Chapter 9
Experimentally Testing Effects of Mycorrhizal Networks on Plant-Plant Interactions and Distinguishing Among Mechanisms

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Abstract Plants of the same and different species are often linked by common mycorrhizal networks (CMNs), and there is substantial disagreement in the literature about whether these linkages have important effects on plant-plant interactions, beyond simply providing mycorrhizal inoculum. Here, I attempt to reconcile opposing viewpoints by reviewing available evidence for three distinct mechanisms by which CMNs can affect plant-plant interactions. I also analyze the details of manipulative field experiments that have been conducted to test CMN effects on plant-plant interactions, and make recommendations for the kinds of future studies that will be most useful in moving forward. I argue that few experiments have unequivocally tested whether CMNs have unique effects on plant-plant interactions, and that these experiments have largely been ignored in favor of debates about the magnitude of resource flows (especially carbon) from plant to plant through CMNs. I suggest that progress on the debate will only be made through more thorough testing of alternative mechanisms besides plant-to-plant carbon flow, especially coupled with experimental manipulations of CMNs to test for consequences on specific aspects of plant community ecological processes.

Keywords Experimental design · Mycorrhizal fungi · Mycorrhizal network · Plant ecology · Plant competition · Plant community

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9.1 Introduction

Many mycorrhizal ecologists agree that common mycorrhizal networks (CMNs), i.e., physical linkages among plant individuals via the mycelia of mycorrhizal fungi, are likely common in nature (Newman 1988; Molina et al. 1992; Francis and Read 1994; Leake et al. 2004; Simard and Durall 2004; Simard et al. 2012; Molina and Horton Chap. 1, this volume; Giovannetti et al. Chap. 2, this volume). The observation that species of arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi are often compatible with multiple host plant species, coupled with the ability of genetically compatible hyphae to anastomose (see Giovannetti et al. Chap. 2, this volume), suggest that CMNs are probably ubiquitous, although confirmation of this conjecture will require direct evidence for these linkages in the field, including careful population genetic studies (Leake et al. 2004; Simard and Durall 2004; Selosse et al. 2006). Hundreds of achlorophyllous plants likely depend on CMN connections with green plants for 100 % of their carbon supply (Leake et al. 2004; Bidartondo 2005), and such 'mycoheterotrophy' also exists to various degrees in some green orchid species, especially at the seedling stage (e.g., Julou et al. 2005; Zimmer et al. 2008). What is less clear is the degree to which these CMN linkages have unique consequences for the ecology of typical green plants, and especially whether mechanisms and consequences (such as community composition) of plant-plant interactions are commonly altered by these linkages. Are physical mycelial linkages between plants per se, beyond just the provisioning of mycorrhizal fungi, generally important for plant ecology?

A variety of approaches, ranging from isotope tracer studies to field manipulations of CMNs, have been used to directly or indirectly address this question, and results of these efforts have generated significant debate. In reviewing some aspects of the evidence, some authors have concluded that CMNs can have "profound effects on plant communities" (Selosse et al. 2006), while others have argued that there is nothing unique about these physical linkages that separates them from other kinds of mutualisms in which plants engage and thus the term "common mycorrhizal networks" is misleading (Bever et al. 2010). Here, I attempt to reconcile these opposing viewpoints and make recommendations for the kinds of future studies that will be most useful in moving forward. I argue that few experiments have unequivocally tested whether CMNs have unique effects on plant-plant interactions, and that these experiments have largely been ignored in favor of debates about the magnitude of resource flows (especially carbon) from plant to plant through CMNs. I suggest that progress on the debate will only be made through more thorough testing of alternative mechanisms besides plant-to-plant carbon flow, especially coupled with experimental manipulations of CMNs to test for consequences on specific aspects of plant community ecological processes. This review is not intended as a comprehensive survey of studies on CMNs; rather, I attempt a conceptual analysis that may be useful as a roadmap for design and interpretation of future studies.

9.1.1 Mechanisms Are Central to the Debate

At least three distinct mechanisms have been hypothesized by which CMNs may affect plant-plant interactions:

Mechanism 1: Flow of resources from one plant to another through the CMN. Under this mechanism, one plant may benefit if it receives a net flow through the CMN of limiting resources including carbon, nitrogen, phosphorus, or water.
Mechanism 2: Unequal contributions of carbon to the CMN by different plants. Under this mechanism, some plants (e.g., recruiting seedlings) may receive the benefits of association with a CMN while contributing a less than proportional share of carbon to the build the CMN.
Mechanism 3: Unequal distribution of a resource by the CMN to different plants. Under this mechanism, a common resource (regardless of where it was obtained by the fungus) may be distributed unequally to different plants.

Newman (1988), in a seminal review that stimulated a great deal of work on the function and significance of CMNs, highlighted five potential "profound implications" of CMNs for the functioning of ecosystems. The first of these implications was that seedlings might be able to join a pre-existing hyphal network and benefit from it at an early stage, which falls under Mechanism 2 above. His second and fourth implications were that organic and mineral nutrients, respectively, could flow from plant to plant and alter the performance of the receiving plant or the balance of plant-plant interactions. These phenomena fall under Mechanism 1 above. Newman's third implication was that plant competition could be altered if competing plants are receiving nutrients from a commonly shared fungal network, rather than taking up nutrients independently, which falls under Mechanism 3 above. Newman's fifth implication was that nutrients could flow from dying plants through CMNs directly to living plants, which has interesting implications for ecosystem cycling of nutrients but does not relate directly to our discussion here on the implications for plant ecology.

Much debate over the importance of CMNs for plant ecology has centered around one specific version of Mechanism 1: net flow of carbon from one plant to another through a CMN (see Simard et al. Chap. 5, this volume). After reviewing early studies that showed radio-labeled carbon could potentially flow both directions from plant to plant through a CMN, Newman (1988) argued that key next steps would be to test whether flow was more significant in one direction than another (i.e., net flow) and to quantify how large is the carbon gain by the receiver plant compared to its gain from photosynthesis. When net flow of carbon through a CMN was first demonstrated in an ectomycorrhizal system, the resulting paper appeared in a high profile international journal (Simard et al. 1997a) and was greeted with significant enthusiasm and discussion. Subsequently, however, there has been significant debate about whether the amounts of carbon flowing through a CMN are likely to be ecologically meaningful (i.e., to have consequences for individual plant growth, populations, or communities), whether the data distinguish

between carbon flow through the CMN versus other pathways (such as respiration from CMN fungi and subsequent fixation by plants linked to the CMN), and why some other studies have failed to find net transfer of carbon (Fitter et al. 1999; Robinson and Fitter 1999; Pfeffer et al. 2004; Bever and Schultz 2005; Whitfield 2007; Bever et al. 2010 but see, e.g., Song et al. 2015). Indeed, it has been argued that since the evidence for net carbon flow from plant to plant is so mixed, CMNs may not have unique consequences for general plant ecology that require recognition of the physical linkages among green plants (Bever et al. 2010).

9.1.2 *Density Effects and Plant-Soil Feedbacks: General Phenomena that Make CMNs Irrelevant?*

Specifically, Bever et al. (2010) argued that the dynamics of plant interactions with mycorrhizal fungi (and other above- and belowground mutualists such as animal pollinators and n-fixing bacteria) can be effectively explained by a traditional population dynamics framework, regardless of whether CMNs form physical linkages among plants (see also Bever and Schultz 2005). Under this view, Mechanisms 2 and 3 may influence plant ecology, but the physical connection among plants through a CMN is not important conceptually. For example, one of two plant species may have a disproportionately positive effect on the density of a particular shared mycorrhizal fungal species in the soil (Mechanism 2), which then has a disproportionately positive effect on the second plant species (Mechanism 3). Although the physical linkage provides the means by which one plant may disproportionately contribute to the density of shared mycorrhizal fungi, or by which a shared fungus may contribute disproportionately to one particular host plant (e.g., Fellbaum et al. 2014), such disproportionate effects on shared interactors is not unique to plant-plant interactions mediated by CMNs. The sharing of mycorrhizal fungi between two plants is conceptually no different than, say, the sharing of the same pollinator by two plants. Species of plants and mycorrhizal fungi simply interact and affect each other's densities, and the strength of indirect interactions among plants and consequent community composition of both plants and fungi depend on the relative strengths of specific direct interactions between particular plant and fungal species (Fig. 9.1). This view has been synthesized more generally, taking into account other soil organisms besides mycorrhizal fungi, as the plant-soil feedbacks approach (Bever 2003).

There are significant advantages to this perspective. Perhaps most importantly, it allows effects of mycorrhizal fungi on plant ecology to be modeled, both conceptually and mathematically, in a very general existing theoretical framework that also applies to other kinds of interactions. It allows us to avoid the messy mixture of concepts (density/population effects and physical engineering/physiological effects) involved when we must consider that mycorrhizal fungi may act as physical conduits for resources among plants. Moreover, plant-soil feedback phenomena can be measured experimentally with straightforward protocols.

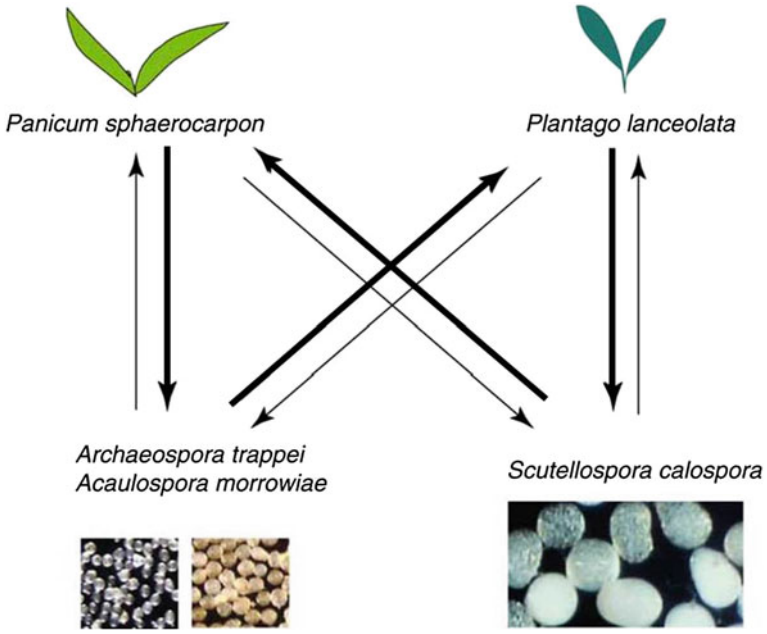

Fig. 9.1 Net pairwise negative feedback between *Panicum sphaerocarpon* and *Plantago lanceolata* generated by changes in composition of AM fungi (Bever 2002). The thickness of arrows represents the relative strengths of benefit between individual species of plants and AM fungi. *Scutellospora calospora* has high fitness with *Plantago*, but *Plantago* does not grow well with *Sc. calospora*. Rather, *Plantago* has highest growth rates in association with AM fungi, *Archaeospora trappei* and *Acaulospora morrowiae*, which themselves have high fitness in association with *Panicum*. The asymmetric fitness relationships generate negative feedback which can contribute to coexistence of these competing plant species. Modified from Fig. 9.2d in Bever et al. (2010), and used with permission from Elsevier

I suggest, however, that available evidence does not support abandoning the idea that CMNs and the physical connections among green plants have unique general effects on plant ecology. Although the plant-soil feedbacks approach can usually account for Mechanisms 2 and 3, it does not capture Mechanism 1. Even if we accept the argument that net carbon flows between green plants through CMNs are usually not significant enough to be ecologically important, and we set aside the examples of achlorophyllous plants and green orchids, compared to carbon flow, relatively little attention has been paid to plant-to-plant flow of other plant resources, such as nitrogen (N), phosphorus (P), and water (Simard et al. 2012; see Simard et al. Chap. 5, this volume); or of non-resource molecules such as defense or stress signaling compounds (e.g., Song et al. 2014, 2015) and allelopathic chemicals (Achatz and Rillig 2014; see Jakobsen and Hammer Chap. 4, this volume). If these flows are important, then CMNs may significantly affect plant ecology via Mechanism 1, and those effects cannot be captured in the population dynamics of mycorrhizal mutualists. Moreover, experiments manipulating CMNs and measuring

plant ecological outcomes (such as the strength of plant-plant interactions) have found evidence that CMNs have unique effects on these outcomes in some contexts, and Mechanism 1 has found support or has not been ruled out in those cases. While such experiments are still relatively few, their importance, design, and strengths and weaknesses have been given short shrift relative to studies of resource flow in the debate about CMNs in plant ecology.

In the remainder of this chapter, I have two objectives. First, I will summarize the potential importance for plant ecology of nitrogen, phosphorus, and water flows from plant to plant through CMNs. Second, I will analyze the details and outcomes of experiments designed to test effects of CMNs on plant-plant interactions, focusing especially on manipulative field experiments, and including discussion of how such experiments can help to clarify the role of CMNs in plant ecology.

9.1.3 *Nitrogen, Phosphorus, and Water Flow Through CMNs*

Nitrogen. N flow from plant to plant through both AM and EM CMNs can sometimes be substantial (reviewed by He et al. 2009; see Simard et al. Chap. 5, this volume). For example, a pair of laboratory microcosm experiments (He et al. 2004, 2005) examined the potential for net N transfer through CMNs between two ectomycorrhizal plants, *Eucalyptus maculata* and *Casuarina cunninghamiana*. The authors grew these plants in compartments separated by 37 μm mesh, which allowed hyphal growth between plants in adjacent compartments, and a 5 mm air gap, which reduced diffusion of water and N between the compartments. Prior studies had used this approach to study one-way N transfer through CMNs (e.g., Bethlenfalvay et al. 1991), but He et al. moved the field forward by applying these methods to test for bi-directional and net flow between two plant species. They also included high water-holding capacity crystals in the soil medium to reduce water diffusion, and included non-mycorrhizal control treatments. As *Casuarina* engages in a nitrogen-fixing symbiosis with *Frankia* bacteria living in root nodules, non-nodulated treatments were also included to test for the effects of nodulation on N transfer. These experiments demonstrated the potential for substantial amounts of N transfer from plant to plant through CMNs, with up to 39 % of ^{15}N-labeled ammonium being transferred from mycorrhizal *Eucalyptus* to *Casuarina* and only 10 % in the reverse direction (He et al. 2005). In the same subset of plants, approximately 30 % of *Casuarina*'s N was derived from transfer through the CMN.

In a field study of an AM system, Moyer-Henry et al. (2006) used ^{15}N natural abundance studies to show that two different AM weed species received up to 80 % of their N via transfers from leguminous crops. Two different non-mycorrhizal weeds in the same experiment received negligible N transfer from the crops, suggesting that the main pathway of N transfer from the crops to the weeds was via AM hyphal networks. Not all studies have found such substantial amounts of N

transferred through CMNs (e.g., Shen and Chu 2004), but these examples show plant-to-plant N transfer in amounts that certainly have the potential to be ecologically significant in N-limited environments, in support of Mechanism 1 above.

Phosphorus. Ecologically meaningful quantities of P have now also been demonstrated to move from plant to plant, with support for the hypothesis that CMNs provide a primary pathway for such flow (see Simard et al. Chap. 5, this volume). For example, Wilson et al. (2006) used a creative combination of CMN-manipulation treatments (No roots/+CMN vs. No roots/No CMN) and mycorrhizal suppression treatments (Benomyl fungicide or no fungicide), along with ^{32}P-labeling, to estimate P flows through multiple pathways between two AM plants, Indian Grass (*Sorghastrum nutans*) and the perennial forb Louisiana Sagewort (*Artemisia ludoviciana*). They found that Indian Grass received more than 50 % of its P via interplant transfers through AM fungal CMNs, whereas Louisiana Sagewort received less than 20 % of its P via CMN transfer, and that transfer through a soil pathway and diffusion through the mesh barrier were negligible. This experimental design and associated results build on earlier studies that used ^{32}P-labeling to show significant P flow from plant to plant, mediated by CMNs (e.g. , Martins and Read 1996; Tuffen et al. 2002). As with N transfer, some studies have shown P transfer among plants to be negligible (e.g., Ikram et al. 1994), but the results highlighted here show that in some systems, P transfer among plants mediated by CMNs can be substantial.

Water. Plants with deep root systems can bring deep water to the soil surface at night and distribute it among fine roots throughout shallow soil layers, through the physiological process of hydraulic lift (Caldwell et al. 1998; Simard et al. Chap. 5, this volume). This process makes water more available during the daytime to shallow roots of the plant conducting hydraulic lift and also to roots belonging to neighboring plants, potentially allowing facilitation among plant individuals of the same or different species (e.g., Dawson 1993). Studies have now shown that plants can transfer hydraulically lifted water directly to the mycelia of their mycorrhizal fungi (Querejeta et al. 2003), and that mycelia of mycorrhizal fungi can then redistribute hydraulically lifted water throughout their masses to multiple plants connected to the same CMN (Egerton-Warburton et al. 2007; Warren et al. 2008; Allen 2009). Lilleskov et al. (2009) used oxygen stable isotope analysis to show that EM sporocarps receive and transpire substantial amounts of hydraulically lifted water, either from host plant roots or via direct mycelial transport from deep water sources. Egerton-Warburton et al. (2007) used fluorescent dye and isotopic tracers, coupled with manipulations of CMNs independent of plant roots in microcosms, to show that CMNs of both AM and EM fungi could distribute hydraulically lifted water from *Quercus agrifolia* seedlings to conspecifics and to different plant species including *Salvia mellifera*, an AM plant. Warren et al. (2008) used dye tracers in a field study to show that CMNs of EM fungi can provide a conduit for hydraulic redistribution of water from adult *Pinus ponderosa* trees to nearby seedlings. Although the narrow diameter of fungal hyphae may restrict mass water flow through CMNs in some cases, the rhizomorphs of some EM fungi may be particularly well suited for this function, especially those that are hydrophobic and possess

large, vessel-like internal hyphae, such as *Suillus* and *Rhizopogon* (Lilleskov et al. 2009; Agerer 2001). Thus, it is possible that mass flow of water may be more substantial through some EM fungal CMNs than through AM fungal CMNs.

These studies on N, P, and water transfer typically cannot rule out the possibility of resource leakage from roots or mycorrhizal hyphae and immediate reabsorption by the CMN, but this caveat does not alter the implications of the results, as these studies do show clearly that CMNs are significantly involved in resource transfer among plants (Wilson et al. 2006). Significant redistribution of water within a CMN, for example, regardless of whether or not it leaks out and is reabsorbed, homogenizes water availability among the shallow root systems of large and small individual plants. When water is a limiting resource, this process effectively creates facilitation of small, shallow-rooted plants by large, deep-rooted plants, mediated by CMNs. Similarly, experiments on N and P flow show clearly that CMNs are mediating significant resource transfer among plants, and additional studies demonstrate an important role for chemical signaling or chemically-mediated interactions among plants (Achatz and Rillig 2014; Song et al. 2014, 2015). These physiological mechanisms are not adequately captured by a simple consideration of the population dynamics of plants and fungi, since facilitation of one plant by another via this mechanism does not require density responses of the fungi to their host plants. Rather, these results suggest the potential for a unique influence of CMNs on plant-plant interactions via Mechanism 1.

9.1.4 Experimental Tests of CMN Effects on Plant-Plant Interactions

Testing whether CMNs affect plant ecological outcomes requires going beyond estimates of resource flows through CMNs. Measurements of plant-plant interaction strengths and/or community level consequences, such as composition and diversity, must be made and compared among treatments differing in the presence of CMN linkages, ideally under field conditions (a CMN field manipulation experiment). Moreover, claims that specific amounts of resource flow are or are not ecologically significant are difficult to justify when we have such a paucity of actual tests for CMN effects (and any associated resource flow) on the plant ecological outcomes of interest. Relatively few such experiments have been published, likely reflecting the difficulty of creating experimental treatments that differ in the presence of CMNs but not in other confounding variables. Despite these challenges, several manipulative field experiments now provide evidence that CMNs formed by mycorrhizal fungi have unique effects on aspects of green plant community ecology. The studies all have unique strengths and weaknesses, an analysis of which may help to inspire and strengthen a new generation of studies that push the field further forward. Below is such an analysis, focusing first on manipulative field experiments before discussing the implications of results from these and other types of studies.

In typical CMN field manipulation experiments, the performance of a target mycorrhizal plant (typically a seedling) is compared among several treatments. In most cases, these experiments have been designed with the possibility in mind that neighbor plant roots may have different (specifically, more negative) direct effects on seedlings than linkages with neighbor plant CMNs, and have utilized at least three treatments to estimate those two separate effects: No roots/+CMN, No roots/No CMN, and +roots/+CMN (Fig. 9.2).

Comparison of target seedling performance in a No roots/+CMN treatment versus a No roots/No CMN treatment (e.g., a trenching treatment) estimates the CMN effect of neighbor plants on a target seedling, in the absence of neighbor roots (Fig. 9.2c vs. d or b vs. d). As detailed below, a No roots/No CMN treatment may (Fig. 9.2c) or may not (Fig. 9.2b) include a control for the effect of the container used to exclude roots in the No roots/+CMN treatment. Comparison between a No roots/No CMN treatment and a +roots/+CMN treatments estimates the net effect of roots and CMNs together (Fig. 9.2a vs. b). By subtraction of these two treatment effects, the effect of roots alone can be estimated. Sometimes, a fourth treatment has been included, +roots/No CMN, which can be used to estimate the effect of CMNs in the presence of roots (when compared to the +roots/+CMN treatment) and to directly estimate the effect of roots alone (when compared to the No roots/No CMN treatment). It is important to note that in all treatments in these studies, both the target plant and the neighbor plant(s) are mycorrhizal. What is being manipulated is whether or not the mycorrhizal fungi of the target and neighbor plants are connected

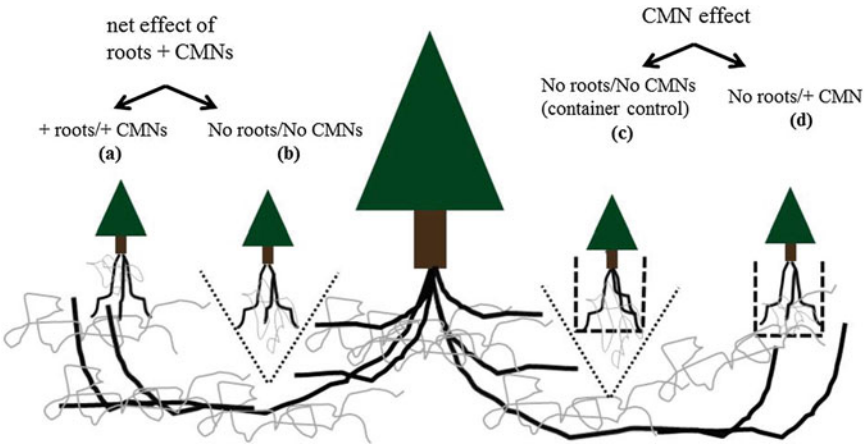

Fig. 9.2 Potential treatments in a field experiment testing separate effects on target plants of common mycorrhizal networks (CMNs) and roots associated with neighboring plants. *Solid black lines* are root, thin *gray lines* are mycorrhizal fungal mycelium, *dotted lines* are trenching treatments or solid barriers, and *dashed black lines* are containers (e.g., bags or PVC cylinders) with mesh openings large enough to allow penetration by fungal mycelium but small enough to exclude roots. **a** +roots/+CMNs. **b** No roots/No CMN. **c** No roots/No CMN (container control). **d** No roots/+CMN

in a CMN and whether or not target and neighbor plant root systems are overlapping. Below, I discuss how these treatments have been implemented, including potential strengths and weaknesses of various approaches. Table 9.1 summarizes the approaches taken in published field studies to date (of which I am aware). Note that all such field experiments so far have studied EM (not AM) fungal CMNs.

No roots/+CMN treatment. This treatment (Fig. 9.2d), when compared with a No Roots/No CMN treatment (Fig. 9.2c vs. d or b vs. d), allows estimation of the effect of neighbor plant CMNs on a target plant, in the absence of neighbor plant roots. Neighbor plant roots may have a different effect on the target plant, so it is ideal to separately estimate root effects and CMN effects. Published studies so far have created No roots/+CMN target plant plots, which lack neighbor plant roots but contain CMNs associated with neighbor plants, using mesh barriers with pore sizes between 20 and 250 μm in cylindrical or rectangular forms ranging in diameter from 10 to 20 cm and in depth from 18 to 40 cm. This pore size range, depending on the plant community, excludes most neighbor roots but allows colonization by fungi. Teste and Simard (2008) and Teste et al. (2009b) included two different treatments, one with 35 μm mesh designed to allow only individual fungal hyphae, and one with 250 μm mesh designed to also allow colonization by rhizomorphs. They observed rare instances of rhizomorphs breaking down into an unstructured form and penetrating the 35 μm mesh. Teste and Simard (2008) also argued that the thickness (not the diameter of the openings) of the mesh used in their study ($\sim$320 μm) likely prevented contact exploration types of EM fungi from colonizing these plots and forming CMNs, although it is unclear how important those contact exploration types are, relative to other types of EM fungi, in forming functional CMNs. An important consideration for this treatment is the volume of the mesh enclosure, which must be sufficient to allow unrestricted target plant root growth for the intended duration of the experiment, but which must not be too large as to be impractical to install.

No roots/No CMN treatment. Early experiments, designed to test how contact with adult tree root systems and associated CMNs may influence the EM fungal community of seedlings, used trenching or coring and subsequent insertion of impermeable barriers to separate outplanted seedlings from the roots and CMNs of neighboring adult trees (reviewed by Deacon and Fleming 1992; see also Simard et al. 1997b). In more recent experiments focused on testing effects of CMNs on plant ecology, two different approaches have been used to establish this treatment, which excludes both roots and CMNs associated with neighbor plants, but in which target plants are mycorrhizal (Fig. 9.2b, c). In one approach, bags or cylinders of the same size as those in the No roots/+CMN treatment are used, but are made of an impermeable material or covered with a mesh of sufficiently small pore size (0.45–4 μm) to exclude roots and fungi but to potentially allow some passage of water and gases (e.g., McGuire 2007; Teste et al. 2009b). An alternative approach has been to repeatedly (every 4–6 weeks) renew a conical trench around the target plant plot, severing roots and CMNs that have grown into the plot (Booth 2004; Booth and Hoeksema 2010). In both cases, target plants establish their own mycorrhizas (not linked to neighbor plants via a CMN) independently, via fungal propagules in the

Table 9.1 Characteristics of field studies used to test CMN effects on plant ecology

Author and year	No roots/+CMN	No roots/No CMN	+roots/+CMN	+roots/No CMN	Mycorrhizal community data	Resource flow data
Bingham and Simard (2012a)	35 µm mesh cylindrical bag (17 cm diam, 32 cm deep)	0.5 µm mesh cylindrical bag (17 cm diam, 32 cm deep)	No barrier	–	–	water and C (^{13}C accumulation in target plant stems)
Bingham and Simard (2013)	35 µm mesh cylindrical bag (17 cm diam, 32 cm deep)	0.5 µm mesh cylindrical bag (17 cm diam, 32 cm deep)	No barrier	–	EM fungi molecular ID (reported in Bingham, and Simard 2012b)	water and C (^{13}C accumulation in target plant stems)
Booth (2004)	44 µm mesh on pvc cylinder (15 cm diam, 18 cm deep)	1. 0.5 m diam. conical trench 2. trench around pvc cylinder	No barrier	AM seedling species planted in EM overstorey	–	–
Booth and Hoeksema (2010)	44 µm mesh on pvc cylinder (15 cm diam, 18 cm deep)	1. 0.5 m diam. conical trench 2. trench around pvc cylinder	No barrier	–	–	water and C (^{13}C accumulation in target plant leaves)
Kranabetter (2005)	250 µm mesh cylindrical bag (10 cm diam, 20 cm deep, open bottom)	4 µm mesh cylindrical bag (10 cm diam, 20 cm deep, open bottom)	No barrier	–	EM fungi morphotypes	–
McGuire (2007)	20 µm mesh pots (30 cm diam, 40 cm deep)	0.45 µm mesh pots (30 cm diam, 40 cm deep)	No barrier	–	–	–

(continued)

Table 9.1 (continued)

Author and year	No roots/+CMN	No roots/No CMN	+roots/+CMN	+roots/No CMN	Mycorrhizal community data	Resource flow data
Teste and Simard (2008)	1. 35 µm mesh cylindrical bag (15 cm diam, 35 cm deep) 2. 250 µm mesh cylindrical bag (15 cm diam, 35 cm deep)	1. 0.5 µm mesh cylindrical bag (15 cm diam, 35 cm deep) 2. Impermeable cylindrical bag (15 cm diam, 35 cm deep)	No barrier	–	EM fungi molecular ID (reported in Teste et al. 2009b)	water (pulsing of deuterated water; reported in Schoonmaker et al. 2007)
Teste et al. (2009a)	1. 35 µm mesh cylindrical bag (15 cm diam, 35 cm deep) 2. 250 µm mesh cylindrical bag (15 cm diam, 35 cm deep)	0.5 µm mesh cylindrical bag (15 cm diam, 35 cm deep)	No barrier	–	EM fungi molecular ID	C and N (pulsing of ^{13}C and ^{15}N)

Columns 2–5 give details of how roots and/or CMNs of neighbor plants were or were not restricted from contact with target experimental plants. All experiments took place in ectomycorrhizal systems.

soil. One major advantage of the first approach (bags or cylinders the same size as in the No roots/+CMN treatment) is that once the bags or cylinders are installed, much less labor is required to maintain the treatment, whereas the trenching treatment requires a great deal of physical labor. In addition, the physical process of trenching could potentially cause disturbance of the target plant plot. On the other hand, the trenching approach has a significant advantage, which is that the volume of soil inside the trenched zone, and thus the soil volume that can be explored by the mycorrhizal fungi associated with the target plant, can be substantially larger than the mesh bag or cylinder used to create the No roots/+CMN treatment. In contrast, if an impermeable barrier or very fine mesh is used to create the No roots/No CMN treatment, then target plants and their associated mycorrhizal fungi in that treatment are restricted to explore the volume of soil inside the bag or cylinder. This situation could be problematic when comparing plant growth with the No roots/+CMN treatment, in which mycorrhizal fungi associated with target plants are theoretically free to explore a much larger soil volume. In addition, the trenching approach allows inclusion of a No roots/No CMN treatment that controls for bag/cylinder effects by growing the target seedling inside a bag or cylinder identical to the one used in the No roots/+CMN treatment (Fig. 9.2c). Regardless of how this treatment is imposed, it is possible it may select for a different subset of the mycorrhizal fungal community than other treatments allowing CMN connections, potentially confounding interpretation of comparisons of plant performance among treatments (also see below under Field CMN-manipulation Experiments).

+roots/+CMN treatment. All of the published CMN manipulation experiments reviewed here took a similar approach to establishing this treatment, which was to plant target plants into intact soil containing roots and compatible CMNs associated with neighbor plants (Fig. 9.2a). In some cases, investigators took the extra step to first impose a disturbance similar to that created in the two No Roots treatments, e.g., installing and then removing a mesh bag or cylinder, in order to control for the effects of this disturbance across the whole experiment (McGuire 2007; Teste et al. 2009b; Booth and Hoeksema 2010; Bingham and Simard 2012a).

+roots/No CMN treatment. This treatment (when compared with a +roots/+CMN treatment) potentially allows estimation of the effect of neighbor plant CMNs on a target plant, in the presence of interactions between target and neighbor plant roots. However, this treatment is the most difficult to establish in a way that does not include confounding effects. Ideally, target and neighbor plants are both mycorrhizal, and their roots and mycorrhizal fungi are intermingled, but no CMN connections form between the mycorrhizas of the two plants. The only way this treatment has been achieved in published experiments is to establish AM target plants in soil colonized by roots and incompatible CMNs of EM neighbor plants (Eason et al. 1991; Booth 2004). In theory another possible approach, if target and neighbor plants are different species, would be to inoculate target and neighbor plants with different host-specific mycorrhizal fungi. All of these approaches have the limitation of confounding the lack of CMNs with one or more other factors that may differ between this treatment and the +roots/+CMN treatment, such as differing densities of compatible mycorrhizal fungi for the target plant, differing species or

types of neighbor fungi, differing neighbor plant species or microenvironments, or differing target plant species.

Additional methodological considerations for CMN field manipulation experiments. Some studies (e.g., McGuire 2007) allowed no time for in-growth of CMNs into No roots/+CMN plots before seedlings were planted, while others allowed up to a year (e.g., Booth and Hoeksema 2010). Although both of these experiments observed positive effects of CMNs on target plant growth and/or survival, I suggest that studies allowing a longer time for establishment of the CMN before planting target plants are more likely to provide accurate estimates of the potential effect of CMNs, since these benefits may be highest when target plants are youngest and join an existing CMN. For the same reason, benefits of CMNs may be less likely to be observed when older seedlings are planted as target plants (e.g., Teste and Simard 2008) compared to when seeds or young seedlings are planted (e.g., McGuire 2007). One study compared results between both of these approaches (Teste et al. 2009b). Bingham and Simard (2012a) demonstrated how the timing of planting can dramatically affect how target seedlings respond to CMNs. Finally, one study (Kranabetter 2005) used open-bottomed bags to implement treatments, making it possible that CMNs and overstorey roots were present in both No roots/No CMN and No roots/+CMN treatments, thereby potentially masking distinct CMN effects. Open-bottomed containers for target plants may be appropriate for implementing these treatments in some systems, but investigators must take care to insure and verify that this approach does not result in confounding colonization of target plant plots by unwanted roots or mycorrhizal fungi from neighbor plants.

9.1.5 What Do Results of Previous Field, Laboratory, and Other CMN Studies Tell Us?

Field CMN-manipulation experiments. Four CMN manipulation studies conducted in EM forests have found substantially higher growth and/or survival of target seedlings in No roots/+CMN treatments compared to No roots/No CMN treatments (Booth 2004; McGuire 2007; Booth and Hoeksema 2010; Bingham and Simard 2012a), providing support for the notion that overstorey trees can have important facilitative effects on seedlings through CMNs (but see Jakobsen and Hammer, Chap. 4, this volume). Two of these studies (Booth and Hoeksema 2010; Bingham and Simard 2012a) reported data that provide a partial test for whether resource transfer (i.e., Mechanism 1) was the mechanism underlying the facilitative effect of CMNs. Booth and Hoeksema (2010) found higher survival of target *Pinus radiata* seedlings over two years in No roots/+CMN plots compared to No roots/No CMN plots, but no differences in target seedling growth, N status, or maximum photosynthetic rates, suggesting that N transfer from overstorey *Pinus radiata* trees was not responsible for facilitative effects. This study did, however, find that leaves of target seedlings connected to overstorey CMNs were significantly depleted in ^{13}C at

the end of the experiment relative to non-networked seedlings. This is the opposite pattern that would be expected if the leaves of networked seedlings contained a substantial proportion of carbon transferred through the CMN from overstorey trees (Hogberg et al. 1999). Rather, this result supports the hypothesis that networked seedlings had access to and transpired more water than non-networked seedlings, as total water transpiration is correlated with depletion in ^{13}C (Farquhar et al. 1989; Fotelli et al. 2003; Querejeta et al. 2006). Moreover, most target seedling mortality occurred during the dry season. Altogether, these results suggest that CMNs associated with overstorey trees had a facilitative effect on understorey seedling dry-season survival through the redistribution of water that was hydraulically lifted by overstorey trees. In the same study, overstorey tree roots were found to have a direct negative (competitive) effect on seedling survival, but much of this competitive effect was offset by the facilitative effect of overstorey CMNs. Bingham and Simard (2012a) found that Douglas fir (*Pseudotsuga menziesii*) seedling survival under a Douglas fir canopy was dramatically higher in a No roots/+CMN treatment compared to a No roots/No CMN treatment. They measured ^{13}C natural abundance in target seedling stems, but did not find significant differences among CMN treatments.

Three field studies (Teste and Simard 2008; Teste et al. 2009b; Bingham and Simard 2013) found no difference in target Douglas fir seedling performance between No roots/+CMN treatments compared to No roots/No CMN treatments. The authors of the two earlier field studies suggested that CMN formation was limited in their No roots/+CMN treatments, and they found increases in target Douglas fir seedling performance in +roots/+CMN treatments compared to No roots/No CMN treatments, which suggests facilitation of seedlings through either roots or CMNs associated with neighbor Douglas fir individuals. One of the field studies (Teste et al. 2009b) tested for C and N transfer from Douglas fir trees to target seedlings, and one (Bingham and Simard 2013) tested indirectly for C and water transfer by measuring ^{13}C natural abundance in target seedling stems; both found little evidence of significant differences among CMN treatments in the amount of resources transferred. In the field experiment by Teste and Simard (2008), however, deuterium-labeled water was used to track water transfer, and it was estimated that target seedlings received more than 21 % of their water through hydraulic redistribution from adult neighbor trees (Schoonmaker et al. 2007). Whether or not the pathway was root-soil-root or root-CMN-root, transfer of water seems to be one of the underlying mechanisms behind this instance of seedling facilitation by trees.

Bever et al. (2010) recently argued that these CMN manipulation experiments do not distinguish between the mechanisms of resource transfer between plants versus changes in density or composition of mycorrhizal symbionts, since treatments that sever the CMN also reduce the density of mycorrhizal fungi available to the target plant and reduce resource availability to the mycorrhizas of the target plant. I disagree with this blanket assessment, and suggest that a careful examination of the details of these experiments can help distinguish between alternative mechanisms. For example, in the Booth and Hoeksema (2010) study, No roots/+CMN

plots and No roots/No CMN both contained cylinders wrapped in mesh that excluded roots but allowed CMN colonization. These cylinders were allowed one year to be colonized by overstorey CMNs before young target seedlings were planted in January during the wet season and trenches were cut to sever overstorey roots and CMNs entering the No roots/No CMN plots. Planted seedlings may have encountered reduced initial densities of mycorrhizal fungi in the latter plots; however, there were no differences among treatments in seedling mortality or growth during the early phase of the experiment. Rather, treatment differences in target seedling mortality appeared later in the experiment, during two subsequent dry summers, when mycorrhizal fungal densities were likely very similar among treatments. Moreover, it seems unlikely that trenching substantially reduced resource availability to mycorrhizas associated with the target seedlings in No roots/No CMN plots (except for resources potentially transferred from overstorey CMNs), as trenches were cut well away from the target seedling cylinders, in a conical shape with a diameter at the soil surface of 0.5 m. As discussed above, stable isotope data from this experiment suggest that seedlings in the No roots/+CMN treatment survived at a higher rate during dry summers at least in part due to great access to hydraulically distributed water from CMNs. Restriction of available resources to mycorrhizas in the No roots/No CMN treatment might be more of a concern in experiments that create this treatment using impermeable or micromesh bags the same size as the mesh bags used in the No roots/+CMN treatment.

As Bever et al. (2010) rightly pointed out, however, the mechanism of resource transfer through CMNs is not mutually exclusive from that of plant-soil feedbacks through altered densities or composition of mycorrhizal symbionts and asymmetric distribution of benefits by the symbionts among different hosts (i.e., versions of Mechanisms 2 and 3) in explaining facilitation of target plants by neighbor plants (see also Simard and Durall 2004; Selosse et al. 2006), and both mechanisms may have been operating in the experiments discussed above that showed facilitation of target seedlings by CMNs of overstorey trees (Booth 2004; McGuire 2007; Booth and Hoeksema 2010; Bingham and Simard 2012a). However, none of those studies reported data on variation in mycorrhizal fungal densities or community composition among treatments. In the experiment by Booth and Hoeksema (2010) one EM fungal taxon, *Tomentella sublilacina*, was most abundant on target seedlings in all treatments, although composition of rare taxa may have differed among treatments (K.J. Hennig, unpublished data). Teste et al. (2009b) found that when Douglas fir seeds (rather than seedlings) were planted in target plant plots, ectomycorrhizal colonization on target seedlings was higher after the first growing season in the +roots/+CMN treatment, but this effect disappeared by the following growth season. In the study by Teste and Simard (2008), treatments had no effect on mycorrhizal colonization, richness, or diversity (reported in Teste et al. 2009a), whereas the field study by Bingham and Simard (2013) found that the similarity of target seedling EM fungal communities to those of neighboring adult trees was significantly altered by CMN manipulations (reported in Bingham and Simard 2012b). In future experimental studies of potential CMN effects on plant ecology, it will be essential to report companion data on how mycorrhizal fungal community

composition is altered by treatments, and ideally also on variation among fungal taxa in how they affect plant growth, so that alternative mechanisms can be clearly distinguished.

Laboratory CMN-manipulation studies. Laboratory (greenhouse or growth chamber) studies across diverse systems have now shown that severing of CMN connections between plants can significantly alter outcomes of plant-plant interactions. In particular, recent experiments in AM systems support the idea that CMNs mediate antagonistic, rather than facilitative, interactions among plants (e.g., Janos et al. 2013; Merrild et al. 2013; Weremijewicz and Janos 2013). Typically, these studies have used restrictive mesh to prevent root overlap between adjacent plants and to allow mycorrhizal hyphae to form a CMN, creating a No roots/+CMN treatment, and have compared target plant performance between this treatment and a No roots/No CMN treatment in which the CMN is severed or prevented. One advantage of these laboratory experiments is that fungal community composition can be controlled, and plant and fungal densities can be either controlled or carefully monitored.

In one example from an AM system, target tomato (*Solanum lycopersicon*) seedlings were found to grow significantly larger and to attain a higher P status when CMN connections to neighbor cucumber (*Cucumis sativus*) plants were severed, suggesting that connections mediate antagonistic effects of cucumber on tomato (Merrild et al. 2013). Fungal densities and root colonization were high across treatments, and the authors argued that asymmetric distribution of P by the AM fungi forming the CMN (Mechanism 3) was responsible for the antagonistic interaction, although the possibility of interplant transfer of P or other resources through the CMN was not explored. Similar growth benefits of severing an AM CMN, despite consistent AM colonization among treatments, were found for *Eucalyptus tetrodonta* (Janos et al. 2013). In yet another AM system, intact CMNs mediated intraspecific competition between big bluestem (*Andropogon gerardii*) individuals, resulting in greater size inequality among plants interconnected by CMNs compared to those with severed CMNs (Weremijewicz and Janos 2013). In this experiment, plants with access to CMNs exhibited higher root colonization by AM fungi, supporting the idea that CMNs mediated plant-plant interactions at least partly through density effects (Mechanism 2). However, the authors argue that CMNs had access to larger volumes of soil for nutrient acquisition, compared to the AM fungi of non-networked plants, allowing higher overall productivity in systems connected by CMNs. This interpretation highlights the problem, highlighted above (under "No roots/No CMN treatment"), that emerges when CMN connections are prevented by using an impermeable container the same size as those in which CMN-connected plants are grown: In this case, the soil volume available for nutrient acquisition by plants disconnected from the CMN is much smaller than the soil volume available to CMN-networked plants, confounding effects of CMN connections per se with differences in resource availability. One laboratory study in an EM system found no difference in target Douglas fir seedling performance between No roots/+CMN treatments compared to No roots/No CMN treatments (Bingham and Simard 2011),

but was notable for its effort to test for C and water transfer between Douglas fir seedlings using stable isotope pulse-labeling.

CMN-inoculation and near-planting experiments. Another type of experiment relevant to the debate about the importance of mycorrhizal networks is one in which the performance of a target plant is compared between two treatments—one in which no mycorrhizal inoculum is provided and one in which mycorrhizal inoculum is provided in the form of the mycelium associated with a companion plant (e.g., Nara 2006). In these experiments, target plant performance is consistently found to be higher when inoculum is provided by a companion plant compared to when it is not (reviewed by van der Heijden and Horton 2009). Although the inoculum in these experiments is indeed provided by the CMN associated with a neighbor plant, I suggest that these results do not provide evidence for unique CMN effects on plant ecology. Since target plant performance is compared between a +CMN treatment and a non-mycorrhizal treatment, the effect of CMN network connections per se cannot be distinguished from the simple effect of mycorrhizal inoculation. This point has been made previously by other authors (Teste and Simard 2008; Bever et al. 2010), and Teste and Simard (2008) suggest that facilitation in these experiments be termed "MN-inoculation" effects rather than direct MN effects. Certainly, these studies provide examples in which the mechanism of indirect plant-plant facilitation is altered density of the mycorrhizal fungal symbiont, as discussed by Bever et al. (2010).

More informative to the debate about unique CMN effects in which linkages per se are important is a comparison of the magnitude of benefit to a target plant when it is inoculated by a CMN versus inoculation by spores or mycelium. Such an experiment would essentially constitute a comparison between a +roots/+CMN or No roots/+CMN treatment versus a No roots/No CMN treatment as described above, in which all target plants are mycorrhizal, but the two treatments differ in the presence of CMN connections. If the mycorrhizal No roots/No CMN treatment is compared with a +roots/CMN treatment, then the relative roles of neighbor plant roots versus CMNs may not be clear. Moreover, the mechanism of interactions (Mechanisms 1–3) would not be clear without careful examination of companion data in each experiment. An example of one such experiment was provided by Kytöviita et al. (2003), who compared the growth of four herbaceous perennial plants in a greenhouse experiment across several treatments, including non-mycorrhizal target plants, target plants inoculated in isolation with spores of one of several AM fungi, or target plants inoculated via mycelial growth of AM fungi from the CMN of an established neighbor plant. They found that growth of the target plants was not improved by inoculation through the CMN compared to inoculation by spores, suggesting either no benefit of a CMN connection, a benefit that was offset by competitive effects of the neighboring plant root system, or a benefit that was offset by competition for resources through the CMN itself (as the authors argue).

In an intermediate approach between CMN manipulations and CMN inoculation studies, a few studies have effectively generated a comparison of target plant performance among +roots/+CMN, No roots/No CMN, and/or +roots/No CMN

treatments by planting an EM target plant in the field near different types of neighbors and/or at different distances from neighbors (e.g., Horton et al. 1999; Dickie et al. 2002, 2005; Teste and Simard 2008). In some of these experiments, +roots/No CMN and +roots/+CMN treatments were effectively established by planting EM target plants in soil colonized by roots and either incompatible CMNs of predominantly AM neighbor plants or compatible CMNs of EM neighbor plants, respectively. Dickie et al. (2002) also created a no roots/+CMN treatment by planting their EM target plants near stumps of dead EM host plants, in soils that presumably lacked roots and CMNs associated with neighbor plants. A key difference between these experiments versus CMN inoculation experiments (reviewed by van der Heijden and Horton 2009) is that the target seedlings in all treatments had access to natural levels of compatible EM inoculum, so that an effect of neighbor CMNs was not necessarily just an inoculation effect. One potential challenge of these studies is that multiple aspects of the microenvironment had the potential to differ among treatments, so interpretation was aided by thorough measurement of microenvironmental variation. Horton et al. (1999) found substantially increased survival of EM *Pseudotsuga* target plants near EM *Arctostaphylos* neighbors compared to those near predominantly AM *Adenostoma* neighbors, and Dickie et al. (2002) found significantly increased nutrient status and growth in *Quercus* seedlings planted near EM *Quercus* neighbors compared to those near AM *Acer* neighbors. In both studies, microenvironmental differences among treatments were minimal, and data on densities and identities of EM fungi on target plants supported the hypothesis that facilitation by neighbors was mediated by altered densities and composition of EM inoculum (as hypothesized by Bever and Schultz 2005; Bever et al. 2010), although resource transfers were not estimated or ruled out, and Horton et al. (1999) also noted available soil P and moisture as potentially explaining treatment differences.

9.2 Conclusions

If we are to make efficient progress in understanding what roles mycorrhizal fungi play in mediating community dynamics and general plant ecology, we need to clearly recognize the kinds of studies that are needed to distinguish among multiple alternative hypotheses, including resource flows through CMNs and altered densities and composition of mycorrhizal fungal communities. First, we need experimental studies that clearly distinguish between effects of physical connections through CMNs per se, versus effects of CMNs on inoculum densities and composition, on strengths of interactions among plants and/or plant community composition. Although these experiments are difficult to execute in a way that allows clear inference, several examples have been published on which future efforts can build. In particular, more such field experiments are needed in AM systems. Most importantly, such studies need to be combined with companion data sufficient to distinguish among multiple types of resource flow through CMNs, as well as data to quantify altered densities, composition, and functions of mycorrhizal fungal symbionts, so that Mechanisms 1–

3 can be effectively tested. In particular, water, phosphorus, and nitrogen flow through CMNs may be more ecologically significant than attention paid to them in previous studies would indicate, and we still understand relatively little about whether or how individual fungal taxa in mixed communities may contribute differently to plant growth or the function of CMNs. Despite decades of progress, we are still in the early stages of determining the general ecological significance of common mycorrhizal networks, and significant advances will only come with careful accumulation and examination of proper evidence.

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