Mycorrhizal facilitation of kin recognition in interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*)

by

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Abstract

Insight into influences on successful seedling establishment could be essential to future regeneration of British Columbia's interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) forests, particularly as climate changes. Areas of harsh climatic conditions have low regenerative capacity and require management decisions leading to enhanced seedling establishment. Variable retention harvesting and natural regeneration from residual trees, for example, may become increasingly important for their locally adaptive traits as climate changes. Kin recognition, mycorrhizal networks, or the combination of the two may be important mechanisms for enhanced seedling establishment in these regions. We examined the effects of relationship (kin vs. non-kin) and mycorrhizal networks on regeneration from seed in greenhouse and field settings. In the greenhouse, kin recognition was evident in differing foliar microelement (Fe, Mo, Al and Cu) and growth variables (total leaf area, volume and stem length) according to relationships between seedlings. Kin recognition was also weakly evident in the field, where it was expressed as differential survivorship among kin versus non-kin seedlings. Kin selection was evident in the greenhouse, where microelement content of kin was greater than non-kin. Greater mycorrhizal colonization of kin compared to non-kin as well as greater donor total leaf area, volume and stem length also suggest kin selection, although not consistently in all experiments. In the field, survivorship was greater among non-kin; however, detection of kin recognition may have been masked by the large effects of site and seed origin on germination and survival. Mycorrhizal networks and carbon transfer occurred within all greenhouse seedling pairs, and enhanced mycorrhization of kin suggests network colonization was involved in kin selection, but our data does not strongly support our hypothesis that kin recognition was facilitated by mycorrhizal networks. While the mechanism of kin recognition is still not well

understood, we provided evidence of kin recognition in interior Douglas-fir seedlings, particularly those that originate from harsh climates, and observed subtle indicators of kin selection or reduction of competition due to a close genetic relationship. Accounting for these phenomena in forest management could be helpful to successful regeneration of interior Douglas-fir forests as stresses associated with climate change increase.

Preface

Contributions by Amanda Asay:

This thesis is an original, unpublished product of the author, Amanda Asay. The identification of the research topic was done by supervisor Dr. Suzanne Simard and modified by Amanda Asay and Dr. Suzanne Simard in conjunction. The design of the research program was conducted by Amanda Asay and Dr. Suzanne Simard with input from committee members Dr. Sally Aitken, Dr. Daniel Durall and Dr. Susan Dudley. Dr. Brian Pickles assisted with the design of the $^{13}CO_2$ labelling design and morphotying procedures. Dr. William Mohn and Roland Wilhelm assisted in the $^{13}CO_2$ labelling design only. The performance of all of parts of the research, both field and greenhouse, were led by Amanda Asay with exception to the DNA analysis, which was sent to the Irving K. Barber School at UBC-Okanagan, and the microwave digestion/ICP (Inductively Coupled Plasma-Optical Emission Spectrometer) and % C, N and S analyses, which were sent to the British Columbia Ministry of Environment Analytical Chemistry Laboratory, and the EA-IRMS (elemental analysis-isotope ratio mass spectrometry), which was sent to the Stable Isotope Facility at UBC-Vancouver. The morphotyping process was shared by Dr. Brian Pickles (Chapter 2) and Amanda Asay (Chapter 2 and 3). The analysis of the research data was done by Amanda Asay with suggestions from Dr. Suzanne Simard and statistical advice from Dr. Valerie LeMay.

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1 Introduction

There are two main questions explored in this thesis: (1) whether kin recognition occurs among interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*-(Beissn.) Franco), and (2) whether kin recognition, if present, is facilitated by mycorrhizal networks.

Study species

The study was conducted on a common and economically valuable species in the interior of British Columbia, Canada: interior Douglas-fir. Interior Douglas-fir forests are widely distributed across western North America, ranging from north-central British Columbia (55^{o}N, up to 760 m elevation) to northern Mexico (19^{o}N, up to 3260 m elevation). Broad variation occurs in climate (precipitation range 410–3400 mm per year; mean July temperature 7–30^{o}C; mean January temperature -9 to 8^{o}C), disturbance regimes (e.g., stand maintaining to stand replacing fires) and site quality (very dry and poor to very moist and rich) (Hermann and Lavender, 1990).

Both pollen and seed from interior Douglas-fir are wind dispersed, which allows for large, continuous populations within its range that can span relatively large, disconnected geographic areas (Hamrick et al. 1992). Douglas-fir is highly genetically diverse compared to other conifers with a considerable amount of genetic variability between varieties correlated with the environmental conditions of the seed's origin (Campbell and Sorensen, 1978, Rehfeldt, 1978, Campbell, 1986, Krutovsky et al. 2009). Population boundaries of Douglas-fir tend to be delineated by areas of low elevation, such as rivers and valleys and high elevation, such as mountain ranges (Rehfeldt, 1989). Elevational topographic features are often only a barrier to seed dispersal but not pollen dispersal (causing the difference between markers of mtDNA,

associated with seed dispersal, and cpDNA, associated with pollen dispersal) and therefore gene flow between populations can persist (Gugger et al. 2010).

Most of the genetic variation of Douglas-fir is due to high regional diversity. There is little variation among populations within the same region (< 1% using mtDNA markers and 4.1% using cpDNA markers) or among individuals within a population (7.8 % using mtDNA markers and 27.5 % using cpDNA markers) when compared with the variation among groups (91.5% and 68.3% respectively) (Gugger et al. 2010). This is consistent with Rehfeldt (1989) who found that most genetic variation occurred over significant differences in geographic distance or elevation, which translated into adaptive differentiation depending on the number of frost free days in the region.

In this study, mature, seed bearing trees from Paska Lake, Farwell Canyon and the Alex Fraser Research Forest, all within British Columbia, were used as well as control cross pollinated seeds from the Kalamalka Research Station. The Alex Fraser Research Forest, Knife Creek block (121.88°W, 52.05°N) and Farwell Canyon sites (122.63°W, 51.79°N) are located outside of Williams Lake in the Cariboo Chilcotin Coast Region. The Paska Lake site (120.67°W, 50.50°N) is located approximately 30 km southwest of Kamloops in the Thompson Okanagan Region. Due to weak differentiation among populations in studies of coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco) in BC, we expected the Alex Fraser Research Forest and Farwell Canyon locations to represent similar 'Cariboo' populations (Krutovsky et al. 2009). The Paska Lake site falls in a different geographic region known as the Thompson-Okanagan, and it likely represents a significantly different population. The two sites in the Cariboo are dry with mild summers and cold winters whereas the site in the Thompson-Okanagan is dry, but has hot summers and mild winters (Spittlehouse 2006) (addition climate data can be found in Tables 4.1-

4.3). Comparisons between these sites were conducted to examine regional and within population or subpopulation effects.

Kin recognition and kin selection

Kin selection describes cooperation between genetically related individuals that can enhance their combined fitness despite the potential individual fitness cost of the cooperative behaviour. The tendency for an individual to participate in these cooperative behaviours, potentially leading to kin altruism, is described in Hamilton's rule [Hamilton 1964]: $rB > C$ where C is the cost to the individual participating in the cooperative behaviour, B is the benefit to the relative or group of relatives and r is the degree of relatedness. A common example of this phenomenon is in social insects such as bees. Due to the high degree of relatedness in the hive from all individuals sharing maternal genes from the queen, each individual can suffer high individual cost to defend or otherwise benefit the hive and still have an overall positive effect on its genes' fitness (Platt and Bever 2009).

Kin selection is not nearly as well understood in plants due to the problem of kin recognition. Kin recognition is the ability to distinguish between kin and non-kin individuals. Animals and many insects have the advantage of context in order to recognize which individuals are their kin, such as bees in a hive, eggs in a nest or, even more clearly, a mother giving birth. Depending on seed dispersal tendencies a plant may just as easily be growing next to a kin individual, non-kin member of the same species or even a different species. Due to this challenge of recognition, before we can establish that kin selection is occurring, we first must establish that there can be recognition of an individual that is closely related. Biedrzycki et al (2010) has provided strong evidence that kin recognition occurs in *Arabidopsis thaliana*, a weedy

herbaceous annual. They also found evidence that kin recognition is conveyed through the active release of soluble root exudates. They determined this by growing seedlings in a medium with the exudates of a kin, a stranger or their own exudates and examined the number of lateral roots developed. The seedlings that were grown in the presence of stranger exudates developed significantly more lateral roots than those grown in either the kin or self-root exudates. They confirmed it was the exudates that were conveying the neighbour identity by adding a root secretion inhibitor that eliminated the differences. This distinct difference suggests that these plants can differentiate between a kin and a stranger by recognizing their root exudates.

These results can be interpreted as kin selection as well. Lateral root development is considered a competitive trait in plants. When a plant produces fewer lateral roots due to its recognition of a kin neighbour, it can be said that it is sacrificing some of its below ground competitive ability to allow its kin to also succeed in close proximity (kin selection). In a review of kin recognition in several plant species by File et al. (2011), nine studies showed that kin groups had outperformed strangers (suggesting kin selection) and eleven studies showed stranger groups outperforming kin groups, with twenty one studies showing no differences between groups. This has sparked a debate over what the dominant process is among plants growing in close proximity. When strangers are more successful, it is suggested that there is a high level of kin competition occurring. Because related individuals are more likely to be phenotypically similar in traits such as rooting depth, kin will be competing in the same niche. Strangers, however, can take advantage of niche partitioning, thus, exploiting slightly different niches. Both individuals are thus provided with enough resources to be successful. There has been evidence that both of these processes are occurring in plant systems. Platt and Bever (2009) suggest that the nature of the competitive behavior that occurs depends heavily on population density. When

space is very limited, kin competition is very high, which could favour niche partitioning, but when there is open space to be utilized, the evolution toward cooperation within a related group may be favoured.

The study of kin recognition and kin selection is still in its infancy. There is still much research to be done before predicting interaction outcomes between plants is possible, if it is ever possible with any certainty. Understanding kin recognition is also complicated by results suggesting that it may be mediated by belowground mechanisms, making it even more challenging for researchers. This study adds to this body of knowledge through examination of kin recognition in a coniferous tree species, interior Douglas-fir, and by the examination of a possible mechanism of facilitation, mycorrhizal networks.

Mycorrhizal networks

Fossil records suggest that fungus-plant symbioses have been occurring ever since plants began to colonize land. It was this symbiosis, where each of the partners used the others' specialized traits, which likely allowed for much of the colonization to occur (Fortin et al. 2009, Smith and Read 1997). Due to its pivotal role in the evolution of land plants, eventually leading to the evolution of land animals, this symbiosis has been considered a more powerful driver for evolution than competition, parasitism or predation (Fortin et al. 2009, Margulis 1981).
The term mycorrhiza comes from the Greek myco, meaning fungus and riza, meaning root. Mycorrhiza symbiosis literally is a root and fungus living in physical contact. It has been estimated that over 90% of plant species form some type of mycorrhiza (Smith and Read 2007, Fortin et al. 2009). Mycorrhizae are thought to have many functions, including providing mineral nutrition and water acquisition, protection against pathogens, resistance to environmental stresses,

soil aggregation, and hormonal activity for the plant as well as providing an essential carbon source for the fungus. There is a wide diversity of specialized mycorrhizal fungi including arbuscular, ecto, ericoid and orchid mycorrhizae. The focus on this thesis is on the ectomycorrhizal fungi. These fungi form associations with the roots of higher plants and these associations are typically characterized by apoplastic growth of the fungus, and formation of a hartig net and mantle. Ectomycorrhizal fungi have been classified into exploration types using characteristics and quantity of emanating hyphae or rhizomorphs (dense groupings of hyphae). They range from contact-exploration types to long-distance exploration types (Agerer 2001). A well-known long-distance exploratory ectomycorrhizal complex is *Rhizopogon vinicolor/vesiculosis*, which associates specifically with Douglas-fir. Long-distance explorers have differentiated rhizomorphs allowing for efficient water and nutrient transport (Agerer 2001).

Mycorrhizae have also been shown to connect roots from different plant individuals of the same or different species (Molina et al. 1992). Resource sharing between individuals through mycorrhizal networks, such as from hub trees to seedlings, has been shown to occur in Douglas-fir forests (Teste et al. 2009, Querejita et al. 2003, Brooks et al. 2006). Resources in some cases can move between plants along source-sink gradients governed by differences in plant physiology, such as photosynthetic rates or nutrient contents in plants, and by fungal factors such as exploration strategy or network density (van der Heijden and Horton 2009, Simard et al. 2012). Regeneration facilitation through mycorrhizal networks appears to increase the regenerative capacity and aid in the self-organization and stability of forests (Simard et al. 2013). A source-sink gradient or size difference has also been shown work in the opposing direction (Merrild et al. 2013). When a size discrepancy was established between two tomato plants connected by a

mycorrhizal network, it resulted in preferential P uptake by the larger plant and P deficiency in the smaller plant.

Mycorrhizal networks can facilitate regeneration either by increasing fungal colonization of new seedlings for greater resource uptake capacity, or by directly transferring resources (water or nutrients) or other compounds from large trees to regenerating seedlings. Most studies show that some resource transfer is occurring through mycorrhizal networks, although some argue that the results are inconclusive (Whitfield 2007). The bigger questions are how much transfer is occurring, which resources or compounds are being transferred, which individual is benefitting, and what are the ecological or fitness consequences? Of the resources examined (mainly carbon, nitrogen, phosphorus or water), carbon transfer has been the central focus in ectomycorrhizal networks. Most studies show that carbon flows through mycorrhizal networks from the source (tree), into to the connecting fungus, and through to the sink (seedling) without significant cost to the source tree. One study showed that bi-directional transfer occurs between source and sink plants, but that there was a net transfer from source to sink plants (Simard et al. 1997). When source-sink relationships between plants shift, such as over the growing season or on an annual basis, the direction of net transfer changes with it (Philip 2006, Deslippe and Simard 2011). When two equivalent plants compete for transferred carbon, cost/benefit considerations of mycorrhizal networks are more complex. Nitrogen transfer also appears to be quite complex. It is still unclear whether nitrogen flows to nitrogen fixing plants or to non-nitrogen fixing plants, and it appears to depend on each of their nitrogen requirements, and therefore the cost-benefit ratio is still unknown (Selosse et al. 2006, Van der Heijden and Horton 2009, Whitfield 2007).

Aside from general function and capability, some scientists have discussed whether plant interactions mediated by mycorrhizal networks are predominantly mutualistic or competitive,

socialist or capitalist (Van der Heijden and Horton 2009). Evidence for a 'socialist' perspective comes from studies showing that resources can be more evenly distributed among plants involved in the network (Perry et al. 1989). On the other hand, evidence for a 'capitalist' perspective show that larger, more resource-demanding or more mycorrhizal-dependent plants benefit more from mycorrhizal networks through larger total biomass gains than smaller plants (Van der Heijden and Horton 2009). The mycorrhizal fungus can also benefit when it connects different plants by increasing the number of healthy hosts from which it acquires carbon (Selosse et al. 2006). These ideas are largely theoretical and more research is necessary.

It is generally accepted that mycorrhizal systems are important to plants and ecosystems but much more research is needed to improve our understanding of processes and patterns. All areas of research involving mycorrhizal networks have been reported as "poorly understood", and even where interaction outcomes could be measured, the underlying process has been "currently unknown" or "not yet clear". Furthermore, network processes that have been studied, such as carbon transfer, have been viewed with a very critical eye by some who are not convinced this is the work of mycorrhizal networks at all. We are still a long way from widely accepted theories and predictions. Due to the infancy of the study of mycorrhizal networks, any and all research in this field will be harshly critiqued but also necessary to increase knowledge of the importance of plant-fungal relationships in ecosystem function.

Plant interactions

Mycorrhizal symbiosis has been found to be a mutualistic relationship in most cases, however, in many systems 'cheaters' evolve to exploit the mutualism without contributing to the relationship (a parasite essentially). Kiers et al. (2011) sought to determine whether

communication occurred between a plant and a fungus to stabilize their mutualistic relationship and to prevent 'cheaters' from exploiting the symbiosis. They found that both the plant and the fungus were able to detect how much benefit they were receiving from their respective fungus/plant partner and preferentially transfer resources in demand to a more cooperative partner. Host plants receiving higher amounts of phosphorus from a particular fungal individual transferred more carbon to that individual. Likewise, a fungus receiving more carbon from a particular host root would provide it with more phosphorus. This is much like a market economy, with higher quality services being rewarded bidirectionally. This study made two novel contributions: first, that there is measurable recognition and reaction in the mycorrhizal symbiosis and, second, that mutualisms are maintained because both partners are important in maintaining the relationship, contrasting with previous theory that one dominant partner held the fate of multiple hosts and essentially forced them into participation (Kiers et al. 2011).

Not only does signaling and communication occur between plant roots and fungi within a single mycorrhiza; evidence is mounting that plants communicate with each other through mycorrhizal networks. The movement of nutrients or water between plants through networks along source-sink gradients can be considered a form of communication. Song et al. (2010) found evidence for biochemical signalling between plants connected by an arbuscular mycorrhizal network. Tomato plants growing near a blight-infected tomato plant were able to "eavesdrop" through mycorrhizal networks that their neighbour had up-regulated defense enzymes to fight off this disease, and as a result were able to up-regulate their own defense enzymes and prime themselves for attack. This reaction decreased disease incidence and severity of the healthy neighbours compared to non-mycorrhizal neighbours, mycorrhizal neighbours that were not connected via a mycorrhizal network, or neighbours connected to another healthy plant.

These treatment comparisons showed that the communication or signal transmission likely occurred through mycorrhizal networks, not through airborne volatiles. Healthy plants connected to the infected individual via mycorrhizal networks were able to increase defense enzyme levels and increase defensive gene regulation previous to any attack on themselves. Although the chemical identity of the signals remains unknown, this study provides needed insight to plant-plant communication and opens the door to further research in identification of signal compounds that may also operate in kin recognition (Song et al. 2010).

Inter-plant communication through mycorrhizal networks does not always benefit all individuals involved. Barto et al. (2011) showed that allelochemicals can also be transmitted through mycorrhizal networks. Allelopathy is the production of compounds used to inhibit the growth of a neighbouring plant. Transfer of allelochemicals from one plant to another through mycorrhizal networks rather than the soil matrix has the advantage of more efficient and faster chemical transmission over greater distances due to the protection of allelochemicals from soil microbes and due to cytoplasmic streaming through hyphal cells. Over the course of two experiments they found significantly decreased biomass and higher allelochemical concentrations in leaves and surrounding soils of receiver plants connected via a mycorrhizal network. The study was conducted using heterospecific plants. This strongly suggests that allelochemicals are being transported through these networks to inhibit neighbouring receiver plants and give the supplier of the allelochemical a competitive advantage (Barto et al. 2011).

These developments provide interesting insight and generate new hypotheses about mechanisms underlying plant ecosystem dynamics. It is conceivable, for example, that a particular plant species could recognize and select for its kin through intraspecific mycorrhizal networks, while simultaneously increasing competitive effects on non-kin neighbours by

releasing allelochemicals or signals or acquiring more nutrients from the network for greater competitive growth.

Overview of the thesis

The main objectives of this thesis, addressed in each of the research chapters (2-4), were to: 1) determine whether kin recognition is detectable in interior Douglas-fir seedlings; 2) determine whether kin recognition, if present, would present in a way that supports the kin selection theory; 3) to determine whether mycorrhizal networks mediated kin recognition between seedling pairs (chapters 2 and 3) or between seedlings and parent trees (chapter 4). The minor objectives that were addressed in specific chapters were to: 1) determine if kin recognition ability varied among distinct genotypes or "families" of seedlings (chapter 2); 2) determine if the region of seed origin affects kin recognition among seedlings grown in a common greenhouse environment (chapter 3); 3) determine if kin recognition occurs along a gradient of relatedness (chapter 3); 4) determine if the region of seed origin affects kin recognition among seedlings grown in the field with a variety of growing conditions (different sites) (chapter 4). Two additional minor objectives were examined across chapters and discussed in the concluding chapter (5); 5) to determine whether full sibling kin pairs from control cross pollination exhibited differing kin recognition effects than did kin pairs collected from open-pollinated parent trees in the field and; 6) to determine whether effects seen in the controlled environment of the greenhouse would be detectable under natural climatic conditions in the field. Chapter 2 was designed to evaluate kin recognition among control cross pollinated sibling pairs in a greenhouse environment. Seedling pairs were sown six months apart in order to encourage a source-sink gradient between the older, "donor" seedling and the younger "recipient" seedling. Mycorrhizal network access was controlled using mesh bags of two pore sizes: one allowing for fungal

hyphae to penetrate the pores and connect the rooting systems of the two seedlings, but preventing root on root contact; and the other pore size preventing fungal hyphae as well as roots from crossing the mesh barrier. Chapter 3 was designed to evaluate kin recognition among open pollinated seeds collected from six parent trees from three field sites (two trees per site) in interior British Columbia in a greenhouse setting. Chapter 4 was designed to evaluate kin recognition in a field setting using naturally pollinated seeds collected from the same parent trees as were used as host centers for kin and non-kin seedlings in the study.

2 Control cross pollinated full-sibling seedling pairs may recognize kin through mycorrhizal networks in a greenhouse setting

Introduction

The forest industry in western North America is benefitted by extensive and productive interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) forests for saw and pulp logs (BC Ministry of Forests, Land and Natural Resource Operations, 2012 annual report). Forest regeneration of interior Douglas-fir has long been problematic due to the harsh growing environment, particularly climatic aridity (Newsome et al. 1991, Huggard et al. 2005, Vyse et al. 2006). This has spawned research on forest regeneration and health for over half a century (Lavender et al. 1990), but these problems are expected to amplify with climate change (Hamann et al. 2011, Wang et al. 2012). Two concepts that may aid the regeneration and stability of these forests are: (1) kin selection, stemming from the ability of plants to recognize others that are closely genetically related to them and support their growth and health (Dudley and File, 2007), and (2) mycorrhizal networks connecting a community of trees and seedlings and facilitating water and nutrient transfer over a source-sink gradient (Perry et al. 1989, Horton and Bruns 2001, Querejeta et al. 2003). We explore both concepts using uneven-aged pairs of interior Douglas-fir seedlings in a greenhouse setting.

Kin recognition in plants is defined as the ability to discriminate kin in competitive interactions (Dudley and File, 2007). This is known to present in at least two ways. Kin selection describes an altruistic behaviour between kin pairs or groups where an individual could suffer a loss to its individual fitness to increase the fitness level of the pair or group (Hamilton, 1964). This theory suggests kin groups will perform better than non-kin groups (File et al. 2011). Niche partitioning theory (or the elbow-room model) describes pairs or groups of individuals that are different genetically and occupy slightly different ecological niches (space and resources), thus

reducing direct competition (Young, 1981). This theory suggests that kin groups will be outperformed by non-kin groups (File et al. 2011). A 2011 review of kin studies in plants found evidence of kin selection in nine studies, evidence of niche partitioning in 11 studies and no evidence of kin recognition in 21 studies (File et al. 2011). The scenarios under which kin recognition could take place are still uncertain. Stress gradients and population densities may contribute to whether individuals put resources toward the recognition of neighbouring plants and responses to them (Platt and Bever, 2009). The mechanisms of recognition are also still largely unknown, although there is evidence that it is a below ground process involving signals found in root exudates (Biedrzycki et al. 2012).

We propose that mycorrhizal networks facilitate the recognition of kin within a connected community. Mycorrhizal networks form from a fungal-root symbiosis (literally "myco"-"rhiza"), where extra-matrical hyphae of a single fungus connect two or more plants of the same or different species (Francis and Read 1984, Perry et al. 1989, van der Heijden and Horton 2009). Mycorrhizal networks comprised of the well-known long-distance exploring ectomycorrhizal fungi, species in the *Rhizopogon vinicolor/vesiculosis* complex, are known to intra-specifically connect most trees in Douglas-fir forests (Kretzer et al. 2003, Teste et al. 2009, Beiler et al. 2010). *Rhizopogon* networks are known to form differentiated rhizomorphs (dense groupings of hyphae) that allow for efficient transport of water and nutrient, such as carbon and nitrogen, particularly along source-sink gradients (Perry et al. 1989, Molina et al. 1999, Agerer 2001, Teste et al. 2010, Bingham and Simard 2011). Resource transfer has been loosely associated with increased regenerative capacity of interior Douglas-fir forests (Teste et al. 2009, Bingham and Simard 2011). Other types of chemical transfer have also been observed in mycorrhizal networks. Song et al. (2010) showed that tomato plants use mycorrhizal networks to detect up-

regulated defense enzymes in blight-infected neighbouring plants, and up-regulate their own genes to constitutively produce defense enzymes to prepare for a potential attack. These other forms of transfer have led us to examine whether kin recognition results from chemical signalling through networks as well.

Chemical signalling is a type of plant interaction that acts as a form of communication (Bruin and Sabelis, 2001). This has been shown to take place both above and below ground but this study will only focus on below ground interactions (Shulaev et al. 1997, Kessler et al., 2006 Song et al. 2010). Some scientists say that chemical signals are used as a means of communication by plants to prime mycorrhizal inoculum to germinate or grow and form a symbiotic relationship (Keirs et al. 2011), to warn neighbours of an imposing threat (Song et al. 2010), to detect the relatedness of a close neighbour (Dudley and File, 2007) or even inhibit a neighbour's growth using allelochemicals (Barto et al. 2011). Biedrzycki et al (2010) showed that the signalling involved in kin recognition in *Arabidopsis thaliana* happens through the root exudates. We wanted to investigate if these root exudates travel through mycorrhizal networks between interior Doulas-fir seedlings. Resources such as water, carbon and nitrogen (Schoonmaker et al. 2007, Teste et al. 2010, Bingham and Simard 2011) as well as defense signals (Song and Simard, unpublished data) have been shown to transfer between interior Douglas-fir trees through mycorrhizal networks, leading us to test whether kin recognition signals also occur along this pathway. If kin recognition signalling and resource transfer is occurring through these pathways, the extent of the colonization of network-forming fungi would likely have an effect on signalling and transfer. File et al. (2012) showed that in *Ambrosia artemisiifolia L.* (common ragweed), the greater the colonization of roots, the greater the growth of each sibling connected by the network.

Research into these areas could inform forest regeneration practices. Managing ecosystems in a way that leaves ecological legacies has been proposed as a means of supporting a more natural regeneration process (Keeton and Franklin, 2005). With this research, identification of ideal ecological legacies to assist forest recovery may be improved. Large hub trees that are highly connected to younger trees through mycorrhizal networks could be used to maintain the below ground fungal community (Beiler et al. 2010). An array of high seed producing, healthy, mature hub trees could also support the germination, survival and growth of their kin, resulting in a naturally regenerated forest community comprised of well adapted, supported and healthy seedlings. If these seedlings can be supported by surrounding mature trees, they may also be better able to deal with changing climate and more extreme conditions such as drought (Bingham and Simard, 2011).

The objective of this study was to determine whether kin recognition occurs between interior Douglas-fir seedlings, whether it is mediated by mycorrhizal networks, and whether it varies among families. We examined kin recognition by comparing performance of full sibling and unrelated pairs using control-pollinated interior Douglas-fir seeds. Our first hypothesis was that the high level of relatedness between sibling pairs would result in shifts in plant behaviour compared to non-sibling pairs. We expected enhanced performance of donor and receiver plants in kin pairs compared to non-kin pairs. Our second hypothesis was that kin recognition would be mediated through mycorrhizal networks. We expected greater kin recognition between donor and receiver plants connected in a mycorrhizal compared to those that were isolated from each other. We also expected the extent of the mycorrhizal network (amount of colonization) to enhance the kin recognition and network effects. Our third hypothesis was that kin recognition would vary genetically, i.e., among families. We expected kin recognition to vary according to the

competitiveness (e.g., shoot or root growth rates) of plant genotypes, where families with faster growth rates are better able to recognize kin. Finally, we expected kin recognition to be mediated through resource transfer between seedlings in a pair, with preferential transfer from parent trees to kin seedlings compared to non-kin seedlings. We expected increased transfer between kin seedlings to be associated with enhanced kin performance.

Methods

Experimental design and treatments

This experiment took place in the University of British Columbia greenhouse in Vancouver, British Columbia, Canada over an 11 month period (March 2012 to February 2013). A total of 100 pots, approximately 2 L, height = 18 cm, diameter = 16.5 cm, were distributed uniformly over half of one greenhouse bench. The pots were re-randomized every two weeks. No supplementary light was provided.

A 2 x 2 factorial design was used where mesh size (two levels) and relationship (two levels) were applied in a completely randomized design. The mesh size factor (two levels) references the pore size of the specialized mesh bags (approximately 20cm x 8 cm) made by Plastok® (Meshes and Filtration) Ltd. (Birkenhead, UK) that separated the root systems of the older, "donor" seedling and the younger, "recipient" seedling. A total of 50 mesh bags had a pore size of 35 μm, which has previously been shown to allow fungal hyphae to penetrate the bag but prevent roots from growing through the barrier (Teste and Simard 2008). The other 50 mesh bags had a pore size of 0.5 μm, which has been shown to prevent fungal hyphae from penetrating and allows only soil water to flow in and out of the bag (Teste et al. 2006). The relationship factor (two levels) references the genetic closeness of the seedling pair grown in a single pot. A total of

40 pots were planted with a kin pairing, where both seedlings originated from the same family (designated 1/1, 2/2, etc.) (Table 2.1). The remaining 60 pots were planted with a non-kin pairing, where the seedlings came from two different families (1/2, 4/3, etc.) (Table 2.1). The seeds were produced by the interior spruce breeding program of the Ministry of Forests, Lands and Natural Resources Operations at the Kalamalka Research Station near Vernon, British Columbia. 1240 control cross-pollinated interior Douglas-fir seeds were used from four pairs of known parents (hereafter referred to as "families" 1 through 4). Family 1 is a control cross pollinated combination of Fdi SA (Salmon Arm, British Columbia) 8069 x SA 8033, family 2 - Fdi SA 8041 x 8016, family 3 - Fdi SA 8047 x SA8037 and family 4 - Fdi SA 8211 x SA 8006 (where Fdi=interior Douglas-fir and SA=Salmon Arm). All of the seedlings that emerged from seeds in the same family were full siblings. The pairings and mesh sizes were combined in a way that resulted in 20 kin pairs with 35 μm mesh, 20 kin pairs with 0.5 μm mesh, 30 non-kin pairs with 35 μm mesh and 30 non-kin pairs with 0.5 μm mesh. The families were equally distributed among all combinations.

Experimental setup

Each pot contained a mesh bag that was placed against the edge and gently packed with a 3:1 mixture of greenhouse standard potting mix: field collected soil (Figure 2.1a). This mixture was thoroughly blended in a mechanical soil mixer to achieve high homogeneity, and autoclaved for one to 1.5 hours at 250°C to kill any fungal inoculum in the field collected soil. Autoclaving was applied to the soil mixture inside the mesh bags to ensure that mycorrhization of recipient seedlings occurred primarily through contact with mycorrhizal networks of donor seedlings. The remainder of the pot was filled with a 1:1 combination of non-autoclaved field collected soil and greenhouse standard potting mix, also thoroughly blended. The soil was collected from an

interior Douglas-fir forest near Princeton, British Columbia (approximately 120.58°W, 49.43°N)(Figure 3.1). The forest occurred in the Dry Cool Interior Douglas-fir subzone (IDFdk), was comprised of pure interior Douglas-fir, and was underlain by a Dystric Brunisol soil with a sandy loam texture and a moder humus form (Canadian System of Soil Classification 1998). The top litter layer was scraped off and the underlying 10 to 15 cm (including the fermentation layer, humus layer and mineral soil) was collected and transported immediately to the UBC greenhouse in Vancouver, BC. This soil was expected to contain a sufficient amount of interior Doulas-fir compatible fungal inoculum to encourage mycorrhizal colonization within the pot.

Each pot was designed to contain a pair of seedlings (Figure 2.1b). One older seedling was established 8 months in advance, outside of the bag, to act as a donor to the younger, recipient seedling. The pairs were subject to the four treatment groups, described above, depending on the relationship of the pair of seeds sown (full siblings or from separate families) and the pore size of the mesh bag installed (0.5 μm or 35 μm).

At the onset of the experiment, all pots were soaked to field capacity with water. A total of 125 seeds from each of the four families described above were sown in 25 pots (100 pots in total), with five seeds per pot. Before they were sown, seeds were sterilized with 10% H_2O_2 for 10 minutes and then allowed to dry. An additional two seeds per pot were stratified and sown in the mixed field soil after six weeks to make up for unsatisfactory germination rates. This involved a 24 hour soak in distilled water followed by drying and refrigeration (4°C) for three weeks. Some seedlings were transplanted from pots with multiple germinants to those yet to germinate with the goal of having at least one seedling per pot. They were all sown in the mixed field soil containing the inoculum. A thin layer of fine gravel known as “forest sand” was spread over all sown seeds to discourage the growth of damping off fungi.

Pots were lightly watered each day until each pot had at least one healthy seedling. There was at least one seedling per pot 10 weeks after the first seeds were sown, at which point the pots were thinned to one healthy seedling as close to the center of the pot as possible. No fertilizer was provided at any point. The watering regime was shifted from a light watering daily to weekly watering to field capacity. The limited watering and absence of fertilization were designed to encourage mycorrhizal colonization. The seedlings were allowed to grow for another two months with periodic thinning when necessary.

When the 'donor' seedlings were 4.5 months old, they were transported into a Conviron PGV36 Plant Growth chamber at UBC to undergo a blackout regime in preparation for an artificial winter. The blackout regime consisted of a 10 day period with 10 hour days at 20°C and 14 hour nights at 15°C. After the blackout period, the seedlings were returned to the greenhouse for three weeks to prepare for winter, and then returned to the growth chamber for the artificial winter. The seedlings experienced 16 hour uninterrupted nights, 8 hour days at low light levels and a temperature of 4°C for six weeks during the artificial winter provided by the growth chamber. The seedlings were then returned to the greenhouse. The soil and seedlings were allowed 24 hours to adjust to the temperature difference, and the second set of 'recipient' seedlings were sown the next day. A total of five seeds from the corresponding kin or non-kin pairing were sown within the inside edges of the mesh bag. The seeds were from the same stock as the seedlings currently established and were stratified in the same manner.

The seeds were sown in accordance with the family they were from and the family of the already established seedling. After the second set of seeds was sown, they were allowed to germinate, grow and were thinned when necessary for the next four months. Heights were recorded for both seedlings every three weeks.

$^{13}CO_2$ labelling

The $^{13}CO_2$ labelling was conducted 8.5 months after the experiment was established. A total of 90 of the original 100 pots remained with a pair of healthy seedlings at the onset of the $^{13}CO_2$ labelling period (18 kin/network (35 μm mesh), 16 kin/no network (0.5 μm mesh), 29 non-kin/network and 28 non-kin/no network). Those 90 pots were split into three labelling treatments (1-day chase, 6-day chase and control). We used 37 pots (9 kin/network, 8 kin/no network and 10 each of the non-kin pots) for a 10-hour labelling period followed by a 6-day chase period before the seedlings were harvested. We used an additional 17 pots (9 non-kin/network and 8 non-kin/no network) for an identical 10 hour labelling period followed by a 1-day chase period before the seedlings were harvested. For cost purposes only the 6-day chase labelled seedlings were used for further analyses in this study and the 1-day chase pots were harvested for RNA analysis for another study being run in parallel. The remaining 36 pots were used as controls.

During the labelling period, the older, donor seedling was sealed inside an inflated plastic Foodsaver® bag (approximately 11'' x 16'') (Figure 2.1c). The bag was transparent on one side and translucent on the other side, so the pots were positioned in way that maximal light would enter the transparent side. The bags were pre-sealed on both sides and open on the ends. We sealed one end using a Seal-A-Meal® food saver, melting the two layers together. After the bag was placed over the donor seedling, the remainder of the bag was sealed along the end and around the base of the seedling with Tuck® Contractors Sheathing Tape. Both the labeled and control seedlings were sealed in the bags and inflated with ambient air. In addition, the labeled seedlings' bags received three injections, at equal time intervals, of ^{13}C labeled CO_2 (99% ^{13}C) (< 1% ^{18}O) totaling 50 ml (Cambridge Isotope Laboratories Inc. Andver, MA). At the end of the 10

hour period, the bags were removed and the labeled and control pots were separated for a 6-day chase period.

Measurements

The growth variables examined in both the donor and recipient seedlings were needle, stem and root weight (g), total and above ground biomass (g), average weight per colonized root tip (g per root tip), percent of total root weight colonized (%), stem length (cm), germination rate (germinants per seeds sown), average growth rate (cm per week), total leaf area (cm^2) and volume, calculated as total leaf area x stem length (cm^3). Average leaf area per needle (cm^2) was examined only in recipient seedlings. The seedlings were removed from the pots after the younger seedlings had grown for four months. The below and above ground portions were separated. The soil was carefully removed from around the roots, which were then washed with tap water. The needles were removed and measured using a LICOR-3100 leaf area meter. The number of needles was also recorded for the younger, recipient seedlings only. The needles were dried and biomass was recorded. The stems were measured for length, number of branches and dry biomass.

The roots were examined for mycorrhizal colonization and the root tips that appeared to be colonized were weighed and visually morphotyped. Sanger sequencing of fungal DNA was performed on a subset of tips at the Irving K. Barber School at UBC-Okanagan. A total of 65 tips were sequenced with at least five tips from each visually morphotyped species and five root tips identified as non-mycorrhizal. Fungal DNA was extracted using the following protocol: 25 µl E7526 SIGMA Extraction Solution (Sigma-Aldrich, ON, Canada) added per individual ECM root tip, incubated at 95 °C for 10 minutes then cooled to 4 °C, 25 µl D5688 Dilution Solution (Sigma-Aldrich) added prior to freezing at -20 °C. The internal transcribed spacer region (ITS)

was amplified for each DNA sample using ITS1 (White et al., 1990) and ITS4/ITS4B primers (Gardes & Bruns, 1993). Amplifications were performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems, ON, Canada) in 12.5 µl volumes containing: Nuclease-free H2O, 1.25 µl of 25 mM MgCl2, 2.5 µl GoTaq® Reaction Mix (Promega, WI, USA), 0.25 µl of 10mM dNTPs, 0.25 µl of 10 mM each primer, 0.65 units GoTaq® DNA Polymerase (Promega), and 100 ng DNA. Thermocycling conditions: 3 minutes denaturation (94 °C), 35 cycles of denaturing, annealing and extension (94 °C for 35 s, 51 °C for 35 s, and 72 °C for 50 s respectively), 10 minutes final extension (72 oC) before cooling to 4 °C. Purification was performed using the USB ExoSAP-IT® PCR Product Clean-Up kit (Affymetrix, CA, USA) then sequenced using ABI BigDye v3.1 Terminator chemistry and an ABI 3130xl Genetic Analyser (Applied Biosystems). Raw sequence data were analysed using the SEQUENCHER software package Version 4.7 (Gene Codes Corp., MI, USA) before comparison with NCBI and UNITE (Abarenkov et al., 2010) databases using the BLAST algorithm. Names were assigned to morphotypes based on the combination of morphological characteristics and minimum 97% sequence matches corresponding to the indicated species. These sequence data are in the process of being submitted to the GenBank database. We identified Rhizopogon by comparing the sequences to NCBI and UNITE databases, only using the Kretzer entries to determine species names. The best matching sequence was a 99% match to Rhizopogon vinicolor (accession number: AF263933) [Kretzer ID].

A suite of foliar nutrients were examined using microwave digestion/ICP (Inductively Coupled Plasma-Optical Emission Spectrometer) and % C, N and S analyses at the British Columbia Ministry of Environment Analytical Chemistry Laboratory, Victoria, BC. The macronutrients examined were C, N, K, CA, Mg, P and S. The micronutrients examined were Al,

B, Cu, Fe, Mn, Mo, Na and Zn. The extrapolated gram content per seedling and average sample concentrations were used in our analysis. C:N and N:P ratios were calculated and examined.

The amount of ^{13}C transferred from the donor seedlings to the recipient seedlings' roots and stems was determined by EA-IRMS (elemental analysis-isotope ratio mass spectrometry) by UBC's (Vancouver) stable isotope facility and examined in terms of excess ^{12}C equivalent. This was calculated according to the modified Button (1991) procedure described by Teste et al. (2009). Three subsamples each of mixed field soil and autoclaved soil mixture were analyzed for total C, N and S, pH, available P and mineral N. Exchangeable cations (Al, Ca, Fe, K, Mg, Mn and Na) and effective cation exchange capacity were also measured (0.1 M barium chloride).

Data analysis

All statistical analyses were run using SAS version 9.3 (Cary, North Carolina). Two-way analysis of variance (ANOVA) using PROC GLM was run separately for each variable. The factors included were relationship, network and relationship x network. An analysis of covariance (ANCOVA) was also run using PROC GLM to examine the effect of family as a covariate. Planned contrasts were carried out to determine the strength of the extreme treatments along the relationship-network gradient: (1) kin/network versus all other treatments, and (2) no-kin/no network versus all other treatments.

Results

^{13}C transfer

There was a difference in the amount of ^{13}C transferred to the recipient seedling stem based on the pore size of the mesh used to separate the seedling root systems. The seedlings grown in mesh bags of the larger pore size (35 μm) allowing fungal hyphae to connect the root

systems received a significantly greater amount of ^{13}C transfer than seedlings grown in mesh bags with smaller pore size (0.5 μm) preventing hyphal connections ($p=0.0290$, Table 2.2, Figure2.2). The genetic relationship between the seedlings in a pair had no effect on in the amount of ^{13}C transferred to the recipient seedling stem. The interaction effect of relationship x network was not significant for the amount of ^{13}C transferred. However, pairwise comparisons showed that differences in ^{13}C transfer between mesh sizes was greater and of higher statistical significance for non-kin than kin pairs (Figure 2.2).

Growth

Relationship and network main effects

There were no significant main effects or strong trends for any of the growth variables due to either the relationship factor or the network factor ($p>0.05$).

Relationship x network effects

There were significant or strong trends in relationship x network interactions for all growth variables measured in the recipient seedlings (Table 2.3). Needle, stem and root weight, total and above ground biomass, total leaf area, stem length, average growth rate and volume (total leaf area x stem length) all showed significant relationship x network interaction effects ($p<0.05$) (Figures 2.3, 2.4). There was also a strong interaction trend for average weight per root tip and average leaf area per needle in recipient seedlings ($p<0.1$) (Table 2.3). The kin/network treatment (kin relationship between the seedling pair where the recipient was grown in a mesh bag allowing for a network formation, 35 μm) and the non-kin/no network (non-kin with 0.5 μm mesh) unexpectedly had consistently lower least square (LS) mean value than the other two

treatments (kin/no network and non-kin/network)(Figure2.3b-f). The only exception was average weight per root tip, where the recipient seedling in the kin/network treatment had a higher LS mean value (2.963 mg) compared to the other three treatments combined (2.078 mg)(p=0.0252) (Figure2.3a)

For donor seedlings, there was a significant relationship x network effect on height growth rate (p = 0.0393) (Table 2.4) with the same pattern among treatments as for the recipient seedlings (Figure 2.4). The planned contrasts showed there was a difference in donor stem length when each extreme treatment ((1) kin/network or (2) non-kin/no network) was compared to the other three treatments combined. Planned contrast 1 showed that kin/network donor seedlings had shorter stem lengths (24.22 cm) than the other three treatments combined (27.07 cm) (p = 0.0406).

Alternatively, donor seedling in the non-kin/no network treatment (planned contrast 2) tended to have longer stems. This effect was coupled with shorter stems of recipient seedlings in the same treatment (non-kin/no network) compared to the three other treatments combined. In planned contrast (2), the donor seedlings also had significantly greater root weight in the non-kin/no network treatment (p = 0.0450). Recipient seedlings, by contrast, tended to have lower root weight, stem weight, and total biomass in the non-kin/no network treatment (p< 0.10). Average growth rate did not follow this trend in the donor seedlings; however, the trend remained when considering just the recipient seedlings.

Family effects

Relationship and network effects were tested for all variables with ANCOVA using the family of the recipient seedling as a covariate. These analyses revealed that family had no

significant effect on any of the variables tested. However, the small sample size (less than 25 units per family) this study may have been provided insufficient power to detect effects.

Nutrients

Relationship effects

There was greater Al ($p<0.0001$) and Fe ($p=0.0850$) content in foliage of donor seedlings of pairs that had a kin relationship compared to those that had a non-kin relationship (Table 2.5). Fe and Mo were significantly higher in recipient seedlings of kin than non-kin pairs (Table 2.2). Relationship had no other effect on the remaining nutrient variables.

Network effects

There was greater foliar Al content in donor seedlings of pairs that had network formation capabilities (35 μm mesh) compared to those that did not (0.05 μm mesh)(Table 2.5). Networking capability had no other effect on the remaining nutrient variables.

Relationship x network effects

There were significant relationship x network interaction effects on C, K, N, S and Zn content of recipient seedlings ($p < 0.05$). There were also strong tendencies for interaction effects on B, Ca, Mg and P ($p < 0.1$) (Table 2.2) (Figure 2.5). In the donor seedlings, there was a relationship x network effect on foliar Al ($p=0.0375$), Fe ($p=0.0680$) and C:N ratio ($p=0.0317$) (Table 2.5).

Donor and recipient foliar nutrient concentrations

Donor seedlings had higher concentrations of S, P, Mg, Ca than recipient seedlings but recipients had higher concentrations of Al, B, Cu, Mo, N and Zn ($p<0.05$). Donors and recipients had the same concentrations of Fe, K and Mn. N content, as well as content of all other macro- and micronutrients, were considerably higher in the larger donor seedlings than recipient seedlings ($p<0.0001$).

Soils

The autoclaved soil had higher percent total C and S and available P but lower pH than non-autoclaved mixed field soil. There was no difference in percent total N or mineral N. Autoclaved soil had greater exchangeable cations (CMol+/Kg), except Mn, which was higher in the mixed field soil and Fe, which did not differ between autoclaved and non-autoclaved soils. The effective cation exchange capacity was also higher in the autoclaved soil (Table 2.6).

Discussion

Kin effects

There was evidence of kin recognition in some of the micronutrient variables. Fe (recipient and donor), Mo (recipient) and Al (donor) were significantly different in the kin seedlings compared to the non-kin seedlings, suggesting that some recognition caused the discrepancy. Interestingly, in all cases where there were significant differences, kin seedlings had greater micronutrient content than non-kin seedlings. Our hypothesis that kin recognition would lead to kin selection is thus supported by the nutrient results. We expected to see that same result in the growth variables, suggesting that kin recognition leads to enhanced kin growth performance. Our results, however, do not support that hypothesis. There were no significant

main kin effects on any of the growth variables. It is difficult to link beneficial kin effects to direct fitness gains in the recipient but instead these benefits have been thought to result from more indirect characteristics such as altered morphology, allocation tendencies or the reduction of resource uptake by donors (File et al., 2011). The favourable growth conditions of the greenhouse used in this experiment may have led to kin recognition that was only detectable in subtle ways, as we observed with Fe, Mo and Al. Fe is important for photosynthesis and as a co-factor in many enzymes, Mo is important in the nitrate reduction reaction, and Al is the most abundant metallic element in the soil and is thought to be important in biochemical pathways involved in signal transduction. Al is known to have toxic effects on plants at certain concentrations but those concentrations were not reached in this study (Guerinot and Li, 1994, Mulder et al., 1959, Soon, 1995). The benefits of Al on plant physiology have not been widely studied and therefore, they are largely unknown.

The differences in "donor and recipient foliar nutrient concentrations" may have resulted from the reduction in pH following autoclaving the soil. The autoclaved soil, present only in the mesh bags surrounding the recipient seedlings, had a significantly lower pH than the surrounding soil at the onset of the experiment. B, Cu and Zn (higher concentrations in recipient seedlings) as well as Fe and Mn (no difference) are more available to plants in mineral soils as pH decreases. More importantly, the content of all nutrients was greater in donor seedlings due to the large discrepancy in size between the donors and recipients. This discrepancy represents a large carbon and nutritional source-sink gradient between donors and receivers that may drive interplant transfers through mycorrhizal networks (Simard et al. 2002).

Mycorrhizal network effects

The DNA sequencing performed on the colonized root tips showed that *Rhizopogon vinicolor* was the most common fungal association colonizing 95.6% of donor roots and 83.3% of recipient roots. This result was as expected; *Rhizopogon vinicolor* is a strong networking fungal species and known to associate closely with Douglas-fir seedlings during forest development (Massicotte et al. 1994, Molina et al. 1999, Tweig et al. 2007, Beiler et al. 2012). Other taxa included *Pyronemataceae* (sp.) and ascomycete endophytes. At least one ectomycorrhizal fungal species was shared between the donor and recipient in all networked treatments where both seedlings were colonized (only 2.2% were not colonized). This provides sufficient evidence that the seedlings in a pair had the potential to form mycorrhizal networks.

Transfer of carbon provided more definitive evidence for the presence of functional mycorrhizal networks in the network treatments. There was significantly greater excess ^{12}C equivalent in recipient seedlings that were grown in mesh bags with the 35 μm pore size, where fungal hyphae could penetrate through the pores. These results agree with Teste et al. (2006), who tested the effect of mesh pore size on mycorrhizal network formation. The ability to detect ^{13}C in the recipient seedling also suggests the potential for transfer of other compounds through networks. Nevertheless, there were no differences in any of the foliar macronutrients, in either donor or recipient seedling, due to a main network effect. It is possible that the carbon and other nutrients are transferred from the donor seedling to networked recipient seedlings but that the nutrients are stored in the seedling stem rather than the roots (tested in excess ^{12}C equivalent analysis) and needles (tested in the foliar nutrient analysis). Teste et al. (2010) also found that excess ^{12}C equivalent measured in the shoots of Douglas-fir seedlings was higher than that

detected in the roots. We also expect that detection of nutrient transfer through comparison of foliar nutrients would be obscured by larger variation and quantities than ^{13}C.

Our hypothesis that formation of networks between donor and recipient seedlings would lead to enhanced growth of recipients was not supported by our data. There were no differences in any growth variables due to a network connection. Several studies have shown an increase in survival, total biomass, root biomass, above ground biomass or height in autotrophic, ectomycorrhizal plants with an opportunity to form networks with other plants (Onguene and Kuyper 2002, Dickie et al 2005, Booth and Hoeksema 2010, Nara 2006, McGuire 2007, Teste et al. 2009, Bingham and Simard 2011). It is possible that some of these same effects may have become apparent in the recipient seedlings had they been allowed to grow for a longer period of time. Some studies have shown network facilitation to occur most strongly or only where seedlings were growing under environmental stress. For example, Bingham and Simard (2011) found improved growth of networked seedlings only under drought stress, McGuire (2007) and Onguene and Kuyper (2002) found increased performance under deep canopy shade, and Nara found increased survival in nutrient poor volcanic soils. Neither donor nor recipient seedlings in our study were water, light or nutrient stressed and therefore may have been less likely to benefit from carbon or nutrient transfer or enhanced nutrient or water uptake through mycorrhizal networks.

Relationship x network effects

Despite few main effects, there was evidence for a significant interaction between network and relationship in colonization, growth and nutrient variables. The interaction patterns were also evident in planned contrasts. Recipient seedling colonization (measured as average

weight per colonized root tip) was greater among networked kin than any other treatment, supporting our hypothesis that mycorrhizal networks would facilitate mycorrhizal colonization of kin. Enhanced colonization by mycorrhizal fungi in kin pairings has also been observed in *Ambrosia artemisiifolia L.* (common ragweed) seedlings (File et al. 2012). However, our hypothesis that mycorrhizal networks would result in enhanced growth performance of recipient kin was not supported. Indeed, we found the opposite. Both the kin/network and non-kin/no network recipient seedlings had the lowest growth rates. This is an interesting result particularly when the donor growth traits are examined in parallel. The kin/network donor seedlings tended to have lower and slower growth (average growth rate and stem length) as observed in the recipients, however, the non-kin/no network donors tended toward to have higher growth values (root weight). This suggests that some recognition of kin is occurring between the kin seedling pairs. However, this appears not to directly enhance recipient performance, but rather to reduce the competitive environment of both kin seedlings (kin cooperation). It is possible that the non-kin/no network donor seedlings, having recognized a stranger seedling in its neighbourhood, only detects a competitor. The donor seedling, having been established earlier, continues to outcompete its unrelated competitor. Dudley and File (2007) identified this same aspect of kin recognition and selection in the root allocation of *Cakile edentula* var. *lacustris* (Brassicaceae), a self-fertilizing annual plant. They found that allocation to fine root mass between stranger (non-kin) pairs did not differ from root allocation of an individual grown alone, but allocation to fine root mass in kin pairs was lower providing a less competitive environment amongst kin.

Family effects

We expected to see genetic differences in both growth and nutrient variables depending on the family in which the seedling came from. That differentiation was also expected to affect the seedling pairs' ability to recognized and cooperate with kin neighbours (Donohue, 2003). Our analysis did not support this hypothesis; there were no differences in the growth or nutrient variables according to the family the seed came from. All seeds were obtained from a seed orchard and received relatively identical treatment before the onset of the experiment, therefore, these seeds may have already been too similar in competitive ability to detect differences among families. Our sample size for families, however, may have been too small to detect family effects in this experiment.

Conclusions

We found subtle evidence of kin recognition expressed in mycorrhizal colonization, foliar micronutrient and growth traits of donor and recipient seedlings. However, our hypothesis that kin recognition would result in greater growth of recipient kin seedlings (i.e., kin selection) was not supported. Instead, kin recognition was evident in reduced growth of both donors and recipient seedlings. It appears that seedlings may have reduced their competitive environment when in the neighbourhood of kin (i.e., increasing elbow room), but donors were increasing their competitive ability when in the neighbourhood of non-kin recipients. We found that mycorrhizal networks formed between donor and recipient seedlings, as shown in shared ectomycorrhizal fungal species and ^{13}C transfer. We also found that networks facilitated mycorrhizal colonization of kin but not non-kin seedlings. However, we did not find any other evidence that kin selection was facilitated by mycorrhizal networks. A competitive advantage was not gained

by any group of kin (family) nor did the high level of genetic relatedness (full sibling pairs) enhance kin recognition. Stronger relationships may have been apparent if we had grown the recipient seedlings for longer or under environmental stress.

While no significant differences translated directly to growth, the enhancement of mirconutrients in kin seedlings may have an effect on other processes involved in seedling health, although that was not a conclusion of our study. It is possible that forest regeneration of interior Douglas-fir forests could improve with management practices that encourage reproduction of kin seedlings near their parents. This could include the retention of healthy, cone-bearing legacy trees to supply seed for natural regeneration. Harvesting practices could be timed to coincide with mast seed years and sites could be prepared in the neighbourhood of legacy trees to provide suitable mixed mineral seedbeds and minimal competition from native grasses (Simard et al. 2003). These practices would also help conserve mycorrhizal networks, which have been shown in other studies to benefit natural and planted regeneration (Horton and Bruns 2001, Teste et al. 2009, Bingham and Simard 2011). Retention of large legacy trees would create strong source-sink gradients with neighbouring seedlings, which could result in greater carbon transfer. This could also create the potential for greater kin selection than we observed between our months-old seedlings. These practices may be particularly effective on sites with low productivity either due to micronutrient deficiencies or drought.

Table 2.1. Family pairings to create kin and non-kin treatments in full sibling greenhouse experiment. All families are control cross pollinated seeds from known interior Douglas-fir parents, both different for each family. Family 1 - Fdi SA 8069 x SA 8033 (1), family 2 - Fdi SA 8041 x 8016 (2), family 3 - Fdi SA 8047 x SA8037 (3) and family 4 - Fdi SA 8211 x SA 8006 (4). Each family is paired with itself (kin), seen in the top row, and each of the other three families both as donors (first number in pair) and recipients (second number in pair) (non-kin), seen in the bottom three rows.

1/1	2/2	3/3	4/4
1/2	2/3	3/4	4/3
1/3	2/4	3/2	4/2
1/4	2/1	3/1	4/1

Table 2.2. Analysis of variance for network, relationship and interaction (network x relationship) effects on foliar nutrient content and ^{13}C transfer in recipient seedlings. * $p < 0.05$.

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Al (g)	2.04	0.1571	1.77	0.184	0.03	0.8659
B (g)	0.23	0.6312	0.83	0.3645	3.47	0.0661
C (g)	0	0.9988	0.5	0.8186	5.15	0.0259*
Ca (g)	0.11	0.7418	0.01	0.9294	3.45	0.0669
Cu (g)	0.36	0.5477	0.05	0.8288	0	0.977
Fe (g)	1.86	0.1767	4.68	0.0333*	2.37	0.1271
K (g)	0.11	0.742	0.02	0.8864	5.28	0.0241*
Mg (g)	0.3	0.5846	0.05	0.8244	3.44	0.0617
Mn (g)	0.16	0.6878	0.58	0.4492	1.13	0.2915
Mo (g)	0.02	0.9012	10.36	0.0019*	0.66	0.4202
N (g)	0.03	0.872	0.12	0.7353	5.47	0.0216*
Na (g)	0.54	0.4647	0.73	0.3963	0.53	0.4705
P (g)	0.04	0.8464	0.11	0.7461	2.87	0.0939
S (g)	0.06	0.8071	0.02	0.885	5.3	0.0238*
Zn (g)	0.11	0.741	0.08	0.7734	5.39	0.0227*
C:N	0.03	0.8553	0.14	0.713	0.04	0.5271
N:P	1.99	0.1623	0.74	0.3917	1.51	0.2224
^{13}C transferred (excess ^{12}C equivalent in stem) (g)	5.76	.0290**	0.03	0.8718	0.82	0.3742

Table 2.3. Analysis of variance for network, relationship and interaction (network x relationship) effects on growth variables in recipient seedlings. * $p < 0.05$.

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Needle weight (g)	0.6	0.4405	0.02	0.8805	4.84	0.0304*
Stem weight (g)	1.02	0.3165	0.38	0.5407	7.19	0.0088*
Root weight (g)	0.05	0.8201	0.27	0.6074	6.06	0.0158*
Total biomass (g)	0.5	0.481	0.01	0.9102	6.47	0.0127*
Above ground biomass (g)	0.72	0.3998	0.07	0.7914	5.57	0.0205*
Total leaf area (cm^2)	0.48	0.492	0.01	0.9189	5.33	0.0234*
Stem length (cm)	0.45	0.5044	0.34	0.5624	5.83	0.0178*
Average weight per colonized root tip (g)	1.36	0.2476	1.01	0.3169	2.98	0.0881
Average growth rate (cm/week)	0.04	0.8335	0.29	0.5943	4.72	0.0326*
Volume (cm^3) -(total leaf area x stem length)	0.59	0.4443	0.19	0.6658	5.04	0.0273*
Average leaf area per needle (cm^2)	0.02	0.8776	0.06	0.8145	2.81	0.0973

Table 2.4. Analysis of variance for network, relationship and interaction (network x relationship) effects on growth variables in donor seedlings. * $p < 0.05$.

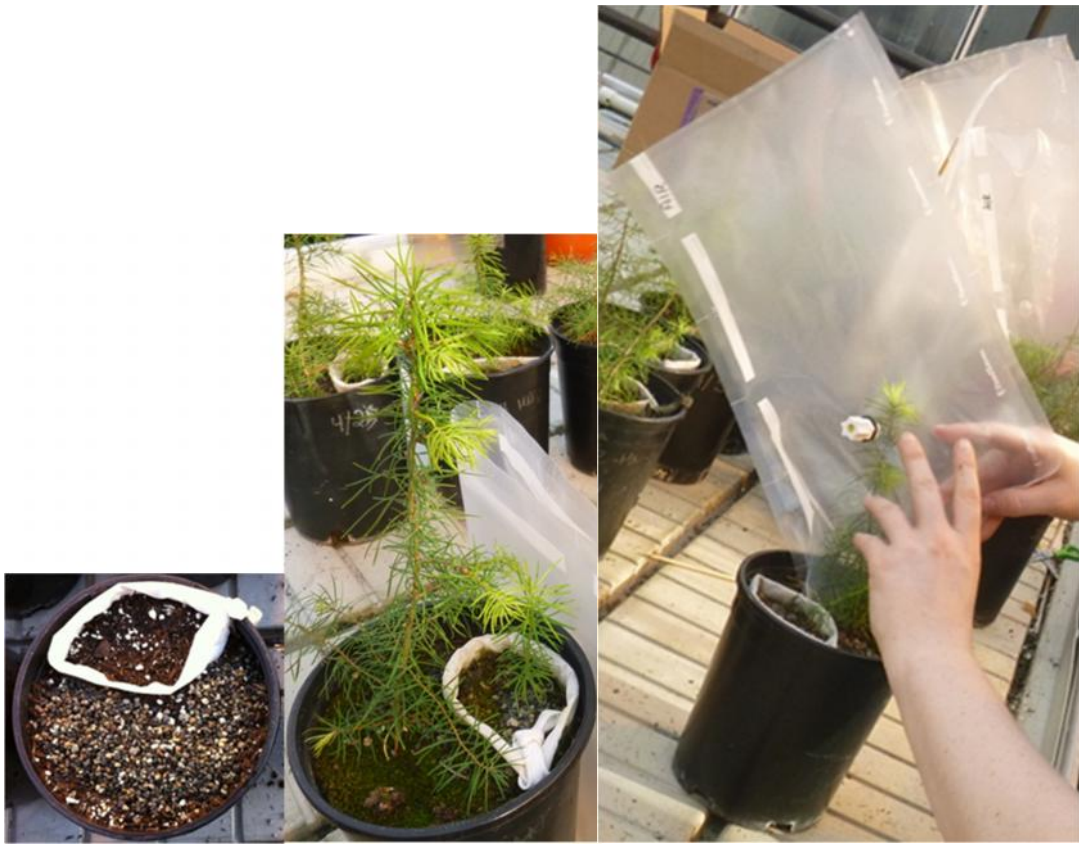
	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Needle weight (g)	0.2	0.6534	0.19	0.6623	1.2	0.2759
Stem weight (g)	0.12	0.7283	1.32	0.254	0.17	0.6843
Root weight (g)	1.52	0.2204	0.79	0.3779	0.94	0.334
Total biomass (g)	0.69	0.4072	0.31	0.5813	0.92	0.3414
Above ground biomass (g)	0.19	0.6662	0.05	0.8153	0.74	0.3925
Total leaf area (cm^2)	0.75	0.3884	0.02	0.8753	0.38	0.5367
Stem length (cm)	2.5	0.1176	1.93	0.1686	0.65	0.4219
Average weight per root tip (g)	1.09	0.3001	0.23	0.6319	0.44	0.5066
Average growth rate (cm/week)	1.03	0.3136	0.66	0.4194	4.38	0.0393*
Volume (cm^3) – (total leaf area x stem length)	1.32	0.2533	0.6554	0.2	0.01	0.9193

Table 2.5. Analysis of variance for network, relationship and interaction (network x relationship) effects on foliar nutrient content in donor seedlings. * $p < 0.05$.

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Al (g)	4.62	0.0345**	38.16	<.0001*	4.47	0.0374*
B (g)	0.03	0.8538	1.59	0.2102	0.2	0.6558
C (g)	0.23	0.6339	0.26	0.6133	1.09	0.2997
Ca (g)	0.44	0.5109	0	0.9662	0	0.9829
Cu (g)	0.75	0.39	0.15	0.695	2.53	0.1155
Fe (g)	0.19	0.6658	3.04	0.0850	3.42	0.068*
K (g)	0.08	0.7724	0.22	0.6378	1.63	0.2047
Mg (g)	0.16	0.6945	0.04	0.8467	0.01	0.9356
Mn (g)	0.08	0.7762	0.33	0.5654	0.14	0.7109
Mo (g)	0.47	0.4961	2.26	0.1367	0.04	0.8372
N (g)	0	0.9653	0	0.9452	0.05	0.8212
Na (g)	0.04	0.837	1.39	0.2409	1.15	0.2856
P (g)	0.07	0.7961	0.16	0.6893	0	0.9642
S (g)	0.06	0.8049	0.02	0.8853	0.82	0.3684
Zn (g)	0.01	0.9049	0.33	0.5654	0.1	0.7511
C:N	0.38	0.541	0.69	0.4072	4.77	0.0317*
N:P	0	0.9983	0.01	0.9356	0.07	0.7987

Table 2.6. Analysis of variance, means ratio, and difference between means for the effect of autoclaving on soil used in mesh bags accessed by recipients (autoclaved) and pots accessed by donors (non-autoclaved) on soil nutrients, CEC (cation excahange capacity) and pH. Positive difference in means values indicate a higher value in autoclaved soil compared to non-autoclaved soil. * $p < 0.05$.

ANOVA – Autoclaving soil	Means ratio	Difference in means	F	P
Al (CMOL+/Kg)	1.21	0.003	4.77	0.0943
Ca (CMOL+/Kg)	1.05	2.240	7.11	0.0560
Fe (CMOL+/Kg)	1.40	0.001	1.73	0.2587
K (CMOL+/Kg)	1.04	0.150	8.01	0.0473*
Mg (CMOL+/Kg)	1.38	3.742	236.87	0.0001*
Mn (CMOL+/Kg)	0.91	-0.124	21.27	0.0099*
Na (CMOL+/Kg)	1.76	0.361	223.6	0.0001*
CEC (CMOL+/Kg)	1.10	6.373	32.14	0.0048*
C (%)	1.12	2.848	11.66	0.0269*
N (%)	0.96	-0.025	3.82	0.1222
S (%)	1.52	0.048	9.91	0.0346*
Available P - PO_4/P (mg/Kg)	1.26	130.446	64.08	0.0013*
Mineral N (mg/Kg)	0.56	-31.469	3.58	0.1315
pH (1:1 H_2O)	0.93	-0.413	549.14	<0.0001*
pH (1:2 $CaCl_2$)	0.94	-0.350	126.72	0.0004*



a **b** **c**

Figure 2.1. Photos of (a) a top view of an experimental unit (pot) just after the donor seedling was sown, (b) experimental unit at the onset of the $^{13}CO_2$ labelling period, recipient age 4 months, with donor age 10 months and (c) fitting a donor seedling with a $^{13}CO_2$ labelling bag.

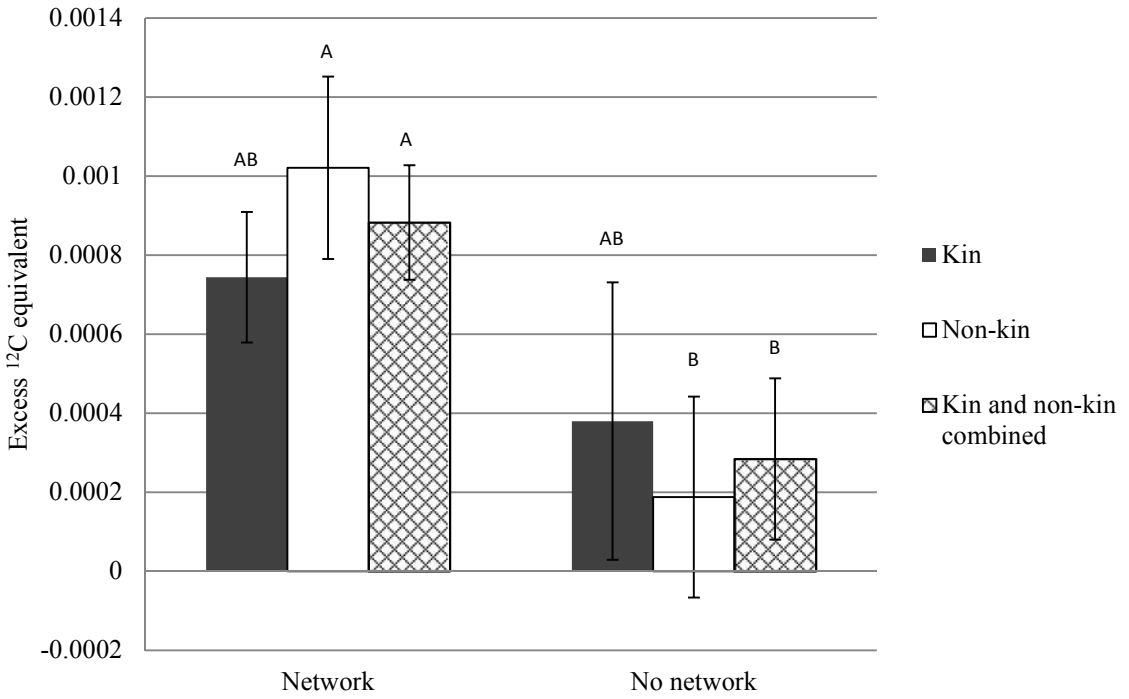


Figure 2.2. ^{13}C transfer to recipient seedlings measured in excess ^{12}C under the four network and relationship combinations as well as all networked compared to all no network recipient seedlings. Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

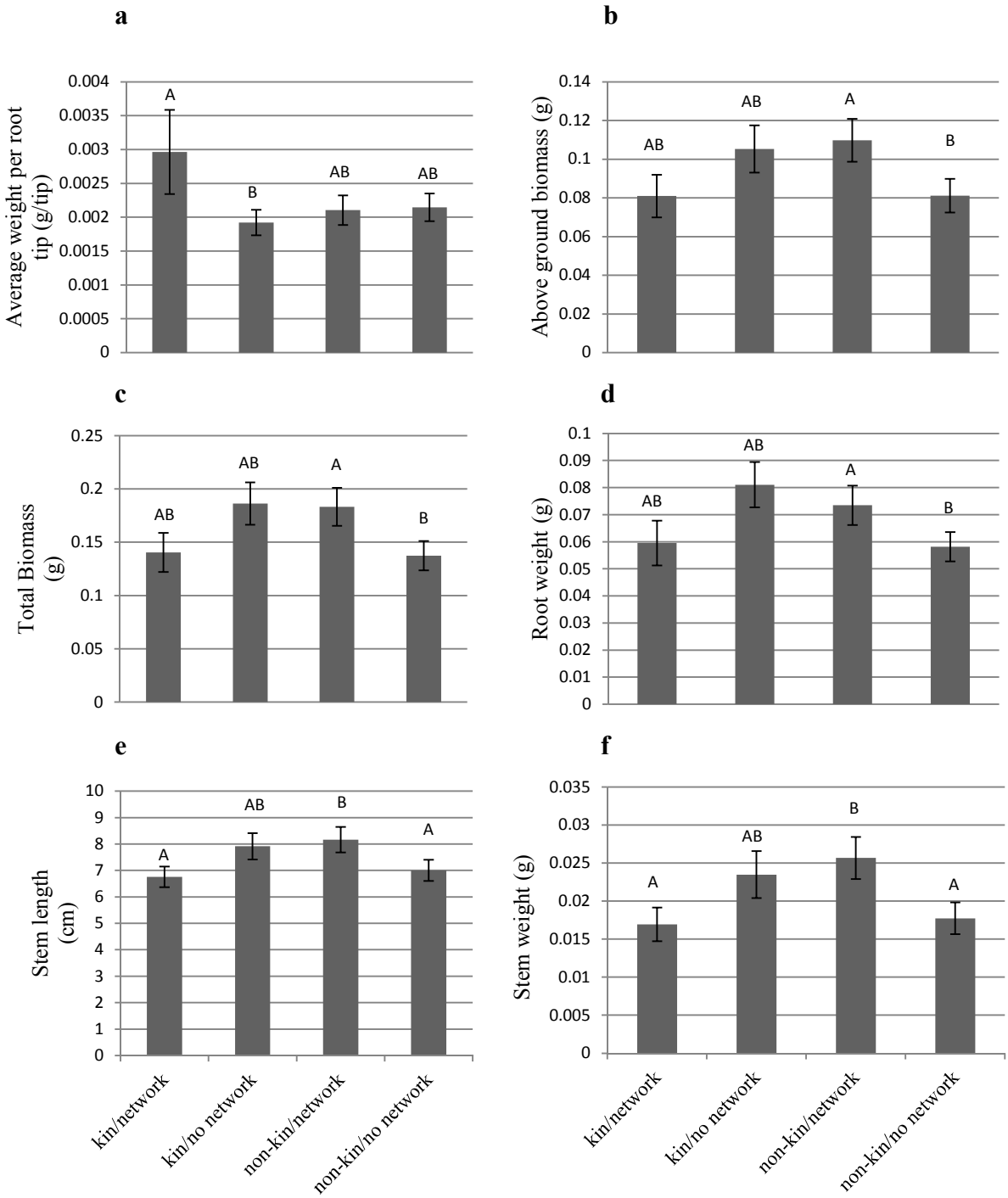


Figure 2.3. Least square means of recipient seedling (a) average weight per colonized root tip, (b) above ground biomass, (c) total biomass, (d) root weight, (e) stem length and (f) stem weight under the four relationship and network combinations (interaction effects). Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

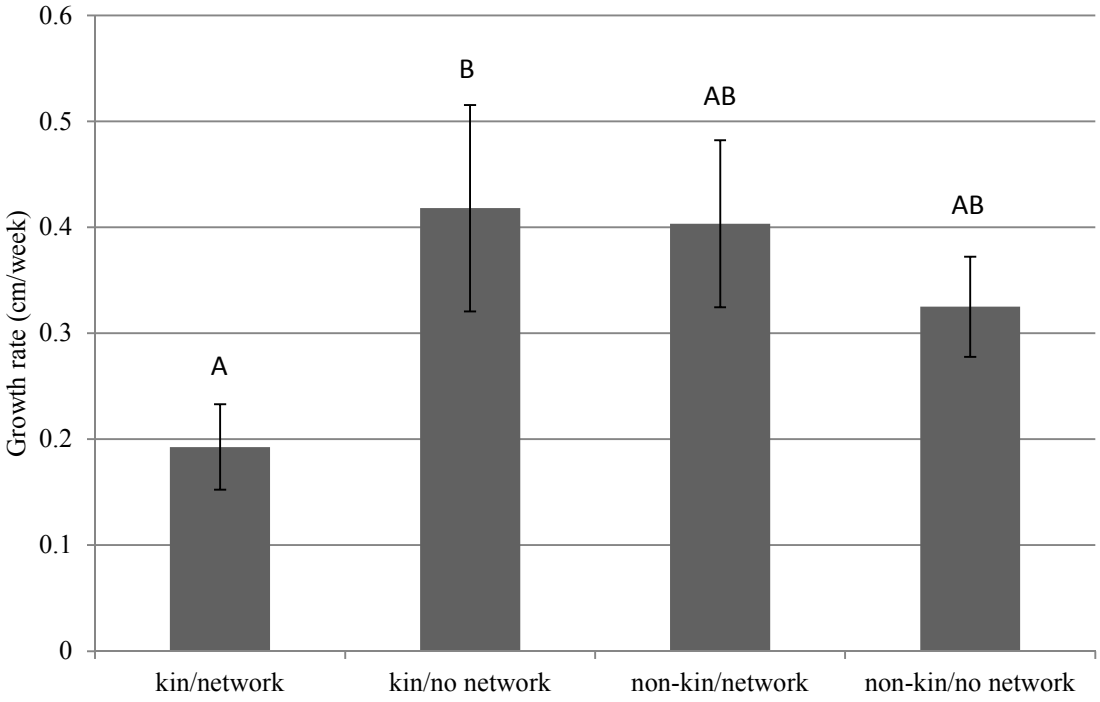


Figure 2.4. . Least square means of height growth rate in donor seedlings under the four relationship and network combinations (interaction effects). Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

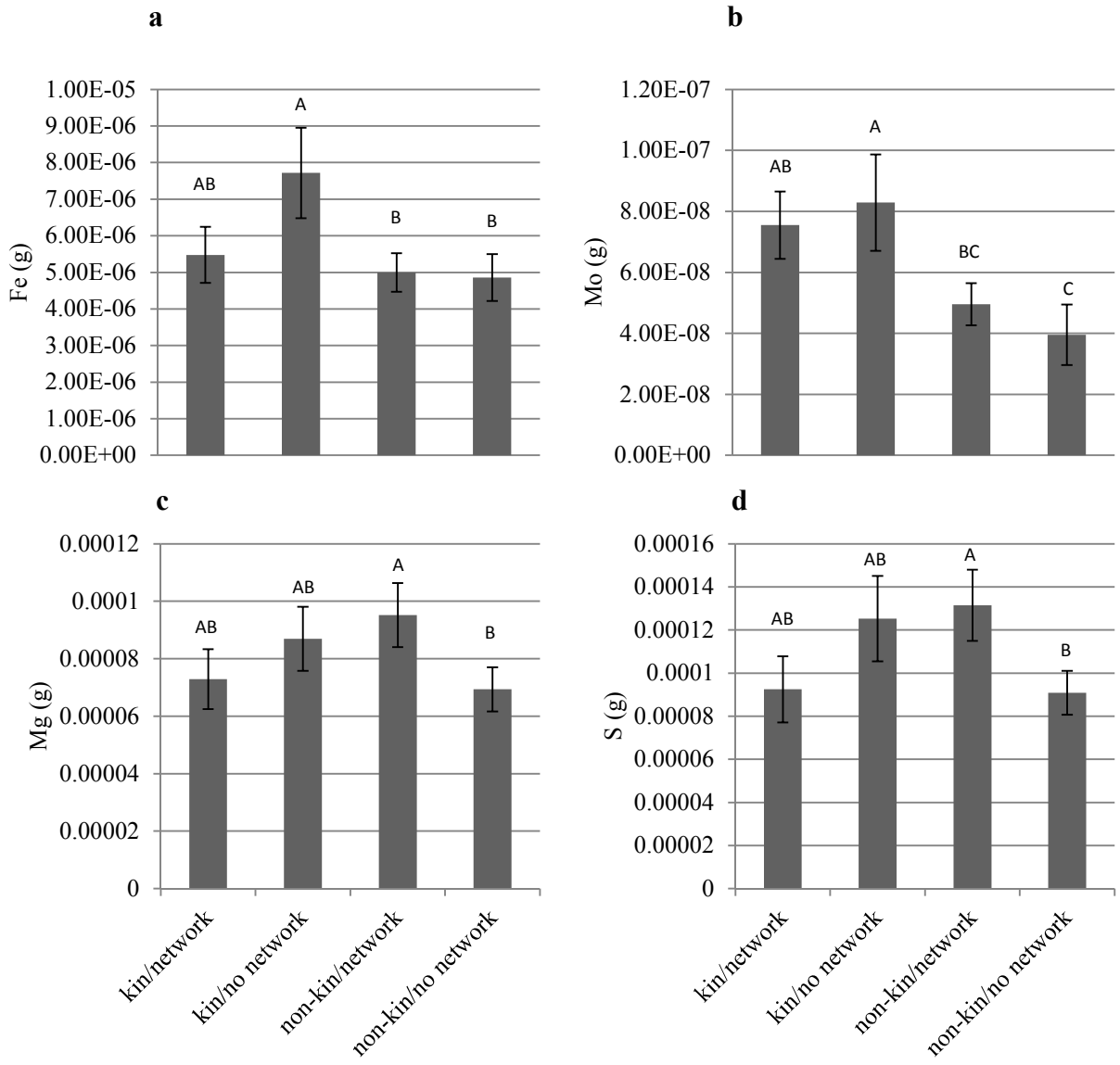


Figure 2.5. Least square means of recipient seedling (a) iron (Fe), (b) molybdenum (Mo) , (c) magnesium (Mg), and (d) sulphur (S) content in grams under the four relationship and network combinations (interaction effects). Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

3 Field collected, greenhouse grown sibling pairs may recognize kin through mycorrhizal networks in a greenhouse setting

Introduction

The interior Douglas-fir (*Pseudostuga menziesii* var. *glauca* (Beissn.) Franco) forests are widely distributed across western North America, ranging from north-central British Columbia (55^{o}N, up to 760 m elevation) to northern Mexico (19^{o}N, up to 3260 m elevation). They vary broadly in climate (precipitation range 410–3400 mm per year; mean July temperature 7–30^{o}C; mean January temperature -9 to 8^{o}C), disturbance regimes (e.g., stand maintaining to stand replacing fires) and site quality (very dry and poor to very moist and rich) (Hermann and Lavender, 1990). The forests are highly sensitive to these climatic, disturbance and site variations, and this is reflected in the composition (relatively pure to mixed stands), structure (multi-storied and uneven-aged to single-storied and even-aged) and species ecophysiology (e.g., shade tolerant to shade intolerant). Within British Columbia, the Interior Douglas-fir (IDF) biogeoclimatic zone includes very dry climatic regions, known as the 'interior drybelt', where interior Douglas-fir is shade tolerant and forms relatively pure, uneven-aged forests, as well as wetter, more productive climatic regions, known as the 'interior wetbelt', where interior Douglas-fir is shade intolerant and grows as an early or mid-seral species in mixture with up to eleven other conifer and broadleaf species. Establishment and growth of interior Douglas-fir is limited in the drybelt primarily by drought and cold temperatures, and in the wetbelt by interspecific competition for light. This variation has led to distinct management considerations (Klenner et al. 2009). For example, selective harvesting and natural regeneration of interior Douglas-fir dominates silviculture systems in the drybelt, whereas clearcutting and planting interior Douglas-fir are favoured in the wetbelt. Natural regeneration is highly variable due to high interannual variation in seed production and climate, and planting success varies from very

low (<50% 5-year survival) in the drybelt to very high (>80%) in the wetbelt (Newsome et al. 1991, Vyse et al. 2006). In addition to climatic, site quality and interspecific variation, there are several other ecological factors that may influence regeneration success, including kin recognition and the presence of mycorrhizal networks, the main concepts explored in this study.

Kin recognition among interior Douglas-fir could be a factor affecting management decisions in IDF forests. Kin recognition is the ability of individuals to discriminate kin, or closely related neighbours (i.e., siblings), in competitive interactions (Dudley and File, 2007). This is a well-known concept among social insects and other animals and is usually translated into kin selection. Kin selection is an altruistic behaviour defined by Hamilton's rule; if the cost of an action to an individual is less than the benefit to another individual multiplied by the level of relatedness, the individual will proceed with the costly behaviour (Hamilton, 1964). This is thought to have evolved from the increase in indirect fitness of the genes shared by the related individuals. Kin recognition and selection are more difficult and nuanced processes to observe in plants than in animals. Nonetheless recent research has shown evidence for kin recognition in some plant species (van der Heijden and Horton 2009, File et al. 2011). Kin recognition has presented in both increased competition (Wilson et al. 1987, Tonsor 1989, Donohue 2003) and increased cooperation between kin individuals (Cheplick and Kane 2004, Boyden et al. 2008, Milla et al. 2009). The ability of interior Douglas-fir to recognize kin and the response to kin within a stand could inform future management decisions.

Interior Douglas-fir has large, continuous populations spanning disconnected geographic areas due to wind dispersal of pollen and seed (Hamrick et al. 1992). Gene flow due to the widespread wind dispersal of pollen, and to lesser degree of seed, has led to relatively little genetic variation for selectively neutral genetic markers within or among populations in the same

region (Gugger et al. 2010). The majority of the among population genetic variation for adaptive traits is due to high regional variation stemming from significant adaptive phenotypic variation linked to the local environmental conditions. We used interior Douglas-fir as a test species in this study because these characteristics may influence the ability or necessity of seedlings to recognize kin; we asked (1) level of relatedness - how genetically close do the seedlings need to be for kin recognition to take place? and (2) seed origin – will seeds coming from areas of differing climatic regions, or levels of environmental stress differ in kin recognition ability? As opposed to simpler self/non-self recognition (i.e. prevention of self-pollination) with a binary outcome, kin recognition involves several possible states along a gradient of relatedness (Chen et al. 2012). Regeneration success may be improved with management practices that consider, for example, the effect of degree of relatedness among individuals on kin recognition. Kin recognition and selection may decrease along a relationship gradient from strong recognition and selection between siblings/progeny (siblings, from the same parent trees for this study) with the highest degree of relatedness, to within-population (different parent trees within the same site), to among-populations (different parent trees from different sites, thus with the lowest degree of relatedness).

The high regional genetic variability and plasticity of interior Douglas-fir allows the species to persist in environmental conditions that vary greatly throughout its range. Due to this variability, populations vary widely in germination, growth and biomass allocation patterns (Kremer 1994, St. Claire et al. 2005, Gugger et al. 2010). The level of environmental stress amplifies the variation. The stress-gradient hypothesis (Greenlee and Callaway, 1996) refers to a conceptual model allowing for greater facilitation among individuals in a community with greater environmental stress (Bertness and Callaway, 1994). Essentially, the harsher the

conditions, the more likely neighbours will benefit from cooperation. Kin selection is a form of cooperation between related individuals in a community, and therefore could be influenced by the amount of stress experienced by the community. Greater environmental stress could lead to greater kin recognition or selection.

Mycorrhizal networks with mature trees have been shown to enhance survival and growth of nearby interior Douglas-fir seedlings more so in the drybelt than the wetbelt of the IDF zone, in keeping with the stress gradient hypothesis (Bingham and Simard 2011). Mycorrhizal networks are underground pathways formed by the hyphae of fungi that symbiotically associate with plant roots (from the Greek "myco" for fungus and "rhiza" for root) and connect two or more plants of the same or different species (Francis and Read 1984, Perry et al. 1989, Horton and van der Heijden 2009). Many studies have documented mycorrhizal networks as mediators for interplant transfer of water, nutrients and other chemicals, such as defense signals or allelochemicals (Agerer 2001, Querejeta et al. 2003, Teste et al. 2010, Bingham and Simard 2011, Barto et al. 2012). Here we investigate whether kin recognition can also be mediated through these networks. Given that access to a mycorrhizal network has been seen to improve interior Douglas-fir regeneration under drought stress (Bingham and Simard, 2011), we examined whether mycorrhizal networks facilitated kin recognition differentially along a stress gradient, where seeds originating from harsh climatic conditions would produce seedlings more apt to form beneficial mycorrhizal associations and result in greater kin recognition than those from more favourable regional climates. In the face of predicted climate change, understanding responses to these environmental stresses could prove important for successful regeneration of future forests.

The objective of this study was to determine whether kin recognition or kin selection would be detectable between greenhouse-grown interior Douglas-fir seedling pairs from field collected seed, whether it is mediated by mycorrhizal networks, and whether seed origin or level of relatedness affects these processes. We used seeds from six mature interior Douglas-fir trees collected from three locations (2 trees per location) that differed in regional climate in interior British Columbia, to yield sibling (seeds originating from the same parent tree (kin)) and non-sibling (seeds originating from different parent trees (non-kin)) pairs. To examine kin recognition along the relationship gradient, we examined the non-kin pairs in two groups where seedlings originated from different parent trees either from (a) the same site or (b) a different site. Our first hypothesis was that kin pairs would have greater growth and foliar nutrient content, indicating kin selection, in comparison to non-kin pairs. The enhancement in growth and nutrition was expected to increase along a relationship gradient, with the greatest values occurring among kin pairs, followed by non-kin originating from the same site (within population), then non-kin originating from different sites (among populations). Our second hypothesis was that kin recognition, and all factors that affect it, would be mediated by mycorrhizal networks. We predicted that kin recognition would be greater in seedling pairs that were connected in a mycorrhizal network compared to those where the seedlings were isolated from each other. We expected that recognition would also be affected by the extent of network formation (amount of colonization). Our third hypothesis was that kin recognition would increase with the competitiveness (e.g., growth rates) of plant genotypes. We expected greater kin recognition among genotypes originating from sites that yielded larger seedlings. We expected seedling root allocation to decrease with increasing regional precipitation of genotype origin, providing seedlings the ability acquire greater soil resources under drought-stressed

conditions through mycorrhizal networks and kin recognition, in agreement with the stress-gradient hypothesis.

Methods

Experimental design and treatments

This experiment took place in the University of British Columbia greenhouse in Vancouver, British Columbia, Canada over an 11 month period (March 2012 to February 2013). Half of one bench was used to equally distribute 180 pots, approximately 2 L with a height of 18 cm and diameter of16.5 cm. The pots were rotated randomly every two weeks. No supplementary light was provided. The seeds were acquired in the fall of 2011 from mature interior Douglas-fir trees in three locations in the interior of British Columbia. The Alex Fraser Research Forest, Knife Creek block (121.88°W, 52.05°N) and Farwell Canyon locations (122.63°W, 51.79°N) are outside of Williams Lake in the Cariboo Chilcotin Coast region. The Paska Lake location (120.67°W, 50.50°N) is approximately 30 km southwest of Kamloops in the Thompson Okanagan Region (Figure 3.1). ClimateBC provided the mean annual temperature (MAT) and precipitation (MAP) for each location in the 1981-2009 climate reference period (Wang et al. 2012). MAT of the Alex Fraser site is 4.8°C and MAP = 470 mm. MAT of the Farwell Canyon site is 3.5°C and MAP = 402 mm. MAT of the Paska Lake site is 3.5°C and MAP = 411 mm. These locations will be discussed in greater detail in chapter 4 (Figure 4.1, 4.2). The top two seed producing trees from each location (six total) were used as the seed source for the donor seedlings for this experiment.

A 2 x 3 factorial design was used, where mesh size (two levels) and relationship (three levels) were applied in a completely randomized design. The mesh size factor (two levels)

references the pore size of the specialized mesh bags (approximately 20cm x 8 cm) made by Plastok® (Meshes and Filtration) Ltd. (Birkenhead, UK) that separated the root systems of the older, "donor" seedling and the younger, "recipient" seedling. A total of 90 mesh bags had a pore size of 35 μm, which has been shown to allow fungal hyphae to penetrate the bag but prevent roots from growing through the barrier (Teste and Simard 2008). The other 90 mesh bags had a pore size of 0.5 μm, which has been shown to prevent fungal hyphae from penetrating but allow soil water to flow in and out of the bag (Teste et al. 2006). The relationship factor (three levels) references the genetic closeness of the seedling pair grown in a single pot. A total of 90 of the pots received a kin pairing, with both seedlings grown from seeds originating from the same tree. These trees were not control pollinated, therefore, the seedling pairs of kin relationship could be full or half siblings. We also examined the relationship of seedling pairs that did not originate from the same tree but came from the same location (i.e., non-kin but from the same site). These were expected to be genetically closer than seeds from different sites but are not guaranteed to be half siblings (because of the lack of controlled pollination, they could be half siblings). The remaining 90 pots received a non-kin pairing (i.e., non-kin from different locations). The relationship pairings and mesh sizes were combined in a way that resulted in 48 kin pairs, 36 non-kin intra-site pairs and 96 non-kin inter-site pairs that were evenly split between the network (35 μm pore size mesh) and no network (0.5 μm pore size mesh) treatments and evenly distributed among the six donor seedling origins.

Experimental setup

Each pot contained a mesh bag that was placed against the pot edge and gently packed with a 3:1 mixture of the greenhouse standard potting mix: field collected soil (Figure 2.1a). This

mixture was blended thoroughly in a mechanical soil mixer to achieve high homogeneity. The mixture was autoclaved for one to 1.5 hours at 250°C to kill any fungal inoculum provided by the field collected soil. The remainder of the pot was filled with a 1:1 combination of non-autoclaved field collected soil and greenhouse standard potting mix that was thoroughly mixed. This design ensured that mycorrhizal colonization of receiver seedlings planted inside the mesh bags occurred through mycorrhizal hyphal growth from the field soil-inoculated donor seedlings. The soil was collected from an interior Douglas-fir stand just outside of Princeton, British Columbia (approximately 120.58°W, 49.43°N) (Figure 3.1). The forest occurred in the Dry Cool Interior Douglas-fir subzone (IDFdk), was comprised of pure interior Douglas-fir, and was underlain by a Dystric Brunisol soil with sandy loam texture and a moder humus form (Canadian System of Soil Classification 1998). The top litter layer was scraped off and the underlying 10 to 15 cm (including the fermentation layer, humus layer and mineral soil) was collected and transported immediately to the UBC greenhouse in Vancouver, BC. This soil was expected to contain a sufficient amount of interior Doulas-fir compatible fungal inoculum to encourage mycorrhizal colonization of donor seedlings within the pot.

Each pot was designed to contain a pair of seedlings (Figure 2.1b). One older seedling was established 8 months in advance to act as a donor to the younger, recipient seedling. The pairs were subject to one of four treatment groups, described above, depending on the relationship of the pair of seeds sown (full siblings or from separate families) and the pore size of the mesh bag installed in the pot (0.5 μm or 35 μm).

At the onset of the experiment, all pots were soaked thoroughly with water. For the first sowing, a total of 150 seeds from each of the six donor seed origins described above were sown in 30 pots (180 pots in total), with five seeds per pot. Before sown, the seeds were sterilized with

10% H_2O_2 for 10 minutes then dried. An additional three seeds per pot were stratified for a second sowing, which entailed a 24 hour soak in distilled water followed by drying and refrigeration (4°C) for three weeks. These were sown after six weeks due to unsatisfactory germination rates in the first sowing. We also transplanted some seedlings from pots with multiple germinants to those yet to germinate with the goal of having at least one seedling per pot. These were all sown in the mixed field soil containing the inoculum. A thin layer of fine gravel known as "forest sand" was spread over the sown seeds to discourage the growth of damping off fungi.

The pots were lightly watered each day until each pot had at least one healthy seedling. The watering regime shifted from a light watering daily to weekly watering to field capacity. The absence of fertilizer and the limited watering regime were meant to encourage mycorrhizal fungal colonization. The seedlings were allowed to grow for another two months with periodic thinning when necessary.

When the seedlings were 4.5 months old they were transported into a Conviron PGV36 Plant Growth chamber at UBC to undergo a blackout regime to induce budset and the onset of dormancy in preparation for an artificial winter. The blackout regime consisted of a 10 day period with 10 hour days at 20°C and 14 hour nights at 15°C. After the blackout period, the seedlings were returned to the greenhouse for three weeks to allow them to become dormant for winter, and then were returned to the growth chamber for an artificial winter. During the artificial winter, the seedlings experienced 16 hour uninterrupted nights, 8 hour days at low light levels and a temperature of 4°C for six weeks to meet their chilling requirement to break dormancy and enter quiescence. The seedlings were then returned again to the greenhouse. The soil and these "donor" seedlings were allowed 24 hours to adjust to the temperature difference,

and the second set of seeds intended as "recipient" seedlings were sown the next day. Five stratified seeds from the corresponding kin, non-kin same site or non-kin different site pairing were sown within the edges of the mesh bag. The seeds were from the same stock as the seedlings currently established.

The seeds were sown in accordance with the tree from which the donor seedling originated and the tree from which the new (recipient) seedling originated. After the second set of seeds was sown, they were allowed to germinate, grow and were thinned when necessary over a four month period. Germination rate for all recipient seeds was recorded. Height was also recorded for both seedlings every three weeks. A total of 89 seedling pairs remained healthy and were harvested at the end of the experiment, which represented 46.4% establishment success rate for the pots. There were 21 kin/network, 20 kin/no network, 19 non-kin/network (5 of which were non-kin from the same site) and 29 non-kin/no network (8 of which were non-kin from the same site).

Measurements

The growth variables examined in both the donor and recipient were needle, stem and root weight (g), total and above ground biomass (g), average weight per colonized root tip (g per root tip), percent of total root weight colonized (%), stem length (cm), germination rate (germinants per seeds sown), average growth rate (cm per week), total leaf area (cm^2) and volume, calculated as total leaf area x stem length (cm^3) in order to estimate the overall effect of the height and conical volume of the seedling. Average leaf area per needle (cm^2) was examined only in recipient seedlings. The seedlings were removed from the pots after the younger seedlings were grown for four months. The below and above ground biomasses were separated.

The soil was carefully removed from around the roots, which were then washed with tap water. The needles were removed and measured using a LICOR-3100 leaf area meter. The number of needles was also recorded for the younger, recipient, seedlings only. The needles were dried and biomass was recorded. The stems were measured for length, number of branches and dry biomass.

The root tips were examined for mycorrhizal colonization and those that appeared to be colonized were weighed and morphotyped. The DNA of a subset of tips was sequenced at the Irving K. Barber School at UBC-Okanagan. For further details refer to Chapter 2 methods.

A suite of foliar nutrients were examined using microwave digestion/ICP (Inductively Coupled Plasma-Optical Emission Spectrometer) and % C, N and S analyses at the British Columbia Ministry of Environment Analytical Chemistry Laboratory. The macronutrients examined were C, N, K, Ca, Mg, P and S. The micronutrients examined were Al, B, Cu, Fe, Mn, Mo, Na and Zn. The extrapolated gram content per seedling and average sample concentrations were used in our analysis. C:N and N:P ratios were calculated. Foliar nutrients were measured only for recipient seedlings.

Data analysis

All statistical analyses were conducted using SAS version 9.3 (Cary, North Carolina). Two-way analysis of variance (ANOVA) was run separately for each variable. The factors included were relationship, network and relationship x network. An analysis of covariance (ANCOVA) was also run to examine the effect of seed origin (location) as a covariate. Significance was determined at $p \leq 0.05$ and trends were noted where $p \leq 0.10$.

Results

Mycorrhizal colonization

Two variables were used to evaluate colonization: percent of total root weight colonized and average weight per colonized root tip. In the donor seedlings, there was a main effect of mesh size (network) (Table 3.1). A higher percent of the donor root weight comprised of colonized root tips and greater average weight per colonized root tip occurred in the 0.5 μm mesh bag where mycorrhizal network formation was prevented ($p<0.05$). In the recipient seedlings, both variables were higher in kin than non-kin seedlings ($p<0.05$) (Table 3.2, Figure3.2). The kin effects were not significant in the donor seedlings and the network effect was not significant in the recipient seedlings. The interaction between network formation and seedling relationship was not significant for either variable in the donors or recipients.

Growth

Relationship effects

In donor seedlings, there was a significant effect of relationship on total leaf area and volume ($p<0.05$) as well as a trend in stem length ($p<0.10$) (Table 3.1). Donor seedlings with a kin relationship with recipients (i.e., seeds of each seedling coming from the same tree) had greater leaf area, volume and stem length than those with a non-kin relationship (Figure 3.3). There were no significant kin effects for any recipient seedling variables (Table 3.2). We also tested the difference between non-kin from same site (both with and without networks) against all other treatments, including non-kin from different sites, for all growth variables. There were no differences between the same site non-kin seedlings and any other treatment. For all other analyses, the non-kin seedlings from the same and different sites were simply considered non-kin.

Network effects

Root weight, total biomass and growth rate were lower in recipient seedlings of pairs that were separated by 35 μm mesh than 0.05 μm mesh ($p<0.05$)(Table 3.2, Figure 3.4). Above ground biomass of networked recipient seedlings also tended to be lower ($p<0.10$) (Table 3.2). Mycorrhizal networks had no significant effect on any of the donor seedling variables (Table 3.1).

Relationship x network effects

The only significant relationship x network interaction effect occurred in root weight of donor seedlings (Table 3.1). Donor seedlings with a kin relationship and no network (0.5 μm mesh) had more root biomass than those with a non-kin relationship and no network (Figure 3.5). Networked non-kin donors also tended to have more root biomass than isolated non-kin donors. ANCOVA showed no origin effects on any growth variables.

Seed origin effects

Seed origin had a significant effect on the growth rate of recipient seedlings ($p = 0.0440$) (Table 3.4). The seedlings from Paska Lake seeds grew faster than seedlings from Alex Fraser Research Forest seeds. The growth rate of seedlings from Farwell Canyon seeds did not different from either seed origin location (Figure 3.6a)

Nutrients

Only recipient seedlings were measured for foliar nutrient content. There was greater aluminum and copper content in recipient seedlings with kin than non-kin relationships (Figure

3.7). The same site, non-kin seedlings were first tested separately from the different site, non-kin seedlings. The kin seedlings, regardless of their ability to form networks, had significantly greater aluminum than non-kin, regardless of whether they came from the same or different sites. The networked kin seedlings had significantly greater foliar copper content than all other treatments (Figure 3.8). There no difference between the same site, non-kin and the different site, non-kin for any variables ($p>0.10$), so all non-kin were combined for all other analyses.

There was lower boron and manganese content in recipient seedlings that were connected by mycorrhizal networks (35 μm mesh) than those isolated (0.5 μm mesh), paralleling network effects on growth responses. The opposite effect occurred with copper, where recipient seedlings had higher copper where they could form a network with the donor.

Copper, zinc ($p<0.05$) and iron content ($p<0.10$) were affected by interactions between relationship and network (Figure 3.9). The origin of the seed was tested as a covariate, but had no effect on any of the nutrient variables.

Germination rates

Germination rate differed among seed origins (locations) ($p<0.0001$) (Table 3.4). The Farwell Canyon site had a significantly higher germination rate than the Alex Fraser Research Forest or Paska Lake sites (Figure 3.6b). There were also significant interaction effects; however, these are not likely ecologically relevant since relationship and network effects are unlikely to affect germination (Figure 3.10).

Soils

The autoclaved soil had higher percent total carbon, sulphur and available phosphorus but lower pH than non-autoclaved mixed field soil. There was no difference in percent total nitrogen or mineral nitrogen. Autoclaved soil had greater exchangeable cations (CMol+/Kg), except manganese which was higher in the mixed field soil, and iron which was unaffected by autoclaving. The effective cation exchange capacity was also higher in the autoclaved soil. The soil in this experiment was identical to that of the full sibling experiment described in chapter 2 (Table 2.6).

Discussion

Kin effects

Evidence of kin recognition was found in both foliar nutrients and seedling growth. We defined kin recognition as a significant difference (greater or lesser) in a variable depending on the seedling's relationship to its pair. We expected the difference to present as kin selection, where kin seedlings, recipients in particular, have greater nutrient content or growth rates compared to non-kin seedlings. We found kin selection expressed in greater foliar content of the micronutrients, aluminum and copper, in kin than non-kin recipient seedlings. Copper is known to be essential for foliar enzyme activity, chlorophyll formation, and is important in seed formation and production (Mengel and Kirkby 2001, Marschner 2012). Aluminum has a potential role in protecting roots against disease (Marschner 2012). Both copper and aluminum are known to be involved in biochemical signaling, particularly ethylene pathways, involved in photosynthesis and plant growth and development (Hirayama et al. 1990). It is possible that increased foliar copper and aluminum among receiver kin played a role via signal transduction or

root physiology in enhancing mycorrhizal colonization and health of receiver roots. Alternatively, increased mycorrhization has been shown to improve copper nutrition in plants (Li et al. 1991). There was no evidence for expression of kin recognition in foliar macronutrients, similar to previous studies showing kin effects on other plant traits such as plant physiology, morphology or growth strategies (File et al 2011, Chen et al. 2012).

We also observed kin selection expressed in greater mycorrhizal colonization of kin than non-kin recipient seedlings (i.e., greater average weight per colonized root tip and greater percent of total root weight colonized). Enhanced mycorrhizal colonization suggests that the kin relationship is providing the recipient seedlings with some advantage, such as improved root physiology, that is recognized and sought by the mycorrhizal fungi. File et al. (2012) also found more extensive mycorrhizal network formation in kin pairs of ragweed seedlings.

Kin recognition was also evident in some donor seedling growth traits. Total leaf area, volume and stem length of donor seedlings were greater where they were paired with kin than non-kin seedlings. In contrast to our expectation, the enhanced growth traits of kin donors did not affect the growth of kin recipients. However, greater growth rates of donor kin may have played a role in the increased mycorrhization and micronutrient content of recipient kin. Donor facilitation of recipient kin, or vise versa, could have occurred through interplant carbon transfer as shown in Chapter 2, or through a priming effect between donor and recipient roots. With no recognition detectable in the recipient seedling growth traits, it is difficult to speculate as to whether the enhanced growth in the donor seedlings was due to kin selection or reduced competition (i.e., niche partitioning) (File et al. 2011).

Seed origin effects

Germination rates were greatest among recipient seeds originating from the driest location, Farwell Canyon (Figure 3.6b). Moreover, kin seedlings originating in Farwell Canyon had significantly higher germination rates than the non-kin seeds (Figure 3.9.). These kin effects were not evident among seed originating from the other two wetter sites. The effect on germination rates suggests that seeds from the harsher climate were better adapted to mass germination in the greenhouse, as many germinants would die in normal field conditions. They are also likely to support greater mycorrhizal colonization for enhanced uptake of limited water and nutrients. These results support our hypothesis that kin selection would be greatest among genotypes that are more competitive in limited resource acquisition. The growth rates among different seed origins corroborated this hypothesis. Seedlings originating from seed at Farwell Canyon grew statistically at the same rate as those from the wetter, Paska Lake origin. Considering the harsh conditions of the Farwell Canyon site, we could expect slower growth rates, but we did not observe this. Rehfeldt (1989) showed that in common garden experiments seedlings from regions with fewer frost free days had reduced growth compared to those from regions with a greater number of frost free days. As the Farwell Canyon site had a shorter frost free period (84 days) than did Paska Lake (98 days), this was an unexpected result (Wang et al. 2012).

This result supports the stress gradient hypothesis, which suggests that environmental stress is a key driver in inter-plant facilitation (Greenlee and Callaway 1996, Callaway et al 2002, Liancourt et al. 2005, Cavieres et al. 2006). It is possible that seeds from Farewell Canyon were genetically primed for kin recognition to facilitate regeneration under climatically stressful

conditions. The transfer of water from donor to recipient plants through mycorrhizal networks has also been shown to increase with environmental stress (Bingham and Simard 2011).

Level of relatedness

We reject our hypothesis that the strength of kin effects would be detectable along a genetic gradient of relatedness, with decreasing strength from kin, to non-kin from the same site, to non-kin from different sites. There was a difference between the kin seedlings and all the non-kin seedlings combined, but no differentiation between non-kin seedlings originating from the same versus different sites. The noted differences were only observed in variables that had kin effects regardless of the separation of non-kin due to site. There could be a number of explanations for this result. Kin recognition could only be sensitive at the sibling/progeny level and therefore relatedness would be undetectable at the population level. If non-kin seedling pairs originating from the same site did not share paternal nor maternal genes, they would not be differentiated from the non-kin pairs originating from different sites at that level of sensitivity. Alternatively, if the recognition process heavily relies on maternal effects and is only sensitive to the sibling/progeny level, the non-kin originating from same versus different sites would be indistinguishable (Donohue, 2003). Due to the wind dispersal process of both the pollen and the seeds of interior Douglas-fir, the populations can be large and continuous, covering significant geographic distances (Hammerick et al. 1992). If these sites are experiencing gene flow amongst them, which is likely the case between the Farwell Canyon and Alex Fraser Research Forest sites as they are relatively close in proximity (approximately 55 km apart), they could be considered one population and therefore, the non-kin originating from the same or different sites would be similar in genetic makeup. Little variation among populations of Douglas-fir has been found

within the same region or among individuals within a population (Gugger et al. 2010) in selectively neutral markers. This is consistent with Rehfeldt (1989), who found that most genetic variation in growth traits such as height and growth strategy occurred over significant differences in geographic distance or elevation, which translated into adaptive differentiation depending on the number of frost free days in the region. However, our results must be considered with caution given the small sample size and the possibility that the condition of the parent tree and the quality or maturation of the seed collected may have influenced the results.

Mycorrhizal network effects

The DNA sequencing performed on the colonized root tips showed that *Rhizopogon vinicolor* was the most common fungal association, colonizing 92.2% of donor roots and 95.6% of recipient roots. This result was expected; *Rhizopogon vinicolor* is a strong networking fungal species that dominates interior Douglas-fir throughout forest development (Tweig et al. 2007, Beiler et al. 2010). The fungal community also included *Pyronemataceae* (sp.) and ascomycete endophytes. In networked treatments, 39 of 40 recipients were colonized by at least one fungal species and had at least one ectomycorrhizal species shared in common between the donor and recipient. This provides sufficient evidence that the seedlings in a pair had the potential to form mycorrhizal networks.

Our hypothesis that the kin effects were facilitated by mycorrhizal networks, where the kin effect would only be detectable where seedlings were connected by a network, was partially supported by our data. Recipient seedlings had higher copper, and to a lesser degree iron content in the kin/network treatment than the other treatments. It is possible that copper and iron were preferentially transferred to kin seedlings through mycorrhizal networks in enzymes or hormones

acting as chemical signals or nutritional supplements for receiver plants. However, the ability to form mycorrhizal networks had no effect on any other nutrient variable where kin recognition was detectable.

Contrary to our expectations, we found that the presence of mycorrhizal networks reduced the growth rate, total biomass and root weight, as well as some foliar nutrients (boron and manganese), of recipient seedlings. We expected that mycorrhizal networks would facilitate recipient seedling growth based on previous research showing enhanced germination, survival and growth of seedlings where they were networked with older trees (Teste et al. 2009, Bingham and Simard 2011). In these studies, increased seedling performance was associated with resource transfer along carbon and nitrogen source-sink gradients. The opposite growth effect that we found agrees with a study by Merrild et al. (2013), who showed a size discrepancy between two tomato plants connected by a mycorrhizal network was associated with preferential P uptake by the larger and P deficiency in the smaller plant. We did not measure foliar P content in both donor and recipient seedlings in this experiment; however, foliar P concentration and content were greater in the larger donors than the smaller recipient seedlings in the full sibling experiment (Chapter 2). The recognition of another nearby seedling via signaling through mycorrhizal networks could prompt the donor seedling to increase its competitive effect on recipient seedlings, thus reducing recipient seedling growth via the niche partitioning or "elbow room" hypothesis (Young 1981, File et al. 2011). In our study, this resulted in reduced root weight, total and above ground biomass, and growth rate of the recipient seedlings with mycorrhizal networks. This did not coincide with network effects on donor seedling growth rates. However, donors that formed networks with recipient seedlings had lower mycorrhizal

colonization rates than those that were isolated, potentially resulting from network-enhanced interplant competition.

Conclusion

Kin recognition was evident through enhanced mycorrhizal colonization and foliar micronutrients of recipient seedlings and greater growth rates of donor seedlings. However our hypothesis that kin recognition would be expressed as kin selection through enhanced growth rates of recipient seedlings was not supported. The effect of seedling relatedness was evident when comparing sibling and non-sibling relationships, but did not appear to act along a gradient of genetic relatedness. Kin recognition appeared to be present in germination rates of seeds originating from the driest location but not the two wetter locations, suggesting that competitive ability of genotype affects kin selection. This finding also supports the stress gradient hypothesis. The variation in germination may be attributed to maternal effects such as the health of the tree and the size of the seeds it produced. Mycorrhizal networks played an unexpected role in kin recognition by reducing growth rate of recipient seedlings and thus increasing asymmetrical competition between donor and recipient seedlings. They also were associated with improved recipient copper and iron nutrition, which perhaps played a role in chemical signaling between plants.

The ability of interior Douglas-fir to recognize kin may have forest management implications. The positive relationship that kin recognition had on aluminum and copper content as well as germination rate and mycorrhizal colonization suggests that, in some cases, particularly where germination is low, or aluminum or copper is scarce, preferentially promoting kin regeneration may be beneficial. Retaining large, healthy legacy trees for natural regeneration

(especially in dry climates), or pre-planning to grow and plant kin seedlings originating from those legacy hub trees (especially in wetter climates), may result in higher germination and survival rates but further research is necessary to substantiate these claims. Variable retention harvesting may be particularly effective in dry sites, such as the Farwell Canyon region, where environmental stress may drive cooperation within the biotic community in accordance with the stress gradient hypothesis (Greenlee and Callaway, 1996). This management approach would also conserve mycorrhizal networks, which have been shown to benefit seedling establishment, particularly in dry sites (Bingham and Simard, 2012). The results from this study also suggest that mycorrhizal networks and kin recognition would enhance growth variation among neighbouring seedlings, perhaps leading to more diverse stand structures.

Table 3.1. Analysis of variance for network, relationship and interaction (network x relationship) effects on growth variables in donor seedlings. *p <0.05

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Needle weight (g)	0.03	0.8723	0.27	0.6075	0.85	0.3604
Stem weight (g)	0.01	0.9318	0.19	0.6671	1.04	0.3115
Root weight (g)	0.10	0.7534	0.58	0.4477	4.11	0.0458*
Total biomass (g)	0.03	0.8560	0.41	0.5226	2.08	0.1531
Above ground biomass (g)	0.01	0.9253	0.26	0.6098	0.98	0.3259
Total leaf area (cm^2)	0.52	0.4747	5.31	0.0237*	0.55	0.4596
Stem length (cm)	0.00	0.9882	2.99	0.0875	0.32	0.5745
Average weight per colonized root tip (g)	7.56	0.0073*	2.33	0.1309	1.20	0.2769
Percent of root weight colonized	5.51	0.0212*	0.01	0.9297	0.24	0.6228
Average growth rate (cm/week)	0.87	0.3539	0.43	0.5130	0.00	0.9945
Volume (cm^3) - (total leaf area x stem length)	0.17	0.6824	4.89	0.0297*	0.26	0.6113

Table 3.2. Analysis of variance for network, relationship and interaction (network x relationship) effects on growth variables in recipient seedlings. *p <0.05

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Needle weight (g)	2.22	0.1403	1.18	0.2797	1.21	0.2738
Stem weight (g)	1.72	0.1929	0.75	0.3899	0.61	0.4378
Root weight (g)	4.21	0.0432*	0.03	0.8676	2.70	0.1043
Total biomass (g)	5.88	0.0174*	0.51	0.4777	0.05	0.8294
Above ground biomass (g)	3.01	00865	1.53	0.2193	1.50	0.2247
Total leaf area (cm^2)	2.04	0.1568	1.14	0.2886	0.61	0.4356
Stem length (cm)	1.24	0.2679	0.37	0.5441	2.13	0.1485
Average weight per colonized root tip (g)	0.40	0.5313	7.06	0.0094*	0.25	0.6215
Percent of root weight colonized	0.04	0.8341	12.59	0.0006*	0.50	0.4834
Average growth rate (cm/week)	4.19	0.0437*	1.33	0.2518	1.18	0.2796
Volume (cm^3) - (total leaf area x stem length)	0.43	0.5149	1.41	0.2386	0.06	0.8135
Average leaf area per needle (cm^2)	0.67	0.4158	0.02	0.8952	1.39	0.2415

Table 3.3. Analysis of variance for network, relationship and interaction (network x relationship) effects on foliar nutrient content and ^{13}C transfer in recipient seedlings. *p <0.05.

	Network		Relationship		Network x Relationship	
	F	P	F	P	F	P
Al (g)	0.27	0.6019	26.07	<0.0001*	1.59	0.2115
B (g)	3.97	0.0496*	2.10	0.151	2.05	0.1555
C (g)	2.11	0.1503	1.03	0.3132	1.20	0.2771
Ca (g)	1.51	0.2222	1.49	0.2263	1.27	0.2637
Cu (g)	4.81	0.0311*	5.48	0.0216*	8.40	0.0048*
Fe (g)	0.13	0.7185	1.09	0.2987	3.35	0.0707
K (g)	1.62	0.2064	1.49	0.2251	1.13	0.2901
Mg (g)	1.21	0.2744	0.65	0.4235	1.32	0.2532
Mn (g)	6.04	0.0160*	0.00	0.9890	0.79	0.3753
Mo (g)	0.18	0.6711	0.27	0.6065	0.96	0.3312
N (g)	1.92	0.1697	1.13	0.2905	0.87	0.3533
Na (g)	0.77	0.3836	0.02	0.8853	0.30	0.5856
P (g)	1.96	0.1647	1.42	0.2369	1.28	0.2606
S (g)	0.67	0.4168	1.43	0.2352	2.00	0.1605
Zn (g)	0.40	0.5293	0.48	0.4911	6.21	0.0147*
C:N	0.00	0.9880	0.23	0.6361	0.56	0.4547
N:P	1.30	0.2579	0.44	0.5073	0.91	0.3438

Table 3.4. Analysis of covariance for network, relationship and interaction (network x relationship) effects, with seed origin included as the covariate and its effects on growth and germination rates recipient seedlings.*p <0.05.

	Growth rate			Germination rate		
	df	F	P	df	F	P
Network	1	4.87	0.0303*	1	1.82	0.1801
Relationship	1	0.00	0.9442	1	1.95	0.1648
Network x relationship	1	3.92	0.0513	1	0.15	0.7018
Seed origin	2	3.25	0.0440*	2	23.99	<0.0001*
Network x seed origin	2	0.51	0.5996	2	0.26	0.7713
Relationship x seed origin	2	4.19	0.0187*	2	21.79	<0.0001*
Network x relationship x seed origin	2	4.51	0.0140*	2	0.14	0.8734

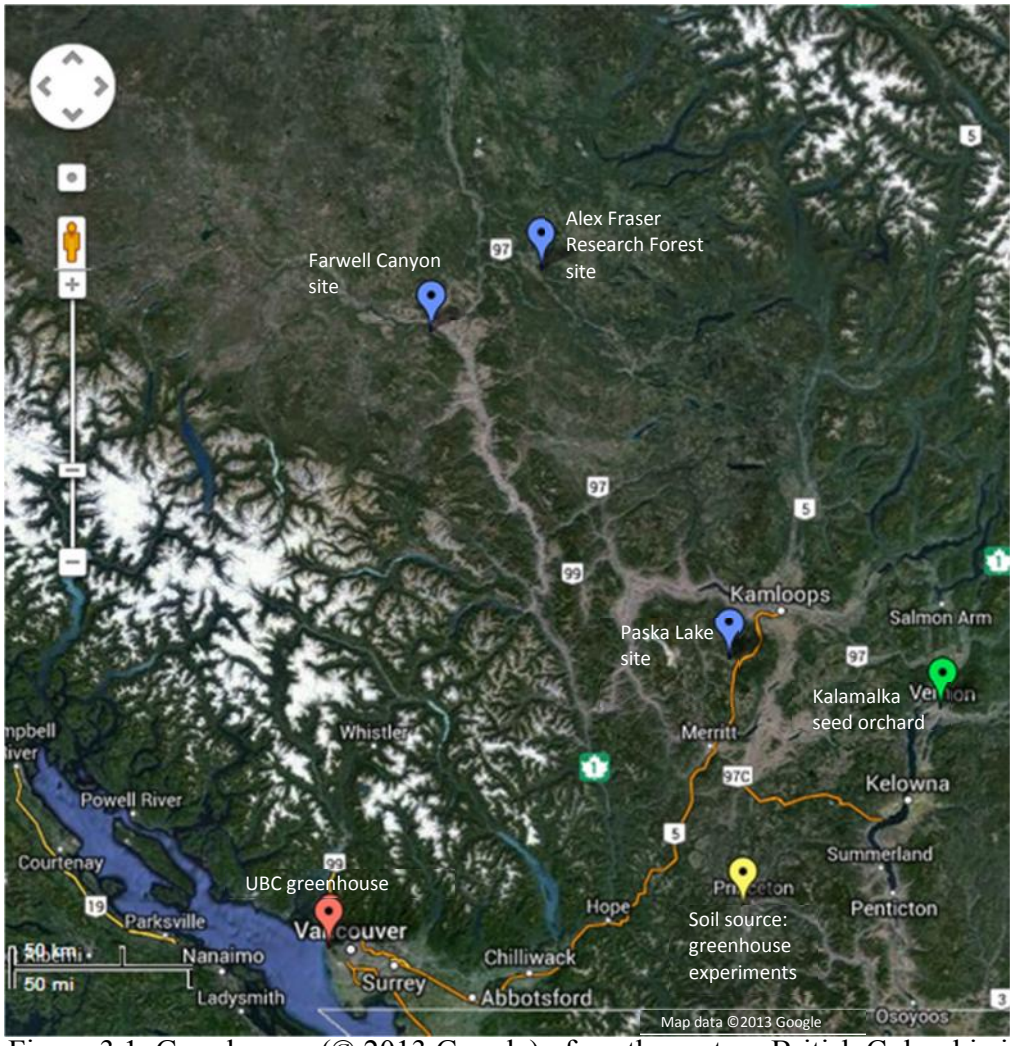


Figure 3.1. Google map (© 2013 Google) of south-western British Columbia including three field research sites (Alex Fraser Research Forest, Farwell Canyon and Paska Lake sites), the seed source for the full sibling experiment (Kalamalka seed orchard), the soil source for the greenhouse experiments (Soil source: greenhouse experiments) and the location of the greenhouse (UBC greenhouse).

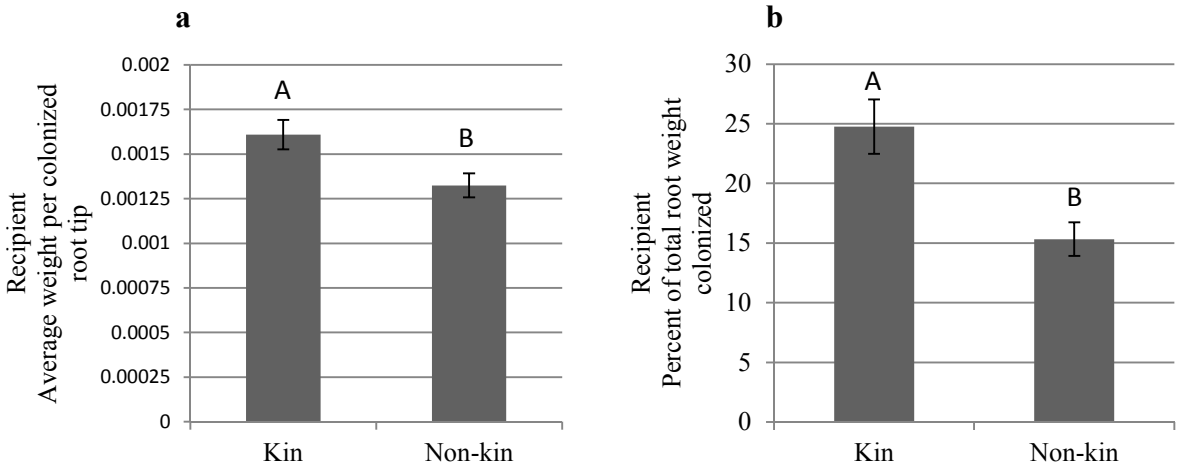


Figure 3.2. Least square means of recipient seedling (a) average weight per colonized root tip and (b) percent of total root weight colonized under the main relationship effects without consideration of the network treatment used. Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

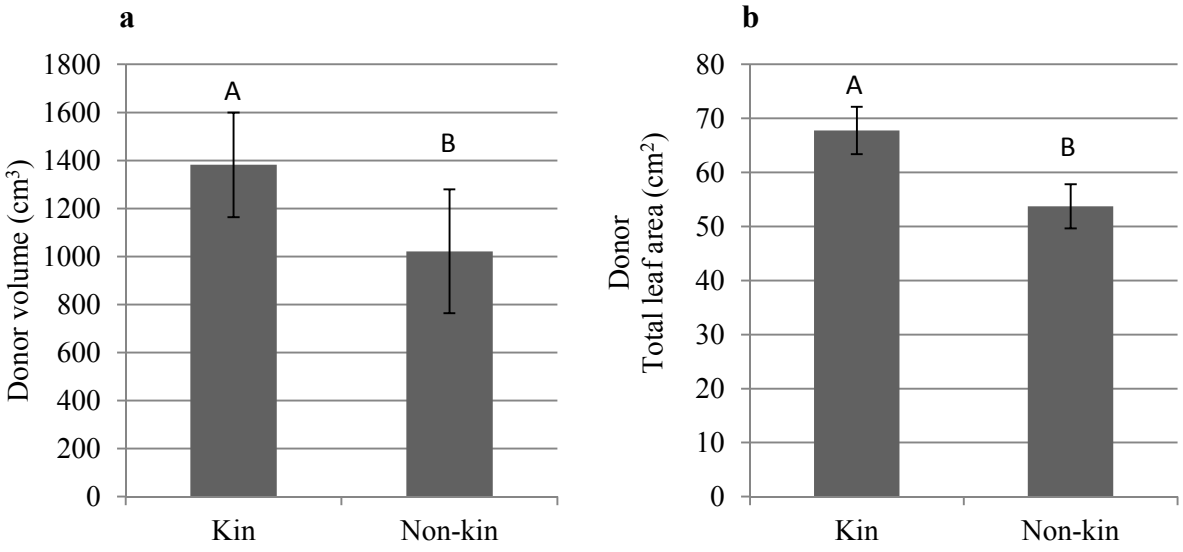


Figure 3.3. Least square means of donor seedling (a) volume (total leaf area x stem length) and (b) total leaf area under the main relationship effects without consideration of the network treatment used. Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

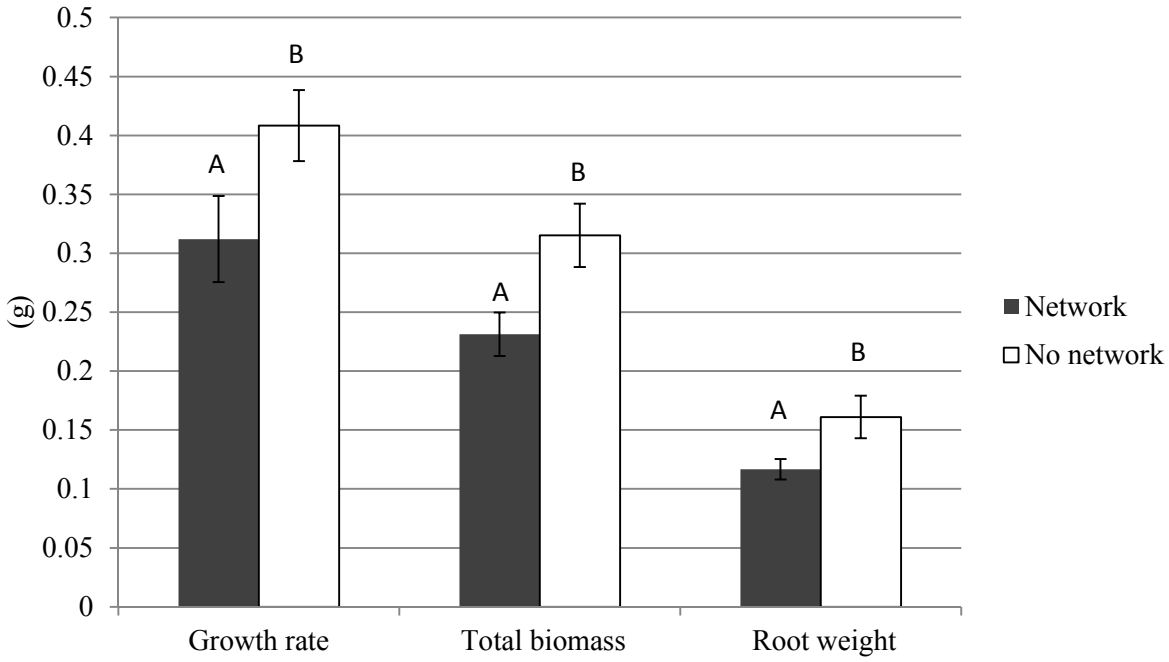


Figure 3.4. The growth rate, total biomass, and root weight least square means of recipient seedlings differentiated by network effect. Different letters indicate bars that are significantly different at $p > 0.05$. Each of the variables were compared by network effect, but were not compared to each other. Error bars are one standard error above and below.

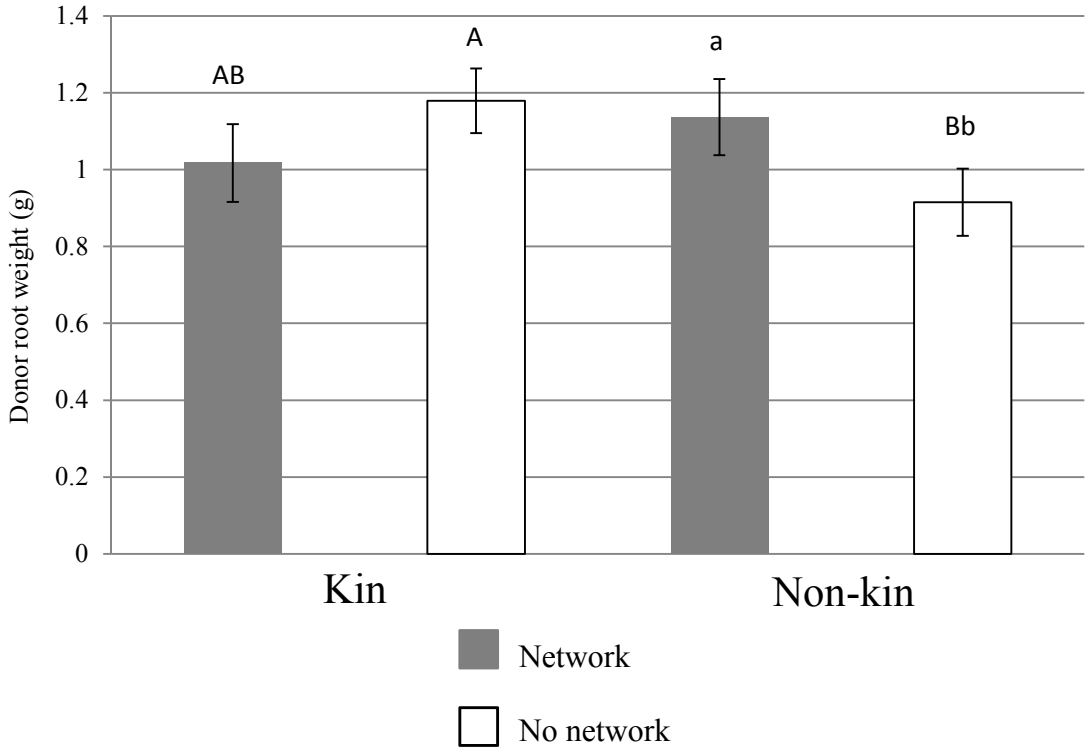


Figure 3.5. Least square means of root weight in donor seedlings under the four relationship and network combinations (interaction effects). Different upper case letters indicate bars that are significantly different at $p > 0.05$. Different lower case letters indicate bars that are significantly different at $p > 0.10$. Error bars are one standard error above and below.

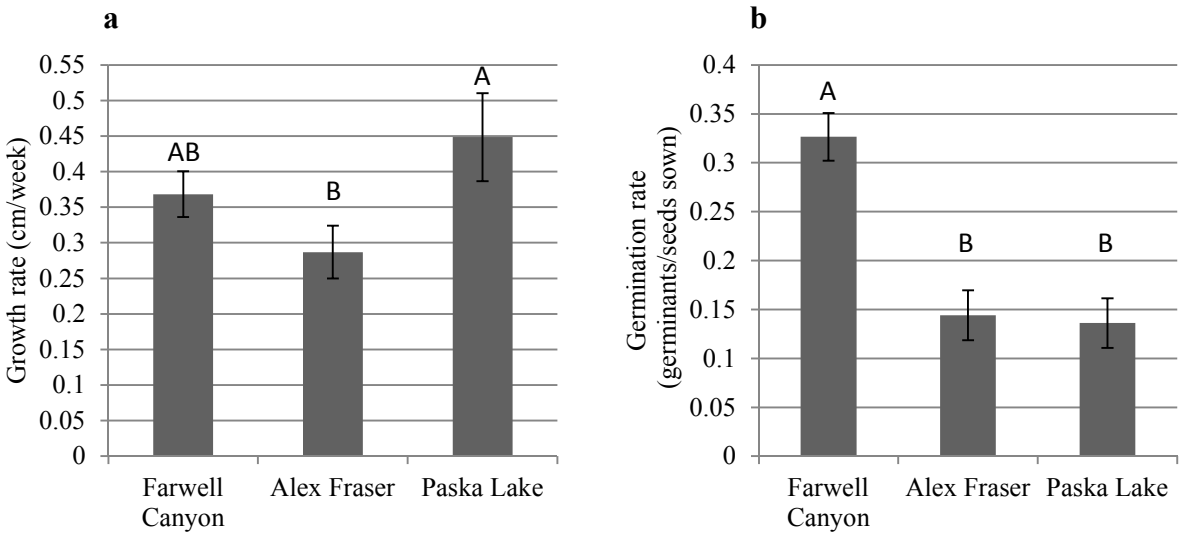


Figure 3.6. The variation of (a) growth rate and (b) germination rate (least square means) between seeds originating from Farwell Canyon, Alex Fraser Research Forest and Paska Lake. Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

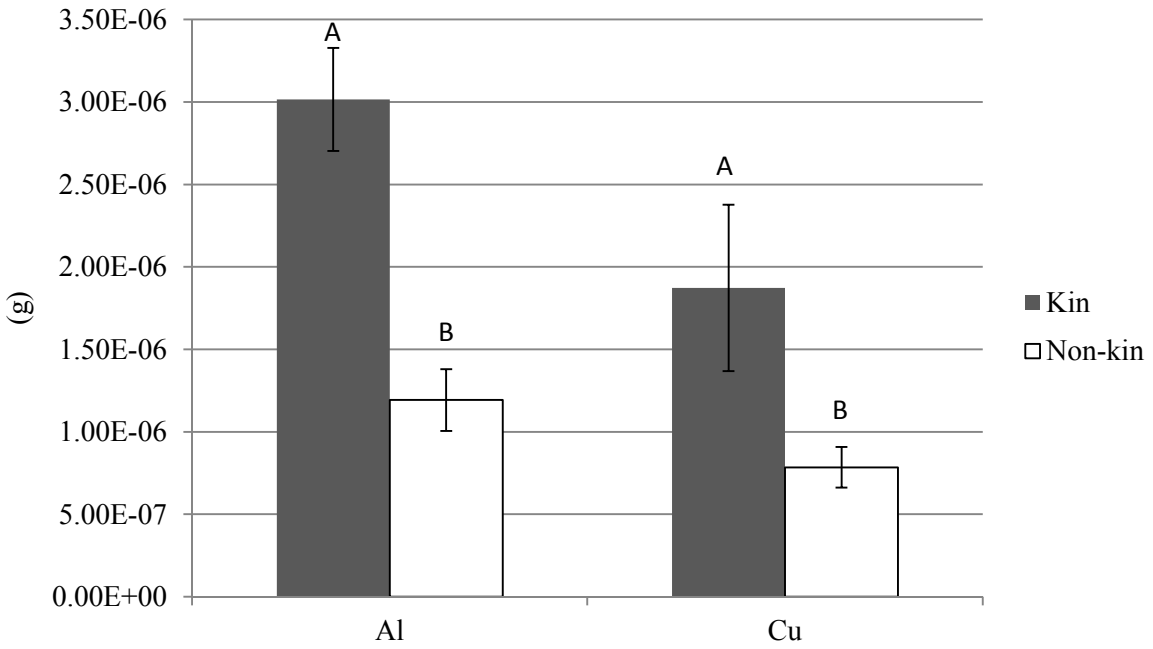


Figure 3.7. Aluminum (Al) and copper (Cu) content least square means of recipient seedlings differentiated by relationship effect. Different letters indicate bars that are significantly different at $p > 0.05$. Each of the variables were compared by relationship effect, but were not compared to each other. Error bars are one standard error above and below.

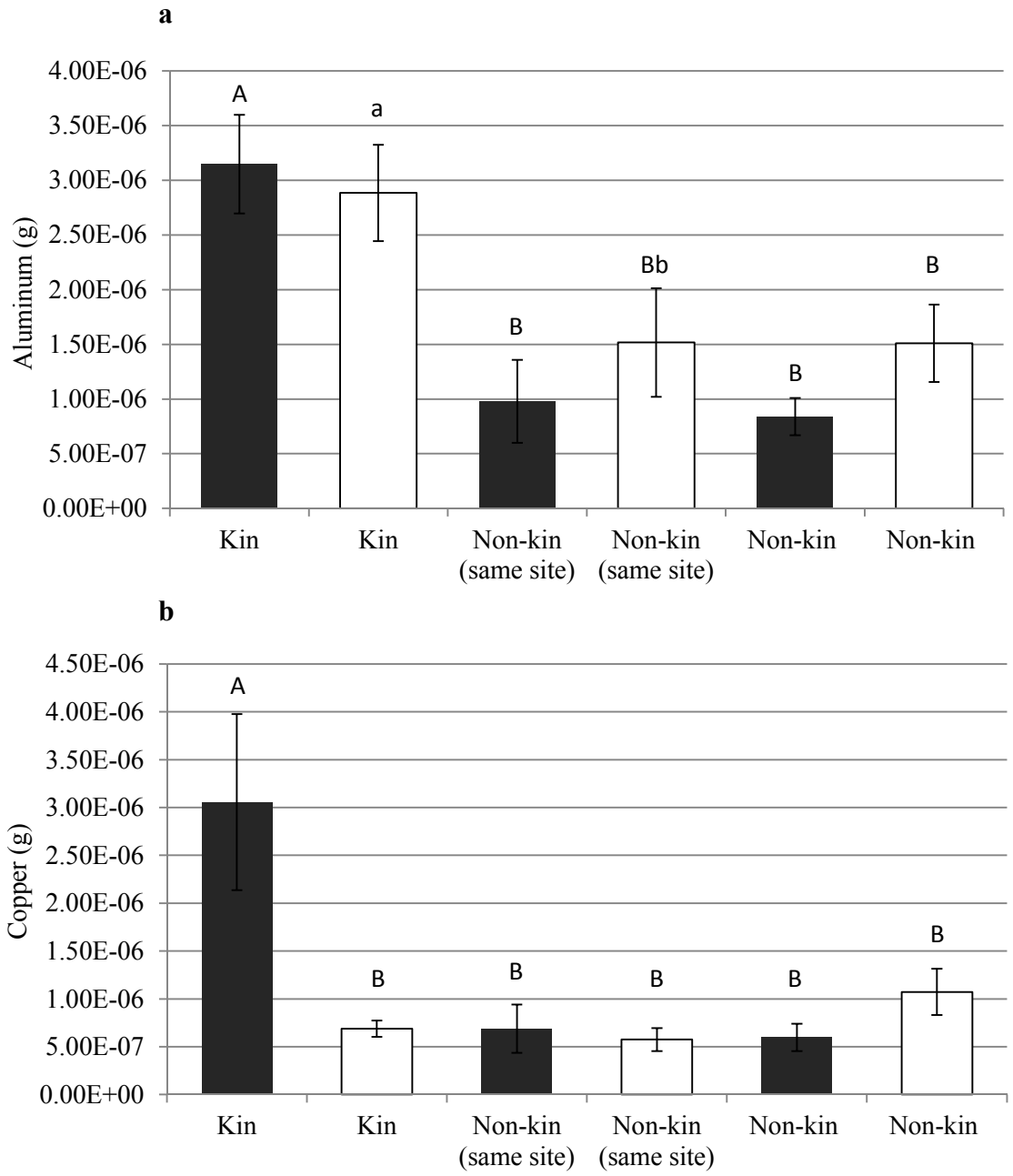


Figure 3.8. same site. Least square means of (a) aluminum (Al) and (b) copper (Cu) differentiated by relationship effect between kin pairs (seeds from same parent trees), non-kin (same site) (seeds from different parent trees within the same site) and non-kin (seeds from different parent trees from different sites). Different upper case letters indicate bars that are significantly different at $p > 0.05$. Different lower case letters indicate bars that are significantly different at $p > 0.10$. Error bars are one standard error above and below.

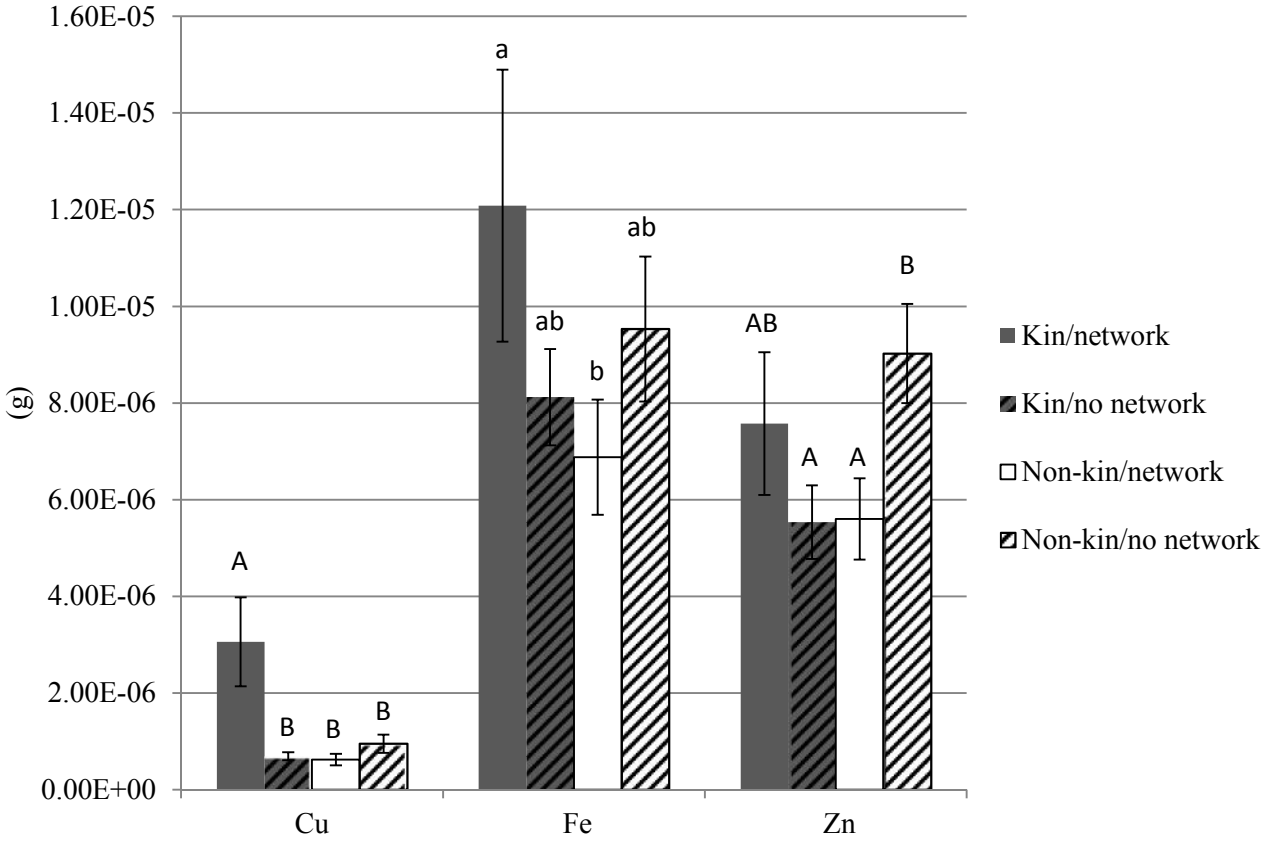


Figure 3.9. Significant least square mean differences in recipient seedlings under the four relationship and network combinations (interaction effects) found in copper (Cu), iron (Fe) and zinc (Zn). Different upper case letters indicate bars that are significantly different at $p > 0.05$. Different lower case letters indicate bars that are significantly different at $p > 0.10$. Each of the variables were compared by relationship effect, but were not compared to each other. Error bars are one standard error above and below.

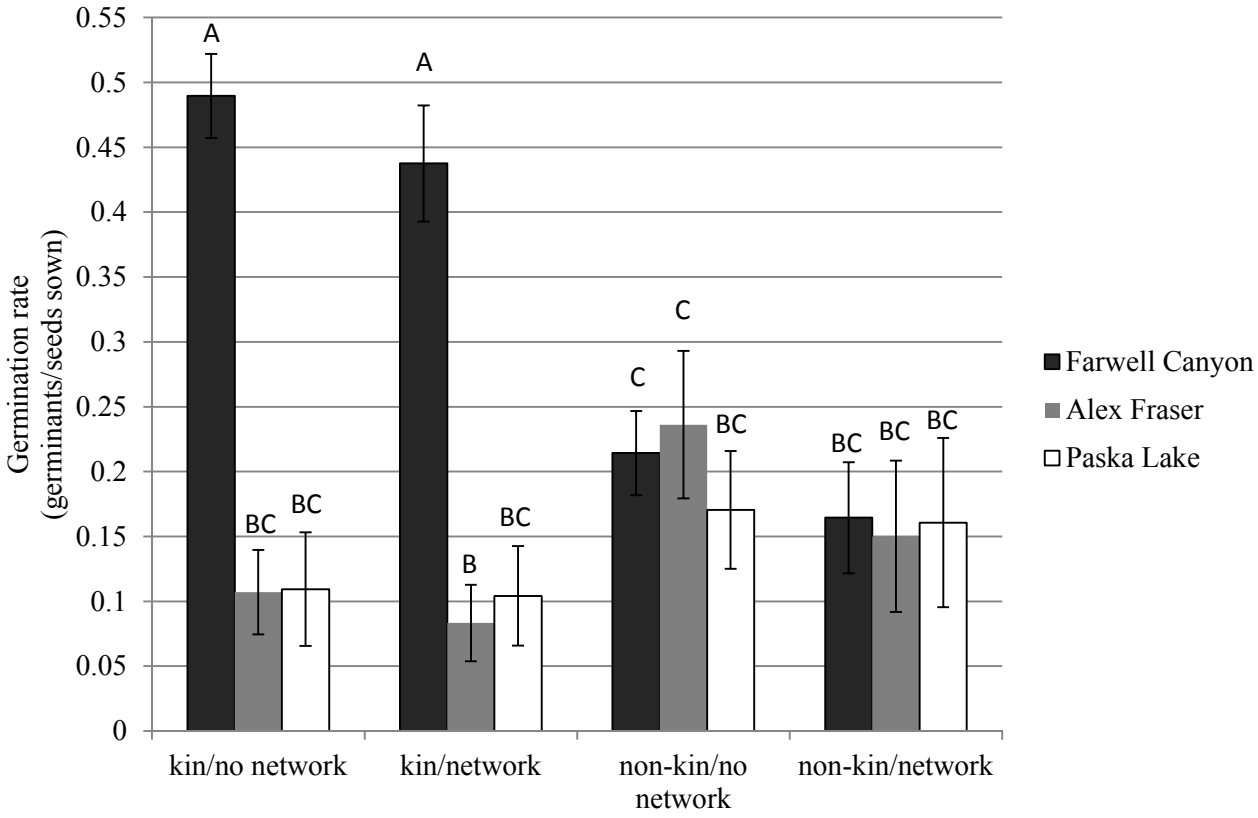


Figure 3.10. Least square means of germination rate in recipient seedlings under the four relationship and network combinations (interaction effects) and seed origin effects. Different letters indicate bars that are significantly different at $p > 0.05$. Error bars are one standard error above and below.

4 Mature trees may preferentially support kin seedlings in germination and survival through mycorrhizal networks

Introduction

Interior Douglas-fir (*Pseudostuga menziesii* var. *glauca* (Beissn.) Franco) grows in a wide variety of climatic conditions and therefore exhibits high genetic variability (Gugger et al. 2010). The variation in climatic conditions has also created distinctive regeneration patterns. In drier sites, seedlings tend to regenerate in clusters around larger, mature trees. In wetter sites, regenerating seedlings are more evenly distributed through disturbed openings (Simard 2009). Since interior Douglas-fir pollen and seed are wind dispersed (Hamrick et al. 1992) and it is unlikely that dispersal would be lower in dry than wet sites, factors other than seed dispersal are likely influencing regeneration patterns. Under identical conditions, clustering would increase competition among regenerating seedlings, suggesting some advantage is gained by regenerating near mature trees. These areas may be reservoirs for water, nutrients and shade, perhaps explaining why a tree grew to maturity there, but other facilitative processes may be present as well. We are interested in the facilitative effect that kin recognition, mycorrhizal networks or the combination of the two may have on regeneration of disturbed sites in the interior Douglas-fir zone (IDF) of British Columbia.

While greenhouse studies can isolate effects and control for growing conditions and genetic variability, it is important to test whether these patterns occur in natural conditions in the field. In this study, we examined kin recognition and mycorrhizal network influence on seedlings grown in the field. Our main hypotheses were identical to those of the greenhouse studies (Chapters 2 and 3). We predicted there would be a difference in germination and survival due to the relationship of the seedling to the mature tree it was sown near (parent tree). We predicted that the kin seedlings would have higher germination and survival than non-kin seedlings (kin

selection). The effects of relationship were predicted to be mediated by mycorrhizal networks. In addition we expected the relationship effect to be stronger where the growing conditions were harsher in accordance with the stress gradient hypothesis.

Methods

Study sites

Three study sites were located in interior British Columbia, Canada. All sites had retention of individual tress due to either partial harvesting (Paska Lake and Alex Fraser Research Forest) or natural disturbances (Farwell Canyon). None of the sites had been site prepared or planted. Understory plant community composition, primarily native grass species, and abundance were similar among all sites. The Paska Lake site (120.67°W, 50.50°N) is approximately 30 km southwest of Kamloops. The Alex Fraser Research Forest site in the Knife Creek block (121.88°W, 52.05°N) is approximately 20 km southeast of Williams Lake in the Cariboo Chilcotin Coast region of British Columbia. The Farwell Canyon site (122.63°W, 51.79°N) is approximately 50 km southwest of Williams Lake also in the Cariboo Chilcotin Coast region of British Columbia. The Alex Fraser Research forest site is the warmest and the wettest of the three followed by the drier and cooler Paska Lake site and the driest site, Farwell Canyon (with same mean annual temperature as Paska Lake).All temperature and precipitation measures were retrieved from ClimateBC, over the most recent 30-year climate period, 1981-2009 (Wang et al. 2012) (Figure 3.1 & Table 4.1). The Alex Fraser Research forest and Farwell Canyon sites are located in the interior Douglas-fir (IDF) biogeoclimatic zone, and Paska Lake is in the Montane Spruce (MS) zone, however all sites are located in interior Douglas-fir forests.

They are found in three separate subzones; IDFdk (Dry Cool IDF), IDFxm (Very Dry Mild IDF) and MSxk (Very Dry Cool MS), respectively.

Experimental design and treatments

In the fall of 2011, cones were collected from 10 to 16 trees in each of the study sites and seeds extracted. Five of these trees were selected at each of the three sites as experimental 'parent trees' based on filled seed production, health and absence of neighbours. In May of 2012, all experimental units were installed at all three sites.

A 2 x 2 factorial design was used where mesh size (two levels) and relationship (two levels) were applied in a randomized complete block design, where the three sites served as blocks. The mesh size factor (two levels) references the pore size of the specialized mesh bags (approximately 20cm x 8 cm) made by Plastok® (Meshes and Filtration) Ltd. (Birkenhead, UK) that separated the root system of the seedling from those of the surrounding below ground community. These bags would provide some protection from below-ground granivores and herbivores as they would not be able to penetrate the mesh from below ground. The relationship factor (two levels) references the genetic closeness of the seedling to the parent tree it was sown near. A kin relationship refers to a seedling grown from seed originating from the same tree in which it was sown near, whereas a non-kin relationship refers to one grown from seed originating from a different mature tree at a different site. A total of 24 nylon mesh bags (Plastok® (Meshes and Filtration) Ltd.), 12 at a pore size of 0.5 μm and 12 at 35 μm, were spaced evenly around each of the 15 trees at a radius of three meters centered on the tree bole. A radius of three meters was selected to accommodate placement of all mesh bags and their seedlings just outside the drip line of the crown. Previous research suggests this distance

represents a zone of optimal trade-off between minimal competition and maximal mycorrhizal colonization (Teste and Simard 2009, Bingham and Simard 2011).

Experimental setup

The mesh bags were placed in holes that were excavated to approximately the size of the bags (approximately 20 cm deep by 8 cm diameter). The soil removed was isolated, mixed and used to fill the mesh bag. The mesh bag was placed into the hole and any remaining soil was used to secure to bag, fill the hole and obscure the view of the bag from wildlife. There was a total of 360 mesh bags installed (24 per tree, five trees per site and three sites). The relationship pairing and mesh size combinations resulted in 180 kin and 180 non-kin relationships evenly split between the network (35 μm pore size mesh) and no network (0.5 μm pore size mesh) treatments.

Five seeds were sown per mesh bag around each of the 15 trees over a three-day period in mid May 2012. For each of the mature trees, 12 of the bags were sown with stratified seeds that were collected from the proximal parent tree (i.e., kin seeds). The remaining 12 bags were sown with seeds collected from the cones of three different mature trees from each of the other two sites (i.e., non-kin non-site seeds). The three trees from each site that provide the seed for the non-kin seedlings were chosen purely by number of seeds obtained in the initial seed collection. The top three seed producers out of the five trees per site were used to provide the non-kin seed. The treatments were split evenly among the following four treatment combinations: (1) kin seeds in 35 μm mesh pore size (kin/network), (2) non-kin seeds in 35 μm mesh (non-kin/network), (3) kin seeds in 0.5 μm mesh (kin/no network) and (4) non-kin seeds in 0.5 μm mesh (non-kin/no

network). We also secured flexible, plastic mosquito netting around each bag in a dome-like shape to reduce seed predation.

Observations and data collection

Over four months in the summer of 2012 (May –August), the sites were checked every three to four weeks for germination, survival, soil moisture content and any disturbance to the treatments. When the first germinant emerged from a bag, the mosquito netting was removed to ensure it would not hinder growth. Soil moisture was measured at three consistent locations around each mesh bag with a HH2 moisture meter with an ML2x Theta probe (Delta-T Devices Ltd.) and averaged for the site (Table 4.2). The most common disturbances were cattle trampling and bag tearing or removal by wildlife (mostly bears). In those cases, if the bag was not damaged, it was returned to its position and, if no seeds were visible, the appropriate seeds for the treatment assigned to that bag were sown again. If the bag was damaged, the appropriate mesh sized bag and seeds were replaced in its original position. At the Paska Lake site, there was some evidence of browsing, but most seedlings that germinated but did not survive appeared to have been under severe drought stress. Mid-way through June 2012, one data logger was installed in each site at representative locations to record ambient air temperature at the surface of the soil and one to record soil temperature at a depth of 20 cm (approximately the maximum bag depth). Ambient air temperature was measured by a HOBO Pendant® Temperature/Alarm Data Logger (UA-001-64) and soil temperature was measured by a HOBO U23 Pro v2 External Temperature Data Logger (U23-004) (Table 4.2). Degree days below 4°C as well and soil temperature:moisture index were derived from the climate data above. Degree days below 4°C are the number of days in which the ambient air temperature dipped below 4°C during the

measurement period (mid-June through August 2012). A more well-known climate variable is degree days below 5°C, however we used 4°C because it better represented climatic differences between sites. Soil temperature:moisture index (T:M) was calculated for each site by dividing the average soil temperature by the average soil moisture (Table 4.3).

Data analysis

Total numbers of germinants and survivors as well as the number of experimental units that contained a germinant and survivor were tallied. A germinant was defined as an emerging seedling with a defined stem. The existence of a germinant(s) for that experiment unit was noted at first observation. If that germinant persisted until the last observation it was considered a survivor for the experimental period. This data was analyzed to detect effects of relationship, network, and the combination of relationship and network using simple statistics. The data was analyzed for site and seed origin effects. Germination and survival rates were calculated by percent. Logistic regression (PROC LOGISTIC) was used to test seed germination probability and probability of presence of a survivor, in response to relationship, mesh treatment (network), the combination of relationship and mesh, as well as site. Odds ratios were calculated using the entire data set. Significance was determined at $p < 0.05$ and trends were noted at $p < 0.10$. All data analysis was conducted using SAS version 9.3 (Cary, North Carolina).

Results

Germination and survival

Of the 1800 stratified seeds sown, 157 germinated (8.72% germination rate) in 96 experimental units (26.67% of experimental units with a germinant). Of the 157 germinants, 40

seedlings survived, yielding a survival rate of 25.5%. Of the 360 experimental units, 26 had at least one surviving seedling, yielding 26.8% of germinating units with a survivor, but only 7.2% of all experimental units with a survivor. 9.8% of seeds with access to a network (35 μm mesh) germinated, and of those, 19.3% survived. 7.7% of seeds without network access (0.5 μm mesh) germinated, and of those, 33.3% survived. 8.9% of seeds with a kin relationship to the parent tree germinated, and of those, 17.5% survived. 8.6% of seeds with a non-kin relationship to the parent tree germinated, and of those, 33.7% survived (Table 4.4).

In the logistic regression, site was a significant factor in both the germination of seeds and the presence of surviving seedlings ($p = 0.0004$ and 0.0001, respectively)(Table 4.5, 4.6). Network tended to have an effect on germination ($p = 0.0699$) (Table 4.5) and relationship tended to have an effect survival ($p = 0.0960$) (Table 4.6). The odds ratios showed that seeds were 2.58 times more likely to germinate in the Paska Lake site than either of the other two sites. The seeds sown at Farwell Canyon were more likely to germinate than those sown at the Alex Fraser Research Forest (Table 4.5, Figure 4.1a). Paska Lake was 7.80 times more likely to have survivors present in the experimental units than either of the other two sites. The Alex Fraser Research Forest was more likely to have surviving seedlings than Farwell Canyon (Table 4.6, Figure 4.1b). Seeds that were grown in a 35 μm mesh bag (network) were 1.54 times more likely to germinate than those in a 0.5 μm mesh bag (no network)(Table 4.5, Figure 4.1a). Seedlings that had a non-kin relationship to the parent tree were 1.98 times more likely to be a survivor than non-kin (Table 4.6, Figure 4.1b). There were no significant interaction (relationship x network) effects.

A separate logistic regression was conducted to determine if seed origin affected germination or survival. There was a significant effect of seed origin on presence of germinants

($p < 0.0001$). The odds ratio indicated that, at present, a germinant was 3.73 time more likely to be from seed originating from Farwell Canyon. None of the other factors tested were significant for germination or survival.

Site and seed origin

The Paska Lake site had the highest germination rate (13.2%) followed by Farwell Canyon (8.8%) and Alex Fraser (3.8%). Paska Lake also had the highest survival rate (40.5%) followed by Alex Fraser (21.7%) and Farwell Canyon (5.6%). The seeds that originated from Farwell Canyon had the highest germination rate (16.7%) when considering all sites, followed by those from Paska Lake (4.8%) and Alex Fraser (4.6%). The seeds originating from Paska Lake had the highest survival rate (41.4%) followed by those from Alex Fraser (32.1%) and Farwell Canyon (19.0%) (Table 4.7). The seeds originating from Farwell Canyon germinated best in the Farwell Canyon and Paska Lake sites, with much less success in the Alex Fraser site. The seeds originating from the Alex Fraser Research Forest site had the most germinants in the Alex Fraser site and Paska Lake sites, with less than half in the Farwell Canyon site. The seeds originating from Paska Lake germinated much better in the Paska Lake site, but with few germinants in either of the other two sites (Table 4.8). Seedlings originating from any site survived best in the Paska Lake site; however, Farwell Canyon seeds had the most survivors in the Paska Lake site followed by Paska Lake seeds and lastly Alex Fraser seeds (Table 4.9).

Climate, soil moisture and relationship

Soil temperature:moisture (T:M) index had an interesting effect on the number of kin germinants versus non-kin germinants (Figure 4.2a). Kin seeds increased in number of

germinants as soil T:M increased, whereas non-kin seeds peaked at the intermediate value (Paska Lake site) and was lower at both the extremes. For both kin and non-kin seedlings, survival peaked at the intermediate soil T:M and had under five survivors at either of the extremes (Figure 4.2b). More non-kin survivors were present at the intermediate soil T:M value at Paska Lake due to the greater number of germinants, however, the survival rate of kin seedlings was actually greater (47.8%) than that of the non-kin seedlings (37.5%) at that site.

For soil moisture content measured over the experimental period, a similar pattern emerged; as soil moisture decreased, kin seed germination increased (Figure 4.3a). At the intermediate soil moisture site, Paska Lake with 0.172 m^3 per m^3, there was greater germination of non-kin seeds, but at the driest site (Farwell Canyon, 0.153 m^3 per m^3) the opposite occurred, with greater germination of kin than non-kin seeds. At the wettest site (Alex Fraser), there was no effect of relationship on number of germinants. The greatest difference in survival between kin and non-kin seedlings (21 non-kin seedlings compared to 11 kin seedlings) occurred at the site with intermediate soil moisture, Paska Lake. The number of survivors at the extremes did not vary by relationship (Figure 4.3b).

Climate, soil moisture and networks

Soil T:M index differentially affected seed germination depending on whether or not they had mycorrhizal network access to the parent tree (Figure 4.2c). Due to the low germination rates and very low survivorship, no significant relationships were found; only trends were noted. At the two wetter sites (Alex Fraser and Paska Lake, respectively), seeds with access to a network germinated at a higher rate than isolated seeds. At the most drought stressed site (highest soil T:M), Farwell Canyon, there was no effect of network on number of germinants. As with

survival, both network and non-network seedling survival peaked at the intermediate soil T:M (Paska Lake) (Figure 4.2d). Isolated seedlings survived in greater numbers than networked seedlings.

Soil moisture content measured over the experimental period had the same effect on survival as soil T:M index. Germination at the driest site, Farwell Canyon, with an average soil moisture content of 0.153 m^3 per m^3, was unaffected by the presence of networks (Figure 4.3c). As soil moisture increased (Paska Lake (0.172 m^3per m^3) then Alex Fraser (0.195 m^3 per m^3)), networks provided germinants an advantage over those that were not networked. The greatest advantage networks provided for germination occurred at the intermediate site, Paska Lake. The greatest relationship effect also occurred at the intermediate site, where 19 non-kin seedlings survived compared to 13 kin seedlings. The number or survivors at the extremes did not vary by relationship (Figure 4.3d).

Discussion

Germination and survival rates

In comparison to the greenhouse experiments discussed in the previous two chapters, germination and survival rates were very low. However, the germination and survival rates we observed are typical for natural regeneration in dry interior Douglas-fir forests (Huggard et al. 2005). In studies examining regeneration from seed in the IDF zone, Teste et al. (2009) found seedling survival rates under 40% and Bingham and Simard (2012) found approximately 50% survival of nursery-grown container stock. Success of natural regeneration is highly variable and considerably lower than artificial regeneration (Simard 2009). Moreover, regeneration success is dramatically reduced with climatic aridity and extreme climate events. As a result, any

advantaged gained by kin recognition or mycorrhizal networks could make a difference in overall seedling establishment in the field. This could allow for regeneration improvements, particularly in regions of high drought stress.

Kin effects

Kin recognition, but not kin selection, was weakly evident as increased probability of survival in kin compared to non-kin experimental units (Table 4.6). The odds ratio showed that non-kin had slightly greater predicted survival than kin (Figure 4.1b); however, this does not necessarily provide evidence of either niche partitioning or non-kin facilitation (*sensu* plant defense hypothesis). By consistently using only the seed from the original 15 parent trees, either as kin or non-kin depending on where it the seed was sown, we were able to compare the survival and germination across sites. Site and seed origin had a greater influence on survival than did relationship. Most survival, regardless of relationship, occurred at the Paska Lake site, and because more non-kin than kin surviving seedlings were present at that site, the results were skewed. Survival rate of kin compared to non-kin, as opposed to the total number of survivors, in our data also contradicts this result. If we take into account higher non-kin germination at the Paska Lake site, kin survival rate (not total number) is actually greater than non-kin (Table 4.8, 4.9). We acknowledge that using the top seed producers may have impacted unmeasured factors, such as seed size, particularly if those trees were producing stress crops, so these results must be interpreted with caution. However, due to the constraints of the experiment, it was necessary to use the top seed producers to acquire enough seed for even the small sample size that we had.

Mycorrhizal network effects

Network effects were weakly evident as increased probability of a germinant being observed in networked versus non-networked experimental units (Table 4.5). The odds ratio also showed that seeds with access to a mycorrhizal network had greater predicted germination (Figure 4.1a). How mycorrhizal networks may affect germination is unclear because colonization is known to occur in these forests only after several months (Barker et al. 2013). Further research is needed to determine whether mycorrhizal networks are involved in biochemical signaling to germinating seeds. We expected to see a difference in survival given that the seedlings could access a mycorrhizal network and therefore nutrients that may otherwise be unavailable with only uncolonized roots. Greater survival of seedlings accessing mycorrhizal networks has been observed in other studies in IDF forests (Teste et al. 2009, Bingham and Simard 2011). Our hypothesis that kin recognition is facilitated by mycorrhizal networks, however, was not supported. There were no significant kin x network interaction effects in the logistic regression. In addition, no network effects were observed when relationship effects were observed (Table 4.6) and no relationship effects were observed when network effects were observed (Table 4.5).

Site and seed origin effects

Seed origin and site had the greatest effect on both germination and survival. The southern-most site, Paska Lake, had the greatest predicted presence of germinants and survivors. Interestingly, the seeds that originated from Farwell Canyon, regardless of sowing site, had significantly higher germination rate than any other seed origin region. Seedlings originating from Farwell Canyon also had the highest number of survivors, but this did not affect survival

rate because of the high germination and high mortality rates (kin and no-kin). As noted in chapter 3, seeds originating from Farwell Canyon and the seedlings they produce may be better adapted to deal with harsh climatic conditions (extreme temperatures and drought stress, Tables 4.1-3) than seeds and seedlings from the other sites. Essentially, Farwell Canyon seeds can germinate in the other sites, but the other seeds cannot germinate at Farwell Canyon. This supports our hypothesis that with greater environmental stress, kin recognition and kin selection becomes more important. It also supports, possibly to a greater extent, that benefit (or detriment) of relation to a parent tree is dwarfed by more significant processes such as regional genetic adaptations.

Climate effects

Climate had interesting effects on both germination and survival. As expected, survival was low in the hottest, driest location. However, it was also low at the coolest, wettest location. This pattern was consistent in kin, non-kin, networked and isolated seedlings. The intermediate site, Paska Lake, was also at the highest elevation. It was the southernmost location and further away from the range limit for interior Douglas-fir; therefore, there may be other site characteristics, such as soil type or availability of soil nutrients, not considered here, that promote greater germination and survival rate. Relation and networking appeared to have a greater influence on germination than survival across the range of climate represented by our sites. As drought and heat increased (higher soil T:M index), germination of kin seeds increased. Again, this may have more to do with regional genetic adaption to the site than actual relation to the parent tree, and an additional study including same site non-kin (different parent tree at the same site) is needed to test this hypothesis. Seeds with access to a network tended to germinate at

a higher rate in the two wetter sites (lower soil T:M index), but these did not differ significantly than the hottest, driest site (Farwell Canyon). This is contrary to the stress gradient hypothesis; however, this may be irrelevant to germination because mycorrhizal fungi require four month-old seedling roots for colonization (Barker et al. 2013).

Conclusion

Relationship and network effects were weakly evident in survivorship and germination, respectively. These effects were slight in comparison to the effect of site on these variables. Site and seed origin were the major determinants of germination and survival, and whether or not the germinant or survivor was kin in relation to the parent tree. Climate interacted with relationship and network factors to affect isolated germinants and survivors in interesting patterns that did not follow a strict stress gradient.

The site and seed origin results have interesting management implications. Seedlings grown in nurseries for outplanting originate from widely diverse seed sources within a seed zone, resulting in considerable variability. Our results suggest this is a good strategy for productive sites, as seedlings from both favourable and harsh growing conditions are able to persist in a productive site. However, sites with harsh growing conditions may benefit from partial harvesting and natural regeneration to ensure a local seed source. Leaving mature trees to act as seed sources, for kin seedlings as well as seedlings with the appropriate genetic adaptations to the region, may facilitate natural regeneration, particularly under harsh conditions. Leave trees would provide shelter from extreme microclimatic conditions and act as reservoirs for mycorrhizal fungi. Due to the low overall germination and survival rates in the IDF zone,

providing seedlings any advantage toward establishment could make a large difference in regeneration success.

Table 4.1. Geographic location and estimates of climatic variables for each of the study sites (Alex Fraser Research Forest, Farwell Canyon and Paska Lake) obtained from ClimateBC (Wang et al. 2012).

Site	Coordinates	Elevation (m)	Mean Annual Temperature (°C)	Mean Annual Precipitaion (mm)	Site Temperature range (°C)
Alex Fraser Research Forest	121.88°W, 52.05°N	862	4.8	470	-10.0 to 22.6
Farwell Canyon	122.63°W, 51.79°N	1184	3.5	402	-12.3 to 21.1
Paska Lake	120.67°W, 50.50°N	1435	3.5	411	-9.4 to 19.3

Table 4.2. Climate data for each study site measured during the experimental period (May-August 2012) including maximum, minimum and average ambient air temperature, soil temperature and soil moisture. Ambient air temperature was measures at ground level, soil temperature was measured at a depth of approximately 20 cm and soil moisture was measured with 10 cm of the soil surface.

Site	Ambient air temperature (°C)			Soil Temperature (°C)			Soil moisture ($m^3.m^3$)		
	Max	Min	**Ave**	Max	Min	**Ave**	Max	Min	**Ave**
Alex Fraser Research Forest	46.5	0.23	**17.9**	21.8	11.8	**15.9**	0.599	0.001	**0.195**
Farwell Canyon	59.1	-1.00	**22.3**	25.7	9.80	**16.2**	0.474	0.001	**0.153**
Paska Lake	44.5	0.01	**16.3**	25.6	8.10	**16.2**	0.489	0.001	**0.172**

Table 4.3. Climate variables for each study site derived from data measured during the experimental period (May-August 2012) including soil temperature:moisture index, degree days 4°C as well as the date range in which those degree days occurred. Soil temperature:moisture index was calculated as soil temperature (°C) divided by soil moisture (m^3per m^3).

Site	Degree days below 4°C	Date range of degree days below 4°C	Soil temperature:moisture index
Alex Fraser Research Forest	10	06/26-08/23	81.5
Farwell Canyon	10	06/30-08/23	105.9
Paska Lake	8	06/18-07/04	94.2

Table 4.4. Germination and survival by total number (#) and percentage (%) for the main effects of network and relationship (kin and non-kin) measured in # or % of treatment units (360 total) and # or % of total seeds sown (1800 total) or germinated (for survival).

	Germination		Survival	
	#	%	#	%
Network units	55	30.6%	13	23.6%
No network units	41	22.8%	13	31.7%
Kin units	45	25.0%	9	20.0%
Non-kin units	51	28.3%	17	33.3%
Total units	**96**	**26.7%**	**26**	**27.1%**
Network seedlings	88	9.7%	17	19.3%
No network seedlings	69	7.7%	23	33.3%
Kin seedlings	80	8.9%	14	17.5%
Non-kin seedlings	77	8.6%	26	33.7%
Total seedlings	**157**	**8.7%**	**40**	**25.5%**

Table 4.5. Logistic regression testing for the seed germination probability in response to relationship, mesh treatment (network), the combination of relationship and mesh as well as site. Odds ratios are given for each category. P values are given for the effect as a whole to test the null hypothesis that the odds ratio is equal to one. * $p < 0.05$.

Logistic regression: c = 0.642		Likelihood ratio P = 0.0007		
Effect	Odds ratios	df	Wald χ^2	P > χ^2
Kin	0.87	1	0.3774	0.5390
Non-kin	1.15			
0.5 µm mesh	0.65	1	3.3457	0.0674
35 µm mesh	1.54			
Paska Lake site	2.58	2	15.4899	0.0004*
Farwell Canyon site	0.73			
Alex Fraser Research Forest site	0.48			

Table 4.6. Logistic regression testing for the probability that a surviving seedling will be present in response to relationship, mesh treatment (network), the combination of relationship and mesh as well as site. Odds ratios are given for each category. P values are given for the effect as a whole to test the null hypothesis that the odds ratio is equal to one. * $p < 0.05$.

Logistic regression: c = 0.776		Likelihood ratio P < 0.0001		
Effect	Odds ratios	df	Wald χ^2	P > χ^2
Kin	0.50	1	2.7709	0.0960
Non-kin	1.98			
0.5 µm mesh	1.00	1	0.0138	0.9064
35 µm mesh	1.00			
Kin x 0.5 µm mesh	0.70	1	0.1935	0.6600
Kin x 35 µm mesh	0.52			
Non-kin x 0.5 µm mesh	1.37			
Non-kin x 35 µm mesh	1.65			
Paska Lake site	7.80	2	18.0632	0.0001*
Farwell Canyon site	0.15			
Alex Fraser Research Forest site	0.34			

Table 4.7. Germination and Survival by total number (#) and percentage (%) for each site and by seed origin location measured in # or % of treatment units (360 total) and # or % of total seeds sown (1800 total) or germinated (for survival).

	Germination		Survival	
	#	%	#	%
Farwell Canyon site	53	8.8%	3	5.7%
Alex Fraser Research Forest site	23	3.8%	5	21.7%
Paska Lake site	79	13.2%	32	40.5%
Farwell Canyon origin	100	16.7%	19	19.0%
Alex Fraser Research Forest origin	28	4.7%	9	32.1%
Paska Lake origin	29	4.8%	12	41.4%

Table 4.8. Total number of seeds that germinated in each site (columns) and which site the germinated seed originated from (rows). Kin germinants (the seed originated and germinanted in the same site, around the same parent tree it came from) are shaded and located along the diagonal. Non-kin germinants (seed origin and site of germination differed) are located off-diagonal. There were equal numbers of kin and non-kin sown in each site, however, the non-kin seed origin was split between the two other sites.

	Farwell Canyon site	Alex Fraser Research Forest site	Paska Lake site	**Total**
Farwell Canyon origin	46	10	44	**100**
Alex Fraser Research Forest origin	5	11	12	**28**
Paska Lake origin	2	4	23	**29**
Total	**53**	**25**	**79**	**157**

Table 4.9. Total number of seedlings that survived in each site (columns) and site of seed origin of the surviving seedling (rows). Kin survivors (the seed originated and the seedling survived in the same site, around the same parent tree it came from) are shaded and located along the diagonal. Non-kin survivors (seed origin and site of survival differed) are located off-diagonal.

	Farwell Canyon site	Alex Fraser Research Forest site	Paska Lake site	**Total**
Farwell Canyon origin	2	3	14	**19**
Alex Fraser Research Forest origin	1	1	7	**9**
Paska Lake origin	0	1	11	**12**
Total	**3**	**5**	**32**	**40**

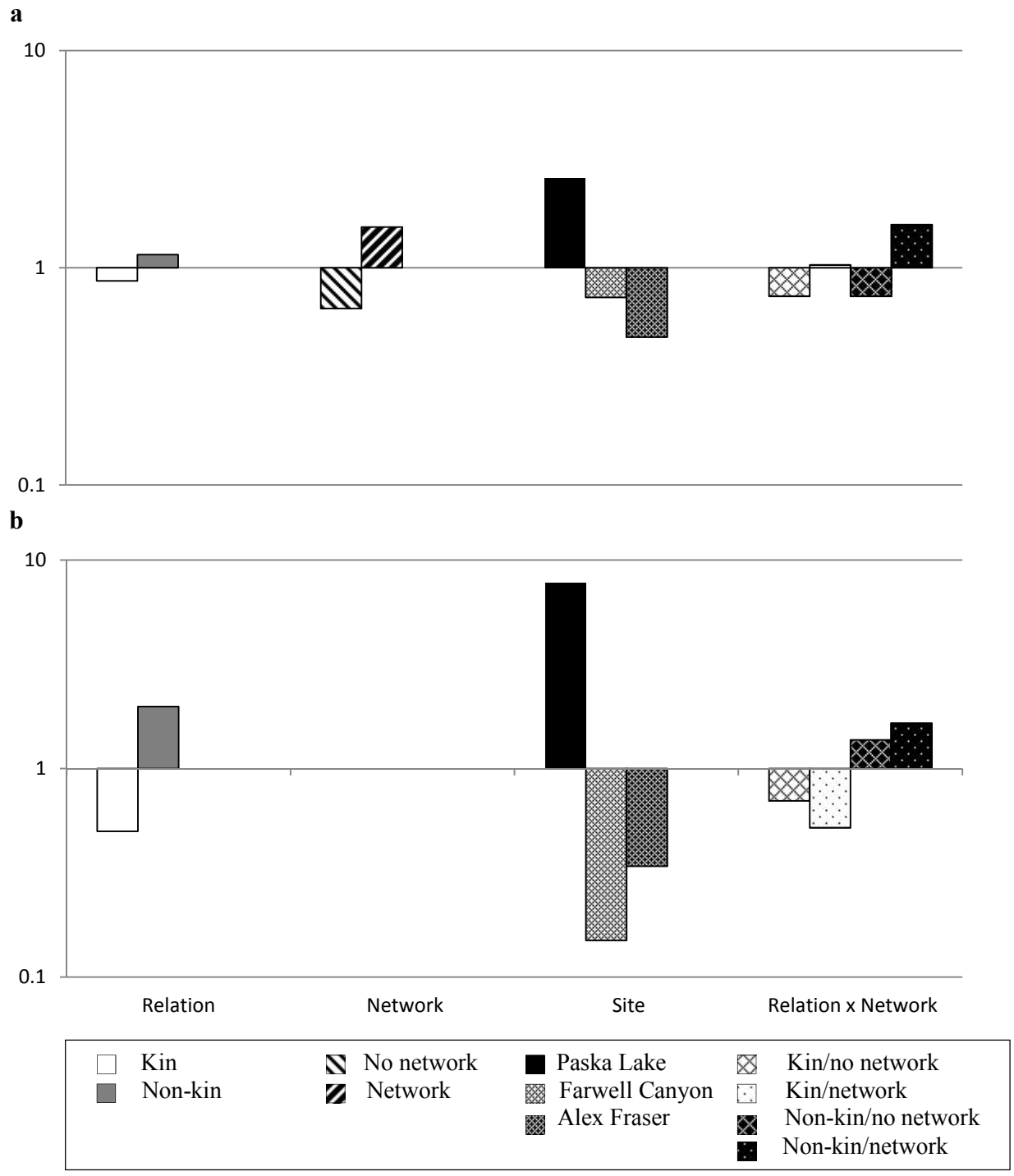


Figure 4.1. Odds ratio values, presented on a logarithmic scale, for relationship, network, site and relationship x network effects in the logistic regression model predicting (a) germination (b) survival.

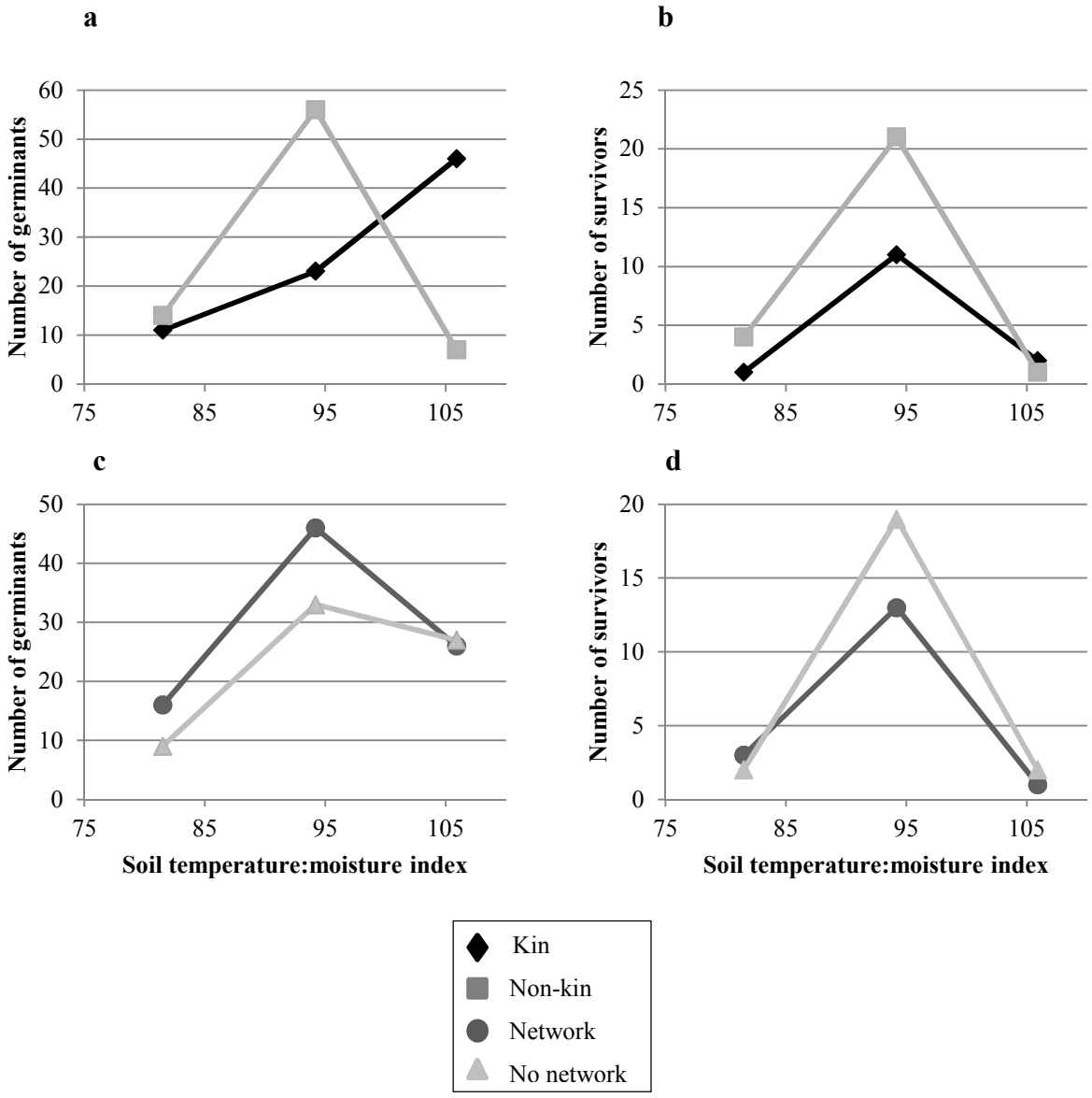


Figure 4.2. The number of (a) kin and non-kin germinants, (b) kin and non-kin survivors, (c) network and no network germinants and (d) network and no network survivors across the range of soil temperature:moisture index values found in the study sites.

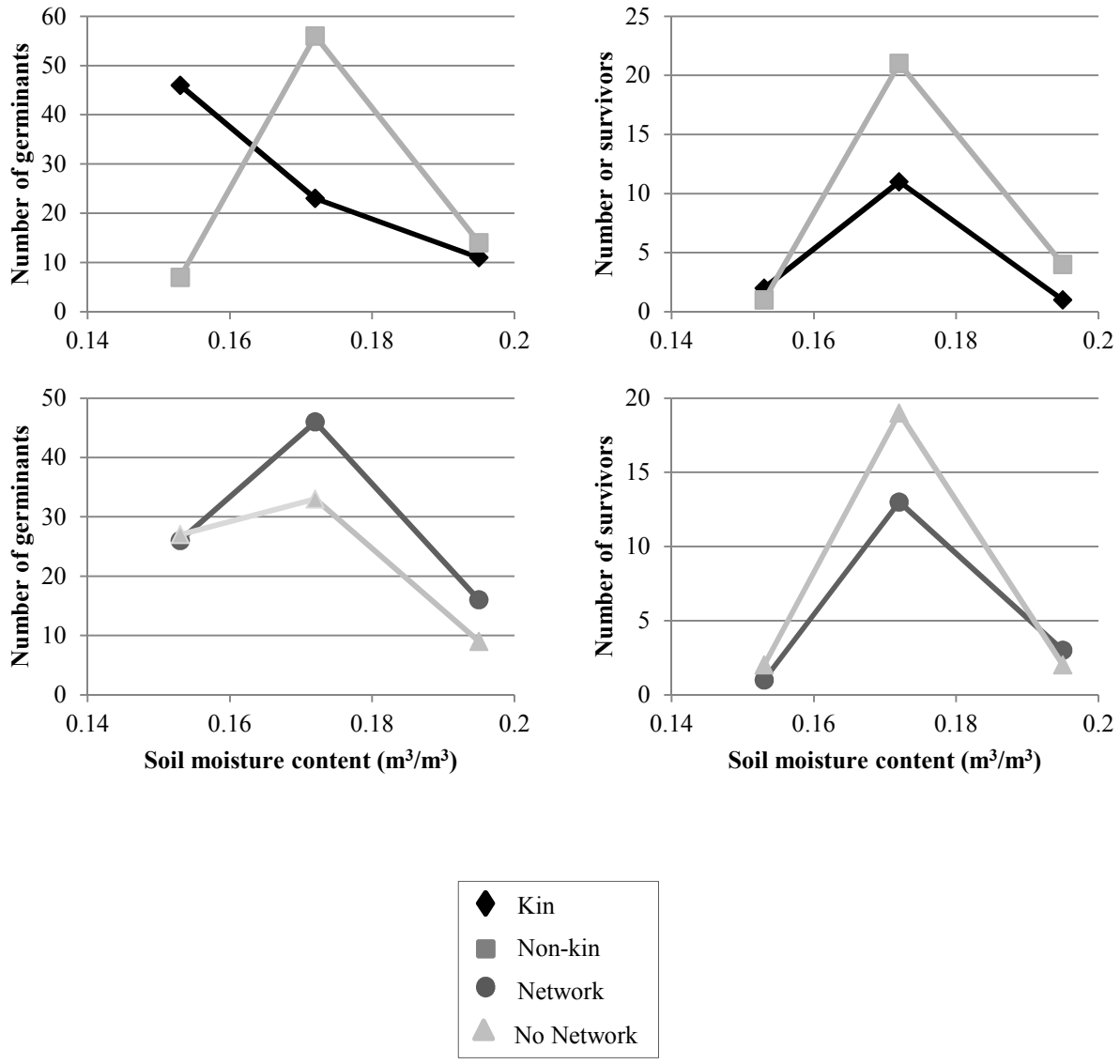


Figure 4.3. The number of (a) kin and non-kin germinants, (b) kin and non-kin survivors, (c) network and no netwrok germinants and (d) network and no network survivors across the range of averaged soil moisture content found in the study sites.

5 Summary and conclusions

The regenerative capacity of interior Douglas-fir in harsh climates has been a concern of forest ecology researchers and managers alike. Insight into influences on successful seedling establishment could be essential to future management decisions as climate changes. Interplant communication has recently generated considerable interest and research results, including evidence of kin recognition and, in some cases, kin selection (File et al 2011). Whether kin recognition occurs and has influence on seedling success in interior Douglas-fir is a new and exciting area of research. While the mechanism of kin recognition is still not well understood, we have provided evidence of kin recognition in interior Douglas-fir seedlings, particularly those that originate from regions of harsh climate, and have observed indirect indicators of kin selection or reduction of competition due to a close genetic relationship.

Review of objectives

There were three main objectives that were addressed in each research chapter. The first was to determine whether kin recognition is detectable in interior Douglas-fir seedlings. The second was to determine whether kin recognition, if present, would present in a way supporting kin selection theory. The third was to determine whether mycorrhizal networks mediated kin recognition between seedling pairs (chapters 2 and 3) or between seedlings and parent trees (chapter 4).

The Chapter 2 minor objective was to determine if kin recognition ability varied among distinct genotypes or "families" of seedlings (chapter 2).

The Chapter 3 minor objectives were to determine if the region of seed origin affects kin recognition among seedlings grown in a common greenhouse environment and to determine if kin recognition occurs along a gradient of relatedness.

The Chapter 4 minor objective was to determine if the region of seed origin affects kin recognition among seedlings grown in the field with a variety of growing conditions (different sites) (chapter 4).

The minor objective comparing Chapter 2 and 3 was to determine whether full sibling kin pairs from control cross pollination exhibited differing kin recognition effects than did kin pairs collected from parent trees in the field with natural pollination.

The minor objective comparing Chapters 2 and 3 to Chapter 4 was to determine whether effects seen in the controlled environment of the greenhouse would be detectable under natural climatic conditions in the field.

Summary of main findings

Major objectives

Objective 1 – Kin recognition

Chapter 2 provided evidence of kin recognition by significant differences found in some foliar micronutrients (Fe, Mo, and Al) according to the relationship, either kin or non-kin, between the seedling pairs. Evidence of kin recognition was found in foliar micronutrients, seedling growth and mycorrhizal colonization in Chapter 3. Kin recognition was weakly evident in Chapter 4 by difference in probability that a survivor would be present in a kin experimental unit compared to a non-kin one.

Objective 2 – Kin selection

Overall the evidence of kin selection was very weak but we were able to detect some significant effects that could be interpreted as kin selection. The significant differences in micronutrients between kin and non-kin seedlings observed in Chapter 2 and 3 suggest kin selection occurred, as all nutrients that differed according to relationship were greater in kin compared to non-kin seedlings. Greater mycorrhizal colonization of kin compared to non-kin seedlings as well as greater donor total leaf area, volume and stem length was observed in Chapter 3. No evidence of kin selection was evident in the growth of recipient seedlings. In Chapter 4, greater predicted presence of a survivor occurred in non-kin experimental units; however, these results must be considered cautiously because of the significantly greater influence of site and seed origin on germination and survival in the field.

Objective 3 – Mediation by mycorrhizal networks

Both donor and recipient seedlings were colonized predominantly by a single mycorrhizal fungus, *Rhizopogon vincolor*, providing sufficient evidence that mycorrhizal networks formed between seedling pairs (Chapter 2 and 3). More definitive evidence for the presence of functional networks was greater transfer of labelled ^{13}C between networked seedlings than isolated pairs (Chapter 2), although this did not result in enhanced performance by networked seedlings. There were significant differences among the four treatment combinations involving relationship and networks. When non-kin seedlings were grown in isolating mesh bags, donor seedlings exhibited enhanced competition toward recipient seedlings. This did not occur with kin seedlings grown with networks, providing evidence that kin recognition could be facilitated by mycorrhizal networks (Chapter 2). Further evidence of facilitation was found in

Chapter 3, where foliar copper and iron concentrations were significantly higher in kin seedlings grown with networks than without. In the field collected sibling seedlings (Chapter 3), the enhanced mycorrhizal colonization of recipient seedlings could only occur through linkage into mycorrhizal networks of donor seedlings. It is unclear if the greater mycorrhizal colonization is evidence for mediation of kin recognition through mycorrhizal networks or is a response due to kin recognition. In Chapter 4, network effects were weakly evident as greater probability that a germinant would be present in a networked experimental unit than an isolated one. In the field experiment, however, kin recognition did not appear to be facilitated by mycorrhizal networks as no interaction effects occurred in the logistic regression. In addition, no network effects were observed when relationship effects occurred and no relationship effects were observed when network effects occurred.

Minor objectives

Objective 1 – Effect of distinct genotypes (families) on kin recognition

Chapter 2 was designed to examine the effect of distinct genotypes, or "families" on kin recognition. The four distinct families did not differ in growth traits or foliar nutrient content, however, which may explain why we were unable to detect a genotype effect on kin recognition. This may have simply been a result of the lack of statistical power. It is also possible these seeds were too similar in competitive ability to influence the growth traits we examined.

Objective 2 – Effect of the region of seed origin on kin recognition

Chapter 3 was designed to examine the effect of seed origin on kin recognition when seedlings were grown in common, favourable conditions (greenhouse setting). Seed origin had a significant effect on germination rate among field collected, greenhouse grown recipient

seedlings. Seeds originating from Farwell Canyon had the greatest germination rate among regions. Seed origin also had an effect on kin recognition in terms of germination rate, as kin originating from Farwell Canyon had significantly higher germination rates than non-kin from Farwell Canyon or any other region tested. Mycorrhizal effects on germination are considered minimal but more research is needed before appropriate conclusions can be drawn from this result. Farwell Canyon had the driest and hottest climate and kin recognition and selection (i.e., cooperation among genetically similar individuals) may be more necessary in harsh climates in accordance with the stress gradient hypothesis.

Objective 3 – Effect of the level of relatedness on kin recognition

Chapter 3 was designed to examine the effect of the level of relatedness on kin recognition. Kin recognition was not affected by the gradient of relatedness we tested. Kin recognition was only detectable at the sibling/ non-sibling level; by contrast, whether the non-sibling originated from a different parent tree within the same site (within population) or a different site (among populations) did not affect any variables with relationship effects, but there was little statistical power to test these effects.

Objective 4 – Effect of growing conditions (site) and the region of seed origin on kin recognition

Chapter 4 was designed to examine the effect of site, or growing conditions due to variable climate, and seed origin, on kin recognition in a field setting. Site and seed origin both had a significant effect on germination. The greatest number of seeds germinated in the southernmost site, Paska Lake. The greatest number of germinated seeds originated from the site that had the harshest growing conditions (most extreme air temperatures, highest soil temperature:moisture index and lowest soil moisture content) during the experimental period,

Farwell Canyon. Site had a significant effect on presence of survivors, with the most survival at Paska Lake. Seed origin did not have a significant effect on presence of survivors. The large significant effects of site and seed origin we observed on the distribution of kin and non-kin germination and survival may have overshadowed potential relationship effects in the field.

Objective 5 – Kin recognition in greenhouse grown full sibling vs. field collected sibling pairs

Despite the fact that control cross pollinated seeds yield full sibling pairs, more evidence of kin recognition was detectable in the field collected seedling pairs. This suggests that the adaptations of seeds originating in different climatic regions are more important to kin recognition than the closer relationship of full siblings originating in similar climatic conditions (all Chapter 2 seeds obtained from the Kalamalka Research station).

Objective 6 – Broad comparison of kin recognition and mycorrhizal networks in a greenhouse vs. a field setting

More evidence of kin recognition was detectable in the greenhouse experiments compared to the field study. At this point, it is difficult to compare the effect of the setting as stated in the objective as low survival rates prevented us from harvesting the field seedlings and therefore comparing all of the same variables. We did see a trend toward kin recognition in the presence of germinants in the field; therefore, we suggest that kin recognition is not a phenomenon only detectable in highly controlled, greenhouse growing conditions. The strongest evidence that mycorrhizal networks are involved in kin recognition was through increased mycorrhizal colonization of kin versus non-kin in the greenhouse (Chapter 3). It is still unclear from this evidence whether mycorrhizal networks have a facilitative role in kin recognition or the enhanced mycorrhizal colonization was a response to kin recognition.

Contributions to the field of study

To our knowledge, this is the first study that examined kin recognition in any coniferous tree species. In a species such as interior Douglas-fir, where there is large variation in climatic conditions across its range and areas of very low regenerative capacity, small advantages to regenerating seedling could prove important to regeneration success. This study contributed evidence that kin recognition occurs among interior Douglas-fir seedlings, although it has very minor effects on the seedlings compared to regional, climatic and other factors. There have been many studies on the effect of mycorrhizal networks on interior Douglas-fir. Our goal was to add to the base of knowledge of the scope of effects mycorrhizal networks have on interior Douglas-fir seedlings. There is evidence that these networks transport water, nutrients and defense signals between conspecific individuals connected by mycorrhizal hyphae. We wanted to determine whether mycorrhizal networks and resource transfer also play a role in facilitating kin recognition. We found increased mycorrhizal colonization of kin compared with non-kin seedlings in the field collected, greenhouse grown sibling greenhouse experiment, which could only have occurred through mycorrhizal network formation, suggestive of kin recognition. Otherwise, there was little evidence that mycorrhizal networks facilitated kin selection in interior Douglas-fir.

The results of this study may have implications for management practices that encourage reproduction of kin seedlings near their parents, including the retention of healthy, cone-bearing legacy trees during harvest to supply seed as well as act as refuges (shelter, nutrients and mycorrhizal inoculum) for natural regeneration. However, more research into kin selection in the field is necessary before these practices could be implemented. These practices may be

particularly effective in areas where natural regeneration and productivity are low, either due to micronutrient deficiencies or drought.

Limitations of studies

The minor objectives included in both greenhouse experiments (Chapter 2 and 3) suffered from small sample size. This was a major limitation both in the greenhouse, but particularly in the field study as we were constrained by the number of seeds produced by the parent trees at the time of collection. More useful information could have also been gathered at the time of collection, such as seed size and weight, which may have proved useful in explaining the high variation in field results. Weather and wildlife also proved to be factors that led to small sample sizes in the field.

Time availability was also a major limitation to these studies. Had these studies been conducted over a longer term, I believe both minor and major objectives could have been tested with much more clarity. More growing time would have allowed for more growth and nutrient differentiation between treatments in the greenhouse. Moreover, greater sample size in the field study would have increased our power to test our hypotheses.

Future directions

More information can be gained from the continuation of the field study to a point where survival is sufficient for harvest and evaluation of all variables. Studies looking into equal sibling compared to non-sibling pairs (seeds sown at the same time) could help to parse out the effects of relationship, network and size discrepancies in a greenhouse study. In the field, studies

examining differential effects of kin recognition and regional adaptation to climate would help distinguish whether kin recognition truly provides an advantage to regeneration in harsh relative to favourable climatic conditions.

References

Agerer R. (2001) Exploration types of ectomycorrhizae: A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* 11 pp.107–114

Barker JS., Simard SW., Jones MD., Durall DM. (2013) Ectomycorrhizal fungal community assembly on regenerating Douglas-fir after wildfire and clearcut harvesting. *Oecologia*. 172:4, pp 1179-1189

Barto EK., Hilker M., Muller F., Mohney BK., Weidenhamer JD., Rillig MC. (2011) The Fungal Fast Lane: Common Mycorrhizal Networks Extend Bioactive Zones of Allelochemicals in Soils. *PLoS ONE* 6:11 pp.880-882 doi:10.1371/journal.pone.0027195

British Columbia Ministry of Forests (2012) Annual report. British Columbia Ministry of Forests, Lands and Natural Resource Operations, Victoria, BC**.**

Beiler KJ., Durall DM., Simard SW., Maxwell SA., Kretzer AM. (2010) Mapping the wood-wide web: mycorrhizal networks link multiple Douglas-fir cohorts *New Phytologist*, 185 pp. 543-553.

Beiler KJ., Simard SW., Lemay V., Durall DM. (2012) Vertical partitioning between sister species of Rhizopogon fungi on mesic and xeric sites in an interior Douglas-fir forest, *Molecular Ecology* doi: 10.1111/mec.12076

Bertness M., Callaway RM. (1994) Positive interactions in communities. *Trends in Ecology and Evolution* 9 pp. 191–193

Biedrzycki ML., Jilany TA., Dudley SA., Bais HP. (2010). Root exudates mediate kin recognition in plants. *Communicative & Integrative Biology* 3:1 pp.28-35

Bingham MA., Simard SW. (2011) Do mycorrhizal network benefits to survival and growth of interior Douglas-fir seedlings increase with soil moisture stress? *Ecology and Evolution* 1 pp. 306-316.

Bingham MA., Simard SW. (2012) Ectomycorrhizal networks of old Pseudotsuga menziesii var. glauca trees facilitate establishment of conspecific seedlings under drought. *Ecosystems*15 pp. 188-199 doi: 10.1007/s10021-011-9502-2

Booth MG., Hoeksema JD., (2010) Ectomycorrhizal networks counteract competitive effects of canopy trees on seedling survival. *Ecology* 91 pp. 2294-2302.

Boyden S., Binkley D., Stape JL. (2008) Competition among Eucalyptus trees depends on genetic variation and resource supply. *Ecology.* 89 pp. 2850–2859. doi:10.1890/07-1733.1

Brooks R., Meinzer FC., Warren JM., Domec J-C., Coulombe R. (2006) Hydraulic redistribution in a Douglas-fir forest: lessons from system manipulations. *Plant Cell Environ.* 29 pp. 138-150.

Bruin J., Sabelis MW. (2001) Meta-analysis of laboratory experiments on plant–plant information transfer *Biochemical Systematics and Ecology* 29:10 pp.1089–1102

Callaway RM., Brooker RW., Choler P., Kikvidze Z., Lortie CJ., Michalet R., Paolini L., Pugnaire FI., Newingham B., Aschehoug ET., Armas C., Kikodze D., Cook BJ. (2002) Positive interactions among alpine plants increase with stress. *Nature*. 417 pp.844–848.

Campbell RK. (1986) Mapped genetic variation of Douglas-fir to guide seed transfer in southwest Oregon. *Silvae Genet.* 35 pp.85-96

Campbell RK., Sorensen FC. (1978) Effects of test environment on expression of clines and on delimitation of seed zones in Douglas-fir. *Theor. Appl. Genet*. 51 pp.233-246

Cavieres LA., Badano EI., Sierra-Almeida A., Gomez-Gonzalez S., Molina-Montenegro MA. (2006) Positive interactions between alpine plant species and the nurse cushion plant Laretia acaulis do not increase with altitude in the Andes of central Chile. *New Phytologist.* 169 pp. 59–69.

Chen BJW., During HJ., Anten NPR. (2012) Detect thy neighbor: Identity recognition at the root level in plants. *Plant Science.* 195 pp.157– 167

Cheplick GP., Kane KH. (2004) Genetic relatedness and competition in Triplasis purpurea (Poaceae): resource partitioning or kin selection? *Int. J. Plant Sci.* 165 pp. 623–630. doi:10.1086/386556

Deslippe JR., Simard SW. (2011) Below-ground carbon transfer among Betula nana may increase with warming in Arctic tundra. *New Phytologist*, 192:3 pp.689-698.

Dickie IA., Schnitzer SA., Reich PB., Hobbie SE. (2005). Spatially disjunct effects of co-occurring competition and facilitation. *Ecology Letters* 8 pp.1191-1200.

Donohue K. (2003) The influence of neighbor relatedness on multilevel selection in the Great Lakes sea rocket. *American Naturalist* 162 pp. 77–92.

Dudley SA., File AL., (2007) Kin recognition in an annual plant. *Biology Letters*. 3, pp.435–438. (doi:10.1098/rsbl.2007.0232)

File AL., Klironomos J., Maherali H., Dudley SA. (2012) Plant kin recognition enhances abundance of symbiotic microbial partner. *PLoS ONE* 7:9 e45648 doi:10.1371/journal.pone. 0045648

File AL., Murphy GP., Dudley SA. (2011) Fitness consequences of plants growing with siblings: reconciling kin selection, niche partitioning and competitive ability. *Proc R Soc B* doi: 10.1098/rspb.2011.1995 pp.1-10

Fortin JA., Plenchette C., Piche Y. (2009) Mycorrhizas: the new green revolution. Éditions MultiMondes

Francis R., Read DJ. (1984) Direct transfer of carbon between plants connected by vesicular–arbuscular mycorrhizal mycelium. *Nature* 307 pp. 53 - 56 doi:10.1038/307053a0

Giovannetti M, Mosse B. (1998) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* 84 pp.489-500.

Greenlee JT., Callaway RM. (1996) Abiotic stress and the relative importance of interference and facilitation in montane bunchgrass communities in western Montana. *American Naturalist*. 148 pp. 386–396.

Guerinot ML., Yi Y. (1994) Iron: Nutritious, noxious, and not readily available. *Plant Physiology*, 104, pp. 815–820

Gugger PF., Sugita, S., Cavender-Bares, J. (2010) Phylogeography of Douglas-fir based on mitochondrial and chloroplast DNA sequences: testing hypotheses from the fossil record. *Molecular Ecology* 19, pp. 1877–1897 doi: 10.1111/j.1365-294X.2010.04622.x

Hamann A., Gylander T., Chen P. (2011) Developing seed zones and transfer guidelines with multivariate regression trees. *Tree Genetics & Genomes*. 7 pp. 399–408 doi:10.1007/s11295-010-0341-7

Hamilton WD. (1964) The genetical evolution of social behaviour. *I. J. Theor. Biol.* 7, 1-16

Hamrick JL., Godt MJW., Sherman-Broyles SL. (1992) Factors influencing levels of genetic diversity in woody plant species, *New Forests* 6 pp.95-124

Hermann RK., Lavender DP. (1990). *Pseudostuga menziesii* (Mirb.) Franco. In: Burns, R.M., Honkola, B.H. (Eds.), Silvics of North America, Volume 1, Conifers. USDA Forest Service, Agricultural Handbook 654, pp. 527–540.

Hirayama T., Kieber JJ., Hirayama N., Kogan M., Guzman P., Nourizadeh S., Alonso JM., Dailey WP., Dancis A., Ecker J R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson Disease–Related Copper Transporter, Is Required for Ethylene Signaling in< i> Arabidopsis</i>. *Cell*. 97:3 pp. 383-393.

Horton TR., Bruns TD. (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* 10 pp.1855-1871.

Huggard DJ., Arsenault A., Vyse A., Klenner W. (2005) The Opax Mountain Silvicultural Systems Project: Preliminary Results for Managing Complex, Dry Interior Douglas-fir Forests. B.C. Ministry of Forests and Range, Forest Sciences Program, Victoria, BC. Extension Note 72.

Keeton, WS. and Franklin JF. (2005) Do remnant old-growth trees accelerate rates of succession in mature Douglas-fir forests? *Ecological Monographs* 75:1 pp. 103-118 doi: 10.1890/03-0626

Kessler A., Halitschke R., Diezel C., Baldwin IT. (2006) Priming of plant defense responses in nature by airborne signaling between Artemisia tridentata and Nicotiana attenuata. *Oecologia* 148 pp. 280–292. doi: 10.1007/s00442-006-0365-8.

Kiers ET., Franken O., Hart MM., Duhamel M., Verbruggen E., Bago A., Vandenkoornhuyse P., Beesetty Y., Fellbaum CR., Palmer TM., Jansa J., Mensah JA., Kowalchuk GA., West SA., Bücking H. (2011) Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis. *Science* 333:880 doi: 10.1126/science.1208473

Klenner W., Walton R., Arsenault A., Kremsater L. (2008) Dry forests of the southern interior of British Columbia: historic disturbances and implications for restoration and management. *Forest Ecology and Management* 256, pp. 1711–1722.

Kremer A. (1994) Genetic diversity and phenotypic variability of forest trees. *Genetics Selection Evolution.* 26:1 pp. S105-S123 doi: 10.1051/gse:19940708

Kretzer AM., Luoma DL., Molina R., Spatafora JW. (2003) Taxonomy of the *Rhizopogon vinicolor* species complex based on analysis of its sequences and microsatellite loci. *Mycologia* 95 pp.480–487.

Krutovsky, KV., St. Clair, JB., Saich, R., Hipkins, VD., Neale, DB. (2009) Estimation of population structure in coastal Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco var. menziesii] using allozyme and microsatellite markers, *Tree Genetics & Genomes,* 5:4 pp.641-658, doi: 10.1007/s11295-009-0216-y

Lavender DP., Parish R., Johnson CM., Montgomery G., Vyse A., Willis RA., Winston D. (1990) Regenerating British Columbia's forests. University of British Columbia Press, Vancouver 1990

Li X., Marschner H., George E. (1991) Acquisition of phosphorus and copper by VA-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant and Soil*, 136:1, pp 49-57

Liancourt P., Callaway RM., Michalet R. (2005) Stress tolerance and competitive-response ability determine the outcome of biotic interactions. *Ecology*. 86 pp. 1611–1618.

Marschner P., (2012) (1st edition by Marschner H., 1986) Marschner's Mineral Nutrition of Higher Plants (Third Edition) Copyright © 2012 Elsevier Ltd. All rights reserved

Margulis, L. (1981) Symbiosis in cell evolution: Life and its environment on the early earth. p.419.

Massicotte HB., Molina R., Luoma DL., Smith JE. (1994) Biology of the ectomycorrhizal genus, *Rhizopogon*. II Patterns of host-fungus specificity following spore inoculation of diverse hosts grown in monoculture and dual culture. *New Phytologist* 126:4 pp. 677-690

McGuire KL., (2007) Common ectomycorrhizal networks may maintain mono-dominance in a tropical rain forest. *Ecology*. 88, pp. 567-574.

Mengel K., Kirkby EA. (2001) Principles of Plant Nutrition. © 2001 Kluwer Academic Publishers

Milla R., Forero DM., Escudero A., Iriondo JM. (2009) Growing with siblings: a common ground for cooperation or for fiercer competition among plants? *Proc. R. Soc. B.* 276 pp.2531-2540. doi:10.1098/rspb.2009.0369

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomenon in mycorrhizal symbiosis: community-ecological consequences and practical implications. In Allen MF (ed) Mycorrhizal Functioning: An Integrative Plant-Fungal Process. Chapman Hall, New York. pp 357-423

Molina R., Trappe JM., Grubisha LC., Spatafora JW. (1999) *Rhizopogon*. In: Cairney, J., Chambers, S.M. (Eds.), Ectomycorrhizal Fungi: Key Genera in Profile. Springer Verlag, Berlin.

Mulder EG., Boxma R., Van Veen WL. (1959) The effect of molybdenum and nitrogen deficiencies on nitrate reduction in plant tissues. *Plant and Soil* 10:4 pp.335-355

Nara K. (2006) Ectomycorrhizal networks and seedling establishment during early primary succession. *New Phytologist* 169 pp. 169-178.

Newsome TA., Sutherland DC., Vyse A. (1991) Establishing Douglas-fir plantations in the dry belt of interior British Columbia. In: Baumgartner, D.M., Lotan, J.E. (Eds.), Interior Douglas-fir: The Species and its Management. Washington State University, Cooperative Extension Service, Seattle, WA, pp. 227–233.

Onguene NA., Kuyper TW. (2002) Importance of the ectomycorrhizal network for seedling survival and ectomycorrhiza formation in rain forests of south Cameroon. *Mycorrhiza* 12 pp.13-17.

Perry DA., Margolis H., Choquette C., Molina R., Trappe LM. (1989) Ectomycorrhizal Mediation of Competition between Coniferous Tree Species. *New Phytologist*. 112:4 pp.501-511

Philip LJ. (2006) The role of ectomycorrhizal fungi in carbon transfer within common mycorrhizal networks. *PhD Dissertation*, The University of British Columbia 152 pp.

Platt TG., Bever JD. (2009) Kin competition and the evolution of cooperation. *Trends in Ecology and Evolution* 24:7 pp.370-377

Querejeta JI., Barea JM., Allen MF., Caravaca F., Rolda´n A. (2003) Differential response of d13C and water use efficiency to arbuscular mycorrhizal infection in two aridland woody plant species. *Oecologia* 135 pp.510–515

Rehfeldt GE. (1978) Genetic differentiation of Douglas-fir populations from the Northern Rocky Mountains. *Ecology* 59 pp.1264-1270.

Rehfeldt GE. (1989) Ecological Adaptations in Douglas-Fir (Pseudotsuga menziesii var. glauca): a Synthesis. *Forest Ecology and Management* 28 pp.203-215

Roche L. (1969) A genecological study of the genus Picea in British Columbia. *New Phytologist* 68 pp.505-554.

Schoonmaker AL., Teste FP., Simard SW., Guy RD., (2007) Tree proximity, soil pathways and common mycorrhizal networks: their influence on utilization of redistributed water by understory seedlings. *Oecologia* 154 pp. 455-466.

Selosse MA., Richard F., He X., Simard SW. (2006) Mycorrhizal network: des liaisons dangereuses? *Trends in Ecology and Evolution* 21:21 pp.621-628

Shulaev V., Silverman P., Raskin I. (1997) Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385 pp.718–721. doi: 10.1038/385718a0.

Simard, SW. (2009) The foundational role of mycorrhizas in the self-organization of the interior Douglas-fir forests. *Forest Ecology & Management* 258 pp.S95-S107

Simard SW., Beiler KJ., Bingham MA., Deslippe JR., Philip LJ., Teste FP. (2012) Mycorrhizal networks: Mechanisms, ecology and modelling. *Fungal Biology Reviews* 26 pp. 39-60

Simard SW, Jones MD, Durall DM. (2002) Carbon and nutrient fluxes within and between mycorrhizal plants. In van der Heijden M, Sanders I (eds) Mycorrhizal Ecology. Springer-Verlag, Heidelberg. Ecological Studies 157 pp.33-61

Simard SW., Jones MD., Durall DM., Hope GD., Stathers RJ., Sorensen NS., Zimonick BJ. (2003) Chemical and mechanical site preparation: effects on Pinus contorta growth, physiology, and microsite quality on steep forest sites in British Columbia. *Canadian Journal of Forest Research* 33 pp.1495-1515.

Simard SW., Martin K., Vyse A., Larson B. (2013) Meta-networks of fungi, fauna and flora as agents of complex adaptive systems Chapter 7, pages 133-164. In: Managing World Forests as

Complex Adaptive Systems: Building Resilience to the Challenge of Global Change. Edited by Puettmann, K, Messier, C, and Coates, KD. Routledge, NY. ISBN 978-0-415-51977. 369 pp.

Simard SW., Perry DA., Jones MD., Myrold DD., Durall DM., and Molina R. (1997) Net transfer of carbon between tree species with shared ectomycorrhizal fungi. *Nature*, 388 pp.579-582

Smith, S.E., Read, D.J. (1997) Mycorrhizal Symbiosis. Copyright © 1997 Elsevier Ltd. All rights reserved

Song, Y.Y., Zeng, R.S., Xu, J.F., Li, J., Yihdego, W.G. (2010) Interplant Communication of Tomato Plants through Underground Common Mycorrhizal Networks. *PloS ONE* 5:10 e13324. doi:10.1371/journal .pone.0013324

Soon YK. (1995) Forms of extractable aluminium in Canadian acid soils and their relations to plant growth. *Developments in Plant and Soil Sciences* 64 pp. 65-70

Sorensen FC. (1979) Provenance variation in *Pseudotsuga menziesii* seedlings from the var. *menziesii*-var. *glaucam* transition zone in Oregon. *Silvae Genetica* 28 pp.37–119.

Spittlehouse, D. (2006) ClimateBC: Your Access to Interpolated Climate Data for BC. *Streamline Watershed Management Bulletin* 9:2 pp.16-20**.**

St. Clair JB., Mandel NL., Vance-Borland KW (2005) Genecology of Douglas Fir in Western Oregon and Washington. *Annals of Botany.* 96:7 pp.1199-1214 doi:10.1093/aob/mci278

Teste FP., Karst J., Jones MD., Simard SW., Durall DM. (2006) Methods to control ectomycorrhizal colonization: effectiveness of chemical and physical barriers. *Mycorrhiza*. 17 pp.51-65

Teste FP, Simard SW., Durall DM., Guy RD., Jones MD. (2009) Access to mycorrhizal networks and roots of trees: importance for seedling survival and resource transfer. *Ecology* 90:10 pp.2808-2822

Teste FP, Simard SW., Durall DM., Guy RD., Berch SM. (2010) Net carbon transfer between *Pseudotsuga menziesii* var. *glauca* seedlings in the field is influced be soil disturbance. *Journal of Ecology* 98 pp.429-439

Teste FP, Simard SW. (2008) Mycorrhizal networks and distance from mature trees alter patterns of competition and facilitation in dry Douglas-fir forests. *Oecologia* 158 pp.193-203.

Tonsor SJ. (1989) Relatedness and intraspecific competition in Plantago lanceolata. *The American Naturalist* 134 pp.897–906 doi:10.1086/285020

Twieg BD., Durall DM., Simard SW. (2007) Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* 176 pp.437-447.

Van der Heijden, MGA., Horton, TR. (2009) Socialism in soil? The importance of mycorrhizal fungal networks for facilitation in natural ecosystems. *Journal of Ecology* 97 pp.1139-1150

Vyse, A., Ferguson, C., Simard, S.W., Kano, T., and Puttonen, P. (2006) Growth of Douglas-fir, lodgepole pine, and ponderosa pine seedlings underplanted in a partially-cut, dry Douglas-fir stand in south-central British Columbia. *Forestry Chronicle* 82 pp.723-732.

Wang T., Campbell EM., O'Neill GA., Aitken SN., (2012) Projecting future distributions of ecosystem climate niches: Uncertainties and management applications. *Forest Ecology and Management* 279 pp.128–140

Wang T., Hamann, A., Spittlehouse, D. (2012) ClimateBC_Map, A Interactice Platform for Visualization and Data Access, Copyright(2012) Wang,T., Hamann, A., Spittlehouse, D. All right reserved., http://www.genetics.forestry.ubc.ca/cfcg/ClimateBC40/Default.aspx, Annual and Seasonal Variables

Whitfield J. (2007) Underground Networking. *Nature* 449 pp.136-138

Willson MF., Hoppes WG., Goldman DA., Thomas PA., Katusic-Malmborg PL., Bothwell JL. (1987) Sibling competition in plants: an experimental study. *The American Naturalist* 129 pp.304–311 doi:10.1086/284636

Young, J. P. W. (1981) Sib competition can favour sex in two ways. *J. Theor. Biol*. 88 pp.755–756. doi:10.1016/0022-5193(81)90249-6