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Root tip competition among ectomycorrhizal fungi: Are priority effects a rule or an exception?

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Abstract. Competition for root colonization among ectomycorrhizal fungi is well documented, but the mechanisms determining competitive outcomes are not clearly understood. In a previous study, we observed that timing of colonization (i.e., a priority effect) had a significant effect on the outcome of competition between two ectomycorrhizal (EM) fungi in the genus *Rhizopogon*. In this study, we explicitly tested the role of priority effects in competition among EM fungi by experimentally manipulating the timing of colonization of four *Rhizopogon* species on *Pinus muricata* seedlings. In a first experiment, we set up 12 two-species combinations, in which seedlings were first inoculated from spores with one species, grown for three months, and then inoculated with an equal density of spores of a second species and grown for an additional three months. Root tip occupation in the two-species treatments was determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of internal transcribed spacer region (ITS) of rDNA. In a second experiment, we further examined competitive interactions between two *Rhizopogon* species using split-root *P. muricata* seedlings. One side of the root system was pre-colonized by one species, and spores of the second species were added to the other side of the root system in all same and different species pair-wise combinations.

We found that for three of the four species (*R. occidentalis*, *R. salebrosus*, *R. vulgaris*), the outcome of competition in the first experiment depended strongly on the timing of colonization, with the first colonizing species always being the competitive dominant. For *R. evadens*, however, initial colonization did not prevent significant subsequent colonization by *R. occidentalis* and *R. vulgaris*. This appeared to be caused by the lower colonization of *R. evadens* compared to the three other species. In the second experiment, we observed that the portion of the split root system that was initially uncolonized remained receptive to colonization when spores were added. The amount of colonization of *R. occidentalis* and *R. salebrosus* on the side of the root system to which they were added was not significantly influenced by species identity on the other side of the seedling. In combination, these results confirm that priority effects do play a major role in dynamics of EM root tip colonization, at least in the early colonization of seedlings, and that the proportion of the root system occupied by a species appears to be a key factor determining competitive success.

Key words: *competition; fungi; mycorrhiza;* Pinus muricata; *priority effects;* Rhizopogon *spp.*

Introduction

Understanding the factors that control the assembly of ecological communities has long interested ecologists (Gleason 1926, MacArthur and Wilson 1967, Connell and Slatyer 1977). The existence of nonrandom patterns of community structure (e.g., Diamond 1975) has driven the search for a broadly predictive set of criteria that defines which combinations of species can and cannot occur together (Wilson and Whittaker 1995). These criteria are often referred to as "assembly rules" (Drake 1990). While there does not appear to be a core set of rules that applies for all ecological communities (Morin 1999), some consistent generalizations have emerged. It is clear, for example, that community composition is often affected by historical events (Ricklefs and Schluter 1993) and the sequence of species arrival can significantly influence composition at later time points (Robinson and Dickerson 1987). These latter kinds of historical effects are commonly referred to as "priority effects," because they often involve early colonists negatively affecting the performance of later arrivals through preemption of shared resources (Alford and Wilbur 1985, Shorrocks and Bingley 1994).

Priority effects have been observed among a wide variety of both macro- and microorganisms (Dix and Webster 1995, Morin 1999, Fukami et al. 2007), but their role in interactions among mycorrhizal fungi is less clear. Mycorrhizal fungi are ubiquitous root symbionts

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of plants that aid in plant nutrient and water acquisition (Smith and Read 1997) and play a major role in determining plant community productivity and diversity (Van der Heijden et al. 1998, Hogetsu and Nara 2004). Strong competitive asymmetries among mycorrhizal fungi have been observed (Wu et al. 1999, Kennedy and Bruns 2005), but the mechanisms by which fungi compete for host root colonization are still poorly understood. Determining these mechanisms is important because many studies have shown that different plant-fungal pairings can result in significant variations in performance for both symbionts (e.g., Bever 2002, Nara 2006).

Two recent experimental studies of mycorrhizal fungi have found contrasting results with respect to the role of priority effects in interspecific interactions. Using experimental spore inoculations, Lilleskov and Bruns (2003) found that pine seedlings initially well colonized by the ectomycorrhizal (EM) fungus *Rhizopogon occidentalis* were eventually dominated by a second EM fungus, *Tomentella sublilacina*. While their study suggested that priority effects were not significant, the experimental design did not include single-species treatments, so the decline in *R. occidentalis* abundance due to factors other than EM competition cannot be discarded. In contrast, Kennedy and Bruns (2005) found a strong negative priority effect determined the outcome of competition between *R. occidentalis* and another *Rhizopogon* species, *R. salebrosus*, in the same experimental host-plant system. Although the latter study indicated that timing of colonization may determine the outcome of mycorrhizal competition, the specific mechanism by which fungi compete was not specified. Competitive outcomes among mycorrhizal fungi may be driven by direct antagonistic interactions among the fungi themselves or by indirect competition for carbon allocation from their plant host. Currently, there is no experimental evidence differentiating these two types of competition, although analogous direct and indirect competitive interactions have been observed in host–parasite systems (Price 1980, Esch and Fernandez 1993).

To explicitly test the role of priority effects in mycorrhizal competitive interactions, the order of colonization among competing species should be experimentally manipulated and experiments should be conducted with multiple species to determine the generality of their conclusions. For many EM fungi, experimentally manipulating the order of colonization is challenging because they do not readily colonize plant roots from spores (Deacon and Fleming 1992, Miller et al. 1993). Species in the genus *Rhizopogon*, however, readily colonize from spores and thus offer an excellent system to address how priority effects influence the outcome of EM competition. In this study, we examined competitive outcomes among four *Rhizopogon* species that are a significant component of the EM assemblages present in early successional *Pinus muricata* forests in coastal California (Peay et al. 2007). By manipulating the timing of spore addition in all pair-wise interactions, we specifically assessed how priority effects influenced the dynamics of EM competition and determined the prevalence of this phenomenon among a group of well-studied species. We also examined the spatial nature of EM competitive dynamics by assessing competitive interactions in a paired split-root experiment.

Methods

Study system and bioassay setup.—Seeds were collected from cones of multiple *P. muricata* individuals at Point Reyes National Seashore in January 2006. In April 2006, surface-sterilized seeds were planted into 160-mL Ray Leach "cone-tainers" (Steuwe and Sons, Corvallis, Oregon, USA), each containing 100 mL of soil. Soil was collected in March 2006 from a mixed scrub–grassland site at Point Reyes National Seashore (38°11′48″ N, 122°57′44″ W). The soil is classified as a Kehoe variant coarse sandy loam, which is a deep, well-drained soil derived from quartz–diorite bedrock moraine. This site was selected because it was previously found to have no *Rhizopogon* inoculum; however, low quantities of inoculum from other EM species were present (Bruns et al. 2008). To eliminate the inoculum from other species, the soil was mixed 1:1 (by volume) with coarse sand and autoclaved on each of two consecutive days at 121°C for one hour. The experiment was conducted in a growth chamber to avoid potential colonization from other EM species (a common phenomenon in greenhouse and field studies). Although the growth chamber does not capture the biotic and abiotic variation present in a field-based experiment, we have previously found that the outcome of competition is similar in field and growth chamber conditions (Kennedy and Bruns 2005, Kennedy et al. 2007*a*). The chamber was set at a light intensity of 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a 16:8 hour light : dark cycle, with temperatures ranging from 18°C to 20°C. Seedlings were watered twice a week to maintain high soil moisture conditions for the duration of the experiment. After one month, in May 2006, seedlings were randomly thinned to one per cone-tainer (initial number of seeds/cone-tainer = 4).

Fungal inoculum for the experiment was created from eight to 12 sporocarps of each *Rhizopogon* species (*R. evadens*, *R. occidentalis*, *R. salebrosus*, and *R. vulgaris*) collected from *P. muricata* forests at Point Reyes. In January 2006, sporocarp collections were made several meters apart and typed by internal transcribed spacer restriction fragment length polymorphism (ITS-RFLP) analysis to verify morphological species identifications. For each species, all of the sporocarps were macerated in distilled water and filtered through cheesecloth to make spore slurries. Spore densities of each species' slurry were quantified with a haemocytometer and stored at 4°C until used. In the first experiment, one month after germination, seedlings were inoculated with spores of a first species at a concentration of 1×10^6 spores/mL. Spores were applied at the top of the cone-tainer and

then lightly watered to facilitate penetration into the soil. Seedlings grew for three months, after which an equal density of spores of a second species was added in the same way. Seedlings were grown for another three months (seven months total) and then harvested for EM root tip analyses. The design of the split-root experiment is detailed in separate section below.

Experimental design.—Our first experiment was designed to explicitly test if the timing of colonization determined the outcome of EM competition. With the four *Rhizopogon* species, we set up six two-species combinations. Each of these was represented twice (reversing the order of species addition the second time) for a total of 12 two-species treatments. As an example, for the *R. salebrosus* and *R. occidentalis* treatments, one treatment had *R. occidentalis* added first and then *R. salebrosus* (denoted throughout in order of spore addition, e.g., *R. occidentalis–R. salebrosus*) and the other treatment had the order reversed (i.e., *R. salebrosus–R. occidentalis*). Each *Rhizopogon* species also had a single-species treatment inoculated at the same time as the two-species treatments. In addition, four-month-old non-mycorrhizal seedlings were inoculated with the same spore slurries as used in the two-species treatments as inoculation timing controls. Finally, we planted a general control treatment in which no spores were added throughout the experiment to insure there was no contamination from spores in the growth chamber or the autoclaved soil. The inoculation controls had seven seedlings per species, the general control had 14 seedlings total, the single-species treatment had 20 seedlings per species, and the two-species treatment had 10 seedlings per pairing ($N = 242$).

EM root tip identification.—In August 2006, three months after the first inoculation, 10 seedlings from each species in the single-species treatment and seven seedlings from the general control treatment were harvested. Seedlings in the two-species and all other treatments were harvested in December 2006, three months after the second inoculation. At harvest, each seedling was removed from its cone-tainer and all the soil was gently rinsed off the root system. Under a 10× dissecting microscope, all live EM root tips were removed from the seedling. Bulked EM root tips from each seedling were flash frozen in liquid nitrogen and then lyophilized. The seedling shoot and remaining portion of the root were separated and individually oven dried at 60°C for 72 hours. All samples were individually weighed and colonization by EM fungi was calculated as (EM root biomass/[EM root biomass + non-EM root biomass]) × 100. This metric of assessing EM colonization is different than those traditionally used in other studies where individual fine roots are scored for colonization and counted. All *Rhizopogon* species have a similar unique coralloid EM morphology that makes the distinction between individual vs. multiple root tips more arbitrary than for other EM fungi (see Plate 1). To avoid this issue, we employed a biomass-based approach, which allows for a standard metric for comparisons across species. Our measure of non-EM root biomass, however, includes the entire root system, not all of which is available for EM colonization (i.e., larger coarse roots). The amount of the root system available for colonization, i.e., fine roots, did not appear to vary in any consistent way across treatments (P. Kennedy, *personal observation*), but our method of assessing colonization did not allow us to specifically define the quantity of non-EM colonizable roots available in the two-species treatments. While the percentage of EM biomass estimates observed here are lower than those in other studies, they do not necessarily indicate that a large percentage of roots were available for colonization by other EM species. To emphasize this distinction, we refer to EM colonization as percentage of EM root biomass, not percentage of EM colonization.

Because the EM roots of the four *Rhizopogon* species in the experiment were morphologically indistinguishable, we used a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis to identify the different species in the two-species treatments. Total genomic DNA was extracted from root tips using a RED-Extract-N-Amp Plant kit (Sigma-Aldrich, St. Louis, Missouri, USA). Individual EM root tips were placed in 10 μL of extraction solution and incubated at 60°C for 10 minutes, and then at 95°C for 10 minutes. After incubation, 30 μL of dilution solution was added and the extraction was kept at room temperature for an additional 30 minutes before being stored at 4°C. The ITS region was amplified from extracted genomic DNA using the fungal-specific primer pair ITS 1F and ITS 4 under conditions previously described (Gardes and Bruns 1993). RFLP digests of positive PCR products were conducted using the restriction enzyme *Hha* I, which was previously found to distinguish all four species (T. Bruns, *unpublished data*). Digests were visualized on 2%/1% agarose gels and the corresponding banding patterns of each species were determined by eye.

To quantify the amount of the root system that was occupied by each species in the two-species treatments, we used the same approach as Kennedy and Bruns (2005). We used this approach rather than the real-time PCR approach we used in other studies because of difficulties encountered with *R. evadens* using the real-time PCR method (Kennedy et al. 2007*b*). From each seedling, we randomly selected 10 root tips for identification (see Appendix A for justification of sample size). The PCR analysis was done in two rounds, with any of the tips that did not amplify in the first round being replaced by new tips and amplified. The average number of tips amplified after two rounds was 9.7 per treatment (minimum = 8, maximum = 10). The ratio of root tips belonging to each species was multiplied by the total EM root biomass of each seedling to calculate respective species' biomasses.

Split-root competition experiment.—To examine how spatial dynamics and competitor identity influence EM

root tip competition, we conducted a second experiment using two of the four *Rhizopogon* species, *R. occidentalis* and *R. salebrosus*. Specifically, we were interested in determining whether prior colonization by one EM species limited the potential for colonization by a second species when direct competition was not occurring. To do this, the root systems of individual *P. muricata* seedlings were trained to grow into two separate compartments of replicate large flat Petri plates (243 × 243 × 18 mm; Nunc Brand Products, Naperville, Illinois, USA; hereafter referred to as microcosms) containing ~350 mL of 2-mm-sieved autoclaved peat potting medium (Premier Horticulture Inc., Red Hill, Pennsylvania, USA). This potting medium was the same used in Lilleskov and Bruns (2003) and was favored over field soil because of its more consistent space filling and stability within the microcosms. The compartments were separated by a sealed Plexiglas barrier that prevented any root or fungal growth from crossing between the two sides of each microcosm (see Appendix B for an example microcosm). After six months of growth, roots in one side of a microcosm were colonized by either *R. occidentalis* (RO) or *R. salebrosus* (RS) by the addition of pre-colonized *P. muricata* donor seedlings (i.e., seedlings that already had EM colonized roots). We chose this method of hyphal inoculation because spore inoculations would have led to differences in the timing of colonization of these two EM species due to differences in their germination speed (as in Kennedy and Bruns 2005), whereas hyphal colonization insured that both *Rhizopogon* species colonized the split-root seedlings at the same time. Both seedlings were grown for another two months, after which EM colonization on the roots of one side of the split-root seedling was confirmed. The donor seedlings were then killed by decapitation, but their root systems were left in the microcosms to minimize disturbance. At that same time, spores of one of the EM species were added to the other non-colonized side of the microcosm in the same manner and concentration as described above. This experiment included four treatments: RS spores added to RS colonized seedlings, RO spores added to RS colonized seedlings, RS spores added to RO colonized seedlings, and RO spores added to RO colonized seedlings, with six replicates per treatment. Split-root seedlings were grown for an additional six months and then harvested to assess EM root tip colonization. Because the identity of the species on both sides of the microcosm was known and no cross-contamination between sides was observed, molecular identification was not necessary. Percentage of EM root biomass was calculated in the same way as above.

Statistical analyses.—To determine how timing of colonization affected EM competition, we compared percentage of EM root biomass across all two-species treatments using one-way analysis of variance (ANOVA). This allowed us to compare the quantity of each EM species in each pair-wise combination (e.g., RO–RS and RS–RO) as well as across species pairings (e.g., RO–RS, RO–RV, RO–RE). Prior to running the ANOVA, data were arcsine transformed to improve variance homogeneity. The shoot, root, and EM biomasses of seedlings in both the single- and two-species treatments were also assessed across both harvest periods with one-way ANOVAs. Percentage of EM root biomass was compared across the single-species treatment using a two-way fixed-factor ANOVA with species (*R. evadens*, *R. occidentalis*, *R. salebrosus*, and *R. vulgaris*) and harvest time (four or seven months) as the predictor variables. For the split-root experiment, percentage of EM root biomass was analyzed using a three-way fixed-factor ANOVA, with species (*R. occidentalis* or *R. salebrosus*), competitor (same or different species), and inoculum source (hyphal or spore) as the model predictor variables. Variances for all the ANOVAs except percentage of EM root biomass were determined to be homogenous based on visual assessments of residual plots. Tukey hsd tests were used for post-hoc comparisons among means. All tests were conducted in JMP 5.0 (SAS Institute, Cary, North Carolina, USA) and considered significant at $P < 0.05$.

Results

In the two-species treatments, we found that initial colonization by one EM species significantly influenced the abundance of the later EM species in 10 of the 12 pairings ($F_{23, 210} = 35.80$, $P < 0.001$, Fig. 1). Early colonization by three of the species, *R. occidentalis*, *R. salebrosus*, and *R. vulgaris*, essentially prevented subsequent colonization by competing species. In fact, in the nine pairings involving these three species, only one had any observed colonization by the later arriving species (*R. occidentalis–R. vulgaris* treatment; Fig. 1). For *R. evadens*, however, early colonization did not prevent subsequent colonization of any of the competing species. In the *R. evadens–R. salebrosus* treatment, *R. evadens* had significantly higher percentage of EM root biomass than *R. salebrosus* at the end of the experiment, but in the *R. evadens–R. occidentalis* and *R. evadens–R. vulgaris* treatments, the final percentage of EM root biomass of the competing species was not significantly different from that of *R. evadens*.

These results appear to be the product of competitive inhibition and not lack of spore viability, as secondary colonization was observed in all of the *R. evadens* treatments and all seedlings in the inoculation control treatment were also colonized (inoculation control percentage of EM root biomass: *R. evadens* 5% ± 1% [mean ± 1 SE], *R. occidentalis* 7% ± 1%, *R. salebrosus* 15% ± 1%, *R. vulgaris* 10% ± 1%). No EM colonization was observed on any of the non-mycorrhizal control seedlings.

In the single-species treatment, we observed significant variation in the percentage of EM root biomass of the four species (species: $F_{3,71} = 3.69$, $P = 0.016$). Overall percentage of EM root biomass was not significantly

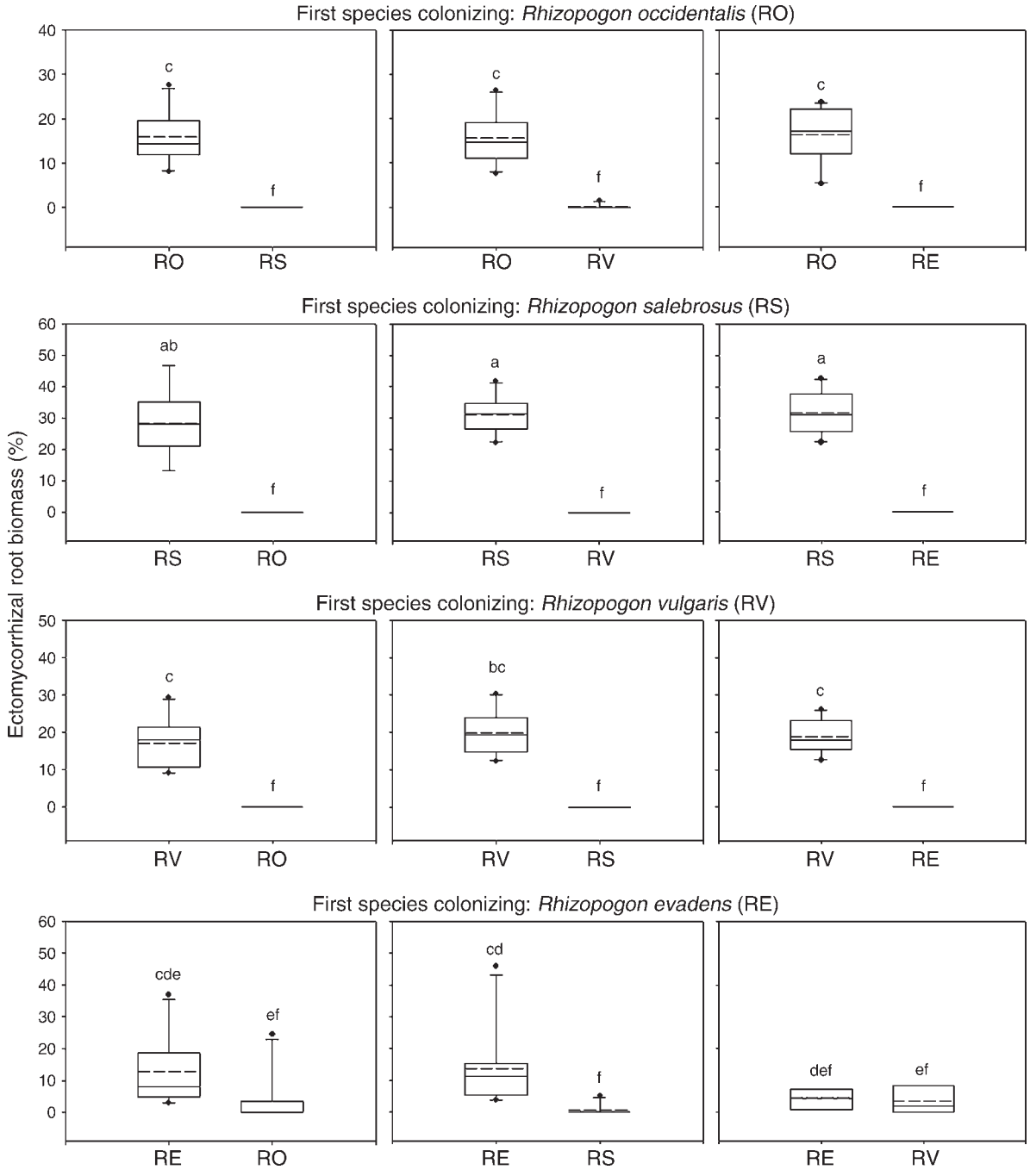

FIG. 1. Percentage of ectomycorrhizal root biomass on *Pinus muricata* seedlings in the two-species treatments. Boxes represent 25–75% of the data, bars (whiskers) represent 10–90%, and black dots represent 0–100%. The median and mean are the solid and dashed lines, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments as determined by Tukey hsd tests.

different between the two harvest periods (time: $F_{1,71} = 0.58$, $P = 0.447$), but there was a significant species by time interaction ($F_{3,71} = 7.21$, $P = 0.003$; Fig. 2). At four months, *R. occidentalis* had significantly higher percentage of EM root biomass than the other three species (Fig. 2). At seven months, however, *R. salebrosus* and *R. vulgaris* had significantly higher percentage of EM root biomass than both *R. occidentalis* and *R. evadens*. At both time periods, *R. evadens* had the lowest percentage of EM biomass and it was the only species in which percentage of EM root biomass stayed constant throughout the duration of the experiment.

Seedling shoot biomass varied significantly across the experimental treatments ($F_{21,188} = 28.27$, $P < 0.001$).

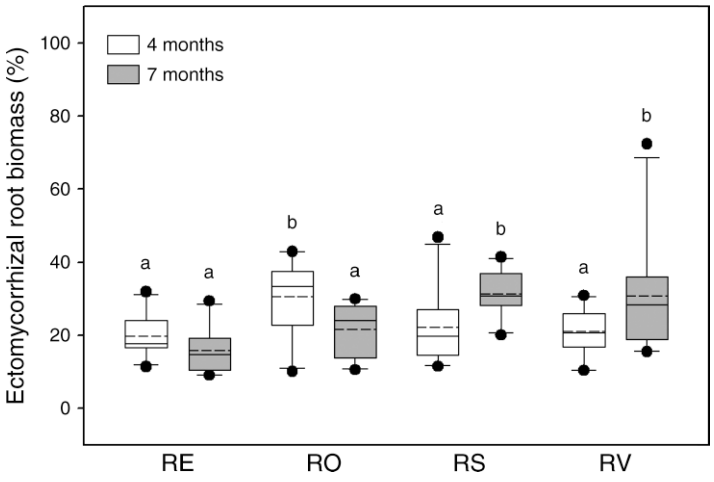

FIG. 2. Percentage of ectomycorrhizal root biomass on *Pinus muricata* seedlings in the single-species treatments. Boxes represent 25–75% of the data, bars (whiskers) represent 10–90%, and black dots represent 0–100%. The median and mean are the solid and dashed lines, respectively. Species abbreviations are: RE, *Rhizopogon evadens*; RO, *R. occidentalis*; RS, *R. salebrosus*; RV, *R. vulgaris*. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments as determined by Tukey hsd tests.

There was no difference among the single-species treatment at four months, but at seven months, shoots of seedlings colonized by *R. salebrosus* were significantly larger than those colonized by *R. evadens*, with *R. occidentalis* and *R. vulgaris* being intermediate (Table 1). For all of the two-species treatments, shoot biomass was not significantly different from that of the single-species treatment of whichever species was added first. Root biomass also varied across treatments ($F_{21, 188} = 22.51$, $P < 0.001$). At four months, the non-mycorrhizal seedlings had significantly greater root biomass than all of the *Rhizopogon* colonized seedlings, except those colonized by *R. evadens*. A similar pattern was seen at seven months, in both the single- and two-species treatments. Like shoot and root biomass, EM root biomass also varied significantly ($F_{21, 188} = 2.17$, $P = 0.004$), however, most treatments at both time periods had statistically similar biomass, with only two of the two-species treatments differing from all others (Table 1).

In the split-root experiment, the mycelium from the donor seedlings successfully colonized the roots of the split-root seedlings in every microcosm. The percentage of EM root biomass on the split-root seedlings was significantly influenced by species, competitor identity, and inoculum source (species $F_{1,41} = 8.29$, $P = 0.0064$; competitor $F_{1,41} = 5.75$, $P = 0.0213$; inoculum source $F_{1,41} = 68.63$, $P < 0.0001$). In 19 of the 24 (79%) microcosms, we observed colonization on the side to which spores were added, but percentage of EM root biomass was generally higher on the hyphal side, which had been colonized for eight weeks longer (Table 2). *R. salebrosus* colonized seedlings more heavily than *R. occidentalis* when inoculated from hyphae, but the two species had equivalent percentage of EM root biomass when inoculated from spore (species × inoculum source interaction $F_{1,41} = 4.77$, $P = 0.0349$). Percentage of EM root biomass on the entire split-root seedlings was significantly higher overall when the two sides were colonized by different species (Table 3), but percentage of EM root biomass of *R. occidentalis* and *R. salebrosus* on the spore side of the microcosm was not significantly influenced by species identity on the other side of the seedling (species × competitor interaction $F_{1,41} = 0.25$, $P = 0.6150$). No other higher order interactions were significant.

DISCUSSION

We found that the presence of one EM species generally had a significant negative influence on the ability of another EM species to colonize *P. muricata* roots. For three of the four *Rhizopogon* species, secondary colonization by a different species was nearly completely inhibited. The patterns of percentage of EM root biomass in the single-species treatments suggest that the ability to prevent other species from colonizing depends on the proportion of the root system occupied by the first colonizer. This was most apparent with *R. evadens*, the only species that did not strongly inhibit secondary colonization by other species. At four months, when the spores of competitors were added, *R. evadens* had an initial percentage of EM root biomass equivalent to two of the three other *Rhizopogon* species (Fig. 2). However, in contrast to the other species, its percentage of EM root biomass did not change during the latter time period of the first experiment (i.e., between four and seven months) and had the lowest average colonization at seven months. The low level of colonization during the second period appears to have allowed spores of the second species to successfully colonize roots of seedlings on which *R. evadens* was already present. Specifically, we found that in two of the three pairings, mean colonization by the competing species was equivalent to that of *R. evadens*, and in some cases, the seedlings in those treatments appeared to be completely dominated by the second species (see Appendix A).

R. evadens has a unique life history among these four *Rhizopogon* species. In nature, it is almost exclusively found in mature forests, at least in the *Pinus muricata* communities of Point Reyes (P. Kennedy, *personal*

TABLE 1. Seedling shoot and root biomasses in the single-species, two-species, and control treatments.

EM treatment, by species	Shoot biomass (g)	Root biomass (g)	EM root biomass
Harvest time four months			
NM	$0.22^{f} \pm 0.01$	$0.15^{bcdef} \pm 0.01$	0 ± 0
RO	$0.23^{f} \pm 0.02$	$0.08^{hi} \pm 0.04$	$0.04^{ab} \pm 0.004$
RS	$0.24^{f} \pm 0.02$	$0.07^{i} \pm 0.01$	$0.02^{ab} \pm 0.005$
RV	$0.24^{f} \pm 0.02$	$0.09^{ghi} \pm 0.01$	$0.02^{ab} \pm 0.005$
RE	$0.20^{f} \pm 0.01$	$0.09^{fghi} \pm 0.01$	$0.03^{ab} \pm 0.004$
Harvest time seven months			
NM	$0.42^{e} \pm 0.03$	$0.27^{a} \pm 0.02$	0 ± 0
RO	$0.52^{de} \pm 0.03$	$0.12^{efgh} \pm 0.01$	$0.03^{ab} \pm 0.005$
RS	$0.67^{abcd} \pm 0.04$	$0.12^{efgh} \pm 0.01$	$0.05^{ab} \pm 0.003$
RV	$0.54^{bcde} \pm 0.03$	$0.15^{bcdef} \pm 0.01$	$0.10^{ab} \pm 0.050$
RE	$0.47^{e} \pm 0.03$	$0.16^{bc} \pm 0.01$	$0.03^{ab} \pm 0.003$
RO–RS	$0.49^{e} \pm 0.03$	$0.13^{defg} \pm 0.01$	$0.02^{ab} \pm 0.003$
RO–RV	$0.47^{e} \pm 0.02$	$0.12^{efgh} \pm 0.01$	$0.02^{ab} \pm 0.002$
RO–RE	$0.49^{e} \pm 0.03$	$0.12^{defgh} \pm 0.01$	$0.02^{ab} \pm 0.003$
RS–RO	$0.69^{a} \pm 0.06$	$0.15^{bcdef} \pm 0.01$	$0.06^{ab} \pm 0.006$
RS–RV	$0.68^{ab} \pm 0.03$	$0.12^{efgh} \pm 0.01$	$0.06^{ab} \pm 0.004$
RS–RE	$0.68^{abc} \pm 0.03$	$0.13^{cdef} \pm 0.01$	$0.06^{a} \pm 0.004$
RV–RO	$0.53^{de} \pm 0.03$	$0.17^{bcd} \pm 0.01$	$0.03^{ab} \pm 0.004$
RV–RS	$0.54^{cde} \pm 0.02$	$0.15^{bcdef} \pm 0.01$	$0.04^{ab} \pm 0.003$
RV–RE	$0.58^{abcde} \pm 0.03$	$0.15^{bcde} \pm 0.01$	$0.04^{ab} \pm 0.003$
RE–RO	$0.44^{e} \pm 0.04$	$0.17^{bc} \pm 0.01$	$0.05^{ab} \pm 0.029$
RE–RS	$0.47^{e} \pm 0.01$	$0.18^{b} \pm 0.01$	$0.04^{ab} \pm 0.019$
RE–RV	$0.47^{e} \pm 0.03$	$0.19^{b} \pm 0.01$	$0.02^{b} \pm 0.003$

Notes: Values reported are means ± SE. Species abbreviations are as follows: RO, *Rhizopogon occidentalis*; RS, *R. salebrosus*; RV, *R. vulgaris*; RE, *R. evadens*; NM, nonmycorrhizal fungi; EM, ectomycorrhizal fungi. Different lowercase superscript letters represent significant differences ($P < 0.05$) based on post hoc Tukey hsd tests.

observation), while the other three species are common colonizers of young pines in the same area (Peay et al. 2007). Among the three pioneer *Rhizopogon* species, *R. salebrosus* is the only one that is also commonly found in the mature forest at Point Reyes (Gardes and Bruns 1996, Taylor and Bruns 1999). Interestingly, *R. salebrosus*, the one species tested that might commonly interact with *R. evadens* in mature forests, was the only tested species with which *R. evadens* showed a priority effect.

The patterns of colonization in the single-species treatment of *R. occidentalis*, *R. salebrosus*, and *R. vulgaris* varied. *R. occidentalis* had significantly higher percentage of EM root biomass at four months than either *R. salebrosus* or *R. vulgaris*, but its abundance declined on a percentage basis in the latter time period of the first experiment. In contrast, the percentage of EM root biomass of *R. salebrosus* and *R. vulgaris* increased on an absolute basis between four and seven months to percentages equivalent to those obtained by *R. occidentalis* after four months. Because all three of these species were able to exclude secondary colonization by other species, it appears that reaching a threshold level of percentage of EM root biomass is key to dominating initial colonization. However, based on the results from the *R. occidentalis* seedlings, it seems

TABLE 2. Ectomycorrhizal root biomass (%) in the split-root experiment, by treatment.

Treatment ($n = 6$)	Pre-colonized side	Spore addition side
RS–RS	$38^{a} \pm 7$	$4^{c} \pm 4$
RS–RO	$42^{a} \pm 7$	$9^{c} \pm 5$
RO–RS	$31^{ab} \pm 3$	$12^{bc} \pm 5$
RO–RO	$18^{bc} \pm 1$	$4^{c} \pm 1$

Notes: Values reported are means ± SE. Species abbreviations are as in Table 1. The ordering of species represents the ordering of each treatment (e.g., RS–RS represents a seedling pre-colonized on one side by RS to which spores of RS were added to the other side). Different lowercase superscript letters indicate significant differences ($P < 0.05$) among treatments as determined by a three-way ANOVA and post hoc Tukey hsd tests.

TABLE 3. Ectomycorrhizal root biomass (%) in the split-root experiment, by predictor variable.

ANOVA predictor variable	Ectomycorrhizal root biomass (%)
Competition	
Same species	$16^{x} \pm 4$
Different species	$24^{y} \pm 4$
Inoculation source	
Hyphae	$32^{l} \pm 3$
Spore	$7^{m} \pm 2$
Species	
RS	$24^{r} \pm 4$
RO	$15^{s} \pm 3$

Notes: Values reported are means ± SE, $n = 24$. Species abbreviations are as in Table 1. Different letters indicate significant differences ($P < 0.05$) among treatments as determined by a three-way ANOVA and post hoc Tukey hsd tests.

PLATE 1. Ectomycorrhizal (EM) root tips of *Rhizopogon salebrosus* in one of the split-root competition experiment microcosms. The material surrounding the EM tips is peat potting medium intermixed with white EM rhizomorphs. Photo credit: P. G. Kennedy.

that maintaining that level of colonization, is less important for determining the outcome of this first phase of root competition. If an EM species does not continue to colonize new roots as the seedling grows, it is not likely to remain dominant over time. This was exactly the pattern observed with *R. occidentalis* in Lillekov and Bruns (2003) when it was paired in competition with *T. sublilacina* and may also explain why *R. occidentalis* is absent from the mature *P. muricata* forests at Point Reyes.

The experimental approach of this study shows that priority effects appear to be a major part of EM colonization dynamics, at least on seedlings, which supports previous work in this system. In particular, the fact that the competitive outcome between *R. occidentalis* and *R. salebrosus* depended on which species was added first confirms our initial observations that timing of colonization can determine the outcome of competitive interactions (Kennedy and Bruns 2005, Kennedy et al. 2007*a*). While we believe these results provide an important addition to our understanding of mycorrhizal root tip competition, we recognize it is not the only factor determining competitive success. Other studies have, for example, shown that direct root tip takeover and replacement is possible (Marks and Foster 1967, Wu et al. 1999) and that initial co-colonization patterns do not necessarily correspond with those assessed at later times (Landeweert et al. 2003, Lilleskov and Bruns 2003). In our own system, we also have seen reversals in competitive interactions that appear to be driven by variation in root density (Kennedy et al. 2007*b*), three-way interactions (Kennedy at al. 2007*b*), and hyphal foraging strategy (Kennedy et al. 2007*a*). We believe another important qualification to the results presented here is that the experimental competition occurred between two different fungal life stages (i.e., hyphal vs. spore). In many forest settings, root tip competition among mycorrhizal fungi is likely to be hyphal; a fungus' ability to prevent secondary colonization by other fungi may be lower in those settings because mycelia connected to other roots have greater carbon reservoirs than spores do. As such, mycelial competitors have the resources to forage more extensively for unoccupied roots. It is also important to note that while we have found similar results between lab and field competition studies in this system (Kennedy and Bruns 2005, Kennedy et al. 2007*a*), fluctuations in light quantity, soil temperature, and other environmental conditions that were held constant in the experiments in this study may significantly influence EM competitive dynamics in the field.

In the split-root experiment, we observed that the portion of the root system that was initially uncolonized remained receptive to colonization when spores were added. This result specifically demonstrates that an uncolonized portion of the root system can be colonized by additional species, which supports the putative dynamics observed in the *R. evadens* treatments above. These findings also suggest that root architecture (e.g., density and/or geometry) and soil heterogeneity (e.g., physical structure) may play important general roles in mediating competitive dynamics among EM fungi, as has been hypothesized previously (Newton 1992, Bruns 1995). A growing number of EM studies have observed spatial patterning among EM fungi with respect to soil depth (Taylor and Bruns 1999, Dickie et al. 2002), which

is consistent with the hypothesis that these factors promote species coexistence. We are unaware of any published experimental work that has tested the specific effects of spatial structure on ectomycorrhizal competitive interactions, but this would seem to be a fruitful area for future research, especially since spatially explicit mechanisms of coexistence have long been thought to be important for the maintenance of species richness in other assemblages (Slatkin 1974, Tilman 1994).

Although the split-root experiment did show that EM competition has a strong spatial component, the quantity of colonization on the side to which spores were added was not affected by the identity of the species present on the other part of the seedling. Seedlings did have the highest overall EM root biomass when both species were present suggesting the potential for resource complementarity, but we have found in previous studies that *R. salebrosus* and *R. occidentalis* appear to provide similar amounts of resources to their host (Kennedy and Peay 2007) and that co-colonization does not increase seedling performance (Kennedy et al. 2007*b*). There are theoretical reasons that plants may choose mycorrhizal symbionts based on their ability to provide resources (Kimmel and Salant 2006), which could drive plant hosts to play an active role in EM competitive dynamics by differentially partitioning carbon or other resources among competitors (Kennedy and Bruns 2005). Some evidence for these types of dynamics has been seen in other plant-microbial interactions (Kiers et al. 2003), but additional studies comparing mycorrhizal species with greater functional differences in varying soil environments are strongly needed to reveal more about the patterns and mechanisms of possible plant–fungal selectivity. In this area, split-root experiments will be particularly important, since they can separate the direct fungal and indirect plant effects of mycorrhizal competition (Kennedy and Bruns 2005).

The combined results of this study suggest that priority effects appear to be a common mechanism determining the outcome of EM competition for seedling root tips. However, the ability to monopolize receptive root tips within the competitive area seems necessary to take advantage of the priority effect and prevent invasion by later arrivals, as evidenced by the performance of *R. evadens* and the split root experiments. This work adds to a growing number of studies demonstrating that competition can significantly influence mycorrhizal interactions (Wu et al. 1999, Landeweert et al. 2003, Mahmood 2003, Koide et al. 2005, Parlade et al. 2007) and also reiterates the importance of assessing mycorrhizal competitive dynamics at multiple time points (Lilleskov and Bruns 2003, Kennedy and Bruns 2005, Kennedy et al. 2007*a*). The vast majority of research on mycorrhizal competition has thus far focused on colonization of host root tips, but assessing competition for nutrients and other soil resources is also needed to understand the factors controlling mycorrhizal competitive dynamics and their influence on mycorrhizal assemblage structure. Given the wealth of competition theory that has been developed for other organisms, we believe this area of mycorrhizal research is primed for further study, especially since only a small subset of plants and fungi involved in this symbiosis have thus far been examined.

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APPENDIX A

An explanation of the sample size used to assess individual species EM colonization in the two-species treatment of the first experiment (*Ecological Archives* E090-146-A1).

APPENDIX B

Photographs showing an example of one of the split-root experiment microcosms (*Ecological Archives* E090-146-A2).