EFFECTS OF PLANT SOCIAL ENVIRONMENT ON MYCORRHIZAE

EFFECTS OF PLANT SOCIAL ENVIRONMENT ON THE MUTUALISTIC INTERACTIONS BETWEEN PLANTS AND MYCORRHIZAL FUNGI

By AMANDA LOUISE FILE, B.Sc

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Department of Biology, McMaster University

Hamilton, Ontario

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Author: Amanda Louise File, B.Sc.

Supervisor: Dr. Susan A. Dudley

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ABSTRACT

Plants and mycorrhizal fungi form a mutualism in which plants donate carbon to the fungus and, in return, receive benefits such as increased nutrient uptake and water. Mycorrhizal fungi colonize plant roots, forming nutrient exchange structures. The fungi also colonize the soil by growing long strands of hyphae that forage for nutrients and attach plants, forming a common mycorrhizal network (CMN). Plants attached to a well-supported CMN will receive greater benefits than those attached to a lesser CMN because the more carbon donations the fungal partner receives, the more it can grow and colonize the soil, accessing hard to reach soil nutrients. Kin selection theory predicts that relatives should donate more carbon to the fungal partner than non-relatives because benefits gained by neighbouring relatives through the CMN lead to inclusive fitness gains. Thus, social environment, i.e. relatedness of the group, could affect the mycorrhizal mutualism. Moreover, the presence of mycorrhizal fungi in the soil could affect plant responses to their social environment.

For my PhD thesis I have investigated whether mycorrhizal fungi respond to plant social environment and whether the presence of mycorrhizal fungi affects plant responses to relatedness. I have addressed these topics in three greenhouse studies and two field studies, using herbaceous plants and trees. I have found strong evidence that siblings have an increased association with their mycorrhizal partner compared to strangers, resulting in greater benefits for siblings. Taken together, the results from this thesis demonstrate that the ability for plants to recognize kin has implications beyond intra-

specific competitive interactions and that plant social environment has important effects on a widespread inter-specific mutualism. Additionally, the recently discovered phenomenon of plant kin recognition has been put into the context of mycorrhizae, and I have shown that mycorrhizal plants respond differently to their social environment than non-mycorrhizal plants.

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CHAPTER 1

GENERAL INTRODUCTION

Plants are social organisms that sense and respond to many aspects of their social environment, including the relatedness of their neighbours. So far most evidence of plant kin recognition is demonstrated in competitive interactions between neighbouring plants (Dudley and File 2007, Murphy and Dudley 2009, Biedrzycki, Jilany et al. 2010, Bhatt, Khandelwal et al. 2011, Karban, Shiojiri et al. 2013). But the finding that plants behave differently depending on social environment has broader implications. Kin recognition could also affect reproductive strategy, cooperation with kin, and interspecific interactions, such as the mycorrhizal symbiosis. Mycorrhizae are intriguing because fungal hyphae attach plants at the roots, providing the opportunity for neighbours to interact via the fungal network and possibly for the fungus to facilitate plant kin recognition by carrying the root exudate signaling molecules more efficiently. However, it is not yet known how plant social environment affects the mycorrhizal mutualism or whether the mutualism affects the ability of plants to recognize the identity of their neighbours.

Most plants live in high-density and relocation is not possible in the face of fierce competition by neighbouring individuals. Neighbours could either be from the same species or different species, and of a similar or different growth form (e.g. grasses, trees, shrubs). It is this combination of proximity and identity of neighbours that creates the

plant social environment. Competition for resources is one of the most important plant social interactions (Goldberg 1990) and depending on the environment, competition could occur aboveground, for light and space, and/or belowground, for nutrients and water. Plants respond to competitive situations using phenotypic plasticity, which is when a given genotype produces a different phenotype in response to a cue. An example of this is stem elongation in response to shading by neighbouring plants, which causes a shift in the ratio of red and far red light (R:FR)(reviewed by Aphalo, Ballare et al. 1999). Elongation is an increase in stem height for a given stem biomass and is therefore a change in stem shape. Phenotypic plasticity can be very costly, as is stem elongation, and not necessarily adaptive. For example, elongated stems are less robust, making them more susceptible to damage by wind or other mechanical forces (Schmitt, Dudley et al. 1999). Nevertheless, having a taller stem may allow an individual to capture more light than its shorter neighbours and thus increase its fitness. Generic cues, such as the R:FR cue, provide an individual with information about the presence of aboveground neighbours, but not who the neighbour(s) might be. However, plants have demonstrated plasticity in response the identity of neighbour roots, showing self/non-self (Mahall and Callaway 1992), species (Huber-Sannwald, Pyke et al. 1996), and kin recognition (Dudley and File 2007, Murphy and Dudley 2009, Biedrzycki, Jilany et al. 2010, Bhatt, Khandelwal et al. 2011, Karban, Shiojiri et al. 2013). Cues for this type of recognition are not yet well understood but involve root exudates (Biedrzycki, Jilany et al. 2010) and possibly volatiles (Karban, Shiojiri et al. 2013).

The ability for plants to recognize their kin is favoured by kin selection. Kin selection is the part of natural selection that acts through indirect fitness (Smith and Wynne-Edwards 1964), which is fitness gained through related individuals. Behaviours and other traits will be favoured in a population by kin selection if rB>C; where r is the relatedness of the individuals, B is the reproductive benefit gained by the recipient and C is the cost of the behaviour/trait to the actor (Hamilton 1964). This is known as Hamilton's rule and explains how altruism, i.e. costly helping, can evolve in social populations. Although kin selection can favour indiscriminate, or blind, altruism, context must be accurate such as in viscous populations (Hamilton 1964). However, many plant species have stochastic dispersal, and grow close to other species, so indiscriminate altruism could be very costly (reviewed in File, Murphy et al. 2012). Kin recognition reduces this cost of altruism since costly helping behaviours can be directed towards relatives. Plants could demonstrate altruism towards related neighbours through decreased competition or other forms of cooperation, such as increased facilitation of a shared symbiotic partner. Mycorrhiza is one such symbiosis where, because the same fungal partner can connect several individuals, there are opportunities for kin selection to act.

The mycorrhizal mutualism is over 400 million years old and occurs in over 80% of all land plant species. The fungal partner is an obligate biotroph because in most cases the fungus cannot survive without the plant as a carbon source (but see Hodge, Campbell et al. 2001, demonstrating possible saprophytic capabilities). For the plant, however, the symbiosis is often facultative, since mycorrhizae may greatly increase the growth and success of the plant but it can also grow and reproduce without the fungus. Mycorrhizal

fungi colonize both the soil and plant roots. The type of root colonization depends on whether the fungus is ectomycorrhizal or endomycorrhizal. Endomycorrhizae form when the fungal partner penetrates the cortical and epidermal cells of the plant roots. Ectomycorrhizae, commonly found on trees, do not penetrate the cortical root cells but form a Hartig net surrounding the root that functions as the main region of nutrient transfer (Peterson, Massicotte et al. 2004).

Aside from the fungi providing benefits to the plant host, the mycorrhizal symbiosis also affects plant-plant interactions through the fungal network. In the simplest scenario, a common mycorrhizal network (CMN) occurs when the same fungal individual, i.e. genet, connects two plants at the roots, causing them to share soil hyphae. However, the CMN can be much more complex, with more than one fungal genet or species connecting several plants of the same or different species (reviewed in Simard and Durall 2004). The CMN may have important effect on competition and evidence suggests mycorrhizal plants have greater asymmetry than non-mycorrhizal groups in size (e.g. Allsopp and Stock 1992, Facelli and Facelli 2002) and fecundity (Shumway and Koide 1995, although see Ayres, Gange et al. 2006).

Alternatively, plants could potentially benefit each other via a CMN through nutrient sharing. Interspecific transfer of nitrogen between plants connected by mycorrhizal hyphae has been demonstrated (Frey and Schuepp 1992, Jalonen, Nygren et al. 2009) and although carbon typically is passed from the plant to the fungi, there is evidence that carbon can also flow from the fungus to the plant (Francis and Read 1984, Simard, Berry et al. 1997, Simard, Jones et al. 1997, Tiwari, Singh et al. 2004, Ren, Lou

et al. 2013, Teste, Simard et al. 2010, Carey, Marler et al. 2004). Carbon transfer between plants via mycorrhizae is likely highly dependent upon a gradient existing, i.e. a source-sink relationship, which will drive the transfer from one plant to another. But could plants preferentially share resources with each other via a CMN? This question has big implications for how plants interact since it provides a potential mechanism for plants to actively donate resources to neighbours.

In this thesis, I present four data chapters that describe six experiments. The overarching theme of these studies is the investigation of how plant social environment affects the mycorrhizal symbiosis. More specifically, I have asked the following questions: 1) How does plant social environment affect root and soil colonization of mycorrhizal fungi? (Chapter 2 & 4); 2) Are there fitness benefits for plants growing with siblings and inoculated with mycorrhizae in the field? (Chapter 3); 3) How does asymmetric stress affect mycorrhizal colonization and plant responses to social environment? (Chapter 4); 4) How do tree seedlings respond to the identity and distance of a nearby adult and does access to mycorrhizal fungi matter? (Chapter 5). The experiments were conducted in the McMaster University Biology Greenhouse and at one of two field sites: 1) Royal Botanical Gardens Arboretum, Hamilton; or 2) Lienhuachih research forest plot, Taiwan. I have used two herbaceous species (Chapters 2-4) and one tree species (Chapter 5).

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CHAPTER 2

PLANT KIN RECOGNITION ENHANCES ABUNDANCE OF SYMBIOTIC MICROBIAL PARTNER.

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ABSTRACT

Background: The stability of cooperative interactions among different species can be compromised by cheating. In the plant-mycorrhizal fungi symbiosis, a single mycorrhizal network may interact with many plants, providing the opportunity for individual plants to cheat by obtaining nutrients from the fungi without donating carbon. Here we determine whether kin selection may favour plant investment in the mycorrhizal network, reducing the incentive to cheat when relatives interact with a single network.

Methodology/Principal Findings: We show that mycorrhizal network size and root colonization were greater when *Ambrosia artemisiifolia L.* was grown with siblings compared to strangers. Soil fungal abundance was positively correlated with group leaf nitrogen, and increased root colonization was associated with a reduced number of pathogen-induced root lesions, indicating greater benefit to plants grown with siblings.

Conclusions/Significance: Plants can benefit their relatives through investment in mycorrhizal fungi, and kin selection in plants could promote the persistence of the mycorrhizal symbiosis.

INTRODUCTION

Many organisms cooperate even though they have the opportunity to cheat. The interaction between plants and mycorrhizal fungi is considered a mutualism because the fungus provides water, nutrients and pathogen defense to the plant in return for carbohydrates. Though most mycorrhizal fungi are obligate symbionts, dependent on plant carbon for growth (Smith and Read 2010) plants may be obligate or facultative in their association with mycorrhizal fungi (Molina 1992). Moreover, mycorrhizal fungi may span the gradient from mutualism to parasitism. Cooperation, conflict, and cheating have all been observed to occur between fungi and plants (Johnson, Graham et al. 1997, Hoeksema, Chaudhary et al. 2010).

The symbiosis is considered by economic models to be a biological market where there is a trade relationship between plant and fungi, each of which specializes on acquiring certain resources (Schwartz and Hoeksema 1998, de Mazancourt and Schwartz 2010, Grman, Robinson et al. 2012). Models show that a mutualism can be stable through a trade relationship (Schwartz and Hoeksema 1998, de Mazancourt and Schwartz 2010). Plants tend to associate more with mycorrhizas when soil nutrients (e.g. Breuillin, Schramm et al. 2010, Omorusi and Ayanru 2011) or plant tissue phosphorus (P) concentration (Menge, Steirle et al. 1978) are low, which supports a simple prediction from the biological market models. Recent experimental evidence indicates that, given a choice, plant and fungal partners can also choose to trade with more cooperative partners, thus promoting a stable mutualism where neither partner is in control of the other (Kiers, Duhamel et al. 2011).

When many plants are connected to a common mycorrhizal network (CMN), tragedy of the commons theory models the mycorrhizal symbiosis as a social good, i.e., a common good that is a shared resource created and/or maintained by the group (Rankin, Bargum et al. 2007). For mycorrhizas, the CMN may be maintained by a group of plants and provides a common resource for that group. The size of the fungal network depends on plant carbohydrate contributions and thus, more soil colonization by fungal hyphae implies more investment by the plant partner (Bever, Richardson et al. 2009). Therefore, the value of the mycorrhizal network as a social good should depend on the summed carbon donations from host plants. Because attached plants will acquire more nutrients from larger networks with greater surface area and increased soil exploration, plants benefit each other by investing in the same fungal partner.

However, as individuals pay a cost to participate in the symbiosis, this creates a conflict. If individuals can escape paying the cost while still reaping the benefits from their partner, there is strong incentive to cheat (Douglas 2008). In the mycorrhizal symbiosis, several plants may be attached to a CMN and many fungal genets or species can simultaneously colonize a single plant. If either the fungus or plant do not identify cheaters and invoke sanctions, the symbiosis is open to non-cooperators since individuals may attach themselves to the mutualism without donating their fair share, ultimately leading to a tragedy of the commons (Hamilton 1964, Hardin 1968, Wade 1980, Rankin, Bargum et al. 2007). A majority of research has concentrated on the potential role of sanctions against cheaters (Weyl, Frederickson et al., Ferriere, Bronstein et al. 2002, Kiers and Denison 2008). However, kin selection among plants offers an alternate

incentive for cooperation between mutualists (Denison, Bledsoe et al. 2003, Nowak 2006) because for a plant, investing carbon in the mycorrhizal network linked to close relatives could increase one's indirect fitness and may remove cheaters from the population (Van Dyken, Linksvayer et al.) preventing a tragedy of the commons (Rankin, Bargum et al. 2007).

Plants frequently live in dense communities where relatedness may be high, providing the opportunity for kin selection (Kelly 1996, Donohue 2003). Kin selection acts more strongly if individuals only demonstrate altruism toward relatives (Gardner and West 2010), which then favours the evolution of kin recognition. Kin recognition has been demonstrated in several species of plants (Dudley and File 2007, Biedrzycki, Jilany et al. 2009, Murphy and Dudley 2009, Bhatt, Khandelwal et al. 2010). Though the mechanism is as yet unknown, root exudates have been demonstrated to convey a signal (Biedrzycki, Jilany et al. 2009). Kin recognition is also manifested as phenotypic plasticity in resource-gathering structures in response to relatedness of the plant group. In *Cakile edentula*, for example, allocation to fine roots was lower among individuals in sibling groups (Dudley and File 2007) relative to groups of non-related individuals. Because fine roots are the sites of nutrient and water absorption, this response suggests that competition for these resources was reduced among siblings (*i.e.*, kin). However, these studies demonstrating kin recognition have been done using non-mycorrhizal plants, and it is possible that the presence of a symbiont could influence interactions among kin.

Although researchers have considered the importance of plant neighbourhood on mycorrhizas, these studies have focused on the benefits of fungal (van der Heijden,

Klironomos et al. 1998) and plant diversity (Miller, Reinhardt et al. 1995, Johnson, Vandenkoornhuyse et al. 2004, Mummey, Rillig et al. 2005, Bingham and Biondini 2009, Hausmann and Hawkes 2009). In the only study that has tested whether the genetic relatedness of neighbours influenced plant interactions with mycorrhizas, Ronsheim & Anderson (2001) found that in the presence of soil fungi, biomass of individuals grown with clones or plants from the same population was greater than individuals grown with plants from a different population (Ronsheim and Anderson 2001). Their study addressed the question of local adaptation to soil fungal communities and they demonstrated benefits of growing with plants from the same population. However, no study has yet measured kin recognition in mycorrhizal plants or tested whether relatedness of a plant population affects mycorrhizal fungal growth. When mycorrhizas are present, greater cooperation among groups of siblings could be manifested through an increase in the CMN. Such an increase could result in greater total nutrient acquisition for the group (Leake, Johnson et al. 2004) or reduce the likelihood of pathogen attack (Maherali and Klironomos 2007), which should enhance the fitness of groups of siblings relative to groups of strangers.

We examined whether the association between *Glomus intraradices* and pairs of *Ambrosia artemisiifolia L.* (common ragweed) seedlings depended on the relatedness of the two plants. *G. intraradices* colonizes plant roots aggressively (Hart and Reader 2002), suggesting that young plants may experience kin selection through mycorrhizas. Because arbuscules are the sites of nutrient exchange and an increase in root colonization by arbuscules indicates a well-established mutualism (Denison and Kiers 2011), we

predicted that plant kin selection would favour the colonization of arbuscules in sibling pairs. To determine whether related seedlings benefited from a potentially enhanced mycorrhizal association, we measured plant growth as well as susceptibility to pathogen attack by measuring the frequency of lesions on roots.

Since an increase in mycorrhizal association in young seedlings may promote a well-developed CMN later in life, we carried out a second experiment to investigate whether plant relatedness and P level affected the symbiosis at the juvenile stage, when the CMN has had time to develop. Hyphae from spores of the same isolate of *G. intraradices* readily fuse together (Croll, Giovannetti et al. 2009), increasing the likelihood of a CMN forming. We predicted that kin selection would favour siblings to donate more carbon to the fungal partner, resulting in greater mycorrhizal association in groups of siblings than in groups of strangers. We also predicted that plants would promote mycorrhizal colonization in lower P environments, where the symbiosis could facilitate plant nutrient acquisition, regardless of the relatedness of the group. We examined whether an enhanced CMN, quantified as the length of the extraradical mycorrhizal hyphae, benefitted plants by measuring the relationship between CMN size and plant growth, as well as between CMN size and leaf nitrogen (N).

We present results that show the mycorrhizal association meets two predictions supported by kin selection theory: plants grown in siblings groups had more mycorrhizal colonization and growth than when they are grown in stranger groups, and the increased mycorrhizal association benefits the plants. Seedlings grown with siblings had more arbuscules and root hyphae and a reduced proportion of lesions on the roots. Juveniles

had longer soil hyphae when grown with siblings, suggesting a more developed CMN, and this was correlated with increased leaf N. We also found that stranger groups had longer soil hyphae in low P, but soil hyphal length and growth was promoted in sibling groups regardless of P level. Alternative hypotheses for these results were explored but these hypotheses were not supported.

MATERIALS & METHODS

A. artemisiifolia L. is a fast growing, wind-pollinated annual plant that readily associates with mycorrhizal fungi, and *G. intraradices* is a widely-distributed arbuscular mycorrhizal fungus (AMF) that has positive effects on ragweed performance (Fumanal, Plenchette et al. 2006). Two greenhouse experiments were conducted at separate times. For both experiments, field pollinated seeds from maternal sibships (families) were stratified on moist sand at 4^{o} C for three weeks. We transplanted to pots containing a soil-free mixture of 3:1 sand and Turface (Profile Products LC, Buffalo Grove, IL, USA) 4 days after germination for experiment 1 and 8 days after germination for experiment 2. Turface is a calcined clay product. A mix of turface and sand provides a substrate that drains well, releases water slowly, and readily separates from roots. Though we did not sterilize the growth medium, it was mixed from un-opened bags and did not include any type of field soil. Moreover, levels of soil fungal hyphae were marked lower in control compared to inoculated treatments. Plants in experiment 1 were measured at the seedling stage and plants in experiment 2 were measured at the juvenile (pre-reproductive) stage.

Experiment 1 (seedlings, pairs)

To test the prediction that social environment affects the mycorrhizal association, we conducted a fully factorial experiment with the following treatments: social environment (siblings vs. strangers) and mycorrhizas (inoculated vs. uninoculated). At this early life-stage, the mycorrhizal hyphal network is not yet established in the soil but plants are colonized by various fungal structures including arbuscules, the sites of nutrient exchange. Each pair of plants was grown in an 8.9 cm diameter, plastic pot. Six families were used to manipulate the social environment with either two siblings (same family) or two strangers (different families) per pot. Fifteen possible stranger combinations were replicated four times, and the six sibling pairs were replicated ten times across the experiment. Half the pots were inoculated with a commercially available product containing spores of *G. intraradices* mixed with a sterile media (30 mL/pot, Myke Annual and Perennial, Premier Tech Biotechnologies, Riviere-du-Loup, QC), spread onto the sand/turface, approximately 2.5 cm below the soil surface, prior to transplanting. Half the pots remained uninoculated. Because we did not add uninoculated media to non-mycorrhizal pots to control for the effect it might have on soil structure and therefore root growth, we were only able to compare belowground plant traits within mycorrhizal treatments.

The experiment was arranged into six blocks, each of which contained 20 randomly arranged pots from all possible treatment combinations. In total, 240 plants were grown in the greenhouse for 4 weeks under natural and supplementary light. Blocks were randomly rearranged on the bench every week. All plants were given a weekly dose

of low P fertilizer (831 ppm, 21-5-20 NPK, Peter's Excel, Scott's Company, Marysville, OH, USA) in solution until the soil was saturated.

Four weeks after transplantation, plants were harvested above- and belowground. Leaves and stems for each plant were dried to constant mass at 37.8^{o} C and weighed. A sample of roots and soil was taken from the bottom 2 cm of the pot. Half of this sample was used for fungal quantification and measurement of root lesions and the other half was washed for root biomass estimation. The rest of the roots in the pot were washed clean of substrate, dried and separated into fine roots (< 1 mm) and coarse roots (>1 mm). Root biomass was quantified as the total from both plants in each pot since it was not possible to identify roots from either plant. Due to the destructive nature of washing roots, root morphological traits were not measured. Mycorrhizal fungi were quantified as percent of the root colonized by arbuscules, vesicles and hyphae. Soil hyphal length was not measured for this experiment because there was not enough time for sufficient soil hyphal colonization. Fungal colonization data used for analysis was the average of two samples taken from each pot. No AMF were found in the uninoculated pots, confirming that our soil did not contain mycorrhizal fungi and there was no cross-contamination across treatments.

Mycorrhizal fungi are known to protect roots from pathogens and other enemies. We assessed the benefit of mycorrhizal colonization for seedlings as the percent of the root affected by lesions. There was no intentional addition of pathogens to the soil for our investigation of the protective effect of the mycorrhizas. Thus, any lesions found on the roots were the result of airborne pathogens commonly found in a greenhouse setting. An

observer who was double blind to treatments quantified lesions. The observer counted any damage sites on the plant roots as a lesion regardless of source because we were interested in the general protective effect mycorrhizas have against lesions, not specific pathogens.

Experiment 2 (juvenile, groups of four)

To test the prediction that older sibling plants grown with mycorrhizas would also increase their association with the fungal partner compared to strangers and to test for mycorrhizal and plant responses to P level, we conducted a second fully factorial experiment that included the following treatments: social environment, mycorrhizas, and P level.

For the social environment treatment, four maternal sibships (families) were used to manipulate the relatedness of each group; either four siblings (same family) in a large (7.3 cm × 7.6 cm × 35.6 cm) pot, four strangers (four different families) in a large pot, or four solitary plants, one from each family, in their own smaller pots (3.8 cm × 3.8 cm × 35.6 cm). Pots were open-ended cellulose bands (Zipset plant bands, Monarch Manufacturing, Colorado), which have a longer rooting depth than the plastic pots used in experiment 1, making them ideal for a longer-term study. To prevent growth of saprobes, a common problem when using these pots, they were soaked in fungicide and dried prior to experimental set up.

To manipulate P level, half the juvenile plants were given high P (3 g/plant, 14-14-14 NPK, Smartcote, Spectrum Brands IP, Brantford, Ont.) and half with relatively low P fertilizer 3g/plant, 15-10-15 NPK, Haifa Multicote, Haifa Chemicals Ltd, FL, USA).

Control release fertilizer (CRF) was applied on the substrate surface and gradually dissolved with each watering. This method was used because of the difficulty of applying nutrients in solution consistently after the canopy closes in high-density stands. Our manipulation of P level was not extreme since we designed the study to investigate plant-plant interactions and plant-fungal interactions rather than response to nutrient stress.

For the mycorrhizal treatment, half the groups were inoculated with spores of a single isolate of *G. intraradices* in solution (50 spores/mL, 10 mL/plant) and the other half were not. Spores were spread onto a layer of compost soil, 5cm from the top of the substrate surface. This layer of compost was covered with sand/turface to fill the pot. The uninoculated groups also had the layer of compost but no spores were applied. This allowed us to control for the effect the compost may have on substrate structure, which could affect root growth. Inoculated and uninoculated pots were randomly arranged within blocks, touching each other. Although AMF colonized roots of inoculated plants (Appendix Fig 2-1), no AMF were found colonizing the roots of uninoculated plants, indicating no cross contamination of fungal spores from inoculated pots. The inoculation protocol in the juvenile experiment differed from that in the seedling experiment because we were able to acquire cultured spores of *G. intraradices*, which allowed more precise control of the number of spores applied to each plant.

The entire experiment consisted of six blocks with at least 30 replicates of each possible treatment combination. Each tray contained 16 pots and two trays were combined to create a block containing 32 randomly arranged groups of four from all possible treatment combinations. Plants were watered every second day until pots were

saturated. In total, 768 plants were grown in the greenhouse under natural and supplementary light. Any seedlings that died within the first three days of transplanting were replaced.

Plants were harvested after 15 weeks of growth, at the juvenile stage. At this point, the soil hyphal network had time to develop and was measured in meters of hyphae per gram of soil. The soil hypha is the fungal structure used to forage for nutrients and consequently, the size of the hyphal network is a strong predictor of nutrient uptake in mycorrhizal plants [44]. Because mycorrhizal fungi are obligate biotrophs, carbon from the plant partner is required for soil hyphal growth and hyphal length is therefore a metric of plant investment. *G. intraradices* has been shown to produce relatively high numbers of vesicles and intra-radical hyphae and low levels of soil hyphae compared to other mycorrhizal fungi (Maherali and Klironomos 2007). However, a previous study suggests there is no trade-off between fungal structures (Powell, Parrent et al. 2009), which may otherwise confound an effect of plant investment on hyphal length.

After harvest, leaves and stems were dried to constant mass at 37.8^{o} C and weighed for each plant. Before roots were cleaned of substrate, a sample of roots and soil was taken from the bottom 2.5cm of the pot. Half of this sample was used for fungal quantification and the other half was washed for root biomass estimation. Once cleaned of substrate, roots were dried to constant mass at 37.8^{o} C and separated into fine roots (< 1 mm) and coarse roots (>1 mm). They were quantified as the total from a large pot or the sum of four solitary pots. Root morphological traits were not measured. Mycorrhizal fungi were quantified as percent of the root colonized by arbuscules, vesicles and hyphae,

and soil hyphal length (m/g soil). An observer who was double blind to treatments carried out fungal quantification.

Leaf N concentration was analyzed for a subset of pots (n=40) given low P, on a 500 – 700 mg sub-sample through dry combustion (900° C) using the Variomax CN Elemental Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). We analyzed leaf N rather than P because of the cost associated with analyzing an appropriate number of samples for statistical analysis and the availability of equipment. Only pots containing three or four plants were included in the analysis. Ten groups from each combination of kin or stranger, inoculated or uninoculated treatments were sampled, for a total of 40 groups. Leaves from each plant in the pot were mixed together providing a pooled estimate of leaf nitrogen for each pot.

Data analysis

SAS (version 9.2; SAD, Cary, NC, USA) was used for statistical analysis. PROC GLM was used to conduct analysis of variance (ANOVA) and covariance (ANCOVA). Biomass variables were log transformed to satisfy the assumptions of GLM. Data presented are the back-transformed least squares means (lsmeans). For aboveground traits of both seedlings and juveniles, the individual was the observation. Because the roots could not be identified to an individual, the pair of two plants (seedling experiment) and the group of four plants (juvenile) was the observation for root, fungal, and allocation traits. In the seedling experiment, all pots were shared. In the juvenile experiment, we summed the root masses of the group of four solitary plants in order to achieve similar statistical distributions for solitary and shared groups. For biomass allocation, we

summed the aboveground masses of the group of plants. For analysis of fungal traits, our null hypothesis for the juvenile experiment was that the measures of fungal colonization for the mix of roots in a large pot would be equivalent to the average of four plants of the same genotypes in solitary pots.

Seedling experiment: To test for effects of treatments on arbuscule, vesicle and hyphal root colonization, ANOVA was done for pairs of inoculated plants only because no fungal structures were found on uninoculated plants. Here, block, social environment, social environment × block, and family were the independent variables (Appendix Table 2- 1). To test for treatment effects on individual aboveground plant biomass, ANOVA was done with log aboveground biomass as the dependent variable and mycorrhizal inoculation, social environment, family and their interactions and block as independent variables (Table 2- 1). To test for an effect of treatments on lesions, ANOVA was conducted; mycorrhizal inoculation, social environment, family and their interactions and block were independent variables, and lesions measured as a percent of root length was the dependent variable. To test the hypothesis that root colonization differed among maternal families, ANOVAs were conducted on the subset of sibling pairs inoculated with mycorrhizal fungi, with plant maternal family as the independent variable, and arbuscule, vesicle and hyphal root colonization as the dependent variables. Effects of treatments on root allocation were measured with ANCOVA with log combined root mass as the dependent variable and log combined aboveground mass as the covariate (PROC GLM).

Juvenile experiment: In this experiment the social environment treatment included a relatedness component (kin vs. strangers) and a root-neighbour component (presence or absence of root neighbours). We therefore carried out ANOVA and ANCOVA in PROC GLM and used pre-planned *a priori* contrast statements (kin vs. stranger and solitary vs. shared) when social environment had a significant main effect or interaction effect. This allowed us to distinguish whether the social environment effects were due to relatedness and/or the presence of root neighbours.

To test for the effect of treatments on soil hyphal length, ANOVA was conducted. Soil hyphal length was the dependent variable; block, mycorrhizas, social environment and nutrient treatments were the independent variables (Table 2- 2). We used contrast statements to distinguish between effects of social environment × mycorrhizas and social environment × mycorrhizas × P level interactions (Table 2- 2). Correlation analysis (PROC CORR) was used to examine the relationship between estimated total leaf nitrogen and soil hyphal length for plants in large pots. PROC REG was used to test for a relationship between estimated total nitrogen and soil hyphae in plants in shared pots, inoculated with mycorrhizas. To test whether family had an effect on soil hyphal colonization, ANOVA was done on groups of siblings with soil hyphal length as the dependent variable and maternal family as the independent variable. To test whether root sample size influenced hyphal length, regression analysis was conducted using soil hyphal length as the dependent variable and root sample mass as the independent variable.

Plants in uninoculated pots served as a control and fungal quantification verified that mycorrhizal fungi were absent from these pots. Thus, when analyzing strictly mycorrhizal structures, arbuscules, vesicles and root hyphae, only plants in mycorrhizal pots were included in the analysis. To test for the effect of treatments on arbuscule and vesicle colonization, ANCOVA was conducted using log fine root as the covariate. Fine root mass was chosen as a covariate to control for plants that had more roots possibly having increased root colonization. Block, social environment and P level were the independent variables. To test for the effect of treatments on log aboveground biomass, ANOVA was conducted using contrast statements to analyze social environment × family, social environment × P level and social environment × mycorrhizas × P level interactions (Table 2- 3). PROC CORR was used to examine the relationships among fungal colonization, belowground biomass and aboveground biomass. Effects of treatments on root allocation were measured in ANCOVA with log combined root mass as the dependent variable and log combined aboveground mass as the covariate (PROC GLM).

RESULTS

Responses in Seedling Pairs

We found evidence that social environment affects mycorrhizal colonization in seedling pairs, as kin selection would predict. Whether or not seedlings were inoculated, there was no evidence of plants responding to the relatedness of their neighbours in log aboveground biomass (Table 2- 1), stem elongation (Appendix Table 2- 2), leaf:stem allocation (Appendix Table 2- 3), and root:shoot allocation (Appendix Table 2- 4).

However, there was an effect of social environment on mycorrhizal root colonization in resource exchange traits; siblings in inoculated pots had 82% more arbuscules and 142% more hyphal colonization compared to strangers (Fig 2- 1, Appendix Table 2- 1). There was a significant effect of family on vesicle colonization (Appendix Table 2- 1), such that some family combinations had significantly more vesicles than others.

Responses in Juvenile groups

Though there was no evidence for juvenile plants responding to the relatedness of their neighbours in biomass (Table 2- 3) and morphology, we did find neighbour relatedness affected their association with mycorrhizas. Whether or not pots were inoculated, social environment did not affect juveniles in allocation to stems controlling for leaf biomass (Appendix Table 2- 5), stem elongation (Appendix Table 2- 6) and branchiness (Appendix Table 2- 7). Low levels of undifferentiated soil hyphae (<1m/g soil) were found in uninoculated pots with juvenile plants (Fig 2- 2, white bars), possibly saprobes feeding on the cellulose pots. There was no difference in soil hyphal colonization across neighbour treatments in the uninoculated pots (Fig 2- 2, white bars). However, in inoculated pots, siblings had more soil hyphae than solitary plants (averaged across four pots), which in turn had more than strangers (Fig 2- 2, black bars). There were no significant differences in hyphal root colonization between kin, strangers and solitary plants (P=0.9679).

Plants in the low P treatment increased allocation to fine roots relative to leaf mass ($F_{1,165}$=29.61, P<0.0001). However, the effect of P on aboveground biomass depended on whether plants were in solitary or shared pots (Table 2- 3). Solitary plants had the highest

aboveground biomass when grown with high P, regardless of inoculation treatment (Fig 2- 3). For plants grown with strangers, aboveground biomass did not differ across treatment combinations with no mycorrhizas × P level interaction (Fig 2- 3). Plants grown with siblings demonstrated a more complex mycorrhizas × P level interaction, with the largest plants from either the uninoculated, high P or inoculated, low P treatment combinations (Fig 2- 3). High P plants had greater stem elongation than low P plants in the absence of mycorrhizas but there was no difference across P levels for inoculated plants (mycorrhizas × P level interaction; Appendix Table 2- 6). In plants inoculated with mycorrhizas, we found more vesicles colonizing the roots for a given fine root mass in the high P treatment compared to low P ($F_{1,79}$=5.80, P=0.0184, Appendix Fig 2- 2).

Soil hyphal length increased in low P (main effect P level, Table 2- 2) but it was entirely due to the difference between high and low P in stranger groups. We found that soil hyphal responses to P level depended on relatedness of the plant group (kin vs. stranger × mycorrhizas × P level, Table 2- 2). Sibling and solitary groups maintained high hyphal length in high and low P (Fig 2- 4). By contrast, strangers in low P had 41 % more soil hyphae than strangers in high P (Fig 2- 4). The effect of P level on arbuscule colonization also depended on social environment ($F_{2,79}$=5.37, P=0.0065, Appendix Fig 2- 3); strangers inoculated with mycorrhizas in low P had more arbuscules colonizing the root than strangers in high P but there were no differences within inoculated solitary and sibling groups.

Benefits to increased mycorrhizal association

Although a field study found positive effects of *G. intraradices* on ragweed after 72 days of growth [43], we found no effects of mycorrhizas on biomass in either seedlings (Table 2- 1, Fig 2- 5) or juveniles (Table 2- 3, Fig 2- 3), possibly because plants were grown with relatively abundant nutrients [3]. However, finding a lack of effect of mycorrhizas on biomass also indicates that inoculated plants were not parasitized by the fungal partner. In the seedling experiment, plants inoculated with mycorrhizas had significantly fewer lesions on their roots compared to plants without mycorrhizas in the same social environment (black bars vs. white bars, Fig 2- 6). Across social environments, siblings inoculated with mycorrhizas had markedly fewer lesions on their roots compared to inoculated strangers (black bars, Fig 2- 6). In the juvenile experiment, total plant biomass was not affected by the mycorrhizal treatment ($P<0.2538$). However, groups in inoculated pots had significantly higher total leaf N estimated from the product of leaf mass and leaf N concentration (percent by mass), than plants in uninoculated pots (inoculated mean= 0.2144, SE=0.0151; uninoculated mean=0.1224, SE=0.0187;$P<0.0007$). Total leaf N was positively correlated with soil hyphal length (correlation coefficient=0.47612; $P< 0.0019$; Fig 2- 7), suggesting that larger mycorrhizal networks were associated with improved plant N uptake.

DISCUSSION & CONCLUSION

We provide the first evidence there is plant kin recognition, *i.e.*, plasticity to relatedness of neighbours, in the mycorrhizal symbiosis, and that siblings can benefit each other through increased mycorrhizal association. Though no evidence of kin recognition

was found in the plants themselves, mycorrhizal colonization and growth may be considered an extended phenotype that responds to the host environmental conditions, including the relatedness of the plant group. In young seedlings, arbuscule and root hyphal colonization responded to relatedness, and pairs of siblings had fewer root lesions than strangers. Juvenile plant investment in the mycorrhizal network depended on the social environment and the nutrient conditions, which translated into a nutritional benefit for plant groups with more soil hyphae. Interestingly, we also found increased fungal colonization in low P, as predicted by the biological market model.

The mycorrhizal response to siblings is supported by kin selection theory. In the presence of likely cheaters, *i.e.*, strangers, mycorrhizal colonization and growth were lower, whereas in the absence of likely cheaters, *i.e.*, in solitary or sibling groups, mycorrhizal colonization and growth was greater. Although we found this pattern in both seedling and juvenile experiments, the mycorrhizal structures that responded were different. In seedlings, we found more arbuscules and root hyphae in siblings than in stranger pairs. Arbuscules, the sites of nutrient exchange, are relatively short-lived (4-10 days) (Smith and Read 2010) and thus the level of root colonization could easily change over a plant's lifetime. In juveniles we found more soil hyphal colonization in groups of siblings compared to strangers. Early in life, the net benefit of associating with mycorrhizas is lower compared to later on because the seedling is donating carbon to the fungal partner that could otherwise be used for its own growth and defence (Johnson, Graham et al. 1997). However, higher root colonization at the seedling stage can have benefits for nutrient uptake at the juvenile and adult stages (Mullen and Schmidt 1993),

which could translate into increased final fitness. This benefit would be even greater if plants were colonizing a CMN connected with related individuals, potentially increasing their inclusive fitness. Our findings from both experiments support this idea since sibling pairs had greater arbuscular colonization than strangers, and at a later life-stage, groups of siblings had increased soil hyphae.

Greater soil hyphal length in juvenile sibling groups implies that the plants growing with siblings actively increased their investment in the mycorrhizal association. Consistent with predictions from the social good model, siblings appeared to contribute more to the symbiosis compared to strangers by supporting increased fungal growth in the soil. Plants have the ability to control their carbohydrate donations to fungi, preferentially allocating carbon to more beneficial fungal partners over more parasitic ones (Kiers, Duhamel et al. 2011), leading to increased fungal fitness (Bever, Richardson et al. 2009), so it is also possible that they could preferentially allocate to a CMN attached to siblings versus one attached to strangers. Similar to previous research (Powell, Parrent et al. 2009), we found no trade-offs between fungal traits (Appendix Table 2-8), supporting the argument that soil hyphae is an indicator of plant contribution to fungal growth. The larger network size in groups of related plants implies that the fungus benefits from plant kin selection. Thus, the plant neighbourhood may be a key influence on the fitness of the fungal partner.

It might be argued that the increased mycorrhizal association in sibling groups is evidence that the fungal partner can more effectively exploit genetically similar groups. In this parasitism hypothesis, finding more arbuscules in seedlings and more soil hyphae

in juveniles can be interpreted as fungal success in sibling groups. Evidence against this parasitism hypothesis would be the observation that plants benefit from increased fungal colonization. We measured two potential short-term benefits that can specifically be attributed to mycorrhizas. First, we found fewer lesions with seedlings associating with mycorrhizas, with sibling pairs having significantly fewer than strangers. This decrease in general lesion number indicates an overall protective effect of mycorrhizas on young seedling roots, suggesting that there are early benefits for siblings who increase their association with mycorrhizal fungi at the seedling stage. The lesions observed on the roots from our seedling study could have come from various sources including fungal pathogens, parasites and root nematodes. However, mycorrhizal fungi are known to benefit plants by protecting them against root lesions through a variety of mechanisms, including competition between pathogens and AM-fungi (reviewed in Smith and Read 2010). The second observation against the parasitism hypothesis is that our data suggests inoculated pots of juvenile plants had higher total leaf N, a result that is consistent with the generally positive effects of soil hyphal length on plant nutrient status (Powell, Parrent et al. 2009). N and P acquisition are often correlated and N is typically the most important limiting nutrient for plant growth (Lambers and Pons 1998) , and pollen and seed production (Lau and Stephenson 1993). Therefore, juvenile plants in sibling groups may have had improved nutrient acquisition ability through an extended mycorrhizal network resulting from their increased investment. Thus for both seedlings and juveniles, there are short-term benefits to having greater mycorrhizal association which could result

in higher survival and fecundity for plants grown with siblings. This is further evidence supporting the argument for kin selection acting on the ragweed-mycorrhizal symbiosis.

Our results suggest that juvenile siblings invested carbon in mycorrhizas even at high P, when the mutualism is likely less necessary for P uptake. Despite a common prediction that plants will have higher association with mycorrhizal fungi in low P (Schwartz and Hoeksema 1998), we found that only strangers had this response. In contrast, siblings and solitary plants maintained consistently higher levels of soil hyphae across P levels. A high level of investment in mycorrhizas, despite high P, could provide multiple benefits including bet hedging against future demand for nutrients, increased water acquisition, and pathogen defense (Smith and Read 2010), all of which could increase the chances of survival and, therefore, final fitness. These benefits could increase one's indirect fitness when attached to the same CMN as relatives.

We were able to reject our alternative hypotheses about the causes of mycorrhizal and plant benefit differences across social environments. Previous studies of plant recognition have found phenotypic plasticity to neighbours in nutrient acquisition traits, including fine roots (Dudley and File 2007, Biedrzycki, Jilany et al. 2009, Murphy and Dudley 2009, Bhatt, Khandelwal et al. 2010). Consequently, one alternative hypothesis is that changes in plant morphology induced by kin recognition caused the differences found in mycorrhizas. However, in neither experiment were there shifts in biomass allocation or aboveground morphological changes in response to social environment. Therefore, plant morphological responses to social environment were not confounded with responses seen in the fungal partner. The only trait showing any social environment interactions

was log aboveground biomass in juveniles. Here, the differences among families in solitary vs. shared effects and in kin vs. stranger effects (social environment × family, Table 2- 3, Appendix Fig 2- 4) were the consequence of more variance among families in stranger than kin or solitary conditions. In the seedling study, we found no effect of family on fungal structures typically associated with strength of the mutualism, arbuscules ($P < 0.8706$) and hyphae ($P < 0.7885$), allowing us to reject the hypothesis that some plant genotypes may have higher specificity for a given fungus. There were no differences in soil hyphal length between the four genotypes of juvenile plants either (Appendix Fig 2- 5). Finding a lack of effect of family on mycorrhizal structures expected to be associated with a stronger symbiosis in both seedling and juvenile studies indicates that the increased colonization in siblings was not due to a particular family having stronger associations with the fungal genotype used in either experiment. We also investigated whether the differences in soil mycorrhizas were the result of soil hyphae being correlated with biomass of the root sample used for fungal quantification, coupled with systematic differences in root biomass between social environments. *Post hoc* analysis revealed no relationship between root sample mass and soil hyphal length (Appendix Fig 2- 6). Above- and belowground biomasses were strongly positively correlated with each other but not with any of the fungal traits. Root hyphal colonization and arbuscular colonization were negatively correlated ($P<0.0278$). No other fungal traits were correlated (Appendix Table 2- 8).

Previous research in *Arabidopsis thaliana* has demonstrated that the mechanism for plant kin recognition involves root exudates (Biedrzycki, Jilany et al. 2009). We

hypothesize that ragweed also uses root exudates to recognize the identity of surrounding plants. If ragweed recognizes that it is growing near siblings and it is also attached to a mycorrhizal fungal partner, it may altruistically donate more carbon to the fungal partner. Kin selection would favour this increased donation since the benefits that could be provided to neighbouring kin would increase the focal individual's inclusive fitness. Alternatively, if a focal individual recognized its neighbours as strangers, it could avoid costly contributions to the CMN that would benefit non-relatives and provide no inclusive fitness rewards.

In conclusion, mycorrhizal colonization and growth was highest in sibling groups, supporting predictions from social good theory that kin selection can stabilize a mutualism (Rankin, Bargum et al. 2007). Though a previous study provided evidence that plants benefit from population level specificity to soil fungal communities (Ronsheim and Anderson 2001), here we demonstrate that the mycorrhizal symbiosis is also affected by plant kin recognition. Low nutrient availability is known to favour mycorrhizal colonization (Valentine, Osborne et al. 2001). However, our results indicate that plant neighborhood may determine the extent of this nutrient effect, since sibling plants invested more in the mycorrhizal network regardless of P level. Moreover, the effect of social environment on soil hyphae was much greater than the effect of increased P. Thus, even in high P where mutualism break down is predicted, plant kin selection may allow fungal populations to persist. Though these results were found in greenhouse studies, natural population structure created through limited seed dispersal can also generate

proximity among siblings (Cheplick 1992), suggesting that kin recognition could be an important mechanism that reinforces the ancient mutualism between plants and fungi.

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Table 2-1: Analysis of variance on aboveground biomass for ragweed seedling pairs.

	Log aboveground biomass (g)		
Source	DF	F	*P*
Social environment	1	0.01	0.9138
Mycorrhizas	1	0.98	0.3244
Family	5	15.67	**<0.0001**
SocialEnv × Myc	1	1.21	0.2683
Myc × Fam	5	0.93	0.4606
SocialEnv × Fam	5	1.20	0.3077
SocialEnv × Myc × Fam	5	1.78	0.1184
Block	1	0.29	0.5911

Log of aboveground biomass is log (abovemass + 1). Social environment and mycorrhizas refer to treatments. Block refers to the experimental unit. Family refers to the specific pairing of maternal sibships within each pot. Significant values are in bold.

Table 2-2: Analysis of variance for groups of four juvenile ragweed plants.

	Soil hyphae		
Source	DF	F	*P*
Social environment	2	52.7	**<0.0001**
Kin vs. stranger	1	95.65	**<0.0001**
Solitary vs. shared	1	12.58	**0.0005**
Mycorrhizas	1	1173.47	**<0.0001**
P level	1	6.10	**0.0146**
SocialEnv × Myc	2	33.79	**<0.0001**
Kin vs. stranger × Myc	1	61.00	**<0.0001**
Solitary vs. shared × Myc	1	8.33	**0.0044**
SocialEnv × P level	2	1.49	0.2282
Myc × P level	1	3.55	0.0612
SocialEnv × M × P	2	5.22	**0.0064**
Kin vs. stranger × M × P	1	9.66	**0.0022**
Solitary vs. shared × M × P	1	0.92	0.3394
Block	5	0.94	0.4568

Social environment, mycorrhizas and P level refer to treatment effects. Where there is a significant effect of social environment in an interaction, PROC GLM with pre-planned contrast statements were used to distinguish between effects of kin vs. stranger and solitary vs. shared. Significant values are in bold.

Table 2-3: Analysis of variance (ANOVA) for individual juvenile ragweed plants.

	Log above mass (g)		
Source	DF	F	*P*
Family	3	7.73	**<0.0001**
Social environment	2	0.03	0.9725
Mycorrhizas	1	0.74	0.3911
P level	1	3.75	0.0532
SocialEnv × Family	6	3.52	**0.0020**
Kin vs. stranger × Fam	3	3.84	**0.0097**
Solitary vs. shared × Fam	3	3.36	**0.0187**
Myc × Family	3	1.95	0.1207
P level × Family	3	3.37	**0.0182**
SocialEnv × Myc	2	0.18	0.8352
SocialEnv × P level	2	4.34	**0.0135**
Kin vs. stranger × P	1	0.18	0.6744
Solitary vs. shared × P	1	8.57	**0.0036**
Myc × P level	1	2.32	0.1279
SocialEnv × M × F	6	0.85	0.5310
SocialEnv × P × F	6	0.62	0.7117
SocialEnv × M × P	2	4.02	**0.0184**
Kin vs. stranger × M × P	1	1.98	0.1600
Solitary vs. shared × M × P	1	5.84	**0.0160**
SocialEnv × M × P × F	6	1.91	0.0766
Block	5	3.23	**0.0070**

Log of aboveground biomass is log (aboveground biomass+0.5). Family refers to maternal sibship. Social environment, mycorrhizas and P level refer to treatment effects. Where there is a significant effect of social environment in an interaction, PROC GLM with pre-planned contrast statements were used to distinguish between effects of kin vs. stranger and solitary vs. shared. Significant values are in bold.

Figure 2-1: Root colonization by arbuscules and hyphae in A. artemisiifolia L seedlings

Ragweed seedlings inoculated with *G. intraradices* were grown in pairs of either siblings (white bars) or strangers (black bars) (n=119). Sibling roots had significantly more arbuscular colonization (P<0.0012) and hyphal colonization (P <0.0001) compared to stranger roots. Error bars represent ± 1 s.e.m.

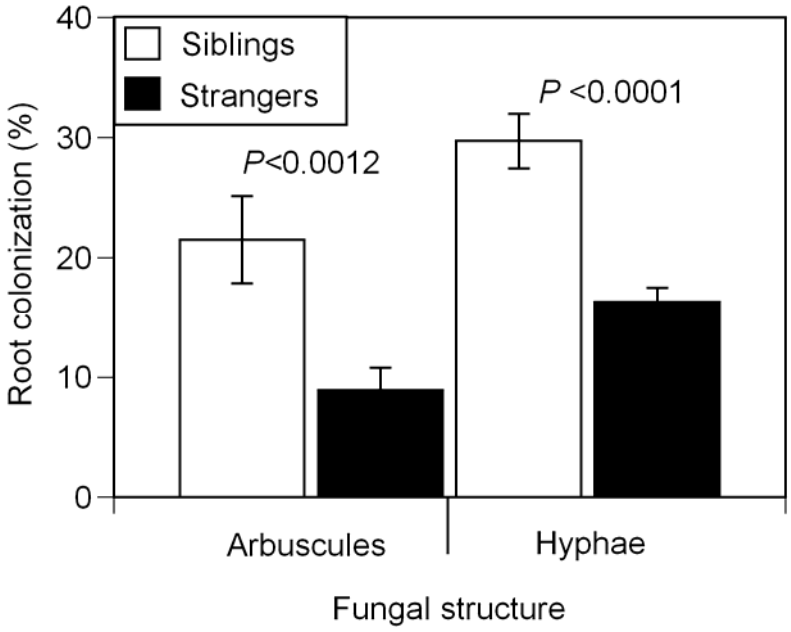

40
30
20
10
0
Root colonization (%)
Siblings
Strangers
P<0.0012
P <0.0001
Arbuscules
Hyphae
Fungal structure

Figure 2-2: Effect of mycorrhizal inoculation and social environment on soil hyphal length for juvenile A. artemisiifolia L plants.

Groups of four plants were either uninoculated (white bars) or inoculated with *G. intraradices* (black bars). Plants were grown solitary, with siblings or with strangers. Soil hyphal length was lower in uninoculated plants and did not differ among social environments; however, soil hyphal length differed markedly among social environments in inoculated groups (social environment × mycorrhizas interaction $P < 0.0001$). Log fine root mass was included as the covariate but had no significant effect. Means that did not differ significantly at $P<0.05$ are represented by the same letter. Error bars represent ± 1 s.e.m.

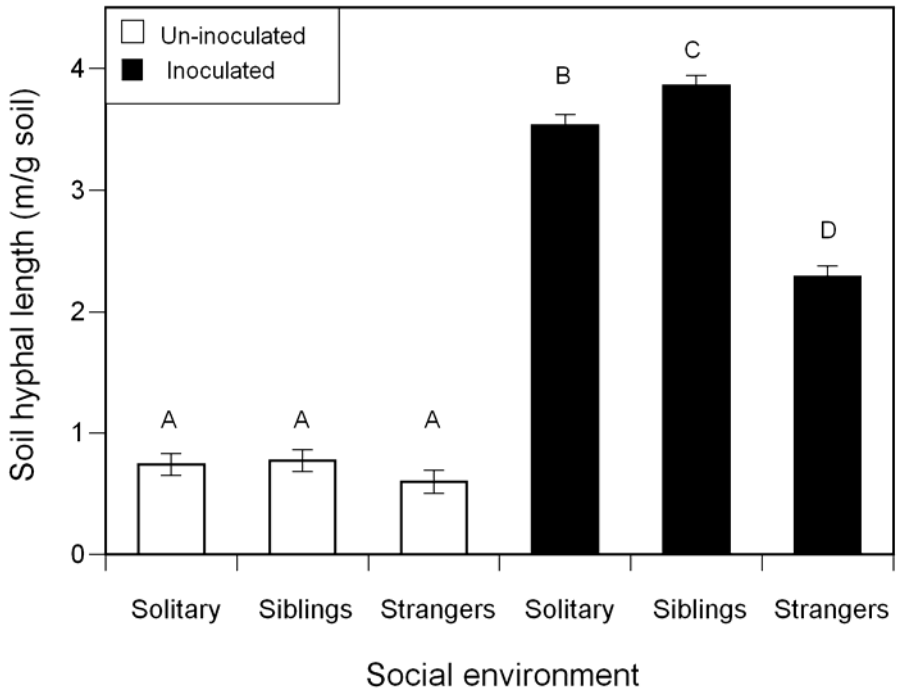

Un-inoculated
Inoculated
Soil hyphal length (m/g soil)
0
1
2
3
4
A
A
A
B
C
D
Solitary
Siblings
Strangers
Solitary
Siblings
Strangers
Social environment

Figure 2-3: Effect of mycorrhizal inoculation, P level and social environment on aboveground biomass for juvenile A. artemisiifolia L plants.

Plants were grown in shared pots (siblings or strangers) or solitary (alone) (n=606). Groups of four plants were either uninoculated and given high P fertilizer (white bars), uninoculated and given low P fertilizer (striped bars), inoculated and given high P fertilizer (black bars) or inoculated and given low P fertilizer (grey bars). Spores of *G. intraradices* were used to inoculate pots. Data presented are the back-transformed lsmeans of log(aboveground biomass +0.5). Error bars represent ± 1 s.e.m.

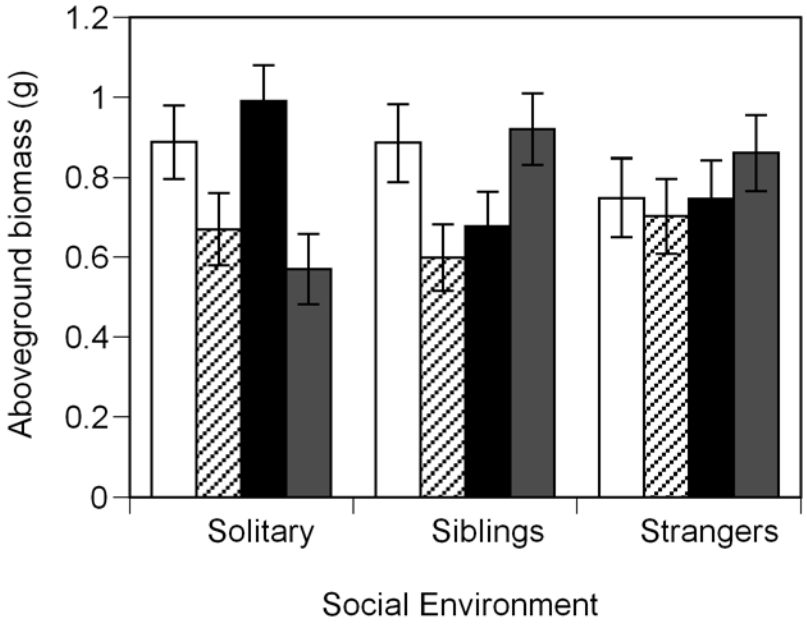

1.2
1
0.8
0.6
0.4
0.2
0
Aboveground biomass (g)
Solitary
Siblings
Strangers
Social Environment

Figure 2-4: Effect of nutrient level and social environment on soil hyphal length for juvenile A. artemisiifolia L plants.

Groups of four were solitary, siblings or strangers and all were inoculated with *G. intraradices* (*n*=93). Solitary and sibling groups had high soil hyphal length in both high and low P, but strangers had low soil hyphal length in high P and increased soil hyphal length in low P (social environment × P level interaction P=0.0338). Log fine root mass was the covariate and had no effect. White bars represent groups that received low P fertilizer, and black bars are groups in high P. Means that did not differ significantly at P <0.05 are represented by the same letter. Error bars represent ± 1 s.e.m.

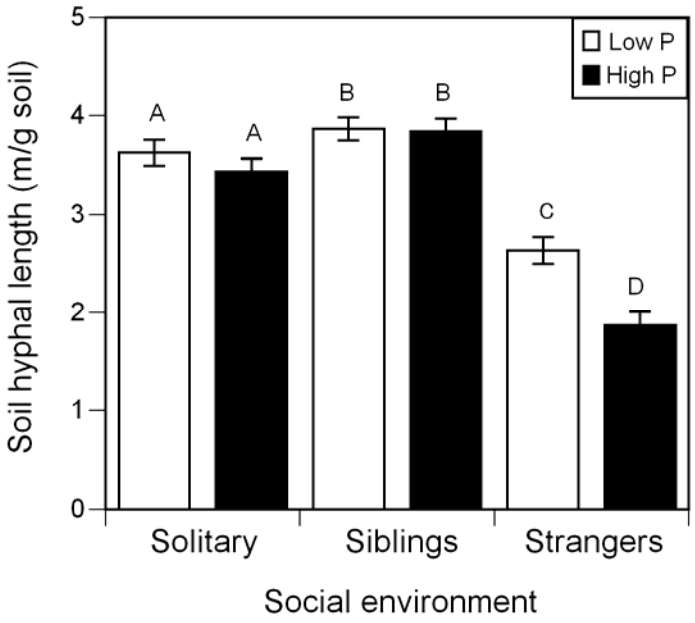

Soil hyphal length (m/g soil)
5
4
3
2
1
0
Low P
High P
A
A
B
B
C
D
Solitary
Siblings
Strangers
Social environment

Figure 2-5: Effect of mycorrhizal inoculation and social environment on aboveground biomass for seedling A. artemisiifolia L plants.

Plants were grown in pairs in shared pots (*n*=238). Pairs were either uninoculated (white bars) or inoculated (black bars) with *G. intraradices*. Data presented are the back-transformed lsmeans of log(aboveground biomass + 1). Means that did not differ significantly at $P < 0.05$ are represented by the same letter. Error bars represent ± 1 s.e.m.

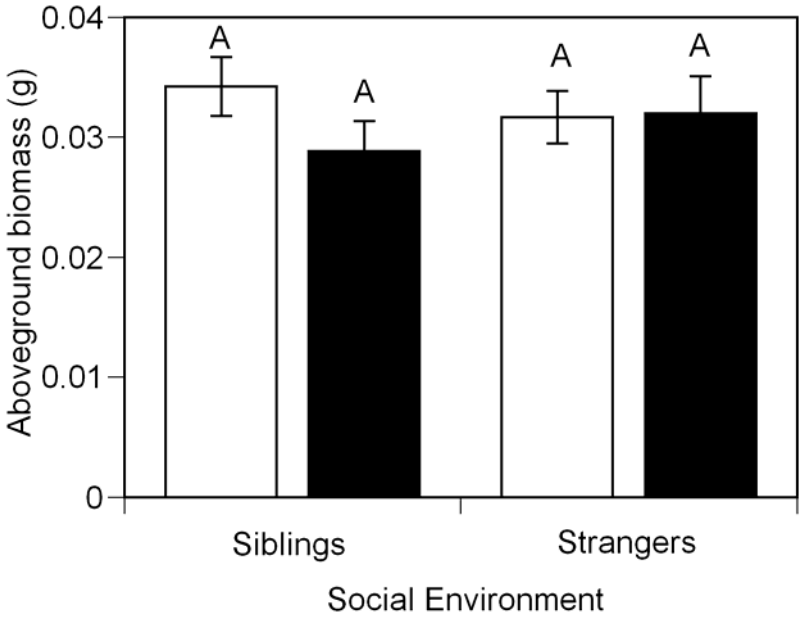

0.04
0.03
0.02
0.01
0
Aboveground biomass (g)
A
A
A
A
Siblings
Strangers
Social Environment

Figure 2-6: Percent of the root afflicted by lesions in pairs of A. artemisiifolia L seedlings

Black bars represent plants inoculated with *G. intraradices* and white bars represent uninoculated plants (*n*=220). Means that did not differ significantly at $P < 0.05$ are represented by the same letter. Error bars represent ± 1 s.e.m.

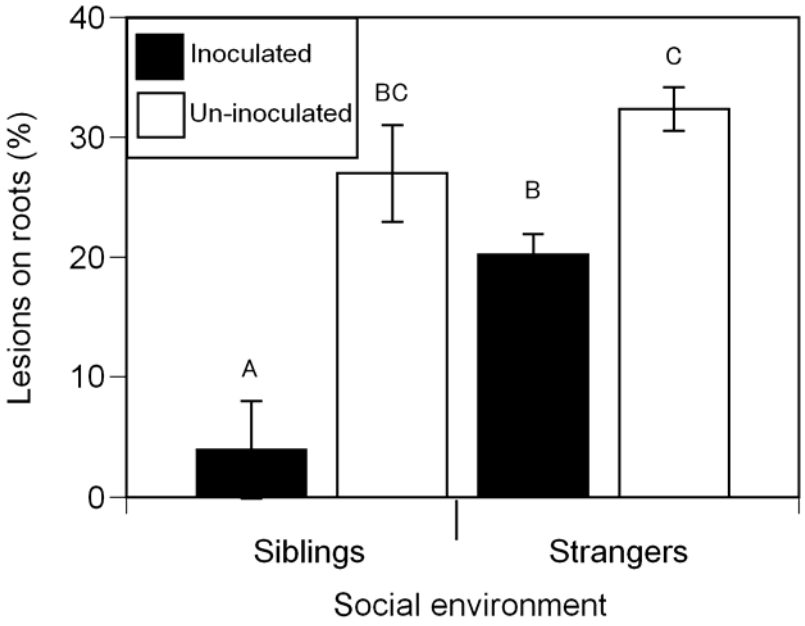
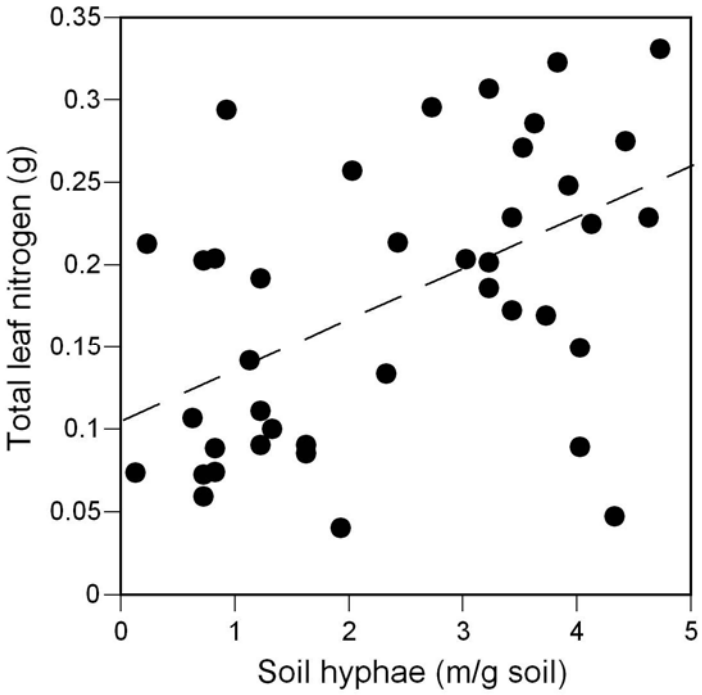

40
30
20
10
0
Lesions on roots (%)
Inoculated
Un-inoculated
A
BC
B
C
Siblings
Strangers
Social environment

Figure 2-7: Effect of soil hyphae on leaf N in juvenile A. artemisiifolia L plants.

Total leaf N is estimated from leaf mass × leaf N concentration. Soil hyphal length was measured in meters per gram of soil (m/g soil), for a subsample of plants grown in low P, with or without *G. intraradices*, with siblings or strangers (n=40). The correlation coefficient is 0.47612 with P=0.0019. Equation of the line is: total nitrogen=0.1088+.0285(soil hyphal length).


Total leaf nitrogen (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
Soil hyphae (m/g soil)

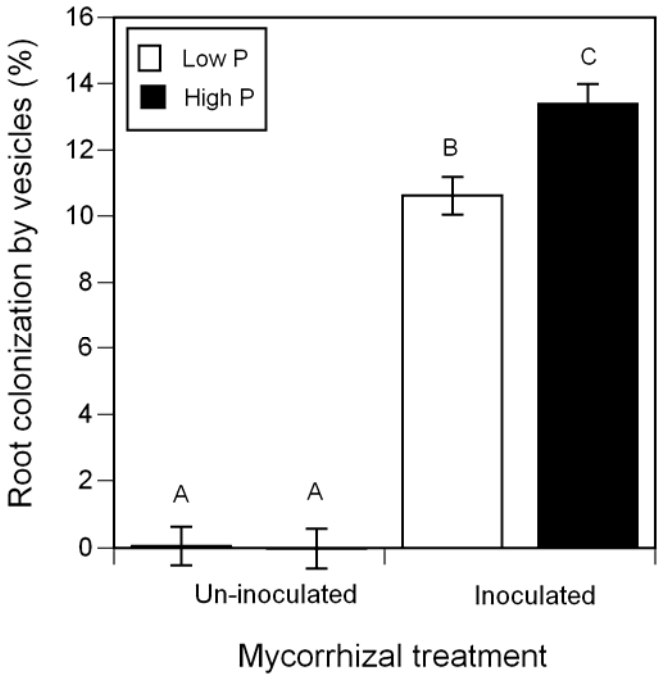
APPENDIX

Appendix figure 2-1. Effect of life stage on mycorrhizal root colonization of *A. artemisiifolia L* roots.

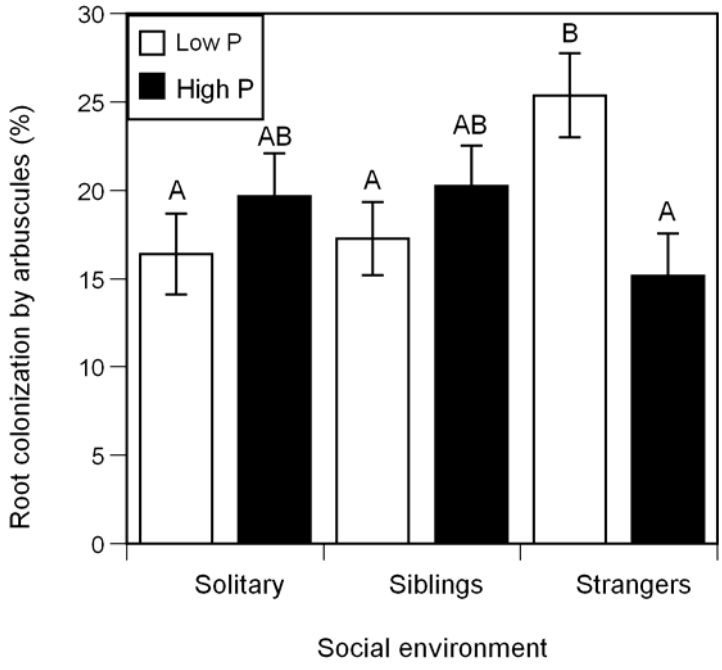
Inoculated plants had vesicles (black bars) and arbuscules (white bars) colonizing the roots of both seedlings and juveniles. Log fine root mass did not affect fungal colonization. Uninoculated plants were not included in this graph because no arbuscules or vesicles were found in soil samples from uninoculated pots. Error bars represent ± 1 s.e.m.


20
15
10
5
0
Root colonization (%)
Seedlings
Juveniles
Life stage

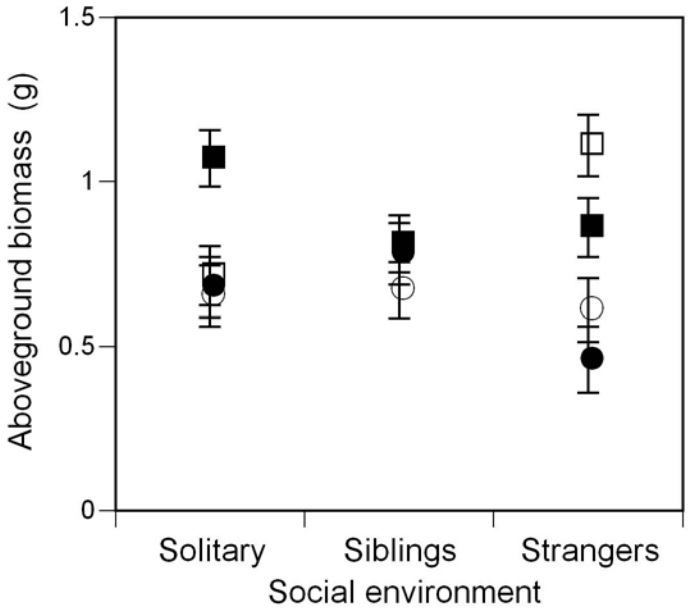
Appendix figure 2-2. **Effect of nutrient level on vesicle colonization on inoculated juvenile *A. artemisiifolia L* roots.** Groups of four plants were inoculated with *G. intraradices*. Uninoculated plants were not included in this graph because no vesicles were found colonizing their roots. Inoculated plants had more vesicles in high P (mycorrhizas × P level interaction, P=0.0177). Log fine root mass is the covariate and had no effect. White bars represent groups that received low P fertilizer, and black bars represent groups that received high P fertilizer. Means that did not differ significantly at P <0.05 are represented by the same letter. Error bars represent ± 1 s.e.m.


Root colonization by vesicles (%)
16
14
12
10
8
6
4
2
0
Low P
High P
A
A
B
C
Un-inoculated
Inoculated
Mycorrhizal treatment

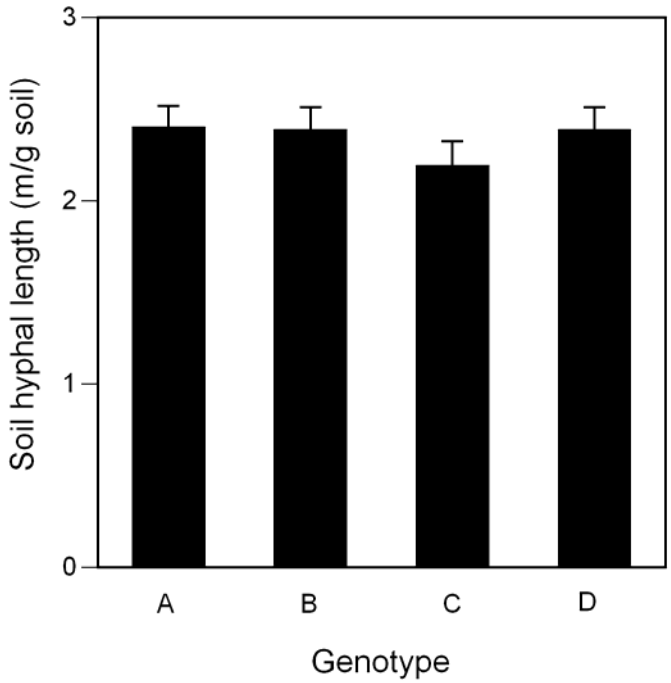
Appendix figure 2-3 Effect of nutrient level and social environment on arbuscule colonization on juvenile *A. artemisiifolia L* roots. Groups of four plants were inoculated with *G. intraradices.* Strangers responded to nutrients but solitary plants and sibling groups did not (Social environment × P level interaction P=0.0065). Log fine root mass is the covariate. Plants were grown alone (solitary), with siblings or with strangers. White bars represent groups that received low P fertilizer, and black bars are groups receiving high P. Means that did not differ significantly at P <0.05 are represented by the same letter.


Low P
High P
Root colonization by arbuscules (%)
30
25
20
15
10
5
0
A
AB
A
AB
B
A
Solitary
Siblings
Strangers
Social environment

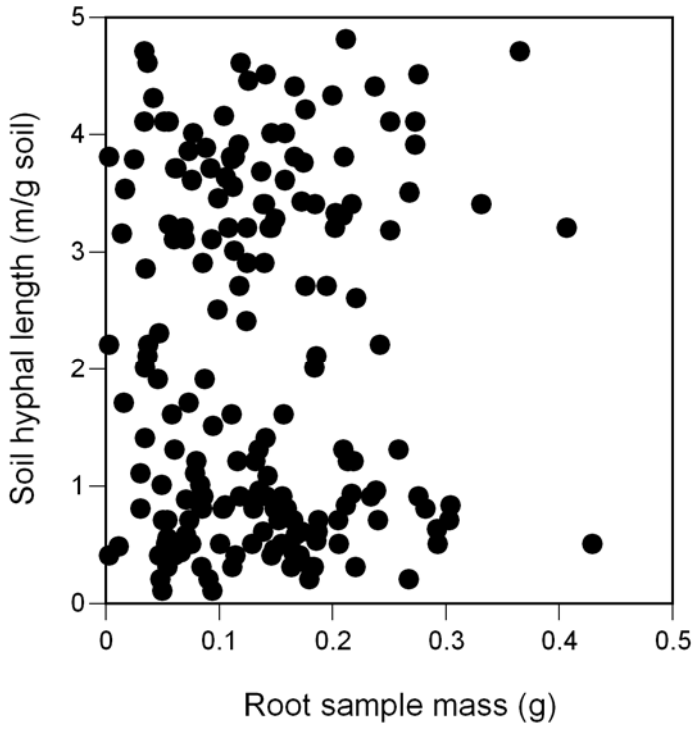
Appendix figure 2-4. Effect of family on aboveground biomass for juvenile *A. artemisiifolia L* plants. Plants were grown in one of three social environments: solitary (alone), kin and stranger (n=606). Each symbol represents a maternal sibship (family). Closed squares: family A; closed circles: family B; open squares: family C; open circles: family D. Data presented are the back-transformed lsmeans of log(aboveground biomass +0.5). Error bars represent ± 1 s.e.m.


1.5
1
0.5
0
Aboveground biomass (g)
Solitary
Siblings
Strangers
Social environment

Appendix figure 2-5. **Effect of juvenile *A. artemisiifolia L* genotype on soil hyphal length.** Analysis was done on groups of four plants grown with siblings (*n*=184). Genotypes (maternal family lines) are represented by letters A-D. There is no statistical difference among families for soil hyphal length (*P*=0.6381). Error bars represent ± 1 s.e.m.


Soil hyphal length (m/g soil)
3
2
1
0
A
B
C
D
Genotype

Appendix figure 2-6: Effect of root sample mass on soil hyphal length. Root sample mass is an estimate of the dried root biomass used for fungal quantification of juvenile *A. artemisiifolia L.* plants. There is no significant relationship between soil hyphae and root sample mass (P=0.6911).


Soil hyphal length (m/g soil)
0
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5
Root sample mass (g)

Appendix table 2-1: Analysis of variance of mycorrhizal structures in ragweed seedling pairs.

	Arbuscules			Root hyphae			Vesicles		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*
Social environment	1	16.70	**<0.0001**	1	48.65	**<0.0001**	1	0.84	0.3620
Block	5	3.35	0.0084	5	1.83	0.1162	5	1.43	0.2208
SocialEnv × block	5	3.84	0.0035	5	1.25	0.2916	5	1.10	0.3656
Family	14	3.85	**<0.0001**	14	3.33	**0.0003**	14	2.75	**0.0022**

Only plants that were inoculated with mycorrhizal spores were analyzed. Social environment refers to kin vs. stranger. Block refers to the experimental unit. Family refers to the specific pairing of maternal sibships within each pot. Significant values are in bold.

Appendix table 2-2: Analysis of covariance indicating stem elongation for ragweed seedling pairs.

	Height (cm)		
Source	DF	F	*P*
Stem biomass (g)	1	322.36	**<0.0001**
Mycorrhizas	1	2.04	0.1549
Social environment	1	0.12	0.7313
Family	5	5.51	**<0.0001**
Myc × SocialEnv	1	0.04	0.8476
Myc × Fam	5	0.40	0.8452
SocialEnv × Fam	5	2.20	0.0551
Myc × SocialEnv × Fam	5	1.00	0.4185

Plants were grown in pairs of either siblings or strangers, with or without mycorrhizal spores. Six maternal sibships (families) were used. Social environment and mycorrhizas refer to treatment effects. Family refers to the specific pairing of maternal sibships within each pot. Significant values are in bold.

Appendix table 2-3: Analysis of covariance for leaf:stem allocation for ragweed seedling pairs.

	Leaf biomass (g)		
Source	DF	F	*P*
Stem biomass (g)	1	237.56	**<0.0001**
Mycorrhizas	1	0.03	0.8693
Social environment	1	0.67	0.4141
Family	5	11.56	**<0.0001**
Myc × SocialEnv	1	0.41	0.5237
Myc × Fam	5	0.09	0.9945
SocialEnv × Fam	5	1.41	0.2236
Myc × SocialEnv × Fam	5	1.18	0.3211

Plants were grown in pairs of either siblings or strangers, with or without mycorrhizal spores. Six maternal sibships (families) were used. Social environment and mycorrhizas refer to treatment effects. Family refers to the specific pairing of families within each pot. Significant values are in bold.

Appendix table 2-4: Analysis of covariance showing root:shoot allocation for ragweed seedling pairs.

	Belowground biomass (g)		
Source	DF	F	*P*
Aboveground biomass (g)	1	504.78	**<0.0001**
Mycorrhizas	1	0.28	0.5943
Social environment	1	0.54	0.4630
Myc × SocialEnv	1	0.02	0.8885

Plants were grown in pairs of either siblings or strangers, with or without mycorrhizas. Social environment and mycorrhizas refer to treatment effects. Family refers to maternal sibship. Significant values are in bold.

Appendix table 2-5: Analysis of covariance showing stem:leaf allocation for groups of ragweed juveniles.

	Log stem		
Source	DF	F	*P*
Log leaf	1	4681.05	**<0.0001**
Social environment	2	0.34	0.7105
Mycorrhizas	1	2.21	0.1371
P level	1	0.80	0.3715
Family	3	8.31	**<0.0001**
Myc × SocialEnv	2	0.33	0.7188
Myc × Fam	3	0.18	0.9132
Myc × P level	1	0.57	0.4518
SocialEnv × Fam	6	0.92	0.4835
SocialEnv × P level	2	0.77	0.4646
P level × Fam	3	0.91	0.4336
SocialEnv × Myc × Fam	6	0.85	0.5346
Myc × P × Fam	3	2.20	0.0868
SocialEnv × P × Fam	6	1.02	0.4086
SocialEnv × Myc × P	2	0.77	0.4617
SocialEnv × Myc × P × Fam	6	0.51	0.8015
Block	5	1.78	0.1160

Plants were grown in groups of four. Social environment, mycorrhizas and P level refer to treatment effects. Family refers to specific maternal sibships within each group. Log stem is log(stem biomass + 1) and log leaf is log(leaf biomass + 1). Significant values are in bold.

Appendix table 2-6: Analysis of covariance showing stem elongation for groups of ragweed juveniles.

	Height (cm)		
Source	DF	F	*P*
Stem biomass (g)	1	385.00	**<0.0001**
Stem × stem	1	160.60	**<0.0001**
Social environment	2	2.80	0.0616
Mycorrhizas	1	0.73	0.3939
P level	1	1.44	0.2299
Family	3	5.69	**0.0008**
Myc × SocialEnv	2	1.68	0.1875
Myc × Fam	3	0.20	0.8992
Myc × P level	1	7.91	**0.0051**
SocialEnv × Fam	6	1.48	0.1843
SocialEnv × P level	2	2.64	0.0725
P level × Fam	3	2.36	0.0710
SocialEnv × Myc × Fam	6	0.55	0.7713
Myc X P × Fam	3	0.65	0.5823
SocialEnv × P × Fam	6	1.69	0.1202
SocialEnv × Myc × P	2	0.91	0.4018
SocialEnv × Myc × P × Fam	6	1.45	0.1916
Block	5	3.56	**0.0035**

Plants were grown in groups of four. Social environment, mycorrhizas and nutrient level refer to treatment effects. Family refers to specific maternal sibships within each group. Significant values are in bold.

Appendix table 2-7: Analysis of covariance indicating branchiness for groups of ragweed juveniles.

	Branch number		
Source	DF	F	*P*
Log above	1	331.61	**<0.0001**
Log above × log above	1	89.11	**<0.0001**
Social environment	2	2.62	0.0736
Mycorrhizas	1	0.82	0.3669
P level	1	2.76	0.0975
Family	3	9.18	**<0.0001**
Myc × SocialEnv	2	0.82	0.4420
Myc × Fam	3	1.55	0.2006
Myc × P level	1	0.60	0.4397
SocialEnv × Fam	6	1.76	0.1059
SocialEnv × P level	2	1.69	0.1857
P level × Fam	3	2.46	0.0618
SocialEnv × Myc × Fam	6	1.82	0.0931
Myc × P × Fam	3	1.69	0.1672
SocialEnv × P × Fam	6	0.39	0.8829
SocialEnv × Myc × P	2	0.98	0.3768
SocialEnv × Myc × P × Fam	6	0.45	0.8468
Block	5	18.52	**<0.0001**

Branch number:log aboveground biomass is a metric of branchiness. Log above is log(aboveground biomass +0.5). Social environment, mycorrhizas and P level refer to treatment effects. Family refers to specific maternal sibships within each group. Significant values are in bold.

Appendix table 2-8: Correlation matrix for juvenile ragweed plants

	Above mass	Total root	VC	HC	AC	Soil hyphae
Total root	**0.95701**	1				
	<0.0001					
VC	0.01632	-0.04623	1			
	0.8773	0.6617				
HC	-0.00990	0.02125	0.05918	1		
	0.9254	0.8406	0.5731			
AC	-0.02896	-0.00969	-0.01234	**-0.22825**	1	
	0.7840	0.9270	0.9066	**0.0278**		
Soil hyphae	0.13395	0.12362	-0.00324	0.11053	0.09898	1
	0.2030	0.2404	0.9754	0.2915	0.3452	

Only plants inoculated with *G. intraradices* were used in this analysis. Spearman correlation was used. Significant values are in bold.

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CHAPTER 3

INVESTIGATING FITNESS RESPONSES TO SOCIAL ENVIRONMENT, DENSITY AND MYCORRHIZAL INOCULATION IN FIELD-GROWN RAGWEED.

ABSTRACT

Plant kinship affects mycorrhizal colonization of both soil and roots. In ragweed, siblings have been shown to benefit from greater fungal colonization through increased nutrient uptake and protection from root enemies. However, the fitness consequences of siblings sharing a mycorrhizal partner have not yet been determined. Here I show that ragweed plants grown in the field have higher performance when grown with relatives and inoculated with mycorrhizae, but this depends on when in the growing season they were planted. This is consistent with siblings better facilitating the common mycorrhizal network, an arguably altruistic behaviour, and achieving greater fitness than strangers. Responses to planting time, herbivory and density illustrate the complexity of interpreting fitness outcomes.

INTRODUCTION

For plants that live in high-density, competition with neighbours is one of the most important social interactions (Kelly 1996). Neighbouring competitors could be of the same or of a different species, and they could be related (kin) or unrelated (strangers). Because of limited seed dispersal, many plants grow in close proximity to their relatives (Cheplick 1992), providing opportunities for kin selection. Kin selection is the

evolutionary force that acts through inclusive fitness, which is fitness gained through relatives (Smith and Wynne-Edwards 1964). Costly behaviours, such as altruism, are predicted to be favoured by kin selection if *r*B>C, where *r* is the relatedness of the individuals, B is the reproductive benefit gained by the recipient and C is the cost of the behaviour/trait to the actor (Hamilton 1964). A common prediction from kin selection theory is that, because groups of related individuals should behave less competitively than groups of strangers, they will suffer a lower cost of competition and out-perform groups of un-related individuals, i.e. groups of kin should achieve higher fitness than groups of strangers. The ability to recognize kin should evolve through kin selection since it allows individuals to direct costly altruism towards relatives, ultimately resulting in inclusive fitness benefits for the actor (Hamilton 1964). Plants can sense and respond to their social environment, i.e. the relatedness among neighbours, by distinguishing between self and non-self roots (Mahall and Callaway 1992), same or different species neighbours (HuberSannwald, Pyke et al. 1996) and whether those neighbours are kin or stranger, i.e. kin recognition (Dudley and File 2007, Biedrzycki, Jilany et al. 2009, Murphy and Dudley 2009, Bhatt, Khandelwal et al. 2010). Plant responses to the social environment have been found in competitive traits, e.g. increased competitiveness with strangers (Murphy and Dudley 2009) or reduced competitiveness with siblings (Dudley and File 2007, Bhatt, Khandelwal et al. 2010). However, recent evidence also shows eavesdropping on herbivore induced volatile signals with siblings (Karban, Shiojiri et al. 2013) and cooperation among siblings attached by mycorrhizal fungi (Chapter 2; File, Klironomos et al. 2012).

But the ability for plants to recognize kin does affect more than just intra-specific competitiveness. Plant social environment affects the inter-specific mycorrhizal symbiosis. In Chapter 2, I show that when common ragweed is grown with siblings, more mycorrhizal hyphae colonize the soil in juvenile plants and more arbuscules and hyphae colonize the roots in seedlings (File, Klironomos et al. 2012). This increased mycorrhizal colonization in ragweed is associated with greater leaf nitrogen content in juvenile plants with increased soil hyphae, and reduced root lesions in seedling plants grown with siblings (File, Klironomos et al. 2012; Chapter 2). These benefits are predicted to increase lifetime fitness, but in Chapter 2 plants were harvested at a young age so lifetime fitness was not measured.

There is evidence for kin selection in plants, but there is controversy over studies measuring fitness. Several plant species do show increased fitness in sibling groups (Tonsor 1989, Andalo, Goldringer et al. 2001, Donohue 2003, Biernaskie 2010). Other species demonstrate either decreased fitness when grown with siblings, presumably due to an increased overlap of resource use among related individuals, i.e. elbow room hypothesis (Young 1981) or they do not differ in fitness whether they are grown with kin or non-kin (reviewed in File, Murphy et al. 2012). Some fitness studies have been used to investigate the consequences of plant kin recognition with one concluding support for the existence of kin recognition (Biernaskie 2011) and others presenting evidence against kin recognition (Milla, Forero et al. 2009, Masclaux, Hammond et al. 2010, Biernaskie 2011). Assessing the fitness benefits of growing with siblings has multiple challenges. Fitness is the total reproductive success of an individual and in plants it can be measured in a

number of different ways. Reproductive structures, such as number of flowers, inflorescence length, flower biomass, seeds or pollen, are commonly measured since they are directly involved in reproductive output. Additionally, performance measures, or measures of non-reproductive plant size, can be used to assess the lifetime success of an individual. Since fitness is the complex outcome of many processes, it should not be used to interpret whether or not kin recognition has occurred (File, Murphy et al. 2012). Nonetheless, a study of lifetime fitness can test hypotheses generated by a trait-based (phenotypic plasticity) study. Siblings attached to a common mycorrhizal network have the opportunity to show altruism to each other by increasing carbon donation and facilitating the growth of the fungal partner. Therefore, kin selection could shape the mycorrhizal symbiosis and siblings inoculated with mycorrhizae are predicted to have higher fitness than inoculated strangers and un-inoculated groups.

Here, common ragweed was grown in pairs of siblings or strangers which were either inoculated or un-inoculated with mycorrhizal fungi, and grown at high- or low-density. I measured aboveground growth and functional traits to ask the following questions: **1) How does social environment (relatedness) affect morphology and fitness of field-grown ragweed? and 2) Are the responses to social environment dependent upon density and mycorrhizal inoculation?**

MATERIALS & METHODS

Study species:

Ambrosia artemisiifolia L (family Asteraceae), common ragweed, is a fast growing annual plant and associates readily with mycorrhizal fungi. Fifteen maternal

(hereafter, “families”) were field collected from three populations in southern Ontario for the first experiment and from one population in southern Ontario for experiment two. Ragweed is wind pollinated and highly out-crossing (Friedman and Barrett 2008) so seeds from the same family were likely half-siblings although they could be as related as full siblings if the same pollen donor pollinated more than one female flower.

Seed germination:

Seeds were removed from their chaff and put on moist sand (Temisca Inc, Quebec) in Petri dishes in the fridge at 4^{o} C. They were removed from the fridge when signs of germination were observed. Petri dishes were put in the growth chamber with 12 hours of light and sand was kept moist. As seeds germinated they were planted into individual cells of plastic 6-cell packs filled with sand, and kept under grow lights on the lab bench. When sufficient numbers of seeds had been transplanted, plants were taken to the greenhouse and watered every second day until transplanting to the field.

Experimental design:

Two experiments were conducted in the same field site at the Royal Botanical Gardens in Burlington, Ontario. Experiment one was done in the summer of 2011 and experiment two was done in the summer of 2012. The goal of both experiments was the same with some minor improvements in the setup for experiment two. Additionally, in experiment one, unknown herbivores severely damaged the plants by removing the upper stem (damage was consistent with mammalian bite) prior to us building an enclosure, so study two was also conducted with the enclosure present for the entire duration. Both

experiments were fully factorial and used the following treatments: 1) social environment 2) mycorrhizal inoculation and 3) density.

Social environment treatment*:* To test whether social environment would affect growth and morphology of ragweed in the field, we manipulated neighbour identity such that half of the pots had plants from the same family (i.e. kin) and half had plants from two different families (i.e. strangers).

Mycorrhizal treatment*:* To test whether siblings inoculated with mycorrhizae would outperform inoculated strangers and un-inoculated plants, half the pots were inoculated with mycorrhizal spores and half were not. In experiment one, 90mL of Mykes (Premiere Tech Biotechnologies, Rivière-du-Loup, Québec), a commercially available mycorrhizae product, was used to inoculate each mycorrhizal pot. In experiment two, the mycorrhizal pots were each inoculated with 400 spores in solution (Premiere Tech Biotechnologies, Rivière-du-Loup, Québec). The commercial product contains a substrate used to carry the spores and makes it easier for homeowners to apply the mycorrhizae to their gardens. However, this substrate could possibly change the soil structure and confound effects of mycorrhizal inoculation. Thus, to improve the methods in the second experiment, we used spores in solution.

Density treatment*:* To test whether effects of social environment and mycorrhizal inoculation were dependent upon density, plants were planted either in high-density or low-density. In each experiment there were four blocks: two high-density and two low-density. Each block had 28 (4 × 7 configuration) 24” PVC pipes, i.e. pots, hammered into the ground and had approximately 1” of the end of the pipe exposed above the soil level.

In high-density arrays, PVC pipes were 0.5cm apart and in low-density arrays, PVC pipes were 20 cm apart.

Soil: Prior to filling the pots in either experiment, the inside of the pipes was thoroughly rinsed with a 5% bleach solution to remove any debris and kill any fungal spores that may have been sticking to the PVC. For experiment one, a 1:3 ratio by volume of sand (Temisca In, St. Bruno-de-Guiges, Quebec) to garden soil (Vigoro, Sure-Gro IP Inc, Brantford, Ontario) was used. This was mixed on a bleached tarp at the field site to avoid contamination. Once soil was mixed thoroughly, the pots were filled. For experiment two, a garden soil containing black earth, peat moss and sand (Garden Gallery Premium Garden Soil, Dundas, Ontario) was used and prior to transport to the field, it was pasteurized to ensure that the soil going into the pots contained no mycorrhizal spores prior to our inoculation. Because of the use of PVC pipes and garden soil, harvesting roots and analyzing mycorrhizal infection were not possible for either experiment.

Planting: *Experiment one:* Seedlings were transplanted to the field at different time points due to availability of germinated seeds. On July 8th, 2011, half of one high-density array and half of one low-density array were planted. After transplanting at the field site, seedlings were watered with a transplant fertilizer (50ppm, 15-30-15 NPK Miracle Grow All purpose fertilizer with micronutrients, The Scotts Company, Marysville, Ohio, USA). On July 14th, 13 seedlings that did not survive the transplantation were replaced. Seedlings were fertilized again with 15-30-15 NPK soluble fertilizer. On July 28th, 2011, the remaining experimental design was planted. Seedlings were initially covered with hardware mesh to protect from herbivory, but once it was removed, large mammalian

herbivores damaged many of the plants. A wire enclosure was built around the experiment preventing further herbivory. On August 10th, 2011, we replaced any dead seedlings from the second planting with new seedlings and fertilized with transplant fertilizer. The experiment was then left to natural conditions for the remainder of the duration. All plants were harvested aboveground on Oct 11th, 2011. Plants were clipped at the soil level and put into paper bags for later processing.

Experiment two: On June 19th 2012, high- and low-density plants were planted in distinct arrays at the North end of the plot (hereafter 'early plants'). Seedlings were well watered after they were transplanted into the field and watered weekly following transplantation. On August 15th, 2012, high- and low-density plants were planted in distinct arrays at the South end of the plot (hereafter 'late plants') and watered well. On August 21st, 2012 all plants were fertilized with a 20-20-20 NPK solution (1tsp/6 ga, Peter's Excel, Scott's Company, Marysville, OH, USA). The wire enclosure from experiment one was left in place for the duration of this experiment but since the roof was not secured, herbivory still occurred to several plants. Once the roof was tied down with zip ties, herbivore damage stopped. All plants were watered every second week during dry weather until plants were big enough to survive dry periods. From then on the experiment was left to natural conditions. On November 19th, 2012, all plants were harvested aboveground. Plants were clipped at the base of the stem and put into paper bags for later processing.

Data collection: Plants were dried at room temperature, then measured and weighed. Height, number of branches, length of the tallest inflorescence, number of inflorescences (experiment two only), mass of all inflorescences (experiment two only), branch mass,

leaf mass, stem mass and seed mass were all recorded. Because the response to herbivores in experiment one was variable, with some plants unable to recover and others that compensated by producing more biomass following the damage, using a score of herbivory as an independent variable was not explanatory enough. Consequently a new independent variable was defined, compensation (Strauss and Agrawal 1999), that combined the presence of herbivory and the response to herbivores. Plants were classified into a) no herbivory, b) partial compensation, where plants with herbivore damage demonstrated growth patterns typical of a release from apical dominance: one or more lateral branches grew vertically into new stems, or c) no compensation, where plants with herbivore damage did not show vertical branch growth. Additionally, the number of new stems (vertical lateral branches) and the length of each new stem were recorded.

Data analysis: SAS statistical software (version 9.2; SAD, Cary, NC, USA) was used for analysis of both experiments using proc GLM and proc GENMOD. Where necessary, biomass and other trait variables were logged to satisfy the assumptions of the GLM. Experiment one and two were analyzed independently.

Experiment 1

To test for treatment effects on morphology and fitness, seed biomass, branch/stem biomass (sum of stem mass and branch mass), realized height (sum of original stem and tallest vertical branch lengths) and total height (sum of all stem and vertical stem lengths of each individual) were analyzed. Analysis of variance (ANOVA) was used with log branch/stem biomass, log seed biomass, log realized height and log total height as dependent variables and compensation, density, social environment,

mycorrhizae, and block (nested with density) as independent variables (Table 1). Block was nested within density since each block had either high or low density but not both. The models included all possible interactions of mycorrhizae, density and social environment. Block and compensation were included only as main effects because there were no predictions about how they would interact with the other treatments. Log vegetative biomass was calculated as ln((branch mass + stem mass) + 0.25) and log seed biomass was calculated as ln(seed biomass + 0.25). Log realized height was calculated as ln(realized height + 1) and total height was calculated as ln(tot height + 1).

To test for effects of treatments on allocation to seeds and stem elongation, analysis of covariance (ANCOVA) was used. Log seed biomass was the dependent variable; log branch/stem biomass was the covariate; and compensation, density, social environment, mycorrhizae, and block (nested with density) were the independent variables (Table 3). Stem elongation was analyzed using total height as the dependent variable and total stem mass as the covariate; compensation, density, social environment, mycorrhizae, and block (nested with density) were the independent variables (Table 3). Three outliers that were removed from the data set after careful consideration.

Experiment 2

To test for effects of treatments on aboveground biomass, seed biomass, height, vegetative biomass and male flower biomass (inflorescence mass), five separate ANOVAs were conducted. Log aboveground biomass, log seed biomass, height, log vegetative biomass and log inflorescence mass were the dependent variables; and density, social environment, mycorrhizal treatment and planting time were the independent

variables, with all possible interactions of the treatments. Log seed biomass was calculated as ln(seeds + 0.0005) and log aboveground biomass was calculated as ln(above mass + 0.02). Log vegetative biomass was calculated as ln(veg mass) and log inflorescence mass was calculated as ln(infl + 0.0005). The raw un-transformed data were analyzed for stem height.

To test for treatment effects on allocation traits, analysis of covariance (ANCOVA) was used. To investigate stem elongation, stem height was the dependent variable and stem biomass was the covariate, with all possible combinations of density, social environment, mycorrhizal treatment and planting time included in the model. A quadratic term for stem mass was added to this model to improve the model fit. Both stem mass and the quadratic term for stem mass were significant. Because of the reduced herbivory in this experiment, we excluded plants that suffered severe herbivory and only analyzed stem elongation in plants that were living and had one main stem at the time of harvest (approximately 15 plants were not included due to herbivory). To investigate allocation to seeds and inflorescences, log seed biomass and log inflorescence mass were the dependent variables and log vegetative biomass, i.e. all non-reproductive aboveground biomass, was the covariate in each model. All possible combinations of density, social environment, mycorrhizal treatment and planting time were included in the models.

To test for treatment effects on whether or not seeds and male inflorescences were produced, generalized linear model (GENMOD) was conducted on the presence or absence of seed biomass and the presence or absence of male inflorescences. Planting

time, mycorrhizal treatment, social environment, and density were the independent variables with all possible two-way interactions included. Log vegetative biomass and the log vegetative biomass × planting time interaction were added to the model to test for effects of size on seed and inflorescence production.

RESULTS

Experiment 1

Effects on performance variables

Performance variables, including branch/stem biomass, seed biomass and either estimate of plant height did not respond to mycorrhizal inoculation or to social environment (Table 1). Density had a significant effect on seed and branch/stem biomass. Plants grown in low density had higher branch/stem biomass than plants grown in high density (Tables 1 & 2). Plants in high density produced lower seed biomass than did plants in low density (Tables 1 & 2). Compensation had a significant effect on seed biomass and both measures of height (realized and total height; Table 1). Plants not affected by herbivores produced the most seed biomass. Partial-compensators, i.e. those plants that grew one or more new stems in response to herbivory, and non-compensators, i.e. plants that did not grow new stems in response to herbivory, produced similar low levels of seed biomass (Table 2). There was significant effect of block nested with density on branch/stem biomass and realized height (Tables 1 & 2). Surprisingly, there were no significant interactions among the independent variables for any of the performance traits analyzed.

Effects on biomass allocation

Allocation to seeds responded to social environment, density and compensation but not to mycorrhizal inoculation (Table 3). Siblings had greater allocation to seeds than strangers (Table 2). Plants grown in high-density allocated less to seeds compared to plants grown in low-density (Table 2). Plants not affected by herbivores had the greatest allocation to seed biomass while non-compensators and partial-compensators did not differ in allocation to seed biomass (Table 2). Stem elongation responded to compensation and block (Table 3). Partial compensators had the highest stem elongation, followed by plants un-affected by herbivores, and then non-compensators (partial compensation mean ± SE=31.8589 ± 2.1381; un-affected mean ± SE=18.3029, ± 0.6472; non-compensator mean ± SE=9.3168 ± 1.2803)

Experiment 2
Effects on performance variables

Early plants had lower seed biomass than late plants (1^{st} mean ± SE=0.0135 ± 0.0028; 2^{nd} mean ± SE=0.0615 ± 0.0144). Otherwise, there were no significant treatment effects on seed biomass (Table 4). Aboveground biomass responded to planting time, density and mycorrhizae (Table 4). Early plants had greater aboveground biomass than late plants (early mean ± SE=0.8134 ± 0.0609; late mean ± SE=0.4670 ± 0.0717). High-density plants had lower aboveground biomass than low-density plants (high-density mean ± SE=1.6833±0.1165; low-density mean ± SE=2.0923, ± 0.1461). Inoculated plants had higher aboveground biomass than un-inoculated plants (inoculated mean ± SE=2.0852 ± 0.1520; un-inoculated mean ± SE=1.6891 ± 0.1114). The effect of mycorrhizal inoculation depended on density, with plants in low-density having similar

high aboveground biomass regardless of mycorrhizal inoculation, but plants in high-density having reduced aboveground biomass in the un-inoculated treatment. Aboveground biomass showed a significant mycorrhizal treatment × density × planting time interaction (Fig 1). Early plants did not differ in aboveground biomass across all inoculation and density treatment combinations. Late plants had similar aboveground biomass across density treatments if they were inoculated with mycorrhizae but if they were un-inoculated, plants in high density had reduced aboveground biomass compared to plants in low-density. Aboveground biomass also showed a significant mycorrhizal inoculation × social environment × planting time interaction (Fig 2). Early plants did not differ in aboveground biomass across social environment and inoculation combinations. Late plants had low aboveground biomass in the un-inoculated treatment regardless of whether the pair was siblings or strangers, but inoculated plants grown with siblings had higher aboveground biomass than inoculated strangers.

Stem height responded to planting time and density. Plants in high-density were taller than those in low density (high-density mean ± SE=32.2419 ± 1.1923; low-density mean ± SE=23.6125 ± 1.2053). Early plants were taller than late plants (early mean ± SE=34.1915 ± 1.0981; late mean ± SE=21.6629 ± 1.2917). The response of stem height to density depended on planting time: early high-density plants were taller than early low-density plants, but late plants did not differ in stem height across density treatments (Fig 3). Stem height showed a significant mycorrhizal inoculation × social environment × planting time interaction: early plants did not differ across social environment and mycorrhizal treatment combinations but in later plants, inoculated siblings were taller

than un-inoculated siblings and strangers did not differ across inoculation treatments (Fig 4).

Vegetative biomass was affected by density, mycorrhizal inoculation, and planting time (Table 4). Plants grown in high-density had lower vegetative biomass than those grown in low-density (Table 5). Inoculated plants had higher vegetative biomass than un-inoculated plants (Table 5). Early plants had higher vegetative biomass than late plants (Table 5). Vegetative biomass showed a significant mycorrhizal inoculation × density interaction. Plants in low-density had high vegetative biomass regardless of mycorrhizal inoculation, but plants in high-density had reduced vegetative biomass in the un-inoculated treatment. Vegetative biomass showed a significant mycorrhizal inoculation × social environment × planting time interaction. Early plants did not differ in vegetative biomass across all inoculation and social environment combinations. Late plants had similar vegetative biomass if they were un-inoculated with mycorrhizae, regardless of social environment. But, if they were inoculated, siblings had higher vegetative biomass than strangers. Vegetative biomass also showed a significant planting time × mycorrhizal inoculation × density interaction. Early plants did not differ in vegetative biomass across density and mycorrhizal inoculation combinations. Late plants grown in low-density did not differ in vegetative biomass across inoculation treatments. However, plants that were inoculated and grown in high-density had higher vegetative biomass than un-inoculated high-density plants. Additionally, inoculated plants did not differ in vegetative biomass across density treatments but un-inoculated plants in low-density had higher vegetative biomass than un-inoculated plants in high-density.

Inflorescence mass responded to density and planting time (Table 4). Plants in high-density had lower male flower mass compared to plants in low-density (high-density mean ± SE=0.0100 ± 0.0020; low-density mean ± SE=0.0302 ± 0.0059). Early plants had lower male flower mass compared to late plants (early mean ± SE=0.0025 ± 0.0052; late mean ± SE=0.1067 ± 0.0225). Inflorescence mass showed a significant social environment × planting time interaction, but it was driven by planting time, i.e. there was no significant effect of social environment on plants within either planting time. Inflorescence mass also showed a significant mycorrhizae × density interaction but it was also dependent upon planting time (3-way interaction; Table 4). For early plants, male flower biomass did not differ within density treatments, regardless of inoculation (Fig 5). However, inoculated plants in low-density had higher male flower mass than inoculated plants in high-density. The same trend was observed for un-inoculated plants. For late plants, inoculated plants had higher male flower mass than un-inoculated plants in high-density but lower male flower mass in low-density. Across density treatments, inoculated plants did not differ in male flower mass but un-inoculated plants had much higher male flower mass in low-density compared to un-inoculated, high-density plants (Fig 5).

Effects on biomass allocation

Stem elongation responded to density (Table 6) with high-density plants being more elongated than low-density plants (high-density mean ± SE=32.2734 ± 0.8087; low-density mean ± SE=25.0313 ± 0.8218). Stem elongation showed a significant density × planting time interaction (Table 6). Early plants responded to density with higher elongation in high-density compared to low-density, but late plants did not differ in

elongation across density treatments. Allocation to seed biomass responded to planting time (Table 6). Early plants had lower seed allocation than late plants (early mean ± SE=0.0094 ± 0.0019; late mean ± SE=0.0776 ± 0.0173). Allocation to male flowers responded to density, planting time, and mycorrhizal inoculation. Early plants allocated less to inflorescences than late plants (early mean ± SE= 0.0017 ± 0.0025; late mean ± SE=0.1387 ± 0.0290). High-density plants had lower allocation to inflorescences than low-density plants (high density mean ± SE=0.0111 ± 0.0021; low-density mean ± SE=0.0258 ± 0.0048). Inoculated plants allocated less to inflorescences than un-inoculated plants (inoculated mean ± SE=0.0131 ± 0.0026; un-inoculated mean ± SE=0.0220 ± 0.0039). Inflorescence mass showed a significant social environment × planting time interaction but this was driven by planting time.

Effects on reproduction

Whether or not plants produced male inflorescences depended on planting time and vegetative biomass (Table 7). Late plants had a higher chance of producing inflorescences than early plants (early planting parameter estimate= -1.8598, mean=0.4498; late planting parameter estimate=0.0000, mean=0.9500; intercept=3.4371). Male flower production also showed a significant density × social environment interaction with strangers in high-density having a lower chance of producing inflorescences than strangers in low-density, but no other differences across treatment combinations (Table 7). Whether or not plants produced female flowers depended on planting time and showed a vegetative biomass × planting time interaction (Table 7). Late plants had a higher chance of producing seeds than early plants (early planting parameter

estimate= -1.1351; late planting parameter estimate=0.0000; intercept=3.1100). Early plants had a greater chance of producing seeds with increasing vegetative biomass, but late plants had a decreasing chance of producing seeds with increasing vegetative biomass (Fig 6).

DISCUSSION

In this study I manipulated social environment and mycorrhizal inoculation of pairs of ragweed grown in high- or low-density. I measured aboveground morphology, performance, (size) and fitness (reproductive output). I had three *a priori* predictions: 1) that plants grown with siblings would outperform strangers 2) inoculated plants would outperform un-inoculated plants; and 3) inoculated siblings would have higher fitness than all other groups due to the combined benefits of mycorrhizae and growing with relatives. Responses of performance traits to mycorrhizal inoculation were dependent upon social environment in experiment two, for plants planted late. Siblings inoculated with mycorrhizae had higher aboveground biomass than all other groups, but only when planted later. Allocation to seed biomass responded to social environment in experiment one, demonstrating a kin recognition response.

When plants were planted later, the aboveground biomass and stem height were greater in siblings with mycorrhizae compared to other groups. This is consistent with increased mycorrhizal colonization of siblings (File, Klironomos et al. 2012; Chapter 2) and my prediction, in Chapter 2, that this would result in fitness benefits for siblings. Siblings may show altruism and compete less than strangers (File, Murphy et al. 2012),

creating a less competitive social environment than that experienced by strangers. Additionally, mycorrhizal inoculation provides many benefits under the right conditions and, increases growth of ragweed (Fumanal, Plenchette et al. 2006). The increased growth and colonization of mycorrhizal fungi in siblings compared to stranger groups in Chapter 2 is presumably because siblings facilitate the symbiosis by donating more carbon to the fungal partner than strangers (File, Klironomos et al. 2012; Chapter 2). This is consistent with altruism among siblings because a common mycorrhizal network (CMN) attaches neighbouring plants and serves as a social good, i.e. a common resource created and maintained by the group (Rankin, Bargum et al. 2007). Although investment in the social good may vary among participants, the benefits received by each are equal so siblings should be selected to invest more in the CMN than strangers. Performance is a predictor of fitness and, here, results indicate that there are lifetime benefits for siblings' increased investment in the social good.

Allocation to seed biomass responded to social environment in experiment one, with siblings allocating more to seeds than strangers. This shift in allocation from vegetative biomass to reproductive biomass in sibling pairs is consistent with the fitness benefit of growing with siblings. If sibling pairs were altruistic and behaved less competitively than stranger pairs, siblings would have been able to invest in reproduction rather than aboveground competitive structures such as branch and stem mass. Although I predicted that fitness or performance would respond to social environment, I may not have found this response due to herbivory, which decreased the sample size and increased trait variation. Additionally, I found no apparent plant responses to the mycorrhizal

inoculation. Thus, if siblings grown with mycorrhizae have lifetime fitness benefits, as I suggested in Chapter 2, I would predict that a lack of response to mycorrhizae, as found in experiment one, should also result in a reduced fitness response to plant social environment.

For many species, mycorrhizal colonization leads to increased biomass since the fungal partner provides numerous benefits such as increased nutrient and water acquisition (Smith and Read 2010) and although benefits are species specific, ragweed growth has been shown to respond positively to mycorrhizae (Fumanal, Plenchette et al. 2006). Here, plants responded to mycorrhizal inoculation in experiment two but not in experiment one. Finding higher aboveground and vegetative biomass in inoculated pots compared to un-inoculated pots in experiment two is indicative of a successful mycorrhizal inoculation treatment. The mycorrhizal inoculation was better controlled in experiment two since I used pasteurized soil in order to kill any mycorrhizal spores already present in the garden soil and I inoculated the soil with mycorrhizal spores in solution, which could explain the difference across experiments. Interestingly, allocation to male inflorescences responded negatively to mycorrhizal inoculation. The effect of mycorrhizae on biomass allocation to reproductive function is species dependent (Philip, Posluszny et al. 2001, Hoffmann, Vierheilig et al. 2011). It remains unclear what causes the decrease in allocation to male reproduction in response to mycorrhizae and a lack of response in female reproduction, but it could demonstrate a cost associated with maintaining the mutualism (Philip, Posluszny et al. 2001).

Density affected performance variables in predictable ways in both experiments and determined responses to mycorrhizal inoculation. The reduced performance and allocation to reproductive function in high-density plants is a result of reduced light availability since belowground density was kept constant across the entire experiment. This is consistent with previous research on density effects (Mustajarvi, Siikamaki et al. 2001). Surprisingly, in experiment one, height and elongation did not respond to density. Height and elongation were difficult to analyze in experiment one because of the release from apical dominance of the main stem and the lack of response is likely due severe herbivory. However, even plants un-affected by herbivores did not respond to density, perhaps because herbivory in high density arrays reduced the actual aboveground density. However, I did find the expected increase in height and elongation in high density in experiment two, in which most plants were not exposed to such damaging herbivory.

Responses to mycorrhizal inoculation were dependent upon density in the second planting time. In experiment 2, inflorescence mass of inoculated early plants showed the typical decreased growth in high-density, indicative of increased competition (Smith, Facelli et al. 2010). But, inflorescence mass and aboveground biomass in inoculated late plants did not respond to density, which I speculate is an indication of mycorrhizae buffering the cost of growing in high density. Differences between planting times could be due to the shift from allocation to vegetative biomass in early plants to allocation to reproductive functions in later plants. Finding increased inflorescence (early and late plants) and aboveground biomass (late plants only) in un-inoculated plants in low density

is consistent with reduced aboveground competition compared to un-inoculated plants grown in high density.

Fitness, performance and morphology responses in experiment two were driven by planting time, which ultimately caused a shift in life history strategy. Early plants allocated more to vegetative biomass, whereas late plants were more likely to produce seeds or inflorescences and allocated more to these reproductive functions. It remains unclear what element of being planted later resulted in higher reproduction but because the second planting happened so late in the summer it is possible that some environmental cue(s), e.g. temperature or photoperiod (day length), or interactions of several cues (Heggie and Halliday 2005) triggered plants to invest earlier in the life cycle in reproduction compared to early plants since the growing season would soon be over (Ims 1990). Intriguingly, planting time also changed the responses to other treatments. Early plants did not respond to social environment, density and mycorrhizae treatment combinations but late plants did. This indicates that field-grown ragweed may only be sensitive to these treatment interactions when they are younger (late plants) and that the strategy may change later on (early plants), resulting in an apparent lack of response in the older plants.

In experiment one, herbivory reduced fitness. This was unsurprising since herbivory was severe and damage to stems was clean cut, consistent medium/large mammals. This resulted in the compensation response. Here we defined plants that were affected by herbivores either as non-compensators, i.e. unable to regain stem biomass, or partial-compensators, i.e. able grow new stems. Increased stem production following the

release of apical dominance is one of the mechanisms that plants use to tolerate herbivory (Strauss and Agrawal 1999). Finding that realized height of partial-compensators did not differ from height of plants un-affected by herbivores demonstrates a tolerance response. However, partial-compensators produced similar seed biomass as non-compensators, demonstrating that they still suffered a cost from herbivory. Because non-compensators were unable to recover sufficient stem biomass, they did not show a tolerance response to herbivory but still managed to produce similar seed biomass to those that did show the tolerance response.

Studies of fitness in plant evolutionary ecology are controversial. This is because regardless of which component of fitness is analyzed, fitness and performance are difficult to interpret if the research question is investigating a process or set of interactions that have occurred during the plant's lifetime. Fitness measures are taken at the final time point of the individual's life and represent the output of all the interactions and forces an individual has experienced including competition, nutrient foraging and reproduction. However, fitness measures don't provide information on any one of these specific interactions (File, Murphy et al. 2012). In fact, some processes could counteract each other, leading to a misinterpretation of what fitness differences among groups really mean. For example, benefits of growing with relatives, i.e. kin selection, could be confounded with the benefits from genetic diversity, i.e. niche partitioning (File, Murphy et al. 2012). Therefore, although fitness outcomes are important in evolutionary biology, it is essential to use caution when interpreting the results of a fitness study. Here, my results from experiment two are interpreted in the context of results from Chapter 2,

which informed us on how social environment affects fungal colonization of ragweed plants at the seedling and juvenile life stages.

In conclusion, late planted siblings inoculated with mycorrhizae outperformed all other late planted groups in experiment two, consistent with lifetime benefits for associating with mycorrhizae when grown with relatives. This supports the notion that siblings donate more carbon to the fungal partner than do strangers, which enhances their social good and, thus, results in greater fitness (File, Klironomos et al. 2012; Chapter 2). Siblings in experiment one allocated more to seed biomass, versus vegetative biomass, than strangers, which is suggestive of reduced competition and possibly increased fitness. However, there was a lack of response to social environment and mycorrhizae by all other traits in experiment one and in experiment two, responses depended upon planting time. This demonstrates the importance of arguments made by File, Murphy and Dudley (2012) in regards to conducting fitness studies; that results should not be interpreted as evidence for or against the demonstration of processes. Future work could explore the quality of offspring produced by siblings compared to strangers in field-grown ragweed to further investigate the extent of fitness benefits for relatives attached by a CMN.

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Table 1: Analysis of variance for *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment one.

	Log seeds			**Log branch/stem**			**Log realized height**			**Log tot height**		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*
Density	1	21.94	**<.0001**	1	10.33	**0.001**	1	0.90	0.34	1	0.22	0.64
Social Environment	1	2.53	0.11	1	0.03	0.86	1	0.17	0.69	1	0.21	0.65
Mycorrhizae	1	1.73	0.19	1	2.46	0.12	1	2.15	0.14	1	2.58	0.11
Compensation	2	9.13	**0.0002**	2	2.09	0.13	2	19.12	**<.0001**	2	36.84	**<.0001**
Density × SocialEnv	1	1.49	0.22	1	0.20	0.65	1	0.07	0.79	1	0.08	0.78
Myc × density	1	0.14	0.71	1	2.97	0.09	1	0.10	0.75	1	0.11	0.75
Myc × SocialEnv	1	1.26	0.26	1	0.46	0.50	1	0.91	0.34	1	1.06	0.31
Myc × SocialEnv × Density	1	0.13	0.72	1	0.12	0.73	1	0.11	0.74	1	0.05	0.83
Block(density)	2	2.33	0.10	2	3.28	**0.04**	2	4.54	**0.01**	2	2.59	0.08

Table 1: Log seed biomass is ln(seeds+0.25). Log branch/stem is ln((branch mass + stem mass) + 0.25). Realized height was calculated as the length of the main stem, plus the length of the longest stem produced following herbivory. Log realized height is ln(realized height + 1). Tot height was calculated as the sum of all stems made following herbivory, i.e. total vertical height. Log tot height is ln(tot height + 1). Analysis was conducted on individual plants that were grown in pairs in the field. Mycorrhizae (Myc), social environment (SocialEnv) and density refer to treatment effects. Compensation refers to whether plants were affected by herbivores and if so, whether they were able to grow additional stems to regain aboveground biomass. Significant values are in bold.

Table 2: Least squared means of aboveground traits of *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment one.

Treatment	Seed mass		Branch/stem mass		Realized height		Total height		Seed allocation	
Social Environment										
Siblings	-0.7086 ± 0.0768	a	-0.6041 ± 0.0752	a	2.6607 ± 0.0588	a	2.8475 ± 0.0679	a	-0.6638 ± 0.0531	a
Strangers	-0.8376 ± 0.0853	a	-0.5897 ± 0.0835	a	2.6860 ± 0.0652	a	2.8800 ± 0.0753	a	-0.7802 ± 0.05883	b
Density										
High	-0.9662 ± 0.0683	a	-0.7268 ± 0.0668	a	2.6434 ± 0.0523	a	2.8466 ± 0.0603	a	-0.8171 ± 0.0484	a
Low	-0.5800 ± 0.0928	b	-0.4670 ± 0.0907	b	2.7033 ± 0.0711	a	2.8808 ± 0.0820	a	-0.6269 ± 0.0640	b
Compensation										
Under-comp	-1.0998 ± 0.1772	a	-0.7754 ± 0.1730	a	2.1160 ± 0.1357	a	2.0983 ± 0.1565	a	-0.9174 ± 0.1226	a
No herb	-0.4157 ± 0.0505	b	-0.4288 ± 0.0501	a	3.0023 ± 0.0386	b	3.0025± 0.0446	b	-0.4964 ± 0.0357	b
Over-comp	-0.8038 ± 0.0971	a	-0.5865 ± 0.0951	a	2.9018 ± 0.0743	b	3.4905 ± 0.0858	c	-0.7522 ± 0.0670	a
Mycorrhizae										
Inoculated	-0.7214± 0.0807	a	-0.5362 ± 0.0789	a	2.7175 ± 0.0618	a	2.9195 ± 0.0712	a	-0.7247 ± 0.0555	a
Un-inoculated	-0.8248 ± 0.0804	a	-0.6576 ± 0.0787	a	2.6292 ± 0.0616	a	2.8080 ± 0.0710	a	-0.7192 ± 0.0559	a

Means presented here are least squared means of ln((branch mass + stem mass) +0.25), ln(seed biomass +0.25), log realized height is ln(realized height + 1), and ln(tot height + 1) ± 1 standard error. Seed allocation means are from ln(seed biomass +0 0.25) with ln((branch mass + stem mass) + 0.25) as the covariate, ± 1 standard error. Mycorrhizae (Myc), density and social environment (SocEnv) refer to treatment effects. Compensation refers to whether plants were affected by herbivores and if so,

whether they were able to grow additional stems to regain aboveground biomass. For each dependent variable and within each treatment, means that did not differ significantly at $P<0.05$ are represented by the same letter.

Table 3: Analysis of covariance for *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment one.

	Log seed biomass (branch/stem mass)			Stem elongation		
Source	**DF**	**F**	***P***	**DF**	**F**	***P***
Social Environment	1	4.27	**0.04**	1	0.24	0.63
Density	1	10.41	**0.002**	1	2.54	0.11
Mycorrhizae	1	0.01	0.92	1	0.06	0.81
Compensation	2	7.48	**0.0008**	2	52.20	**<0.0001**
Density × SocEnv	1	0.79	0.38	1	0.19	0.67
Myc × Density	1	2.58	0.11	1	0.63	0.43
Myc × SocEnv	1	0.47	0.49	1	0.04	0.83
Myc × Density × SocEnv	1	0.09	0.76	1	0.46	0.50
Block(density)	2	0.31	0.74	2	6.06	**0.003**

Allocation to seed biomass and stem elongation for individual plants that were grown in pairs in the field. The sum of the height of all stems on a plant was used to analyze elongation. Mycorrhizae (Myc), density and social environment (SocEnv) refer to treatment effects. Compensation refers to whether plants were affected by herbivores and if so, whether they were able to grow additional stems to regain aboveground biomass. Significant values are in bold.

Table 4: Analysis of variance for *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment two.

	Log above			**Log seeds**			**Height**			**Log veg**			**Log inflorescence**		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*
Density	1	5.24	**0.02**	1	0.11	0.75	1	25.91	**<0.0001**	1	6.17	**0.01**	1	18.61	**<0.0001**
Social Environment	1	2.48	0.12	1	0.36	0.55	1	0.90	0.35	1	1.76	0.19	1	0.30	0.58
Mycorrhizae	1	4.91	**0.03**	1	0.69	0.41	1	0.83	0.36	1	5.89	**0.02**	1	0.60	0.44
Planting time	1	13.56	**0.0003**	1	29.38	**<0.0001**	1	54.61	**<0.0001**	1	38.84	**<0.0001**	1	205.55	**<0.0001**
Density × SocialEnv	1	0.01	0.94	1	0.39	0.53	1	0.57	0.45	1	0.17	0.68	1	0.35	0.56
Myc × Density	1	10.54	**0.001**	1	0.47	0.50	1	0.36	0.55	1	10.90	**0.001**	1	7.31	**0.008**
Myc × SocialEnv	1	1.97	0.16	1	2.29	0.13	1	0.01	0.94	1	2.18	0.14	1	1.32	0.25
Density × Plnt	1	0.60	0.44	1	0.06	0.80	1	15.29	**0.0001**	1	0.94	0.33	1	0.52	0.47
SocialEnv × Plnt	1	0.05	0.82	1	0.04	0.84	1	0.14	0.71	1	0.01	0.91	1	4.04	**0.05**
Myc × Plnt	1	1.40	0.24	1	1.02	0.32	1	2.77	0.10	1	1.46	0.23	1	2.37	0.13
Myc × Density × SocialEnv	1	0.03	0.87	1	0.59	0.45	1	0.05	0.83	1	0.07	0.79	1	1.68	0.20
Density × SocialEnv × Plnt	1	0.10	0.76	1	1.02	0.32	1	0.90	0.34	1	0.03	0.87	1	2.39	0.12
Myc ×Density × Plnt	1	13.76	**0.0003**	1	0.01	0.93	1	2.22	0.14	1	13.95	**0.0003**	1	5.34	**0.02**
Myc × SocialEnv × Plnt	1	7.89	**0.006**	1	0.22	0.64	1	6.36	**0.01**	1	8.58	**0.004**	1	3.36	0.07
Myc × Density × SocialEnv × Plnt	1	0.21	0.65	1	0.47	0.49	1	1.01	0.32	1	0.48	0.49	1	1.62	0.21

Log above is ln(aboveground biomass +0.02), log veg is ln(veg), log seeds is ln(seed biomass +0.0005) log loginflorescence is ln(male flower mass + 0.0005). Analysis was conducted on individual plants that were grown in pairs in the field. Mycorrhizae,

density and social environment refer to treatment effects. Planting time (Plnt) refers to whether plants were planted in June or August, 2012. Significant values are in bold.

Table 5: Least squared means of vegetative biomass for *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment two.

Treatment	Vegetative mass	
Social Environment		
Siblings	0.4122 ± 0.0748	a
Strangers	0.2693 ± 0.0773	a
Density		
High	0.2072 ± 0.0756	a
Low	0.4744 ± 0.0765	b
Planting time		
Early	0.6760 ± 0.0697	a
Late	0.0057 ± 0.0820	b
Mycorrhizae		
Inoculated	0.4714 ± 0.0797	a
Un-inoculated	0.2102 ± 0.0722	b

Means presented here are the least squared means of the log transformed vegetative biomass (ln(veg mass)). Mycorrhizae, density and social environment refer to treatment effects. Planting time refers to whether plants were planted in June (1) or August (2), 2012. Significant values are in bold.

Table 6: Analysis of covariance for *Ambrosia artemisiifolia* plants grown in pairs in the field in experiment two.

	Seed allocation			**Stem elongation**			**Allocation to male flowers**		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*
Density	1	1.47	0.23	1	39.48	**<0.0001**	1	11.68	**0.0008**
Social Environment	1	0.03	0.87	1	0.53	0.47	1	0.00	0.96
Mycorrhizae	1	0.00	0.99	1	0.00	0.95	1	4.31	**0.04**
Planting time	1	52.09	**<0.0001**	1	0.79	0.37	1	255.29	**<0.0001**
Density × SocialEnv	1	0.66	0.42	1	0.06	0.80	1	0.98	0.32
Myc × Density	1	0.20	0.66	1	0.60	0.44	1	0.46	0.50
Myc × SocialEnv	1	1.14	0.29	1	0.00	0.95	1	1.13	0.29
Density × Plnt	1	0.37	0.54	1	15.16	**0.0001**	1	1.26	0.26
SocialEnv × Plnt	1	0.10	0.75	1	0.69	0.41	1	5.28	**0.02**
Myc × Plnt	1	0.40	0.53	1	2.94	0.09	1	0.87	0.35
Myc × Density × SocialEnv	1	0.72	0.37	1	0.04	0.84	1	1.94	0.17
Density × SocialEnv × Plnt	1	0.10	0.75	1	0.26	0.61	1	0.99	0.32
Myc ×Density × Plnt	1	1.45	0.23	1	0.34	0.56	1	0.00	0.94
Myc × SocialEnv × Plnt	1	2.28	0.13	1	0.73	0.39	1	1.13	0.29
Myc × Density × SocialEnv × Plnt	1	0.23	0.63	1	0.41	0.52	1	1.25	0.26

Analysis was conducted on individual plants that were grown in pairs in the field.
Mycorrhizae (Myc), density and social environment (SocEnv) refer to treatment effects.
Planting time (Plnt) refers to whether plants were planted in June or August, 2012.
Significant values are in bold.

Table 7: Generalized linear model of male and female reproduction for pairs of ragweed grown in the field (experiment two).

Source	Male reproduction *P*	Female reproduction *P*
Log vegetative biomass	**0.03**	0.18
Density	0.09	0.71
Planting time	**<0.0001**	**0.03**
Mycorrhizae	0.48	0.24
Social environment	0.99	0.98
Log veg × plnt	0.09	**0.0018**
Plnt × density	0.22	0.19
Plnt × Myc	0.44	0.71
Density × SocEnv	**0.05**	0.93
Plnt × SocEnv	0.30	0.69
Myc × SocEnv	0.35	0.21

Mycorrhizae (Myc), density and social environment (SocEnv) refer to treatment effects. Planting time refers to whether plants were planted in June or August, 2012. Log vegetative biomass was calculated as (ln(veg mass)). Significant values are in bold.

Fig 1: Effect of planting time, density and mycorrhizal inoculation on aboveground biomass of plants grown in pairs in the field. 1st planting (left panel) occurred in June and 2nd planting (right panel) occurred in August, 2012. Aboveground density was either high or low, with belowground density remaining constant. Pairs of plants were either inoculated with *Glomus intraradices* (black bars) or left un-inoculated (white bars). Within each panel, bars with different letters are statistically different at the $P<0.05$ level.

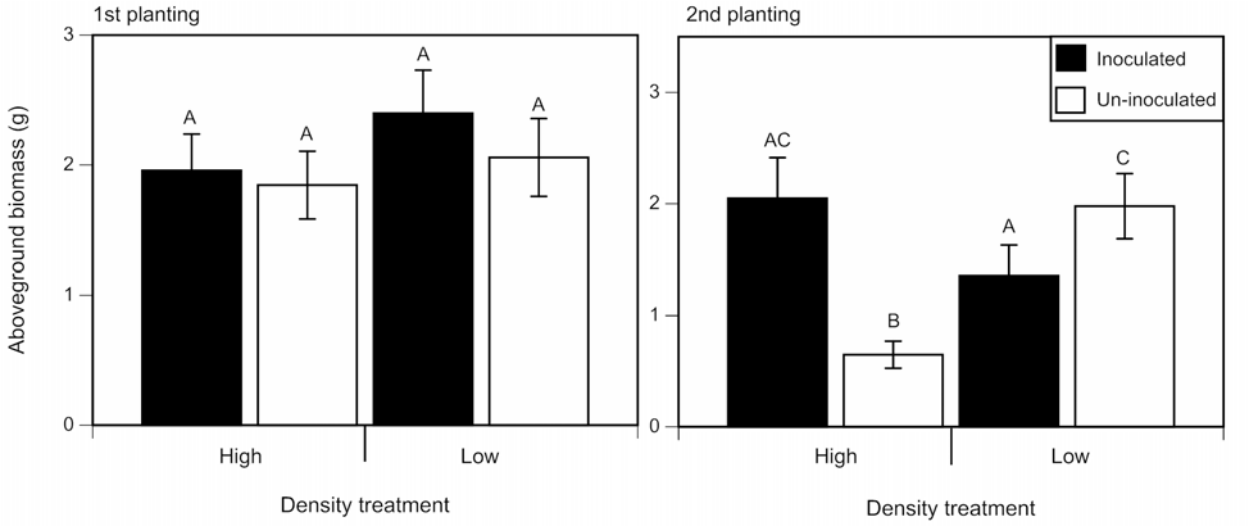

1st planting
2nd planting
Aboveground biomass (g)
Inoculated
Un-inoculated
A
A
A
A
AC
B
A
C
High
Low
Density treatment
0
1
2
3

Fig 2: Effect of planting time, social environment and mycorrhizal inoculation on aboveground biomass of plants grown in pairs in the field. 1st planting (left panel) occurred in June and 2nd planting (right panel) occurred in August, 2012. Plants were grown in pairs of siblings or strangers and were either inoculated with *Glomus intraradices* (black bars) or left un-inoculated (white bars). Within each panel, bars with different letters are statistically different at the $P<0.05$ level.

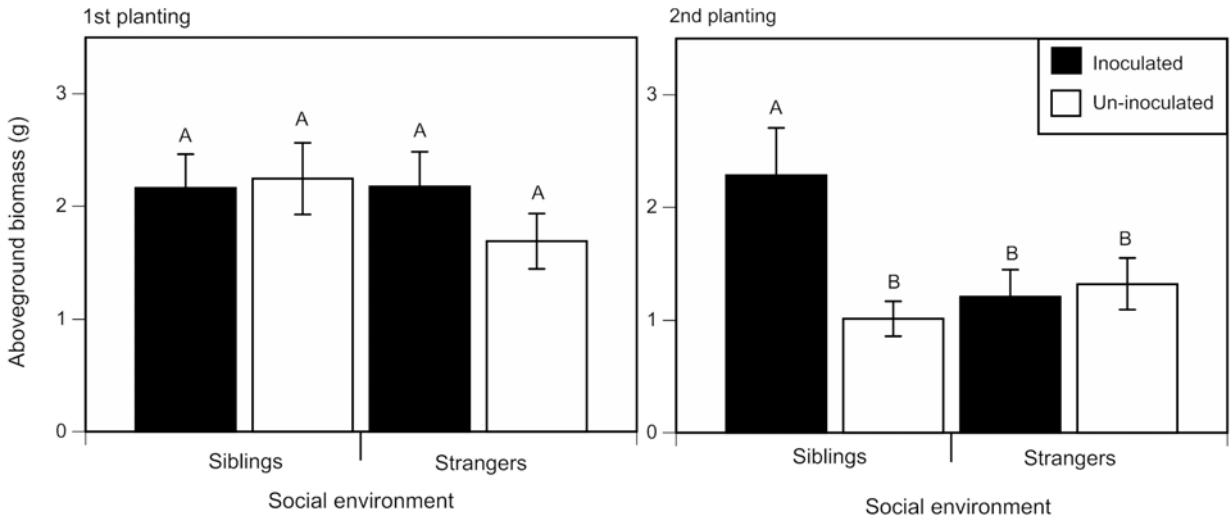

1st planting
2nd planting
Aboveground biomass (g)
0
1
2
3
A
A
A
A
B
B
B
Inoculated
Un-inoculated
Siblings
Strangers
Social environment

Fig 3: Effect of planting time and density on stem height of plants grown in pairs in the field. Plants were either planted in the field in June (1^{st}) or August (2^{nd}), 2012. Aboveground density was either high (black bars) or low (white bars), with belowground density remaining constant. Bars with different letters are statistically different at the $P<0.05$ level.

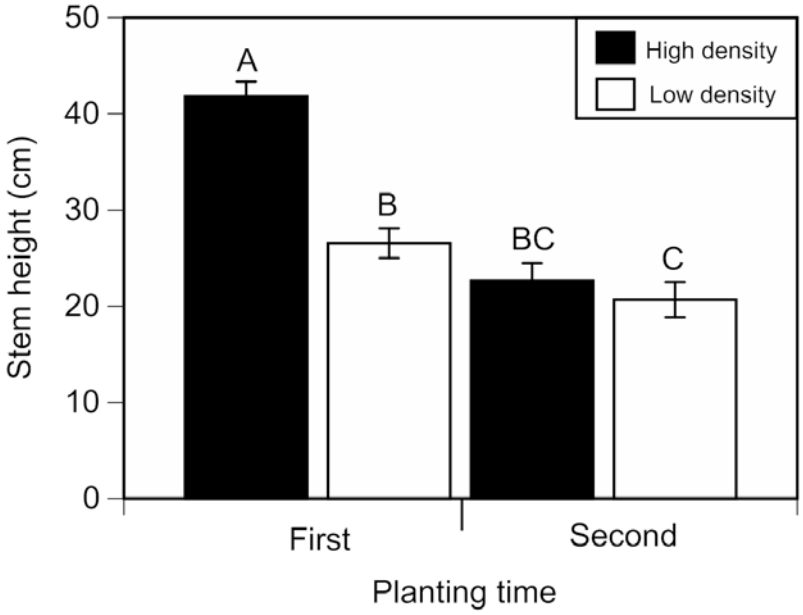

50
40
30
20
10
0
Stem height (cm)
A
B
BC
C
High density
Low density
First
Second
Planting time

Fig 4: Effect of planting time, social environment and mycorrhizal inoculation on stem height of plants grown in pairs in the field. 1st planting (left panel) occurred in June and 2nd planting (right panel) occurred in August, 2012. Plants were grown in pairs of siblings or strangers and were either inoculated with *Glomus intraradices* (black bars) or left un-inoculated (white bars). Within each panel, bars with different letters are statistically different at the $P<0.05$ level.

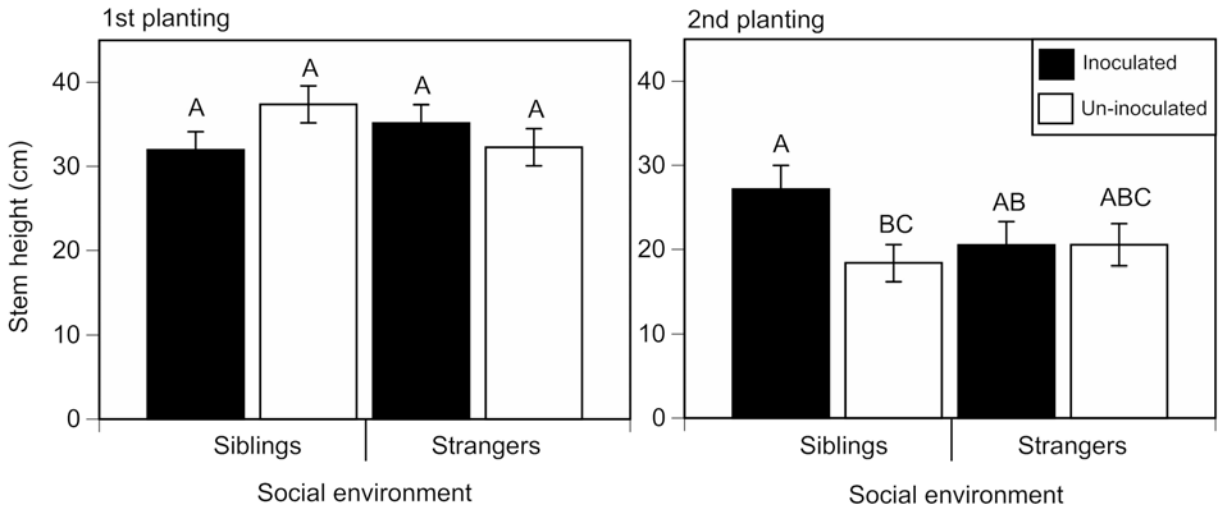

1st planting
2nd planting
Inoculated
Un-inoculated
Stem height (cm)
0
10
20
30
40
A
A
A
A
A
BC
AB
ABC
Siblings
Strangers
Social environment
Siblings
Strangers
Social environment

Fig 5: Effect of planting time, density and mycorrhizal inoculation on male flower (inflorescence) mass of plants grown in pairs in the field. 1st planting (left panel) occurred in June and 2nd planting (right panel) occurred in August, 2012. Aboveground density was either high or low, with belowground density remaining constant. Pairs of plants were either inoculated with *Glomus intraradices* (black bars) or left un-inoculated (white bars). Within each panel, bars with different letters are statistically different at the $P<0.05$ level. Note the different scales on the y-axes for 1st and 2nd panels.

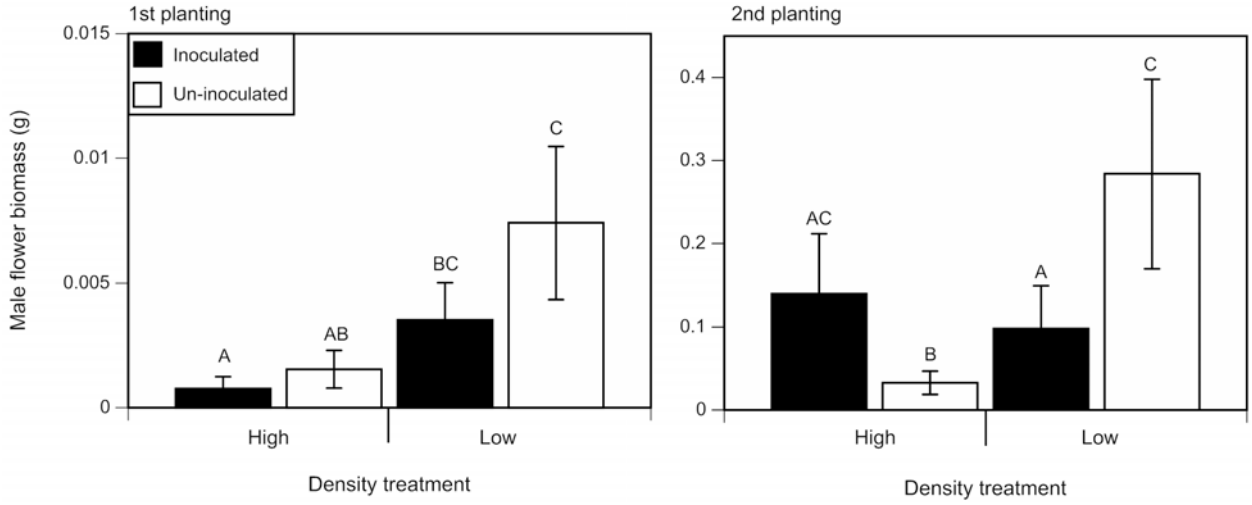
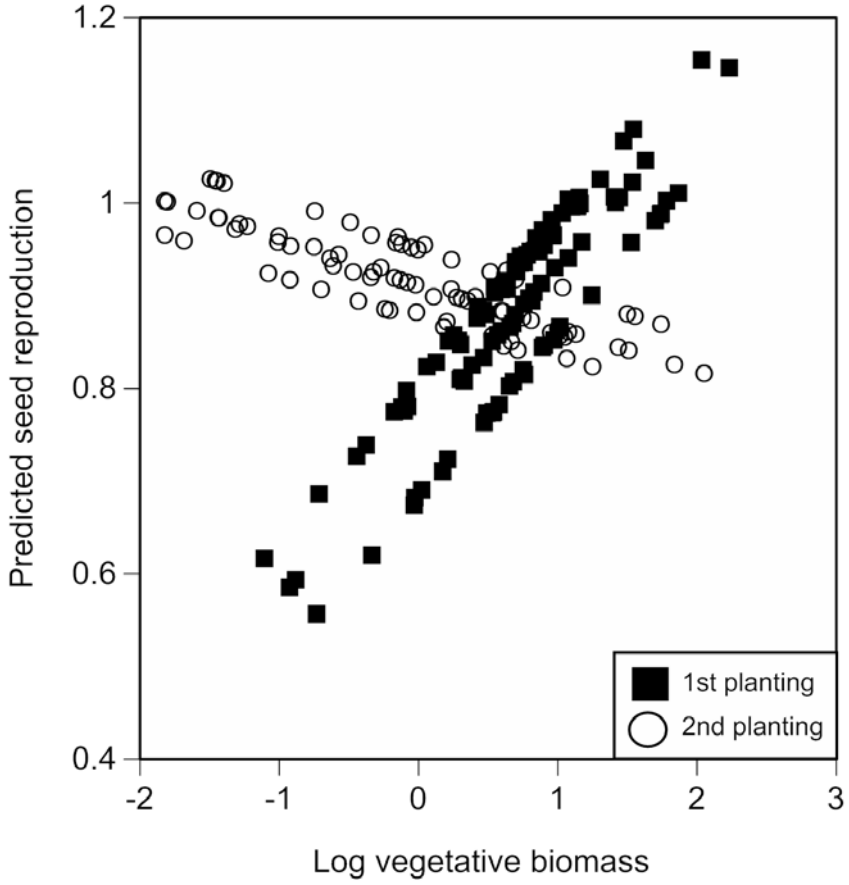

1st planting
Inoculated
Un-inoculated
Male flower biomass (g)
0.015
0.01
0.005
0
A
AB
BC
C
High
Low
Density treatment
2nd planting
0.4
0.3
0.2
0.1
0
AC
B
A
C
High
Low
Density treatment

Fig 6. Predicted probability of seed production across increasing vegetative biomass. Ragweed plants were planted in June (1st planting, i.e. early) or August (2nd planting, i.e. late). Predicted probabilities were calculated using ANOVA.


1.2
1
0.8
0.6
0.4
Predicted seed reproduction
-2
-1
0
1
2
3
Log vegetative biomass
1st planting
2nd planting

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CHAPTER 4

HOW DO ASYMMETRIC STRESS AND SOCIAL ENVIRONMENT OF PLANTS AFFECT THEIR GROWTH AND ASSOCIATION WITH MYCORRHIZAL FUNGI?

ABSTRACT

Mycorrhizal colonization of soil and plant roots is dependent upon the social environment of the group of plants. However, the physiological status of the plants may also be important.

Whether or not plants are stressed, and whether that stress is asymmetric, e.g. one plant is more stressed than its neighbour, could have implications on the mycorrhizal mutualism. A stressed plant could effectively cheat the symbiosis by paying less carbon and reaping benefits from its neighbour's contributions. Here, I show that mycorrhizal colonization of pairs of plants depends on whether one plant, neither plant, or both plants are shaded. Additionally, I show that specific leaf area (SLA), an aboveground competitive trait, of *Plantago lanceolata* responds to neighbour relatedness, demonstrating kin recognition. Interestingly, this response was dependent upon the presence of mycorrhizal fungi. Results are consistent with kin selection and demonstrate that plant kin recognition could be facilitated by a common mycorrhizal network.

INTRODUCTION

Mycorrhizal fungi provide several benefits to their plant hosts including increased nutrient uptake, greater access to water and protection from root enemies; all in exchange

for carbon, which allows the fungus to grow and reproduce (Smith and Read 2010). Fungal hyphae colonize the soil, foraging for soil nutrients and form a common mycorrhizal network (CMN) among plants. Because the CMN acts as a shared resource among connected plants, and it is created and maintained by the group, it can be considered a social good (Rankin, Bargum et al. 2007). If a plant donates carbon to the fungal partner, it supports the social good, which directly benefits the plant and also benefits attached neighbours. Equally, if one plant cheats by not contributing as much carbon as other members, the CMN will be less developed but that cost is shared among the entire group. The better supported by plant carbon the CMN is, the greater the benefits received by attached plants since a more extensive fungal network can more effectively explore the soil for nutrients. How much carbon plants donate to the CMN could depend on their social environment, i.e. whether they are growing next to relatives or non-relatives, and therefore may be shaped by kin selection. Plants are expected to donate more carbon to their fungal partner when the CMN is attached to siblings since relatives should gain inclusive fitness from the better supported social good. Non-relatives, however, are expected to donate less carbon since the incentive to cheat is higher due to a lack of inclusive fitness benefits. In Chapter 2, I show that fungal growth and colonization responds to plant social environment (File, Klironomos et al. 2012), but what if neighbouring plants are under differential, i.e. asymmetric, light availability?

Abiotic stress shapes plant-mycorrhizal fungal interactions. Fungal colonization of plant roots is most successful when soil nutrients, especially phosphorus, are low since the plant partner is dependent upon the fungus to capture limiting nutrients (Smith and

Read 2010). However, when plants are exposed to low light they produce less photosynthate, and therefore have less available carbon to donate to the fungus (reviewed in Smith and Read 2010), reducing mycorrhizal infection (e.g. Hayman 1974, Tester, Smith et al. 1986, Son and Smith 1988, Pearson, Smith et al. 1991, Whitbeck 2001; although see Furlan and Fortin 1977, Graham, Leonard et al. 1982, Smith and Gianinazzipearson 1990). If one plant attached to a CMN has lower light availability, it can gain carbon from the plant with greater light availability, apparently through passive transport, i.e. a source-sink relationship (reviewed in Simard, Durrall et al. 2002). In a source-sink scenario the donor plant has more carbon than the receiver because the receiver plant is either under different resource conditions or at an earlier life stage. In this circumstance, the plant with fewer resources, e.g. in low light, could be considered a cheater (*sensu* Brosnan and Bshary 2010) since it can pay less carbon to the fungal partner but still receives benefits, causing an increased carbon cost to the high light neighbour. Thus, supporting mycorrhizae would be costly to the plant with high light unless it could reap inclusive fitness benefits, i.e. the neighbour is a relative. In animals, there are opportunities for reciprocal altruism, i.e. helping another individual with the expectation that help will be given to them in the future (Trivers 1971). But in annual plants, this is unlikely since environmental conditions are similar over a plant's lifetime and size asymmetries between individuals tend to persist (Schwinning and Weiner 1998, Nagashima 1999).

Plants often live in high-density and, depending on the dispersal mechanism (Wade 1980), close to relatives (reviewed by Kelly 1996). Thus, plants attached by a

CMN can be kin. This creates the opportunity for kin selection to act on plant investment in the fungal partner since kin would benefit each other through increased investment in the fungal partner when sharing a CMN. In Chapter 2, I showed that relatedness, i.e. social environment, of common ragweed affects mycorrhizal growth and colonization. Groups of ragweed siblings have greater fungal colonization and growth of soil hyphae compared to groups of strangers, resulting in increased nutrient uptake and reduced pathogen lesions (File, Klironomos et al. 2012; Chapter 2). Though several studies indicate passive transport of carbon to a carbon-poor plant via the CMN, none have looked at how the social environment (relatedness of the group) affects plant performance and fungal colonization of plants under asymmetric light availability, i.e. whether plants change their behavior if the mutualism becomes more costly, and if so, whether those changes are predicted by kin selection. In this study, inoculated and un-inoculated plants were grown in sibling or stranger pairs, with both plants, only one, or neither plant shaded, and we measured fungal colonization and plant functional traits. I asked the following questions: **1) How does asymmetric light availability of a pair of plants affect growth and colonization of *Glomus intraradices* and does plant social environment affect the response? 2) Does *Plantago lanceolata* respond to social environment and are the responses dependent upon shade, and mycorrhizal inoculation? 3) Are plant responses affected by shading of neighbours?**

MATERIALS & METHODS

Study species

Plantago lanceolata L. (family Plantaginaceae) is a perennial herb which is widely distributed across North America. It has a rosette growth form, which means it has a modified stem with very small internodes and as such the leaves grow in a circle, close to the ground. *Plantago lanceolata* forms strong mycorrhizal associations and has been used extensively in studies of mycorrhizal ecology. Previous research has shown flower production of *P. lanceolata* responds to relatedness of the group (Tonsor 1989) and that this species can transfer carbon from one plant to another when connected by mycorrhizal network (Fitter, Graves et al. 1998).

Seeds were collected from the Royal Botanical Gardens, Burlington Ontario (Rock Chapel trail) in August 2010. Individuals were found along the trail edge as *P. lanceolata* grows well in disturbed areas such as ditches. *P. lanceolata* is self-incompatible and wind-pollinated (Kuiper and Bos 1992) so depending on the pollen donor, the seeds collected from one mother plant were either half sibs (same mother, different father; r=0.25) or full sibs (same mother and father; r=0.50). Entire mother plants, with seeds attached, were stored in individual paper bags, dried at room temperature and stored until seeds were needed for this experiment.

Seed germination

On April 23rd, 2012, seeds from eight maternal sibships (hereafter, families) were removed from the inflorescences and freed from the hulls (or chaff). Seeds were put on moist sand in petri dishes in the growth chamber. Petri dishes were kept covered overnight to avoid drying out but lids were taken off during the day so to allow for

airflow. As seeds germinated they were transferred to individual cells in 6-cell gardening packs filled with sand. These were kept under grow lights in the lab until several trays were full. Transplanted seedlings were taken to the greenhouse on May 7th, 2012. Because the number of germinated seedlings was inadequate for sufficient sample size, more seeds (10 families) were prepped for germination on Petri dishes on May 15th, 2012. These were transplanted to cell packs as they germinated and transported to the greenhouse on May 24th, 2012. All seedlings in the greenhouse were watered every second day and fertilized weekly. By the time the first blocks of the experiment were planted, seedlings were roughly the same size despite their difference in germination time.

Setup and experimental design

A 1:1 ratio by volume of play sand (Premier Tech Home & Garden Inc, Brantford, ON) and turface (Profile Products LC, Buffalo Grove, IL, USA) was used for this experiment. The sand/turface was thoroughly mixed on a bleached tarp before pasteurizing. Seven blocks were set up, each using four trays containing nine plastic 2.83L pots (Tall Ones, Stuewe & Sons, Tangent, Oregon), for a total of 252 pots across three greenhouse benches. Pots were filled with the sand/turface mixture up to 3 inches (7.62 cm) below the top of the pot. 50mL of pasteurized garden soil (Garden Gallery Premium Garden Soil, Dundas, Ontario) was spread across the surface of the sand/turface mix, to serve as a substrate for mycorrhizal spores. This topsoil layer was added to all pots to avoid confounding effects of mycorrhizal inoculation and substrate composition. More sand/turface was used to fill up the remaining pot volume.

Social environment treatment: To test the hypothesis that social environment would affect growth and biomass of *Plantago lanceolata* and mycorrhizal colonization, two seedlings were planted in each pot. Seedlings were either from the same family (kin) or from two different families (strangers). Because there was ere not enough seedlings to plant the entire experiment at once, planting took place on three separate dates: June 8th, June 13th and July 5th, 2012.

Mycorrhizal treatment: To test whether plant responses to social environment were dependent upon mycorrhizal inoculation and to test for fungal responses to plant social environment, half of the pots were inoculated with 400 spores of *Glomus intraradices* (Premier tech biotechnologies, Riviere-Du-Loup, Quebec) and half of the pots were left un-inoculated. Pots that were inoculated with spores were randomly interspersed among un-inoculated pots within a block but care was taken to not cross-contaminate.

Shade treatment: To test whether asymmetric light availability affects growth and colonization of *Glomus intraradices* and plant responses to social environment, pots were assigned one of three shade treatments: 1) both plants were un-shaded; 2) one plant was shaded, one was un-shaded; or 3) both plants were shaded. Shades were cylinders (diameter=3”; height=12”) made from black fiberglass insect screening (Phifer wire products, USA), placed over top each plant and secured in the soil with a bamboo stake, so that the plant was shaded from all sides, but not from the top. This allowed water to reach the shaded plants and was meant to minimize microclimate differences between shaded and un-shaded plants. Shades were installed on earlier planted plants on July 24th and on later planted plants on August 10th. A light meter was used to confirm that shades

reduced photosynthetically active radiation (PAR). PAR was reduced most at the bottom of the shades, near the soil surface (Shaded PAR=94.3 μmol/photons/m^2/second; un-shaded PAR=182.3 μmol/photons/m^2/second).

Fertilization

Seedlings were initially given 20-20-20 NPK fertilizer (1tsp/gal) bi-weekly before they were transplanted into experimental blocks. After transplanting, on July 23rd and August 9th, all seedlings were fertilized with 15-15-30 NPK soluble fertilizer with chelated micronutrients (1tsp/gallon, Plant Products Company Ltd, Brampton, Ontario). Plants were watered with the fertilizer solution until the pots were saturated to ensure equal nutrient application.

Harvest and data collection

Aboveground biomass was harvested from August 28-Aug 30th, 2012. Rosettes were clipped at the soil level and the longest leaf was determined. The width and length of the longest leaf was recorded as well as the total number of leaves, number of clones, number of flowers and length of the longest inflorescence. Leaf area of the tallest leaf was measured using a leaf area meter (Analytical Development Company Limited, AM100). All aboveground biomass was dried in a drying oven for 24 hours at 100 degrees F (37.8 degrees Celsius). After aboveground biomass was removed, pots were put in a 5° Celsius cold room until root washing occurred to prevent growth of saprobes. Prior to washing the roots of a given pot, the entire column of soil and roots was carefully removed from the pot. A sample of soil and roots was taken from the bottom of the column, and then cut in half so that approximately 140mL of roots/soil was packaged for later fungal

quantification and the other 140mL sample was washed free of substrate to estimate the biomass of the sample sent for fungal analysis. The rest of the roots in each pot were washed free of substrate and dried in a drying oven at 100 degrees F (37.8 degrees Celsius). All plant parts were weighed to a resolution of 4 decimal places (0.0001 g) to obtain dried biomass.

Data analysis

SAS 9.2 (version 9.2; SAD, Cary, NC, USA) was used for statistical analysis using PROC GLM and PROC PRINCOMP. Biomass and leaf area variables were logged to satisfy the assumptions of the GLM. To test for treatment effects on vegetative biomass, analysis of variance (ANOVA) was done on individual plants with social environment, mycorrhizae, shade and neighbour shade as the independent variables (Table 1). To test for treatment effects on specific leaf area (SLA), analysis of covariance (ANCOVA) was conducted with leaf area of the tallest leaf as the dependent variable and dry mass of that leaf as the covariate; mycorrhizal inoculation, social environment, shade and neighbour shade were the independent variables (Table 2). A quadratic term for leaf dry mass was added to this model to improve the model fit. To test for treatment effects on allocation to fine roots, ANCOVA was conducted with log fine root mass as the dependent variable and log vegetative biomass as the covariate; mycorrhizal inoculation, social environment and shade were the independent variables (Table 3). Analyses for fungal structures were done at the pot level since root and soil samples were collected from the bottom of each pot without the possibility of knowing the identity of the roots. To test for treatment effects on mycorrhizal fungal colonization, ANOVAs were

conducted on pots that had been inoculated with fungal spores. Root colonization of arbuscules, vesicles and hyphae, and soil colonization of hyphae were dependent variables, and social environment, shade treatment and block were the independent variables (Table 4). Here, block reflects planting time and environmental heterogeneity in the greenhouse and thus responses to block are generally uninteresting.

RESULTS

Effects on mycorrhizal structures

Social environment had a significant main effect on arbuscules, root hyphae and soil hyphae (Table 4). Plants grown with siblings had over 1.5 times the arbuscules colonizing their roots compared to plants grown with strangers (Table 5). Siblings had greater hyphal root colonization than strangers (Table 5) and pots with siblings had more soil hyphae than pots of strangers. However, vesicles were not affected by social environment. Shade had a significant effect on all mycorrhizal structures. When both plants were shaded, colonization of arbuscules, vesicles, root hyphae and soil hyphae was lowest; when one plant was shaded, fungal colonization intermediate; and when neither plant in the pot was shaded, colonization was highest (Table 5).

Mycorrhizal colonization demonstrated a significant interaction between shade treatment and social environment that differed among fungal structures. When both plants were shaded, there was no difference in arbuscular colonization between sibling and stranger pairs. When one plant was shaded, siblings had twice the arbuscular colonization compared to stranger pairs and when neither plant was shaded, siblings had 1.5 times the arbuscular colonization of that of strangers (Fig 1). Social environment did not affect root

hyphal colonization when both or neither plants were shaded, but when one plant was shaded, siblings had significantly more root hyphae than strangers (Fig 2). When both plants were shaded, siblings had lower vesicular colonization than stranger pairs; when one plant was shaded siblings had greater vesicular colonization than stranger pairs; and colonization did not differ across social environments when neither plant was shaded (Fig 3). Soil hyphal length was not affected by social environment when both plants were shaded, but when one or neither plants were shaded, siblings had significantly more soil hyphal growth compared to strangers (Fig 4). Block had no significant effect on colonization by any of the mycorrhizal structures.

Effects on plant traits

Vegetative biomass was affected by shade treatment and block (Table 1). Shaded plants had lower vegetative biomass than un-shaded plants (shaded mean=0.7783, SE=0.0277; un-shaded mean=1.0955, SE=0.0324). Allocation to fine roots was affected by shade treatment and block (Table 3). Pots with two shaded plants had the lowest allocation to fine roots; pots with one shaded plant had intermediate fine root allocation; and pots with both plants un-shaded had the highest fine root allocation (Fig 7).

Specific leaf area (SLA) was affected by social environment, shade treatment, neighbour shade and block (Table 2). Plants grown with siblings had lower SLA than plants grown with strangers (sibling mean=27.8574, SE=0.3067; strangers mean= 28.7244, SE=0.3084). Shaded plants had higher SLA than un-shaded plants (shaded mean=30.3340, SE=0.3175; un-shaded mean=26.2477, SE=0.3134). If the neighbour was shaded, the focal plant also had increased SLA compared to those with un-shaded

neighbours (shaded neighbour mean=29.2077, SE=0.3116; un-shaded neighbour mean=27.3741, SE=0.3053). The response to shade depended on neighbour shade such that un-shaded plants with a shaded partner had significantly higher SLA than those with an un-shaded partner, whereas shaded plants had similar high SLA regardless of their neighbour's shade treatment (Fig 5). The effect of social environment depended on mycorrhizal inoculation with un-inoculated plants not differing in SLA across social environments. However, inoculated strangers had higher SLA compared to inoculated siblings. Inoculated strangers also had higher SLA than un-inoculated strangers, whereas inoculated siblings had similar SLA regardless of mycorrhizal treatment (Fig 6). SLA showed a significant shade × mycorrhizal inoculation interaction with un-shaded plants having slightly higher SLA when inoculated, but shaded plants having slightly higher SLA when un-inoculated, though the effects of inoculation within shade treatments were not themselves significant (Table 2).

DISCUSSION

In this study, I manipulated mycorrhizal inoculation and social environment of pairs of plants that experienced either the same or asymmetric light availability. I measured fungal colonization and plant functional traits. I predicted kin recognition responses in both plant traits and fungal colonization, and asked how such responses were affected by manipulating the light availability of one or both plants. Both functional traits and fungal colonization demonstrated responses to social environment. In particular, one plant functional trait, SLA, demonstrated a kin recognition response, but only if pots were inoculated with mycorrhizae. Sibling pairs had higher fungal colonization even when

light availability was asymmetric. In contrast, stranger pairs maintained low fungal colonization unless both plants were un-shaded.

Fungal growth and colonization were increased in sibling pairs, which is consistent with altruism towards siblings as demonstrated in Chapter 2. The response to siblings was affected by the shade treatment. When one or neither plant in a sibling pair was shaded, I found high fungal colonization and growth. This is consistent with un-shaded sibling pairs donating high amounts of carbon, and therefore producing the strongest mutualism with the fungal partner, i.e., high resource exchange opportunities and a larger CMN. Strangers were expected to behave selfishly by not donating carbon to their fungal partner in order to avoid the cost of helping non-relatives. This was true for pairs with one or two shaded plants. However, strangers did have increased fungal colonization and growth when neither plant was shaded, with hyphal and vesicle colonization as high as un-shaded sibling pairs. This is consistent with both plants contributing to the fungal partner and implies reciprocity between neighbours (Trivers 1971, Nowak 2006). When one plant was shaded, I found that siblings maintained high levels of fungal colonization and growth, e.g. siblings with asymmetric light availability had more than double the arbuscule colonization than a shaded pair. However, strangers had decreased fungal colonization when under asymmetric light availability. Shaded plants received less PAR than un-shaded plants and likely photosynthesized less, leaving them with less available carbon to donate to a fungal partner. This is supported by our finding of reduced vegetative biomass in shaded plants. Assuming that the shaded plant had low enough light availability that it could not donate much carbon to the fungus, this

is equivalent to the shaded plant cheating and the un-shaded sibling showing altruism by donating enough carbon to maintain high fungal growth and colonization. If the un-shaded stranger were to donate as much carbon as the un-shaded sibling, it could potentially provide a large benefit to the shaded neighbour, which would ultimately lead to no inclusive fitness gains due to the lack of relatedness. Low levels of fungal colonization and growth in pots with both plants shaded indicates reduced carbon availability and an inability of siblings to increase carbon donations.

Vesicles responded similarly to other fungal structures when the plants were grown with siblings but when plants were grown with strangers, the vesicle response was unexpected. Vesicles are widely accepted as the storage units for the fungus (Denison and Kiers 2011), and may serve a reproductive function (Peterson, Massicotte et al 2004), i.e. they are strictly beneficial to the fungus. Therefore, an increase in vesicle colonization may indicate either that the fungus has access to excess carbon or that the fungus senses reduced carbon availability and is storing carbon in anticipation of poor conditions in the future. Rather than making short-lived arbuscules, storing lipids could allow fungi to produce spores even in poor conditions. There is no functional argument for why strangers with one shaded plant have reduced vesicle colonization compared to the other shade treatments.

Mycorrhizal inoculation affected plant responses to social environment in specific leaf area, an aboveground competitive trait, consistent with kin recognition. SLA was higher in inoculated strangers than inoculated siblings and un-inoculated strangers suggesting that strangers were able to be more competitive when associating with

mycorrhizal fungi. Plants use vertical structures, or spacers, to reach available light and when plants are competing for light they may preferentially invest in these vertical spacers to grow taller than their neighbours (reviewed in Weijschede, Berentsen et al. 2008). Since *P. lanceolata* is a rosette, stem elongation is not possible so the leaves themselves act as the vertical spacers. A shift in morphology of these vertical spacers, i.e. changes in SLA, is arguably competitive since higher leaf area for a given mass means greater light interception, and possibly increased neighbour shading (Schieving and Poorter 1999, Vermeulen, Anten et al. 2013). However, higher SLA comes at a cost since greater surface area means increased transpiration from the leaves. Because mycorrhiza increases water uptake for the plant, strangers associating with mycorrhiza could compensate for increased water loss and not suffer the cost of competition compared to their un-inoculated counterparts. Plants grown with siblings had decreased SLA indicating reduced aboveground competition compared to plants grown with strangers, consistent with sibling altruism. Plant performance and allocation to roots did not respond to mycorrhizal inoculation as expected but since *Plantago lanceolata* is a facultative mycorrhizal host, meaning that it does not require mycorrhizal fungi to grow and survive, more severe nutrient conditions may be required to see significant differences in size across inoculation treatments.

Specific leaf area increased in response to both the shade treatment of the focal individual and the shade treatment of its neighbour. Increased SLA in low light or low light quality (reduced red light) has been found in other species and allows plants to acquire more light (Poot, Pilon et al. 1996, Weijschede, Martinkova et al. 2006, Murphy

and Dudley 2007). As expected, shaded plants had higher SLA than un-shaded plants, and neighbour's shade treatment did not matter. However I did find a response of SLA to neighbour shade in un-shaded focal plants, demonstrating that the neighbour's shade reduced the light of the focal plant. In this study, plants were grown at high density and although the shades were installed around individual plants, they were tall and could have cast shadows on un-shaded plants. This also indicates that the shade material caused a reduction in light such that when the focal individual was shaded, any added reduction in light caused by the neighbour's shade was small enough not to cause a further change in SLA compared to those with un-shaded neighbours. Reduced light availability has been shown to cause a shift of carbon into light acquisition traits such as lower root:shoot or root:leaf allocation (Sultan and Bazzaz 1993, Meziane and Shipley 2001), and here I found the expected decrease in allocation to fine roots.

In conclusion, I have shown that plant responses to social environment, consistent with kin recognition, were dependent upon mycorrhizal inoculation. Additionally, I have shown that mycorrhizal colonization of *P. lanceolata* is dependent upon the relatedness of the pair of plants in a pot. The fungal responses to plant social environment were dependent upon light availability of each of the plants, and imply that an un-shaded plant may altruistically support the fungal partner when attached to a relative with less carbon to donate. Strangers behaved selfishly unless both had high light availability, making it less risky to donate carbon to the fungal partner when with a stranger. Overall, these results show that the importance of plant kin recognition in maintaining the mycorrhizal

mutualism (File, Klironomos et al. 2012; Chapter 2) may be highly dependent upon the aboveground resource conditions of each of the connected plants.

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Table 1: Analysis of variance on vegetative biomass of *Plantago lanceolata* plants.

	Log vegetative biomass		
Source	**DF**	**F**	***P***
Social environment	1	0.07	0.80
Shade	1	56.96	**<0.0001**
Mycorrhizae	1	0.00	0.99
Neighbour shade	1	0.34	0.56
SocialEnv × Shade	1	1.87	0.17
SocialEnv × Myc	1	0.24	0.63
Shade × Myc	1	0.01	0.91
SocialEnv × Nshade	1	1.43	0.23
Shade × Nshade	1	1.65	0.20
Myc × Nshade	1	3.07	0.08
SocialEnv × Shade × Myc	1	0.28	0.59
SocialEnv × Shade × Nshade	1	1.16	0.28
SocialEnv × Myc × Nshade	1	1.25	0.26
Shade × Myc × Nshade	1	0.10	0.75
SocialEnv × Shade × Myc × Nshade	1	0.04	0.85
block	6	8.55	**<0.0001**

Log vegetative biomass is ln(veg+1). Analysis was conducted on individual plants that were grown in pairs in the greenhouse. Mycorrhizae (Myc), social environment (SocialEnv), shade and neighbour shade (Nshade) refer to treatment effects. Significant values are in bold.

Table 2: Analysis of co-variance on specific leaf area (SLA) of *Plantago lanceolata* plants.

		Leaf area (cm^2)	
Source	**DF**	**F**	***P***
Single leaf	1	221.92	**<0.0001**
Single leaf × single leaf	1	30.21	**<0.0001**
Social environment	1	3.98	**0.05**
Shade	1	80.07	**<0.0001**
Mycorrhizae	1	0.20	0.66
Neighbour shade	1	17.59	**<0.0001**
SocialEnv × Shade	1	0.00	0.96
SocialEnv × Myc	1	5.44	**0.02**
Shade × Myc	1	3.81	**0.05**
SocialEnv × Nshade	1	2.67	0.10
Shade × Nshade	1	11.37	**0.0008**
Myc × Nshade	1	0.51	0.47
SocialEnv × Shade × Myc	1	0.10	0.75
SocialEnv × Shade × Nshade	1	1.19	0.28
SocialEnv × Myc × Nshade	1	1.54	0.22
Shade × Myc × Nshade	1	0.05	0.82
SocialEnv × Shade × Myc × Nshade	1	0.05	0.82
block	6	7.26	**<0.0001**

Analysis was conducted on individual plants that were grown in pairs in the greenhouse. Single leaf biomass is the co-variate. Social environment (SocialEnv), mycorrhizae (Myc), shade and neighbour shade (Nshade) refer to treatment effects. Significant values are in bold.

Table 3: Analysis of co-variance on fine root allocation for pairs of *Plantago lanceolata* plants.

		Log fine root biomass	
Source	**DF**	**F**	***P***
Logveg	1	0.08	0.78
Logveg × Logveg	1	20.45	**<0.0001**
Social environment	1	2.33	0.13
Shade	1	22.48	**<0.0001**
Mycorrhizae	1	0.67	0.41
SocialEnv × Shade	1	0.71	0.49
SocialEnv × Myc	1	0.51	0.48
Shade × Myc	1	1.12	0.33
SocialEnv × Shade × Myc	1	1.04	0.36
block	6	6.49	**<0.0001**

Log fine root is ln(fine roots + 0.01). Logveg is the co-variate and is ln(veg +1). Shade, social environment (SocialEnv) and mycorrhizae (Myc) refer to treatment effects at the pot level. Significant values are in bold.

Table 4: Analysis of variance for pairs on fungal colonization by *Glomus intraradices* of *Plantago lanceolata* roots.

	Arbuscules			Log root hyphae			Log vesicles			Soil hyphae		
Source	**DF**	**F**	***P***	**DF**	**F**	***P***	**DF**	**F**	***P***	**DF**	**F**	***P***
Social environment	1	35.59	**<0.0001**	1	4.69	**0.03**	1	1.37	0.24	1	42.76	**<0.0001**
Shade	2	55.23	**<0.0001**	2	30.26	**<0.0001**	2	17.17	**<0.0001**	2	97.94	**<0.0001**
SocialEnv × Shade	2	10.44	**<0.0001**	2	3.18	**0.05**	2	47.67	**<0.0001**	2	8.09	**0.0005**
Block	6	0.35	0.91	6	0.65	0.69	6	0.72	0.63	6	1.36	0.24

Arbuscules, root hyphae and vesicles were measured as the percent of the root colonized by each structure. Soil hypha was measured in centimeters per gram, i.e. length of hyphae growing in the soil. Log root hyphae is ln(hyphal root colonization + 0.8) and log vesicles is ln(vesicle colonization + 2). Shade, social environment (SocialEnv) and mycorrhizae (Myc) refer to treatment effects at the pot level. Significant values are in bold.

Table 5: Table of means for colonization by *Glomus intraradices* of *Plantago lanceolata* plants grown in pairs.

Source	**Arbuscules**	**Soil hyphae**	**Hyphae**	**Vesicles**
Social Environment				
Siblings	24.0476 ± 1.0047 a	3.6889 ± 0.1071 a	27.2631 ± 1.5122 a	17.3320 ± 0.8445 a
Strangers	15.8232 ± 1.0324 b	2.6845 ± 0.1101 b	23.0435 ± 1.3213 b	18.7721 ± 0.9330 a
Shade treatment				
Both	10.9981 ± 1.2661 a	1.8786 ± 0.1350 a	17.2994 ± 1.2376 a	14.1801 ± 0.8957 a
One	19.1905 ± 1.2465 b	3.1524 ± 0.1312 b	24.8680 ± 1.7041 b	17.7525 ± 1.0619 b
Neither	29.6176 ± 1.2465 c	4.5292 ± 0.1330 c	36.4566 ± 2.5069 c	23.1784 ± 1.3712 c

Soil hypha was measured in centimeters per gram, i.e. length of hyphae growing in the soil. Arbuscules, hyphae and vesicles were measured as the percent of the root colonized by each structure. Means for hyphae and vesicles presented here are the back transformed means from ln (hyphae +0.8) and ln(vesicles+2) transformations respectively. Within each fungal trait and within treatments, different letters represent means that are statistically different from each other at the $P<0.05$ level or lower. Shade treatment refers to whether both plants in a pot were shaded, one was shaded or neither plant was shaded.

Figure 1: Effect of social environment and shade treatment on root colonization by arbuscules. Pairs of plants were either kin or strangers. Shade treatment was imposed at the pot level with either both plants were shaded, one plant was shaded or neither plant was shaded. Bars with different letters are statistically different at the $P<0.05$ level.

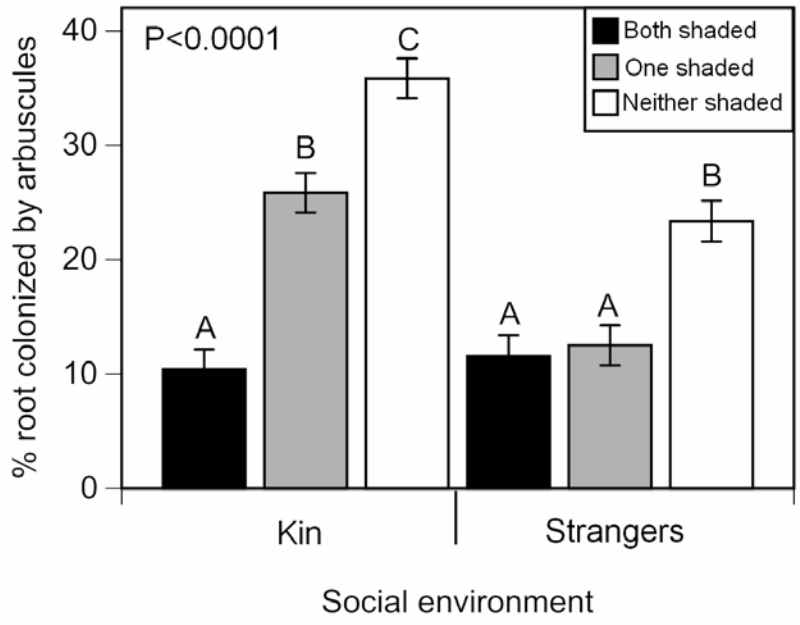

P<0.0001
Both shaded
One shaded
Neither shaded
% root colonized by arbuscules
40
30
20
10
0
A
B
C
A
A
B
Kin
Strangers
Social environment

Figure 2: Effect of social environment and shade treatment on hyphal root colonization. Pairs of plants were either kin or strangers. Shade treatment was imposed at the pot level with either both plants were shaded, one plant was shaded or neither plant was shaded. Bars with different letters are statistically different at the $P<0.05$ level.

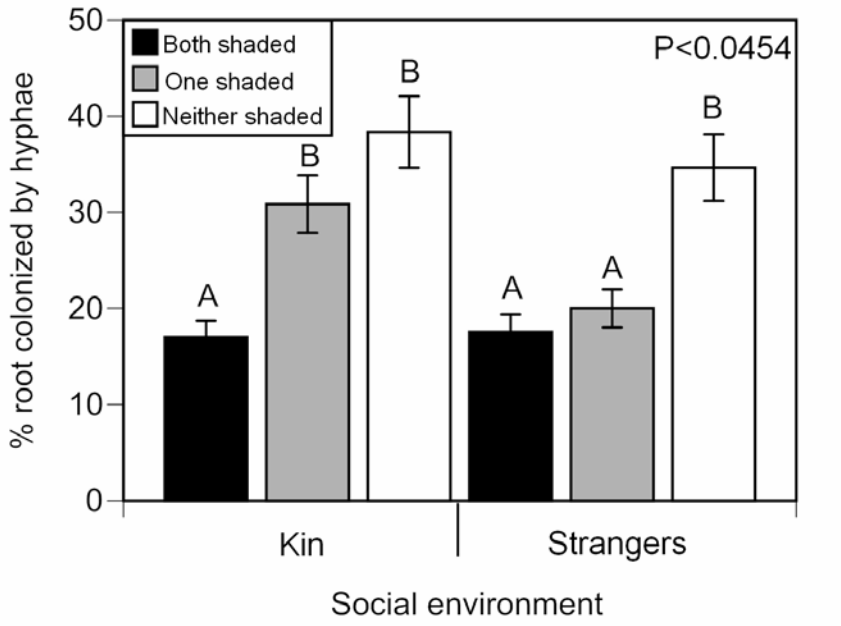

50
40
30
20
10
0
% root colonized by hyphae
Both shaded
One shaded
Neither shaded
P<0.0454
A
B
B
A
A
B
Kin
Strangers
Social environment

Figure 3: Effect of social environment and shade treatment on root colonization by vesicles. Pairs of plants were either kin or strangers. Shade treatment was imposed at the pot level with either both plants were shaded, one plant was shaded or neither plant was shaded. Bars with different letters are statistically different at the $P<0.05$ level.

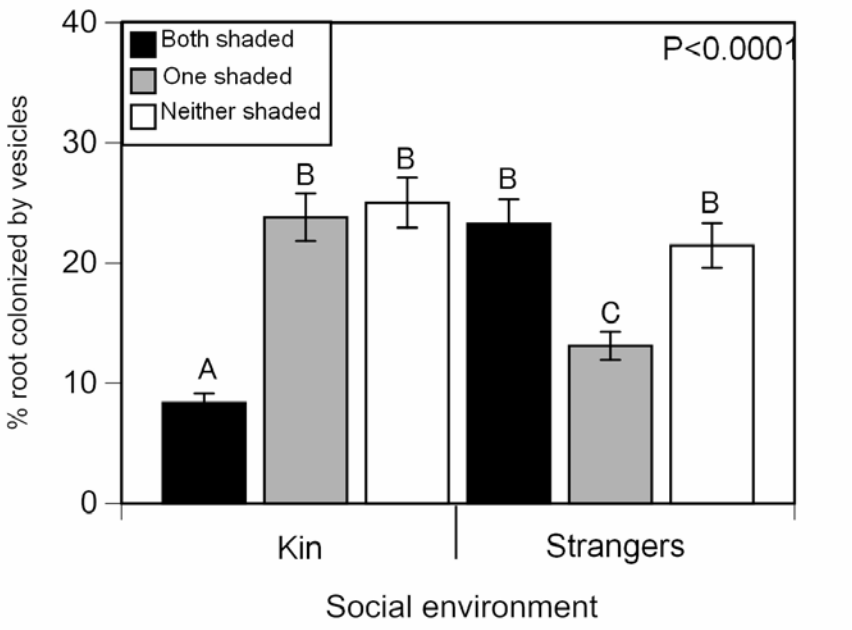

Both shaded
One shaded
Neither shaded
P<0.0001
% root colonized by vesicles
40
30
20
10
0
A
B
B
B
C
B
Kin
Strangers
Social environment

Figure 4: Effect of social environment and shade treatment on soil hyphal length. Soil hypha was measured in cm per gram of soil. Pairs of plants were either kin or strangers. Shade treatment was imposed at the pot level with either both plants were shaded, one plant was shaded or neither plant was shaded. Bars with different letters are statistically different at the $P<0.05$ level.

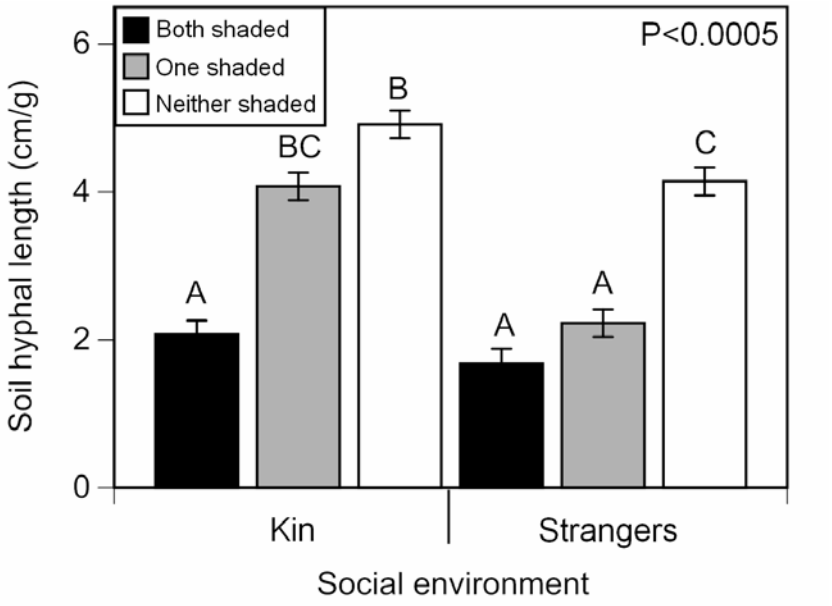

Both shaded
One shaded
Neither shaded
P<0.0005
Soil hyphal length (cm/g)
6
4
2
0
A
BC
B
A
A
C
Kin
Strangers
Social environment

Figure 5: Effect of shade and neighbour's shade on specific leaf area of plants grown in pairs in the greenhouse. Specific leaf area is the area of the tallest leaf relative to the biomass of that leaf. Bars with different letters are statistically different at the $P<0.05$ level.

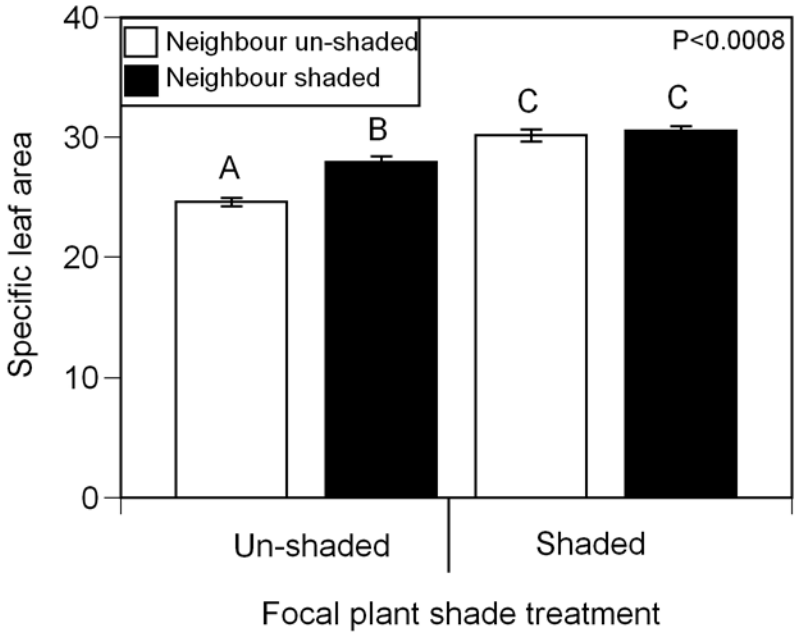

Neighbour un-shaded
Neighbour shaded
P<0.0008
Specific leaf area
40
30
20
10
0
A
B
C
C
Un-shaded
Shaded
Focal plant shade treatment

Figure 6: Effect of social environment and mycorrhizal inoculation on specific leaf area of plants grown in pairs in the greenhouse. Specific leaf area is the area of the tallest leaf relative to the biomass of that leaf. Plants were either grown with a sibling or a stranger and were either inoculated with spores of *Glomus intraradices* (black bars) or left un-inoculated (grey bars). Bars with different letters are statistically different at the $P<0.05$ level.

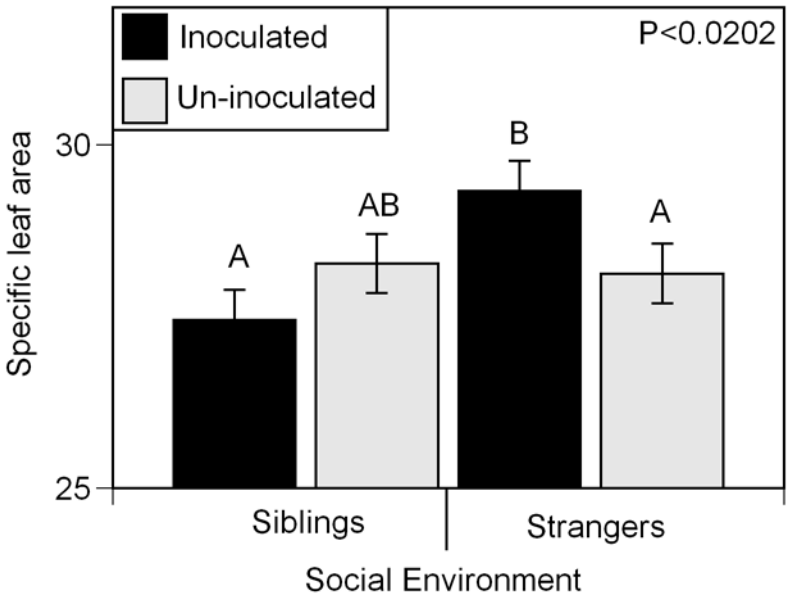
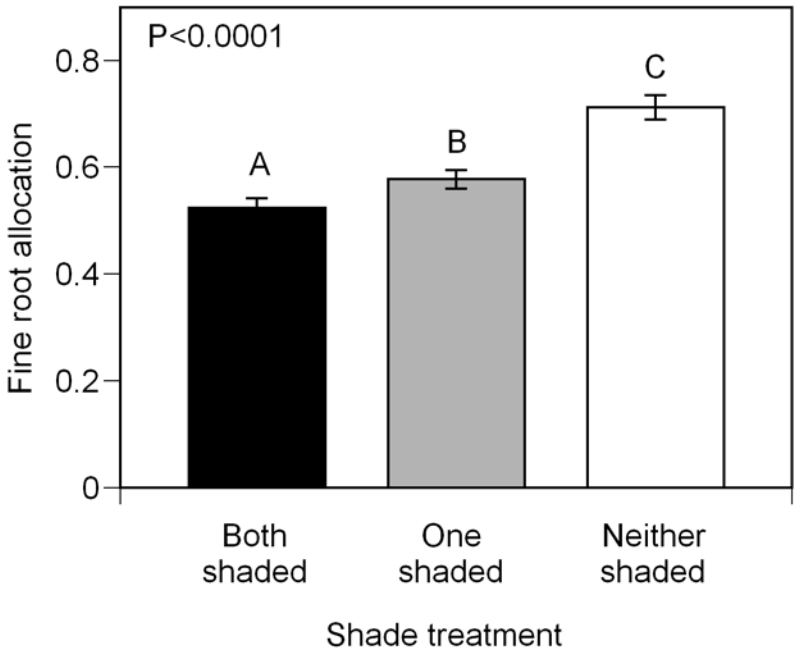

Inoculated
Un-inoculated
P<0.0202
Specific leaf area
30
25
A
AB
B
A
Siblings
Strangers
Social Environment

Figure 7: Effect of shade treatment on allocation to fine roots of pairs plants grown in the greenhouse. Shade treatment was imposed at the pot level with either both plants were shaded, one plant was shaded or neither plant was shaded. Bars with different letters are statistically different at the $P<0.05$ level.


P<0.0001
0.8
0.6
0.4
0.2
0
Fine root allocation
A
B
C
Both shaded
One shaded
Neither shaded
Shade treatment

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CHAPTER 5

TESTING FOR MATERNAL CARE IN TREES: HOW DOES SOCIAL ENVIRONMENT AND ACCESS TO MYCORRHIZAE AFFECT SEEDLING SUCCESS?

ABSTRACT

The environment into which a seed disperses has important consequences on germination and seedling establishment. Two different theories offer opposing predictions about the effect of adult tree identity on seedling success. Seedlings may perform better farther away from relatives or conspecifics due to harmful pathogens associated with the adult's local environment, or they may perform better when closer to related adults due to associations with beneficial soil symbionts. Here, I investigated the effects of social environment (relatedness), distance from nearby adults and access to mycorrhizal fungi on seedling growth and morphology in a natural forest. I show evidence for kin recognition in seedling morphology and species recognition in mycorrhizal infection of seedling roots. Aboveground morphology responded to light environment and mycorrhizal manipulations. Results show that seedlings are very sensitive to their surroundings and responses to biotic and abiotic factors are not easy to predict.

INTRODUCTION

The establishment and success of tree seedlings is important for forest dynamics because it plays a key part in determining community structure. Seedling establishment and growth are not only affected by abiotic factors such as light and nutrient availability,

but also biotic factors including interactions with other plants and associations with symbionts (Leck, Simpson et al. 2008). The benefit for seedlings of growing closer to related or un-related established adults, i.e. the social environment, is an important aspect of biotic environment. Seedlings may be more successful when they grow farther away from the host-specific enemies of their relatives (Janzen 1970, Connell 1971). Conversely, they may be more successful when growing closer to a related adult since kin selection favours altruism towards relatives (Hamilton 1964). Other aspects of the seedling neighbourhood, such as distance from mature adult trees, nutrient availability, and presence of microbes are also predicted to be important factors in determining seedling growth and success.

The Janzen-Connell hypothesis predicts that tropical tree seedling recruitment should be lower in close proximity with conspecific adults due to host-specific pathogens, resulting in the high species diversity found in tropical forests (Janzen 1970, Connell 1971). That is, the farther away seeds germinate from conspecific adults, the farther away from the negative effects of species-specific pathogens they should be. However, studies testing the Janzen-Connell predictions at the community-level show mixed results. Condit, Hubbell et al. (1992) found that Janzen-Connell effects were species specific, with some species showing increased seedling recruitment near conspecifics, some showing decreased recruitment near conspecifics and with some species proximity of conspecifics did not matter. Thus, Janzen-Connell predictions are likely only met under certain circumstances for relatively few species. Others have found evidence supporting Janzen-Connell predictions (Connell, Tracey et al. 1984, Hubbell, Condit et al. 1990,

Harms, Wright et al. 2000). The results of these studies and the development of the theory have created debate over the relevance of the Janzen-Connell predictions and to what strength this effect has on maintaining diversity (Wright 2002).

Kin selection theory offers alternate contrasting prediction to that of the Janzen-Connell hypothesis; that there will be altruism between biological parent and offspring, which would lead to seedlings growing close to their parent having greater success than those growing near un-related conspecifics or trees of other species. That is, trees could demonstrate parental care towards seedlings. Parental care is any behaviour by the parent that appears to increase offspring fitness. In animals, parental care includes caring for eggs, feeding offspring, preparing nests and defending a territory (Clutton-Brock 1991). But do plants provide parental care? There are many non-genetic, maternal effects that can be seen well into the developing seedling's life, with diminishing importance as the offspring matures (Roach and Wulff 1987). These include environmental effects (reviewed by Roach and Wulff 1987), resource partitioning to developing seeds and effects of the seed coat, a maternally derived tissue (reviewed by Donohue 2009). Many of these effects may not be due to maternal choice and therefore cannot be considered maternal care. However, choosing how much of a given resource to give to particular seeds or choosing whether or not to abort a seed are under maternal control and could be considered maternal care. After offspring have been dispersed, there may be other opportunities for parental care, especially if dispersal is local and the offspring germinates in close proximity to the parent. One way that post-dispersal parental care could occur is through the mycorrhizal symbiosis.

Trees are highly mycorrhizal and are connected by networks of mycorrhizal fungi, known as common mycorrhizal networks (CMN). CMNs are important in seedling establishment since seedlings can tap into an existing mycorrhizal network, providing them with increased access to water and nutrients at a relatively low cost (reviewed in Horton and van der Heijden 2008), but see (Horton, Bruns et al. 1999, Dickie, Koide et al. 2002, Booth 2004). Seedlings may also gain carbon from older, more dominant individuals in the community through the CMN, which could assist in their early growth and success (reviewed in Horton and van der Heijden 2008). Thus, adult trees could pass nutrients or carbon to their offspring via a CMN, or donate more carbon to the mycorrhizal partner, facilitating the growth and success of the fungi. In this way, the parent could gain indirect fitness since the growing seedling would benefit either due to direct donations from the parent or indirectly through a well-supported fungal partner.

In order for parents to preferentially direct care towards their offspring, they need the ability to differentiate them from unrelated seedlings. Plant kin recognition has been demonstrated in herbaceous species (Dudley and File 2007, Biedrzycki, Jilany et al. 2009, Murphy and Dudley 2009, Bhatt, Khandelwal et al. 2010, Karban, Shiojiri et al. 2013) and in Chapter 2, I show that mycorrhizal fungi can serve as an extended phenotype in response to plant social environment. When common ragweed is grown with related individuals, there are increased mycorrhizal soil hyphae and/or fungal root colonization compared to those grown with strangers (File, Klironomos et al. 2012; Chapter 2). I argued that plants could behave altruistically towards kin by increasing carbon donations to the CMN attached to siblings. Most plant kin recognition has been demonstrated in

annual plants and as of yet very little work has been done to investigate kin recognition in perennials. However, long-lived perennials, such as trees, offer an interesting system to study kin recognition and altruism because trees have ample opportunity to interact across generations.

In this study I manipulated the social environment of seeds planted under adult trees at varying distances, with and without access to mycorrhizal fungi in a sub-tropical forest. I asked the following questions: **1) Does species and relatedness of the nearby adult affect seedling growth and morphology? 2) Are effects of the nearby adult dependent upon whether the seedling has access to mycorrhizal fungi? 3) How does distance from the adult tree affect the influence adults have on young seedlings?**

MATERIALS & METHODS

Study site

This experiment was conducted at the Lienhuachih (LHC) research forest plot in Taiwan, which is part of the Smithsonian institute Centre for Tropical Forest Science global network of research forests. Taiwan is a sub-tropical country, allowing for an extended growing season compared to temperate forests and since tropical trees readily associate with mycorrhizal fungi, this was an ideal location. LHC is located within the Lienhuachih experimental forest in the central mountain range in Nantou County ($23^{o}54'49"$N, $120^{o}52'43"$E), 667-845 meters above sea level (Chang, Hwong et al. 2010). The research plot was established in 2008 and covers an area of 25 hectares (500m × 500m). LHC is a natural forest consisting of broad-leaf evergreen trees.

Study Species

The species used in this study, *Cyclobalanopsis pachyloma* Schottky *(*or *Quercus pachyloma;* family Fagaceae), was chosen based on availability of healthy, mature adults, seed production in the year of the study, and topographical distribution of the adult trees in the forest plot. I used the existing LHC species distribution data to select a number of species that would be ideal for the study and then examined the size and health of the adult trees upon visiting the plot. There were eight mature *C. pachyloma* individuals with adequate seed availability at approximately the same elevation, along the same ridge. Elevation and topography may have an effect on seedling success due to temperature, light and moisture gradients, so it was necessary to select adults that were in the same topographical conditions. The Fagaceae (beech) family is a dominant family in LHC (Chang, Hwong et al. 2010). *C. pachyloma* is a canopy species and is in the top ten species in LHC for importance values (IV), derived from the following calculation: IV= [(relative density + relative basal area)/2] (Chang, Hwong et al. 2010). *C. pachyloma* is monoeceious species (male and female flowers on the same individual) and produces acorns with cupules that cover up to 2/3 of the nut (see appendix Image 1 & 2).

Seed collection and stratification

In October 2011, focal individuals were selected and fruits were protected from predation using fine hardware cloth. Groups of fruits were wrapped in the cloth and left on the tree to further mature. At the end of November 2011, seeds had matured and were harvested. They were stored in breathable mesh bags until transported to the lab to be prepared for stratification. Prior to stratification, seeds were soaked in a 10% bleach solution for 10 minutes following the removal of cupules. Seeds were then rinsed with

water and soaked in fresh tap water overnight. They were put in large plastic bags with moist peat moss in an approximate 1:1 seed/peat volume ratio. Bags were sealed but not entirely void of air to prevent anoxic conditions, and then put in the fridge at approximately 6 degrees Celsius. Seeds were stored in the fridge from November 25, 2011 until Feb 13, 2012. After this stratification period, each seed was weighed and put in its own poly bag with a small amount of moist peat and an ID tag, then stored at 6 degrees Celsius until planting. Seeds from each mother tree are considered a maternal sibship (hereafter, "family").

Experimental design

Seeds were planted in a factorial design that manipulated relatedness of the nearby adult tree, distance from that adult tree and access to local mycorrhizal fungi.

Social environment: To test whether *C. pachyloma* seedlings would be more successful when planted under their own mother, or under another mother of the same species, 99 seeds were planted under each of the eight adult focal trees. Fifty seeds were offspring from the focal tree (i.e. kin), and 49 seeds were from the seven other focal trees (seven from each other family, i.e. strangers). To test whether seedlings would be more successful under another species compared to the focal species, ten randomly chosen treatment combinations (family and mycorrhizal treatment) were planted under eight trees from two different species: *Schefflera octophylla* (family Araliaceae) and *Cinnamonmum subavenium* (family Lauraceae). We chose non-focal adults that were as close to the focal adults as possible to reduce environmental effects such as elevation and slope that may

confound the results. However, because the distribution of these two species is slightly different than that of *C. pachyloma*, several of the individuals were not on top of the ridge where the focal individuals were found, but down the slope either north or south of the ridge. The other species treatment was not fully factorial due to time constraints.

Mycorrhizal exclusion: To test whether the seedling responses to social environment were dependent upon their access to mycorrhizal fungi, i.e. whether maternal care is facilitated by mycorrhizae, the seeds were planted in one of three bag treatments: 1) 250 micron bag: pore size large enough to let mycorrhizal hyphae into the bag but not roots from surrounding vegetation (36 per focal tree); 2) 0.5 micron bag: pore size small enough to keep out roots and mycorrhizal hyphae (36 per focal tree); or 3) no bag: seed was planted directly in the ground allowing it to contact roots and soil microbes (27 per focal tree).

Distance from adult: To determine whether the strength of the effect of nearby adult tree depended on distance from that tree, seeds were planted in one of three arcs: 2 meters, 3 meters or 4 meters from the base of the focal tree. Within each arc, seeds were planted 28.5cm from each other. The design under each of the 8 focal trees was fully factorial and treatment combinations were randomly placed across the three arcs.

Seeds were planted from February 28th to March 1st, 2012. The mesh bags for the bag treatment were 3.25”wide and 14” long (Knight Corporation, Ardmore, PA, USA) and had a drawstring at the top. Bags were pre-filled with 350mL of soil (Sondermischung Kultursubstrat, Gramoflor GmbH & Co, Germany) and tied shut before transport into the field site to prevent contamination of airborne mycorrhizal spores.

Holes were dug with a hand auger and each bag was buried with only the top one inch of the bag exposed. Bags were left closed until planting. Seeds that were in the no bag treatment were planted in a hole that was dug and then filled with 350mL of the same soil as that in the bags. Each seed was labeled with a unique ID tag and covered with a fine white mesh to prevent seed predation. In April 2012, seeds had germinated and the mesh was removed from all the seeds. The experiment was then left to natural conditions until October 2012.

Data collection and harvest

Germination was scored two months after planting, in April, 2012. We collected data on all surviving plants and harvested seedlings from October 4^{th}-6^{th}. Any germinated seedlings in mesh bags were harvested, with the leaves stored separately from the stems. Leaf number, length of the longest leaf, height and diameter of the stem one centimeter from the base of the stem were recorded. Any roots that were identifiable as belonging to the seedling were collected. Any seedlings that were planted directly in the ground (no bag) were left in the field with only non-destructive measures taken. Although the mesh bags were supposed to keep roots from coming into contact with the seedlings, almost all of the bags were damaged, presumably from animals or from neighbouring tree roots. The soil within the bags contained roots from many different plants, making it difficult to identify the study seedlings' roots and impossible to retrieve all of the roots from a given seedling. Additionally, seedling roots often grew into the fabric of the 0.5 mesh bags, making it impossible to retrieve the roots without damaging them. Roots from half of the seedlings were washed the same day that they were harvested in a bleach solution and

dried for weighing. Roots from the other half of the seedlings were kept at 6 degrees Celsius and then later stained with dye to quantify mycorrhizal colonization. Upon return to Canada, all collected biomass was dried in a drying oven at 100 degrees F (37.8 degrees Celsius) for 24 hours then placed in a sealed container. All stems, leaves and roots collected were weighed to obtain dry biomass.

Canopy gap photos

Because the 16 adult trees were spread out over distance and some elevation, light availability was observably variable among adults. Light availability is important for seed germination and seedling success and thus could confound any effects due to the experimental manipulations. To determine the light availability at each of the adult trees, canopy photos were taken using a fish eye lens mounted on a Nikon E4500 camera. All photos were taken on Oct 15th, 2013, either before 8:30 am or after 2:30pm to avoid overexposed photos. Five photos were taken along the 3m arc at approximately equal distance under each of the focal trees. One photo was taken under each of the non-focal trees. Photos were thresholded using SideLook 1.1.01 (M. Nobis, 2005). Thresholding is the process of turning the colour photo taken in the field into a binary image (black and white). After photos were thresholded, they were loaded into Gap Light Analyzer (GLA, version 2.0, Simon Fraser University, Burnaby, BC). A sample image with a point indicating which direction is north was loaded into GLA and then registered so the software used that same configuration for each canopy photo. GLA then calculated canopy openness of each photo. See appendix Fig 1 for average canopy openness under each adult tree.

Mycorrhizal quantification

A red dye was mixed using Ponceau S as per Daughtridge, Boese et al. (1986). Root samples that had been stored in the fridge were gently washed in tap water to remove soil and debris. The sample was then soaked in the dye solution for 15 minutes and rinsed off in 10% acetic acid solution. Finally, the root was rinsed in distilled water briefly before observing under the dissecting microscope. The length of the root sample was measured since the size of the sample collected varied depending on the mesh bag and presence of roots from neighbouring vegetation. Infected and un-infected root tips were counted for each root sample. Root tips infected by mycorrhizae appeared swollen and bright red. Un-infected root tips were not swollen and were brown/yellow in colour. Only un-broken root tips were counted since it was not possible to tell whether a broken root had been infected or not.

Leaf area calculation and herbivory quantification

Leaves from each harvested individual were photographed (Olympus, Camedia C-5060, 5.1 megapixel). Many individuals suffered from herbivory, including missing leaf area. To estimate the original leaf area, edges of herbivore-affected leaves were drawn in by hand using templates created from intact leaves from each family. This was done blind to treatments. A ruler was included in every photo so each photo could be scaled appropriately once loaded into the software. Photos were opened in ImageJ (National Institute of Health, version 1.46) and thresholded. Once the appropriate contrast was found, the analyze particles function was used with the “include holes” and “masks” features so the software would turn the hand-drawn portions into solid objects. The

objects of interest were selected and ImageJ calculated the total area of those objects. I also measured the actual area of the harvested leaves for each individual.

To calculate surface damage of the leaves, I used the threshold feature of ImageJ to adjust the green contrast of the photos so that only un-damaged leaf matter was highlighted and ImageJ could measure the area of that un-affected area. I subtracted un-damaged area from actual area to get an estimate of surface damage area.

Data analysis

SAS statistical software (version 9.2; SAD, Cary, NC, USA) was used with proc GLM, proc GENMOD and proc LOGISTIC. Where necessary, biomass and other trait variables were logged to satisfy the assumptions of the GLM. Where possible, analysis was conducted on the entire data set but since some traits could only be measured on harvested plants, analyses of those traits were conducted on harvested plants only. Seed biomass was included as a covariate when it was significant since larger seeds typically produce larger plants. Proc GENMOD was used to analyze seed germination, seedling survival and emergence data because they are binomial, rather than continuous, variables. We defined a new variable, emergence, to indicate if plants germinated in April and whether or not they survived to harvest time in October. If plants germinated in April and survived to the time of harvest in October, they were scored as “emerged”. If they germinated but did not survive to October then they were counted as “not emerged” and if they did not germinate they were excluded from emergence analysis. In the GENMOD models, germination, survival and emergence were the dependent variables and seed biomass, arc, tree, family and bag treatment were the independent variables. Proc

LOGISTIC was used to create predicted probability plots for germination and survival, each as a function of seed biomass. To test for treatment effects and random effects, analysis of covariance (ANCOVA) was conducted using proc GLM.

To test for effects on performance or size, analysis of covariance (ANCOVA) was used to examine aboveground biomass, height, and leaf mass from harvested plants and stem diameter from all emerged plants. Since social environment, nearby adult tree and family contain overlapping information, all three factors could not be included in the same model. Therefore, I used the same pre-determined method to decide which two of the three factors would be included in each model. I fit models according to the following protocol: I first fit a model with tree and family. I then fit models with social environment and tree, and social environment and family. The model with the best fit (highest r-square) was used. Interactions among treatments were only included in the model when they were significant and when there was ample sample size of each treatment combination to conduct a meaningful analysis since the final harvested data did not include all possible treatment combinations due to death and imbalanced experimental design.

To test whether seed biomass varied across families, ANOVA was used with seed biomass as the dependent variable and family as the independent variable. For aboveground biomass, tree, family, distance from the adult tree and bag treatment were included as independent variables. In the analysis of height, social environment, family, bag treatment and distance were included in the model as independent variables. In the analysis of leaf biomass, the log of leaf mass was used as the dependent variable to satisfy

the assumptions of the GLM. Log leaf mass was calculated as ln (leaf mass + 0.5). Log seed biomass (ln(seed mass)) was used as the covariate. Family, tree, bag treatment arc and herbivory were included as independent variables. Herbivory was calculated as 1-(actual leaf area/estimated original leaf area) to provide us with an estimated percent of the leaf missing, i.e. a measure of herbivory, which should have big effects on traits like leaf biomass. Percent herbivory was included in models involving leaf mass since loss of leaf area significantly reduced leaf mass. To analyze stem diameter, family, tree, distance and bag treatment were included as independent variables. The arc × bag treatment interaction was also included in this model. To investigate morphology on all surviving plants, we analyzed leaf length: stem diameter on plants that had leaves at the time of harvest. Length of the longest leaf was the dependent variable and stem diameter was the covariate. Tree, family, distance, and bag treatment were included in the model as independent variables.

To test for effects on seedling morphology, ANCOVA was used. Seedling stem elongation was examined using stem height as the dependent variable and stem biomass as the covariate. A quadratic term for stem mass was added to the model to improve model fit. Tree, distance, family and bag treatment were included as independent variables. A regression model with elongation was also run to determine the effect of canopy openness. Leaf:stem allocation was analyzed with log leaf mass as the dependent variable and log stem mass as the covariate. Family, distance, social environment, bag treatment and herbivory were included as independent variables. Leaf: stem allocation was also analyzed with only plants un-affected by herbivores included and the same

significant results were obtained. Specific leaf area (SLA) was analyzed with log actual leaf area (ln(area+0.01)) as the dependent variable and log leaf mass (ln(leaf mass +0.05)) as the covariate. Family, tree, distance and bag treatment were independent variables. A quadratic term for log leaf mass and a cubic term for log leaf mass were added to the model and both were highly significant. A regression model to test for effects of canopy openness on SLA was run using proc GLM. Leaf number was analyzed using proc GENMOD with distance, social environment, family and bag treatment and independent variables. Only plants under focal trees were included in this analysis. To test for treatment effects on estimated leaf area, harvested plants were analyzed. Log estimated leaf area was the dependent variable; log seed mass, tree, family, distance and bag treatment were independent variables. To investigate leaf length all living plants at harvest time were analyzed. Leaf length was the dependent variable; family, distance, and bag treatment were independent variables.

To investigate leaf surface damage, I conducted ANCOVA with the damaged leaf area ((ln(damaged area)), as the dependent variable. Log transformed leaf surface area ((ln(actual leaf area + 0.01))) was the covariate and tree, family, distance and bag treatment were the independent variables.

To analyze total herbivore damage to leaves, I defined a new variable that included both estimated missing leaf area and surface damage: total herbivory= (estimated area-undamaged area)/estimated area. We used ANOCOVA with log transformed total herbivory (ln(total herbivory +0.1)) as the dependent variable; log seed

biomass as the covariate; and species, family, distance and bag treatment and independent variables.

To analyze fungal colonization on harvested plants I calculated the rate of infection: number of infected root tips/total number of root tips. I conducted an arcsin of the square root transformation on this data. This was then analyzed using ANCOVA with rate of infection as the dependent variable; root length as the covariate; and social environment and bag were the independent variables with the interaction of the two included in the model. This model was not significant so I interpret these results with caution.

RESULTS

Effects on germination, survival and emergence

Seed germination responded to bag treatment (Appendix Fig 2). Seed germination was variable among families, i.e. maternal sibships (Table 1, Appendix Fig 3). Probability of survival responded to bag treatment (Appendix Fig 4), depended upon the nearby adult tree (Appendix Fig 5) and varied across families (Table 1; Appendix Fig 6). Bag treatment affected emergence, i.e. probability of survival if the seed germinated (Fig 1), depended upon the nearby adult tree (Fig 2) and varied across families (Fig 3) (Table 1). Seeds planted directly into the ground (no bag) had lower germination, survival and emergence compared to seeds planted into either of the mesh bags. Seeds planted under non-focal trees varied in survival but not significantly so because of very small sample size. Larger seeds had a higher chance of germinating, surviving and emerging

(germination parameter estimate=0.5946, intercept=-1.5638; survival parameter estimate= 0.7485, intercept=-2.6259; emergence parameter estimate=0.7640, intercept=-1.7968).

Effects on performance/size

Total aboveground biomass of harvested plants varied across family (Table 2, Fig 4). Height varied across family and responded to distance from the adult tree (Table 2). Seedlings closest to the adult tree (2 meters) were taller than seedlings 3 meters and 4 meters from the adult (Table 3). Stem diameter responded to bag treatment and family (Table 4). Plants in no bag had smaller stem diameter than plants in either mesh bag (Table 3). Stem diameter showed a significant bag × distance effect (Table 4, Fig 5). Within arcs, plants in no bags had the smallest stem diameter. Plants 2 meters from the adult tree had the largest stem diameter if planted in a 250-micron bag but this was not statistically different from plants in 0.5 micron bags. Plants 3 meters from the adult tree had the largest stem diameter if grown in 0.5micron bag. Plants 4 meters from the adult tree had the largest stem diameter if grown in the 250 micron bag. Leaf mass responded to bag treatment and varied across family (Table 2). Leaf mass also depended upon the nearby adult tree (Fig 6) and percent herbivory (Table 2). Seedlings in 0.5-micron bags had higher leaf mass than plants in 250-micron bags (Table 3). Leaf length responded to bag treatment and varied across families (Table 4). Plants in 0.5-micron bags had higher leaf length than plants in no bag or 250-micron bags (Table 3). Estimated leaf area was affected by bag treatment and depended upon nearby adult tree, and varied across families (Table 2). Plants in 0.5-micron bags had greater estimated leaf area than plants in 250-micron bags (Table 3). Number of leaves was responded to social environment for plants

grown under focal trees (Table 5). Strangers produced more leaves than kin (Table 3). Seed biomass varied across families (Fig 7).

Effects on morphology

Stem elongation responded to distance from adult tree and depended on the nearby adult tree (Table 6; Fig 8). Seedlings closest to the adult tree had higher stem elongation than seedlings in the more distant arcs (Table 7). Elongation decreased with increasing canopy openness (parameter estimate=-0.2031, intercept=7.9655). Leaf: stem allocation responded to bag treatment, distance from the adult tree, percent herbivory and social environment (Table 6). Seedlings in 0.5 micron bags had higher allocation to leaves for a given stem mass than seedlings in 250 micron bags. Seedlings planted at 2 meters allocated less biomass to leaves compared to seedlings planted at 4 meters (Fig 9; Table 7). Seedlings planted under their biological mother (kin) had lower allocation to leaf biomass than seedlings planted under an unrelated conspecific adult (Fig 10; Table 7). Seedlings planted under a non-conspecific adult did not differ in leaf: stem allocation from either kin or stranger seedlings. Leaf:stem allocation was reduced with increasing herbivory. Specific leaf area (SLA) varied across families (Table 6) and decreased with increasing canopy openness (parameter estimate=-0.0116, intercept=7.0522). Leaf length: stem diameter morphology responded to bag treatment and varied across families (Table 6). Seedlings in 0.5 micron bags allocated more to leaf length than seedlings in 250 micron bags. Seedlings planted directly in the ground (no bag) did not differ from seedlings in either bag treatment (Table 7). Root: leaf allocation responded to bag treatment, social environment and varied across families (Table 6). Seedlings planted

under non-focal trees (other species) had lower allocation to roots than seedlings planted under focal trees regardless of whether they were kin or strangers (Table 7). Seedlings in 0.5 micron bags allocated less to roots than seedlings planted in 250 micron bags (means Table 7).

Effects on herbivory

Total leaf herbivory responded to species (Table 8). Seedlings under the focal species had higher total herbivory damage than seedlings under a non-focal species tree (focal mean=0.1850, SE=0.0164; non-focal mean=0.0938, SE=0.0382).

Effects on fungal infection

Rate of mycorrhizal infection responded to bag treatment but it was dependent upon social environment. Plants grown under conspecifics (kin or stranger) trees did not differ in fungal infection across bag treatments but plants grown under other species had higher fungal infection if grown in a 250 micron bag (Fig 11). Longer root segments had higher rate of infection and all roots longer than 20cm had some mycorrhizal infection.

DISCUSSION

In this experiment, I manipulated social environment, distance from the adult tree and access to mycorrhizal fungi in tree seedlings to test for maternal care and its dependence upon mycorrhizae. I took non-destructive measures of functional plant traits of all surviving seedlings and measured above- and belowground biomass, allocation traits and fungal colonization of harvested seedlings. I predicted that social environment would affect growth and morphology of seedlings and that this would be dependent upon their access to mycorrhizae, via the bag treatment. I also predicted that responses would

depend on distance from the adult tree since adult influence is expected to decrease over space and seedling environment should differ with distance from the base of the adult trunk. Plant morphology responded to social environment, distance from the adult tree and bag treatment. Mycorrhizal infection responded to social environment, depending on the bag treatment. However, results were more complicated than those predicted by either the Janzen-Connell or kin selection hypotheses.

Social environment

I found responses to social environment in two plant morphology traits: allocation to leaf biomass and root allocation. Seedlings grown near a stranger conspecific allocated more to leaves for a given stem mass and higher leaf number than those grown near a related conspecific. The functional biology and fitness consequences of this trait are uncertain but the results are consistent with an increased ability to capture light in strangers. This could be a benefit for strangers since increased leaf biomass is associated with increased carbon acquisition. Alternatively, siblings may have been building more robust stems, which could lead to increased chance of survival in the future. Root allocation was decreased in plants that grew near non-conspecific adults compared to those that grew near conspecific adults. Increased allocation to roots in conspecifics indicates greater belowground nutrient foraging, whereas seedlings grown with other species increased their aboveground nutrient capture.

Bag treatment

The bag treatment was only partially successful in excluding or allowing mycorrhizae but responses were found in both mycorrhizal colonization and plant growth and morphology. Mycorrhizal root infection responded to bag treatment depending on

species of the nearby adult. Firstly, seedlings in both types of mesh bags had mycorrhizal infection indicating that the 0.5 micron bags, meant to prevent inoculation, were not completely successful and by the time of harvesting, allowed just as much colonization as 250 micron bags. I expected that related seedlings or conspecifics would have greater colonization than seedlings from another species because of adaptation to local fungal genotypes. However, seedlings grown under non-focal species adults in 250 micron bags had a higher infection rate than seedlings in all other treatments. This is consistent with a benefit to growing farther away from biological adults, since mycorrhizal networks are commonly associated with greater seedling establishment (Horton and van der Heijden 2008). I speculate that this increase in fungal colonization in seedlings planted under non-focal adults is because the seedlings are not affected by host-specific pathogens and can form better associations with mycorrhizal fungi. This would be interesting to explore in a future experiment, either with a larger sample size in the field, or in a greenhouse experiment using field soil from under different adult trees.

Size and morphology of harvested seedlings responded to bag treatment. Interestingly, seedlings in 0.5 micron bags were larger and allocated more to leaves than seedlings in 250 micron bags. Although the mycorrhizal infection rate results do not suggest a reduced mycorrhizal association in either of these bag types, the bags may have manipulated mycorrhizal access earlier in the experiment before seedling roots could grow out of the bags and before roots of neighbouring vegetation could grow in. If this was the case, the increased performance in seedlings in 0.5 micron bags is consistent with a reduced cost for seedlings not associating with mycorrhizae at this early life stage.

Although there are many benefits for seedlings to tapping into an established CMN, seedlings are relatively carbon poor and may suffer a high carbon cost to associate with a mycorrhizal partner. An alternate explanation for these results is that the bags themselves affected growth responses of the seedlings. Perhaps the two bag types had differential drying or access to water. Bags made from 0.5 micron mesh are much thicker than 250 micron mesh so they may keep the soil inside moister and/or prevent ground water from entering. To address this, I conducted a small bag drying trial experiment with bags buried in pots in the greenhouse without plants. Over the course of one week, soil in both bag types dried at a similar rate but a larger-scale study looking at just effects of bag type of seedling growth would need to be done to confirm this. Across all seedlings in the experiment, bag treatment also had interesting effects on emergence. Seeds planted in either type of bag had much higher chance of emerging, for a given seed size, than seeds planted directly in the ground. Thus, the bags may have protected seeds and young seedlings from herbivory and damage from soil enemies.

The effect of bag treatment on stem diameter, a measure of plant size, depended on the distance from the adult tree. Seedlings located two and four meters from the adult tree were larger if they were in either of the bag treatments, i.e. compared to no bag, but those located three meters from the adult tree were larger if grown in a 0.5 micron bag. Assuming that the 0.5 micron bags prevented mycorrhizal infection early on in the experiment, this result is consistent with a benefit of not associating with mycorrhizae and/or evidence that the 0.5 micron bags reduced exposure to harmful soil pathogens. Finding plants grown in no bag had significantly reduced stem diameter is consistent with

these plants suffering in some way or that they were elongated (thinner, taller stems). However, there is no other evidence that plants in no bags were smaller since they had similar leaf number and leaf length to seedlings in 250 micron bags. Because these seedlings were planted directly in the ground, measures of stem diameter were taken at the soil level, which may not have been the same point on the stem that harvested seedlings were measured. Thus, this difference in stem diameter could be due differences in how plants were measured rather than a response to treatments.

Distance from adult tree

As predicted, seedling morphology responded to distance from the adult tree, indicative of increased light availability closer to the edge of the adult canopy. Seedlings allocated more to stem biomass and were taller the closer they were to the nearby adult, demonstrating a typical shade avoidance response (Aphalo, Ballare et al. 1999). Seedlings that were planted at the edge of the adult canopy, where adult branches and leaves are less dense, allocated more biomass to leaves and grew longer leaves; both carbon-acquiring traits which would presumably give these plants size benefits in the future. Stem elongation responses to identity of nearby adult tree and the decreased SLA found with increasing canopy openness indicate the importance of the light environment on seedlings. This plasticity to light availability by young seedlings is adaptive since seedlings can experience a variety of environments depending on where they germinate (Schmitt, Stinchcombe et al. 2003).

Maternal effects and variation among families

Many size and morphology traits varied across families, consistent with genotypic variation and maternal effects. Seed mass varied across mother trees, which was expected

because, although focal species trees were located on the same ridge, they varied widely in size and seed production. However, mean seed biomass was not correlated with a measure of mother tree size, diameter at breast height. Seed size determined seedling size, demonstrating that maternal investment in seeds plays an important role in seedling success. Controlling for seed size, aboveground biomass varied across families, indicative of genotypic variation.

Conclusion

I have shown that tree seedling morphology responds to social environment, consistent with kin recognition, and distance from the nearest adult tree, consistent with light availability effects. I have also demonstrated that mycorrhizal infection of seedling roots responds to species, depending on the bag treatment, which implies that there could be mycorrhizal benefits to growing farther from conspecifics. There were strong maternal effects, i.e. seed size, on seedling responses to treatments. Overall, this study shows that seedling growth and morphology are very sensitive to the seedling's neighbourhood. Rather than one major force acting, e.g. kin selection or Janzen-Connell effects, there are several aspects of the plant neighbourhood that determine seedling establishment and growth. Seedlings not grown in mesh bags were left in the field site for future measurement. It would be valuable to return to the field site and measure these plants, possibly over various future time points, to further investigate some of the effects found through the current analysis.

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Table 1: Generalized linear model (GENMOD) for survival, germination and emergence of *Cyclobalanopsis pachyloma* grown in the field.

Source	Germination *P*	Survival *P*	Emergence *P*
Seed biomass	**<0.0001**	**<0.0001**	**0.0011**
Distance	0.1028	0.6872	0.9860
Tree	0.0870	**0.0025**	**0.0023**
Family	**0.0101**	**<0.0001**	**0.0035**
Bag	**<0.0001**	**<0.0001**	**0.0001**

Analysis was conducted on individual seeds and seedlings grown in the field. Distance refers to the distance of the seedling from the nearby adult. Tree refers to the identity of the nearby adult. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Significant values are in bold.

Table 2: Analysis of covariance for size traits of *Cyclobalanopsis pachyloma* seedlings.

	Height			Log leaf mass			Log above			Estimated leaf area		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*
Seed	1	17.66	**<0.0001**	1	12.37	**0.0006**	1	8.88	**0.0033**	1	22.29	**<0.0001**
Tree	14	0.98	0.4729	14	1.86	**0.0350**	14	1.09	0.3665	14	2.07	**0.0170**
Bag	1	0.00	0.9495	1	11.40	**0.0009**	1	3.27	0.0723	1	16.33	**<0.0001**
Family	7	2.27	**0.0307**	7	4.06	**0.0004**	7	3.06	**0.0045**	7	7.50	**<0.0001**
Distance	2	3.46	**0.0334**	2	0.65	0.5227	2	0.17	0.8430	2	1.08	0.3419
Percent herbivory	-	-	-	-	27.70	**<0.0001**	-	-	-	-	-	-

Analysis was conducted on harvested seedlings. Distance refers to the distance of the seedling from the nearby adult. Tree refers to the identity of the nearby adult. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Percent herbivory is a covariate calculated from estimated original leaf area. Significant values are in bold.

Table 3: Means for size traits of *Cyclobalanopsis pachyloma* seedlings

Source	Leaf number	Estimated leaf area	Leaf mass	Stem diameter	Height	Leaf length
Bag						
No bag	2.8142 ± 1.0347 ab	-	-	1.4124 ± 0.1067 a	-	52.0035 ± 4.497
250 micron	2.3976 ± 0.8745 a	13.2971 ± 1.2910 a	0.0700 ± 0.0094 a	1.8072 ± 0.0714 b	12.7654 ± 0.4911 a	53.0186 ± 3.179
0.5 micron	2.9057 ± 1.0667 b	18.2644 ± 1.6212 b	0.0979 ± 0.0106 b	1.8120 ± 0.0658 b	12.7375 ± 0.4911 a	62.2580 ± 2.894
Distance from adult						
2m	2.4190 ± 0.0963 a	15.5969 ± 1.4330 a	0.0802 ± 0.0095 a	1.6719 ± 0.0757 a	13.6837 ± 0.4954 a	56.5842 ± 3.051
3m	2.7621 ± 0.0795 a	14.4723 ± 1.7237 a	0.0796 ± 0.0123 a	1.6785 ± 0.0921 a	12.4225 ± 0.6236 b	53.0839 ± 3.889
4m	2.9343 ± 0.0662 a	16.6626 ± 1.7958 a	0.0902 ± 0.0121 a	1.6813 ± 0.0826 a	12.1483 ± 0.5778 b	57.6119 ± 3.576
Social environment						
Kin	2.4869 ± 0.0674 a	-	-	-	-	-
Stranger	2.9236 ± 0.0681 b	-	-	-	-	-

Means presented here are the raw data or the back transformed means if a log transformation was used. Within each treatment and trait, different letters represent means that are statistically different from each other at the $P<0.05$ level or lower.

Table 4: Analysis of covariance for size traits of *Cyclobalanopsis pachyloma*

	Stem diameter			Leaf length		
Source	DF	F	*P*	DF	F	*P*
Seed	1	10.47	**0.0014**	1	13.82	**0.0003**
Tree	14	1.30	0.2069	14	0.96	0.4944
Bag	2	10.01	**<0.0001**	1	7.30	**0.0009**
Family	7	2.09	**0.0460**	7	5.11	**<0.0001**
Distance	2	0.01	0.9937	2	1.18	0.3105
Bag× Distance	4	3.19	**0.0143**	-	-	-

Analysis was conducted on seedlings alive at time of data collection. Distance refers to the distance of the seedling from the nearby adult. Tree refers to the identity of the nearby adult. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Significant values are in bold.

Table 5: Generalized linear model (GENMOD) for leaf number of *Cyclobalanopsis pachyloma* seedlings grown in the field.

	Leaf number
Source	***P***
Distance	0.2089
Social environment	**0.0466**
Family	0.0599
Bag	0.0730

Analysis was conducted on seedlings alive at the time of harvest. Distance refers to the distance of the seedling from the nearby adult. Social environment refers to whether seeds were planted under their mother, another conspecific adult or an adult from a different species. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Significant values are in bold.

Table 6: Analysis of covariance on *Cyclobalanopsis pachyloma* seedlings.

	Stem elongation (height: stem mass)			Log leaf mass: log stem mass			SLA (log leaf area:log leaf mass)			Leaf length: stem diameter			Log roots: log leaf mass		
Source	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*	DF	F	*P*
Tree	14	2.61	**0.0018**	-	-	-	-	-	-	14	0.85	0.6119	-	-	-
Bag	1	1.90	0.1697	1	20.43	**<0.0001**	1	2.07	0.1522	2	7.24	**0.0010**	1	5.34	**0.0222**
Family	7	1.73	0.1049	7	1.26	0.2745	7	2.03	**0.0456**	7	3.71	**0.0009**	7	4.39	**0.0002**
Distance	2	4.45	**0.0130**	2	3.80	**0.0246**	2	1.19	0.3083	2	2.11	0.1240	-	-	-
Percent herbivory	-	-	-	1	21.06	**<0.0001**	-	-	-	-	-	-	-	-	-
Social environment	-	-	-	2	4.12	**0.0181**	2	0.41	0.6613	-	-	-	2	4.25	**<0.0001**
Seed biomass	-	-	-	-	-	-	-	-	-	1	7.02	**0.0088**	-	-	-

Analysis was conducted on harvested seedlings except for leaf length: stem diameter, which was conducted on all seedlings alive at time of harvest. Distance refers to the distance of the seedling from the nearby adult. Tree refers to the identity of the nearby adult. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Social environment refers to whether seeds were planted under their mother, another conspecific adult or an adult from a different species. Percent herbivory is a covariate calculated from estimated original leaf area. Significant values are in bold.

Table 7: Means for morphology traits of *Cyclobalanopsis pachyloma* seedlings

Source	**Stem elongation**	**Leaf mass:stem mass**	**Leaf length: stem diameter**	**Root: leaf**
Bag				
No bag	-	-	58.5726 ± 4.3371 ab	-
250 micron	13.1074 ± 0.3476 a	0.0806 ± 0.0053 a	53.0037 ± 2.9468 b	0.0646 ± 0.0032 a
0.5 micron	12.7032 ± 0.3249 a	0.1070 ± 0.0058 b	62.4174 ± 2.6832 a	0.0573 ± 0.0025 b
Distance from adult				
2m	13.5852 ± 0.3262 a	0.0845 ± 0.0056 a	59.1753 ± 2.8676 ab	-
3m	12.7187 ± 0.4167 b	0.0907 ± 0.0073 ab	54.6140 ± 3.6160 a	-
4m	12.4118 ± 0.3862 b	0.1051 ± 0.0070 b	60.2046 ± 3.3487 b	-
Social environment				
Kin	-	0.0839 ± 0.0043 a	-	0.0682 ± 0.0027 a
Stranger	-	0.1016 ± 0.0130 b	-	0.0636 ± 0.0026 a
Other	-	0.0946 ± 0.0051 ab	-	0.0519 ± 0.0048 b

Means presented here are the lsmeans of the raw data or the back transformed means if a log transformation was used. Within each treatment and trait, different letters represent means that are statistically different from each other at the $P<0.05$ level or lower.

Table 8: Analysis of covariance on herbivore damage of *Cyclobalanopsis pachyloma* seedlings

		Total herbivory	
Source	DF	F	*P*
Seed	1	0.03	0.8665
Family	7	1.56	0.1522
Distance	2	1.16	0.3173
Bag	1	1.16	0.2832
Species	1	4.20	**0.0420**

Analysis was conducted on harvested seedlings. Distance refers to the distance of the seedling from the nearby adult. Family refers to the maternal sibship, i.e. focal tree, from which the seed came. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Species refers to whether the seed was planted under the focal species (*Cyclobalanopsis pachyloma)* or under one of the two non-focal species (*Schefflera octophylla* or *Cinnamonmum subavenium)*. Significant values are in bold.

Table 9: Analysis of covariance on fungal infection of *Cyclobalanopsis pachyloma* seedlings.

	Rate of infection		
Source	DF	F	*P*
Root length	1	6.70	**0.0113**
Social environment	2	2.13	0.1246
Bag	1	7.68	**0.0068**
SocEnv × Bag	2	4.06	**0.0206**

Analysis was conducted on harvested seedlings. Social environment (SocEnv) refers to whether seeds were planted under their mother, another conspecific adult or an adult from a different species. Bag refers to whether the seed was planted directly in the ground, in a 250-micron mesh bag or a 0.5-micron mesh bag. Significant values are in bold.

Fig 1: Effect of Bag treatment on the predicted probability of emergence with increasing seed size. Seed biomass is on the x-axis and probability of emergence is on the y-axis. Each line represents one of three bag treatments: no bag (solid blue line), 0.5 micron bag (red dashed line) or 250 micron bag (green dashed line).

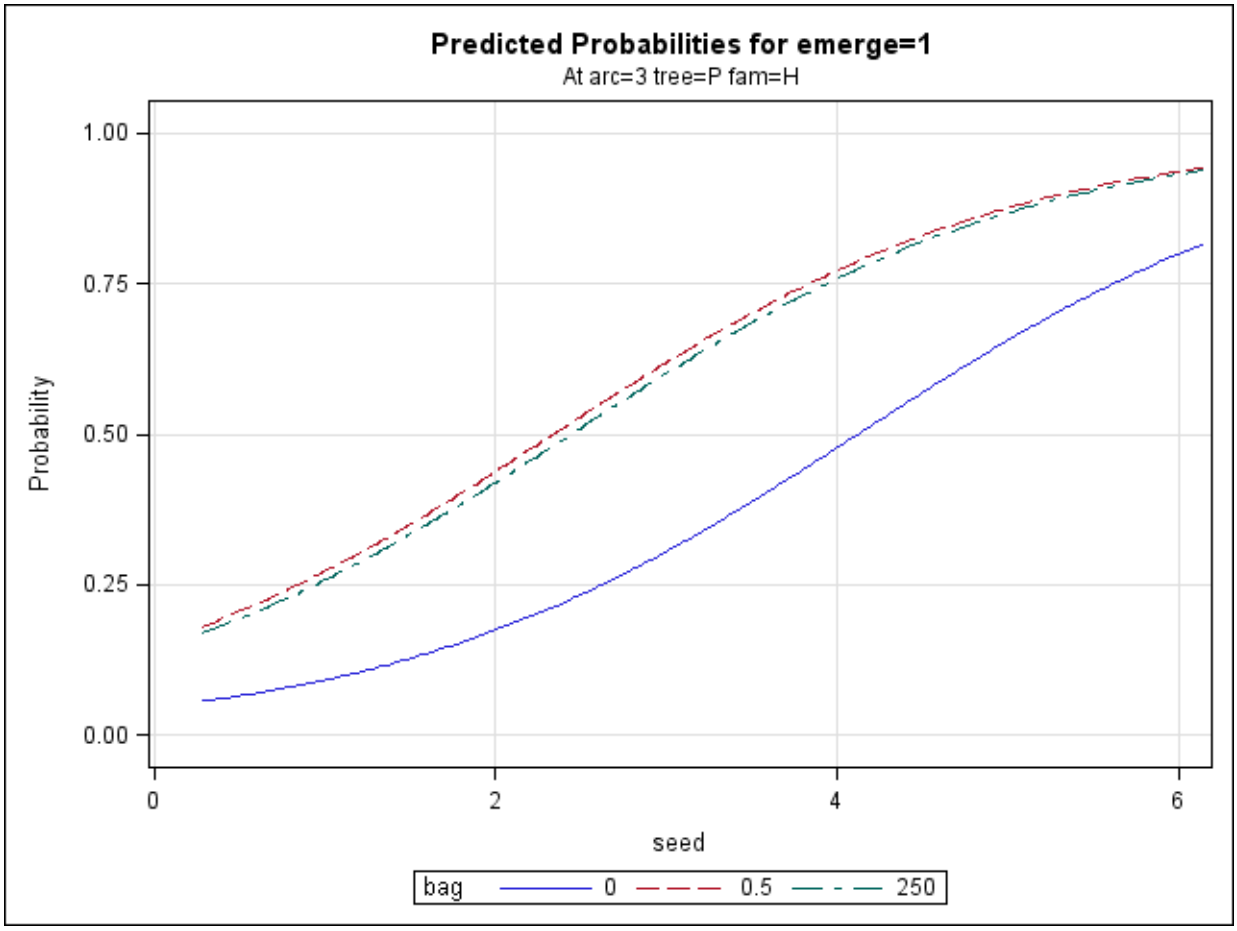

Predicted Probabilities for emerge=1
At arc=3 tree=P fam=H
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
bag
0
0.5
250

Fig 2: Effect of adult tree on the predicted probability of emergence with increasing seed size. Seed biomass is on the x-axis and probability of emergence is on the y-axis. Each line represents a different adult tree under which seeds were planted. Trees A-H are from the focal species, *Cyclobalanopsis pachyloma,* trees I-L are from the species *Schefflera octophylla* and trees M-P are from the species *Cinnamonmum subavenium*.

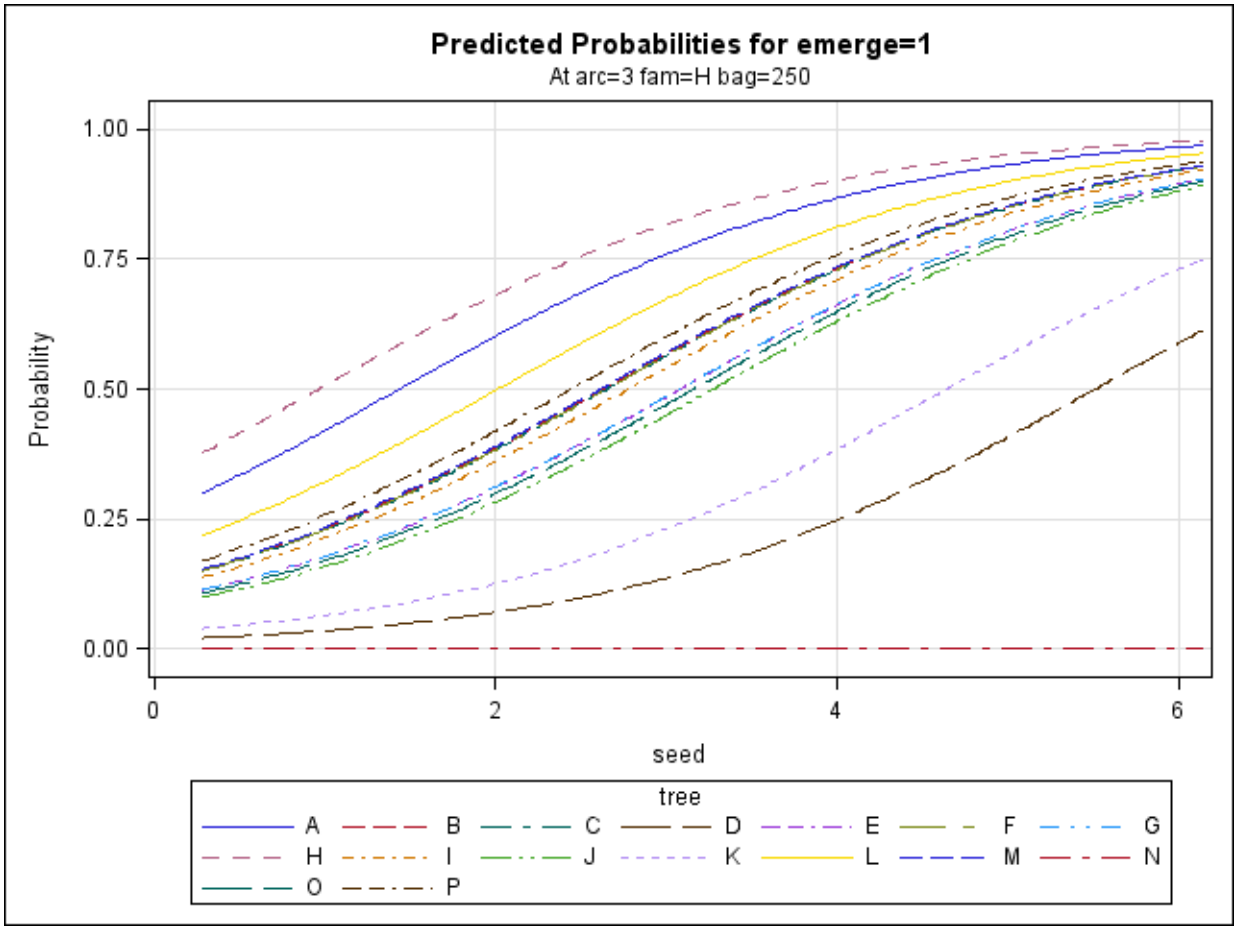

Predicted Probabilities for emerge=1
At arc=3 fam=H bag=250
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
tree
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Fig 3: Effect of family on the predicted probability of emergence with increasing seed size. Seed biomass is on the x-axis and probability of emergence is on the y-axis. Each line represents a different family from the focal species, *Cyclobalanopsis pachyloma.*

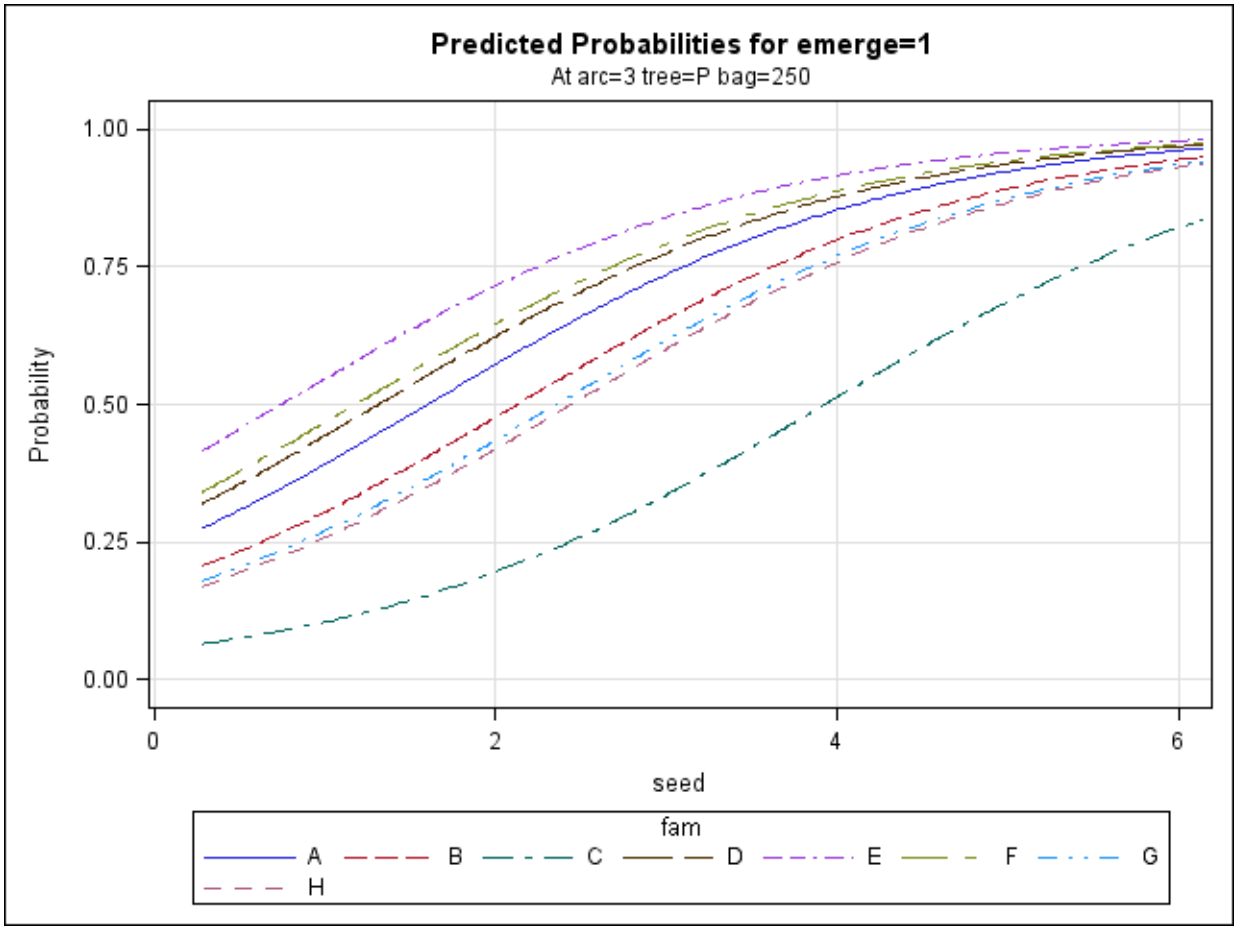

Predicted Probabilities for emerge=1
At arc=3 tree=P bag=250
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
fam
A
B
C
D
E
F
G
H

Fig 4: Above ground biomass for a given seed mass of harvested *Cyclobalanopsis pachyloma* seedlings from eight maternal families.

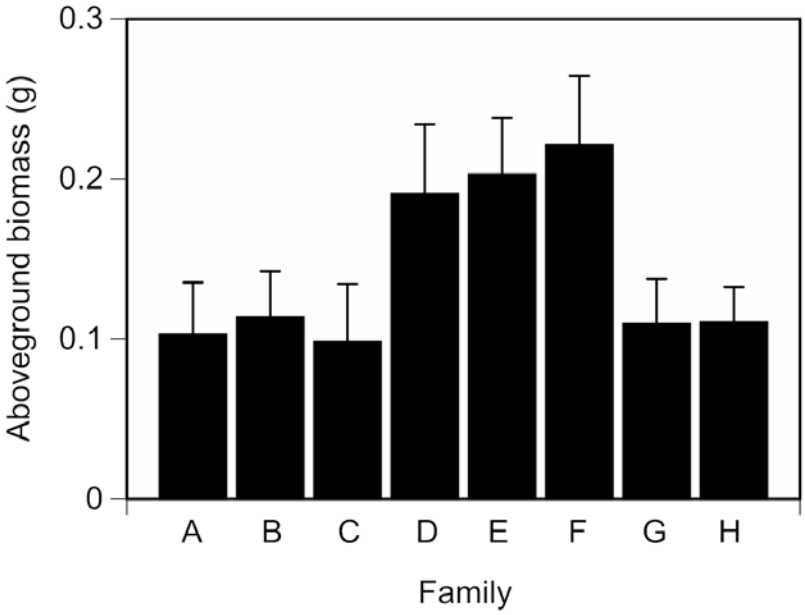

0.3
0.2
0.1
0
Aboveground biomass (g)
A
B
C
D
E
F
G
H
Family

Fig 5: Effects of distance from adult tree and bag treatment on seedling stem diameter. Seedlings of *Cyclobalanopsis pachyloma* were grown two, three or four meters from the trunk of the adult tree. Seeds were planted in one of three bag treatments: no bag (open bars); 250 micron bag (grey bars); or 0.5 micron bag (black bars).

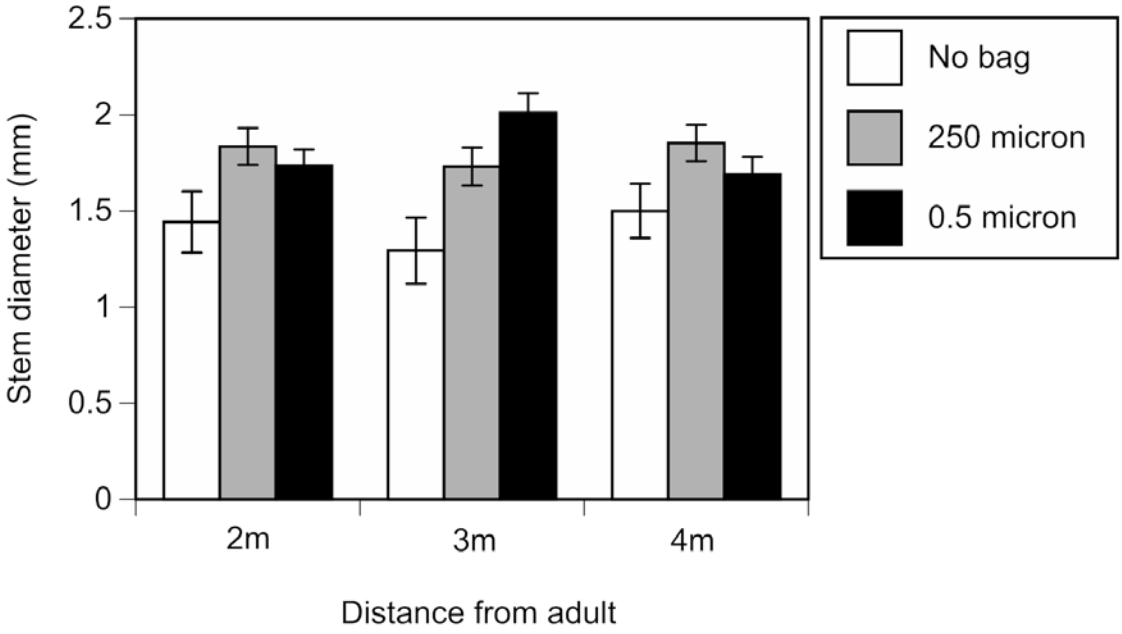

2.5
2
1.5
1
0.5
0
Stem diameter (mm)
2m
3m
4m
Distance from adult
No bag
250 micron
0.5 micron

Fig 6: Leaf biomass of harvested *Cyclobalanopsis pachyloma* seedlings grown under one of 16 adult trees. Adult trees were either from the focal species, *Cyclobalanopsis pachyloma,* or from one of two other species: *Schefflera octophylla* and *Cinnamonmum subavenium*.

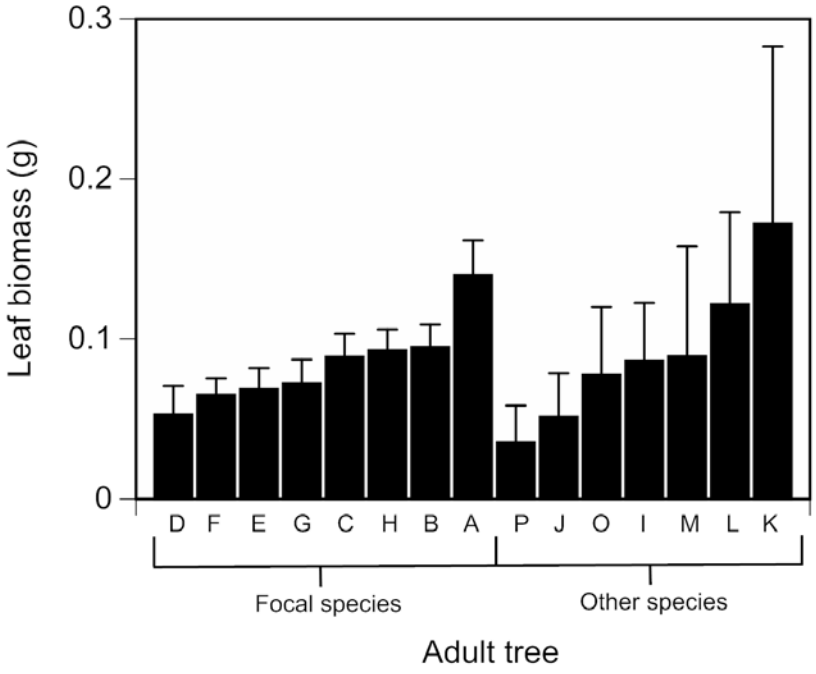
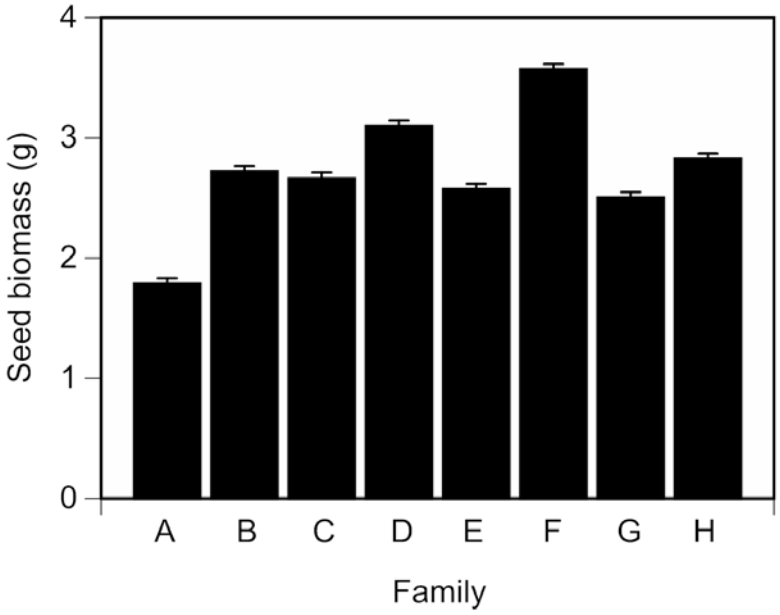

0.3
0.2
0.1
0
Leaf biomass (g)
D F E G C H B A P J O I M L K
Focal species
Other species
Adult tree

Fig 7: Biomass of seeds collected from eight different maternal families of *Cyclobalanopsis pachyloma*


Seed biomass (g)
0
1
2
3
4
A
B
C
D
E
F
G
H
Family

.

Fig 8: Affect of nearby adult tree on seedling stem elongation (height for a given stem biomass). Adult trees were either from the focal species, *Cyclobalanopsis pachyloma,* or from one of two other species: *Schefflera octophylla* and *Cinnamonmum subavenium*

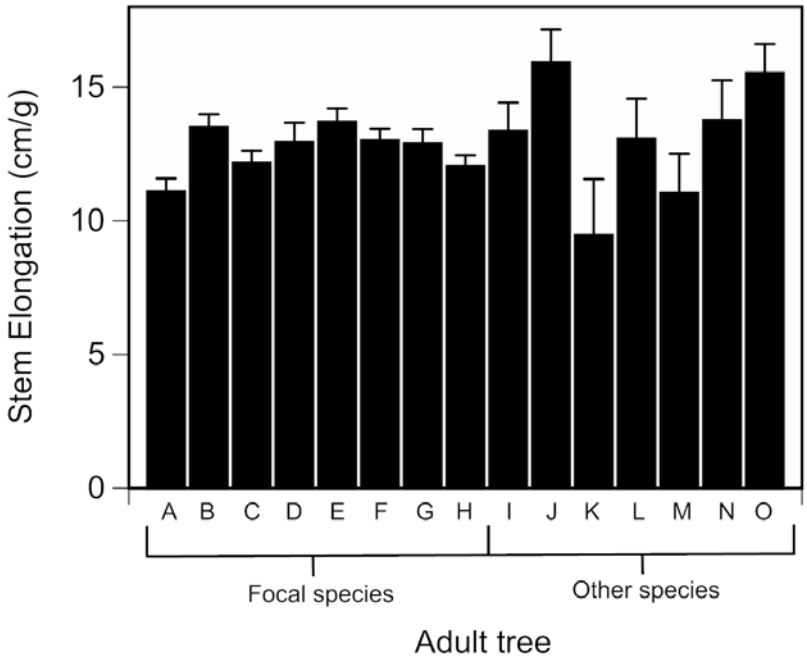

Stem Elongation (cm/g)
15
10
5
0
A B C D E F G H I J K L M N O
Focal species
Other species
Adult tree

Fig 9: Effect of distance from adult tree on allocation of leaf biomass relative to stem biomass of harvested plants. Plants were located at increasing distances from the adult tree: 2m, 3m or 4m. Bars with different letters are statistically different at the $P<0.05$ level.

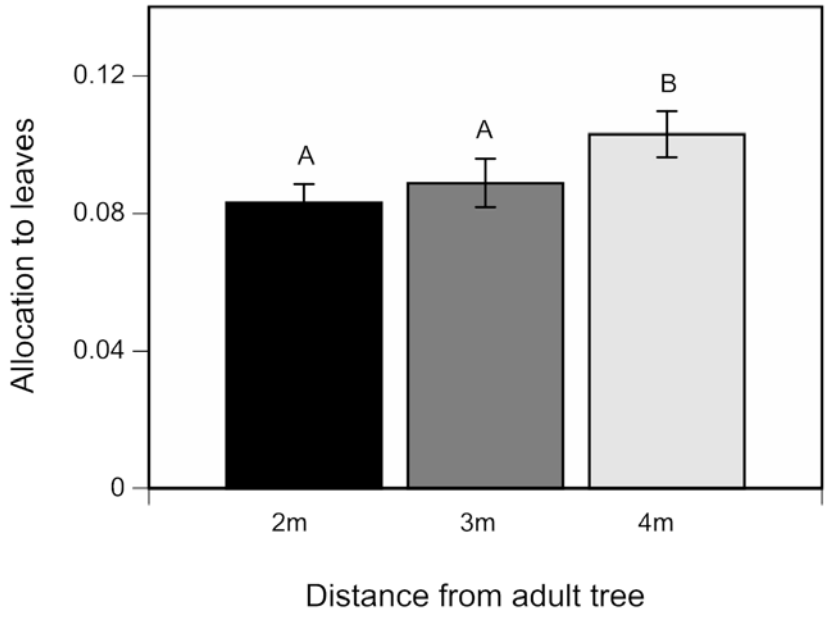

0.12
0.08
0.04
0
Allocation to leaves
A
A
B
2m
3m
4m
Distance from adult tree

Fig 10: Effect of social environment on allocation of leaf biomass relative to stem biomass of harvested plants. Plants were grown under their mother tree (kin), an un-related focal species tree (stranger) or under an adult tree from another species (other species). Bars with different letters are statistically different at the $P<0.05$ level.

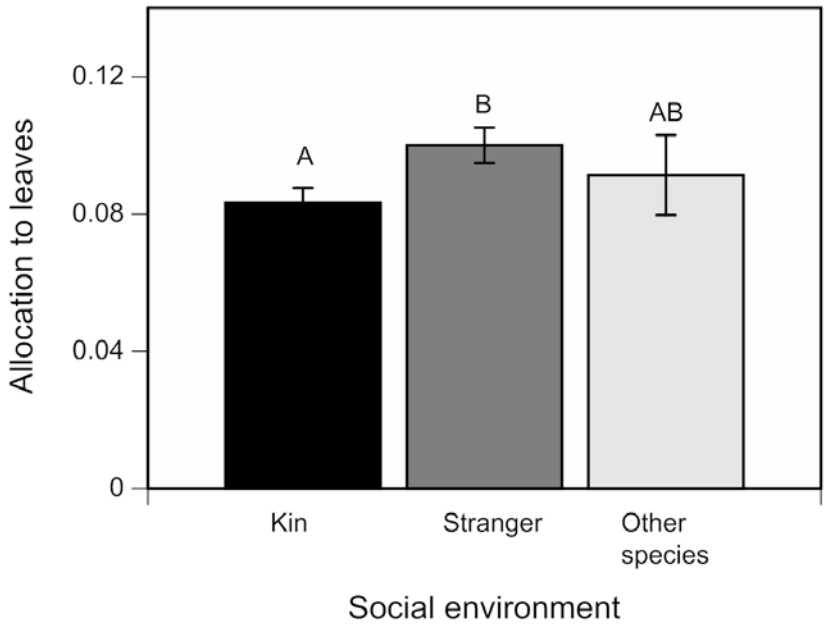
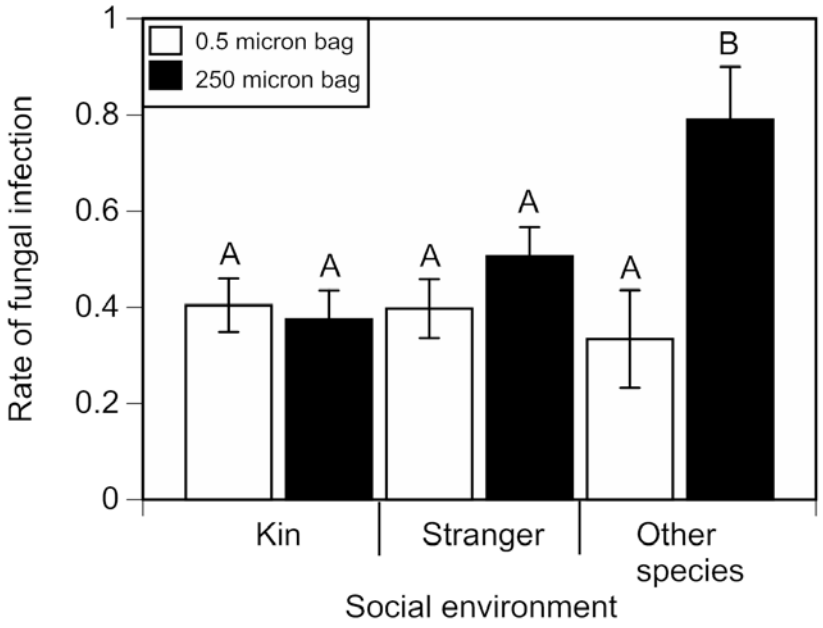
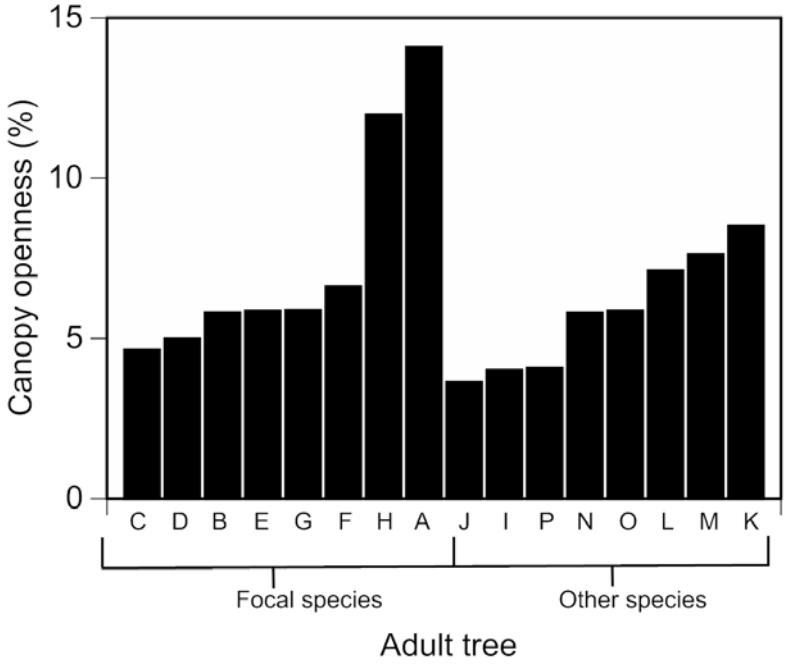

0.12
0.08
0.04
0
Allocation to leaves
A
B
AB
Kin
Stranger
Other species
Social environment

Fig 11: Effect of social environment and bag treatment on rate of mycorrhizal fungal infection of *C. pachyloma* seedling roots.

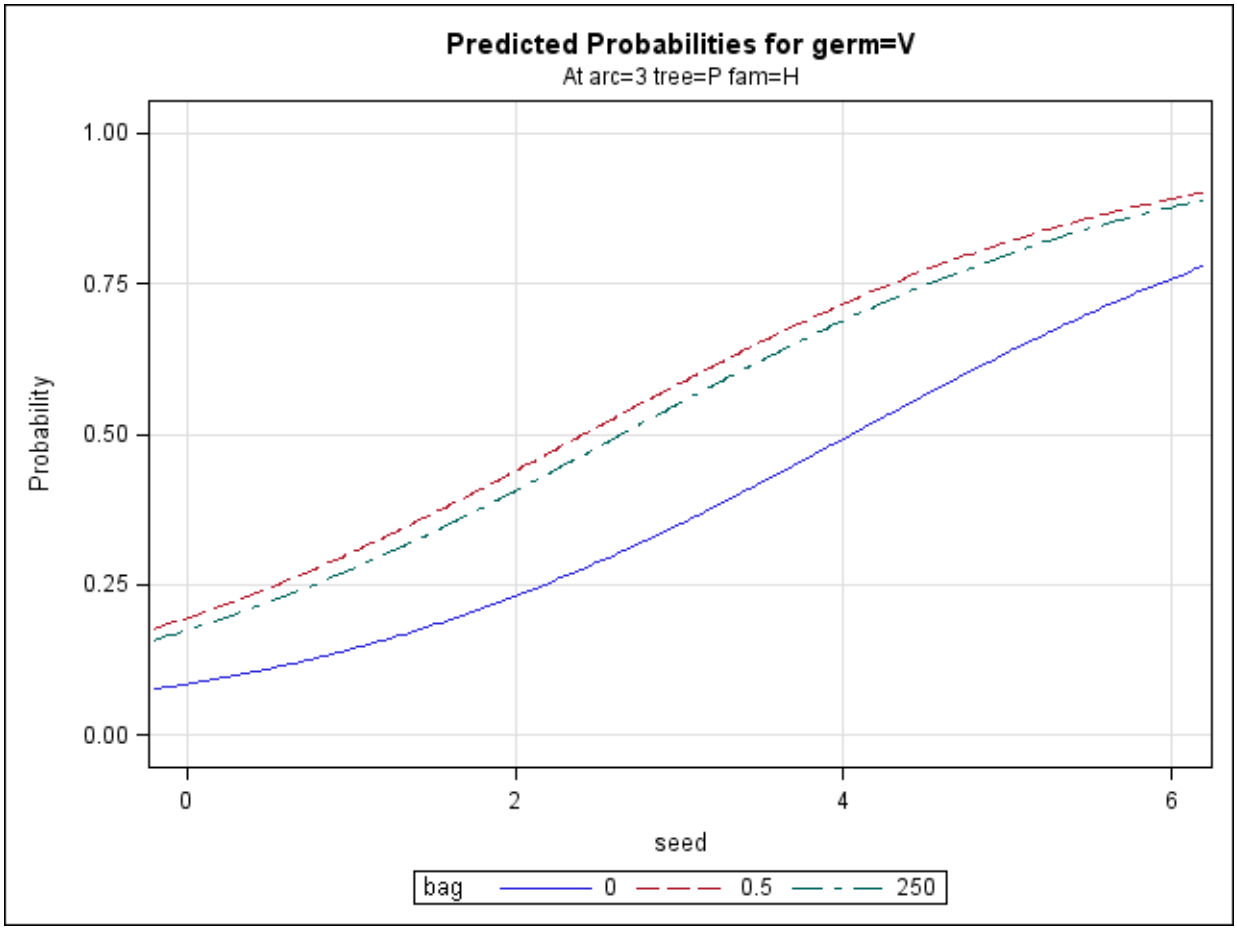

0.5 micron bag
250 micron bag
Rate of fungal infection
1
0.8
0.6
0.4
0.2
0
A
A
A
A
A
B
Kin
Stranger
Other species
Social environment

APPENDIX

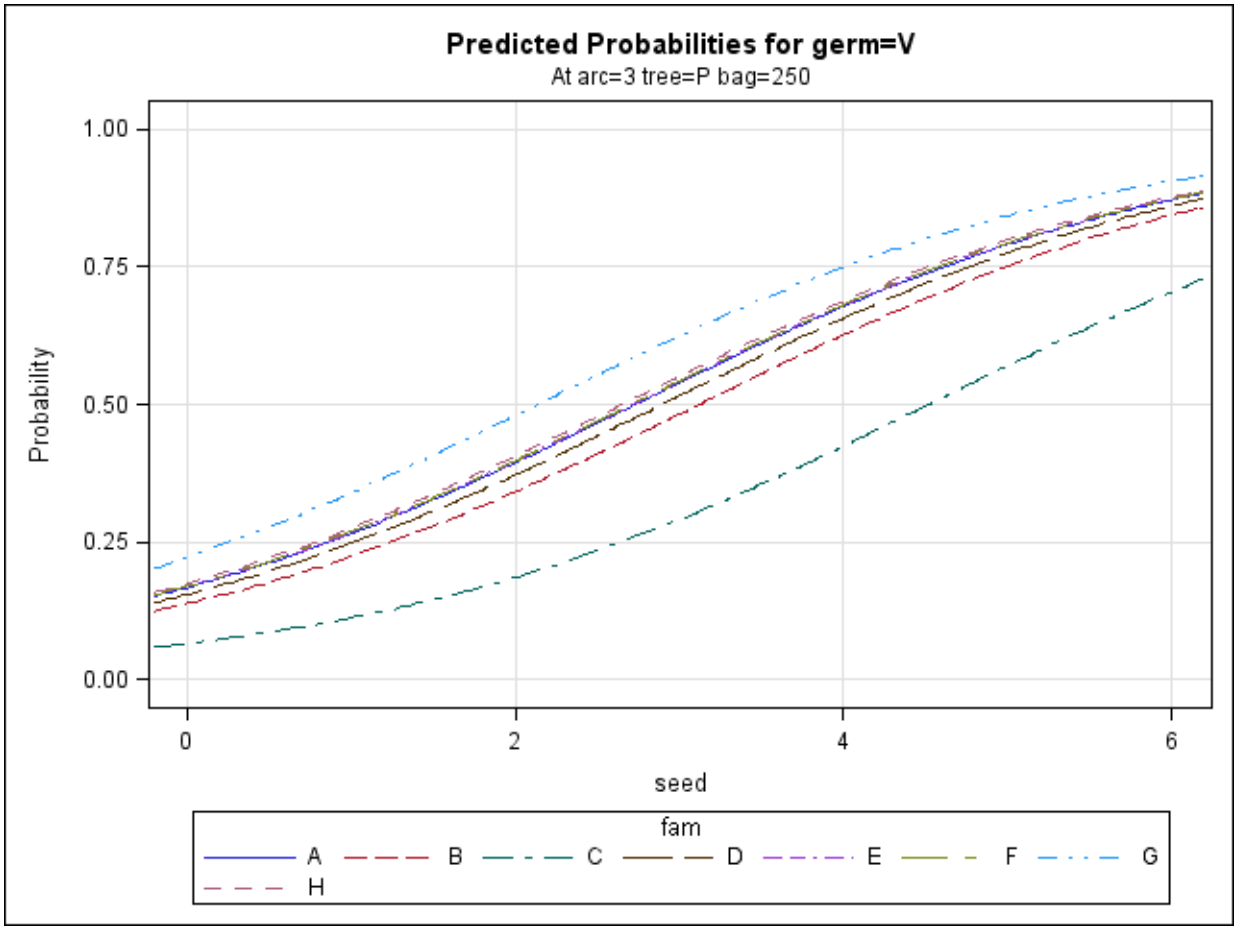
Appendix Fig 1: Canopy openness for each of the 16 adult trees. Adult trees were either from the focal species, *Cyclobalanopsis pachyloma,* or from one of two other species: *Schefflera octophylla* and *Cinnamonmum subavenium.*


15
10
5
0
Canopy openness (%)
C D B E G F H A J I P N O L M K
Focal species
Other species
Adult tree

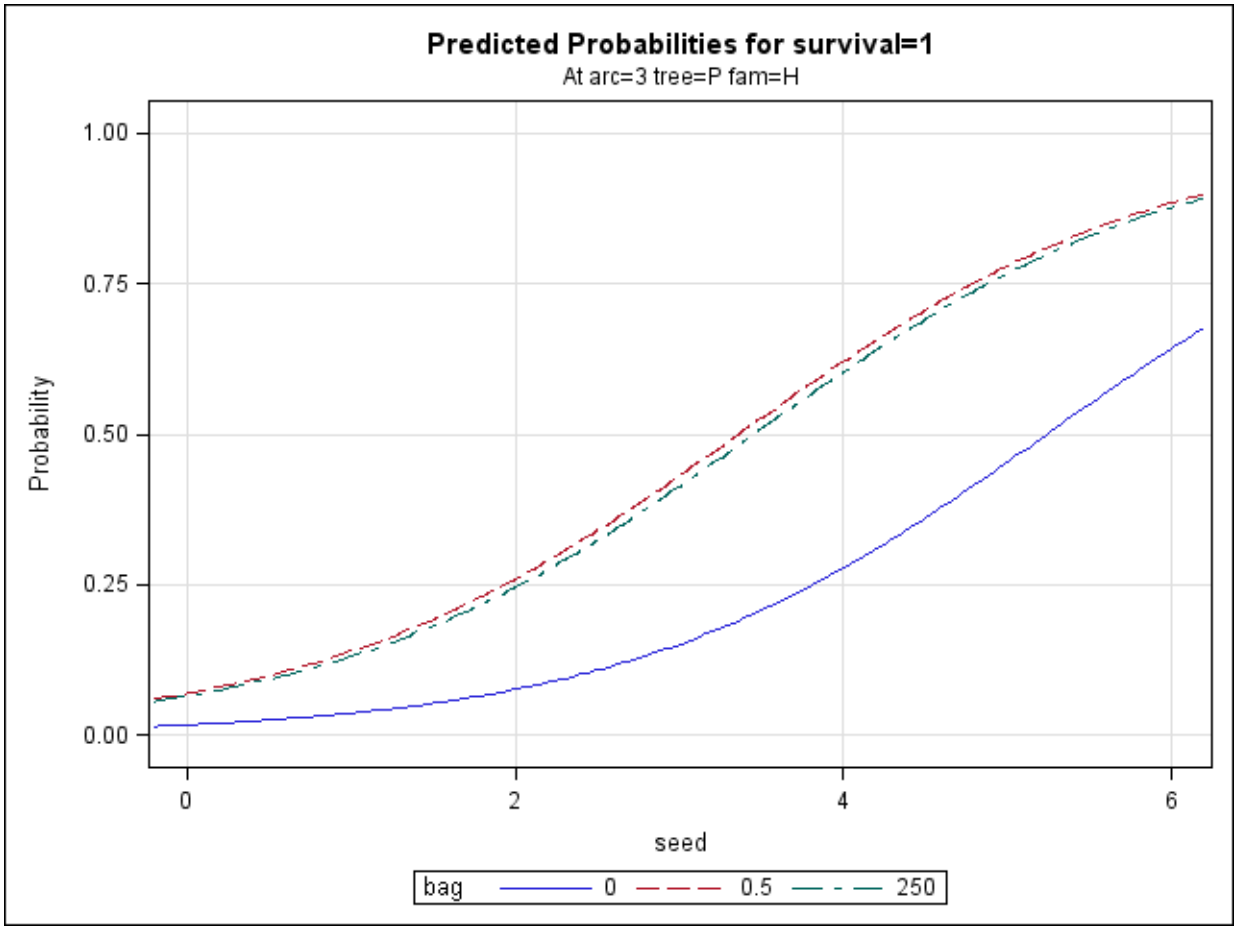
Appendix Fig 2: Effect of bag treatment on the predicted probability of germination with increasing seed size. Seed biomass is on the x-axis and probability of germination is on the y-axis. Each line represents a different bag treatment: no bag (solid blue line), 0.5 micron bag (red dashed line) or 250 micron bag (green dashed line).


Predicted Probabilities for germ=V
At arc=3 tree=P fam=H
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
bag
0
0.5
250

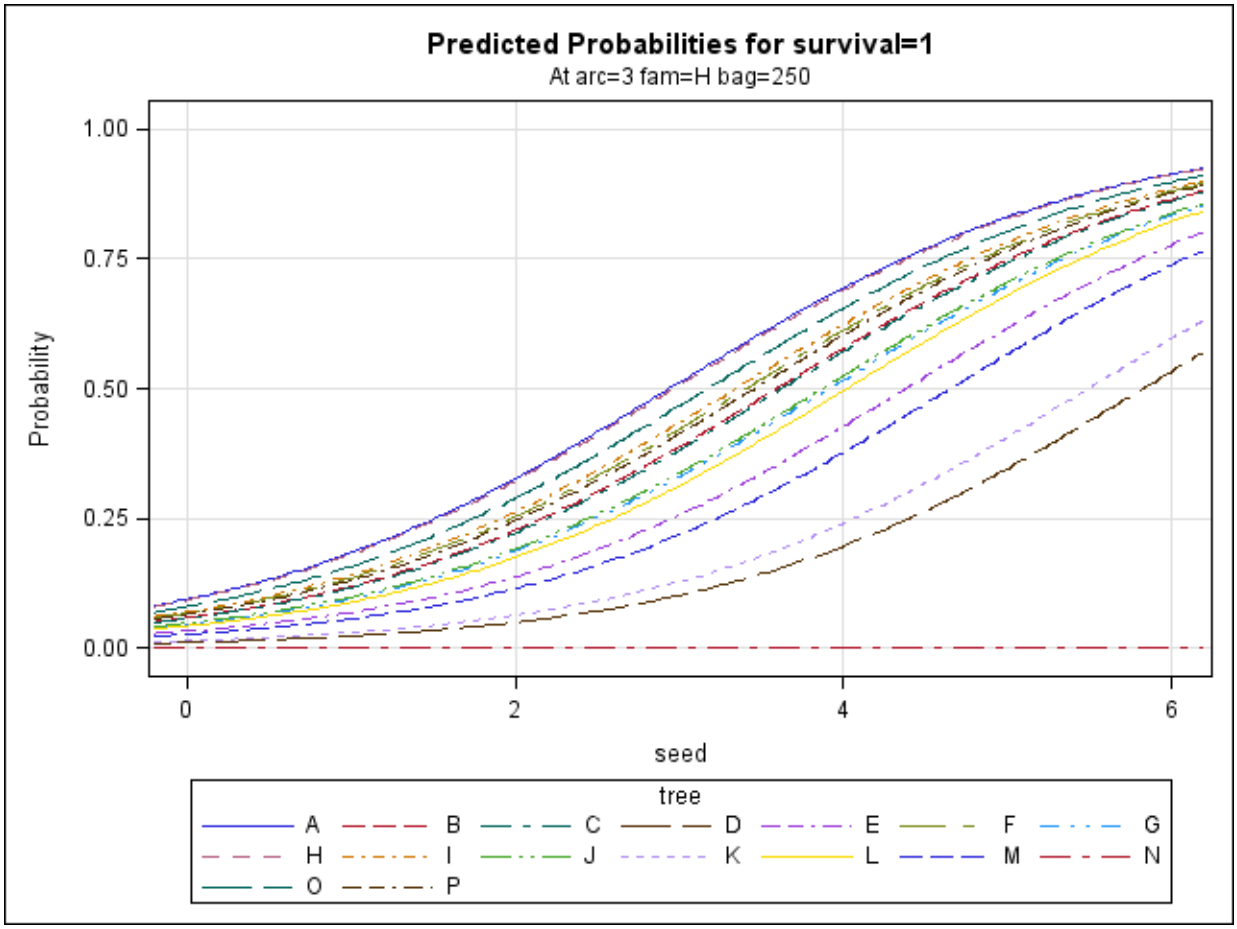
Appendix Fig 3: Effect of family on the predicted probability of germination with increasing seed size. Seed biomass is on the x-axis and probability of germination is on the y-axis. Each line represents a different family from the focal species, *Cyclobalanopsis pachyloma.*


Predicted Probabilities for germ=V
At arc=3 tree=P bag=250
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
fam
A
B
C
D
E
F
G
H

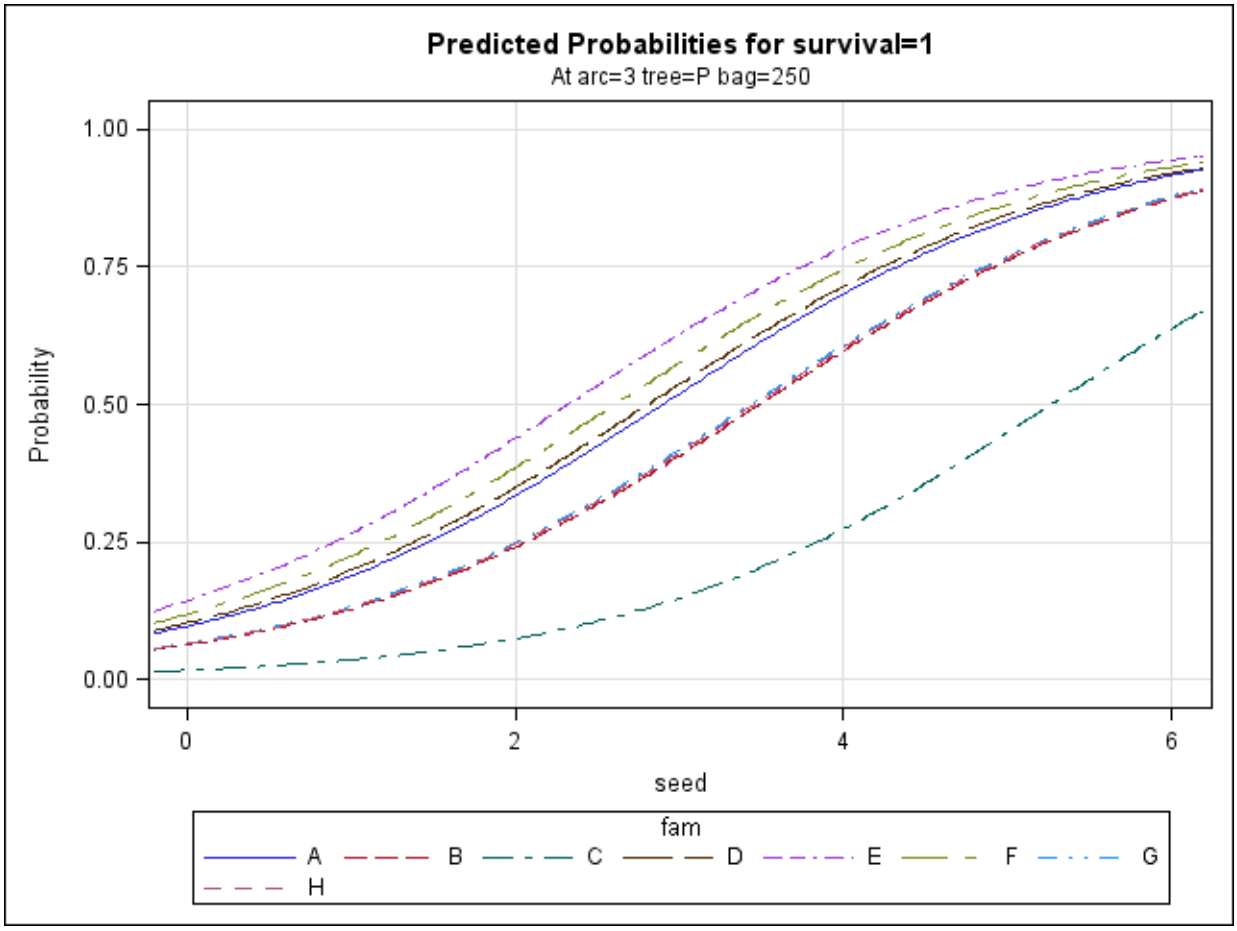
Appendix Fig 4: Effect of bag treatment on the predicted probability of survival at the time of harvest with increasing seed size. Seed biomass is on the x-axis and probability of survival is on the y-axis. Each line represents one of three bag treatments: no bag (solid blue line), 0.5 micron bag (red dashed line) or 250 micron bag (green dashed line).


Predicted Probabilities for survival=1
At arc=3 tree=P fam=H
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
bag
0
0.5
250

Appendix Fig 5: Effect of adult tree on the predicted probability of survival at the time of harvest with increasing seed size. Seed biomass is on the x-axis and probability of survival is on the y-axis. Each line represents a different adult tree under which seeds were planted. Trees A-H are from the focal species, *Cyclobalanopsis pachyloma,* trees I-L are from the species *Schefflera octophylla* and trees M-P are from the species *Cinnamonmum subavenium*.


Predicted Probabilities for survival=1
At arc=3 fam=H bag=250
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
tree
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Appendix Fig 6: Effect of family on the predicted probability of survival at the time of harvest with increasing seed size. Seed biomass is on the x-axis and probability of survival is on the y-axis. Each line represents a different family from the focal species, *Cyclobalanopsis pachyloma.*


Predicted Probabilities for survival=1
At arc=3 tree=P bag=250
Probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
seed
fam
A
B
C
D
E
F
G
H

Appendix Image 1: *Cyclobalanopsis pachyloma* acorn with cupule attached.

Appendix Image 2: *Cyclobalanopsis pachyloma* seeds after the cupules have been removed. The seeds range in colour depending on their ripeness. Lighter green/yellow coloured seeds (on the left) are less mature than darker, purple/brown coloured seeds (on the right).

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CHAPTER 6

GENERAL CONCLUSION

In this thesis I have investigated how plant social environment, i.e. relatedness of the neighbours, affects the mycorrhizal symbiosis and whether mycorrhizae determine plant responses to neighbour relatedness. I have addressed this topic through a series of greenhouse and field experiments, within and across generations, in three different species. In the data chapters presented here, I have shown evidence addressing four questions: 1) How is plant kin recognition affected by the mycorrhizal symbiosis? 2) Do siblings benefit each other by promoting the mycorrhizal symbiosis? 3) Could plant kin selection affect the stability of this mutualism? 4) How are responses to mycorrhizae and social environment dependent upon abiotic and biotic factors?

Kin recognition

I hypothesized that mycorrhizae could facilitate plant kin recognition because fungal hyphae connect plants at their roots. In Chapter 4, I found some support for this hypothesis. I show that whether or not plants are inoculated with mycorrhizal fungi affects their response to the relatedness of their neighbour in an aboveground competitive trait, specific leaf area (leaf area for a given leaf biomass; SLA). Sibling pairs were expected to show altruism and behave less competitively than stranger pairs. *Plantago lanceolata* demonstrated the predicted increased SLA with strangers, allowing them to better shade their neighbours, but only when inoculated with mycorrhizae. This is consistent with kin recognition being facilitated by mycorrhizae, perhaps through

increased transport of signaling molecules. However, it could also be evidence that mycorrhizae make the environment more favourable for plants, allowing them respond to social environment. The experiment in Chapter 4 was not designed to distinguish between these two possible mechanisms but it would be interesting to explore this in a future experiment. Additionally, I found that mycorrhizal inoculation affected plant competitive responses to social environment only in *P. lanceolata*, not in ragweed or *C. pachyloma*, so it would also be interesting to explore whether this response occurs in other species.

Kin selection

I hypothesized that plants could demonstrate altruism towards siblings by increasing their support of a shared mycorrhizal fungal partner. This hypothesis is predicted by kin selection since a well-established common mycorrhizal network (CMN) can result in inclusive fitness benefits for relatives. I have shown evidence of this hypothesis in Chapters 2-4. In Chapters 2 and 4, I show that siblings had increased soil hyphal growth compared to strangers, which is consistent with altruism among siblings since increased hyphal growth implies greater plant carbon donations to the fungal partner (Bever, Richardson et al. 2009). Moreover, in Chapter 2, I found reduced root damage in siblings and increased nutrient uptake with increased hyphal growth, demonstrating benefits for siblings increasing their mycorrhizal association. In Chapter 3, I conducted two experiments, a year apart. In the first experiment, most traits responded to herbivory and I found no evidence for an effect of mycorrhizae. However, in the second experiment, pairs of inoculated siblings had higher performance than all other groups, though this depended on when they were planted. Thus, the results from Chapters 2-4 show that not

only can the CMN be thought of as an extended phenotype that responds to plant social environment, but also that plant kin selection may shape the mycorrhizal symbiosis.

Mutualism stability

Although the plant-mycorrhizal association is commonly thought of as a mutualism, it likely ranges from parasitism to mutualism depending on the species involved (Johnson, Graham et al. 1997). And since there is no evidence of one partner controlling the mutualism, an important evolutionary question is what keeps the mutualism from slipping into a parasitism, i.e. what stabilizes the mutualism and allows it to persist? In Chapter 2 and Chapter 4, I show evidence of increased mycorrhizal growth and increased root colonization in groups of related plants compared to groups of un-related plants. Specifically, both common ragweed and *Plantago lanceolata* had more arbuscules colonizing their roots when grown with siblings compared to strangers. Arbuscules are the sites of nutrient exchange between fungal and plant partners and are considered an indicator of how well established the symbiosis is (Denison and Kiers 2011). Therefore, more arbuscules in siblings indicates a stronger mycorrhizal relationship among kin. Depending on the dispersal mechanism, plants often live close to relatives (Cheplick 1992) and several species can identify kin from non-kin (Dudley and File 2007, Biedrzycki, Jilany et al. 2009, Murphy and Dudley 2009, Bhatt, Khandelwal et al. 2010, Karban, Shiojiri et al. 2013) Thus, the stability of the symbiosis as a mutualism could be maintained through mycorrhizal responses to plant social environment.

Responses to other environmental factors

In nature, the mycorrhizal symbiosis is not isolated from the environment and both abiotic and biotic factors, such as light availability and plant density, are known to

have important effects on fungal growth and colonization of plant roots. Thus, we should also expect that any responses of social environment to mycorrhizae and responses of mycorrhizal colonization to social environment would depend on other environmental factors. This has been addressed in Chapters 3-5. In Chapter 3, I show the importance of density, herbivory and planting time on ragweed morphology and performance responses to relatedness and mycorrhizal inoculation. Herbivory and density are particularly relevant since they are components of the natural environment. Plants experienced herbivory in experiment one, causing decreased sample size and increase variance in aboveground traits. In experiment two, herbivory was less widespread and responses of performance and fitness to inoculation depended on density. In Chapter 4, I show that the effect of social environment on mycorrhizal colonization of pairs of *P. lanceolata* was dependent upon light availability. Whether one plant in a pair was shaded, both plants were shaded or neither plant was shaded mattered for fungal growth and colonization of all fungal structures in response to plant relatedness. In nature, neighbouring plants could have asymmetric stress due to differences in age, size or because of subtle differences in their environments, e.g. light or nutrient gradients. In Chapter 5, I have demonstrated the importance of distance from nearby adult tree, canopy openness and maternal investment on seedling growth. Although I was looking for responses to social environment and mycorrhizal access, my results show that they are just part of the many environmental agents acting on the sensitive seedling's germination, establishment and growth.

Synthesis and future research

Overall, results from the four data chapters presented here show a comprehensive illustration of how plant social environment interacts with the mycorrhizal symbiosis. Importantly, this thesis shows strong evidence for the mycorrhizal symbiosis serving as an extended phenotype that responds to plant kin recognition, and that plants can show altruism towards kin through mycorrhizae. Moreover, results indicate that any responses to mycorrhizae and plant social environment should be considered in the context of natural conditions since other environment factors have important consequences. My results offer new questions about the evolutionary ecology of mycorrhizae: Can plants control carbon donation to the fungus and/or to neighbouring plants? Is plant kin recognition facilitated by mycorrhizal hyphae or plastic to the presence of the fungal partner? Are offspring from inoculated siblings more successful than inoculated strangers or un-inoculated plants? Over time, how do mycorrhizae, social environment and other environmental factors affect tree seedling growth? What is the importance of mycorrhizae in social interactions across generations of herbaceous plants? The plant-mycorrhizal fungal relationship is shaped by plant responses to their social environment, which research in mycorrhizal ecology should now take into consideration.

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