

Chapter 8

Interspecific Mycorrhizal Networks and Non-networking Hosts: Exploring the Ecology of the Host Genus *Alnus*

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Abstract While the dominant ectomycorrhizal (ECM) fungi in most temperate and tropical forests have low host specificity, a commonly cited exception to this pattern is the ECM fungal community associated with the host genus *Alnus*. In this chapter, we discuss multiple hypotheses that have been put forth to explain the specificity of the *Alnus* ECM symbiosis and consider their strengths and weaknesses in light of current research on the topic. In addition to reviewing the range of suggested explanations, we also propose and discuss a new alternative explanation of *Alnus* ECM specificity involving three-way interactions among *Alnus* plants, ECM fungi, and *Frankia* bacteria. With specific regard to common mycorrhizal networks (CMNs), we believe they may play an important role in the specificity observed in the *Alnus* ECM system. To understand that role in the larger context of research on *Alnus* ECM fungal communities, we begin our chapter with a synopsis of the studies documenting the unique specificity pattern. From there, we discuss why it appears to be advantageous for *Alnus* plants not to participate in interspecific CMNs. Finally, we elaborate on how specificity may be established and maintained in the *Alnus* ECM system and suggest what we consider to be promising future research directions.

Keywords Mycorrhizal specificity · Mutualisms · Partner choice · Common mycorrhizal networks · Tri-partite interactions

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8.1 Introduction

A fundamental prerequisite for the formation of interspecific common mycorrhizal networks (CMNs) is the ability of mycorrhizal fungi to associate with multiple species of co-occurring host plants. Many ectomycorrhizal (ECM) fungi have broad host ranges (Molina et al. 1992; Molina and Horton, Chap. 1, this volume) and form compatible mycorrhizal relationships with many distantly related plant genera and species. At the same time, ECM fungi specific to a particular host plant genus are common, even on ECM host plants with broad fungal compatibility (Molina et al. 1992). ECM hosts also have variable degrees of receptivity to fungal associates. For example, some ECM hosts, such as *Pseudotsuga menziesii*, are reported to be receptive to colonization by thousands of fungal species (Trappe and Fogel 1977); others, such as *Pisonia grandis*, appear to associate with only a very limited number of ECM fungi (Suvi et al. 2010).

Based on a range of recent studies, it appears that the most frequent and/or abundant ECM fungi in most temperate and tropical forests have low host specificity (Horton and Bruns 1998; Horton et al. 1999; Cullings et al. 2000; 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; but see Smith et al. 2009). A commonly cited exception to this pattern, however, is ECM fungi associated with the host genus *Alnus*. Unlike other ECM fungi-plant host systems, *Alnus* ECM fungal communities have been consistently characterized by low species richness and a high proportion of genus-specific species (Molina 1979; Tedersoo et al. 2009; Walker et al. 2014). While other ECM hosts do associate with ECM fungi that are also genus-specific (e.g. *Rhizopogon* and ECM hosts genera within the Pinaceae), they are rarely the dominant fungi present in mature forests.

The factors contributing to the reciprocal specificity of the *Alnus* ECM system have been the subject of considerable speculation. In this chapter, we highlight a number of hypotheses that have been put forth to explain this specificity and consider their strengths and weaknesses in light of current research on the topic. We begin with a synopsis of the studies documenting the unique specificity pattern. From there, we discuss why it appears to be advantageous for *Alnus* plants not to participate in interspecific CMNs. Finally, we elaborate on how specificity may be established and maintained in the *Alnus* ECM system and suggest what we consider to be promising future research directions.

8.2 Documenting the *Alnus* ECM Specificity Pattern

Frank (1888) was the first to determine that fungal colonization of the roots of *Alnus* trees was ectomycorrhizal in nature (Table 8.1). It took many more years, however, before distinct morphologies were identified (Masui 1926) and fungal species identities were reported (Favre 1948, Singer 1950). An early review by Trappe

(1962) cited 14 ECM fungal species associated with *Alnus* hosts based on morphological characterization, including members of the genera *Alnicola*, *Russula*, *Lactarius*, *Gyrodon*, and *Cenococcum*. Additional ECM fungal morphotypes were later observed on field-collected *Alnus* roots (Horak 1963; Neal et al. 1968; Mejsstrik and Benecke 1969), some of which were initially identified as *Cortinarius*, *Paxillus*, and *Alpova*. Molina (1979, 1981) and Godbout and Fortin (1983) found that fungal species consistently observed with *Alnus* trees as sporocarps (Neal et al. 1968; Trappe 1975) formed ectomycorrhizas in pure culture synthesis assays, while others not observed to be associated with *Alnus* did not typically form ectomycorrhizas, or formed ones that were anatomically anomalous. Intriguingly, the presence of *Paxillus involutus* (now recognized as a species complex; Jargeat et al. 2014) as an *Alnus* associate in field settings remained unclear, but additional pure culture work indicated that this species could form functional ectomycorrhizas with *Alnus* species in lab settings (Chatarpaul et al. 1989; Arnebrant et al. 1993; Massicotte et al. 1999). Despite further detailed morphotyping analyses of ECM root tips (Miller et al. 1991), the global total of ECM fungal species thought to associate with *Alnus* trees by the mid-1990s was fewer than 50 (Molina et al. 1994).

DNA-based analyses of the *Alnus* ECM system have largely confirmed previous work based on other methods. Pritsch et al. (1997) were the first to use these methods by matching RFLP patterns of *Alnus* ECM morphotypes with sporocarps present in *Alnus* forests. Although those authors did not detect any new *Alnus*-associated ECM fungal genera, they did increase the number of species present on *Alnus* roots. In subsequent studies where DNA extraction was followed by sequencing of the fungal ITS and/or LSU region, some new genera and lineages were identified (Tedersoo et al. 2009; Kennedy and Hill 2010; Kennedy et al. 2011a; Bogar and Kennedy 2013). Those studies also increased the number of ECM fungal species associated with *Alnus* hosts, but not in a way that significantly altered the general pattern of low richness and high specificity. Interestingly, Tedersoo et al. (2009) found that the majority of ascomycete species (4 of 6) associated with *A. glutinosa* and *A. incana* in Estonia were also found in association with other ECM hosts, although those species made a minor component of the communities identified in that study. Rochet et al. (2011) summarized much of this molecular work by noting that there appear to be six dominant Basidiomycete genera (*Tomentella*, *Alnicola*, *Lactarius*, *Cortinarius*, *Alpova*, and *Russula*), a few other Basidiomycete genera not consistently found as ectomycorrhizas (e.g. *Paxillus*, *Hebeloma*, *Inocybe*, and *Pseudotomentella*), and a number of unknown members of the Helotiales associated with *Alnus* hosts. At present, the best estimate of the number of *Alnus*-associated ECM fungal species comes from the global-scale study by Polme et al. (2013), which suggested total richness to be around 200 species.

Table 8.1 Chronological summary of observations and experiments on ectomycorrhizal (ECM) fungi associated with *Alnus* species, with a focus on those reporting new species or genera

Publication	<i>Alnus</i> species	Number of taxa ^a	New genera ^b	Reported identity of fungal associate ^c	Identification method	Study type
Frank (1888)	<i>A. viridis</i>	~	~	the presence of fungal taxa described	ECM morphology	Field
Masui (1926)	<i>Alnus</i> spp.	6	~	6 mycorrhizal types distinguished	ECM morphology	Field
Favre (1948)	<i>A. viridis</i> , <i>A. incana</i>	~	1	several <i>Alnicola</i> spp. observed	Fruitbody	Field
Singer (1950)	<i>A. acuminata</i>	~	2	survey included <i>Russula</i> spp.	Fruitbody	Field
Trappe (1962)	<i>A. incana</i> , <i>A. nigra</i> , <i>A. viridis</i> , <i>A. jorullensis</i> , <i>A. crispa</i> , <i>Alnus</i> spp.	14	5	<i>Alnicola diplocystis</i> , <i>A. melinoides</i> , <i>A. umbrina</i> , <i>Cenococcum graniforme</i> , <i>Lactarius controversus</i> , <i>glycosmus</i> , <i>lilacinus</i> , <i>obscuratus</i> , <i>Gyrodon lividus</i> , <i>montana</i> , <i>monticola</i> , <i>Russula emetica</i> , <i>montivaga</i> , <i>decolorans</i>	Reported associations	Review
Horak (1963)	<i>A. viridis</i>	8	8	<i>Alnicola melinoides</i> , <i>submelinoides</i> , <i>suavis</i> , <i>Hydrocybe</i> (sic) <i>atropusillus</i> , <i>Lactarius tabidus</i> (<i>theiogalvus</i>), <i>Paxillus involutus</i> , <i>Phlegmacium moseri</i> = <i>Cortinarius</i> , <i>Russula alnetorum</i>	ECM morphology	Field
Trappe (1964)	<i>A. rubra</i>	1	8	<i>Cenococcum graniforme</i> = <i>geophilum</i>	ECM morphology	field
Neal et al. (1968)	<i>A. rubra</i>	2	9	Dark brown clavate = <i>Alpova diplophloeus</i> , Pale brown glabrous = <i>Lactarius obscuratus</i>	ECM morphology	Field
Mejstrik and Benecke (1969)	<i>A. viridis</i>	3	9	Subtype B, F, K	ECM morphology	Field/bioassay
Froidevaux (1973)	<i>A. rubra</i>	1	9	<i>Lactarius obscuratus</i>	ECM morphology	Field

(continued)

Table 8.1 (continued)

Publication	Alnus species	Number of taxa ^a	New genera ^b	Reported identity of fungal associate ^c	Identification method	Study type
Molina (1979, 1981)	<i>A. rubra</i> , <i>A. glutinosa</i> , <i>A. incana</i> , <i>A. rhombifolia</i> , <i>A. sinuata</i>	6	13	<i>Alpova diplophloeus</i> , <i>Astraeus pteridis</i> , (<i>Laccaria laccata</i>), (<i>Paxillus involutus</i>), (<i>Pisolithus tinctorius</i>), <i>Scleroderma hypogaeum</i>	ECM formation	Pure culture assay
Godbout and Fortin (1983)	<i>A. crispa</i> , <i>A. rugosa</i>	10	15	<i>Alpova diplophloeus</i> , <i>Cenococcum geophilum</i> , <i>Cortinarius</i> cf. <i>subporphyropus</i> , <i>Hebeloma</i> cf. <i>crustuliniforme</i> , <i>Laccaria laccata</i> , <i>Leccinum subleucopheum</i> , <i>holopus</i> , <i>Paxillus involutus</i> , <i>Pisolithus tinctorius</i> , <i>Scleroderma</i> (sic) <i>citrinum</i>	ECM formation	Pure culture assay
Miller et al. (1991)	<i>A. rubra</i>	11	16	<i>Alpova diplophloeus</i> , <i>Cortinarius bibulus</i> , <i>Hebeloma</i> cf. <i>crustuliniforme</i> , <i>Laccaria laccata</i> , <i>Lactarius obscuratus</i> , <i>Paxillus involutus</i> , <i>Thelephora terrestris</i> = <i>Alnicola</i> spp., <i>Tomentella subilacina</i>	ECM morphology	Field/bioassay pure culture assay
Pritsch et al. (1997)	<i>A. glutinosa</i>	16	16	<i>Alnirhiza cystidiobrunnea</i> = (<i>Tomentella</i> aff. <i>subilacina</i> , <i>ilacina</i> , <i>violaceae</i> , <i>suffusa</i> , <i>cana</i> , <i>texta</i> , <i>cremicolor</i> , <i>atroverrucosa</i> , <i>Cortinarius</i> cf. <i>helvelloides</i> , <i>alneus</i> , <i>Lactarius obscuratus</i> , <i>omphaliformes</i> , <i>ilacinus</i> , <i>Naucoria escharoides</i> = <i>Alnicola melinoides</i> , <i>subconspersa</i> , <i>Russula pumila</i>	ECM formation/RFLP	Field
Becerra et al. (2002, 2005)	<i>A. acuminata</i>	10	16	<i>Alnirhiza silkacea</i> = <i>Gyrodon monticola</i> , <i>Cortinarius helodes</i> , <i>tucumanensis</i> , <i>Lactarius omphaliformis</i> , sp., <i>Naucoria escharoides</i> , <i>Russula abnjojullensis</i> , <i>Tomentella</i> spp.	ECM formation/RFLP	Field

(continued)

Table 8.1 (continued)

Publication	Alnus species	Number of taxa ^a	New genera ^b	Reported identity of fungal associate ^c	Identification method	Study type
Tedersoo et al. (2009)	<i>A. glutinosa</i> , <i>A. incana</i>	40	21	<i>Alnicola</i> spp., <i>Corinarius alnetorum</i> sp., (<i>Geopyxis</i> cf. <i>carbonaria</i>), <i>Gyrodon lividus</i> , <i>Hebeloma</i> aff. <i>helodes</i> , <i>Helvella</i> aff. <i>elastica</i> sp., unknown <i>Humaria</i> sp., <i>Inocybe</i> sp., <i>Lactarius cyathuliformis</i> , <i>lilacinus</i> , sp., unknown <i>Pachyphloeus</i> spp., <i>Paxillus filamentosus</i> , <i>rubicudatus</i> , <i>Peziza michelii</i> , <i>Pseudotomentella</i> spp., <i>Russula pumila</i> , <i>Tomentella</i> aff. <i>subtilacina</i> , <i>stuposus</i> , <i>coerulea</i> , <i>ellisi</i> , <i>lateritia</i> , <i>terrestris</i> , sp.	DNA sequencing of ITS and LSU	Field
Kennedy and Hill (2010)	<i>A. rubra</i>	14	22	<i>Alnicola escharoides</i> sp., <i>Alpova diplophloeus</i> , <i>Corinarius</i> spp., <i>Inocybe</i> sp., <i>Lactarius</i> cf. <i>obscuratus</i> , <i>Pseudotomentella</i> sp., <i>Tomentella</i> spp., <i>Xerocomus</i> sp.	DNA sequencing of ITS	Field
Kennedy et al. (2011)	<i>A. jorullensis</i> , <i>A. acuminata</i>	23	24	<i>Alnicola</i> sp., <i>Alpova</i> sp., <i>Clavulina</i> sp. , <i>Corinarius</i> spp., <i>Hymenogasteraceae</i> sp., <i>Inocybe</i> spp., <i>Lactarius</i> spp., <i>Sebacinacea</i> spp. , <i>Tomentella</i> spp.	DNA sequencing of ITS and LSU	Field
Bogar and Kennedy (2013)	<i>A. rhombifolia</i>	13	25	<i>Alnicola</i> spp., <i>Alpova diplophloeus</i> spp., <i>Boletaceae</i> sp., <i>Clavulina</i> sp., <i>Laccaria laccata</i> , <i>Russula</i> sp., <i>Tomentella ellisi</i> spp., <i>Thelephorales</i> sp., <i>Tuber</i> sp.	DNA sequencing of ITS	Field/bioassay

^aNumber of unique groups, morphotypes, or species reported

^bRunning total of new genera is an estimate, and has been adjusted based on corrected reports

^cFirst reports of new genera are in bold type; species in brackets formed incomplete mycorrhizas

8.3 Could the *Alnus* ECM Specificity Pattern Be an Artifact?

The atypical specificity of the *Alnus* ECM system has been observed in many different studies and experimental settings. The consistency of those results presented a striking pattern, but some aspects of previous work leave open the possibility that the currently accepted specificity paradigm could be artifactual. For example, the study of *Alnus* ECM fungal communities has focused largely on temperate geographic regions, but *Alnus* species also occur at tropical latitudes in Central and South America. Studies from many groups of organisms have shown that species richness tends to be higher in tropical regions and decreases as one moves towards the poles (i.e. the latitudinal gradient of species diversity—LGD) (Townsend et al. 2008). As such, part of the current perception that *Alnus* ECM fungal communities are species poor may be related to the temperate bias of past *Alnus* studies. Similarly, nearly all previous studies involving comparisons between *Alnus* and other ECM fungal communities have involved distantly related ECM hosts, such as *Pseudotsuga menziesii* (Miller et al. 1992), multiple Pinaceae species (Massicotte et al. 1994), and *Pinus montezumae* (Kennedy et al. 2011a). Because ECM fungal community similarity has been shown to be lower when comparing more distantly related hosts (Ishida et al. 2007), the observed specificity of the *Alnus* system could also be an artifact of the types of host comparisons made thus far. Below we discuss three of our own studies that recently examined these issues to test the robustness of the *Alnus* ECM specificity pattern (Kennedy et al. 2011a; Bogar and Kennedy 2013).

Two *Alnus* species, *A. jorullensis* and *A. acuminata*, grow in montane tropical forests in central Mexico, either alone or with other ECM host species such as *Pinus montezumae*. Kennedy et al. (2011a) sampled the ECM fungal communities present at multiple sites for each *Alnus* species. We found that, like their temperate counterparts, the *Alnus* ECM fungal communities in Mexico had relatively low species richness. Interestingly, many of the ECM fungi present in the Mexican *Alnus* forests were strikingly similar to those present in *Alnus* forests in other parts of the world. For example, in the genus *Tomentella*, the five most abundant species in Mexico had sequences that matched much better to sequences of *Tomentella* species sampled in forests in the United States, Europe, and Argentina than to other *Tomentella* species sampled in Mexico (Fig. 8.1). The sequence matches were very high (>97 %), suggesting that *Alnus* species may associate with many of the same ECM fungi globally. A similar pattern was also evident in the ECM fungal genera *Cortinarius*, *Lactarius*, and *Inocybe*. In addition, we identified notably higher species richness on *P. montezumae* in ten-fold fewer ECM fungal root tips; 24 ECM fungal species were identified from 42 *Pinus montezumae* ECM root tips, compared to only 21 ECM fungal species detected on over 400 concurrently sampled *Alnus* ECM root tips. This result reinforces the depauperate nature of *Alnus* ECM fungal communities compared with other ECM hosts. More importantly, despite a clear intermingling of root systems at two of the study sites, there were no species shared

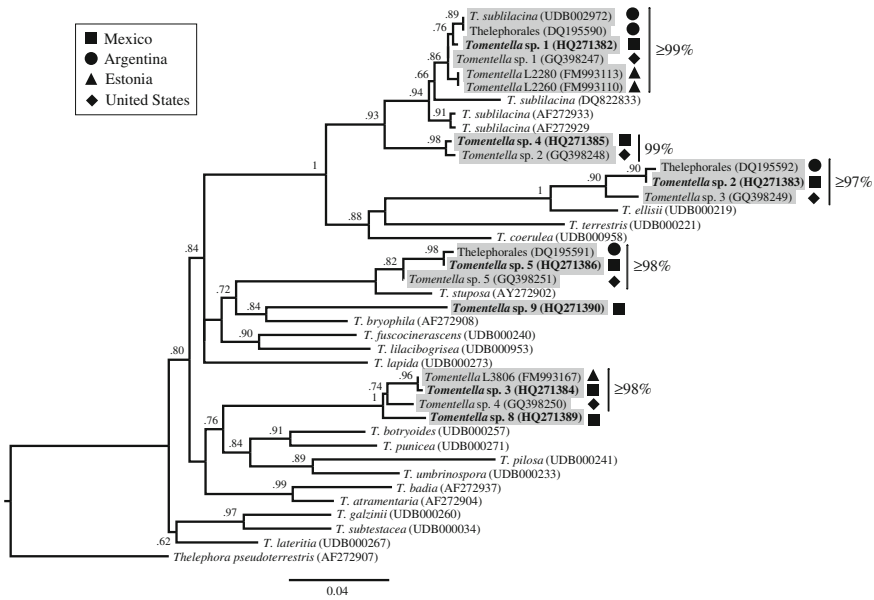


Fig. 8.1 Phylogenetic reconstruction of taxa in the ECM genus *Tomentella* based on rDNA ITS sequences. Nodes are labeled with aLRT scores from the maximum likelihood analysis above 0.60. Species are labeled with species names or unique identifier and GenBank or UNITE number in parentheses. *Alnus*-associated species are designated in gray boxes, with the Mexican *Alnus*-associated species in bold. Symbols next to selected *Alnus*-associated species indicate the geographic area from which they were obtained. The percentage values for the selected groups represent pair-wise comparisons between all group members. *Thelephora pseudoterrestris* was designated as the outgroup for rooting

between the ECM fungal communities on *A. jorullensis* and *P. montezumae*. The low species richness and reciprocal specificity observed on *Alnus* species in this tropical-based study, suggests that the unique pattern present in the *Alnus* ECM system is consistent regardless of geographic location of study. Polme et al. (2013) confirmed this conclusion with a comprehensive spatial sampling of 22 *Alnus* species over 96 geographic locations covering a wide range of latitudes.

We also examined whether host evolutionary relationships might explain *Alnus* ECM specificity by comparing *Alnus* and *Betula* ECM fungal communities. We predicted that if *Alnus* ECM fungi are specific to the family Betulaceae, rather than only to *Alnus*, they would be expected to associate with both genera. To test this hypothesis, we examined the ECM fungal communities on *Alnus rhombifolia* and *Betula occidentalis* in western Idaho, USA (Bogar and Kennedy 2013). We characterized the communities in a riparian habitat where the hosts co-occur, comparing the ECM fungal communities present when *Alnus* and *Betula* roots overlapped to those present on each host alone. In order to accomplish this, we collected soil cores at the base of trees of each host species in pure stands (i.e. from solitary host trees >2 m from the other host type) and soil cores from

between the two host species (i.e. we dug directly in between alternate hosts that were ≤ 30 cm from each other) in mixed stands. We found that the ECM fungal community associated with *A. rhombifolia* was relatively species poor and host-specific as compared to the ECM fungal community on *B. occidentalis* (i.e. overall, there were fewer ECM fungal species on *A. rhombifolia*, and the same ECM fungal species were not found on *B. occidentalis* in this study, Fig. 8.2). This was true even when overlapping root systems of the two hosts were sampled (Fig. 8.2). In comparison to the results of Kennedy et al. (2011a), the specificity of the *Alnus*-associated ECM fungal community was not as strong: the two host species associated with six of the same ECM fungal species across the site. However, overlapping root systems (i.e. those found in the same core) of *A. rhombifolia* and *B. occidentalis* almost never associated with the same fungal species. Thus, even though host specificity may not be absolute for some *Alnus*-associated ECM fungi, it appears that association with *Alnus* may preclude simultaneous association with *Betula*. In short, this study suggests that the unique specificity observed in the *Alnus* ECM system is not a byproduct of previous comparisons involving more distantly related hosts.

More recently, we also examined the ECM fungal communities associated with *Alnus glutinosa* in New Zealand, which is a non-native tree invader on the North and South Islands (Bogar et al. 2015). We speculated that by sampling outside the native range of *Alnus* trees, the *A. glutinosa* individuals present in New Zealand might be 'forced' into associating with a broader suite of ECM fungi, particularly those present on native ECM hosts (e.g. *Nothofagus* spp.). We found, however, that the ECM fungal communities present on *A. glutinosa* in New Zealand were notably species poor (only 9 species present across over 300 root tips sampled) and completely dominated by European *Alnus*-associated ECM species (which is the native range of *A. glutinosa*). In fact, we found no ECM fungal species present on *A. glutinosa* that appeared to be associated with native New Zealand ECM hosts. This result further reiterates the globally anomalous nature of the *Alnus*-associated ECM system and suggests that even well outside their native ranges, the specificity of the plants and fungi involved in this symbiosis remains intact.

Taken together, we believe there is abundant evidence that *Alnus* ECM fungal communities are both species poor (Masui 1926; Horak 1963; Neal et al. 1968; Mejstrik and Benecke 1969; Brunner et al. 1990; Miller et al. 1992) and highly host specific (Molina 1979, 1981; Molina et al. 1992; Godbout and Fortin 1983; Pritsch et al. 1997; Tedersoo et al. 2009; Kennedy and Hill 2010; Bent et al. 2011) and that this unique pattern is not based on sampling artifact (Kennedy et al. 2011a; Bogar and Kennedy 2013; Bogar et al. 2015).

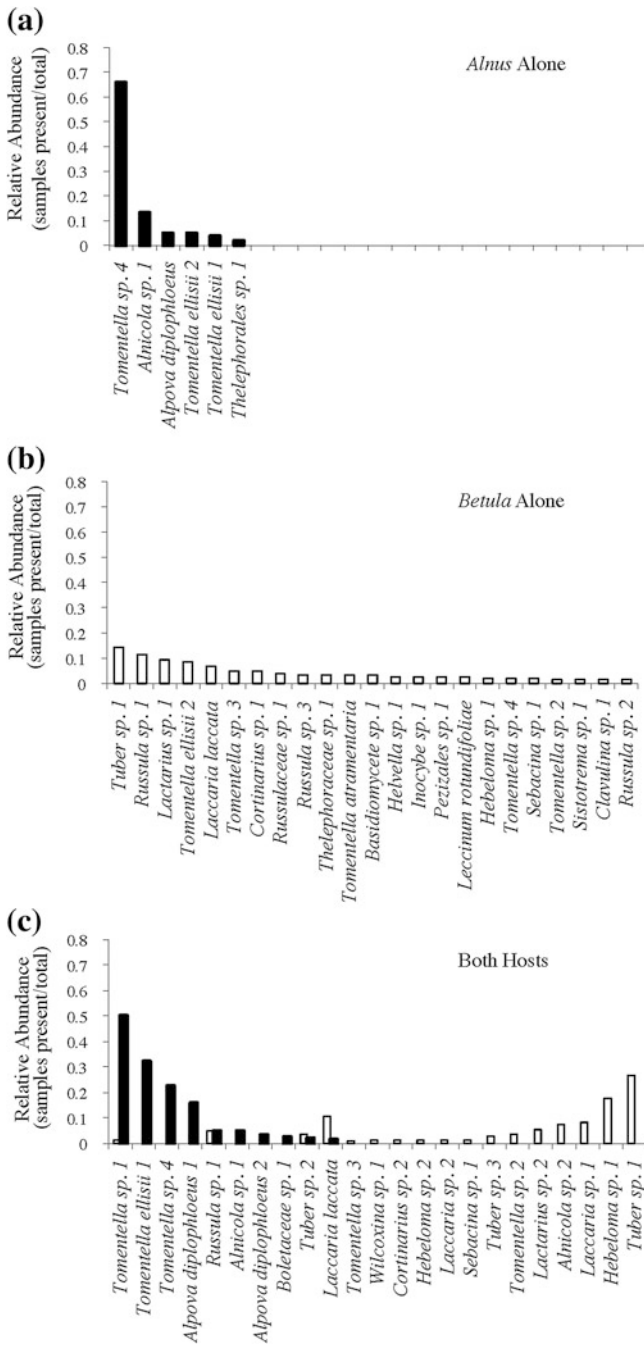


Fig. 8.2 Ranked relative abundance of ECM species richness on *Betula occidentalis* (open bars) and *Alnus rhombifolia* (filled bars) at a field site in western Idaho, USA. Sampling found six fungal taxa that were present on both *Betula* and *Alnus* (names in bold)

8.4 Why or Why not Participate in CMNs?

To better understand why reciprocal specificity among *Alnus* and their associated ECM fungi makes them unlikely to participate in interspecific CMNs, it is helpful to briefly revisit the ecological benefits provided by CMNs. One of the most widely cited benefits of CMNs for both plants and fungi is access to a larger resource pool. By joining an extensive established mycelial network, connected plants have the ability to draw from a much larger soil volume than unconnected plants (Newman 1988). This benefit appears to be particularly important for seedlings, which lack well-developed root systems (van der Heijden and Horton 2009). With connections to a variety of host species (Booth 2004), or to the same host species at a range of growth stages (Teste et al. 2010; Beiler et al. 2010; Booth and Hoeksema 2010), ECM fungi also receive carbon from multiple sources. This redundancy may provide an important buffer against spatially or temporally variable host inputs (e.g. deciduous versus evergreen hosts, canopy versus understory individuals).

A related proposed benefit of CMNs is inter- or intraspecific plant facilitation (Molina and Horton, Chap. 1, this volume; Nara Chap. 6, this volume). Molina and Trappe (1982) hypothesized that the resprouting ability of certain plant species in forests of the Pacific Northwest, USA allows the ECM fungal community to be maintained directly after fire or clear-cutting. The presence of compatible fungi benefits subsequent colonization of later seral plants by providing those individuals with access to established mycelial networks. Seedlings of the resprouting plants then reciprocally establish in the understory of those later seral forests and therefore benefit from CMNs in the same way. Evidence supporting CMN-mediated interspecific plant facilitation has been documented in California (Horton et al. 1999), Japan (Nara and Hogetsu 2004) and Corsica (Richard et al. 2009), and recent work in the dry forests of western Canada indicates that CMNs can also facilitate the establishment of conspecific *Pseudotsuga menziesii* seedlings (Teste et al. 2010). Fungal benefit in the above scenarios comes from the ability to maintain a constant carbon source during disturbance-associated host species regeneration.

CMNs may also benefit plants by mediating nutrient transfer among connected individuals (Simard et al. Chap. 5, this volume). This benefit has been most clearly documented among mycoheterotrophic plants, which received all of their carbon from CMNs connected to adjacent autotrophic plants (Bidartondo 2005). The transfer of carbon has also been documented among autotrophic plants, although the levels of movement among autotrophic individuals appear to be much lower than to both mycoheterotrophic or mixotrophic plants (Simard et al. 2012). In addition to carbon, other resources can also move among CMN-linked plants, including nitrogen (Arnebrant et al. 1993; He et al. 2004, 2005), phosphorus (Finlay and Read 1986), water, and defense compounds (Song et al. 2010; Johnson and Gilbert 2015). Although we are unaware of studies demonstrating beneficial movement of resources among fungal individuals through linked plants, that pathway may exist, especially for a resource that would be lost or not transferable through soil. Finally, we believe it is important to stress that the three

aforementioned benefits are not mutually exclusive; plants and fungi may benefit in multiple simultaneous ways from CMNs (Simard et al. 2012).

Notwithstanding potential intraspecific networks (i.e. connections among *Alnus* individuals) and given the aforementioned benefits provided by CMNs, why do *Alnus* plants and their associated ECM fungi remain unconnected to co-occurring non-*Alnus* ECM hosts? One reason is likely related to the general life history of this host genus. As typically pioneer successional species, *Alnus* individuals establish in habitats where, in many cases, other ECM hosts are not already present. Doing so reduces or eliminates the opportunity for *Alnus* plants to join established mycelial networks or to benefit from CMN-mediated facilitation. (Because *Alnus* seedlings are shade intolerant, and do not occur under an established *Alnus* overstory, they also do not have immediate access to intraspecific CMNs.) While *Alnus* forests tend to be mono-dominant initially, there is establishment by other ECM hosts (e.g. those in the Pinaceae) over time (Miller et al. 1992). While the presence of other ECM host species provides the potential for CMNs and the transfer of resources between connected individuals, the dynamics of CMN nutrient transfer appears to be unfavorable for *Alnus* plants. Simard et al. (1997) showed that interspecies CMN-mediated resource transfer follows a source-sink pattern, with net carbon movement towards shaded individuals. Connecting to CMNs with understory species would therefore represent a carbon loss for *Alnus* individuals, as it would for other pioneer species. In addition, since *Alnus* seedlings tend not to establish under canopies, there are also no reciprocal opportunities for this genus to regain carbon benefits from CMNs (unlike the scenario discussed by Molina and Trappe (1982) above).

The forests of central Mexico provide an interesting exception to this pattern. At some locations, *Alnus jorullensis* persists under a *Pinus montezumae* canopy, resulting in *Alnus* as the potential carbon sink (i.e. a favorable situation for *Alnus*; Kennedy et al. 2011a). As noted earlier, however, *Alnus* and *Pinus* individuals at those sites do not appear to share any common ECM fungi, therefore no CMNs between *Alnus* and *Pinus* are possible. This finding suggests the absence of CMNs among *Alnus* trees and other ECM hosts is not solely driven by unfavorable carbon-based source-sink dynamics.

We believe that a second key factor discouraging the formation of CMNs for *Alnus* plants is their co-association with nitrogen-fixing *Frankia* bacteria. These bacteria provide *Alnus* with a unique source of nitrogen relative to co-occurring ECM host plants. Although interspecific CMNs involving *Alnus* plants appear to be functionally non-existent in natural settings, in a laboratory study, Arnebrant et al. (1993) showed that substantial amounts (~20 %) of fixed nitrogen could move through CMNs from *Alnus glutinosa* to *Pinus contorta*. Similar results were obtained by Ekblad and Huss-Danell (1995), who observed that up to 9.5 % of the nitrogen in CMN-linked *Pinus sylvestris* seedlings was derived from *Frankia*-based nitrogen fixation. Given the substantial carbon allocation by *Alnus* plants towards *Frankia* bacteria (see below), and the value of nitrogen as a resource, the absence of CMNs between *Alnus* and non-*Alnus* individuals would prevent co-occurring plants from directly accessing this commodity. Intriguingly, He et al. (2004, 2005) used

labeled isotopes to show a net movement of nitrogen through CMNs from non-*Frankia*-associated *Eucalyptus maculata* to *Frankia*-associated *Casuarina cunninghamiana* individuals. Since these latter results conflict with the findings of Arnebrant et al. (1993) and Ekblad and Huss-Danell (1995), additional studies, particularly in field settings, are needed to further define the patterns and drivers of nitrogen transfer dynamics.

8.5 Establishment and Maintenance of the *Alnus*-ECM Fungus Specificity Pattern

Although the absence of CMNs may be selectively advantageous for *Alnus* plants based on their life history and relationship with *Frankia* bacteria, questions remain about how specificity in the *Alnus*-ECM fungus system is established and maintained. 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 evidence to support its role in driving patterns of co-speciation (Rochet et al. 2011). Our interests, however, lie in the more proximate causes of the observed specificity. As such, we focus the remainder of the chapter on a number of hypotheses that may explain how current interactions among *Alnus* trees and their associated ECM fungi reinforce their unique specificity pattern.

8.5.1 *Alnus*-ECM Fungus Specificity: Signaling and Sanctioning Hypothesis

Before ECM host plants and fungi begin to interact with one another, each symbiont is confronted with incomplete information about the other partner. For example, how do *Alnus* plants identify which of the fungi in the ECM community pool have the right characteristics to meet their needs? Similarly, how do ECM fungi differentiate *Alnus* roots from those of other co-occurring hosts? The latter issue is partially resolved by the fact that *Alnus* trees often establish in mono-dominant stands, but there are a number of situations in which *Alnus* individuals do co-occur with other host species (Tedersoo et al. 2009; Kennedy et al. 2011a; Bogar and Kennedy 2013). This problem of asymmetric information can be resolved in two ways (Archetti et al. 2011). The first is to choose partners before the interaction is established. This mechanism, known as partner choice (Bull and Rice 1991), can be accomplished by signaling. Under this scenario, *Alnus* plants would broadcast information about their own attributes, and the ECM fungi would respond by associating or not based on that signal. The experimental study of Massicotte et al. (1994) showed strong indirect support for chemical signaling between *Alnus* plants and ECM fungi. Those authors observed that *Alpova diplophloeus*, an *Alnus*-specific ECM fungus, germinated

readily in the presence of *Alnus* roots (as determined by subsequent root tip colonization) but never in the sole presence of roots of a number of other ECM host species. Conversely, no non-*Alnus* associated ECM fungus germinated in the presence of *Alnus* roots alone, but most did germinate in the presence of their preferred host, infrequently colonizing a secondary host. Collectively, these data suggest that *Alnus* roots release a unique chemical cue that induces spore germination of only the fungi having attributes beneficial to *Alnus* (and perhaps only eliciting a response from those fungi that may also benefit from resources associated with *Alnus*). Analogous signaling that induces partner germination has been observed among mycoheterotrophic plants and their associated ECM fungi (Bruns and Read 2000; Bidartondo and Bruns 2005) as well as with the conifer-induced germination of other host-specific ECM fungi in the genus *Suillus* (Fries et al. 1987).

A second way that the asymmetric information problem can be resolved is by monitoring the interaction after it has been established. This kind of monitoring is commonly referred to as host sanctioning and typically involves some form of punishment of “misbehaving” symbionts (Kiers and Denison 2008). One example of host sanctioning comes from the soybean-rhizobia symbiosis, where the soybean host is able to selectively decrease oxygen availability to nodules that are not fixing nitrogen (Kiers et al. 2003). While nitrogen fixation is a tightly controlled anaerobic process mediated by plant leghemoglobin, oxygen is still required by these bacteria as a terminal electron acceptor, therefore reduced oxygen impedes rhizobial performance (Kiers et al. 2003). In the *Alnus* ECM system, some of the results of Molina (1979) are consistent with host sanctioning. He found that two ECM fungi not typically associated with *Alnus rubra*, *Paxillus involutus* and *Astraeus pteridis*, were able to establish mycorrhizas with this host species in pure culture synthesis assays. Interestingly, Molina (1979) found that cross-sections of the *A. rubra*-*P. involutus* mycorrhizas had high concentrations of phenolics in root cortical cells that were not present in the comparable mycorrhizas of *Alpova diplophloeus*. This was interpreted as the result of *Alnus* recognizing *P. involutus* as the “wrong” symbiont and attempting to decrease subsequent colonization. Similar results were reported by Malajczuk et al. (1982) involving interactions between multiple *Eucalyptus* host species and *Pinus*-specific ECM fungi. However, if this mechanism of sanctioning was the primary way that *Alnus* plants avoid significant colonization by the “wrong” fungi, a similar pattern should have also been observed in the mycorrhizas of *Astraeus pteridis*. Instead, phenolic concentrations in *A. rubra*-*A. pteridis* mycorrhizas were low, suggesting that this “wrong” symbiont (1) was able to meet host needs and prevent sanctioning, (2) was subject to sanctioning at some other time or under some environmental condition not captured in that experiment, or (3) had some way of remaining undetected despite being the “wrong” symbiont.

These two mechanisms, partner choice and host sanctioning, could also work in concert to create the unique specificity observed in the *Alnus* ECM system. The collective results of the two aforementioned studies suggest that partner choice likely plays a significant role in preferentially inducing the germination of ECM fungi recognized by *Alnus* as beneficial, while host sanctioning might be an

important mechanism available to minimize or eliminate any “incorrect” *Alnus* ECM interactions. Considering the relative strengths of the two mechanisms, colonization of *Alnus* plants by the “wrong” ECM fungi seems either non-existent or to occur only very rarely in field settings (Kennedy et al. 2011a; Bogar and Kennedy 2013; Polme, personal communication). If host sanctioning were the dominant mechanism driving specificity, one would expect to find more ECM fungi forming mycorrhizal associations with *Alnus* trees, at least initially. Since this is not normally the case, it seems that pre-interaction partner recognition is most likely the dominant mechanism affecting *Alnus* ECM specificity, with interaction-based host sanctioning playing a limited secondary role.

8.5.2 *Alnus*-ECM Fungus Specificity: Interspecific Competition Hypothesis

The specificity of the *Alnus*-ECM fungus system may also be mediated by competition between *Alnus* and co-occurring ECM host plants either directly or via ECM fungi. Both hosts and fungi could escape a certain amount of competitive pressure by restricting the set of symbionts with which they associate. This applies particularly to situations in which hosts or fungi are adapted to colonize soon after disturbance events. To fully appreciate why a set of symbionts might not participate in local CMNs, it is important to consider selection acting on both the hosts and the fungi individually since it occurs at distinctly different spatial and temporal scales.

As noted previously, CMN connectivity could be helpful to ECM host species that establish under the canopy of other trees. By maintaining broad receptivity to many different ECM fungi, a later successional ECM host has a greater chance of joining an established mycelial network early in development. This scenario was discussed by Kropp and Trappe (1982) with respect to *Tsuga heterophylla*, a late-successional, broadly receptive ECM host in northwest North America. In contrast, by denying later successional seedlings access to CMNs by associating with host-specific ECM fungal communities, early successional hosts would suppress the establishment of competing hosts and maintain their own dominance in a stand. In the case of *Alnus*, whose dominance is limited by its short-lived nature, not participating in CMNs would also prevent any ‘facultative epiparasitism’ (sensu Bruns et al. 2002) of fixed nitrogen by co-occurring ECM host plants. Intraspecific competition, of course, would be unaffected by this specificity, and would remain an important ecological force in these situations (also noted by Bruns et al. 2002). Kropp and Trappe (1982) and Molina et al. (1992) both noted that pioneer tree species often do associate with communities of host-specific ECM fungi (e.g. *Pseudotsuga*, *Alnus*), supporting the hypothesis that these early successional settings encourage specialization.

Selection on the fungi must also influence whether or not a set of symbionts will participate in local CMNs. As discussed above, in most situations, ECM fungi

would benefit by connecting to multiple host species. This would expand the effective resource pool available to a given fungal genet, and provide the fungus with insurance in the event that resources were no longer provided by a primary host. Competitive dynamics, however, have led some ECM fungi to specialize on particular hosts. Bruns et al. (2002) discuss the case of the genus *Rhizopogon*, species of which dominate both the “spore bank” and the below-ground communities of their ECM hosts (*Pinus* and *Pseudotsuga*) early in forest succession at Point Reyes, CA, USA. Over time, this group of fungi becomes less common on their hosts, suggesting that they are weaker competitors relative to the other fungi with which the hosts associate (Bruns et al. 2002). It seems possible, then, that these fungi have specialized on early successional hosts as a consequence of competition: a combination of long-lived propagules (Bruns et al. 2009) and well-timed, host-specific germination (Massicotte et al. 1994) could allow these fungi to guarantee themselves a host with relatively little competition from other fungi, at least early in succession (see further discussion of this dynamic in Kennedy 2010; Kennedy et al. 2011b). While the competitive dynamics of *Alnus*- and non-*Alnus*-associated ECM fungi have not been examined, a similar spore longevity pattern to *Rhizopogon* has been noted for the *Alnus*-specific species *Alpova diplophloeus* (Miller et al. 1994).

On the whole, both ECM hosts and fungi may experience competitive pressure to specialize—and thus, evade CMN participation—under a number of circumstances, but particularly early-successional situations and settings in which a symbiont has enhanced access to a particular set of resources.

8.5.3 *Alnus*-ECM Fungus Specificity: Soil Chemistry Hypothesis

One of the ways that fidelity (see Molina and Horton, Chap. 1, this volume) could be reinforced is by some form of environmental filtering. A widely noted environmental parameter with respect to *Alnus* forests is their soil chemistry (Hibbs et al. 1994; Becerra et al. 2005; Tedersoo et al. 2009; Yarwood et al. 2010). *Alnus* soils are typically characterized by low pH, which is a byproduct of the hydrogen production associated with nitrification (Bormann et al. 1994). Both high acidity and high nitrate levels may represent a formidable combination of environmental filters, as both have been shown to limit the growth of a variety of ECM fungi (Hung and Trappe 1983; Lilleskov et al. 2002; Avis et al. 2003; Trudell and Edmonds 2004; Cox et al. 2010). To experimentally test their effects in the *Alnus* ECM system, Huggins et al. (2014) manipulated the pH and nitrate concentrations present in the liquid media of a suite of *Alnus*- and non-*Alnus* ECM fungal species. They found that the growth of *Alnus* ECM fungi were not, on average, affected by high acidity, while non-*Alnus* ECM fungi had a significantly negative growth response under the same conditions. Similarly, when grown at high nitrate, non-

Alnus ECM fungi also generally performed more poorly. Taken together, the results of Huggins et al. (2014) are consistent with soil pH and nitrate concentrations being important environmental filters that may underlie the specificity in the *Alnus* ECM system. At the same time, multiple lines of other evidence do not clearly support this mechanism. Sites initially dominated by *Alnus* trees are readily replaced by other ECM host species over time and if high soil acidity and nitrate levels are strongly inhibitory to non-*Alnus* ECM fungi, one would expect that ECM fungal colonization of other hosts to be low in *Alnus*-influenced soils. Miller et al. (1992), however, observed that *Pseudotsuga menziesii* seedlings grown in soils from both young and older *Alnus* forests were similarly well colonized with a diverse range of ECM fungi as *P. menziesii* seedlings grown in young and older *P. menziesii* forest soils. Data from the recent field study of *Alnus* and *Betula* ECM fungal communities also indicates that non-*Alnus* ECM fungi can survive on their preferred hosts even when occupying the same soil as *Alnus* roots (Bogar and Kennedy 2013). If the specificity of *Alnus* ECM fungal communities is strongly driven by soil chemistry alone, the ECM fungal community on *Betula* roots should have been substantially changed when overlapping with *Alnus* roots relative to the community on *Betula* roots in the absence of *Alnus*. It was not, however, suggesting that either *Betula* ECM fungal associates are tolerant of similar soil conditions as *Alnus* ECM fungal associates or conditions were not changed enough in mixed settings to shift community composition significantly. It also appears that at least some *Alnus*-associated ECM fungi are negatively affected by high nitrate concentrations. For example, Koo et al. (1995) found that colonization of *Alpova diplophloeus* on *Alnus rubra* seedlings was significantly decreased in highly mineral nitrogen-amended soils and Huggins et al. (2014) also showed that some *Alnus* ECM fungi performed poorly at high nitrate levels. Taken together, these studies suggest that *Frankia*-induced changes in pH and soil nitrogen concentrations can affect ECM fungal colonization and community structure, but do not appear to be solely responsible for the atypical composition of *Alnus* ECM fungal communities.

Along with pH and nitrogen, *Alnus* trees are known to influence other aspects of soil chemistry as well. Specifically, soils in *Alnus* forests can be low in inorganic phosphorus (Giardina et al. 1995; Compton and Cole 1998, 2001), and enriched in organic phosphorus (Zou et al. 1995). Tedersoo et al. (2009) hypothesized that ECM fungal communities associated with *Alnus* species may be strongly associated with soil phosphorus concentrations due to the phosphorus demands of co-occurring *Frankia* (see below). Their community analyses, however, indicated that soil phosphorus levels had no statistically significant effects on ECM fungal community structure. Koo et al. (1996) also found that mineral phosphorus fertilization did not decrease mycorrhizal colonization in a greenhouse study. Collectively, these studies suggest that soil phosphorus concentration does not strongly influence *Alnus* ECM fungal community composition or colonization, however, our more recent work suggests that *Alnus* ECM fungal communities may have a unique physiological response to soil phosphorus availability (see below).

8.5.4 *Alnus-ECM Fungus Specificity: Host Metabolic Hypothesis*

A different way that the presence of *Frankia* bacteria may affect *Alnus* ECM fungal communities is by shifting host nutritional needs in a way that favors fungi adept at acquiring nutrients aside from nitrogen. In particular, nitrogen-fixing plants are often limited by phosphorus (Benson and Clawson 2002), so *Alnus* individuals may selectively associated with ECM fungi that have enhanced enzymatic abilities towards phosphorus acquisition. Indirect support for this hypothesis was shown by Ekblad et al. (1995), who found that in microcosms containing *Alnus incana* and *Pinus sylvestris* seedlings colonized by *Paxillus involutus*, fungal biomass peaked in low phosphorus soils. Another study found that *Alnus* seedlings colonized by both *Frankia* and ECM fungi could have higher phosphorus tissue concentrations when grown in certain types of soils than seedlings colonized by just *Frankia* alone (Yamanaka et al. 2003). However, because the presence of ECM fungal colonization tends to raise seedling phosphorus levels on other hosts (Smith and Read 2008), it is unclear whether the response seen in those *Alnus*-based studies is due simply to ECM fungal colonization or colonization by ECM fungi specialized on greater phosphorus acquisition.

To test whether *Alnus* ECM fungi have different enzymatic capabilities relative to ECM fungi associated with other host trees, direct assays of enzyme production from ECM root tips are necessary. The logistics and throughput capacities of ECM root tip enzyme assays have improved significantly in recent years (Courty et al. 2005; Pritsch et al. 2011) and a growing body of literature is developing around these techniques (Courty et al. 2010; Pritsch and Garbaye 2011; Jones et al. 2012). With regard to the *Alnus*-ECM fungus system, we recently compared the enzyme activity of ECM fungal root tips sampled from pure stands of *Alnus rubra* and *Pseudotsuga menziesii* at the Cascade Head and H.J. Andrews Experimental Forests in Oregon, USA (Walker et al. 2014). Excised ECM fungal root tips were tested for acid phosphatase (phosphorus) and leucine aminopeptidase (nitrogen) activity and DNA was extracted for molecular identification based on the rRNA ITS gene region.

From those samples, we were able to molecularly identify 62 and 75 % of the ECM fungal root tips sampled from *A. rubra* and *P. menziesii* plots, respectively. The ITS sequences of 18 different ECM fungal species were recovered from *A. rubra* root tips, while 76 ECM fungal species were detected on *P. menziesii* root tips, and an additional four species were shared. These levels of species richness correspond well with previous studies of both *A. rubra* and *P. menziesii* ECM fungal communities (Kennedy et al. 2003; Cline et al. 2005; Horton et al. 2005; Kennedy and Hill 2010). In support of the aforementioned hypothesis, the *A. rubra*-associated ECM fungal community had significantly higher acid phosphatase activity than the ECM fungi associated with *P. menziesii*, while the leucine aminopeptidase of *A. rubra*-ECM fungal root tips was significantly lower at the nitrogen-rich site (Fig. 8.3). Collectively, these results indicate that *A. rubra*-associated ECM fungi appear to

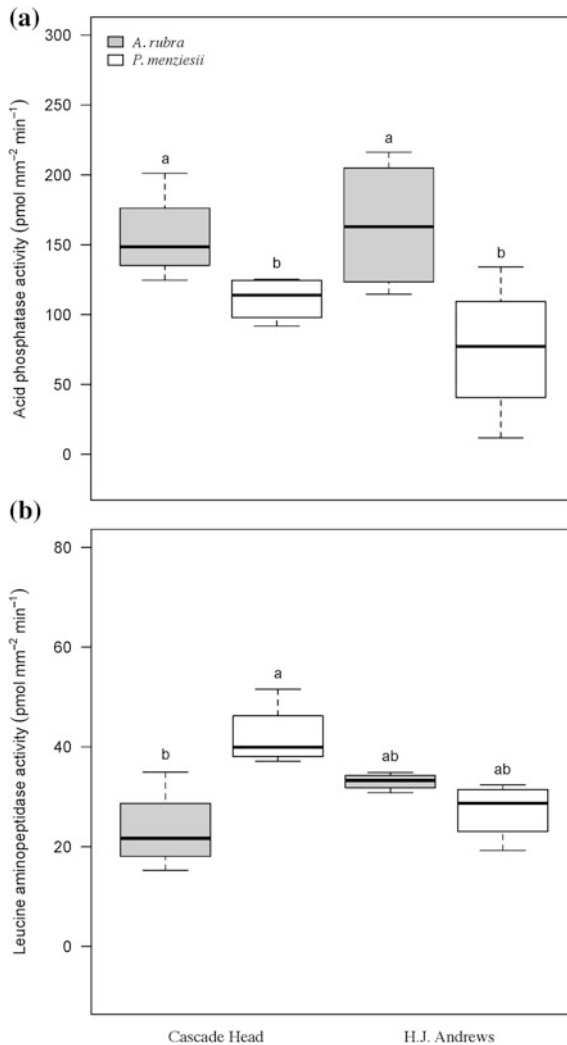


Fig. 8.3 Differences in potential (a) acid phosphatase and (b) leucine aminopeptidase activity between the ECM fungal community on *Alnus rubra* (grey) as compared to *Pseudotsuga menziesii* (white) at Cascade Head and H.J. Andrews, Oregon, USA. Raw data is presented in the figure, but all data were cube root transformed in order to meet assumptions of normality for statistical analyses. Lower case letters designate significant differences at $P \leq 0.05$ detected by univariate ANOVAs and subsequent Tukey's HSD tests. Boxes surrounding median values represent the first and third quartiles, while whiskers show the smaller (and larger) of either the maximum (and minimum) values or $1.5 \times$ the interquartile range (approximately ± 2 SD); $N = 2$

have enhanced phosphorus acquisition abilities, and that host nitrogen status may mediate ECM fungal physiological response as demonstrated by the elevated levels of organic nitrogen acquisition by the *P. menziesii* ECM fungal community in the absence of *Frankia*-derived nitrogen.

8.5.5 *Alnus*-ECM Fungus Specificity: A Host-Fungus Reward System Based on Nitrogen?

We formalize an additional hypothesis regarding *Alnus* ECM specificity: that *Alnus* plants may also provide a reward to ECM fungi to help maintain specificity in this system. A reward system may be particularly important for *Alnus* individuals because they may provide less carbon to ECM fungi than other hosts, due to their simultaneous interaction with *Frankia* bacteria. While this speculation about carbon allocation has yet to be tested, the photosynthetic rates of *Alnus* species are similar to non-*Frankia*-associated broad-leaved species (Agren and Ingestad 1987; Koike 1990). As such, *Alnus* individuals do not appear to have a larger carbon pool from which to allocate to their dual symbionts. Since carbon allocation to *Frankia* and ECM fungi has been estimated at $\sim 15\%$ per symbiosis (Tjepakema et al. 1986; Smith and Read 2008), it seems likely that, relative to other hosts, *Alnus* plants may provide less carbon to ECM fungi. In light of this carbon dynamic, what might *Alnus* plants offer to prevent defection to more carbon generous hosts?

We suggest that *Alnus* may provide its chosen ECM fungi with direct access to the nitrogen fixed by the *Frankia* bacteria. While this would represent a reversal of the way nitrogen is typically traded between plants and ECM fungi (nitrogen is usually provided to the plant by the fungus), the unique ecology of this tri-partite symbiosis may favor this change in partner trading dynamics. From the fungal perspective, getting nitrogen from the host would decrease the need to scavenge nitrogen from the soil. Although *Alnus* individuals may provide less carbon to the fungi, the fungi may not need to invest as much carbon in nitrogen-scavenging enzymes as they would when colonizing a non-nitrogen-fixing host. Furthermore, since organic matter may be limited in early successional settings, it would be easier for the fungi to get nitrogen from the host instead of relying on organic sources in soil. From the host perspective, it may be advantageous to provide *Frankia*-derived nitrogen to ECM fungi unlikely to participate in networks so that no nitrogen is lost through CMNs to other host species. While providing nitrogen to their fungi would represent a cost to *Alnus* plants (because of their carbon investment in the *Frankia* bacteria) all else being equal, multiple studies have shown that *Alnus* individuals colonized by both *Frankia* bacteria and ECM fungi can be larger than plants with only a single symbiont (Chatarpaul et al. 1989; Koo et al. 1995). This suggests the putative benefits of host-ECM nitrogen provisioning outweigh its costs. It has also been noted that plants can have higher phosphorus concentrations in their tissues when colonized by *Frankia* bacteria and ECM fungi (Yamanaka et al. 2003). Since

phosphorus has been demonstrated to be a limiting resource for nitrogen fixation (Jha et al. 1993; Uliassi and Ruess 2002), the presence of ECM fungi would be beneficial to the *Frankia* bacteria as well, if the plant is able to allocate greater phosphorus to bacterial nodules.

In support of this nitrogen reward hypothesis, Arnebrant et al. (1993) found that many of the amino acids in the ECM fungus used in their study, *P. involutus*, contained nitrogen originally fixed by *Frankia*. Given the short-time scale of their experiment (ten weeks, seven day labeling period) and the fact that live *Frankia* nodules are not known to excrete nitrogen into their external environment, it seems very likely that the nitrogen was passed from *Frankia* to the plant and then onto the ECM fungus. Ekblad and HussDanell (1995) obtained comparable results, although amino acids were not directly assayed in that study. The results of two additional studies are also consistent with a potential transfer of nitrogen from host to fungus in *Alnus* ECM interactions. Koo et al. (1995) found that *Alpova diplophloeus* colonized only 10 % of the root systems of *Alnus rubra* seedlings when they were non-nodulated, but 65 % when *Frankia* nodules were present. Similarly, Yamanaka et al. (2003) observed no colonization by *Alpova diplophloeus* on non-nodulated *Alnus tenuifolia* seedlings, but between 75 and 100 % colonization when *Frankia* nodules were present. Although neither of these studies directly indicates that nitrogen is the resource responsible for higher ECM fungal colonization, the results are consistent with a significant benefit provided to the fungus by co-colonization with *Frankia* bacteria. It should also be noted that in experimental settings, *Frankia* are the first of the two microbial symbionts to colonize *Alnus* seedlings (Miller et al. 1992; Koo et al. 1995), and that all *Alnus* individuals in field conditions are colonized by *Frankia* bacteria (Benson and Dawson 2007). These latter findings indicate that the ability of *Alnus* individuals to readily access nitrogen for a reward system for ECM fungi appears to be the default state in nature.

If nitrogen is provided from the plant to the fungus, monitoring its consumption could also be a way in which *Alnus* individuals control which fungi are sanctioned. Presumably, the nitrogen demands of ECM fungi engaged in CMNs with other hosts would be higher than for non-networked species [due to demand from the other hosts and the source-sink dynamics of CMN resource transfer (Simard et al. 1997)]. By limiting colonization of CMN-forming fungi, *Alnus* individuals may be able to prevent any facultative epiparasitism via CMNs. It should be noted, however, that this method of sanctioning would not prevent host generalist ECM fungi from colonizing *Alnus* plants. In fact, if a host generalist ECM fungus were only associating with an *Alnus* individual, its nitrogen demands should be similar to that of typically *Alnus*-associated species and therefore it would likely avoid sanctions. Data from our study of *Alnus rhombifolia* and *Betula occidentalis* ECM fungal communities show some support for this scenario (Bogar and Kennedy 2013). We found that there were six ECM species associated with both *Alnus rhombifolia* and *Betula occidentalis*. Five of the six ECM species were, however, never found on the roots of both hosts within the same soil core. This suggests while some fungal species could associate with both hosts, different individuals of those fungal species were present on each host. We did, however, find one species, *Laccaria laccata*, that

was present on both *Alnus rhombifolia* and *Betula occidentalis* ECM root tips in the same soil core. If those tips were colonized by the same fungal individual and our logic about sanctioning based on nitrogen allocation is accurate, we would expect that *Alnus* ECM roots in that core would begin to reject colonization by *L. laccata* over time due to excess nitrogen consumption. A key untested assumption of this logic is the spatial scale over which sanctioning is occurring. If it occurs at the individual tip scale, it seems unlikely that *L. laccata* would be able to establish since excess nitrogen consumption should begin immediately if source-sink dynamics drive CMN resource transfer (Simard et al. 1997). In contrast, if it occurs at the multi-tip scale, it seems possible that *L. laccata* could establish on *Alnus*, but once sensed as a significant nitrogen drain, would be rejected as a preferred symbiont.

8.6 Future Research Directions

As shown in this chapter, the reciprocal specificity of the *Alnus*-ECM fungus system is well established. The mechanisms responsible for creating and maintaining this specificity and how it may reinforce non-participation in CMNs, however, still require further study. Based on the hypotheses discussed above, we believe research in the following areas will be particularly important: (1) determining the full carbon budget for *Alnus* plants colonized with both *Frankia* and ECM fungi (to test the primary assumption of the rewards system hypothesis), (2) further examining the enzymatic capacities of *Alnus*-associated and non-*Alnus*-associated ECM fungal root tips (to reinforce our recent findings supporting the host metabolic hypothesis), (3) defining the signal used by *Alnus* plants to induce specific spore germination (to validate the role of partner recognition), (4) exploring the growth of more *Alnus*-associated and non-*Alnus*-associated fungi under a range of acidity and nitrogen concentrations in the same experimental setting (to better test the role of environmental filtering), and (5) assessing the competitive dynamics between *Alnus*- and non-*Alnus*-associated fungi (to assess the influence of inter-specific competition). In addition, if *Frankia* bacteria play a central role in the specificity patterns observed in the *Alnus* ECM system, a similar pattern should be seen in other systems where all three symbionts are present. Members of the plant genera *Allocasuarina*, *Casuarina*, *Cercopcarpus* and *Dryas* are known ECM fungal hosts that also associate with *Frankia* bacteria. Therefore, examining patterns of ECM fungal richness and host specificity in these host systems would be helpful in generalizing about the putatively distinctive nature of the *Alnus*-*Frankia*-ECM fungus tri-partite symbiosis.

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