# Plant-mediated partner discrimination in ectomycorrhizal mutualisms

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**Abstract**

Although ectomycorrhizal fungi have well recognized effects on ecological processes ranging from plant community dynamics to carbon cycling rates, it is unclear how actively plants are able to influence the structure of these fungal communities. To address this knowledge gap, we performed two complementary experiments to determine 1) whether ectomycorrhizal plants can discriminate among potential fungal partners, and 2) to what extent the plants might reward better mutualists. In experiment 1, split-root *Larix occidentalis* seedlings were inoculated with spores from three *Suillus* species (*S. clintonianus, S. grisellus,* and *S. spectabilis*). In experiment 2, we manipulated the symbiotic quality of *Suillus brevipes* isolates on split-root *Pinus muricata* seedlings by changing the nitrogen resources available, and used carbon-13 labeling to track host investment in fungi. In experiment 1, we found that hosts can discriminate in multi-species settings. The split-root seedlings inhibited colonization by *S. spectabilis* whenever another fungus was available, despite similar benefits from all three fungi. In experiment 2, we found that roots and fungi with greater nitrogen supplies received more plant carbon. Our results suggest that plants may be able to regulate this symbiosis at a relatively fine scale, and that this regulation can be integrated across spatially separated portions of a root system.

Keywords: ectomycorrhiza, *Larix occidentalis*, partner choice, *Pinus muricata*, stable isotope enrichment, *Suillus*

## Introduction

Ectomycorrhizal fungi play critical roles in forest ecosystems, both as major drivers of nutrient cycling and carbon storage in soils (Averill et al. 2014; Clemmensen et al. 2015) and as key mediators of plant community dynamics (Nara and Hogetsu 2004; Bennett et al. 2017). Given the plant costs involved in the symbiosis (in terms of root occupancy, immune activation, plant carbon, or other resources) (Smith and Read 2008; Van Wees et al. 2008; Kennedy 2010; Corrêa et al. 2012; Pringle 2016), hosts should prefer to invest in more beneficial ectomycorrhizal mutualists when presented with several possible partners. This scenario has been well documented in the arbuscular mycorrhizal symbiosis, where plants can reward greater nutrient-providing symbionts with increased carbon supplies (Bever et al. 2009, Kiers *et al.*, 2011; Argüello *et al.*, 2016), and in the legume-rhizobial mutualism, where plants can punish ineffective nitrogen fixers, apparently by withholding oxygen (Kiers et al. 2003). These mechanisms—rewards and punishment—likely fall along a continuum of host control strategies that encompasses many possible mechanisms (Kiers and Denison 2008).

Because mechanisms of host control should only be required when a symbiotic interaction is costly to the plant, the cost of the ectomycorrhizal mutualism has been the subject of much debate. Since the fungi themselves principally benefit from host-derived carbon, this resource is often assumed to be the main cost to the plant host (Smith and Read 2008; Pringle 2016). This view has been challenged recently by the finding that carbon is an excess resource for plants (Corrêa et al. 2012), and that carbon allocation does not always directly correspond to nitrogen provisioning from the fungi (Corrêa et al. 2008; Valtanen et al. 2014; Hasselquist et al. 2016; Hortal et al. 2017). Carbon may still be important, however, when considering the potential costs of ectomycorrhizal mutualism. It might be most realistic to think of the "cost" of supporting any given ectomycorrhizal fungus as an *opportunity* cost: although the plant is typically not carbon-limited, any carbon that goes to one fungus is carbon could have been invested in another, potentially superior, symbiotic partner. Similarly, any root tip occupied by one fungus is a root tip that could have been occupied by another, with different and perhaps preferable symbiotic abilities. A host should, in theory, allow an interaction to continue only while it is receiving

mutualistic services that are at least as beneficial as the average mutualist in the available species pool (Johnstone and Bshary 2008), although recent work has demonstrated that environmental fluctuations can induce hosts to support lower-quality mutualists (Moeller and Neubert 2016). Maintaining this kind of control over the fungi should require an ectomycorrhizal plant to adjust its investment in mutualists according to their relative performances, even when carbon is not limiting *per se*.

Although discrimination among partners by plants has been demonstrated in other root symbioses, the ectomycorrhizal mutualism may be controlled in different ways. Unlike arbuscular mycorrhizal and rhizobial mutualisms, the ectomycorrhizal symbiosis is consistently extracellular and involves dozens of independently evolved fungal lineages (Smith and Read 2008; Tedersoo and Smith 2013). Additionally, the magnitude of variation in partner quality may be more extreme: ectomycorrhizal fungi vary dramatically in enzymatic capabilities, biomass production, and environmental tolerances (Agerer 2001; Jones et al. 2003; Talbot et al. 2013). Arbuscular mycorrhizal fungi, by contrast, may contain less functional variation within their monophyletic guild (Powell et al. 2009), although the full extent of their functional diversity has yet to be adequately characterized (Chagnon et al. 2013; Behm and Kiers 2014). Similarly, while there has been well-documented variation in nitrogen provisioning by different rhizobial strains (Friesen 2012), they provide a single nutrient compared to the more diverse services provided in mycorrhizal symbioses (e.g. multiple nutrients and water acquisition, protection from heavy metals and pathogens) (Colpaert et al. 2011; Bennett et al. 2017). Given the phylogenetic diversity and the breadth of symbiotic benefits provided by ectomycorrhizal fungi, their plant hosts may need to regulate them using different mechanisms from those employed by arbuscular mycorrhizal or rhizobial hosts.

Plant hosts have at least two opportunities to discriminate among fungal partners. Prior to initiating an interaction, a plant may respond to fungal signals and accept or reject potential partners based on their identities or anticipated benefits. This phenomenon is well documented in rhizobial symbioses (Oldroyd et al. 2011), and may also be important in mycorrhizal

associations (Schmitz and Harrison 2014; Garcia et al. 2015). In ectomycorrhizal associations, the strongest evidence for pre-interaction screening by plants is the phenomenon of host specificity. Among fungi, many are specific to particular families or genera of plants (Molina and Horton 2015); among plants, although strict specificity to one or a few species of fungi is almost entirely restricted to mycoheterotrophs (Bruns et al. 2002), the suite of symbionts a plant supports often varies predictably with plant taxonomy (Molina and Horton 2015). In many cases, this specificity may be exerted at the spore germination stage – that is, host-specific fungi will not germinate until appropriate roots are present, despite being capable of broader associations as hyphae (Massicotte et al. 1994; Lofgren et al. 2018). Additionally, recent research into ectomycorrhizal fungal gene expression has identified many small secreted peptides that appear to be expressed at the initiation of symbiosis, and whose identities can vary considerably among fungal taxa (Plett et al. 2011; Liao et al. 2016). This points strongly to pre-interaction signaling as an important checkpoint in this symbiosis.

Once an interaction has been initiated, the plant may also be able to reward or sanction its partner according to the benefits it provides. This is often cited as a key prerequisite for mutualism stability (Hoeksema and Kummel 2003; Frederickson 2013), since pre-interaction signaling may not truthfully convey the quality of the potential partner. It is far from clear, however, to what extent and on what basis ectomycorrhizal plants may discriminate among fungal partners according to their symbiotic benefit. Theory predicts that plants in nitrogen-limited systems should reward nitrogen-providing fungi with carbon resources (Franklin et al. 2014; Moeller and Neubert 2016). This would be consistent with the way that plants distribute resources among non-symbiotic roots, investing resources to encourage the growth of nitrogen-providing roots into patches of valuable soil resources (Chen et al. 2018). Importantly, these soil resources may be available to both roots and soil fungi, creating competition between the symbionts (Peay 2016). These competitive interactions could complicate rewards for cooperation, as hosts may direct photosynthate to portions of the root system that provide nitrogen, regardless of their symbiotic status. Adding to this complexity, theory suggests that, even with plant preferential allocation, a stable community of fungi on a root system may include

some non-mutualists, assuming that the plant will invest initially in fungi of unknown quality (Christian and Bever 2018). Thus, the degree of plant discrimination that exists in any given symbiosis may be important in explaining the diversity of ectomycorrhizal fungi that coexist on a fine scale.

Reflecting this complexity, evidence for plant rewards of cooperative ectomycorrhizal fungi has thus far been equivocal. Hasselquist et al. (2016) found idiosyncratic effects of nitrogen addition and shading on resource trading between ectomycorrhizal fungi and host trees in a boreal forest, suggesting that rates of carbon and nitrogen exchange are context-dependent and may not always benefit host plant growth. In particular, fungal retention of nitrogen may be greatest when little nitrogen is present, with transfer to host plants increasing as more becomes available (Näsholm et al. 2013). Comparing three species of *Pisolithus* associated with *Eucalyptus grandis,* Hortal et al. (2017) did not find evidence for plant carbon rewards of fungal nitrogen supply under conditions where nitrogen was not limiting. As soil nitrogen declines, plants have been shown to invest more in mycorrhizas, but they do not necessarily experience greater nitrogen transfer to their own tissues with greater fungal colonization (Corrêa et al. 2008). Taken together, it appears that plants may, under certain conditions, reward fungal nitrogen provisioning with carbon resources, but the relationship between fungal nitrogen transfer rates and plant investment in the fungus is complex.

To examine the importance of pre-colonization screening (based on fungal identity) and post-colonization selection (for symbiotic benefit) in mediating plant discrimination among ectomycorrhizal fungi, we performed two experiments. Both used host plants in the Pinaceae and fungi in the Pinaceae-specific genus *Suillus*. In Experiment 1, we grew split-root *Larix occidentalis* seedlings associated with each of three *Suillus* species, in single- or pair-wise combinations, to investigate whether the presence of another fungus elsewhere in the root system would affect the timing and extent of colonization from spores in an isolated root compartment. We hypothesized that, if the plant could discriminate prior to associating with a fungus, the initiation of colonization would vary depending upon the presence and identity of an

158 indirect competitor elsewhere on the root system. In experiment 2, we grew paired split-root
159 *Pinus muricata* seedlings with isolated genotypes of *Suillus brevipes* that varied only in access to
160 organic nitrogen. We then tracked plant photosynthate investment in fungi and roots of
161 artificially varying nutritional qualities using $^{13}C$ labeling. If rewards for nutrient provisioning were
162 important in this system, we expected nitrogen-providing roots and fungi to receive more
163 photosynthate from the plant than those with access to fewer nitrogen resources.
164
165 **Materials and Methods**
166
167 *Experiment 1:* Larix-Suillus
168
169 ***Plant propagation***
170 In March 2014, *Larix occidentalis* seeds (Silva Seed Company, Roy, WA, USA) were soaked in
171 distilled water for 24 hours and then stratified at 4°C for four weeks. The seeds were germinated
172 in Petri dishes containing moistened filter paper and then transferred into 19.5 cm x 19 cm x 0.5
173 cm plexiglass chambers. Each chamber was filled with 180 ml of a twice-autoclaved mix of forest
174 soil, peat moss, and sand (2:2:1 by volume). We added ten germinated seeds to the upper soil
175 surface. The microcosms were placed in a growth chamber at 21°C with a 16:8 hr light-dark cycle
176 and watered regularly with distilled water. After four weeks, the chambers were opened and the
177 roots of each seedling were pruned to facilitate division into two primary root segments. The
178 chambers were closed and the seedlings were grown for another 8 weeks under the same
179 conditions prior to their transfer into the experimental microcosms.
180
181 ***Microcosm set-up***
182 The experimental microcosms (hereafter referred to as microcosms) included the same soil mix,
183 but consisted of two separate square 10 cm x 10 cm x 1 cm Petri dishes that were glued together
184 (Fig. S1). Using a soldering tool, a small hole was notched into the upper sides of the Petri dishes
185 to plant each half of the seedling's root system inside each of the two Petri dishes while the shoot
186 grew above them. This allowed isolation of the two halves of the root system for each seedling

into the separate but immediately adjacent sides of the microcosm. A 1 mL pipette tip was inserted in a second small hole in each Petri dish, which allowed for controlled addition of water while preventing cross contamination between the two sides of the microcosm. For each seedling, both halves of the root system were checked for viability and size equivalency during transfer. If necessary, some roots were trimmed to make the two halves of the root system as equal as possible. Because the seedlings were not completely uniform in size, different sized seedlings were evenly distributed across treatments (i.e. all treatments received a similar range of seedling sizes).

***Experimental treatments***

To investigate how single- versus two-species ectomycorrhizal fungal inoculation affected both fungal colonization patterns and plant performance, we added inoculum of one of three *Larix*-associated *Suillus* species on one side of the microcosm: *S. clintonianus, S. grisellus,* or *S. spectabilis*. The ectomycorrhizal species inoculum was added as homogenized single-species spore slurries at a concentration of 5 x $10^5$ spores/ml of soil, which falls within the range of previous assays that have observed consistent ectomycorrhizal colonization (Kennedy and Bruns 2005). Spores were obtained from multiple sporocarps collected in Minnesota, USA in fall 2013 (see Kennedy et al. (2011) for additional details on spore inoculum preparation). In half of the microcosms, a second species was inoculated at the same time on the other side of the microcosm. The resulting treatments, which contained 6 replicate microcosms initially, were: 1) *S. clintonianus*/non-ectomycorrhizal, 2) *S. grisellus*/non-ectomycorrhizal, 3) *S. spectabilis*/non-ectomycorrhizal, 4) *S. clintonianus/S. grisellus*, 5) *S. clintonianus/S. spectabilis*, 6) *S. grisellus/S. spectabilis*. In order to prevent algal growth and shield the roots and fungi from light, each microcosm was wrapped in aluminum foil. The seedlings were grown in the microcosms for 20 weeks under the same light and temperature conditions used prior to inoculation, watering to saturation two or three times per week.

***Data collection***

After a total of 28 weeks of growth, seedlings were harvested to quantify ectomycorrhizal fungal colonization and seedling performance. During harvest, the stem of each seedling was cut immediately above the point where the root system split into two halves. All parts of above this splitting point were designated as shoot biomass and everything below as root biomass. Shoots were dehydrated at 65°C for 72 hours and then weighed. Needles from each seedling were also analyzed for leaf nitrogen content. For that analysis, 2 mg of leaf material was ground to a fine powder by shaking at 1500 rpm for 5 minutes in 2 mL screw-cap tubes with a Tungsten bead on a Geno/Grinder 2010 (SPEX, Mutchen, NJ, USA). The percent leaf nitrogen by mass was quantified on an elemental analyzer (Vario PyroCube, Elementar, Mt. Laurel, NJ, USA) at the University of Minnesota. The root systems of each seedling were rinsed free of adhering soil and stored in tap water at 4°C until scoring for ectomycorrhizal colonization (all treatments were processed within 10 days of harvest). Prior to scoring, each root sample was cut into one cm segments and mixed to equalize ectomycorrhizal root tip density across the sample. We then selected pieces of the root sample at random and visually scored ectomycorrhizal colonization under a dissecting microscope. For each root system sample, a minimum of 200 root tips were assessed. After scoring, all portions of the root sample (i.e. scored and un-scored) were dehydrated at 65°C for 72 hours and weighed for dry biomass. Because the microcosms were constructed with clear plastic and their depths were thin enough that much of the root system was visible, we were also able to visually inspect each microcosm and record the presence of ectomycorrhizal root tips without disturbing the root systems or the fungi during the course of the experiment. To quantify time to initial ectomycorrhizal fungal colonization, we unwrapped the microcosms every two weeks and searched each side for ectomycorrhizal root tips. We note that these time measurements are low-resolution estimates because initial colonization could have happened any time in the two-week window before the observations were recorded.

***Statistical analyses***

We used two-way analyses of variance (ANOVA) to compare differences across ectomycorrhizal fungal species identity and competitor treatments in a) % ectomycorrhizal colonization at the final harvest and b) time with ectomycorrhizal fungal colonization. We performed another two-

way ANOVA to compare compartment root mass across treatments, as a proxy for plant investment in the fungus. We also assessed differences in the benefits provided by each fungus by comparing plant biomass and % leaf nitrogen by mass in single-species treatments using two separate one-way ANOVAs. Following each significant ANOVA, differences among treatment means were determined using Tukey HSD tests. Prior to interpreting the ANOVAs, models were checked for adherence to homoscedasticity and normality assumptions, and data were log-transformed if necessary.

*Experiment 2:* Pinus-Suillus brevipes

***Plant and fungal propagation***

*Pinus muricata* seeds were obtained from cones collected at Point Reyes National Seashore, CA, USA in November 2013. Fungal inoculum was produced using 12 *Suillus brevipes* cultures that were originally isolated from sporocarps collected in Yosemite National Park and Mendocino County, CA, USA, as well as Alberta, Canada (Branco et al. 2015). Each plant received just one of these isolates to ensure genetic homogeneity of fungi across the root system, while allowing us to capture intraspecific variation in fungal behavior across seedlings with different fungi (see table S1 for distribution of isolates among treatment groups). We cultivated these isolates on modified Melins-Norkran medium (Marx 1969) with sterile cellophane membranes for transfer to seedlings. Pine seeds were surface-sterilized, germinated on moist filter paper, and planted into twice-autoclaved soil (50% sand, 50% low-ectomycorrhizal inoculum soil from Point Reyes) (Peay 2018) in February 2014. Throughout the experiment, seedlings were maintained in a growth room with a 16:8hr light:dark cycle at a temperature between 20-27 °C. In late 2014 (July through December), seedlings were gently uprooted, rinsed in deionized water, and moved into ectomycorrhizal synthesis chambers consisting of clean zip-top plastic bags containing Ingestad solution-moistened paper towels (Nylund and Wallander 1989). Cellophane membranes covered in *Suillus brevipes* mycelium, representing 8-12 weeks of growth from a plug, were gently pressed against the roots, and the bags were zipped closed (roots inside, shoot outside) and wrapped in foil to prevent light penetration. Seedlings remained in synthesis chambers for one week, and

then were transplanted (with adhering mycelia) into a twice-autoclaved artificial soil containing 50% sieved perlite, 40% vermiculite, and 10% peat by volume, in which they grew until harvest in early March (total 12-32 weeks). Uninoculated seedlings were rinsed in deionized water and then replanted into artificial soil, skipping the synthesis chambers step. Although the staggered mycorrhization schedule meant that seedlings were not at a uniform age when fungi were introduced, all mycorrhizal seedlings used in the experiment had been colonized for at least 12 weeks and were observed to have mature ectomycorrhizas in several places on their root systems when they were transplanted into split-root microcosms.

*Microcosm set-up*

In March 2015, all seedlings were uprooted, checked for colonization, and replanted into split-root microcosms (two 631-mL Anderson Tree Band pots taped together) containing the same sterile artificial soil medium (Fig. S1). The root system of each seedling (inoculated with a single genotype) was divided in two halves. Each half of the root system was planted in one of the two pots of the microcosm with the shoot above them. Seedlings were watered to saturation once per week with deionized water during the four-month period during which they grew in the split root microcosms.

***Nitrogen treatments to manipulate fungal partner quality***

During the replanting of seedlings into the split-root microcosms, we planted nitrogen-containing mesh bags into both compartments of each microcosm (ANKOM technology, NY: R510 bags, 50µm pore size). These bags allowed us to manipulate the symbiotic quality of the genetically identical isolates on either side of a given seedling's root system, by changing the amount of nitrogen available to them. Bags were filled with twice-autoclaved sand mixed with casein (Thermo Fisher Scientific product S25238), an extract of nitrogen-rich milk proteins, in three concentrations: no casein, 0.5 g casein, or 1 g casein per bag, creating a gradient of nitrogen availability to plants and fungi. Seedlings were divided into three nitrogen treatment groups: Sand on each side of the root system (sand/sand), an identical amount of casein on each side (nitrogen/nitrogen), and sand on one side, casein on the other (nitrogen/sand). The sand/sand

(S/S) and nitrogen/nitrogen (N/N) treatments allowed the genetically identical isolates on each side of a given root system to perform as plant symbionts of similar quality. The nitrogen/sand (N/S) treatments were intended to force one of the genetically identical isolates (provided with only a sand bag) into the role of a putatively less effective symbiont than its competitor (provided with casein) on the other side of the root system. Because the plant had the opportunity to associate with symbionts of different quality, we called this the "choice" treatment.

***$^{13}$C labeling***

We used stable isotope enrichment to track plant allocation of carbon resources to roots and genetically identical isolates on each side of their root systems. In July 2015, plants were placed in $CO_2$ enrichment chambers at the Oxford Tract greenhouses at the University of California, Berkeley (Herman et al. 2012). We used an automated system to introduce $^{13}CO_2$ into the chamber each time the plants drew down chamber $CO_2$ below 400ppm, maintaining $CO_2$ concentrations between 400-800 ppm during the six hour labeling period. Plants were labeled in five batches, one per day, due to space limitations within the labeling chambers. The first batch was labeled using 99% $^{13}CO_2$ (Sigma-Aldrich product 364592). For subsequent batches, we used a 10% enriched $^{13}CO_2$ source (Sigma-Aldrich product 600180). (See "Statistical Analysis" for details on our treatment of batch effects introduced by different $^{13}$C concentrations.) Plants were harvested exactly seven days after $^{13}$C enrichment, and harvested root tissues were stored up to a week in cold tap water before dissection and drying.

***Data collection***

At the end of the experiment, we harvested 53 labeled seedlings. These included 5 colonized and 12 uncolonized nitrogen/nitrogen plants, 7 colonized and 11 uncolonized nitrogen/sand plants, and 6 colonized and 12 uncolonized sand/sand plants. (Some plants were not successfully colonized by fungi, so replication of uncolonized plants was greater in all treatments.) Twelve additional plants, which had been in the growth room with other plants, but had not received $^{13}$C label, were harvested as unenriched controls (3 uncolonized and 1 colonized nitrogen/nitrogen plants, 3 uncolonized and 1 colonized nitrogen/sand plants, and 3 uncolonized and 1 colonized

sand/sand plants). For each seedling, we removed the shoot, separated the root compartments, and thoroughly rinsed the roots from each compartment separately in tap water. After clipping the roots from each compartment into 2 cm fragments, we used randomly selected pieces to determine the ectomycorrhizal colonization (counting at least 100 fine roots per plant). For colonized plants, we collected both ectomycorrhizas and uncolonized fine roots (<1 mm diameter) from the fragments for $^{13}C$ concentration measurements; because uncolonized plants had no ectomycorrhizas, we collected only uncolonized fine root tissue. All plant and fungal tissues were oven-dried and weighed as in experiment 1. Dried uncolonized fine roots were homogenized into a fine powder in a Minibeadbeater (Biospec products, Inc., Bartlesville, OK, USA) for stable isotope analysis; due to low mass of available tissue per plant, ectomycorrhizas were analyzed without grinding (approximately 15-25 whole ectomycorrhizas per sample tin, typically 1-3 mg dry mass). Most colonized plants yielded enough ectomycorrhizal dry mass for mass spectrometry, but three nitrogen/sand, two nitrogen/nitrogen, and one sand/sand plant did not, bringing replication for ectomycorrhizal tissue down to 12 plants total. Elemental (carbon/nitrogen) and stable isotope analysis were performed using a Costech Elemental Analyzer (Costech Analytical Technologies, Valencia, CA, USA) coupled with a Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS) (Thermo Fisher Scientific, Waltham, MA, USA) at the Carnegie Institute of Global Ecology, Stanford, California, USA. The signal was processed as described in Kornfeld et al. (2012).

***Data processing***

$^{13}C$ label concentration was calculated from IRMS data as mmol $^{13}C$ excess per mol $^{12}C$, relative to unenriched pine seedlings (Slater et al. 2001). Using these concentration values, we calculated a log ratio carbon allocation metric to express differences in the amount of $^{13}C$ allocated by the plant to each half of its root system (Ji and Bever 2016), allowing us to consider the distribution of $^{13}C$ in a root system with respect to a compartment of interest. A positive allocation metric indicates greater [$^{13}C$] in the focal compartment relative to the other half of the root system, while a negative value indicates a lesser [$^{13}C$] than in the other half. This ratio was calculated separately for each tissue of interest (ectomycorrhizas and uncolonized fine roots). When root

systems were divided between nitrogen (casein) and non-nitrogen (sand) compartments, we used the nitrogen-provisioning half of the root system as the focal compartment, calculating the allocation metric as $\ln([^{13}C]_{N\ compartment}/[^{13}C]_{Sand\ compartment})$. Otherwise, when nitrogen treatments on each side were identical, the numerator position was assigned at random to the value from one of the compartments, with the value from the other compartment serving as the denominator (i.e. $\ln([^{13}C]_A/[^{13}C]_B)$), resulting in values arbitrarily assigned to positive or negative quantities. When the mmol $^{13}C$ excess value was zero or slightly negative (one value, -0.03 mmol $^{13}C$ excess per mol $^{12}C$ in the mycorrhizas of a nitrogen/nitrogen plant), we forced it to a very small positive value for log-transformation (+0.000001 mmol $^{13}C$/mol $^{12}C$). To compare the nitrogen contents of ectomycorrhizas or fine root tissues in paired root compartments, we employed the same log ratio procedure as we did for $^{13}C$ allocation, comparing the percent nitrogen contents of each tissue across paired root compartments.

***Statistical Analyses***

To assess differences in $^{13}CO_2$ enrichment across labeling batches, we examined patterns in overall enrichment and in the carbon allocation metric. This analysis included only fine roots from uncolonized plants, a reference tissue that was well-represented in all labeling batches. To examine overall enrichment, we performed a Kruskal-Wallis test with a post-hoc Dunnett test to compare the mean mmol $^{13}C$ excess in fine roots across the two halves of each split root system. To test for batch effects in the carbon allocation metric, we used an analysis of variance (ANOVA). To determine whether total nitrogen supplied to the plant (0g, 0.5g, or 1g times the number of nitrogen-containing bags planted near the root system), average % ectomycorrhizal colonization across the root system, or their interaction affected seedling biomass or fine root nitrogen concentration, we built two linear models after log-transformation to improve homoscedasticity and normal distribution of residuals for each outcome. We performed a two-way ANOVA to see how nitrogen treatment (N/S, N/N, or S/S) and addition level (0g, 0.5g, of 1g casein) affected carbon allocation to root tissues. For this analysis, we used data from the primary nutrient-absorbing organ in colonized and uncolonized plants: ectomycorrhizas for colonized plants, and fine roots from uncolonized plants. To see how these nitrogen treatments may have affected

tissue nitrogen concentrations, we performed the same set of tests using the log-transformed ratio of nitrogen concentrations in paired root compartments as the dependent variable. In each case, if a predictor (nitrogen treatment or addition level) did not significantly predict the dependent variable, we performed a subsequent one-way ANOVA using only the significant factor, followed by a Tukey HSD test to identify the pairwise differences among groups. To examine how root nitrogen provisioning might have influenced carbon allocation, we performed linear regression predicting the log carbon allocation ratio – that is, the difference in carbon quantities allocated to each side of a split root system – to the log-transformed nitrogen concentration ratio (the difference in tissue nitrogen concentrations between sides of a split root system), including root tissue type (ectomycorrhiza vs. uncolonized fine root) as a covariate. All statistical analyses for both experiments were conducted in R v.3.3.3 (R Core Team, 2017) and considered significant at $P < 0.05$.

## Results

### *Experiment 1: Can* Larix *discriminate among three* Suillus *species?*

All the uninoculated halves of the one-species treatment microcosms remained non-mycorrhizal throughout the experiment. As such, we are confident the ectomycorrhizal root tip colonization observed was the result of our spore inoculation and that there was no cross contamination between sides of the microcosms. The extent of colonization by each *Suillus* species depended upon the interaction between fungal species identity and competitor identity (two-way interaction: $F_{3, 34} = 3.475$, p = 0.027; Table S2A). When growing alone, all three species colonized seedling root systems to similar extents, occupying on average 38% of available roots per seedling (Fig. 1A). When a second species was present on the other side of the microcosm, *S. clintonianus* and *S. grisellus* colonized the root systems to a similar extent as when growing alone (Fig. 1A). *S. spectabilis*, however, was sensitive to the presence of a second species, occupying a much smaller proportion of the root system in the two-species treatments (0.9%) than in the single-species treatment (34.7%).

418

419 The timing of root system colonization by the three *Suillus* species also varied by treatment,
420 although with statistical significance that was marginal (two-way interaction: $F_{3, 34}$ = 2.363, p =
421 0.088; Table S2B). Without an ectomycorrhizal fungal competitor, all three *Suillus* species
422 colonized *Larix* seedlings at approximately the same time (Fig. 1B). When a second species was
423 present on the other side of the microcosm, however, *S. spectabilis* established much later,
424 forming ectomycorrhizas only in the last 1-3 weeks of the experiment. In contrast, time with
425 ectomycorrhizal colonization by *S. clintonianus* or *S. grisellus* was approximately the same
426 whether growing alone or when a second species was present (Fig. 1B).
427
428 In the single species treatments, different fungal species appeared to be equally effective
429 mutualists, producing plants with statistically indistinguishable biomass and foliar nitrogen
430 fractions (Table 1; Table S3). We also observed no significant differences in root biomass among
431 *Larix* seedlings root compartments with different fungal treatments (Table S2C).
432
433 *Experiment 2: Can* Pinus *reward N-provisioning roots and fungi with carbon resources?*
434
435 Colonization by *Suillus brevipes* isolates ranged from 0-86% across all root compartments, with a
436 median of 17% among colonized plants. Although the mesh bags were intended to make added
437 nitrogen available only to fungi, they did not completely exclude roots. Nitrogen from casein,
438 then, was available both to plants and to fungi through direct contact as well as possible leaching
439 from the bags into the surrounding soil during watering. Although nitrogen addition level (0g,
440 0.5g, or 1g casein ingrowth bags) significantly affected fine root nitrogen concentrations (i.e.
441 mean % nitrogen across the two halves of a split root system) (Table S4A), % ectomycorrhizal
442 colonization had only a marginally significant effect, and neither factor had a significant impact
443 on plant biomass (Table S4B). All labeled plants were substantially enriched for $^{13}C$ (range -20.2
444 to +358‰, with mean $\pm$ standard deviation (sd) = +54.8 $\pm$ 88.4 ‰, compared to unenriched plant
445 tissues with a range of -30.3 to -27.1‰, mean $\pm$ sd = -28.1 $\pm$ 1.0‰). The extent of fine root
446 enrichment (mean mmol $^{13}C$ excess across the two halves of a split root system) in uncolonized

plants was significantly predicted by labeling day (Kruskal-Wallis: $X^2 = 28.015$, df = 3, $p < 0.001$), but this batch effect disappeared when we analyzed the log-ratio carbon allocation metric (ANOVA: $F_{3,35} = 0.396$, P = 0.756).

Nitrogen treatment (N/N, S/S, or N/S), but not nitrogen addition level (0g, 0.5g, or 1g casein), significantly affected plant carbon allocation to the primary absorptive belowground tissue: fine roots in uncolonized plants, and ectomycorrhizas in colonized plants (Table S5A). The "choice" treatment (sand(S)/nitrogen(N)) exhibited the most pronounced preferential allocation (most positive allocation metrics towards the nitrogen compartment) (Fig. 2A) (ANOVA: $F_{2,50} = 3.707$, P = 0.0315; Tukey HSD test, N/N-S/S p = 0.661, N/S-S/S p = 0.174, N/S-N/N p = 0.028). This pattern was mirrored by the effect of nitrogen treatment on log-transformed ratios of tissue nitrogen concentrations in paired root compartments (Fig. 2B; Table S5B) (ANOVA: $F_{2,50} = 4.58$, P = 0.0149; Tukey HSD test, N/N-S/S p = 0.616, N/S-S/S p = 0.114, N/S-N/N p = 0.013). There was also a strong positive association between the log-transformed ratio of $^{13}C$ allocation to root tissues and the log-transformed ratio of percent nitrogen detected in those tissues. Specifically, as the difference (log ratio) in percent nitrogen between tissues in paired root compartments increased, so did the difference in $^{13}C$ allocation to those paired tissues. Root tissue type – ectomycorrhiza, from colonized plants, or uncolonized fine root, from uncolonized plants – was not a significant predictor of the carbon allocation metric (Fig. 3 and Table S6: adjusted $r^2 = 0.8155$, df = 49, $p < 0.001$).

**Discussion**

Collectively, our results suggest that ectomycorrhizal plants have the ability to discriminate among potential fungal partners both prior to colonization (experiment 1) and during a symbiotic interaction (experiment 2). In the first experiment, we found that *Larix occidentalis* seedlings associated less with *Suillus spectabilis* when presented with an alternative ectomycorrhizal symbiont than when no alternative was available (Fig. 1). That is, the extent of *S. spectabilis* colonization was heavily reduced in the two-species treatments relative to when it colonized alone. This likely reflected delayed onset of root colonization by this fungus (Fig. 1B), suggesting

the mechanism at work acted prior to an active symbiosis with *S. spectabilis*. While our results do not identify the specific mechanism, we believe they are most consistent with some kind of pre-colonization signaling. One potential type of signaling could be the activation of plant defense genes, as Hortal et al. (2017) recently demonstrated that defense genes can be upregulated when multiple ectomycorrhizal fungal species colonize *Eucalyptus* seedlings. Specifically, they found only the least effective ectomycorrhizal mutualist triggered defense gene activation, suggesting that plants may have localized control on colonization. When the least effective mutualist was the only fungus available for colonization, however, they showed that defense genes were not upregulated against that symbiont, consistent with our observations in the present experiment. This effect need not solely reflect plant control of colonization, however, as it is equally plausible that both *S. clintonianus* and *S. grisellus*, but not *S. spectabilis*, induced a plant immune response across the entire root system as they colonized the host roots. This activation of defense pathways could have inhibited infection by *S. spectabilis*, but not either of the other fungi. If plant defense genes were responsible for inhibiting colonization by *S. spectabilis* in our system, however, they were only active for a limited period of time. After 20 weeks, seedlings with high colonization by *S. grisellus* or *S. clintonianus* on one side of the microcosm were eventually colonized (at low levels) by *S. spectabilis* on the other side. Since there was no nutrient supplementation throughout the experiment, it is possible that local depletion of nutrients on the initially colonized side created an incentive for host plants to allow *S. spectabilis* onto their roots in order to exploit additional nutrients on the other side of the microcosms. Or, perhaps, nutrient depletion could have weakened colonization on the initially colonized side, leading to a downregulation of the plant's defense response. Future work that carefully manipulates soil nutrient content and subsequently tracks resource movement and plant defenses would be required to explore this scenario further.

Although our results could be consistent with other mechanisms, we think these alternative explanations are less likely to be true. One is that this effect was mediated by plant resource allocation. For example, the earlier-establishing fungus may have siphoned plant resources to its side of the split-root microcosm, resulting in the *S. spectabilis* side accumulating fewer spore-

germination cue molecules simply because it received fewer plant resources. We believe this possibility is unlikely, however, as all three *Suillus* species were equivalently effective mutualists in terms of plant biomass gain and leaf nitrogen content, and root biomass was equivalent across both sides of all paired microcosms (Table 1, Table S2). Furthermore, even if *S. spectabilis* were an inferior mutualist, the plant did not have much opportunity to withhold resources, because that fungus did not establish until the end of the experiment in the presence of a competitor. A second possibility is fungal control on root colonization. In order to respond to a reduction in host quality – for instance, if the host shifted photosynthate to the competing fungus – *S. spectabilis* would need to be engaged in active symbiosis with a root as the competitor became established. In our experiment, we detected no active symbiosis until very late in the trial. It also seems unlikely that an obligate symbiont such as *S. spectabilis* would reduce its own growth so profoundly, in the absence of a suitable alternative host. Taken together, these results suggest the possibility that *S. spectabilis* was actively discriminating against *L. occidentalis* is also an unlikely explanation for our results.

In experiment 2, we found that *Pinus muricata* seedlings directed recent photosynthate to roots that contained more nitrogen, but made no distinction between ectomycorrhizal roots of colonized plants and roots of uncolonized plants (Fig. 3). Although it is well established that resource-providing roots receive greater investment from a plant (Eissenstat et al. 2015; Chen et al. 2016; Cheng et al. 2016), this is to our knowledge the first demonstration of carbon investment tracking tissue nitrogen concentrations equally well in mycorrhizal and uncolonized fine roots. This mechanism may allow for plant rewards of resource-providing fungi, a phenomenon known to occur in the arbuscular mycorrhizal symbiosis (Kiers et al. 2011). The design of our experiment, however, makes it difficult to establish the time frame in which plant rewards may be important: The $^{13}C$ data represent allocation after a week-long chase period, while the nitrogen concentration data likely reflect a long-term average of nitrogen flux through the tissue. Despite operating on different time scales, the close correspondence between differences in tissue nitrogen and recent carbon concentrations across the split-root microcosms indicates that these resources may be tightly coupled. In contrast with our results, however, Valtanen et al. (2014)

found that these resources may not to be strictly coupled over the long-term in diverse fungal communities. Future work should aim to clarify the influence of experimental time frame and mutualist diversity on the coupling of plant and fungal resource exchange in this symbiosis. Finally, we note that if fine spatial carbon-nitrogen coupling (root tip to root tip) does exist, this would allow the plant to regulate its own root foraging efforts while also encouraging cooperative fungi, suggesting that fundamental plant processes could reward cooperation even without symbiosis-specific mechanisms (Frederickson 2013).

We clearly recognize that the interpretation of both our experiments are constrained by experimental and methodological limitations. Although the patterns we observed provide intriguing evidence that ectomycorrhizal competition can be strongly influenced by host plant signaling, including independent replicates for each fungus/competitor combination would further increase our confidence in the interpretation of the experiment 1 results. With regard to experiment 2, the nitrogen source we used (casein protein) was not solely fungus-accessible, although it was contained in mesh bags that partially inhibited root growth. To fully disentangle the influence of fungal partner quality from the effects of competition between plant roots and soil fungi, it would be necessary to fully separate roots from the nitrogen source and render it solely accessible to the fungi, for example by introducing an air gap (He et al. 2005; Fellbaum et al. 2014). Using a labeled nitrogen source would also illuminate resource trading dynamics that we could not capture here. Finally, it also would be useful to be able to directly control the rates of $^{13}C$ uptake across plants (rather than tracking allocation metrics in split root plants), which would require stricter control of label concentration and timing than we were able to achieve in this study.

In aggregate, our results support the hypothesis that ectomycorrhizal host plants, like those engaged in other root symbioses, have the ability to discriminate among potential microbial partners (Bever et al. 2009; Kiers et al. 2011; Argüello et al. 2016; Hortal et al. 2017). Further, our results suggest that plant-based discrimination can occur both prior to symbiosis, based on signals relevant to fungal identity, as well as after symbiosis has begun, based on the services

provided by a fungal partner. Although signaling has been suggested to be an important factor regulating partner recognition in this system (Plett et al. 2014), our results suggest that fungal-plant signaling can affect the outcomes of co-colonization among ectomycorrhizal fungi. While our isotope-based results apply equally well to roots associated with fungal partners and to uncolonized roots, they are consistent with plant allocation patterns that would encourage symbiotic cooperation. By influencing which fungi succeed and in which contexts, a host plant may not only affect its own performance, but also alter the pool of symbionts available to nearby plants. This process may in turn influence the way that the forest community develops and how quickly resources like carbon and nitrogen move through the system.

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**Author contributions**

P.K. designed experiment 1, which was conducted by S.H. and J.H. L.B. designed and carried out experiment 2 with support from K.P. and A.K. L.B. analyzed the data and composed the manuscript, with writing and editing assistance from P.K., and additional revisions from all co-authors.

**Compliance with ethical standards**

Conflict of interest: The authors declare that they have no conflict of interest.

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**Figures and Tables**

Figure 1: Extent of ectomycorrhizal % colonization (A) and timing of ectomycorrhizal fungal colonization (B) of *Larix occidentalis* seedlings varies based on the interaction between fungal species identity and competitor treatment. Letters indicate significant differences identified among the interacting factors (fungus and competitor identity) by a post-hoc Tukey HSD test on the two-way ANOVA results. The midline of each box represents the mean value for that fungus and competitor combination, hinges illustrate the boundaries of the first and third quartiles, and whiskers extend to the most extreme points no more than one and a half times the interquartile range away from the hinge. All data are plotted as individual points overlain on the boxplots.

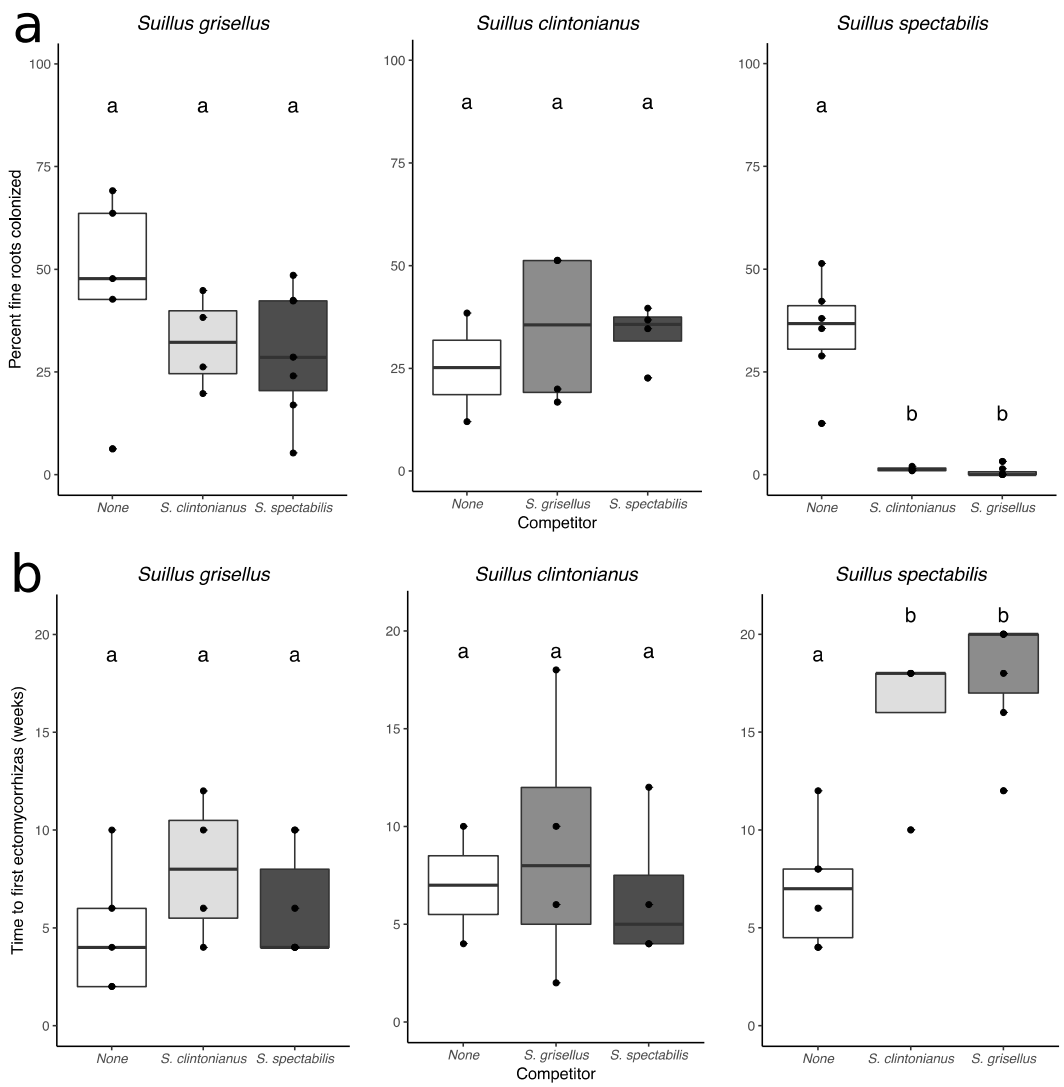


Figure 2: A) The log-transformed $^{13}C$ allocation ratio, representing carbon allocation to across the split root systems of *Pinus muricata* seedlings, was significantly higher in the choice (nitrogen/sand) treatment than in the nitrogen/nitrogen treatment (significance determined by a post-hoc Tukey test). This result mirrors the pattern in B): the log-transformed ratio of nitrogen content in root tissues – whether absorptive roots or ectomycorrhizas – was greatest in the choice treatment. Boxes, points, and significance labels are plotted as in Figure 1. Closed circles illustrate data from ectomycorrhizal tissue; open circles indicate values from uncolonized fine root tissue. The dashed line illustrates a log-ratio of 0 (no difference between paired root compartments).

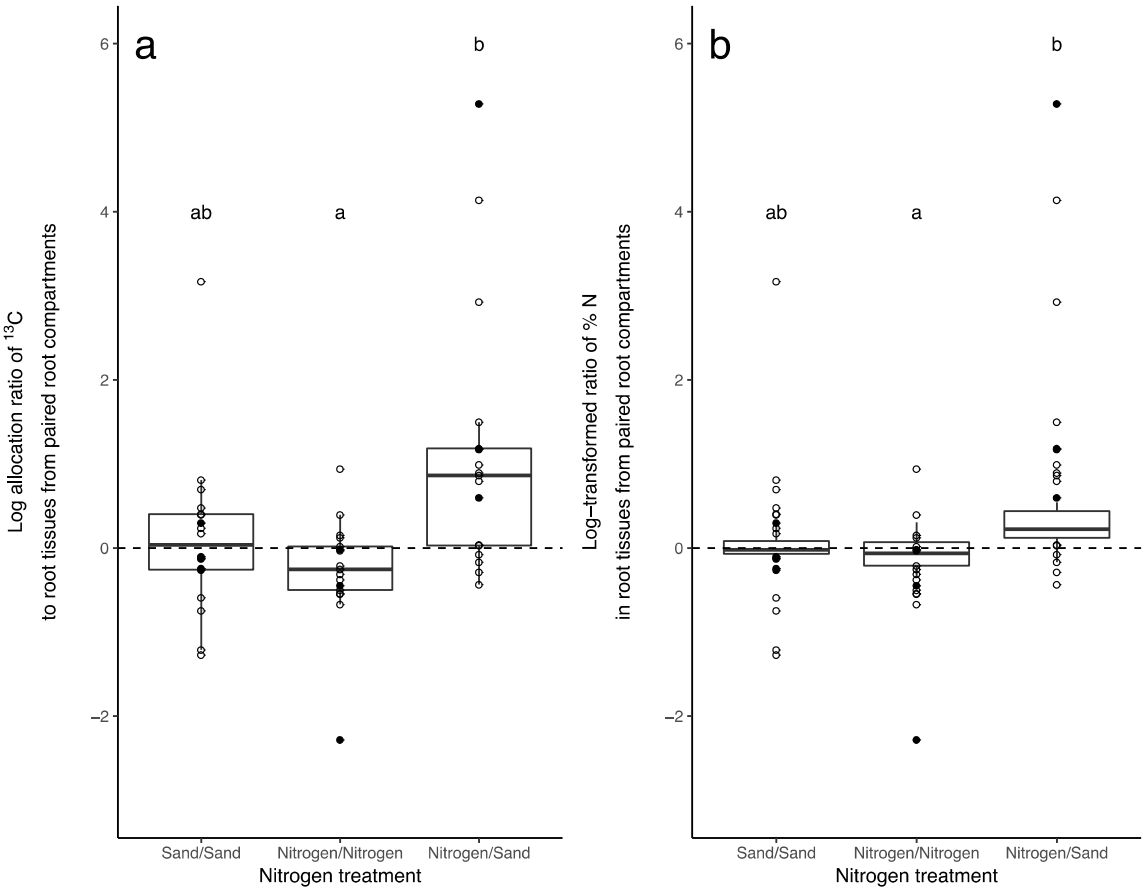
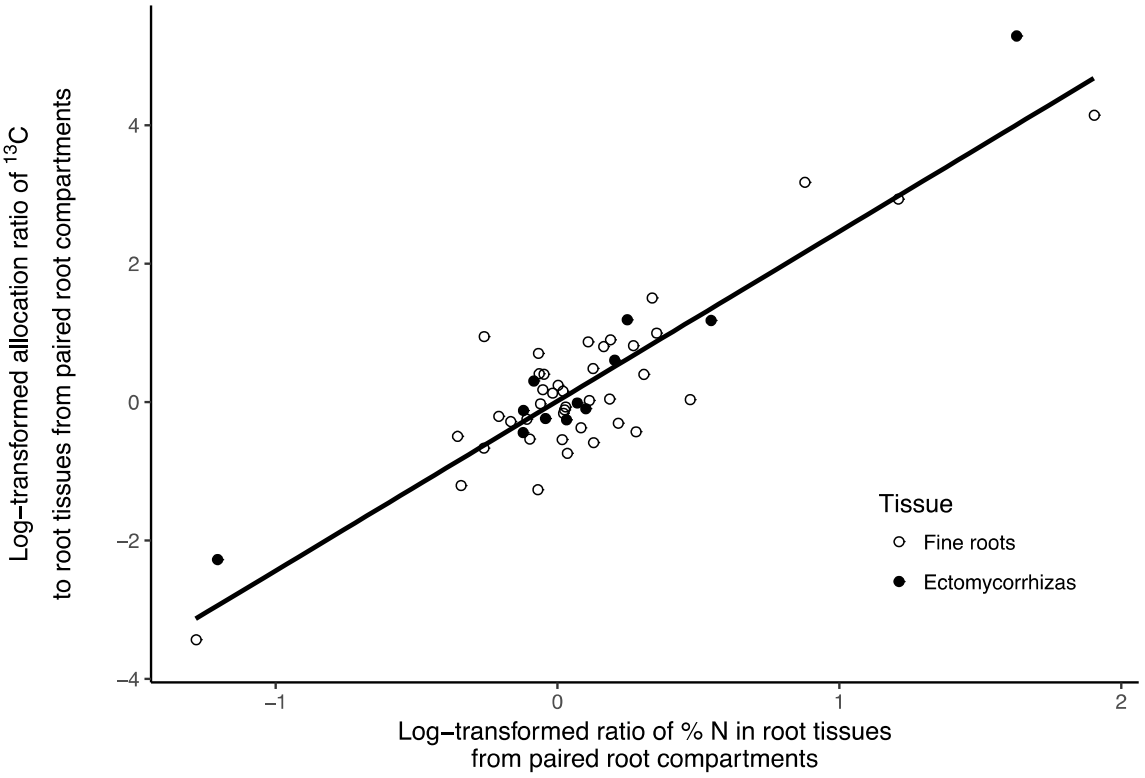


Figure 3: The difference in nitrogen content within a tissue type in paired root compartments, measured as a log ratio, was significantly correlated with the difference in $^{13}C$ allocation to those tissues, measured as the log-ratio $^{13}C$ allocation metric described in Materials and Methods. When comparing paired root compartments A and B from a single microcosm, the metric is calculated as $\ln([^{13}C]_A/[^{13}C]_B)$, so that a negative value indicates more $^{13}C$ on side B, while a positive value indicates more $^{13}C$ on side A. Open circles illustrate values for uncolonized fine root tissue; closed circles are from ectomycorrhizas. The line illustrates the predicted relationship between the variables as modeled by linear regression, with shading indicating the 95% confidence interval. Although percent nitrogen represents a long-term average of nitrogen flux through a root, while the $^{13}C$ allocation measures carbon flux over the course of the week long chase period, the amount of carbon these plants would allocate to their root tissues – whether ectomycorrhizas or absorptive roots – was related to the amount of nitrogen those roots contained.



797 Table 1: *Larix* seedling biomass and foliar % nitrogen do not vary significantly by fungal identity
798 in single-species treatments. Values presented are mean plus or minus one standard deviation.

| | Focal species (no competitor): | | |
|---|---|---|---|
| | *S. clintonianus* | *S. grisellus* | *S. spectabilis* |
| Total biomass (g) | 1.29 ± 0.05 | 1.27 ± 0.25 | 1.26 ± 0.11 |
| Foliar nitrogen (%) | 1.34 ± 0.16 | 1.43 ± 0.21 | 1.28 ± 0.16 |

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