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Unlocking environmental keys to host specificity: differential tolerance of acidity and nitrate by *Alnus*-associated ectomycorrhizal fungi

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ABSTRACT

The ectomycorrhizal (ECM) fungal communities associated with the host genus *Alnus* have been widely noted for their low richness and high proportion of host-specific species, but the processes underlying their atypical structure remain poorly understood. In this study, we investigated whether the high acidity and nitrate concentrations characteristic of *Alnus* soils may act as important environmental filters that limit the membership in *Alnus* ECM fungal communities. Using a pure culture approach, we grew four species from two host groups (*Alnus* and non-*Alnus*) in liquid media containing different acidity and nitrate concentrations. We found that the growth of the *Alnus*-associated ECM fungi was not, on average, affected by high acidity, while the non-*Alnus*-associated ECM fungi had a significantly negative growth response under the same conditions. Similarly, when grown at high nitrate, the non-*Alnus*-associated ECM fungi also generally performed more poorly. Growth responses of the *Alnus*-associated ECM fungi in both the high acidity and high nitrate treatments indicated tolerance rather than preference for those chemical conditions. The mechanism underlying the differential acidity tolerance may involve active hyphal buffering of local acidity environments. Taken together, our results suggest that soil chemical conditions likely do act as significant environmental filters that, along with other ecological and evolutionary factors, drive the atypical specificity of *Alnus* ECM interactions.
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Introduction

A number of recent studies indicate that the most abundant ectomycorrhizal (ECM) fungi in many temperate and tropical forests have low host specificity (Horton and Bruns, 1998; Kennedy et al., 2003; Nara and Hogetsu, 2004; Ishida et al., 2007; Twieg et al., 2007; Tedersoo et al., 2008; Richard et al., 2009; Smith et al., 2011; Kennedy et al., 2012; but see Smith

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et al., 2009). A commonly cited exception to this pattern is the ECM fungal community associated with the host genus *Alnus*. Unlike on other ECM hosts, the ECM fungal communities present on young and mature *Alnus* trees have been consistently characterized by both low species richness and a high proportion of species that only grow in association with this host genus (Molina, 1979; Pritsch et al., 2000; Tedersoo et al., 2009; Kennedy and Hill, 2010; Kennedy et al., 2011; Roy et al., 2013; Põlme et al., 2013; Bogar and Kennedy, 2013). While other ECM hosts do associate with ECM fungi that are host-genus specific (e.g. *Rhizopogon* and ECM hosts genera within the Pinaceae, Grubisha et al., 2002), they are rarely the dominant fungi present in mature forests.

The factors underlying the specificity of the *Alnus* ECM system have been the subject of considerable speculation (Horton et al., 2013). Many authors have discussed this system from a co-evolutionary standpoint (Molina et al., 1994; Moreau et al., 2006; Kennedy and Hill, 2010) and there is some evidence to support co-evolution driving patterns of speciation within certain *Alnus*-associated ECM fungal lineages (Rochet et al., 2011). A number of other researchers have noted differences in the biotic and abiotic conditions present in *Alnus* and non-*Alnus* dominated host systems (Molina et al., 1994; Kennedy et al., 2014). In particular, many environmental explanations have centered on the presence of *Frankia* bacteria, which form nodules on the roots of host plants and fix nitrogen into plant-available forms in exchange for carbohydrates derived from photosynthesis (Huss-Danell, 1997). The genus *Alnus* is the lone ECM host of *Frankia* bacteria in temperate and boreal forests and the co-presence of *Frankia* bacteria appears to significantly alter this host system for ECM fungi from both a biotic and abiotic perspective.

Biotically, one way in which *Frankia* bacteria may influence ECM fungal community structure is by affecting the nutrient status of their shared host. Specifically, the provisioning of nitrogen by *Frankia* bacteria may shift the nutritional needs of *Alnus* individuals towards association with ECM fungi adept at acquiring other nutrients (Molina et al., 1994; Kennedy and Hill, 2010; Horton et al., 2013). It has been demonstrated that nitrogen-fixing plants are often limited by phosphorus (Uliassi and Ruess, 2002), so *Alnus* individuals may selectively associate with ECM fungi that have enhanced enzymatic abilities towards phosphorus acquisition. Walker et al. (2014) recently tested this hypothesis by comparing the organic phosphorus and nitrogen acquisition abilities of ECM fungi associated with *Alnus rubra* and the non-*Frankia* host *Pseudotsuga menziesii* at two field sites in the western United States. They found the ECM fungal communities on *A. rubra* had significantly greater organic phosphorus acquisition abilities than those on *P. menziesii* at both sites, while the organic nitrogen acquisition abilities of *P. menziesii*-associated ECM fungal communities were significantly higher at one of the two sites. Taken together, those results are largely consistent with the hypothesis that the presence of *Frankia* bacteria alters the composition of *Alnus*-associated ECM fungal communities towards species with specific nutrient acquisition abilities. This process may thus act as one environmental filter (*sensu* Koide et al., 2011 – a biotic or abiotic environmental factor that selects for fungi with a given set of physiological traits) that limits ECM fungal community membership on *Alnus* trees.

Frankia bacteria are also known to strongly influence the abiotic soil environment. In particular, soils in which *Alnus* individuals are abundant are characterized by both high acidity and nitrate concentrations (Miller et al., 1992; Martin et al., 2003), due to elevated nitrification associated with the decomposition of nitrogen-enriched leaf litter (Van Miegroet and Cole, 1985). In non-*Alnus* systems, both acidity and nitrate have been shown to have significant effects on ECM fungal growth and community composition. For example, Hung and Trappe (1983) and Yamanaka (2003) demonstrated that some ECM fungi grew relatively poorly in high acidity media (initial pH = 3), suggesting the high acidity conditions in *Alnus* soils may be unfavorable for most species. Similarly, both Lilleskov et al. (2002a) and Kjøller et al. (2012) found that ECM fungal richness deceased in response to increasing soil nitrogen concentrations, with the decrease of ECM fungal species being strongly correlated with increasing levels of soil nitrate. Other studies have found that several ECM fungal taxa show significant sporocarp reduction in response to relatively short-term nitrogen fertilization (Termorshuizen, 1993; Brandrud, 1995). Because these responses occur prior to subsequent acidification, these effects have also been attributed to increased nitrate levels (Lilleskov et al., 2002b).

While the abiotic conditions present in *Alnus* soils may act as another environmental filter limiting the membership of ECM fungi in this host system, to date there have been no direct tests of their effects on *Alnus* versus non-*Alnus* ECM fungal communities. In addition, although high acidity and high nitrate may act synergistically in nature, the relative effect of each of these variables on the performance of *Alnus*-associated ECM fungi remains unclear. To address these gaps, we experimentally tested the effects of acidity and nitrate on the growth of a suite of *Alnus*-associated and non-*Alnus*-associated ECM fungi. We chose to conduct our experiment in a pure culture system (i.e. without the host present) to be able to precisely manipulate the environmental conditions of interest, have high levels of replication within treatments, and determine the direct fungal response to changed acidity and nitrate concentrations. Although we recognize that the choice of experimental systems may raise concerns about ecological relevance (Erland et al., 1990), multiple previous studies using pure culture approaches have found their results correlate with those observed in field settings (Lilleskov et al., 2002b; Yamanaka, 2003). As such, we think the approach used in this study provides an important first test of the role of soil chemical conditions in contributing to the atypical richness and specificity patterns observed in *Alnus* ECM fungal communities.

We hypothesized that *Alnus*-associated ECM fungi would have significantly greater growth at high acidity and nitrate compared to non-*Alnus*-associated ECM fungi. Given the generally negative effects of increased levels of both of these variables on ECM fungal growth, we speculated that a differential response between host groups (i.e. *Alnus* versus non-*Alnus*) would be the result of greater tolerance rather than preference for high acidity and nitrate. For our purposes, we considered tolerance to be the ability to grow similarly in the high acidity and nitrate treatments compared to growth on

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standard media, while we considered preference to be a significant increase in growth due to an ability to utilize higher levels of nitrate or grow more rapidly at high acidity. We also assayed the chemical conditions present following fungal growth to try to better understand the mechanisms driving the responses observed.

Materials and methods

Species selection

Eight species of ectomycorrhizal (ECM) fungi were used; four exclusively associated with *Alnus* host species (*Alpova diplophloeus*, *Melanogaster luteus*, *Gyrodon lividus*, and *Paxillus rubicundulus*) and four associated with other ECM hosts. Of the four non-*Alnus*-associated ECM fungal species, *Rhizopogon vesiculosus*, *Suillus lakei*, and *Truncocolumella citrina* are all ECM fungi that associate exclusively with the ECM host genus *Pseudotsuga*, while *P. involutus* has a broad host range that includes both gymnosperm and angiosperms (Ek et al., 1994). The *P. involutus* strain used in this study was isolated from a sporocarp associated with a single *Betula papyrifera* individual in western Oregon, USA in 2011. Four of the other eight species (*A. diplophloeus* associated with *A. rubra*, *R. vesiculosus* associated with *P. menziesii*, *T. citrina* associated with *P. menziesii*, *S. lakei* associated with *P. menziesii*) were isolated from fungal sporocarp collections made in 2011 from a variety of locations in the western Oregon, USA. The three additional species (*M. luteus* associated with *A. incana*, *G. lividus* associated with *A. incana*, and *P. rubicundulus* associated with *A. glutinosa*) were isolated from fungal sporocarp collections from *Alnus* forests in the French Alps and Pyrenees mountains. All eight species belong to the order Boletales, but the four *Alnus*-associated species and *P. involutus* belong to the family Paxillaceae, while both *S. lakei* and *T. citrina* belong to the family *Suillaceae*, and *R. vesiculosus* belongs to the family *Rhizopogonaceae*. Culture species identity was confirmed by ITS rDNA sequencing from mycelium from all eight species using the same methods described in Kennedy and Hill (2010).

Acidity and nitrate media manipulations

To assess the effect of acidity and nitrate concentrations on ECM fungal growth, two experiments were conducted. Both were done at the same time and for the same duration, but involved independent samples and different experimental treatments. Published ECM culture experiments were first analyzed as well as studies of soil abiotic conditions in Pacific Northwest *Alnus* and non-*Alnus* forests (see Supplementary Appendix 1). Because field nutrients are usually reported in milligrams/grams per dry kilogram of soil, values from the literature were first converted into units of molarity before considering them for our liquid culture-based study (see Supplementary Appendix 1 for full details of this conversion). Based on that survey, a series of experimental treatments was designed with a range of acidity and nitrate concentrations similar to those in *Alnus* and non-*Alnus* soils. All other media conditions were held constant across all treatments, which were based on the standard Modified Melin-Norkans (MMN) media on which all eight species were originally isolated (Supplementary Table 1). In the acidity and nitrate experiments, there were two manipulation treatments: high pH (initial pH = 3.1) and low pH (initial pH = 6.3) and high nitrate (1×10^{-3} mol l^{-1}) and low nitrate (1×10^{-5} mol l^{-1}), respectively. Fungal growth in those manipulation treatments was compared to that in a fifth treatment with standard media acidity and nitrogen conditions (pH = 4.5 and nitrogen provided as ammonium at 1×10^{-4} mol l^{-1}). Because the standard nitrogen source (ammonium) was replaced with nitrate in the nitrate treatments, an additional nitrate treatment was also included with the same total amount of nitrogen as the standard treatment (1×10^{-4} mol l^{-1}) (to differentiate growth responses to due to nitrogen source versus differences in total available nitrogen). In each treatment ($N = 6$), five replicate cultures of each species were grown, resulting in a total of 240 samples across both experiments.

Fungal growth conditions

Sterile 16 mm diameter glass test tubes were inoculated with tissue from the eight ECM fungal species and 10 ml of liquid media. For each species, the tissue used for inoculation was derived from a single isolated strain of mycelium that had been maintained in pure culture on standard solid media for multiple months. To ensure consistent starting ages and amounts of mycelial inoculation, 5 mm diameter plugs were taken from the growing edge of the actively growing mycelium of each species. The plugs were cut into eighths and the individual pieces placed directly into the replicate tubes. The small size of the cut plugs (1 plug = 0.75 cm diameter by 0.4 cm depth = ~0.18 ml: 1/8th = ~0.02 ml) ensured that the majority of the nutrients available to the growing fungal tissue came from the new liquid media and not from the solid media in the inoculum plug. Following inoculation, the tubes were capped to prevent entry of airborne material but not restrict oxygen flow. The tubes were maintained in a shaded laboratory environment with stable temperature (24–26 °C) and humidity (33–35 %) for 50 d (with no shaking). In addition to the tubes with inoculum, a set of blanks (i.e. tubes with each treatment but no inoculum) that were exposed to the same growth conditions were set up to account for changes in filtrate nutrients or tissue growth due to possible contamination or evaporation. Finally, for every set of plugs used to inoculate the media tubes, five identical plugs were plated on standard solid media to confirm the viability and reliability of the plugs used to inoculate the liquid media. All of the experimental blanks had no or very little variation from their expected state and all of the extra inoculum plugs that were plated on solid media showed viable and contaminant-free growth. This verified that any lack of growth was due to treatment conditions and not the inoculum.

Experimental harvest and measurement

After 50 d, the solid and liquid contents of each test tube were separated with vacuum filtration through a 2.5 μm pore diameter filter disc (Whatman Grade 5, Sigma–Aldrich, St. Louis, MS, USA). Following filtration, the filter paper discs were placed into drying pans and the filtrate pipetted into

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15 ml capped centrifuge tubes. The filter paper discs were dehydrated for at least 48 hr at 60 °C. The mass of each filter disc was measured both before and after tissue harvest and the absolute fungal tissue mass calculated for all samples by subtracting the original mass of each disc from the combined mass of the disc with tissue on it. The filtrate was stored at −20 °C prior to chemical analyses. The pH of the liquid media was measured before and after 50 d incubation using a digital pH meter, and the concentrations of inorganic nitrogen ions (NH_4^+ and NO_3^-) in the filtrate were quantified with a discrete analyzer (SmartChem 200, Unity Scientific, Brookfield, CT, USA).

Prior to our liquid culture experiments, the eight different species of ECM fungi were seen to grow at different rates on standard solid media, indicating they have intrinsically unequal growth rates. To account for this variation, the fungal tissue mass data were transformed to represent the percent change in biomass during our experiments. Specifically, the values represent the percent mass difference between the growth of a species in manipulated media relative to its growth in standard media (pH = 4.5 and with nitrogen as ammonium). This value is hereafter referred to as relative fungal growth. Unlike absolute biomass, the relative growth values of fungal cultures (expressed as positive or negative percentages) can be directly compared among species.

Statistical analyses

To analyze fungal growth responses in the different acidity and nitrate treatments, a two-way fixed-factor analysis of variance (ANOVA) was used for each experiment. In each ANOVA, the predictor variables were set as host group (*Alnus* or non-*Alnus*) and either acidity or nitrate concentration (high or low), respectively. Following the ANOVAs, significant differences were determined among treatment means using Tukey HSD tests. The relative growth in each treatment was also compared with a null hypothesis of zero growth for each host group and for each individual species using post-hoc Dunnett's tests. Differences in the filtrate pH across host groups and acidity treatments were tested using the same two-way fixed-factor ANOVA described above. To compare differences in the relationship between relative growth of *Alnus*-associated and non-*Alnus*-associated ECM fungi and nitrate consumed in the high nitrate treatment, analysis of covariance (ANCOVA) was used. Prior to each analysis, that residuals were homoscedastic was visually confirmed. All figures were generated in R (R Development Core Team 2013) and all statistical analyses conducted in JMP v.10 (Cary, NC, USA). Test results were considered statistically significant at $P < 0.05$.

Results

There was a significant interaction between acidity and host group on relative fungal growth (Acidity * Host interaction: $F_{1,75} = 15.74$, $P < 0.001$, Fig 1). While the average growth responses of the *Alnus*-associated ECM fungi were similar in the low and high acidity treatments and not significantly different from zero (Dunnett's test, $P > 0.05$), the average relative growth of the non-*Alnus*-associated ECM fungi decreased significantly at high acidity compared to the low acidity, approximately three-fold (Tukey HSD test, $P < 0.05$). The mean response of the non-*Alnus*-associated ECM fungi in the high acidity treatment was also significantly lower than growth at standard acidity (Dunnett's test, $P < 0.05$).

When analyzed at the species level, there were species with positive as well as negative growth responses in both host groups in the low acidity treatment (Fig 2). However, in the high acidity treatment, the non-*Alnus*-associated ECM fungal species showed consistently negative growth responses, whereas the *Alnus*-associated ECM fungal species had mixed responses. *Gyrodon lividus* and *Melanogaster luteus* had slightly positive growth responses and *A. diplophoeus* and *P. rubicundulus* had slightly negative growth responses. Comparing across all the species in both acidity treatments, only *T. citrina* at low and high acidity, and *S. lakei* and *P. involutus* at high acidity had growth responses significantly different from growth at standard acidity (Dunnett's tests, $P < 0.05$).

The pH values of the final culture filtrate decreased relative to the initial pH values in both acidity treatments (Fig 3). This acidification occurred in both the experimental controls (i.e. samples with no fungi present, the final pH of which is shown by red lines in Fig 3) as well as those with fungi present. Because this acidification occurred in both samples with and

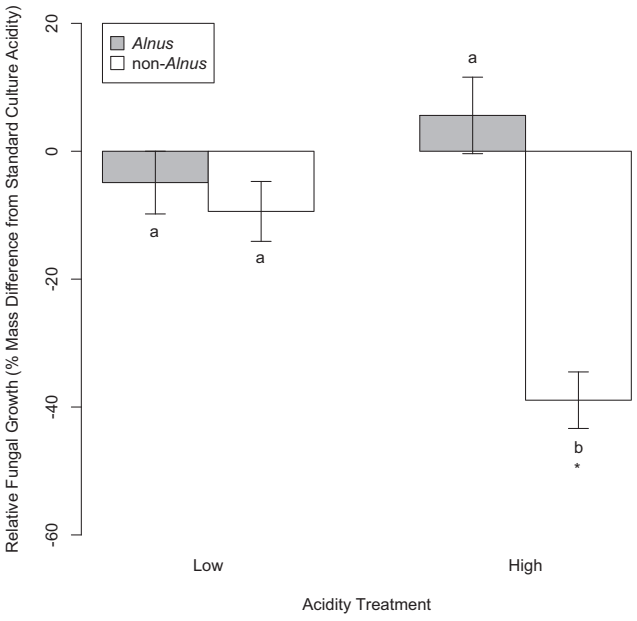

Fig 1 – Average growth response of the *Alnus*-associated and the non-*Alnus*-associated ectomycorrhizal (ECM) fungi when grown in liquid media with low acidity (pH 6.3) and high acidity (pH 3.1). Relative growth was calculated as percent difference in biomass accumulation over 50 d relative to each species' biomass accumulation under standard conditions (pH 4.5). Error bars denote one standard error. Letters indicate statistically significant differences between means as determined by a Tukey HSD test. Asterisks indicate values significantly different from zero (i.e. growth response significantly different from that in standard acidity) as determined by a Dunnett's test.

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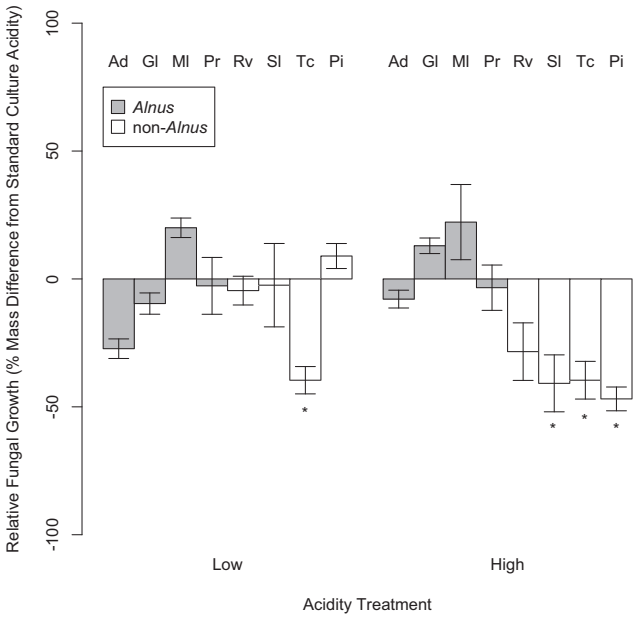

Fig 2 – Average growth response of each individual species of the *Alnus*-associated and the non-*Alnus*-associated ectomycorrhizal (ECM) fungi when grown in liquid media with low acidity (pH 6.3) and high acidity (pH 3.1). Ad = *Alpova diplophoeus*, Gl = *Gyrodon lividus*, Ml = *Melanogaster luteus*, Pr = *Paxillus rubicundulus*, Rv = *Rhizopogon vesiculosus*, Sl = *Suillus lakei*, Tc = *Truncocolumella citrina*, Pi = *Paxillus involutus*. Relative growth was calculated as percent difference in biomass accumulation over 50 d relative to each species' biomass accumulation under standard conditions (pH 4.5). Error bars denote one standard error. Asterisks indicate values significantly different from zero (i.e. growth response significantly different from that in standard acidity) as determined by a Dunnett's test.

without fungi present, and because the final media volumes of all samples were consistently lower than the initial 10 ml (data not shown), it appears that much of the change was driven by evaporation over the duration of the experiment. However, in the low acidity treatment there was a significant difference between host groups in the amount of acidification that occurred. While the filtrate pH values from the non-*Alnus*-associated ECM fungi were similar to that of the low acidity control, the filtrates from the *Alnus*-associated ECM fungi were significantly less acidic (i.e. pH values closer to the starting value of 6.3) (Acidity * Host interaction: $F_{1,75} = 328.87$, $P < 0.001$). This difference, if present, was not detectable in the high acidity treatment.

There was also a significant interaction between nitrate concentration and host group on relative fungal growth (Nitrate * Host interaction: $F_{1,76} = 4.37$, $P = 0.039$, Fig 4). At low nitrate, both the *Alnus*-associated and non-*Alnus*-associated ECM fungi grew significantly less than they did on standard culture nitrogen (relative growth was reduced by about 20% for both groups) (Dunnett's tests, $P < 0.05$), and these growth responses were not significantly different from each other (Tukey HSD test, $P > 0.05$). At high nitrate, however, the average growth response of the *Alnus*-associated ECM fungi was not significantly different from that in standard media (i.e., relative growth was not significantly different from zero), while the non-*Alnus*-associated ECM fungi had a significantly negative growth response (relative growth reduced, on average, by about 50% and was significantly different from zero).

When analyzed at the species level, the responses were more variable (Fig 5). Unlike the acidity treatments, in both the low and high nitrate treatments, both host groups had species with positive growth responses and others with negative growth responses. At low nitrate, growth responses of both the *Alnus*-associated and non-*Alnus*-associated ECM fungal species were mixed, and only *A. diplophoeus* had a growth response that was significantly different from growth on standard nitrogen. At high nitrate, the growth responses of both the *Alnus*-associated and the non-*Alnus*-associated ECM fungal species were again variable, but only one of the *Alnus*-associated species (*G. lividus*) had a growth response significantly different from growth on standard nitrogen, while three of the four non-*Alnus*-associated ECM fungal species (*R. vesiculosus*, *S. luteus*, and *T. citrina*) had significantly negative growth responses and only one (*P. involutus*) had a significantly positive growth response (Dunnett's tests, $P < 0.05$).

The amount of nitrate remaining in the filtrate media was strongly influenced by nitrate treatment. In the low nitrate

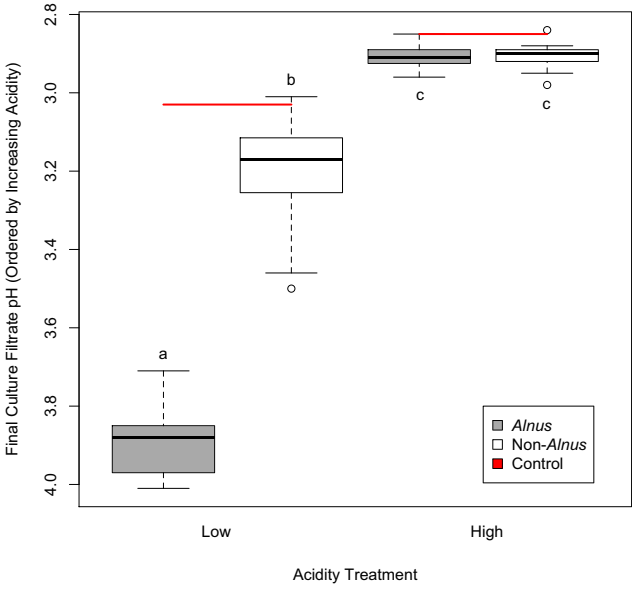

Fig 3 – Final pH of liquid media from the low acidity (starting pH 6.3) and high acidity (starting pH 3.1) treatments after 50 d growth. Red lines represent the final pH of the controls (which had the same initial pH as the low and high treatments, but no fungal inoculum added). Boxes surrounding median values represent the first and third quartiles, while whiskers show the smaller (and larger) of either the maximum (and minimum) vales or 1.5× the interquartile range (approximately ± 2 standard deviations). Letters indicate statistically significant differences between means as determined by a Tukey HSD test.

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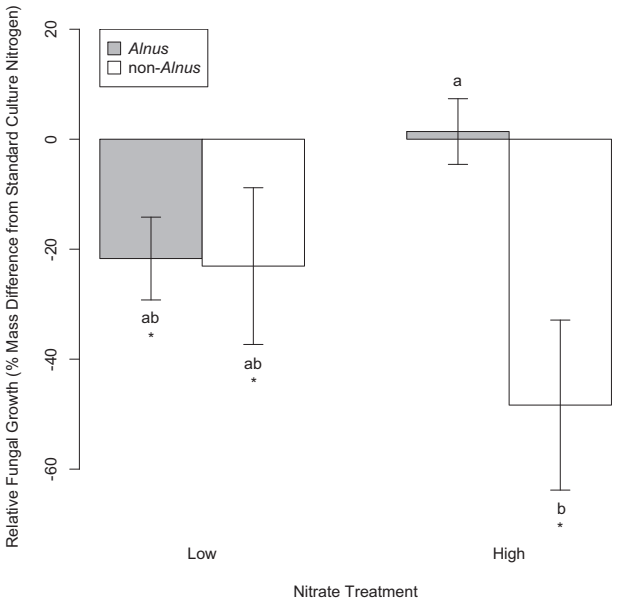

Fig 4 – Average growth response of the *Alnus*-associated and the non-*Alnus*-associated ectomycorrhizal (ECM) fungi when grown in liquid media with nitrogen available as nitrate at low concentration (1.0×10^{-5} mol l^{-1}) and high concentration (1.0×10^{-3} mol l^{-1}). Relative growth was calculated as percent difference in biomass accumulation over 50 d relative to each species' biomass accumulation under standard conditions (nitrogen available as ammonium at medium concentration (1.0×10^{-4} mol l^{-1})). Error bars denote one standard error. Letters indicate statistically significant differences between means as determined by a Tukey HSD test. Asterisks indicate values significantly different from zero (i.e. growth response significantly different from that in standard acidity) as determined by a Dunnett's test.

treatment, no detectable nitrate remained in the media at the end of the experiment (data not shown). In the high nitrate treatment, the amount of nitrate consumed was significantly positively correlated with the relative growth of species in both host groups (Nitrate Consumed: $F_{1,20} = 14.64$, $P = 0.001$, Fig 6). Although the amount of nitrate consumed per unit of relative growth was significantly lower for the *Alnus*-associated than non-*Alnus*-associated ECM fungal species (Host: $F_{1,20} = 4.88$, $P = 0.039$), the slope of the relationship between nitrate consumed and relative growth was not significantly different between host groups (Host * Nitrate Consumed Interaction: $F_{1,20} = 1.20$, $P = 0.26$).

Discussion

Our results are largely consistent with field patterns of ECM fungal specificity and differences in the abiotic conditions present in *Alnus* and non-*Alnus* soil environments. We found that the growth of the *Alnus*-associated ECM fungi were not, on average, affected by high acidity, while the non-*Alnus*-associated ECM fungi showed a significantly negative growth response under those conditions. Similarly, when grown at high nitrate, the non-*Alnus*-associated ECM fungi also generally performed more poorly than *Alnus*-associated ECM fungi. Since *Alnus* soils are known to have significantly higher acidity and nitrate than non-*Alnus* soils (Miller et al., 1992; Martin et al., 2003; Walker et al., 2014), these findings support the hypothesis that soil chemical conditions may act as an important environmental filter that inhibits non-*Alnus*-associated ECM fungi from thriving in *Alnus* environments. The growth responses we observed are also consistent with previous studies showing that many other non-*Alnus*-associated ECM fungi have very limited abilities to grow at the acidity and nitrate concentrations that are common in *Alnus* soils (Hung and Trappe, 1983; Lilleskov et al., 2002b).

While ECM fungal growth responses were significantly different when analyzed by host group, the patterns were more varied when looking at individual species. In particular, the growth of *P. involutus* differed from that of the other non-*Alnus*-associated ECM fungi. *Paxillus involutus* showed a negative growth response in the high acidity treatment (consistent with other the non-*Alnus*-associated ECM fungi), but had a

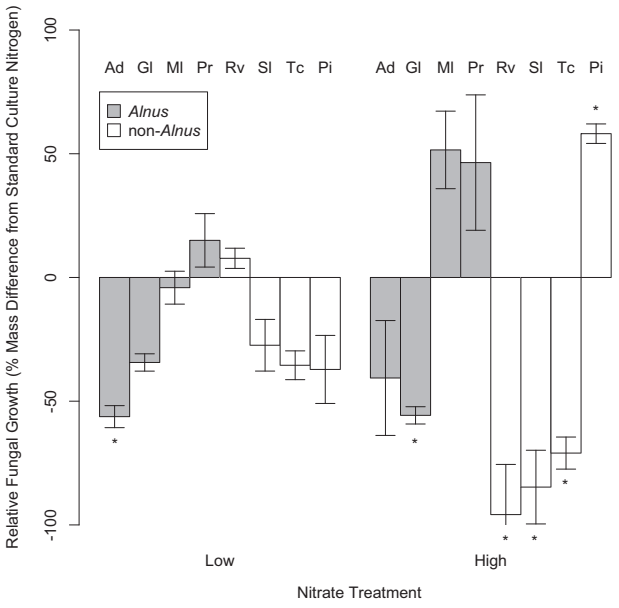

Fig 5 – Average growth response of each individual species of the *Alnus*-associated and the non-*Alnus*-associated ectomycorrhizal (ECM) fungi when grown in liquid media with nitrogen available as nitrate at low concentration (1.0×10^{-5} mol l^{-1}) and high concentration (1.0×10^{-3} mol l^{-1}). Ad = *Alpova diplophoeus*, Gl = *Gyrodon lividus*, Ml = *Melanogaster luteus*, Pr = *Paxillus rubicundulus*, Rv = *Rhizopogon vesiculosus*, Sl = *Suillus lakei*, Tc = *Truncocolumella citrina*, Pi = *Paxillus involutus*. Relative growth was calculated as percent difference in biomass accumulation over 50 d relative to each species' biomass accumulation under standard conditions (nitrogen available as ammonium at medium concentration (1.0×10^{-4} mol l^{-1})). Error bars denote one standard error. Asterisks indicate values significantly different from zero (growth response significantly different from that in standard media).

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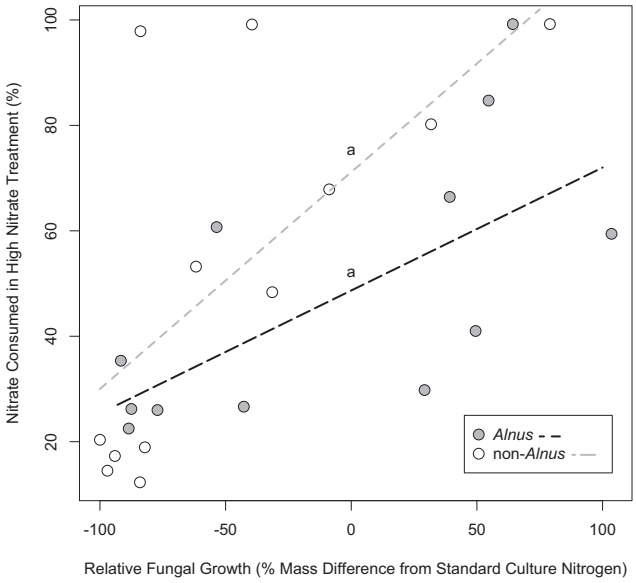

Fig 6 – Percentage of nitrate consumed by the ectomycorrhizal (ECM) fungi over 50 d growth from the high nitrate liquid media (initial nitrate concentration 1.0×10^{-3} mol l^{-1}). For each of the four *Alnus*-associated and the four non-*Alnus*-associated species, we analyzed the filtrate for only three of the five replicates, resulting in $n = 24$. Relative growth was calculated as percent difference in biomass accumulation over 50 d relative to each species' biomass accumulation under standard conditions (nitrogen available as ammonium at medium concentration (1.0×10^{-4} mol l^{-1})). Shared letters indicate the difference between slopes was not significant as determined by an analysis of covariance.

positive growth response in the high nitrate treatment. We interpret this positive growth response as evidence that this species could tolerate the higher nitrate in the media, which is a characteristic that we expected only the *Alnus*-associated ECM fungi to exhibit. Although this differed from our expectation, it is similar to the results of Lilleskov et al. (2002a), who found that *P. involutus* was one of the few species that dominated zones of high nitrification in a non-*Alnus* forest; specifically, its abundance (both as colonized root tips and as above ground fruit bodies) was positively correlated with the ambient levels of nitrate. *Paxillus involutus* was also shown to grow well on nitrate relative to many other ECM fungal species in a previous pure culture study (Keller, 1996). Interestingly, *P. involutus*, unlike most other ECM fungi, including congeners of the *Rhizopogon* and *Suillus* species tested in the present study, have been shown to associate with *Alnus* hosts under laboratory settings (Molina, 1979) and form fully functional mycorrhizas (Arnebrant et al., 1993). Early reports suggested *P. involutus* may also be associated with *Alnus* hosts in the field (Laiho, 1970), but a number of recent molecular-based studies of *Alnus* ECM fungal communities throughout the world have found no evidence to support that assertion (Tedersoo et al., 2009; Kennedy and Hill, 2010; Kennedy et al., 2011, Bogar and Kennedy, 2013; Roy et al., 2013; Põlme et al., 2013). The results we obtained here may help resolve this discrepancy. While *P. involutus* has the capacity to grow well at high nitrate concentrations, it appears that it is not able to do so at high acidity, which may limit its ability to successfully colonize *Alnus* roots in field settings. We note that Ek et al. (1994) observed successful growth of *P. involutus* on both *Picea* and *Betula* seedlings in peat with an initial pH of 4.0. Although the conditions in that experiment are more acidic than our standard pH treatment (4.5), Yamanaka (2003) found that many fungal species that grew successfully at pH 4 grew very little or not at all at pH 3. Since our high acidity treatment had an initial pH of 3.1, it appears that *P. involutus* growth decreases rapidly in environments below pH 4. Taken together, our results suggest the soil chemical conditions in *Alnus* soils appear to act as a set of environmental filters that sequentially limit the membership of *Alnus* ECM fungal communities (Koide et al., 2011). While we imagine that the two variables we assessed here have synergistic effects in nature, our results suggest that in relative terms soil acidity acts as a stronger filter for *Alnus* ECM community specificity than soil nitrate.

The fact that the *Alnus*-associated ECM fungi did not grow better at high acidity compared to at standard media acidity indicates they did not have a preference for a high acidity environment. Instead, it appears that the *Alnus*-associated ECM fungi were simply better able to tolerate higher acidity than the non-*Alnus*-associated ECM fungi. An important remaining question is whether this differential tolerance ability of the *Alnus*-associated ECM fungi is driven by an active or passive mechanism. We think our filtrate data provides partial insight regarding this issue, although we readily acknowledge that the following inference involves some speculation. On average, the media containing the *Alnus*-associated ECM fungi in the low acidity treatment underwent a considerably smaller change in proton concentration (an increase of approximately $1 \times 10^{-3.9}$ mol H^+ per liter) than the baseline change (an increase of approximately $1 \times 10^{-3.3}$ mol H^+ per liter) of the media without any ECM fungi present. In contrast, the change in proton concentration of the media in which the non-*Alnus*-associated ECM fungi were grown was not notably different from the baseline change (an increase of approximately $110^{-3.2}$ mol H^+ per liter) (see Appendix 2 for details about these calculations). We think these results indicate that the *Alnus*-associated ECM fungi can actively respond to acidity conditions, and more effectively neutralize local growing environment than the non-*Alnus*-associated ECM fungi. While this magnitude of difference in proton concentration was discernible in media filtrate of the low acidity treatment, the logarithmic nature of pH measurements obscured the magnitude of difference in the high acidity nutrient media. We assume that the physiological traits of these fungi were consistent across treatments, but more spatially explicit measurements (e.g. using microelectrodes) of acidity directly next to mycelium versus in the bulk medium are needed to better assess this supposition. Active hyphal buffering of environmental proton concentrations have been noted in other fungal systems (Dix and Webster, 1995), but more research into their prevalence among ECM fungi, particularly *in situ*, are needed to better understand this mechanism.

In regards to nitrate, the pattern of overall average growth responses was similar to the high acidity responses (i.e. the

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non-*Alnus*-associated ECM fungi were negatively affected and the *Alnus*-associated ECM fungi were relatively unaffected), but the individual species' responses in that treatment were more variable in both host groups. While the presence of negative growth responses indicate that the differential effect of nitrate is at least partially due to tolerance, several species also had positive growth responses in the high nitrate treatment, at least one of which was statistically significant (*P. involutus*). This is consistent with the possibility that, instead of simply tolerating high nitrate concentrations, some of the ECM fungi examined may have actually preferred growing in that condition. It is also possible, however, that the positive growth responses were simply due to the increased amount of nitrogen made available to the ECM fungi, not a unique ability to use nitrate more efficiently. We think that the filtrate data are more consistent with the latter supposition. Specifically, we observed that the *Alnus*-associated and non-*Alnus*-associated ECM fungi did not differ significantly in their ratio of relative growth to amount of nitrate consumed. This suggests that differences in the growth of the *Alnus*-associated and non-*Alnus*-associated ECM fungi were not the product of intrinsic differences in the efficiency with which they assimilate and/or metabolize nitrate as a nitrogen source. Instead, these differences were simply the product of their different abilities to tolerate high nitrate concentrations, and thus have access to the extra nitrogen in solution. We acknowledge, however, that there is a trend in the filtrate data for the *Alnus*-associated ECM fungi to grow more than the non-*Alnus*-associated ECM fungi per amount of nitrate consumed. We therefore stress that additional research is needed to confirm the mechanism(s) by which *Alnus*-associated ECM fungi achieve their differential growth under high nitrate conditions.

Nygren et al. (2008) surveyed a large number of ECM fungal species and determined that the ability to grow when nitrogen was provided only as nitrate was highly variable. We recognize that the presence of nitrate as the sole source of nitrogen in the nitrate manipulation treatments may, therefore, complicate the interpretation of our results (i.e. the fungi may be responding to either the presence of nitrogen as nitrate or to changes in its abundance). To address this issue, we compared the ability of the *Alnus*-associated and non-*Alnus*-associated ECM fungal species to use nitrate by replacing the standard media source of nitrogen (i.e. ammonium) with nitrate. Importantly, we kept the total amount of nitrogen available (1×10^{-4} mol N per liter) in those two treatments constant, which isolated the difference to source of nitrogen rather than quantity. On average, the change from ammonium to nitrate reduced the growth of both the *Alnus*-associated and non-*Alnus*-associated ECM fungi by approximately 10 % (Supplemental Fig 1). However, all eight fungal species were able to successfully grow on nitrate and the mean growth of each host group was not significantly different from zero (Dunnett's test $P > 0.05$). These results suggest that while nitrate may have been harder to use (i.e. there is a greater energetic cost associated with growing on nitrate versus ammonium (Smith and Read, 2008)), it was a viable source of nitrogen for the fungi examined. The finding that growth was generally reduced rather than improved also further supports our assertion that none of these ECM fungi grow preferentially on nitrate as a nitrogen source.

Although the results of this study provide information regarding the ecological drivers of *Alnus* ECM specificity, we recognize there are aspects of our experiment that limit inference. Aside from the issue of working in a pure culture system, we readily acknowledge that our experimental design lacked a key treatment, i.e. both high acidity and high nitrate, which would have best mimicked the chemical conditions actually found in natural settings. We chose to analyze these two variables separately to experimentally assess their independent effects, but as noted above, we speculate, and our results are consistent with, their effects interacting in the same direction (i.e. towards limiting *Alnus* ECM fungal community membership). A second limitation to our study was the use of only a single strain for each fungal species. While we chose to focus on replication at the species rather than strain level, intraspecific differences have been reported in response to changing acidity and nitrate levels in previous work (Hung and Trappe, 1983; Finlay et al., 1992). As such, future research incorporating greater strain richness will help validate that the results obtained here are generalizable for each species examined. A final consideration is the phylogenetic relationships of the *Alnus*-associated and non-*Alnus*-associated ECM fungi examined. All eight species belonged to the order Boletales, but the four *Alnus*-associated species were members of the same family (Paxillaceae), while three of the four non-*Alnus* associated species belong to different families (*Suillaceae* and *Rhizopogonaceae*). The individualistic responses of the species examined in both treatments suggest that our results are not strongly influenced by phylogenetic affinity. However, assessing the acidity and nitrate responses of species in additional ECM fungal lineages containing both *Alnus*- and non-*Alnus*-associated species (e.g. *Tomentella*, *Lactarius*) will be important for determining whether the results we found are both phylogenetically independent and broadly representative.

Conclusions

In summary, our results indicate that *Alnus*-associated ECM fungi generally appear to be well adapted to growth in *Alnus* soil conditions due to their differential tolerance for high acidity and high nitrate. These findings help explain why *Alnus* hosts associate with specific ECM fungi, but given that in the absence of these conditions (i.e. low acidity and low nitrate) the non-*Alnus*-associated ECM fungi did not grow significantly better than the *Alnus*-associated ECM fungi, it appears that other factors must be involved in driving the dual specificity of this system. As noted above, the nutrient demands of the host appear to be one biotic environmental filter contributing to community specificity. Kennedy et al. (2014) discuss a range of additional factors, including specificity in spore germination cues, host sanctions, and interspecific competition. Determining the relative importance of these factors in the *Alnus* system as well as looking for similar patterns in other host specific ECM systems (e.g. *Pisonia* (Hayward and Horton, 2012) or *Gnetum* (Tedersoo and Polme, 2012)) represents important next steps to more fully understanding the ecological dynamics influencing ECM community composition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2014.04.003.

REFERENCES

Arnebrant, K., Ek, H., Finlay, R.D., Soderstrom, B., 1993. Nitrogen translocation between *Alnus glutinosa* (L) Gaertn Seedlings inoculated with *Frankia* sp and *Pinus contorta* Doug Ex-Loud seedlings connected by a common ectomycorrhizal mycelium. *New Phytologist* 124, 231–242.

Bogar, L.M., Kennedy, P.G., 2013. New wrinkles in an old paradigm: neighborhood effects can modify the structure and specificity of *Alnus*-associated ectomycorrhizal fungal communities. *FEMS Microbiology Ecology* 83, 767–777.

Brandrud, T.E., 1995. The effects of experimental nitrogen addition on the ectomycorrhizal fungus flora in an oligotrophic spruce forest at Gårdsjön, Sweden. *Forest Ecology and Management* 71, 111–122.

Dix, N.J., Webster, J., 1995. Fungal Ecology. Chapman and Hall, London, p. 549.

Ek, H., Andersson, S., Arnebrant, K., Soderstrom, B., 1994. Growth and assimilation of NH_4^+ and NO_3^- by *Paxillus involutus* in association with *Betula pendula* and *Picea abies* as affected by substrate pH. *New Phytologist* 128, 629–637.

Erland, S., Soderstrom, B., Andersson, S., 1990. Effects of liming on ectomycorrhizal fungi infecting *Pinus sylvestris* L. II. Growth rates in pure culture at different pH values compared to growth rates in symbiosis with the host plant. *New Phytologist* 115, 683–688.

Finlay, R.D., Frostegard, A., Sonnerfeldt, A.M., 1992. Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl Ex Loud. *New Phytologist* 120, 105–115.

Grubisha, L.C., Trappe, J.M., Molina, R., Spatafora, J.W., 2002. Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences. *Mycologia* 94 (4), 607–619.

Hayward, J.A., Horton, T.R., 2012. Edaphic factors do not govern the ectomycorrhizal specificity of *Pisonia grandis* (Nyctaginaceae). *Mycorrhiza* 22, 647–652.

Horton, T.R., Bruns, T.D., 1998. Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). *New Phytologist* 139, 331–339.

Horton, T.R., Hayward, J.A., Tourtellot, S.G., Taylor, D.L., 2013. Uncommon ectomycorrhizal networks: richness and distribution of *Alnus*-associating ectomycorrhizal fungal communities. *New Phytologist* 198, 978–980.

Hung, L., Trappe, J., 1983. Growth variation between and within species of ectomycorrhizal fungi in response to pH in vitro growth. *Mycologia* 75, 234–241.

Huss-Danell, K., 1997. Actinorhizal symbioses and their N_2 fixation. *New Phytologist* 136, 375–405.

Ishida, T.A., Nara, K., Hogetsu, T., 2007. Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer-broadleaf forests. *New Phytologist* 174 (2), 430–440.

Keller, G., 1996. Utilization of inorganic and organic nitrogen sources by high-subalpine ectomycorrhizal of *Pinus cembra* in pure culture. *Mycological Research* 100 (8), 989–998.

Kennedy, P.G., Hill, L.T., 2010. A molecular and phylogenetic analysis of the structure and specificity of *Alnus rubra* ectomycorrhizal assemblages. *Fungal Ecology* 3 (3), 195–204.

Kennedy, P.G., Izzo, A.D., Bruns, T.D., 2003. There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. *Journal of Ecology* 91 (6), 1071–1080.

Kennedy, P.G., Garibay-Orijel, R., Higgins, L.M., Angeles-Arguiz, R., 2011. Ectomycorrhizal fungi in Mexican *Alnus* forests support the host co-migration hypothesis and continental-scale patterns in phylogeography. *Mycorrhiza* 21, 559–568.

Kennedy, P.G., Smith, D.P., Horton, T.R., Molina, R.J., 2012. *Arbutus menziesii* (Ericaceae) facilitates regeneration dynamics in mixed evergreen forests by promoting mycorrhizal fungal diversity and host connectivity. *American Journal of Botany* 99 (10), 1691–1701.

Kennedy, P.G., Bogar, L.M., Walker, J.K.M., 2014. Interspecific mycorrhizal networks and non-networking hosts: exploring the ecology of the host genus *Alnus*. In: Horton, Tom R. (Ed.), The Ecology of Mycorrhizal Networks. Springer-Verlag in press.

Kjøller, R., Nilsson, L., Hansen, K., Schmidt, I.K., Vesterdal, L., Gundersen, P., 2012. Dramatic changes in ectomycorrhizal community composition, root tip abundance and mycelial production along a stand-scale nitrogen deposition gradient. *New Phytologist*, 1–9.

Koide, R.T., Fernandez, C., Petprakob, K., 2011. General principles in the community ecology of ectomycorrhizal fungi. *Annals of Forest Science* 68 (1), 45–55.

Laiho, O., 1970. *Paxillus involutus* as a mycorrhizal symbiont of forest trees. *Acta Forestalia Fennica* 106, 1–72.

Lilleskov, E.A., Fahey, T.J., Horton, T.R., Lovett, G.M., 2002a. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* 83 (1), 104–115.

Lilleskov, E.A., Hobbie, E.A., Fahey, T.J., 2002b. Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. *New Phytologist* 154 (1), 219–231.

Martin, K.J., Posavatz, N.J., Myrold, D.D., 2003. Nodulation potential of soils from red alder stands covering a wide age range. *Plant and Soil* 254 (1), 187–192.

Miller, S., Koo, C.D., Molina, R., 1992. Early colonization of red alder and Douglas fir by ectomycorrhizal fungi and Frankia in soils from the Oregon coast range. *Mycorrhiza* 2, 53–61.

Molina, R., 1979. Pure culture synthesis and host specificity of red alder mycorrhizae. *Canadian Journal of Botany* 57, 1223–1228.

Molina, R.J., Li, C.Y., Myrold, D.D., 1994. Root symbioses of red alder: technological opportunities for enhanced regeneration and soil improvement. In: Hibbs, D.E., Tarrant, R. (Eds.), The Biology and Management of Red Alder. OSU Press, Corvallis, OR, pp. 23–46.

Moreau, P.A., Peintner, U., Gardes, M., 2006. Phylogeny of the ectomycorrhizal mushroom genus *Alnicola* (Basidiomycota, Cortinariaceae) based on rDNA sequences with special emphasis on host specificity and morphological characters. *Molecular Phylogenetics and Evolution* 38 (3), 794–807.

Nara, K., Hogetsu, T., 2004. Ectomycorrhizal fungi on established shrubs facilitate subsequent seedling establishment of successional plant species. *Ecology* 85 (6), 1700–1707.

Nygren, C., Eberhardt, U., Karlsson, M., Parrent, J., Lindahl, B.J., Taylor, A.F.S., 2008. Growth on nitrate and occurrence of nitrate reductase-encoding genes in a phylogenetically diverse range of ectomycorrhizal fungi. *New Phytologist* 180, 875–889.

Põlme, S., Bahram, M., Yamanaka, T., Nara, K., Dai, Y.C., Grebenc, T., , et al.Tedersoo, L., 2013. Biogeography of ectomycorrhizal fungi associated with alders (*Alnus* spp.) in relation to biotic and abiotic variables at the global scale. *New Phytologist* 198, 1239–1249.

Pritsch, K., Munch, J.C., Buscot, F., 2000. Identification and differentiation of mycorrhizal isolates of black alder by sequence analysis of the ITS region. *Mycorrhiza* 10 (2), 87–93.

Richard, F., Selosse, M.-A., Gardes, M., 2009. Facilitated establishment of *Quercus ilex* in shrub-dominated communities within a Mediterranean ecosystem: do mycorrhizal partners matter? *FEMS Microbiology Ecology* 68, 14–24.

Rochet, J., Moreau, P.-A., Manzi, S., Gardes, M., 2011. Comparative phylogenies and host specialization in the alder ectomycorrhizal fungi *Alnicola*, *Alpova* and *Lactarius* (Basidiomycota) in Europe. *BMC Evolutionary Biology* 11, 40.

Roy, M., Rochet, J., Manzi, S., Jargeat, P., Gryta, H., Moreau, P.-A., Gardes, M., 2013. What determines *Alnus*-associated ectomycorrhizal community diversity and specificity? A comparison of host and habitat effects at a regional scale. *New Phytologist* 198, 1228–1238.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis. Academic Amsterdam, Boston.

Smith, M.E., Douhan, G.W., Fremier, A.K., Rizzo, D.M., 2009. Are true multihost fungi the exception or the rule? Dominant ectomycorrhizal fungi on *Pinus sabiniana* differ from those on co-occurring *Quercus* species. *New Phytologist* 182 (2), 295–299.

Smith, M.E., Henkel, T.W., Aime, M.C., Fremier, A.K., Vilgalys, R., 2011. Ectomycorrhizal fungal diversity and community structure on three co-occurring leguminous canopy tree species in a Neotropical rainforest. *New Phytologist* 192 (3), 699–712.

Tedersoo, L., Polme, S., 2012. Infrageneric variation in partner specificity: multiple ectomycorrhizal symbionts associate with *Gnetum gnemon* (Gnetophyta) in Papua New Guinea. *Mycorrhiza* 22, 663–668.

Tedersoo, L., Jairus, T., Horton, B.M., Abarenkov, K., Suvi, T., Saar, I., Koljalg, U., 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. *New Phytologist* 180 (2), 479–490.

Tedersoo, L., Suvi, T., Jairus, T., Ostonen, I., Põlme, S., 2009. Revisiting ectomycorrhizal fungi of the genus *Alnus*: differential host specificity, diversity and determinants of the fungal community. *New Phytologist* 182, 727–735.

Termorshuizen, A.J., 1993. The influence of nitrogen fertilisers on ectomycorrhizas and their fungal carpophores in young stands of *Pinus sylvestris*. *Forest Ecology and Management* 57, 179–189.

Twieg, B.D., Durall, D.M., Simard, S.W., 2007. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist* 176 (2), 437–447.

Uliassi, D.D., Ruess, R.W., 2002. Limitations to symbiotic nitrogen fixation in primary succession on the Tanana River floodplain. *Ecology* 83 (1), 88–103.

Van Miegroet, H., Cole, D.W., 1985. Acidification sources in red alder and Douglas-fir soils – importance of nitrification soil. *Soil Science Society of America Journal* 49, 1274–1279.

Walker, J.K.M., Cohen, H., Higgins, L.M., Kennedy, P.G., 2014. Testing the link between community structure and function for ectomycorrhizal fungi involved in a tri-partite symbiosis. *New Phytologist* 202, 287–296.

Yamanaka, T., 2003. The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi in vitro. *Mycologia* 95 (4), 584–589.

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