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

Julia A. HUGGINS[a], Jennifer TALBOT[b], Monique GARDES[c], Peter G. KENNEDY[a,d,*]

[a]Department of Biology, Lewis & Clark College, Portland, OR, USA
[b]Department of Biology, Stanford University, Palo Alto, CA, USA
[c]Laboratoire Evolution et Diversite Biologique, Universite Toulouse 3 Paul Sabatier, UMR 5174 UPS, ENFA, CNRS, 118 route de Narbonne, 31062 Toulouse Cedex, France
[d]Departments of Plant Biology & Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN, USA

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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.

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

* *Corresponding author*. 250 Biological Science Building, 1445 Gortner Ave., St. Paul, MN 55108, USA. Tel.: +1 612 624 8519; fax: +1 612 625 1738.
E-mail address: kennedyp@umn.edu (P.G. Kennedy).
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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