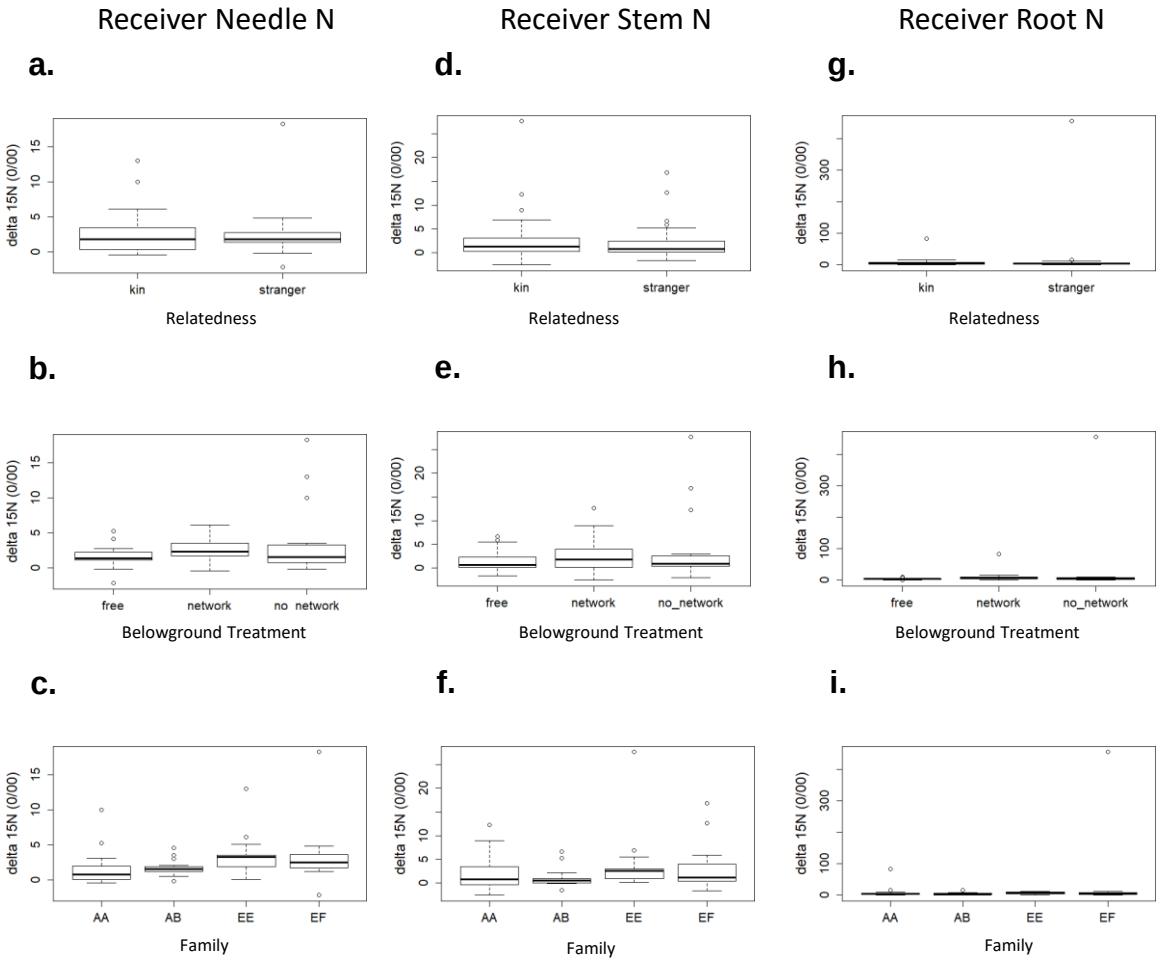

Figure 9. Box and whisker plots of ^{15}N nitrogen content in receiver tissues in Experiment 2 with no defoliation treatments applied (all experimental units equivalent to herbivory-control), organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle ^{13}C content is shown in the left-most column by a.) relatedness, b.) belowground treatment, and c.) family. Stem ^{13}C content is presented in the same order in the middle column: d.) relatedness, e.) belowground treatment, f.) family; and root ^{13}C is shown in the right-most column, spanning the same set of fixed effects g.) relatedness, h.) belowground treatment, and i.) family.

Table 6. ANOVA table of effect of **family**, and **belowground** treatments on the movement of pulse-labeled ^{15}N from donor to receiver seedlings grown in pots based on linear mixed-effects modeling of ^{15}N content in roots, stems and needles of receiver seedlings, as determined using the Satterthwaite approximation for degrees of freedom.

Receiver-NeedleN

	df	F-value	p-value
Intercept	1, 42.350	39.433	**0.000**
Donor needle C	1, 29.00	0.550	0.464
Relatedness	1, 29.00	0.719	0.404
Belowground	2, 29.00	1.581	0.223
Family	1, 29.00	7.932	**0.009**
Related x Below	2, 29.00	1.065	0.358
Related x Family	1, 29.00	3.089	*0.089*
Below x Family	2, 29.00	0.650	0.529

Receiver-StemN

	df	F-value	p-value
Intercept	1, 42.350	39.433	**0.000**
Donor needle C	1, 25.49	0.589	0.450
Relatedness	1, 24.86	0.014	0.906
Belowground	2, 26.44	0.881	0.426
Family	1, 26.37	2.320	*0.140*
Related x Below	2, 24.83	0.915	0.414
Related x Family	1, 24.66	0.351	0.559
Below x Family	2, 26.39	1.645	0.212

Receiver-RootN

	df	F-value	p-value
Intercept	1, 37.88	4.499	**0.000**
Donor needle C	1, 29.63	1.588	0.217
Relatedness	1, 29.36	2.217	*0.147*
Belowground	2, 30.63	4.582	0.018
Family	1, 30.57	2.820	*0.103*
Related x Below	2, 29.51	0.820	0.450
Related x Family	1, 29.78	1.996	*0.168*
Below x Family	2, 30.61	2.171	*0.131*

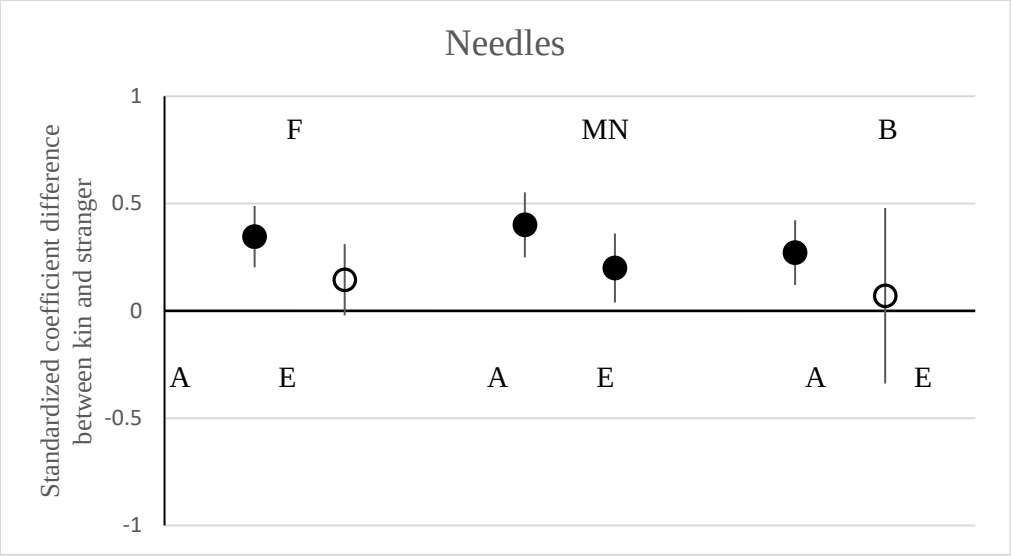

Figure 10. Standardized coefficient difference in receiver seedling needle ^{13}C content in *kin* (A to A or E to E) vs *stranger* (A to B or E to F) seedlings. All seedling are herbivory controls. Positive values indicate more ^{13}C in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

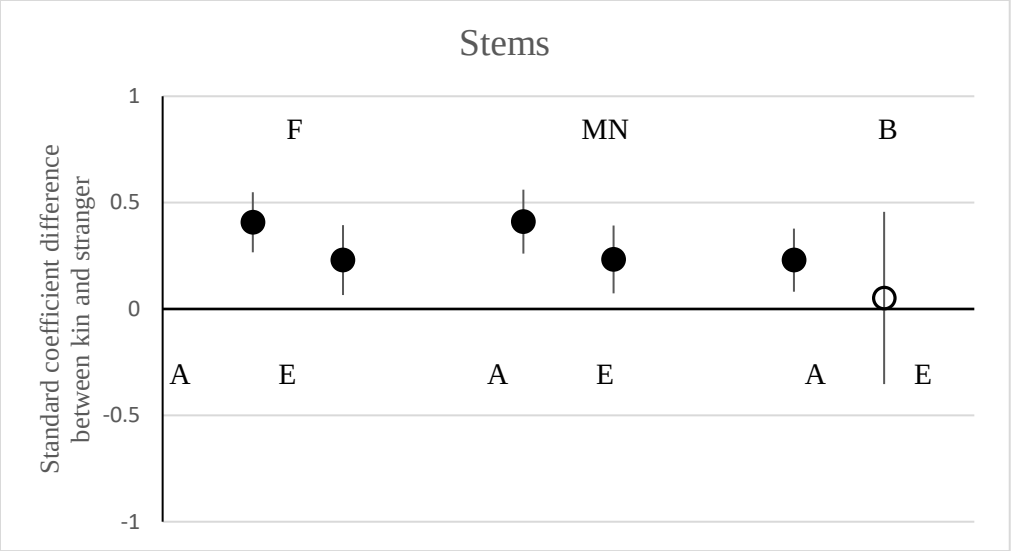

Figure 11. Standardized coefficient difference in receiver seedling stem ^{13}C content in *kin* (A to A or E to E) vs *stranger* (A to B or E to F) seedlings. All seedling are herbivory controls. Positive values indicate more ^{13}C in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

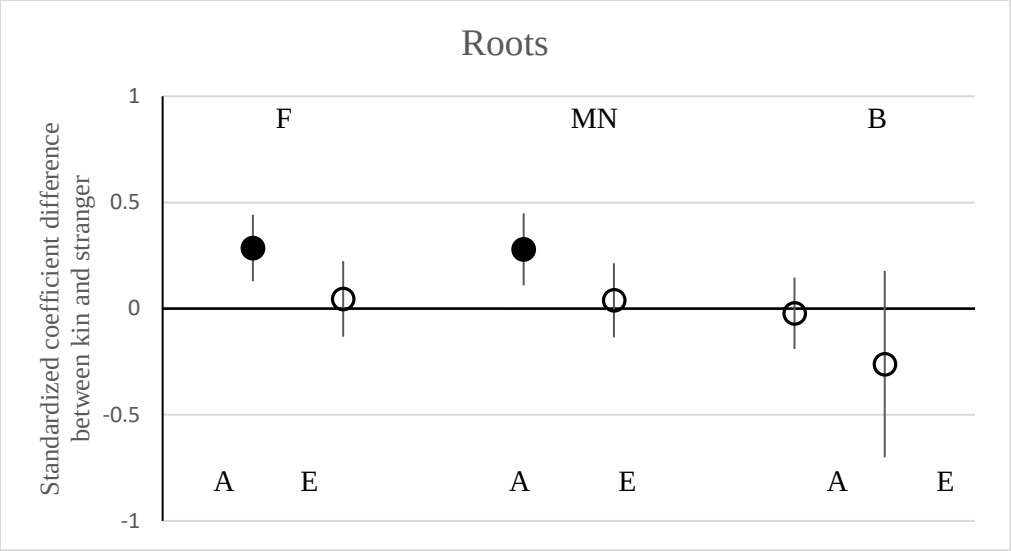

Figure 12. Standardized coefficient difference in receiver seedling root ^{13}C *kin* (A to A or E to E) vs *stranger* (A to B or E to F) seedlings. All seedling are herbivory controls. Positive values indicate more ^{13}C in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

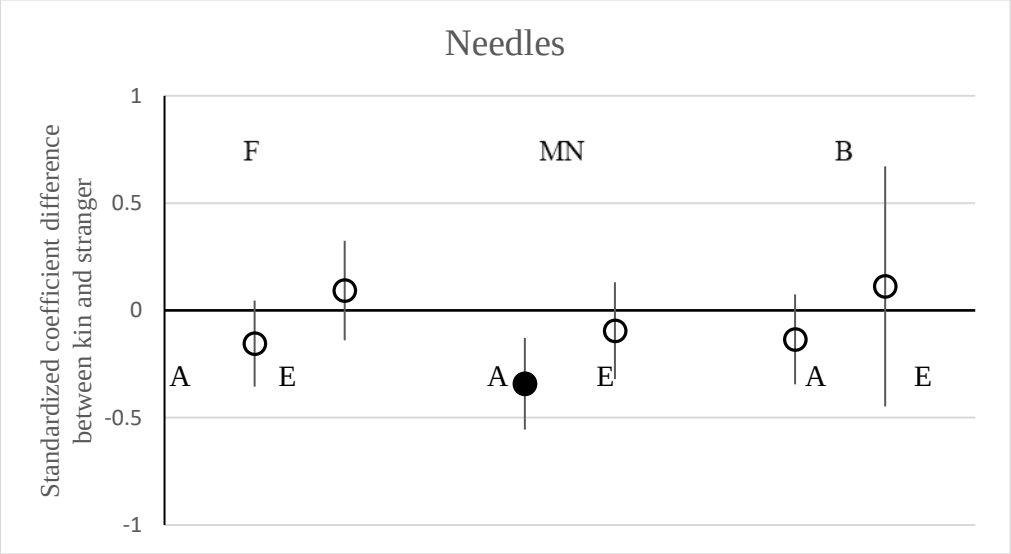

Figure 13. Standardized coefficient difference in receiver seedling needle ^{15}N content in *kin* (A to A or E to E) vs *stranger* (A to B or E to F) seedlings. All seedling are herbivory controls. Positive values indicate more ^{15}N in kin needles. Error bars indicate the 95% confidence interval. F= free to interact (no bag); MN=Mycorrhizal network; B=blocked. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

Table 7. Summary of LME models used to investigate ^{13}C movement from donor to receiver seedlings in response **relatedness** and **family** treatment with **belowground** treatments of *MN*, *F*, and *B*. Note that the Shapiro-Wilk statistic and associated p-value refer to the test for normality of residuals.

LME model	Marginal R^2	Conditional R^2	Shapiro-Wilk statistic	p-value
Receiver-Needle	0.646	0.804	0.988	0.830
Receiver-Stem	0.681	0.804	0.983	0.602
Receiver-Root	0.564	0.652	0.970	0.158

Table 8. Summary of LME models used to investigate ^{15}N movement from donor to receiver seedlings in response **relatedness** and **family** treatment with **belowground** treatments of *MN*, *F*, and *B*. Note that the Shapiro-Wilk statistic and associated p-value refer to the test for normality of residuals.

LME model	Marginal R^2	Conditional R^2	Shapiro-Wilk statistic	p-value
Receiver-Needle	0.297	0.591	0.959	0.050
Receiver-Stem	0.220	0.662	0.958	0.047
Receiver-Root	0.375	0.658	0.983	0.591

Discussion

In these experiments, ^{13}C-CO_2 was used to track a photosynthetic product synthesized by the donor seedling, through a mycorrhizal network, to either or both kin and stranger seedlings residing in the same pot. Simultaneously, a donor branch was immersed in a solution of ^{15}N-ammonium nitrate for the same duration, to allow for tracking of nitrogen movement in the system. With the application of herbivory (defoliation) treatment, this pulse-chase experiment was designed with the idea that a mycorrhizal network may act as a conduit for the transfer of ^{13}C and ^{15}N tracer molecules, representing, potentially, a transfer of communication molecules. A significant effect size represents the difference between the amount of 13C or 15N received by kin over stranger, with positive values indicating more transfer to kin, with a 95% confidence interval.

The overall hypothesis, that there would be preferential ^{13}C transfer to kin over stranger through MNs in response to herbivory, is supported. With some exceptions and nuance, which

are discussed below, there was significantly more ^{13}C in kin over strangers with a significant ANOVA result (Table 1). Of the hypotheses, the first that there would be a constitutive small amount of transfer regardless of treatment is also accepted. In the ^{13}C needle data, most treatment combinations resulted in a significant effect size that was greater in kin over strangers (Figure 3). The second hypothesis that transfer between kin and stranger would not differ between manual and worm defoliation treatments is also supported, as in almost all cases (with the exception of ^{15}N stems-Figure 7), effect sizes were very similar in both manual and worm defoliation, and followed similar patterns, even if some were not significant (as in Figure 5, similar pattern between manual and worm defoliation, however, only the worm-MN treatment combination is significant). The third sub-hypothesis that transfer would occur through the MN and not through the blocked (B) and free (F) belowground treatments was not supported as there was no clear pattern and transfer occurred in many B and F cases. Finally, the hypothesis that all families would behave in the same manner is also rejected. Family E showed different ^{13}C and ^{15}N patterns. Furthermore, Family E and F transferred more than Family A and B (Figure 1).

Kin recognition

Greater transfer of ^{13}C and ^{15}N to kin over strangers was found in all linear mixed-effects models within these Douglas-fir greenhouse experiments. In the first experiment, the only exception to this was stem ^{15}N (Figure 7), and then only in the MN belowground treatment with manual defoliation. In the second experiment, Family E showed significant effect sizes less often than Family A, however, the assimilation of ^{13}C was not equal between families (Figure 1). However, in the needle ^{13}C (Figure 10), family E had a significant effect size to kin over stranger in the MN belowground treatment, but not in the F or B treatments. Furthermore, the effect size was smaller than that of family A. Comparing the two families under these conditions suggests that both families preferentially transfer ^{13}C to kin over stranger, however, family A tends to transfer more. That tendency to transfer more can also be seen with significant effect sizes in the F and B belowground treatments, whereas these treatments do not produce a significant effect size in family E.

Kin selection in plants has been identified in Douglas-fir (Pickles *et al.*, 2017), Great Lakes sea rocket (*Cakile edentula*) (Dudley & File, 2007), and the model plant *Arabidopsis thaliana* (Biedrzycki *et al.*, 2010; Crepy & Casal, 2015), for example. There is increasing

evidence of kin recognition in plants (File *et al*., 2012b). Regardless of the complexity of the responses to the different treatments tested, consistent preferential transfer to kin over stranger under many treatment combinations in this experiment adds evidence to support the ability of Douglas-fir to recognize and respond to kin, implying that kin recognition is indeed occurring within these trees.

The present study found differences between the amounts of isotope transferred between different families of Douglas-fir, all of which are from the same provenance, and thus all families are assumed to have experienced similar historical environmental conditions. Differences in the ability of families to transfer more C to kin versus strangers are thus unlikely due to having experienced vastly different environments. Pickles *et al.*, (2017) also found differences in the abilities of different Douglas-fir families to transfer photosynthetically derived ^{13}C. This evidence suggests that Douglas-fir is capable of kin recognition, but it may not be advantageous to be responsive to kin under all conditions. The differential abilities of the seedlings in this study and in the Pickles *et al.*, (2017) study may reflect a legacy of interactions. Douglas-fir are long-lived. Evolutionary changes recorded in the genome cannot be responsive to shorter-term conditions and smaller scale environmental legacies. It could be that the capacity to respond to kin exists within the genetic makeup of Douglas-fir, however, the expression of that capacity may vary.

Western spruce budworm herbivory

Interestingly, in the free (F) to interact treatment, there was a ^{13}C significant effect size with kin greater than stranger only under western spruce budworm defoliation, and not manual defoliation or control (Figure 3). This result implies that there is something unique about the western spruce budworm chewing that elicits a response that cannot be induced by physical damage to needles. This ability of a plant to discriminate based on the identity of the defoliator has been demonstrated in *Arabidopsis* (Stotz *et al.*, 2000). This effect is observable only in the free treatment, which, most closely resembles natural conditions.

Mycorrhizal networks

The belowground treatments did not function as expected in this study. Using mesh bags of different pore sizes to control hyphae and root interactions has been demonstrated to be effective in isotope transfer studies (Teste *et al.*, 2010; Philip *et al.*, 2010), although Pickles *et al.*, (2017) found transfer in the presence of mycorrhizal network-blocking (B) mesh. No difference in terms

of transfer of ^{13}C was observed between the MN and blocked (B) belowground treatments in needles (Figure 3) and stems (Figure 4). Occasionally, significant differences were observed (e.g. in roots, Figure 5) where transfer from donor to receiver plants was affected by worm defoliation treatment in the MN and free (F) belowground treatments, but not in the root and hyphal blocking (B) mesh. It is surprising that this effect is not replicated in the stem and needle ^{13}C SES figures. Mesh bags that restrict root and hyphal growth may permit transfer nonetheless, especially in this type of experiment where the seedlings are grown within mesh bags from seed. Roots are very strong (Tosi, 2007), and given enough time, may have penetrated the bags. Furthermore, roots and hyphae were pressed up to the sides of the bags when the seedlings were harvested, it is therefore possible that both roots and hyphae may have been transferring exudates across the thin mesh bag membrane by virtue of close proximity, however, it is more likely that mesh bags were compromised by roots penetrating them. A more robust system, like that in Babikova *et al.*, (2013), would employ a sturdy metal cylinder that could be rotated to cut off any hyphae or thin roots that may have grown across the intended barrier.

It has long been known that ectomycorrhizal fungi forage for nitrogen to trade for plant-derived photosynthates in the symbiosis (Smith & Read, 2008). Indeed, nitrogen structures communities of ectomycorrhizal fungi (Cox *et al.*, 2010), and impacts soil carbon sequestration (Averill *et al.*, 2014). While carbon and nitrogen dynamics are tightly linked (insufficient nitrogen will reduce primary productivity), in this experimental system they do not appear to follow each other under all conditions. The stable isotope of nitrogen used in this system was much more variable in its dynamics than the carbon isotope, the data was not normally distributed (carbon data was normally distributed), and the models resulting from this data are thus less reliable at describing what was happening in the system. This difference in and of itself points to complicated nitrogen movement, potentially mediated by ectomycorrhizas and driven by processes not controlled for nor considered in these experiments. Nitrogen movement may be governed by hierarchical rules depending on resource availability, competition and network status (Croft *et al.*, 2012), which were not explicitly tested in this experiment.

The aim of this study was to find whether there was evidence for plant communication in conifers via the transfer of carbon and/or nitrogen through mycorrhizal networks. Examining the movement of both nitrogen and carbon in the system simultaneously indicates that nitrogen transfer did not follow the same pattern as carbon transfer, which could indicate that a

nitrogenous compound was not involved in signaling. For example, the compound of interest is unlikely to be systemin, which is a mobile peptide-based information molecule involved in wound response (Bowles, 1998; Wisniewska *et al.*, 2003), because, as a peptide, it contains nitrogen. Granted, insect feeding and wounding produce very complex reactions in plants (Gatehouse, 2002), and nitrogen containing molecules may nonetheless play a role and simply be undetectable with this coarse application of stable isotopes. It can at least be confirmed that the movement of nitrogen is not tightly coupled to carbon, and while that does not exclude a nitrogenous signaling compound (as trace amounts might be masked by the technique), it suggests this is less likely.

Conclusion

In conclusion, these experiments show that there is evidence of a higher rate of transfer of ^{13}C to kin. Furthermore, this transfer can occur without herbivory stimulus, that is, there is evidence of a greater constitutive transfer to kin over strangers. Both manual and western spruce budworm defoliation resulted in a higher rate of uptake by kin over stranger, and effect size did not differ significantly between the two treatments, in most cases. Transfer was predicted to occur only when mycorrhizal connections were permitted, in the MN belowground treatment, and at a lower rate in the free (F) treatments, however, evidence of transfer was also found in the blocked (B) belowground treatments. And finally, Douglas-fir families appear to differ in their ability to transfer carbon and nitrogen through MNs, implying that while they may be capable of kin selection, they express this ability differently depending on family identity.

Chapter 3: Kin recognition and herbivory field experiment

Introduction

Mycorrhizal networks (MNs) can serve as agents for plant communication belowground and mediate complex interactive behaviour of plants (Gorzelak *et al.*, 2015). In addition to resource transfer (Simard *et al.*, 1997c; Klein *et al.*, 2016), defense chemicals are thought to travel through MNs between different plant hosts, eliciting differential defense responses to insect and pathogen attacks compared with healthy networked plants (Song *et al.*, 2010, 2015; Babikova *et al.*, 2013). There is evidence to show that mycorrhizal association are an extended phenotype of plants which depend on relatedness (File *et al.*, 2012a). Plants respond to belowground resources, but also integrate that resource-space knowledge with neighbor presence (Cahill *et al.*, 2010), modifying their competitive abilities depending on identity of neighbor (Semchenko *et al.*, 2007) and also relatedness in the case of conspecifics (Kelly, 1996; Semchenko *et al.*, 2007; File *et al.*, 2012a). In previous work (Chapter 2), I demonstrate that greater C and N transfer to kin over strangers through MN in Douglas-fir seedlings in greenhouse pots. However, it is still not known whether such transfers occur in nature.

Several studies have demonstrated resource transfer through mycorrhizal networks (MN) in the field. For example, Teste *et al.*, (2010) showed that soil disturbance affected carbon transfer differently if trees were planted or regenerated naturally, with implications for forest regeneration and planting practices. Simard *et al.*, (1997b) showed that paper birch transferred 10% of its carbon to shaded Douglas-fir neighbors, an amount that is energetically sufficient for seed production, but only 1% when the neighbours were in full sun. Field studies have also shed light on other ecological factors regulating transfer through MNs. For instance, Philip, (2006) and Lerat *et al.*, (2002) both found evidence for seasonal bi-directional transfer in response to phenology in temperate forests. A recent paper by Klein *et al.*, (2016) demonstrated the importance of MN to resource transfer in mature trees. Using stable isotope analysis with ^{13}C depleted CO_2 applied at the canopy scale, these authors demonstrated that 40% of carbon photosynthesized by a spruce tree was transferred to adjacent heterospecific trees through mycorrhizal networks. This study demonstrates that carbon transfer was of sufficient magnitude to be considered ecologically important to the productivity of the trees. While resource transfer

through mycorrhizal has been demonstrated in forests, the potential for defense signal transfer has yet to be tested in the field.

Testing the potential for MN-mediated chemical information transfer and kin favouring such transfer in the field is needed to verify that this phenomenon occurs *in situ* and is ecologically relevant to plant communities. Douglas-fir forests are ideal for systems for field experiments owing to their extensive distributions in the Pacific Northwest (from Mexico to northern British Columbia) (Hermann & Lavender, 1999; Lavender & Hermann, 2013), high genetic variability (S*t Clair et al*., 2005), potential for adaptation to a changing climate (Aitken *et al.*, 2008) and importance to the economy (Barnes, 2015). Evidence of the capacity for Douglas-fir to recognize kin could have implications for harvesting, planting and management practices for this species. The importance of MNs to Douglas-fir forest structure and self-organization has been discussed (Simard, 2009), and this forms the basis for further questions regarding the impacts of genetic relationships among trees connected together in MNs. If kin selection in Douglas-fir facilitates growth and health through resource sharing and defense signal transfer with neighbours, then simple modification to forestry practices, such as retaining old trees for their seed sources and planting families together in patches, may result from this work. Indeed, demonstrating the potential for defense and information chemical transfer in a field setting is an important addition to the greenhouse study described in Chapter 2.

Accomplishing a field test of signal transfer in response to herbivory stress between MN linked Douglas-fir was not without challenge. The use of live defoliating insects was determined to be too risky to the natural environment due to potential escapees causing deleterious effects. As a result, this field experiment focuses on manual rather than budworm defoliation, even though an earlier result indicated a muted response to manual compared with budworm defoliation. Chapter 2 results demonstrated that western spruce budworm defoliation could elicit a greater transfer response than manual defoliation in some cases (Figure 3: more ^{13}C was transferred to kin than stranger neighbours needles from budworm-treated compared to manually-treated donors through the free belowground pathway; and Figure 5: more ^{13}C was transferred to kin than stranger needles from budworm than manually defoliated donors through the MN belowground pathway), and may elicit a targeted defense response in Douglas-fir (see Chapter 4), that might not be replicated by manual defoliation. Furthermore, greenhouse grown seedlings were required in the field, versus seed origin plants in the greenhouse study, in order to

increase survival in the field and to manipulate Douglas-fir of known families to test kin recognition.

Using pairs of *kin* and *strangers* (**relatedness** treatment) planted in the field, this experiment is analogous to Chapter 2, and considers **belowground** (*MN* and *B*) and **defoliation** treatments (*manual* and *no-herbivory control*) to address the central hypothesis outlined below. 1) The hypothesis of the present study was that belowground carbon and nitrogen transfer to kin Douglas-fir seedlings would be greater to kin than to strangers in response to simulated herbivory stress (manual defoliation) in the field. Furthermore, 2) Would carbon transfer be greater in response to manual defoliation as compared to no-herbivory control plants?, and 3) greater between plants with *MN* **belowground** treatment over the *blocked (B)*? And finally, 4) would all **families** tested exhibit similar patterns?

Methods

A 2 x 2 x 10 fractional factorial set of treatments with two **belowground** treatments (*mycorrhizal network (MN)* and *blocked roots and fungal hyphae (B)*), two **defoliation** treatments (*no-herbivory control* and *manual defoliation*) and 10 combinations of 4 *kin* (*AA, BB, CC, DD*) and 6 *stranger* pairs (*AB, AC, AD, BC, BD, CD*), were replicated four times in a completely randomized design for a total of 160 seedling pairs. Full sibling Douglas-fir seedlings were grown from seeds obtained from the British Columbia Ministry of Forests, Land, and Natural Resource Operations (MFLNRO) at the Kalamalka Research Forest Station in a greenhouse in cone-tainers™ (Stuewe and Sons, Inc, Tangent Oregon) containing 75% peat, and 25% perlite potting mix (West Creek Farms, Fort Langley, BC, Canada). A fine gravel layer was applied to soil surfaces to prevent "damping-off" fungus. Once germinated and established, seedlings were grown in the greenhouse in an 8hr dark, 16hr light cycle with natural day length supplemented by artificial light. Seedlings were placed outside to harden off for 6 weeks prior to planting in the field in late April and early May 2016. Nine-month old seedlings were planted into a 3 year-old cutblock near Revelstoke, BC (50°50'33.4"N 118°03'04.8"W). This site was located approximately 6.5km down the Akokolux Forest Service road at the end of Airport Road in Revelstoke BC, with permission from MoFLNRO in partnership with Stella-Jones (Revelstoke, BC) who held tenure over the cutblock. This block had been planted 3 years prior with cedar, larch, ponderosa pine, and Dougas-fir. Early succession species were found in this study site

including: naturally regenerating *Rosa woodsii*, *Vaccinium* sp., and *Chamaenerion angustifolium* in addition to the planted seedlings. Naturally regenerating seedlings did not appear to be a significant source of seedlings at this site. The site was located within the Interior Cedar-Helmlock biogeoclimatic zone, very wet variant. It was chosen for its climactic variables, specifically, significant rainfall, which was expected to facilitate survival of transplanted seedlings during the critical first season. Seedlings were removed from cone-tainers, placed into either 35µm (*MN*) or 0.5µm mesh bags (*B*) (Plastok® Ltd. Birkinhead, UK), extra space in the mesh bags was filled with onsite soil, and seedlings in mesh bags were then planted in pairs (separated by not more than 30cm between seedlings) with a minimum 5m separation between experimental units. Seedling pairs received the manual defoliation or control treatments immediately prior to being pulse-labelled and were destructively harvested in late September and early October 2016.

Pulse-labelling

Pulse-labelling was performed by sealing the donor seedling in a 50cm by 30cm plastic bag (FoodSaver®) in which an injection valve had been installed. Each bag was filled with ambient air to capacity, sealed completely at the base of the stem using Plastalina modelling clay (Craft Smart®, Irving Texas USA) and Tuck® Contractors sheathing tape (Cantech, Montreal QC, Canada) and tested for air tightness by gently squeezing the bag. A wooden gardening stake was attached to the bag with Tuck tape to provide additional support and prevent the entire weight of the bag from being supported by the stem alone. Only air-tight bags were used and each donor seedling received 50ml of 99 atom% ^{13}C-CO_2 (Cambridge Isotopes Laboratory, Inc. Tewksbury, MA, USA) before being allowed to photosynthesize for 24 hours.

Each donor seedling was additionally provided with 1mL of 20mM ^{15}N-ammonium nitrite (Cambridge Isotopes Laboratory, Inc. Tewksbury, MA, USA) in the form of a 1.5mL microcentrifuge tube taped securely to a healthy branch, with needles dipped into the labelling liquid. The ^{15}N label was allowed to absorb for the same 24 hour period as the ^{13}C-CO_2 label. Seedlings were bagged and pulse-labelled at different times throughout the day. The time of the pulse was noted and converted into a categorical variable (categories spanned 2 hour blocks of time throughout the day). These categories were later used during analysis as the random variable in LME models, in an effort to account for possible variation in the response variable due to the timing of the treatment application/pulse-labelling (e.g. diurnal processes and weather

related variation such as changes in the solar irradiance reaching seedlings) that might influence photosynthetic efficiency.

Seedling sampling and elemental analysis

Immediately after the 24 hour pulse labelling period, seedlings were destructively harvested, placed in plastic freezer bags and transported to the lab in coolers. Seedlings were stored in a cold room until processed, for a maximum of 3 months. Roots were separated from aboveground biomass, and needles were separated from stems. Root tips were harvested for mycorrhizal DNA extraction (see Chapter 5) and stored at -20°C. Roots, needles, and stems were dried in a 60°C oven for 3 days (until no change in mass). For carbon and nitrogen isotopic content analysis, samples of each tissue type were ground in liquid nitrogen and then transferred to tin cups, using 3mg sub-samples for low enrichment plants (receiver seedlings) and 2mg sub-samples for high enrichment plants (donor seedlings). Carbon and nitrogen isotopic content was determined using combustion analysis with elemental analysis (Elementar, Hanau, Germany), in C, N mode, paired with an isotope ratio mass spectrometer (IRMS, Isoprime, Cheadle, UK). The C or N isotope ratio ($\delta^{13}C$ or $\delta^{15}N$) was calculated as:

(eq. 1) $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1)$

Where $R_{standard}$ = Vienna-PeeDee Belemnite (VPDB) standard (1.1237×10^{-2}) for C, and N_2 atmospheric gas (3.677×10^{-3}) for N.

Statistical analysis

All statistical analyses were performed in R 3.3.3 (R core team, 2017). Linear mixed effects (LME) models were constructed using the nlme (Pinheiro *et al.*, 2017) and lme4 (Bates *et al.*, 2015) packages in R. ANOVA results were determined using lmerTest (Kuznetsova *et al.*, 2016), which implements the Satterthwaite (1946) approximation for denominator degrees of freedom, based on the SAS proc mixed theory (SAS, 1978). Data were split into the movement of carbon and nitrogen in the system and were treated separately.

Stable carbon isotope values of both donor and receiver needles, stems, and roots were normally distributed. The fixed effects used in the models were **relatedness** (*kin* vs *stranger*), **family**, **belowground** pathway (*MN,* and *blocked (B)*), and **defoliation** treatment (*manual*

defoliation or *no-herbivory control*). The random effect was the categorical time slot in which pulse-labelling occurred. **Relatedness**, *kin* vs. *stranger* (eq. 1), and **family** effects (eq. 2) were investigated with separate LME models. **Family** is not a replicated level of "*kin*" and "*stranger*", rather, they are two ways to categorize the data, effectively asking a similar question whether transfer to kin is greater than to stranger. Linear models require factorials (the same treatments repeated for all levels). Kin contains 4 levels which are different to the six stranger levels, that is they are not factorial. Defined in this way, it is not possible to test both kinship and family in the same model. Rather, the "kin" effect is tested by replicating multiple examples of kin (as well as replicating multiple examples of stranger pairs) to determine that the effect remains true regardless of the genetic identity of the individuals.

(eq. 1) *Receiver C ~ relatedness + defoliation + belowground + relatedness*defoliation + relatedness*belowground, random =1|time of pulse*

(eq. 2) *Receiver C ~ family + defoliation + belowground + family*defoliation + family*belowground, random =1|time of pulse*

Stable nitrogen isotope values were log transformed to achieve a normal distribution. Receiver needle nitrogen was square-root transformed. Fixed and random effects were the same as for the ^{13}C models. Again, kinship (eq. 3) and family effects (eq. 4) were investigated.

(eq. 3) *Receiver N ~ relatedness + defoliation + belowground + relatedness*defoliation + relatedness*belowground, random =1|time of pulse*

(eq. 4) *Receiver N ~ family + defoliation + belowground + family*defoliation + family*belowground, random =1|time of pulse*

Donor needle, stem, and root ^{15}N did not covary with receiver ^{15}N content and were not used in the models. All models used were tested for normality of the resultant residuals to ensure the models were valid (Gurka *et al.*, 2006). Model fit was determined using the R package piecewiseSEM (Lefcheck, 2016).

LME models were used to generate standard effect sizes (SES) (Schielzeth, 2010), of the response variables (^{13}C and ^{15}N tissue content of kin vs stranger-including roots, stems, and needles). SES was calculated based on standardized coefficients derived from the LMEs. Briefly, the intercept of a regression coefficient (or the response variable) was removed to allow all levels of the treatments to be compared, and then divided by 2x the standard deviation (SD). Dividing by 2x SD sets the variance to 1, thereby standardizing the value and rendering it comparable to other response variable values. Standard effect size was determined by subtracting the standardized coefficients of kin from stranger, allowing for the determination of size and directionality of the difference. A 95% confidence interval was determined for each comparison by using the pooled standard error for all the terms considered. Thus the SES was interpreted as the additional change in treatment level, in standard deviations, for one standard deviation change in the predictor variable (Schielzeth, 2010; Bates *et al.*, 2015). Standardizing in this manner allowed for comparing the results between models, as several models were used. A more detailed explanation on how to interpret SES figures and effect sizes is given in Chapter 2, Methods. The SES figures in this experiment compare manual defoliation and no-herbivory control treatments, where standardized regression coefficients for the no-herbivory control treatment were subtracted from the manual defoliation treatment, thus positive values not crossing zero (at a 95% confidence interval) indicate a significant effect in the manual defoliation treatment.

Results

Boxplots summarizing the data the ^{13}C data (Figure 14) and ^{15}N (Figure 15) data by fixed effects (relatedness, belowground treatment, defoliation treatment, and family) are included for data visualization. The relatedness response variable is decomposed either into kin and stranger categories, or broken down by family: kin (AA, BB, CC, DD) and stranger (AB, AC, AD, BC, BD, CD) (Figure 14, Figure 15). This delineation is maintained throughout the results. There was no significant transfer of ^{13}C and ^{15}N to kin over stranger, however, family pairs differed significantly, some displaying preferential transfer to kin.

There was no difference in ^{13}C means detected between kin and stranger when all seedlings were considered together (Table 9). LME models testing kin vs stranger explained very little of the variation in the data, yielding conditional R^2 values of 7%, 6%, and 9% for needles, stems, and root receiver ^{13}C tissue content (Table 10). None of the LME generated ANOVAs

were significant (Table 9). There was no difference in ^{15}N means detected between kin and stranger seedlings. LME models testing the categories of kin vs stranger explained very little of the variation in receiver ^{15}N tissue content, yielding conditional R^2 values of 16%, 10%, and 4% for needles, stems, and roots, respectively (Table 10). No LME generated ANOVAs were significant (Table 10).

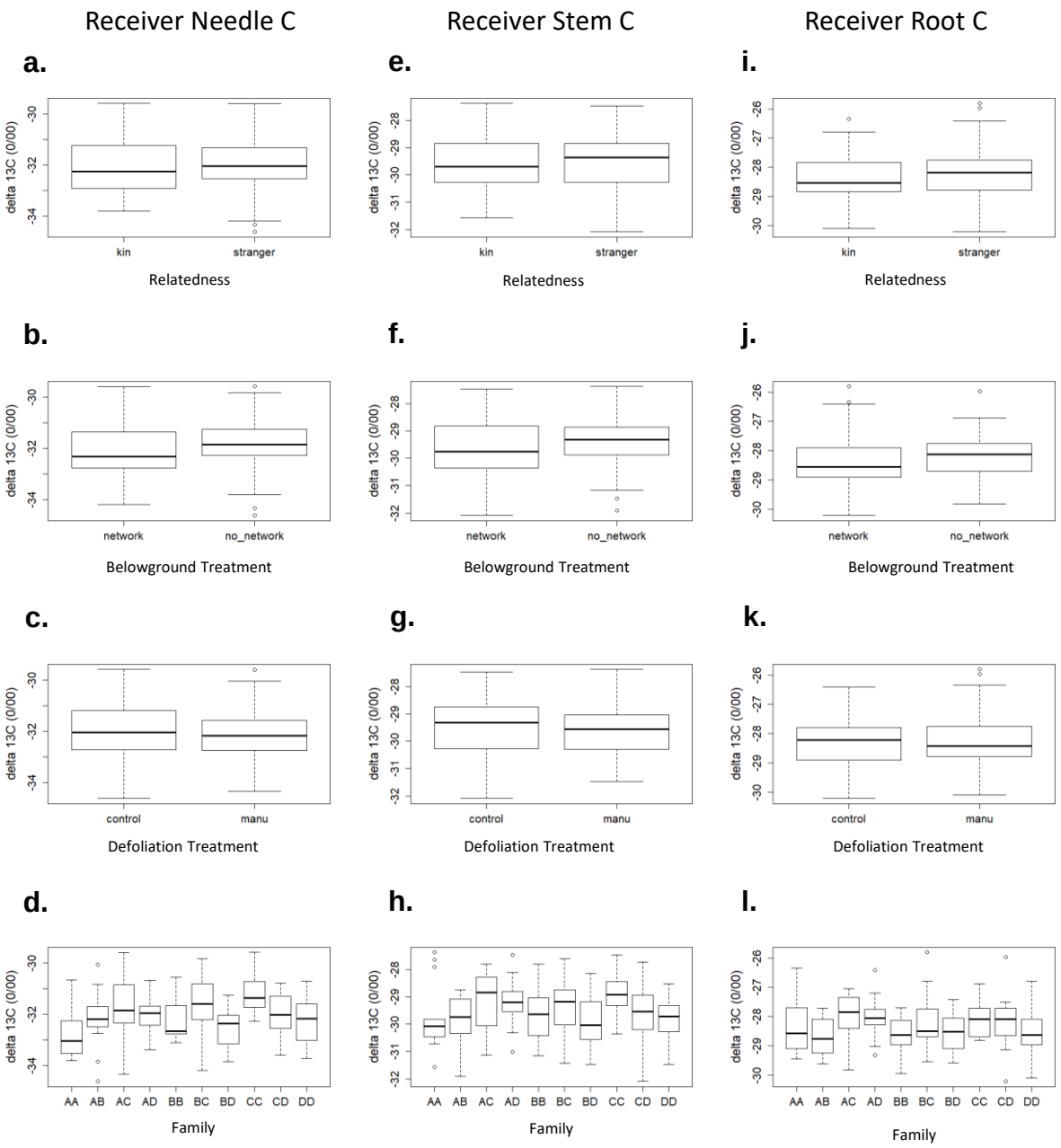

Figure 14. Box and whisker plots of ^{13}C carbon content in receiver tissues, organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle ^{13}C content is shown in the left-most column by a.) relatedness, b.) belowground, c.) defoliation, and d.) family. Stem ^{13}C content is presented in the same order in the middle column: e.) relatedness, f.) belowground, g.) defoliation, and h.) family; and root ^{13}C content is shown in the right-most column, spanning the same set of fixed effects i.) relatedness, j.) belowground treatment, k.) defoliation, and l.) family.

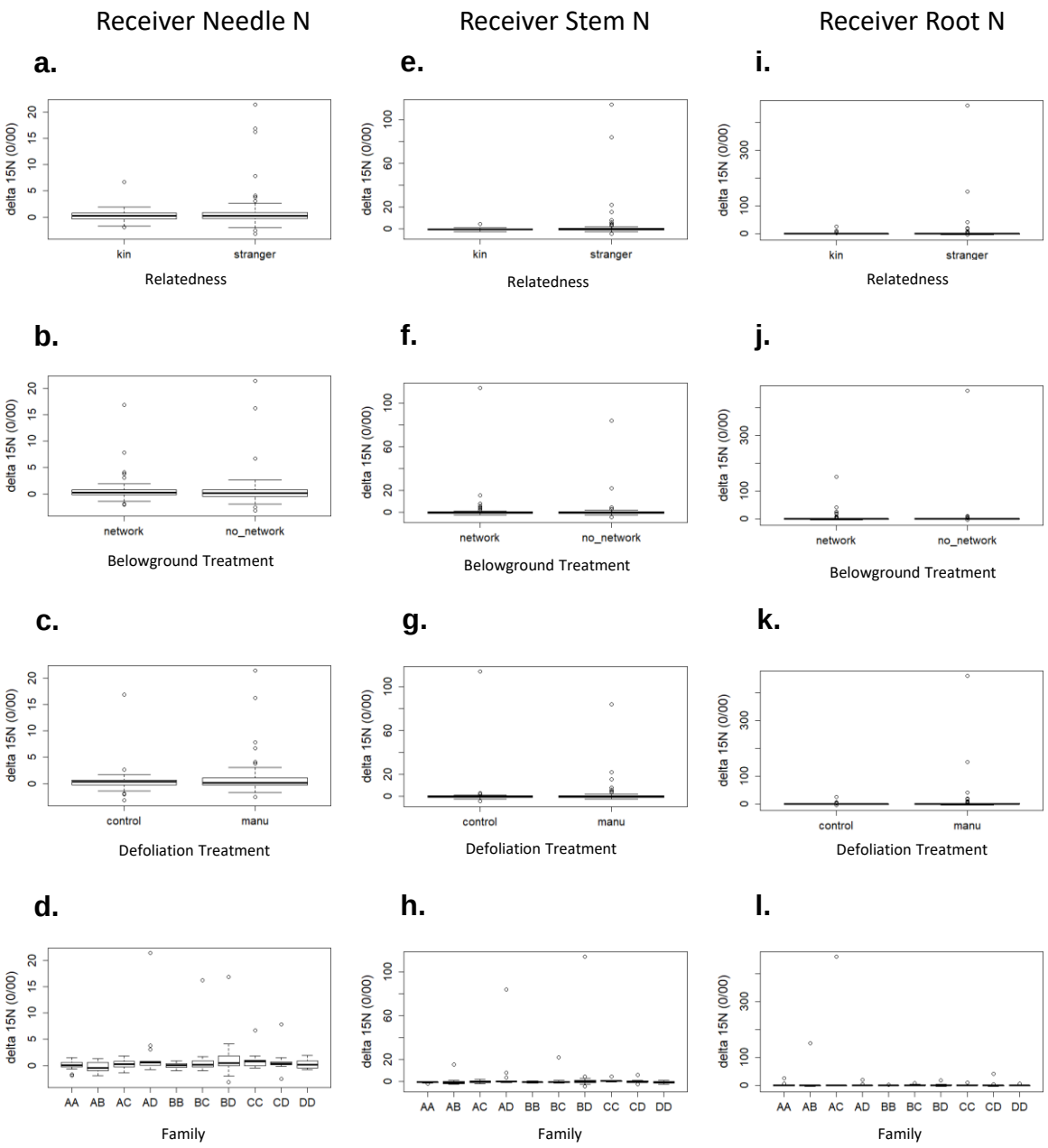

Figure 15. Box and whisker plots of ^{15}N carbon content in receiver tissues, organized by fixed effects in LME models. The dark line represents the median value, the box indicates the upper and lower quartile of the spread of the values (interquartile range), and the whiskers indicate the extent of the extreme values of the data. Outliers, represented by open circles, denote values outside 1.5x the interquartile range. Needle ^{15}N content is shown in the left-most column by a.) relatedness, b.) belowground, c.) defoliation, and d.) family. Stem ^{15}N content is presented in the same order in the middle column: e.) relatedness, f.) belowground, g.) defoliation, and h.) family; and root ^{15}N content is shown in the right-most column, spanning the same set of fixed effects i.) relatedness, j.) belowground treatment, k.) defoliation, and l.) family.

Table 9. ANOVA values derived from LMEs testing the effects of receiver tissue stable isotope content (^{13}C and ^{15}N) in response to the **relatedness** between seedling pairs, that is whether the pair were *kin* or *stranger*. Significant **belowground** treatment effects were detected in the *MN* in stem ^{13}C and needle ^{15}N content only, as determined using the Satterthwaite approximation for degrees of freedom.

	Receiver Needle ^{13}C			Receiver Needle ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1,133.41	119.673	**0.000**	1, 130.00	0.777	0.442
Donor needle				1, 133.22	11.715	**0.001**
Relatedness	1, 134.61	0.195	0.659	1, 129.25	1.367	0.244
Herbivory	1, 139.76	0.293	0.589	1, 136.31	0.251	0.617
Belowground	1, 138.15	2.107	*0.149*	1, 134.39	0.230	0.632
Herbiv x Below	1, 132.20	0.005	0.944	1, 128.62	1.096	0.297
Related x Herbiv	1, 139.88	1.805	*0.181*	1, 136.70	0.282	0.596
Related x Below	1, 138.36	0.179	0.673	1, 134.13	0.061	0.805

	Receiver Stem ^{13}C			Receiver Stem ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1, 138.91	111.371	**0.000**	1, 142.00	0.266	0.791
Donor needle				1, 142.00	12.853	**0.000**
Relatedness	1, 140.79	0.058	0.810	1, 142.00	1.257	0.264
Herbivory	1, 145.00	0.411	0.522	1, 142.00	0.270	0.604
Belowground	1, 143.24	3.952	**0.049**	1, 142.00	0.268	0.605
Herbiv x Below	1, 137.59	0.679	0.411	1, 142.00	1.062	0.304
Related x Herbiv	1, 144.95	0.752	0.387	1, 142.00	0.000	1.000
Related x Below	1, 144.23	0.029	0.866	1, 142.00	0.001	0.982

	Receiver Root ^{13}C			Receiver Root ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1, 132.81	134.890	**0.000**	1, 139.00	0.431	0.667
Donor needle				1, 139.01	0.074	0.785
Relatedness	1, 134.15	0.944	0.333	1, 139.01	1.099	0.296
Herbivory	1, 140.57	0.036	0.851	1, 139.01	1.422	0.235
Belowground	1, 138.56	2.915	*0.090*	1, 139.01	0.203	0.653
Herbiv x Below	1, 131.33	3.036	*0.084*	1, 139.01	0.495	0.483
Related x Herbiv	1, 140.09	0.466	0.496	1, 139.01	1.495	0.224
Related x Below	1, 139.39	0.046	0.831	1, 139.01	0.278	0.599

Table 10. Summary of marginal and conditional R^2 values generated by LME models testing ^{13}C and ^{15}N tissue content with either family or relatedness as fixed effects. Shapiro-Wilks are included to indicate normality of residuals.

LME model	Covariate	Marginal R^2	Conditional R^2	Shapiro-Wilk	p-value
13C-Family pairs					
Receiver-Needle	none	0.315	0.359	0.995	0.914
Receiver-Stem	none	0.244	0.244	0.991	0.529
Receiver-Root	none	0.228	0.232	0.990	0.406
13C-Related	**(Kin vs Stranger)**				
Receiver-Needle	none	0.039	0.073	0.994	0.884
Receiver-Stem	none	0.046	0.062	0.992	0.580
Receiver-Root	none	0.049	0.091	0.991	0.478
15N-Family pairs					
Receiver-Needle	Donor needle N	0.259	0.305	0.755	0.000
Receiver-Stem	none	0.193	0.193	0.446	0.000
Receiver-Root	none	0.178	0.178	0.368	0.000
15N-Related	**(Kin vs Stranger)**				
Receiver-Needle	Donor needle N	0.119	0.162	0.615	0.000
Receiver-Stem	Donor needle N	0.109	0.109	0.355	0.000
Receiver-Root	Donor needle N	0.041	0.041	0.231	0.000

Families

Tissue concentrations of ^{13}C and ^{15}N in Douglas-fir differed between families (Figure 14, Figure 15: d., h., and i. in both figures). Further illustrating this difference between families, some Douglas-fir families demonstrated significant effect sizes of ^{13}C in kin over strangers, whereas others did not. Significantly more ^{13}C-carbon was transferred between certain family pairs, and not always between kin. Two kin family pairs (AA and BB) as well as two stranger pairs (AB and CD) had significant effect sizes with more ^{13}C following manual defoliation treatments (Figure 16), in needle tissue. It is possible that other kin effects were also occurring as the 95% confidence interval for effect size of CC pairs only just includes zero (Figure 16), meaning that, given slightly less stringent criteria, the increased transfer in this kin family might be considered significant. ^{13}C within DD pairs after manual defoliation was not significant in the needle tissue (Figure 16), however, it was significantly higher in both roots and stems (Figure 17). Thus, all

four family groups could be described as transferring more ^{13}C to kin plants in the MN belowground treatment following manual defoliation, however, this did not occur in all tissues tested. A pattern similar to that established in needle tissue was observed in stem (Figure 17) and root (Figure 18) tissue ^{13}C content, however, significant effect sizes were detected in ^{13}C in DD stems, whereas effect size was not significant in the CD pair (Figure 12). Using the Satterthwaite approximation for degrees of freedom, ANOVA results are reported in Table 11. While the ^{15}N data yielded non-signicant ANOVA results, the ^{13}C data showed several significant fixed effects. The family fixed effect was significant in needle, and marginally significant in the stem and root 13C. Belowground treatment was significant in needles and stems, and marginally significant in roots. Some interactions were also marginally significant including family x herbivory in stems and herbivory x belowground in roots (Table 11).

Networks

Almost all of the significant differences that were observed in this experiment occurred between seedlings in the MN treatment. No significant transfer occurred in the blocked treatment needles (Figure 19); blocked treatment stems (Figure 20), with a single exception: root tissue of the family pair AB had a significant effect size of ^{13}C in manual defoliation treatments over controls (Figure 21). This corresponds with the results of the ANOVAs, which found that the belowground treatment was a significant fixed effect in the family-level analyses (see Table 11).

Nitrogen

A significant effect size of ^{15}N in response to manual defoliation, was in the root tissues of some families (Figure 24) but not needle (Figure 22) or and only in CC in stem tissues (Figure 23). Significantly more ^{15}N was detected in the following family pairs: AA, AB, AC, CC, and CD. Transfer was only significant in the MN mesh treatment, and not in the blocked treatment (Figure 25, Figure 26, and Figure 27). LME model results are summarized in Table 10.

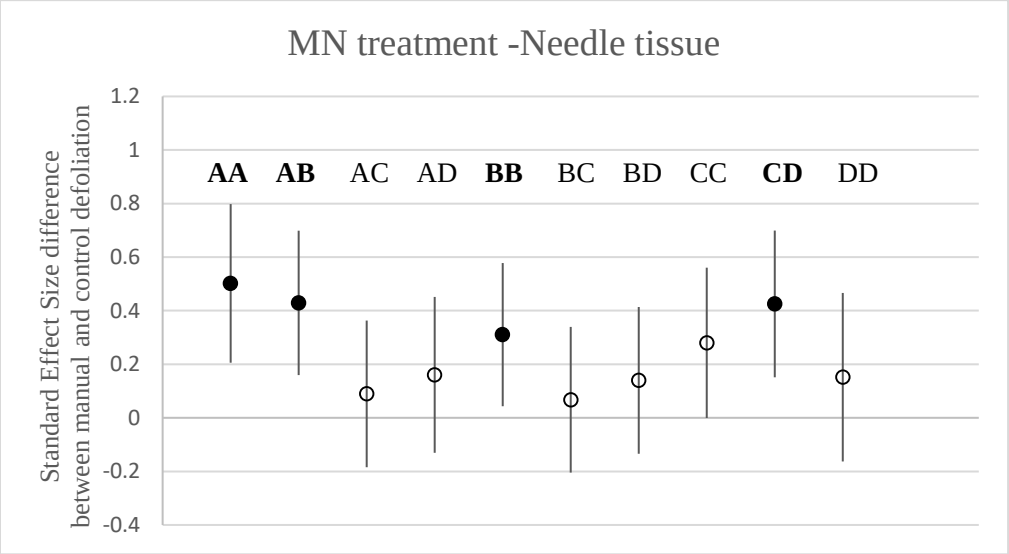

Figure 16. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{13}C needle content. Positive values indicate more ^{13}C in manually defoliated needles. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

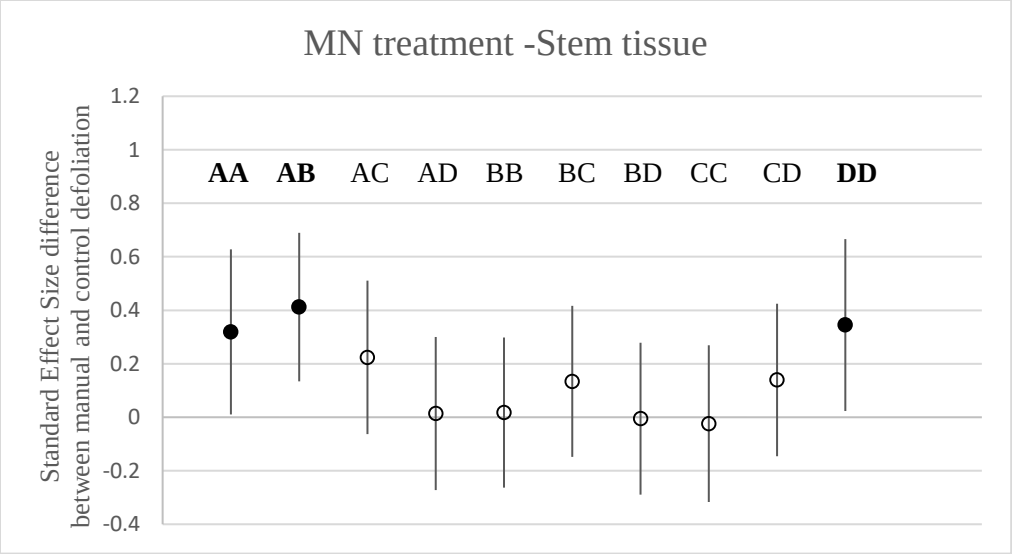

Figure 17. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{13}C stem content. Positive values indicate more ^{13}C in manually defoliated stem. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

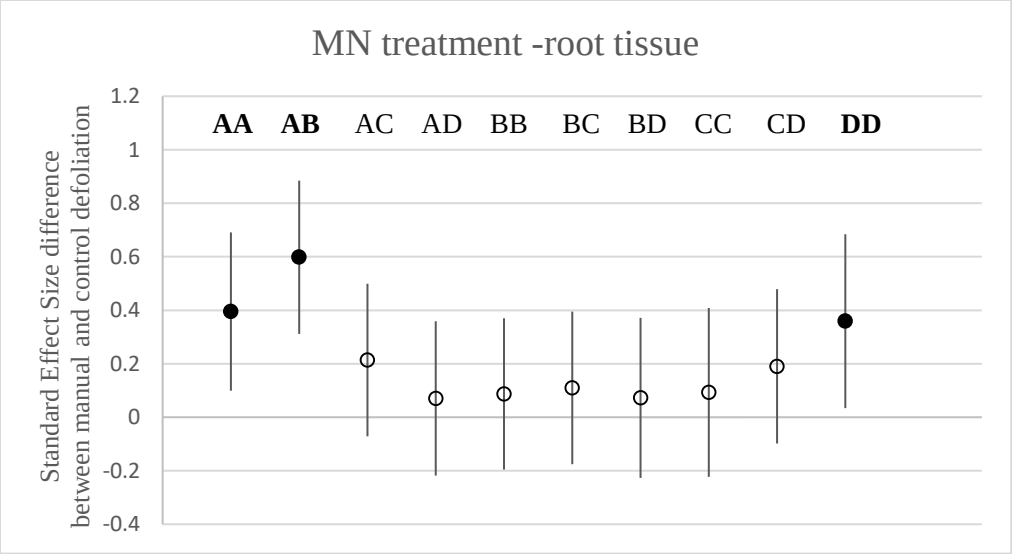

Figure 18. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{13}C root content. Positive values indicate more ^{13}C in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

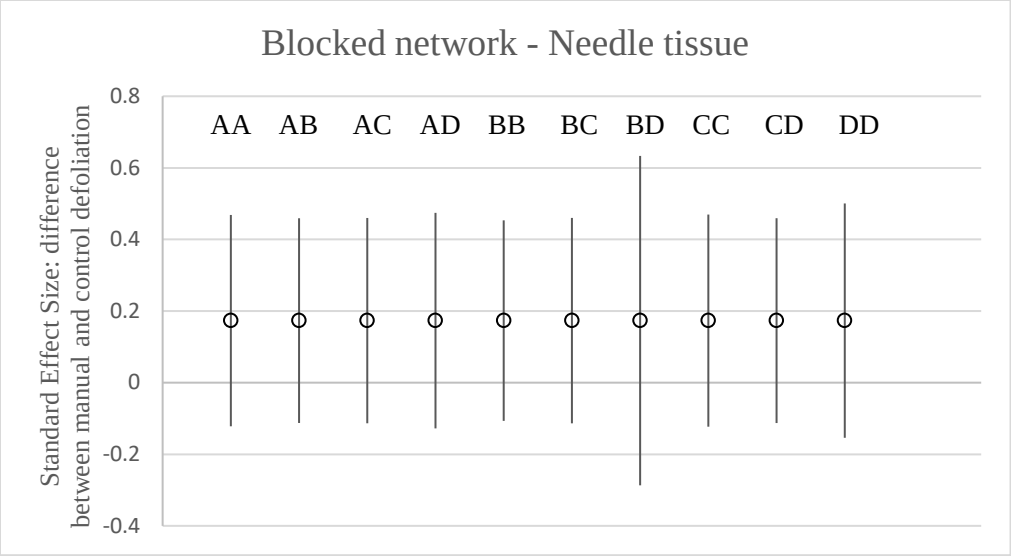

Figure 19. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the B **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver $^{13}C_{needle}$ content. Positive values indicate more ^{13}C in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

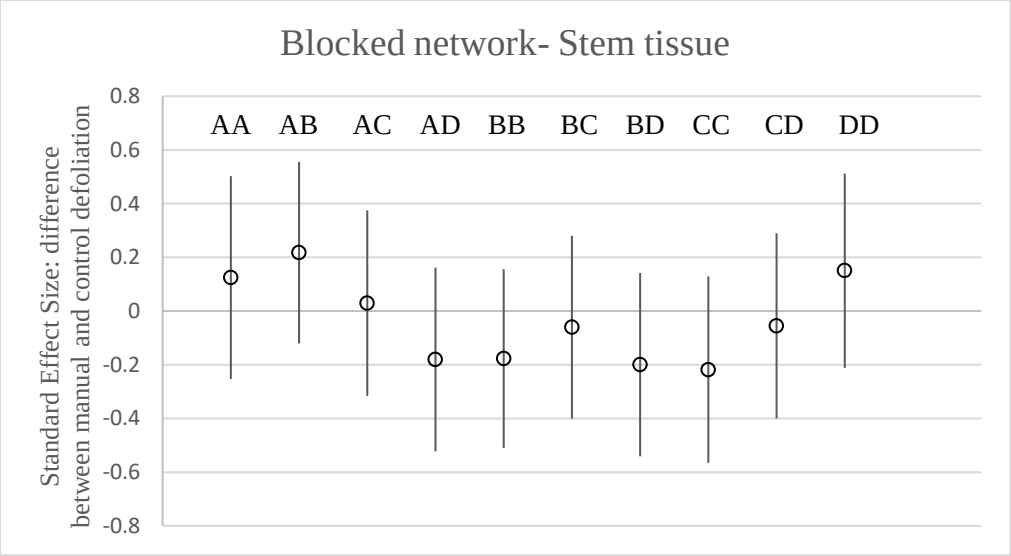

Figure 20. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the B **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver $^{13}C_{stem}$ content. Positive values indicate more ^{13}C in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

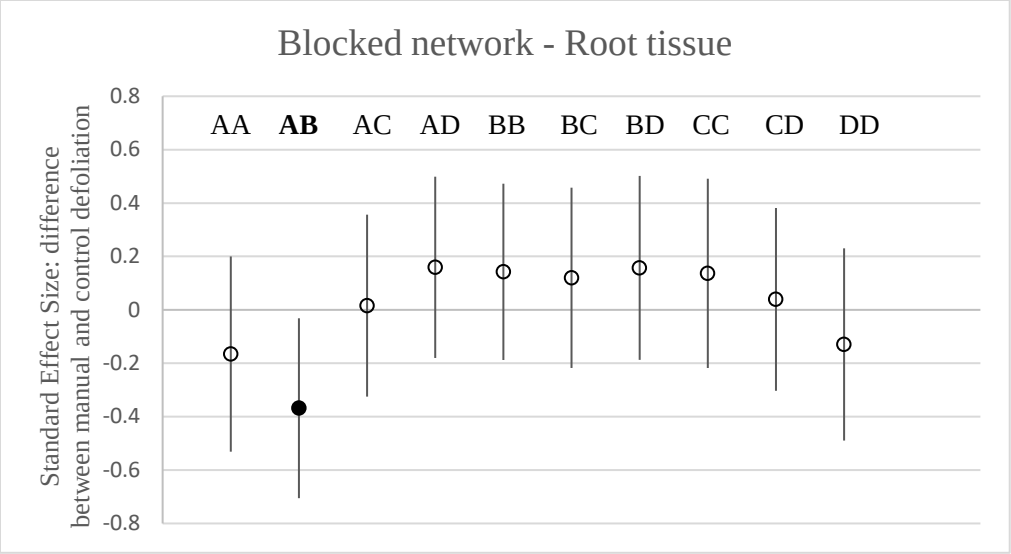

Figure 21. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{13}C uptake in receiver seedlings in the B **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{13}C root content. Positive values indicate more ^{13}C in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

Table 11. ANOVA values derived from LMEs, as determined using the Satterthwaite approximation for degrees of freedom, testing the effects of receiver tissue stable isotope content (^{13}C and ^{15}N) in response to the relationship between seedling pairs, that is whether the pair were kin or stranger.

	Receiver Needle ^{13}C			Receiver Needle ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1, 139.66	77.16	**0.000**	1, 136.50	0.395	0.693
Donor needle				1, 131.67	14.375	**0.000**
Family	1, 136.48	3.05	**0.002**	1, 132.29	1.232	0.281
Herbivory	1, 138.36	1.02	0.315	1, 135.04	0.253	0.616
Belowground	1, 139.25	3.11	**0.080**	1, 136.48	0.254	0.615
Herbiv x Below	1, 128.33	0.00	0.977	1, 124.01	1.288	0.259
Family x Herbiv	1, 136.11	2.64	**0.008**	1, 132.87	0.585	0.808
Family x Below	1, 137.94	0.70	0.711	1, 135.06	1.310	0.237

	Receiver Stem ^{13}C			Receiver Stem ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1, 145.00	67.41	**0.000**	1, 145.00	0.167	0.867
Family	1, 145.00	1.848	*0.064*	1, 145.00	0.931	0.501
Herbivory	1, 145.00	0.787	0.377	1, 145.00	0.030	0.862
Belowground	1, 145.00	4.940	**0.028**	1, 145.00	0.083	0.774
Herbiv x Below	1, 145.00	0.572	0.451	1, 145.00	1.893	0.171
Family x Herbiv	1, 145.00	1.775	*0.078*	1, 145.00	1.196	0.302
Family x Below	1, 145.00	0.743	0.669	1, 145.00	1.276	0.254

	Receiver Root ^{13}C			Receiver Root ^{15}N		
	df	F-value	p-value	df	F-value	p-value
Intercept	1, 140.90	88.80	**0.000**	1, 142.01	0.441	0.660
Family	1, 136.47	1.534	*0.142*	1, 142.01	0.875	0.549
Herbivory	1, 140.98	0.008	0.931	1, 142.01	2.163	0.144
Belowground	1, 140.61	2.358	*0.127*	1, 142.01	0.261	0.610
Herbiv x Below	1, 123.24	2.582	*0.111*	1, 142.01	0.537	0.465
Family x Herbiv	1, 137.69	1.358	0.213	1, 142.01	0.955	0.480
Family x Below	1, 137.98	0.858	0.565	1, 142.01	1.126	0.348

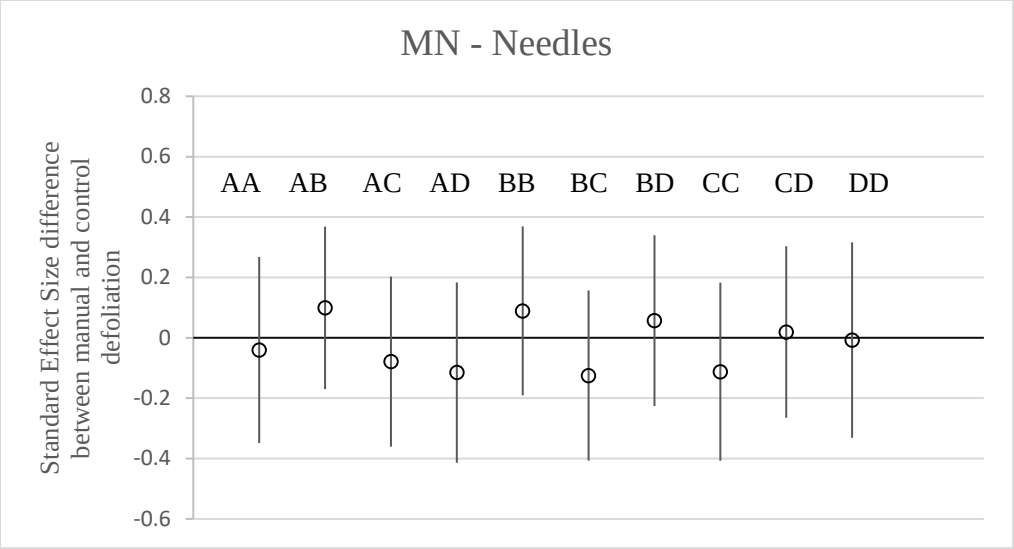

Figure 22. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N needle content. Positive values indicate more ^{15}N in manually defoliated needles. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

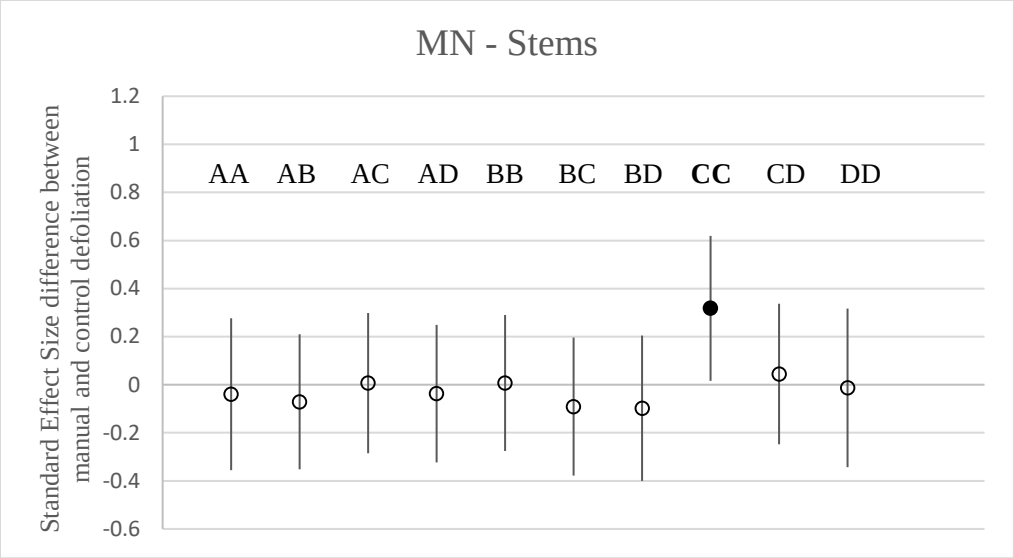

Figure 23. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N stem content. Positive values indicate more ^{15}N in manually defoliated stem. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

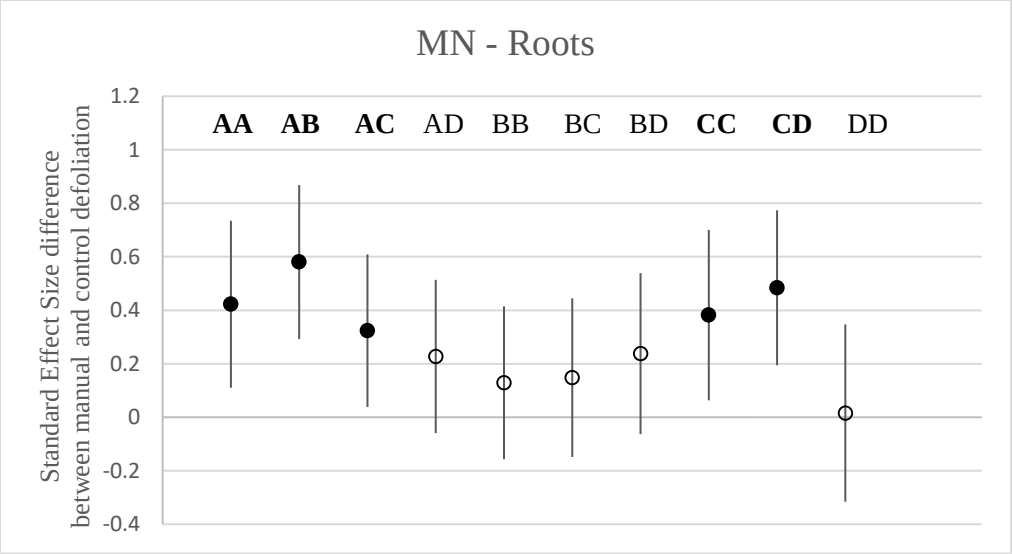

Figure 24. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *MN* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N root content. Positive values indicate more ^{15}N in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

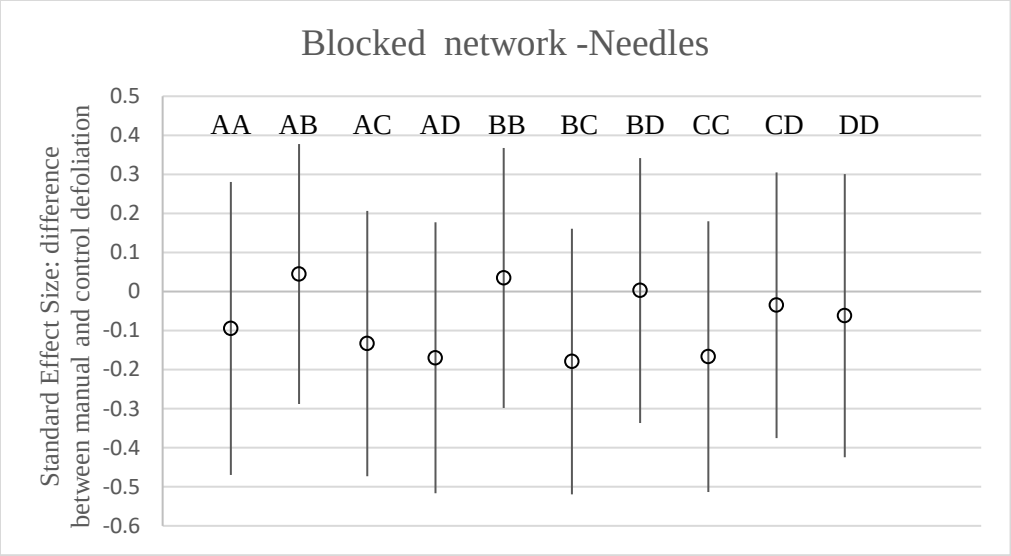

Figure 25. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *B* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N needle content. Positive values indicate more ^{15}N in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

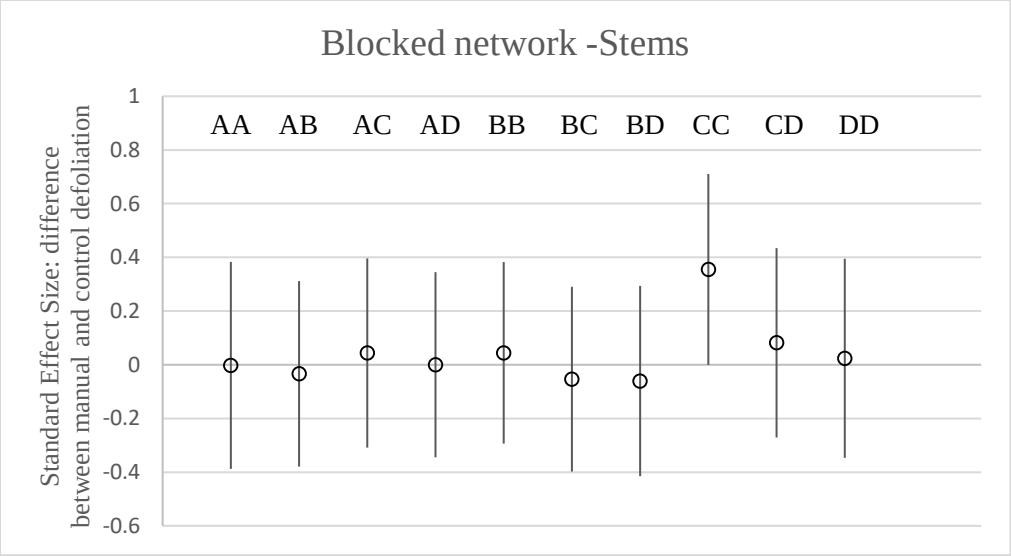

Figure 26. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *B* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N stem content. Positive values indicate more ^{15}N in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

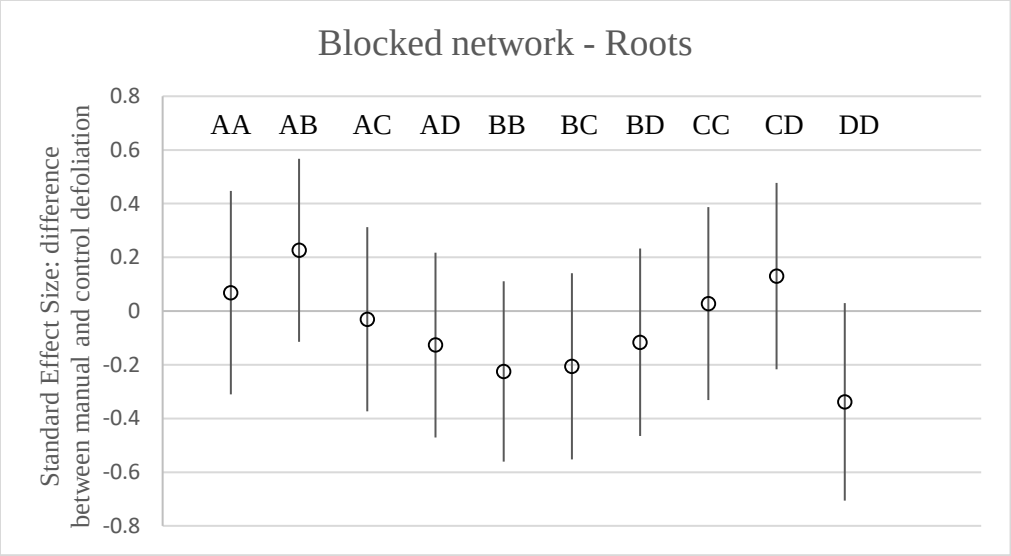

Figure 27. Variation among family pairs in response to *manual defoliation* as SES subtracted from *no-herbivory controls* SES of ^{15}N uptake in receiver seedlings in the *B* **belowground** treatment. Standard coefficient differences are based on a linear mixed-effects model and are expressed as the difference between manually defoliated and control treatment receiver ^{15}N root content. Positive values indicate more ^{15}N in manually defoliated root. Error bars indicate the 95% confidence interval. Solid circle (●) represents a significant effect, whereas open circles denote non-significance (○).

Discussion

The hypothesis that belowground carbon and nitrogen transfer to kin Douglas-fir seedlings would be greater in kin is rejected. The hypothesis that carbon and nitrogen would be transferred in response to the manual defoliation treatment is supported. The hypothesis that transfer would occur in MN belowground treatment groups is supported, as most significant effect sizes were found in this treatment. And finally, the hypothesis that all families would transfer carbon and nitrogen to kin over stranger at the same rate is rejected. There were significant differences between the carbon effect sizes between families.

When taking a more nuanced approach in which family pairs are considered individually, it becomes clear that some families generate a significant effect size whereas other do not. Thus it can be inferred that some families are more likely to transfer through MNs regardless of the relationship to their neighbor, and some do transfer to kin. Furthermore, when a signal transfer response in a family pair was detected, it was always greater in the manually defoliated treatment, showing that manual defoliation can trigger signal transfer in the field. In all cases but

one (Figure 21), an isotopic signal was transferred in the MN mesh treatment, indicating that the ability to form mycorrhizal networks with neighbours does appear to be important for belowground transfer of stable isotopes to aboveground (^{13}C and ^{15}N) and belowground (^{13}C and ^{15}N) plant tissues.

The case for kin recognition

Tissues that were tested for ^{13}C and ^{15}N included needles, stems and roots. Because this work was primarily interested in testing transfer responses after simulated herbivory, thereby implicating defense chemical compounds as the potential signals being transferred, needle tissue is potentially the most compelling tissue to consider. If it is indeed a defense signal being sent from one seedling to the next, then it ought to be localized in the needles to become effective at activating defenses against a needle defoliating pest. This was indeed observed in the case of ^{13}C in kin combinations AA and BB, with CC pairs also displaying greater ^{13}C content in needles following manual defoliation, but at a level for which significance could not be conclusively confirmed. Considering all the tissue types (Figure 16,Figure 17, and Figure 18for ^{13}C), the kin combination DD did have significantly more ^{13}C in both root and stem tissues, but not needles. In this case, perhaps transfer occurred more slowly and was yet to be detectable in the needle tissues of DD pairs. No evidence of elevated receiver needle ^{15}N was observed in any of the family combinations, suggesting that it was not incorporated into the signal molecules. Therefore, considering all the tissues examined, the case can be made that there is evidence of kin recognition, via a signal containing ^{13}C, in all the families studied. One important challenge for signal detection may have been the timing at which seedlings were harvested after pulsing with the stable isotopes; either earlier or later may have resulted in better detection. Additional replicates would likely have helped dramatically in determining whether preferential transfer was occurring in some cases (particularly in the case of CC kin pairs), but due to the limited number of seeds available from known kin families this was unfortunately not possible.

Mycorrhizal network effects

Unlike in the greenhouse experiment described in Chapter 2, this field experiment did not detect evidence of signal transfer when the potential to form mycorrhizal networks was inhibited by using blocked (B) belowground treatment (with only one anomaly, see Figure 21). In the previous greenhouse experiment, seedlings were grown in bags for 8-10 months, potentially creating a situation where the seedling may have become root-bound generating opportunities for

roots to penetrate the bags and/or for mycorrhizal fungi to grow on the mesh surface adjacent to each other, potentially transferring compounds through exudation while in close proximity to each other. In their greenhouse experiment Pickles *et al.*, (2017) found that ^{13}C was present in receiver fungal tissues regardless of B or MN belowground treatment, indicating that a viable pathway for the movement of seedling derived photosynthates is possible under these conditions. In this field experiment, seedlings were planted into bags in the field and given only one growing season to expand their roots into the mesh bags (4 months), reducing the possibility of unintended sharing in the blocked belowground treatments. Thus it appears that this field experiment was successful in its use of mesh bags for blocking or allowing the passage of mycorrhizal fungal hyphae, while restricting the transfer of stable isotopes through exudation.

Defense signal transfer

This study does not explicitly test whether the ^{13}C or ^{15}N enrichment of receiver seedlings represents a defense signal, although it does provide circumstantial evidence in support of this hypothesis (see Chapter 4 for non-target LCMS identification of potential molecules involved in transfer). Small amounts (*circa* 0.1% of total recovered label) of ^{13}C have been documented to be transferred via ectomycorrhizae connecting Douglas-fir seedlings in the lab (Pickles *et al.*, 2017), and also in the field (Teste *et al.*, 2009, 2010), this transfer was reported as constitutive (that is, not in response to a stimulus). As a response to simulated herbivory (using manual defoliation as in this study), Song *et al.*, (2015) demonstrated defense signal compound transfer from Douglas-fir to Ponderosa pine (*Pinus ponderosa* Douglas ex. C. Lawson) seedlings. We know from Song *et al.*, (2015) that Douglas-fir are capable of 'warning' connected inter-specifics about a potential defoliating threat in a greenhouse environment. The present study is the first to report transfer between conspecific Douglas-fir of known families in response to manual defoliation in the field. Plant responses to potential signalling compounds passed through arbuscular mycorrhizal networks have also been documented in *Alternaria solani* and *Vivica faba* (Babikova *et al.*, 2013; Song *et al.*, 2014).

Furthermore, according to the results in Chapter 2, which utilized western spruce budworm defoliation in addition to manual defoliation, there is evidence that more ^{13}C is transferred in response to insect defoliation. Insect defoliation was not used in the field experiment because of concerns of the impacts of potential budworm escapees on the surrounding forest. While manual defoliation has been demonstrated to be effective by Song *et*

al., (2015), the use of insects could potentially have elicited a stronger response in this experiment. Differential plant defense responses based on the identity of the insect have been shown in *Arabadopsis thaliana* (Appel *et al.*, 2014), suggesting that the same may be possible in Douglas-fir. Perhaps the seedlings are able to differentiate between a human wielding experimental scissors and its evolutionarily engrained (Axelson *et al.*, 2015) response to a deleterious leaf chewing insect. The latter is likely to transfer a number of chemicals into the tissue surrounding the wounds caused by its mouthparts, unlike the clean and sterilised cuts of the former.

Nitrogen

Ectomycorrhizas are adept at exchanging soil-derived nitrogen for plant-host derived sugars (Parrent, J.L. and Vilgalys, 2007; Cox *et al.*, 2010). When tracing the transfer of nitrogen and carbon at the plant-root-mycorrhizal interface, carbon and nitrogen should theoretically move in the opposite direction. Of course, the actual movement of these two nutrients is much more complex (Jones *et al.*, 2009). Here, it was found that only the root tissue ^{15}N content of receiver plants differed in effect size in response to manual defoliation (Figure 24), whereas in needles and stems, although the models explained comparatively similar amounts of variation (Table 10), significant effect sizes were not found for any of the factors tested. The seedling pairs showing more ^{15}N transfer in response to manual defoliation included two kin pairs (AA and CC) and three stranger seedling pairs (AB, AC, and CD), indicating that relatedness was not the only factor playing a role in terms of nitrogen transfer in this system. Notably, both families A and C appear to be involved in greater and more significant nitrogen movement than B and D. Thus the individual genetics of Douglas-fir may play a role in determining how much nitrogen is acquired, at least in root tissues.

The nitrogen detected within the roots of these seedlings may have been found exclusively in fungal tissues. While most active root tips were removed prior to drying and sampling for ^{15}N EA-IRMS, no substantial effort was made to ensure mycorrhizal fungi were completely removed from the roots. Nor was any effort made to identify the amount of ^{15}N found in fungal tissues such as fungal specific phospholipid-derived fatty acids (PLFAs), as was done by Pickles *et al.*, (2017). Thus the values of ^{15}N obtained for root tissue here may actually represent the ^{15}N present in both plant and fungal tissues. Nonetheless, differential amounts of ^{15}N were found in the roots of different family pairs, indicating that nitrogen may not be equally

distributed between adjacent conspecifics in a natural setting. This in itself is extremely interesting and a novel result of this experiment.

In conclusion, this experiment showed that kin selection is possible in Douglas-fir, but the effect size differs between families, such that, when family pairs are considered together as kin and stranger factors, no significant differences are found between kin and stranger seedlings. Furthermore, carbon rather than nitrogen compounds move through a MN in response to manual defoliation in the field.

Chapter 4: Identification of signal transferred

Introduction

Many studies have demonstrated transfer of carbon through MNs (Francis & Read, 1984; Simard *et al*., 1997c; Leake *et al*., 2006; Teste *et al*., 2010; Deslippe & Simard, 2011; Song *et al*., 2015; Pickles *et al*., 2017). Often, the net amount of carbon transferred is small (e.g. Teste *et al.*, (2010)), which raises the question of whether the transfer of carbon through MNs has ecological significance in terms of any nutritional value. Owing to the complexity of pulse-chase experimental designs required to capture evidence of carbon movement through a MN, there are a number of factors that may contribute to a detection failure. For example, it is possible that labelled carbon will not be captured during the chase as a result of experimental timing issues (e.g. short pulse durations), failure to localize the label within a particular plant tissue (e.g. subsampling from biomass unlikely to receive new carbon inputs), or the movement of labelled carbon into unanticipated endpoints (e.g. non-target plants or microorganisms), particularly in field experiments. Indeed, transfer is often bidirectional (Simard *et al.*, 1997c; Philip, 2006), seasonal (Philip, 2006), and may be miniscule during summer months (Teste *et al.*, 2010). The size of the experimental plants may also contribute to the small amounts of transfer detected. In a recent study by Klein *et al.*, (2016) in a mature forest, a Norway spruce (*Picea abies*) was continuously labelled with ^{13}C depleted CO_2 over a 5 year period, resulting in a demonstration that 40% of photosynthesized carbon was transferred to neighbouring non-conspecific trees. In earlier studies, Simard *et al.*, (1997) found a net transfer of 10% of photosynthetic carbon from *Betula papyrifera* to *Pseudostuga menziesii* through MNs, and Deslippe & Simard, (2011) found 11% transfer among *Betula nana* individuals through belowground pathways, amounts equivalent to reproduction costs of these species. Thus, in the long term, the transfer of carbon through MNs is likely to be ecologically significant.

Even small amounts of carbon-based molecules, transferred between plants through belowground pathways, can have a large ecological impact if their presence generates a functional response in the receiver. Biochemical information transmitted at critical times has the potential to enhance plant fitness under stress. However, with only a few exceptions (e.g. Song *et al.*, (2010); Babikova *et al.*, (2013)), most studies have not attempted to discern the identity of the molecules being transferred in this manner. An information molecule such as a defense

chemical need not be transferred in large quantities to have a significant functional impact on a receiving plant, and several studies have provided evidence that defense chemicals can be transferred through MNs. Song *et al.*, (2010) demonstrated the activation of a defense response in a receiver tomato plant linked via MNs to a donor tomato plant being attacked by a fungal pathogen. Repeating this experiment with jasmonate-knockout mutant donor tomato plants, Song *et al.*, (2010) showed that knockout tomatoes were unable to send a signal, whereas wild-type tomatoes with the jasmonate signaling pathway intact were able to elicit a response in receiver plants. Jasmonates are employed by plants as signaling and defense compounds (Wasternack, 2007). A subsequent experiment found that conifers may also transfer defense signals through MNs, as (Song *et al*., 2015) found that manual defoliation of Douglas-fir triggered a defense response in MN-linked ponderosa pine receivers. However, the identity of the defense signal was not investigated in this case, nor were knockout mutants available for conifers.

Metabolite profiling is a powerful technique that can be used to identify unknown biological compounds in complex mixtures. Liquid chromatography (LC) coupled with tandem mass spectroscopy (MS) and electrospray ionization is particularly useful for untargeted profiling (Smith *et al.*, 2006). Liquid chromatography, unlike gas chromatography, can process water soluble molecules in addition to volatiles, thereby targeting a larger array of potentially important biological molecules such as proteins (Linscheid, 2005; De Vos *et al.*, 2007). While no technique would cover the entire metabolome of a plant, owing to the complexity of the molecules involved, LCMS is well suited to analyzing plant secondary metabolism (De Vos *et al.*, 2007). While the analysis of LCMS can be very onerous and technically challenging, the online tool XCMS (Tautenhahn *et al.*, 2012) is widely adopted for this purpose by non-specialist users. This tool provides data processing, statistical analysis, and identification of compounds of interest by linking the mass spectroscopy profiles of significant peaks to Metlin (Smith *et al.*, 2005), a metabolite mass spectroscopy database.

The hypothesis of this study was that the carbon compound transferred preferentially to kin over strangers through MN in response to herbivory treatment in the greenhouse experiment (Chapter 2) could be identified through non-target whole metabolome profiling with LCMS. It was surmised that the signaling molecule would be a jasmonate compound, and following the results of Chapter 2, that more of that compound would be found in the needle tissues of kin than stranger seedlings following herbivory treatment.

Methods

Needles sampled from Douglas-fir seedlings in the greenhouse experiment described in Chapter 2 were used in untargeted metabolomic analysis using LCMS. The experimental design of the Chapter 2 greenhouse experiment is briefly reiterated below for reference.

Experimental design

A 3 x 3 x 2 factorial set of treatments, with three belowground pathways (naturally planted with roots and hyphae "free (F)" to interact; mycorrhizal network only "MN" with roots blocked; soil pathway only with both hyphae and roots "blocked (B)"), three defoliation treatments (control, insect defoliation, and manual defoliation) and two relatedness levels (kin, stranger) was replicated 10x for families A and B. Each experimental unit consisted of three Douglas-fir seedlings (two seedlings of one family (kin) and the third seedling from a different family (stranger)) grown from seed in a 4L pot containing 50% natural forest soil mixed with 50% greenhouse potting mixture consisting of 75% peat, and 25% perlite (West Creek Farms, Fort Langley, BC, Canada). For the belowground pathway treatments, the three seedlings in each pot were individually grown in either 0.5µm pore-sized mesh (blocked "B"), 35µm pore-sized mesh (MN) (Plastok Meshes and Filtration Ltd. Birkenhead UK), or with no mesh bags (free "F"), with each seedling in its own bag (when applicable). Both mesh sizes prevent root passage, however the 35µm pore-sized mesh allows the passage of ectomycorrhizal hyphae (MN treatment) and thus formation of a MN, whereas the 0.5µm pore-sized mesh does not (blocked treatment), although soil pathway solute transfer is also possible through this mesh size (Pickles *et al*., 2017). The no bag treatment is referred to as "free (F)".

Experimental units were grown for 8 months prior to treatment. Of the three seedlings grown per pot, two were kin and one was a stranger. Herbivory treatments were applied immediately before pulse-labelling with 99% ^{13}C-CO_2. One of the two kin in an experimental unit was selected as a 'donor' to which a herbivory treatment was applied, immediately followed by pulse-labelling. For the herbivory treatments, donors were either untreated controls, had their needles cut with scissors (to a level of approximately 50% of needles cut in half for a total of 25% defoliation), or were inoculated with western spruce budworm (at a level of ten 2nd instar budworms placed on fresh buds per seedling). The 2nd instar western spruce budworms were kindly provided by John Dedes at the Great Lakes Forestry Research Centre, Forestry Canada, Sault Ste. Marie, Canada. It is important to note that, to prevent airborne signal transfer, each

individual seedling within a pot was sealed into an airtight plastic bag immediately prior to defoliation of the donor seedling (the donor's bag was sealed immediately after budworm application or manual defoliation).

Samples used for LCMS

Twelve needle samples from seedlings from the greenhouse experiment described above were used in LCMS. The twelve seedlings from 4 pots represent one replicate each of a WSB herbivory and manual defoliation treatments, and 2 replicates of no herbivory healthy plant controls, all with kin and stranger receivers, and all with the MN belowground treatment. No blocked or free belowground treatment pots were used for LCMS. High intra-seedling variability prevented statistical comparisons of 10 of the 12 seedling needle samples (addressed in the Results and Discussion sections). In order to address this issue, growth chamber experiments were attempted (described below). The growth chamber Douglas-fir kin and stranger pairs failed to become colonized, and were not analyzed for plant secondary metabolites using LCMS.

Liquid chromatography-mass spectroscopy (LCMS)

Experimental units were destructively harvested for analysis as described in Chapter 2. A random sampling of approximately 3g of needles was collected from each seedling harvested in the greenhouse experiment, frozen in liquid nitrogen, then stored at -80°C until processing. Four experimental units from the greenhouse experiment were selected for HPLC, for a total of 12 seedlings, representing each of the three herbivory treatments (WSB defoliation, manual defoliation, and control), and only the MN belowground pathway treatment. To extract whole metabolite profiles, triplicate technical replicates of 30mg of needles were ground and shaken in 70% HPLC grade methanol at 4°C for 24 hours. Aqueous methanol was removed after centrifugation at 13,000 x g for 10min and preserved at -80°C. Benzoic acid at 1mg/mL was used as an internal standard. Samples were run on Agilent 1100/ LCMSD Trap XCT Plus mass spectrometer at Michael Smith Laboratories at UBC under the following conditions: A Zorbax SB-C18, 4.6mm ID x 150mm, 3.5 um pore size packing material column run at 30°C with a flow rate of 0.8mL/min and an injection volume of 5µL was used; the detector was a diode array (DAD) with wavelength 190-600nm; the mobile phase was (A) water + 0.2% formic acid and (B) acetonitrile + 0.2% formic acid; a run time of 23min; run in alternating negative/positive electrospray with nebulizer pressure of 60psi, a dry gas flow rate of 12L/min at 350°C; a mass range of 100-800; and output analyzed by Bruker LCMSD software Trap version 3.2 (build 121).

Metabolite profiles generated by the above method were analyzed using the online service 'XCMS online' (Smith *et al.*, 2006; Tautenhahn *et al.*, 2012; Huan *et al.*, 2017), which identifies metabolites of interest by comparing replicated LC profiles in treatment vs control samples to identify peaks that are significantly different. Mass spectroscopy values of identified peaks of interest were compared to ReSpect (RIKEN Center for Sustainable Resource Science, Hirosawa, Wako, Saitama, Japan), a database of phytochemical spectra.

Growth chamber

A pilot study showed that most of the needle samples from the four experimental units did not produce comparable profiles within the technical replicates. Variation within the needle samples of each seedling was the likely culprit. In an effort to reduce variability of metabolite peaks between needle samples, several methods for cultivating Douglas-fir seedlings in a simplified system were attempted. For example, since a mixed community of fungi in forest soil may have contributed to variation in receiver responses in a seedling-network-seedling system, soil was eliminated and replaced with agar medium. All attempts to grow Douglas-fir seedling pairs were done in an ATC40 Conviron (Conviron® Winnepeg, Manitoba, Canada) growth chamber at 21°C for daylight length of 18 hours and 17°C for 6 hours of dark. Furthermore, a pure culture of *Laccaria bicolor* (generously donated by Dr. Melanie Jones UBC-Okanagan, BC, Canada) was used to generate the MN. Modified Melin-Norkrans medium (as in (Rossi & Oliveira, 2011)) diluted to 1/10 strength was found to support slow growth rates of *L. bicolor*. Minimal nutrients in the agar were thought to encourage mycorrhization as a carbon source for the fungus. Douglas-fir seeds were germinated on the agar which was simultaneously inoculated with *L. bicolor*; this method resulted in 100% contamination with a penicillin-like fungus, despite 2 hours of seed pre-treatment in 10% H_2O_2, and despite high germination rates (83%). Seeds were also germinated in potting mix consisting of 75% peat, and 25% perlite (West Creek Farms, Fort Langley, BC, Canada) until 2 weeks old, then triple washed in distilled water and placed on MMN agar plates with a 2 week-old *L. bicolor* colony. In this second approach, roots failed to become colonized by *L. bicolor* even after 6 weeks of growth. In each approach, pairs of kin and stranger Douglas-fir seedlings were used with the intent of applying two herbivory treatments (manual defoliation and control) to donor seedlings and examining the LCMS profiles of both donors and receiver seedlings. However, due to lack of colonization, these approaches were abandoned.

Results

A comparison of profiles (Figure 28) from kin and stranger receiver seedling needles linked via a MN, with the kin donor treated with WSB defoliation, yielded 3 peaks of interest which were increased in size in kin (over stranger) with triplicate technical replicates. There were a 4.45-fold increase in a peak identified as a flavonoid (Figure 29); a 3.3-fold increase in a peak identified as co-enzyme A (Figure 30); and a 5.6-fold increase in a peak identified as piperacillin (Figure 31). Charge to mass ratios, retention times, and p-values are listed in Table **12**.

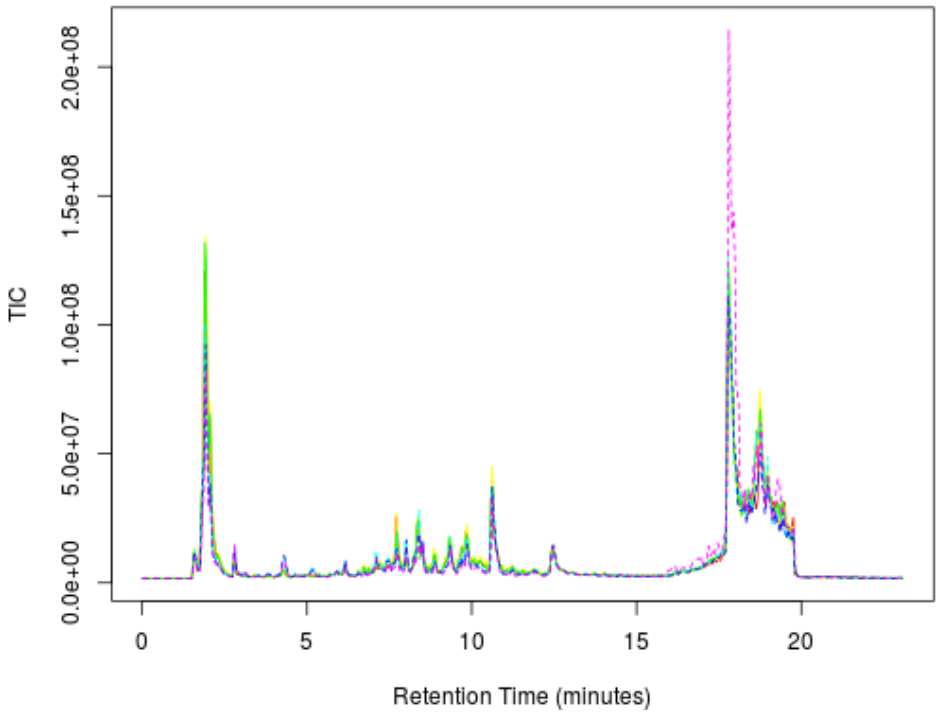

Figure 28. Total metabolite profiles from LCMS, in technical triplicate, for kin and stranger receiver needles in a MN belowground treatment with a WSB treated donor Douglas-fir seedling. Different colours represent technical triplicate replicates of each kin and stranger needle sample.

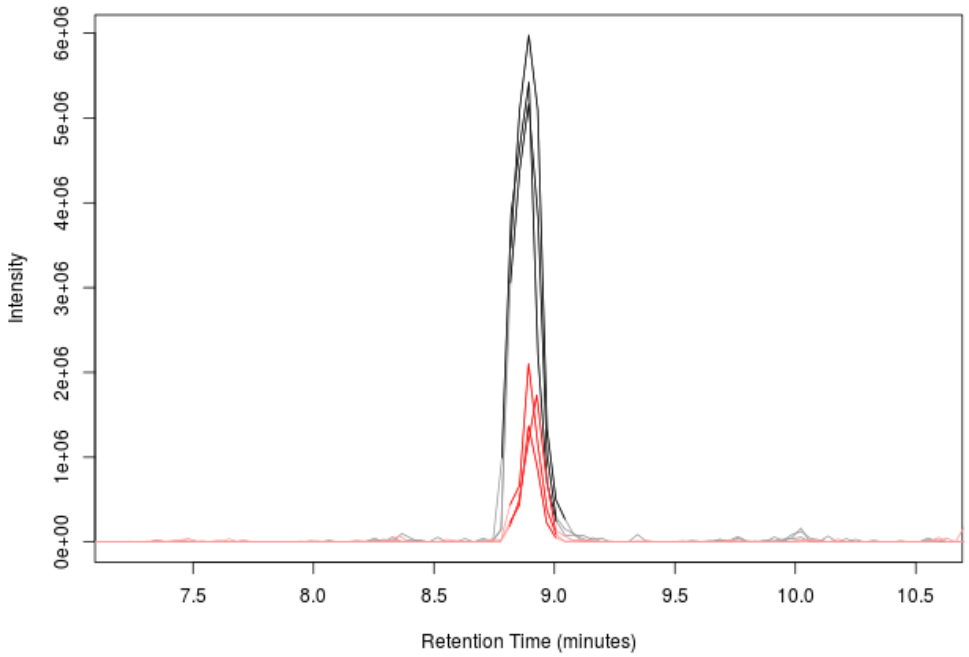

Figure 29. Overlaid triplicate peaks derived from LCMS analysis of needle tissue of kin receiver (black lines) and stranger receiver (red lines) with a median retention time of 8.89min, and tentatively identified by mass spectroscopy charge/mass ratios as a flavonoid molecule.

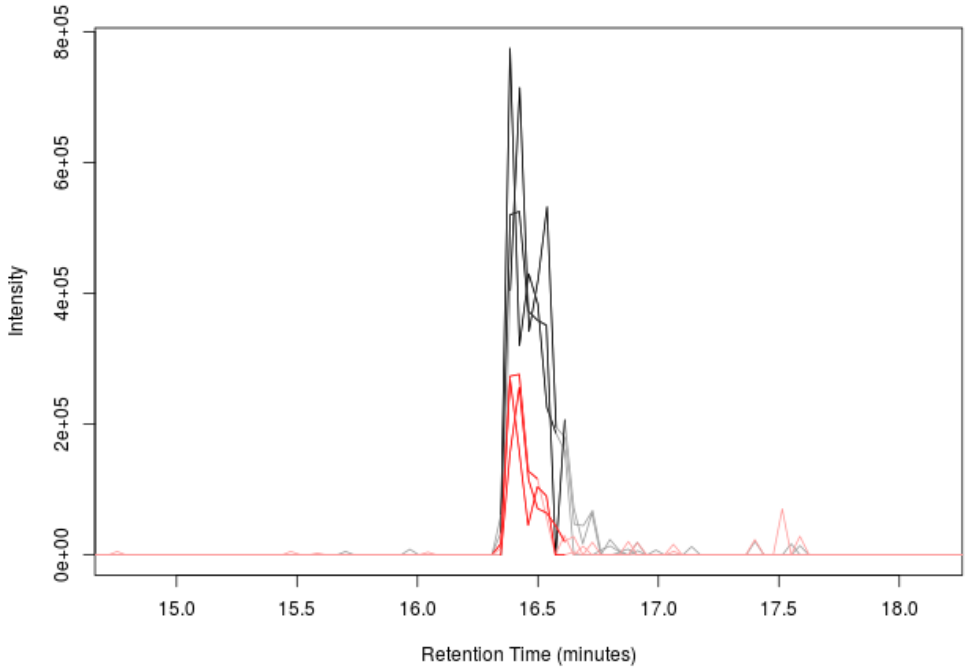

Figure 30. Overlaid triplicate peaks from LCMS analysis of needle tissue of kin receiver (black lines) and stranger receiver (red lines) with a median retention time of 16.4min, and tentatively identified by mass spectroscopy charge/mass ratios as CoEnzyme A.

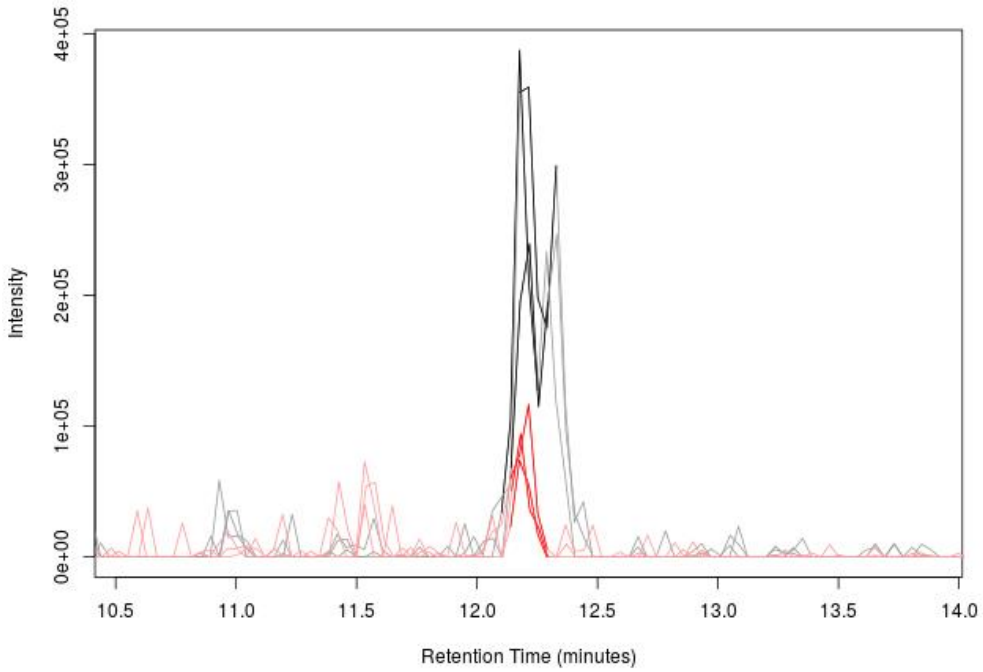

Figure 31. Overlaid triplicate peaks from LCMS analysis of needle tissue of kin receiver (black lines) and stranger receiver (red lines) with a median retention time of 12.2min, and tentatively identified by mass spectroscopy charge/mass ratios as piperacillin.

Table 12. Summary of XCMS comparison of WSB defoliated kin and stranger receiver seedlings in the MN belowground treatment.

XCMS name	fold	p-value	m/z	retention time	ReSpect ID	ReSpect accession
M479T9	4.4538	0.0002	479.0469	8.8932	Flavonoid	PS091102
M769T16	3.3120	0.0005	769.1501	16.4228	Co enzyme A	PS071101
M519T12	5.6175	0.0030	519.0764	12.2141	Piperacillin	PS032201

Discussion

Samples from a WSB herbivory and MN belowground treated experimental unit were comparable using XCMS online. As seen in Chapter 2, significantly more ^{13}C was transferred to kin over strangers in that treatment, thus the compounds identified could be part of a defense response triggered by transfer of defense signal. Three compounds were found to be elevated in

kin vs stranger needles: they are tentatively identified as a flavonoid, coenzyme A, and piperacillin.

Insufficient replication and high within-sample variability made it difficult to conclusively identify the transferred compound through non-target metabolite profiling. Comparisons of seedling needle whole metabolite LCMS profiles were hampered by high variability within the technical replicates. High within-sample variability was likely due to the sampling strategy at the time of harvest of the greenhouse experiment (Chapter 2): needles were randomly sampled from the entire seedling. Following reflection on the results obtained in this experiment, it seems reasonable to conclude that a response in a receiver seedling would most likely have been localized to the buds, and not detected systemically throughout all the needles of the plant. *Arabadopsis thaliana* is capable of localized responses based on oviposition of defoliating insect eggs (Little *et al*., 2006), and defense is costly to the plant in the short and long term (Strauss *et al.*, 2002), thus it is likely that the activation of defense response would be localized to herbivore targeted tissues only. Ultimately, the high variability is likely due to mixing tissues actively preparing a defense response with tissues that were less likely to be targeted by the specific herbivore (in this case WSB). An effort was made to replicate a simplified version of the transfer experiment in controlled growth chamber experiments, however, this was unsuccessful.

Flavonoid

The more than four-fold increase in a flavonoid compound (Figure 29) (Isorhamnetin-3-Galactoside-6"-Rhamnoside) detected in kin vs stranger (networked; budworm treated), as originally uploaded to the ReSpect databse by Sawada *et al*., (2009), provides compelling evidence that defensive information is preferentially transferred to kin through MNs. This corresponds well with the significant results in Chapter 2, which show that the ^{13}C stable isotope of carbon is preferentially transferred to kin vs strangers in this treatment group (networked; budworm treated). The specific compound, while not originally identified in a conifer (Sawada *et al.*, 2009), nonetheless is a member of a class of molecules that have been extensively studied in conifers (Feucht *et al.*, 2012; Lee *et al.*, 2014). Flavonoids occur in high levels in *Pseudotsuga menziesii* (Feucht *et al.*, 2012). Furthermore, they have been implicated in synergizing insecticide synthesis in *in vitro* studies (Wang *et al.*, 2016). They are also associated with stress

responses, specifically in cases of heat stress and drought in conifers (Feucht *et al.*, 2016). At least in white spruce (*Picea glauca*), flavonoids are synthesized in response to jasmonate signaling from wounding (Richard *et al.*, 2000). Increasing jasmonate concentrations trigger an increase in production of flavonoids (Richard *et al.*, 2000), thus providing circumstantial support for the suggestion that the information molecule transferred in this case was a jasmonate. In order to trigger any defense response, including flavonoid production, the phytohormone jasmonic acid must first be produced. In sitka spruce (*Picea stikensis*), wounding triggers AOE allene oxide cyclase, which converts 12,13-epoxy-octadecatrienoic acid into 12-oxo-phytodienoic acid (or OPDA) (Ralph *et al.*, 2006), triggering the octadeconoid synthesis pathway that triggers jasmonic acid synthesis (Ralph *et al.*, 2006). The jasmonate pathway is complex, with many potential points of regulation feeding into jasmonic acid expression (Wasternack, 2007).

Coenzyme A

Coenzyme A (Baddily *et al*., 1953) is a ubiquitous compound that binds to enzyme substrates, promoting reactions involved in the synthesis and oxidation of fatty acids (Nelson & Cox, 2005). Coenzyme A was also found to be elevated (approximately a 3-fold increase) in a kin versus stranger receiver seedling connected to its WSB-treated donor through a MN (Figure 30). Terpenes, implicated in conifer defense against insect herbivory, among other stimuli, are synthesized from fatty acids utilizing coenzyme A in the process (Huber & Ralph, 2004; Huber *et al.*, 2005). The finding of elevated coenzyme A may denote the early stages of terpene synthesis in the receiver kin seedling. Karst *et al.*, (2015) found that monoterpenes in stems and needles of *Pinus contorta* depended on the identity of the fungal community associating with the trees; those fungi sourced from pine-beetle killed stands resulted in lower monoterpene tissue content. Thus mycorrrhizal fungi may be mediating defense signaling and defensive chemistry in conifers, both in the short term, as illustrated by this study, and as a legacy effect of previous insect outbreaks.

Piperacillin

Piperacillin is a semi-synthetic broad spectrum β lactam antibiotic that gained favour in the 1970s and 80s (Fu & Neu, 1978; Eliopoulos & Moellering, 1982), but since has become less

clinically useful due to bacterial antibiotic resistance (Drawz & Bonomo, 2010). Finding this compound in kin Douglas-fir needles is most likely a misidentification, as it is highly unlikely that Douglas-fir needles have the capacity to produce a semi-synthetic antibiotic. However, as an indirect defense mechanism (Heil, 2008), perhaps Douglas-fir defense signals trigger a fungal foliar endophyte to produce an antibiotic targeting the members of the gut microbiome of WSB in order to reduce its feeding efficiency. Recruiting carnivores that prey on plant pests is an example of indirect plant defense (Heil, 2008), perhaps triggering endophytes to effectively hobble defoliator digestion. The presence of endophytes on plant leaves has been shown to affect foraging behaviour of herbivores (Vicari *et al.*, 2002; Afkhami & Rudgers, 2009). While WSB gut microbiomes have not been studied, it is known that other Lepidopteran species' gut microbiomes contain certain bacterial species that are crucial to a worm's proper digestion of plant material (Chen *et al.*, 2016; Xia *et al.*, 2017). A multi-partite symbiosis of this nature was not addressed in this experiment, however compelling it may be. It is unfortunate that, lacking biological replication, this result is tenuous. However, the analysis conducted here does provide a good hypothesis for future testing, and provides intriguing hints as to the defensive processes that ectomycorrhizal fungi may play a role in mediating through signal transfer.

Non-target whole metabolome profiling is a powerful technique for identifying metabolites of interest in plants (De Vos *et al.*, 2007). Online tools reduce the learning curve required to analyze the large datasets (Tautenhahn *et al.*, 2012), thereby rendering LCMS techniques accessible to a wider researcher audience. Some compelling molecules were identified in this study, elevated in kin over stranger, and tentatively linked to a complex defense chemistry in conifers. The main barrier to using this technology is the high cost of sample processing, which can be reduced by designing experiments with low replicate numbers with a reduced number of treatments. The greenhouse factorial experiment (3 x 3 x 2 with 10 replicates) was too large to analyze using LCMS. With a simplified experimental design, non-target whole metabolome profiling has the potential to identify the molecule being transferred between paired Douglas-fir seedlings linked via MNs, which would be a significant contribution to the field, given that all evidence for defense molecule transfer, has, to date, been indirect evidence.

Chapter 5: Mycorrhizas

Introduction

Using mesh bags to manipulate the presence of a mycorrhizal network was first demonstrated in a lab study by Simard *et al.*, (1997), which documented reciprocal carbon transfer between paper birch (*Betula papyrifera*) and Douglas-fir. This approach was adapted by (Fitter *et al.*, 1998) to test transfer of stable isotope (^{13}C) carbon between a grass and a herb through arbuscular MNs. Subsequent work refined this approach and explicitly tested its efficacy in preventing root interaction while permitting fungal hyphae to interact, thereby allowing for delineation of transfer through soil-only, soil and hyphae-only, and roots-hyphae-and soil pathways (Teste *et al.*, 2006; Philip *et al.*, 2010). For example, Teste *et al.* (2006) showed that the rhizomorphs of ectomycorrhizal fungi (thick aggregations of multiple hyphae that enable transport of water and nutrients over 10's of cm or even meters) were blocked by mesh with pore sizes < 1 μm. Application of the mesh bag method for control of MN formation between plants has been successfully applied to field experiments (Johnson *et al.*, 2001, 2005; Schoonmaker *et al.*, 2007; Teste & Simard, 2008; Teste *et al.*, 2009).

Mycorrhizal fungal hyphae vary in their longevity, but are typically ephemeral and turn over at a faster rate than fine roots (Allen & Kitajima, 2013). Direct observation of a MN would be an onerous task, but can be inferred by examining the distribution of fungal genets (the mycelia of a single fungal individual) on multiple host plants (Beiler *et al.*, 2010). Descriptions of the MN in transfer experiments have relied on assessments of root tip communities using morphotyping (Agerer, 2001), as in Philip *et al.*, (2010) or additionally cloning, and Sanger sequencing of these morphotypes as in Teste *et al.*, (2009). Observation and identification of colonized root tips may be considered indirect evidence of MN formation; however, taken together with the evidence from more direct observations (e.g. the identification of genets via genotyping, and the transfer of isotopically labelled compounds between plants that could only access them via fungal rhizomorphs), provide strong support for the formation of MNs through appropriately-sized mesh in belowground bag treatments.

Ectomycorrhizal fungal colonization of both variants of Douglas-fir (coastal and interior) has been well documented (Borchers & Perry, 1989; Molina & Trappe, 1994; Horton & Bruns, 1998; Twieg *et al.*, 2007) Indeed, the high fungal diversity observed in forests dominated by

Douglas-fir led Trappe, (1977) to speculate that this host may associate with over 2000 fungal species. Most of these initial observations were based on sporocarp data, yet observations of the fungal communities of Douglas-fir root tips are also suggestive of high diversity. Twieg *et al.*, (2007) found 105 fungal OTUs within an area of 30m x 30m, and demonstrated that fungal community structure differed between age classes of Douglas-fir, with the youngest cohort supporting the least diverse community. Furthermore, younger cohorts supported more Douglas-fir specialist symbionts, whereas older Douglas-fir tree fungal communities had more generalists, many of which (~40%) were shared with neighbouring paper birch.

Sequences of the internal transcriber region (ITS) are used for the identification of most ectomycorrhizal fungi (Koljalg *et al.*, 2005, 2013). As opposed to sequencing single root tips or morphotypes, using high through-put techniques such a Illumina MiSeq, generates millions of sequences for a single run that can be split between multiple samples and can represent a community of fungi (Chu *et al.*, 2016; Glassman *et al.*, 2017). The ability to detect and identify ecotmycorrhizas has recently been greatly improved by the development of new fungal specific primers (Ihrmark *et al.*, 2012). In addition, the development of PIPITS (Gweon *et al.*, 2015) brings together many disparate open-access tools optimized for use in fungal community identification. While tools such as QIIME (Caporaso *et al.*, 2010) provide users with the flexibility to modify parameters to suit community analysis in fungi, QIIME does not facilitate analysis of the length variability found in fungal ITS regions. Thus fungal datasets must be moved from one environment to another to apply tools such as ITSX (Bengtsson-Palme *et al.*, 2013), which is specifically design to handle length variable ITS amplicon data. PIPITS incorporates ITSX, as well as 8 other dependencies in a Linux environment to provide users with a streamlined pipeline for analysis of fungal communities from Illumina sequencing datasets.

The purpose of this study was to verify whether Douglas-fir seedlings used in the greenhouse and field experiments were colonized by ectomycorrhizal fungi. This would lend support to the assertion that the belowground mesh bag treatments (MN) were likely to have formed a MN. Furthermore, the community structure of fungi colonizing Douglas-fir seedling root tips was hypothesized to vary depending on the genetic identity of Douglas-fir, providing a plausible mechanism for kin selected transfer through MN via preferential MN formation with select symbionts.

Methods

Douglas-fir seedling root tips from the field experiment were analyzed for ectomycorrhizal fungal community structure. The experimental design is recounted in brief below, for full details, see Chapter 3.

Experimental design

A 2 x 2 x 10 factorial set of treatments with two **belowground** treatments (*mycorrhizal network (MN) and blocked roots and fungal hyphae (B)*), and two **defoliation** treatments (*no-herbivory control* and *manual defoliation*) was replicated over 10 **relatedness** treatment combinations of 4 *kin* (*AA, BB, CC, DD*) and 6 *stranger* pairs (*AB, AC, AD, BC, BD, CD*, with 4 replications for a total of 160 seedling pairs.

Full sibling Douglas-fir seedlings were grown from seed and planted into mesh bags (35µm or 0.5µm pore size) in the field in late April and early May 2016 near Revelstoke, BC (50°50'33.4"N 118°03'04.8"W). Seedling pairs received the manual defoliation or control treatments immediately prior to being pulse-labelled with ^{13}C-CO_2 and destructively harvested in late September and early October 2016. For full details see Chapter 3.

Fungal community

All colonized root tips were harvested from all seedlings in both the greenhouse and field experiments and randomly sampled for fungal community analysis. Randomization was performed by floating root tips, 30 at a time, in a petri dish containing distilled water. A sheet of 3cm grid paper was placed underneath the petri dish and only those root tips that crossed a line were selected. Approximately 1-2 g of roots (wet) were harvested from each seedling in this manner and frozen at -80°C. DNA was extracted from frozen samples by first grinding in liquid nitrogen and subsequently extracting using DNeasy® Plant Mini Kit (Qiagen). DNA was diluted 1/10 for PCR amplification using new fungal ITS2 primers (Ihrmark *et al*., 2012), which provide a significant improvement in specificity for fungal community analysis over older classic primers (Gardes & Bruns, 1993). Extracted DNA was prepared for Illumina® MiSeq as per Gweon *et al.*, (2015). Briefly, a two-step PCR was used first to amplify the target community and attach over-hangs on both forward and reverse primers which are targets for the second set of primers containing DNA barcodes (indexes). The product of the first PCR was used as template in the second, and each amplified sample was gel extracted using QIAquick gel extraction kit (Qiagen ®), and added to a single library in equal proportions. Sequencing was performed on the

Illumina® MiSeq platform (Microbiome Insights Inc, UBC, Vancouver Canada for the greenhouse seedlings; and The Applied Genomics Core-TAGC, University of Alberta, Edmonton, Canada for the field seedlings). The raw data generated was analyzed using the PIPITS pipeline (Gweon *et al.* 2015), which was designed for the analysis of mixed fungal communities by amalgamating a suite of open-source tools into a stand-alone package implemented in a Linux environment. Data was quality filtered, taxonomy assigned based on the curated ectomycorrhizal fungal database "UNITE" (Koljalg *et al.*, 2013) and checked for chimeras with a trained database based on fungal ITS sequences. Variable length ITS sequences were extracted and utilized for taxonomic assignments and OTU table generation (Gweon *et al.*, 2015). The OTU table was used to generate non-metric multidimensional scaling (NMS) graphs for visualizing fungal community structure using PC-ORD 5.0 (MjM Software Design). Statistically significant differences between treatment groups were assessed using permutational multivariate ANOVA (PERMANOVA) for balanced groups, and multi-response permutation procedures (MRPP) when groups were not balanced (MjM Software Design).

Results

A total of 17, 841, 553 raw sequences generated by two Illumina MiSeq runs, were consolidated into 10, 070 operational taxonomic units (OTUs) at a level of 97% similarity, which were further sorted into 1876 phylotypes (binning based on the same taxonomic assignments). Examination of sequence abundance revealed that 95% of the sequences were represented by 105 phylotypes, each containing 10, 000 sequences or more (Figure 32). These were used to run NMS and MRPP analysis. No NMS solution was found (when running 105 phylotypes, 1876 phylotypes, and the full 10,070 OTU table) and MRPP found no ecologically significant differences between any of the treatment groups when all fungi were considered together, however, some interesting patterns were found when ectomycorrhizal fungi only were considered. When considered together as pairs, significant MRPP differences were discovered between the DD pair and 5 out of 9 of the other pairs of seedlings planted (non-significant comparisons not shown). However, grouping the data by family D alone yielded marginally significant MRPP differences when comparing D to A, and D to B, but no other comparisons (Table 14).

For overall fungi, each seedling bore a similar community to that observed across all seedlings, consisting of 79% Ascomycota, 20% Basidiomycota and 1% Zygomycota, based on sequence abundances (Figure 30). Genus level identification was possible for most phylotypes.

Ectomycorrhizal fungi, and putative mycorrhizal fungi, comprised 32% of all sequences and are summarized in Figure 31. Phylum Basidiomycota contained the highest proportion of ectomycorrhizal fungi (71%), whereas only 21% of Ascomycota sequences were from ectomycorrhizal fungi, and only 9% from Zygomycota. Species accumulation curves of the entire data set, as well as the 95% subset of fungal and ectomycorrhizal sequences used to analyze this data set, show that sampling was sufficient to capture the diversity of fungi associating with roots of Douglas-fir in this experiment (Figure 34). Species accumulation curves (Figure 34) indicate an alpha diversity of approximately 90 unique sequences between all seedlings representing approximately 25 phylotypes. Phylotypes correspond to a group of sequences that bin to the same taxonomic assignments based on the UNITE database (Koljalg *et al.*, 2005) of curated fungal sequences.

Indicator species analysis indicated *Rhizopogon* sp. and *Rhizopogon parksii* was associated with the blocked belowground treatment, occurring significantly less often in the other belowground treatments. In addition, the free belowground treatment harboured *Geomyes auratus* more often than the other treatments. Finally, *Tomatella* sp. was associated with Family C but not the other three families tested. No other families had statistically significant indicator species associated with their roots. All treatment levels were considered, and only statistically significant results are reported in Table 13.

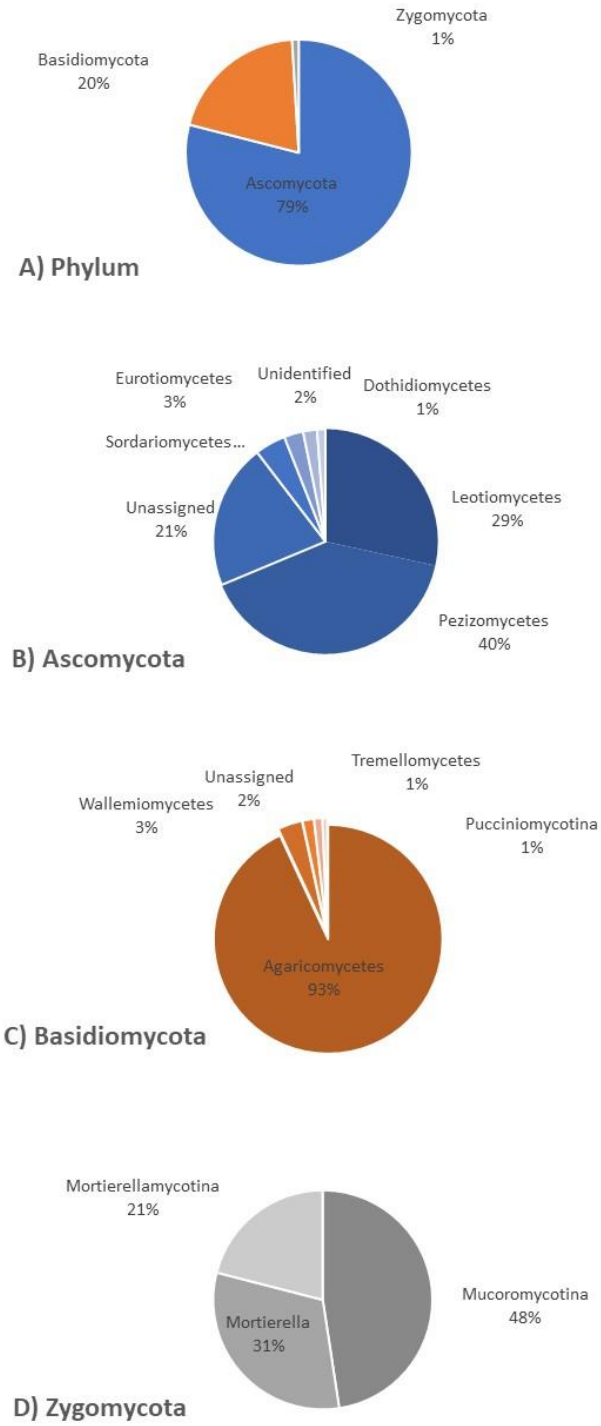

Figure 32. Pie chart of fungal sequences from all field experiment seedling root tips showing breakdown by A) Phylum and Class by B) Ascomycota, C) Basidiomycota and D) Zygomycota.

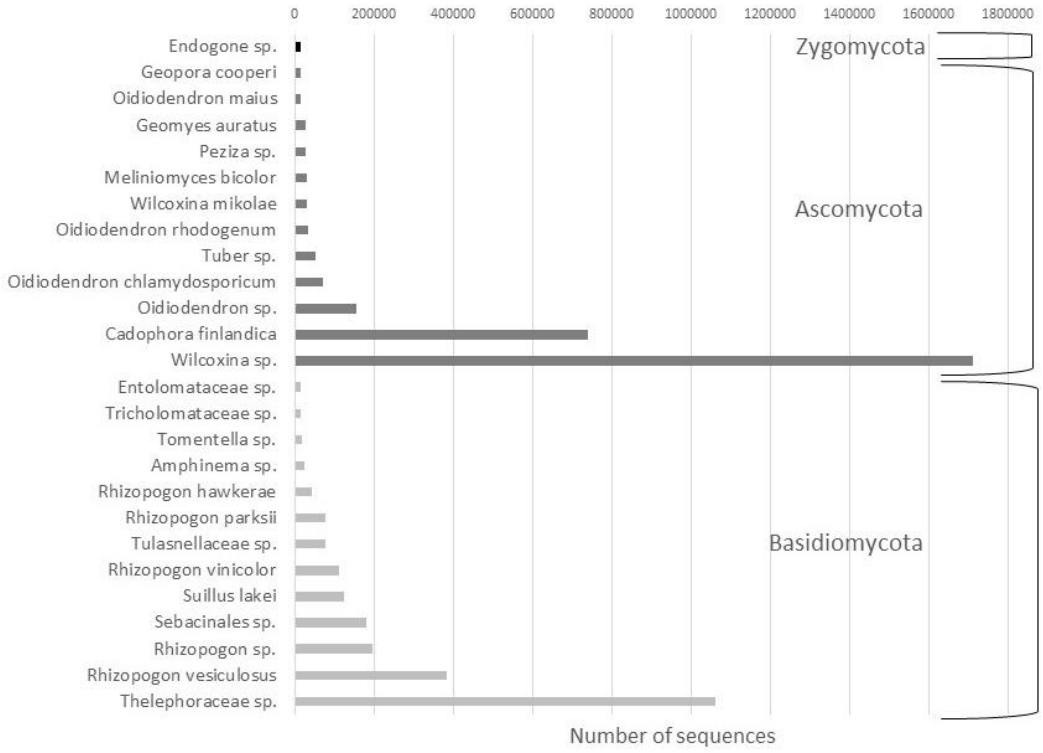

Figure 33. Numbers of sequences of all ectomycorrhizal species and putative ectomycorrhizal species identified from root tips of field samples, accounting for 32% of all fungal sequences (71% of all Basidiomycota, 23% of all Ascomycota, and 9% of all Zygomycota sequences).

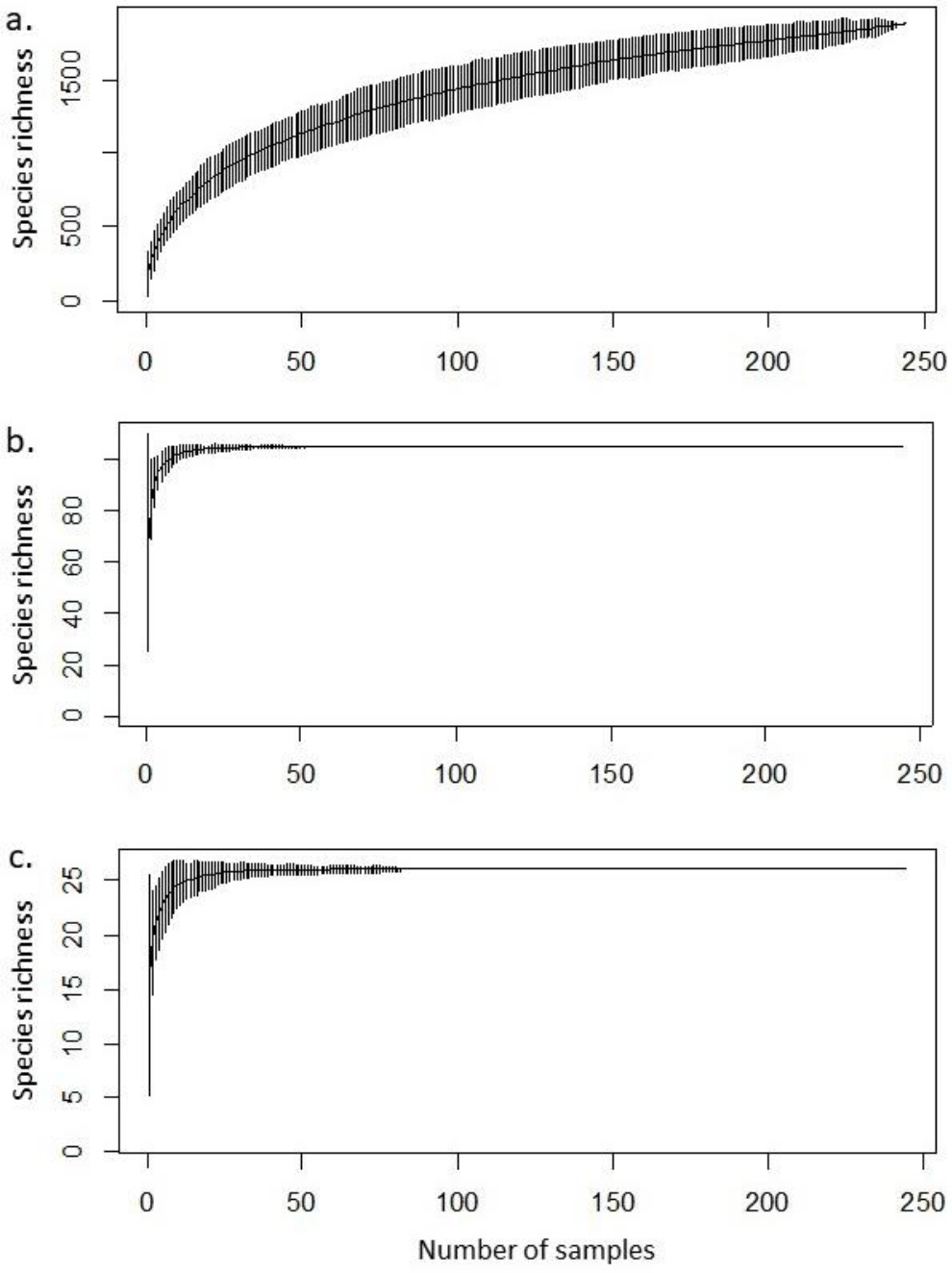

Figure 34. Species accumulation curves for phylotypes based on PIPITS process. a. All fungal phylotypes. b. Fungal phylotypes with ≥ 10k sequences. c. EMF phylotypes with ≥ 10k sequences. Curves based on 100 permutations of Chao richness estimator. Bars represent 95% CI of mean species richness estimate.

Table 13. Indicator Species analysis results by treatment group. Only statistically significant results within treatment levels are included. Treatments without statistically significant indicator species include herbivory treatment, as well as the identity of the family pairs (family in this case indicates the identity of the seedling itself, rather than the identity of the family that it was planted along-side with).

Treatment	Level	Species ID	Indicator Value	p-value
Belowground Treatment				
	Blocked			
		Rhizopogon sp.	37.9	0.028
		Rhizopogon parksii	34.4	0.018
	Free			
		Geomyes auratus	37.5	0.013
Family				
	C			
		Tomentella sp.	6.5	0.016

Table 14. MRPP comparisons of fungal community structure of family pairs, as planted in the field, and individual families. For brevity, only 6 out of a possible 45 pairs of comparisons for family pairs are presented (the remaining 39 combinations were not significantly different at a level of $p>0.05$).

As pairs

comparison	t-values	p-values
DD vs AA	-3.022	**0.012**
DD vs AC	-2.860	**0.015**
DD vs AD	-2.214	**0.034**
DD vs CD	-2.871	**0.014**
DD vs BD	-2.476	**0.024**
AB vs CD	-1.825	**0.054**

As single family

comparison	t-values	p-values
A vs B	-0.332	0.297
A vs C	-0.425	0.277
A vs D	-1.451	*0.088*
B vs C	-0.265	0.326
B vs D	-1.555	*0.077*
C vs D	0.400	0.594

Discussion

As expected, ectomycorrhizal species were found on root tips of Douglas-fir seedlings, implying the potential for the formation of MN between planted seedlings. The second hypothesis that Douglas-fir ectomycorrhizal colonization would be structured based on the genetic family identity of Douglas-fir was partially accepted, as there were indications that the ectomycorrhizal community may differ based on based on pairings of families, particularly in the case of the DD pairing. Most other pairs were not significantly different to each other, nor was family composition different when considered individually (with the exception of a marginally significant difference in the case of two comparisons: A vs D and B vs D). These observations indicate a particular family pairing of Douglas-fir can structure the ectomycorrhizal community, but not all pairs have the capacity to generate this effect. In addition, the effect seems to be amplified depending on the identity of the seedling planted adjacent to it. Family D is implicated in most of these interactions. Taken as a whole, the fungal community (ectomycorrhizas at 30% and all other fungi at 70% of the total community) did not differ under any of the treatments. Thus the hypothesis, as presented, can only be partially accepted. The data reveal more nuance and point to genetically-derived differences in complex interacting systems.

Indicator species analysis added another layer of complexity to the interpretation of ectomycorrhizal community structure. Family D, while implicated in the MRPP analysis failed to yield a significant indicator species. Thus there is no particular fungus that can be implicated in this effect, rather, it is the structure of the entire community that differs. *Tomentella* sp. was found to be an indicator species for family C (Table 13). No other families demonstrated statistically significant indicator species. In addition, while *Tomentella* sp. was found to be associated with family C most often, and absent from the other families, it is not one of the more abundant species sequences found (Figure 34). Perhaps a longer residence time in natural soils would have allowed more ectomycorrhizal species selection to occur, however, during the duration of this experiment, there is scant evidence to support the hypothesis that families harbor unique ectomycorrhizal fungi.

In studies that presuppose the formation of a MN by growing seedlings in mesh bags, it is vital to confirm that colonization by ectomycorrhizal fungi took place. In this field experiment, 32% of all sequences were ectomycorrhizal or putative ectomycorrhizal species, including *Rhizopogon* sp. which are long distance contact types (Agerer, 2001) known to form

rhizomorphs and implicated in transfer between plants in MN (B*eiler et al*., 2010), although transfer can occur through other types of fungi (Deslippe *et al.*, 2016).

Finding no difference in community structure between genotypes of Douglas-fir indicates that all Douglas-fir root tip communities in this experiment have the same potential to form a MN. The kin effects noted in both studies are not likely to be caused by Douglas-fir preferentially associating with a particular fungal community. The role of the fungi in the kin effect is simply to provide a conduit or physical pipeline for transfer of information chemicals. File *et al.*, (2012) found an increase in MN size and abundance of symbiont when *Ambrosia artemisiifolia* L. was grown with kin. It appears as though selectivity for ectomycorrhizal fungal symbionts is not a mechanism involved in kin selection in Douglas-fir, unlike that found in *A. artemisiifolia* by File *et al.*, (2012).

Interestingly, there is some evidence to suggest that growing Douglas-fir seedlings in mesh bags has the potential to select for particular ectomycorrhizal species. While the overall community structure did not differ significantly between belowground treatment groups, some species were identified as indicator species (Table 13). In the blocked (0.5µm mesh) belowground treatment, two *Rhizopogon* species (or rather, possibly the same species, as the phylotype *Rhizopogon* sp. could not be identified down to species level) occurred more often in this treatment and not in the free (no bag) or MN (35µm). *Rhizopogon parksii* is a known mycorrhizal associate of Douglas-fir and forms many sized rhizomorphs (Massicotte *et al.*, 2000). If the formation of a mycorrhizal network is the preferred state of a seedling-fungus-seedling interaction, then perhaps attempting to block a mycorrhizal network with small pore-sized mesh may select for ectomycorrhizal fungi that have the capacity to form hyphal connections of variable width. Thus rather than blocking the network, this belowground treatment may instead be selecting for fungi that have the capacity to penetrate through artificial barriers. Indeed, in Chapter 2, the blocked network belowground treatment did not appear to block the MN at all. In those experiments, however, the network had 8 months to establish, whereas in the field experiment, the establishment of the MN was reduced to only 4 months. And finally, *Geomyces auratus* was an indicator species for the free (no bag) belowground treatment. This species, also known as *Geomyces pannorum*, is capable of forming ericoid mycorrhizas (Vohník *et al.*, 2007) and its presence may indicate connections to the wider understory ecosystem at the field site. It was not found in high abundance (Figure 33). Nonetheless, of the

25 phylotypes identified in this study, only 3 (or 4, assuming the *Rhizopogon* sp. phylotype represents a different species) exhibited preferences for any of the treatments. Thus the overall null hypothesis of no difference in community composition between all treatments is accepted.

In these studies, colonization frequency was estimated from the number of fungal nuclear ribosomal ITS sequences recovered using high-throughput Illumina sequencing. Although this approach is not without problems, it is becoming a more accepted method (Taylor *et al.*, 2016) owing in large part to improvements fungal primers (Ihrmark *et al.*, 2012; Taylor *et al.*, 2016). The large numbers of sequences generated by a MiSeq run (10 million) increase the chances of sequencing even those species present in very low numbers. Determining percent colonization of a target ectomycorrhizal community is difficult regardless of the methods employed, depending more on the extent and depth of sampling (Taylor, 2002) and less on the methods used to count. More importantly to studies considering the formation of a MN, it is the presence of fungi capable of forming a MN, with representative ectomycorrhizal fungi identified in sufficient numbers to form the network.

Conclusion

Kin selection in Douglas-fir was the strongest effect found in these studies. Seedlings given the choice to transfer ^{13}C derived from a ^{13}C-CO_2 pulse to either a full-sibling kin or a known genetic non-relative (stranger) preferentially transferred carbon to kin. This kinship response could be amplified with herbivory treatment. Specifically, preferential transfer to kin over stranger always occurred at low levels, even in untreated controls, and this transfer could be made to increase with western spruce budworm defoliation or manual defoliation of the 'donor' seedlings. Douglas-fir were assigned designations of 'donor' and 'receiver' with the donor being herbivory treated and pulsed with stable isotopes. The amount of transferred ^{13}C was compared between receiver kin and stranger seedlings grown in the same greenhouse pot to test for any evidence of a donor choice or preference. Transfer occurred through belowground pathways, including through mycorrhizal networks, in all cases, as all above-ground transfer pathways were blocked.

Distinct families of Douglas-fir seedlings behaved differently under identical conditions. Some families preferentially transferred a signal to kin, some transferred to strangers as well, whereas some showed no preference. "Families" in this case were groups of full-sibling kin derived from known parents within the same seed provenance. In addition, one of the families recruited a unique suite of ectomycorrhizal fungi to form their mycorrhizal network. All seedlings were inoculated by a mixed set of ectomycorrhizal fungi from soils found in their original home range. Each seedling harboured between 15-25 unique phylotopes of fungi, as determined by high-throughput sequencing approaches of a random sampling of colonized root tips. Hypothetically, each seedling was exposed to the same mixed community and given the same opportunity to 'select' from potential ectomycorrhizal partners. Most families hosted very similar communities, except for family D, which hosted a unique suite of root-associated fungi, and interestingly, this effect was amplified when D was grown with its kin. Family D also showed significant preferential transfer of signal to kin over strangers. However, other families that showed significant preferential transfer to kin over strangers did not harbor a unique ectomycorrhizal community structure. Thus some families may rely on a particular mycorrhizal network, but kin-selection is not necessarily dependent on the identity of the fungi forming the mycorrhizal network.

The presence of a mycorrhizal network was required for transfer in a field experiment, but was not necessary for transfer in a greenhouse experiment. This result may point to problems with using mesh bags of varying pore size to permit or restrict the formation of a mycorrhizal network. Or alternatively it may reflect the different residence times of seedlings in those bags (in the greenhouse experiment, the Douglas-fir seedlings were grown for an additional 4 months, and thus the roots may have had opportunity to perforate the mesh bags, skewing results). Thus a mycorrhizal network is sufficient for transfer, but not necessary. Transfer of signal molecules in response to a herbivory stimulus may take place through the soil matrix as root exudates, not necessarily through fungal hyphae. Other soil denizens, such as bacteria, may facilitate signal transfer. Additionally, transfer may also occur as diffusion along a gradient in the soil.

Signal transfer, in this thesis, is defined as the belowground transfer of newly photosynthesized carbon compounds. The identity of the transferred compounds was not determined. Whole tissue metabolomics was attempted to identify signals, and some compounds, possibly implicated in defense-signaling, were identified. It was hypothesized that defense signals would be transferred in response to herbivory stress, but no common plant defense signals were identified. While the movement of carbon in similar experiments has been demonstrated to respond to source-sink dynamics and thus considered resource-sharing in previous work, transfer in the context of these experiments was considered to be communication. In the case of herbivory control treatments (that is, no herbivory applied), transfer occurred nonetheless. This transfer may represent a constitutive sharing, effectively indicating that lines of communication are open belowground, much like aboveground pathways. Once herbivory stress was applied, a pulse of information regarding the nature of the attack was expected to move quickly from the attacked donor to the receiver through a belowground pathway, and indeed, the amount of ^{13}C transfer increased in those cases.

Thus kin-selected signal transfer through mycorrhizal networks, as well as alternative belowground pathways, occurs in some families of Douglas-fir, and is stronger in some families. Hence it appears that in the case of the conifer Douglas-fir, as is found with human mammals, some families are nicer than others.

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