

# Priority effects determine the outcome of ectomycorrhizal competition between two *Rhizopogon* species colonizing *Pinus muricata* seedlings

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## Summary

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Received: 25 October 2004

Accepted: 14 December 2004

• Competition is often considered a fundamental process influencing assemblage structure, yet little is known about competition among ectomycorrhizal (EM) fungi. Here, we examine competitive interactions between *Rhizopogon occidentalis* and *Rhizopogon salebrosus* in a 6-month microcosm study.

• *Pinus muricata* seedlings were grown in three EM treatments: *R. occidentalis*, *R. salebrosus*, and *R. occidentalis* and *R. salebrosus*. At 2, 4, and 6 months, five seedlings per treatment were harvested and the EM root tip biomass of each species was determined. Root tips in the two-species treatment were identified using molecular techniques.

• *R. occidentalis* had similar EM root tip biomass when grown alone or in the presence of *R. salebrosus*. By contrast, *R. salebrosus* had significantly lower EM root tip biomass when grown with *R. occidentalis* than when grown alone, indicating it was a competitive inferior under the conditions tested. Competition was driven by differences in timing of colonization resulting in a strong priority effect for *R. occidentalis*.

• Our results, together with two earlier studies, indicate competition may play a more important role in EM interactions than previously recognized.

**Key words:** competition, ectomycorrhizal fungi, *Pinus muricata*, priority effect, *Rhizopogon*.

*New Phytologist* (2005) **166**: 631–638

© *New Phytologist* (2005) doi: 10.1111/j.1469-8137.2005.01355.x

## Introduction

A number of generalizations are beginning to emerge about the structure of ectomycorrhizal (EM) assemblages (Dahlberg, 2001; Horton & Bruns, 2001). The first is that most EM assemblages are highly diverse, with many rare species and few dominants (Danielson, 1984; Visser, 1995; Dahlberg *et al.*, 1997; Gehring *et al.*, 1998; Horton *et al.*, 1999; Kennedy *et al.*, 2003). Second, the distribution of most EM species is patchy, even at the scale of centimeters (Bidartondo *et al.*, 2000; Dickie *et al.*, 2002; Tedersoo *et al.*, 2003; Lilleskov *et al.*, 2004). Third, at larger spatial scales (i.e. between forest stands and vegetation types) certain higher

taxonomic groups (e.g. Russulaceae and Thelephoraceae in temperate coniferous forests) appear to be dominant (Horton & Bruns, 2001), but disturbances such as nitrogen deposition or stand-replacing fire can cause major changes in assemblage structure (Dahlberg *et al.*, 1997; Horton *et al.*, 1998; Peter *et al.*, 2001; Lilleskov *et al.*, 2002; Avis *et al.*, 2003).

Despite the emerging generalizations about the structure of EM assemblages, our understanding of the factors that determine EM assemblage structure remains rudimentary. Abiotic factors such as soil chemistry and soil moisture affect EM species composition (Erland & Taylor, 2002), but little is known about the effects of biotic interactions other than host specificity (Molina *et al.*, 1992). Of the potential biotic

factors, interspecific competition has long been considered to be an important factor in structuring natural assemblages, particularly for sessile organisms (Hutchinson, 1959; Connell, 1983; Schoener, 1983; Goldberg & Barton, 1992). While little is known about the occurrence of interspecific competition among EM fungi in nature, a number of recent experiments suggest that competition may play a significant role in EM interactions (Wu *et al.*, 1999; Jonsson *et al.*, 2001; Landeweert *et al.*, 2003; Lilleskov & Bruns, 2003).

There are only two studies of EM fungi that explicitly tested competition using single-species and two-species treatments. Wu *et al.* (1999) examined interspecific competition among three EM species colonizing *Pinus densiflora* and found that interactions were generally antagonistic but species dependent. In one species pairing, they found that mycelial overgrowth and root tip replacement of one species by the other occurred. In a second species pairing, there appeared to be mutual inhibition of mycelial spread, but no effect on root tip colonization. Landeweert *et al.* (2003) observed a significant increase in *Suillus bovinus* and decrease in *Paxillus involutus* in the two-species treatment, despite relatively constant densities of *P. involutus* in the single-species treatment through time. While these studies provide direct evidence of competition, more studies are needed to assess the frequency, intensity, and mechanisms of competitive interactions among EM fungi.

In this study, we examined EM competition between two *Rhizopogon* species grown in microcosms for 6 months. Using a microcosm approach allowed us to observe root tip colonization in real time. We focused on two species that naturally co-occur in overlapping niches in the *Pinus muricata* forests of coastal California: *R. occidentalis* and *R. salebrosus*. In earlier studies by T. Bruns and colleagues, *R. occidentalis* and *R. salebrosus* were referred to as *R. ochraceorubens*, and *R. subcaerulescens*, respectively (Gardes & Bruns, 1996; Horton *et al.*, 1998; Baar *et al.*, 1999; Taylor & Bruns, 1999; Grogan *et al.*, 2000), but internal transcribed spacer (ITS) sequences from holotype specimens have changed the nomenclature (Bidartondo & Bruns, 2002; Kjølner & Bruns, 2003). From earlier studies, we know *R. salebrosus* accounts for about 3% of the colonized roots in 35–45 yr-old mature *P. muricata* forests (the dominant EM fungus, *Russula amoenolens*, accounted for 27% of colonized roots in Gardes & Bruns (1996)), while *R. occidentalis* has never been encountered on roots of mature *P. muricata* trees. However, *R. occidentalis* does fruit at the borders and in small disturbed areas of the mature forest (T. Bruns, pers. obs.). Both species are common in the spore bank of forested areas (Kjølner & Bruns, 2003) and are codominants on seedlings following stand-replacing fire (Horton *et al.*, 1998; Taylor & Bruns, 1999). In addition, both species are frequently codispersed to the same places in rodent fecal pellets (T. Bruns, unpublished data). From these results and observations we can conclude that following disturbance, these two species are likely to be competitors for seedling root

colonization in nature, and that this competition is likely to be initiated by spore colonization.

Here we tested the interactions between *R. occidentalis* and *R. salebrosus* competing for seedling roots under one set of controlled conditions. Our purpose was to look for experimental evidence of competition, to examine the structure of this interaction, and to see if the outcome was correlated with observed natural patterns. Specifically, we expected that *R. occidentalis* might have an initial advantage, but that *R. salebrosus* would dominate over time.

## Materials and Methods

### Definition of competition

Here we define competition as the negative effects that one species has upon another by consuming, or controlling access to, a limited resource (Birch, 1957; Keddy, 1989). Since EM fungi require plant carbon to survive and roots comprise the site of nutrient exchange between the fungus and the plant, we will use occupation of roots as our measure of EM competitive performance.

### Experimental design

In spring 2003, seeds were collected from multiple *Pinus muricata* individuals at Pt. Reyes National Seashore in northern California. On 12 July 2003, the seeds were surface sterilized in hydrogen peroxide for 20 min and soaked in distilled water for 48 h. Following soaking, the seeds were planted onto sterile 2% water agar plates and germinated in a growth chamber with 14 : 10 h light:dark cycles and 85% relative humidity. Seedlings were grown for 58 d under sterile conditions and then transplanted into plexi-glass microcosms (20 × 10 × 0.5 cm) on 8 September 2003. The soil used in the microcosms was nonsterile and collected from a mixed scrub/grassland site on Tomales point (38°11.807' N, 122°57.736' W) within Pt. Reyes National Seashore. The site is 9 km from the nearest *P. muricata* individual, and the soil was determined to be free of *Rhizopogon* spores by bioassay (T. Bruns, unpublished data). The soil is classified as a Kehoe variant coarse sandy loam, which is a deep, well-drained soil derived from Quartz-diorite bedrock. It is similar to the soils in which the two *Rhizopogon* species naturally occur 15 km to the south. In early June 2003, *R. occidentalis* and *R. salebrosus* spores were added to the soil at  $9.1 \times 10^4$  spores ml<sup>-1</sup> of soil. The spores came from slurries from 17 separate sporocarp collections of each species at Pt. Reyes National Seashore. Each collection was made several meters apart, and all collections were typed by ITS-RFLP analysis to verify morphological species identifications. The same spore slurries were used for an ongoing spore longevity study. The viability of each species' slurry was quantified by serially diluting soils initially containing  $9.1 \times 10^4$  spores ml<sup>-1</sup> with sterile soil in

two-fold steps to a final concentration of  $0.17 \text{ spore ml}^{-1}$ , and then bioassaying each of the 19 dilutions with 12 replicate seedlings. The soils were air-dried and stored at room temperature before being used in the microcosms.

On 8 September 2003, seedlings were planted into three treatments: first soil with *R. occidentalis*; second soil with *R. salebrosus*; and third soil with both *R. occidentalis* and *R. salebrosus*. Before planting, the single-species treatments were mixed 1 : 1 with inoculated soil and field soil containing no *Rhizopogon* spores to ensure equal spore densities ( $4.55 \times 10^4$  spores  $\text{ml}^{-1}$  per species) across treatments. Fifteen replicates were planted for each treatment and five randomly selected replicates were harvested approximately 2 (27 November 2003), 4 (16 January 2004), and 6 (21 March 2004) months after planting ( $n = 45$  total). The seedlings were watered weekly to maintain high soil moisture and grown under the growth chamber conditions described above. Three weeks before the second harvest, there was a 7-d power outage in the growth chamber; however neither the seedlings nor the EM fungi appeared to be adversely affected. When harvested, seedlings were washed to remove adhering soil and examined under a dissecting microscope. All live EM root tips were removed from each seedling and flash frozen with liquid nitrogen. EM root tips were then lyophilized for 48 h and weighed. NonEM fine roots were also removed and dried at  $80^\circ\text{C}$  for 72 h and weighed. Mycorrhizal colonization was calculated as  $(\text{EM biomass} / (\text{EM biomass} + \text{nonEM fine root biomass})) \times 100$ .

We used molecular techniques to ascertain the identity of the species colonizing seedlings in the two-species treatment. Twenty randomly selected root tips were taken from each seedling in this treatment (one seedling had only 15 tips) and DNA was extracted using the Qiagen DNEasy tissue kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions. Twenty root tips ranged from five to 33% (mean = 18%) of the total EM biomass of a seedling. DNA was PCR amplified using the ITS 1F and four primer pair under conditions previously described (Gardes & Bruns, 1996). The PCR reactions were done in two rounds, so that if any of the 20 samples did not amplify, they were replaced with new root tips and then amplified. After two rounds, the mean positive amplification rate was 17 root tips (range: 15–20). The amplified DNA was imaged on 1% agarose gels. Because *R. occidentalis* and *R. salebrosus* have very different ITS sizes (> 100 base pairs), we were able to score the vast majority of the samples by eye. For those samples that were not clear, the amplified DNA was cut with the restriction endonuclease Hha 1, which had been previously shown to distinguish *R. occidentalis* from *R. salebrosus* (L. Grubisha, unpublished result). Restriction digests were imaged on 1.5% agarose gels and band size differences were determined by eye. The proportion of root tips belonging to each species was multiplied by the total EM biomass of that seedling to estimate the respective biomasses. In a small number of cases, root tips were colonized by both species and these tips were

counted equally towards the proportion of each species. We confirmed the identity of single-species treatments by randomly selecting two root tips and conducting the same analyses. In all cases, seedlings in the single-species treatments were colonized by the species with which it was originally inoculated.

In addition to sampling root tips, we had planned to analyze the mycelial dynamics of EM competition as well. To identify the mycelia of each species in the two-species treatment, we needed to use molecular methods because the mycelia of *R. occidentalis* and *R. salebrosus* were similar morphologically. We took multiple soil samples from each microcosm, extracted the total soil DNA with Mo Bio Ultra-Clean soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA), and PCR amplified the fungal DNA using the ITS 1F and four primer pair. In many samples, there was no evidence of any hyphae under a dissecting scope, however, those samples had positive PCR reactions, indicating that the DNA came from either very small hyphae or from the spores used for initial inoculations. Since *Rhizopogon* spores are known to have considerable longevity (Horton *et al.*, 1998; Kjølner & Bruns, 2003), we could not rule out the possibility that the DNA came from spores instead of mycelia and therefore we did not include these data.

### Statistical analyses

Over the course of the experiment, some seedling mortality occurred that appeared to be independent of treatment. As a result, we sampled the following number of seedlings at each time point: at 2 months we harvested five seedlings for each treatment, at 4 months we harvested four seedlings for the *R. occidentalis* treatment, and five seedlings from both the *R. salebrosus* and two-species treatment, and at 6 months we harvested five seedlings for the *R. occidentalis* treatment and the two-species treatment and four seedlings for the *R. salebrosus* treatment. During the last two harvests, we also found that five seedlings (two *R. occidentalis* seedlings, one *R. salebrosus* seedling, and two two-species treatment seedlings) were cocolonized with a *Thelephora* species. Because *Thelephora* colonization may have influenced the colonization and competitive dynamics of the *Rhizopogon* species, those seedlings were eliminated from all statistical analyses. Therefore the number of seedlings analyzed at 4 months was three in the *R. occidentalis* treatment, four in the *R. salebrosus* treatment, and five in the two-species treatment and at 6 months we analyzed four seedlings in the *R. occidentalis* treatment, five in the *R. salebrosus* treatment and three in the two-species treatment.

To evaluate the effects of competition on mycorrhizal colonization and root tip biomass, we used two three-way fixed factor analyses of variance (ANOVAs). The model predictor variables were treatment (single- vs two-species), time (2, 4, or 3 months), and species (*R. occidentalis* and *R. salebrosus*). Before running the ANOVAs, the mycorrhizal biomass data were  $\log(x + 1)$  transformed and the variances were determined to

be homogenous (Cochran's  $C$ -test,  $P > 0.05$ ). Post-hoc Tukey HSD tests were used for *a posteriori* comparisons among means. All statistical tests were performed using SYSTAT 10.2 (SYSTAT Software Inc., Richmond, CA, USA) and considered significant at  $P < 0.05$ .

## Results

Serial dilutions showed that the inoculum potential of *R. occidentalis* and *R. salebrosus* was very similar (Fig. 1). At concentrations above 2800 spore  $\text{ml}^{-1}$ , both species generally colonized 100% of the seedlings. At concentrations between 1400 and 43 spores  $\text{ml}^{-1}$ , EM colonization declined rapidly. The slopes of the lines of the two species between the inflection points were similar, and at concentrations below 43 spores  $\text{ml}^{-1}$ , seedlings were only rarely colonized (and then only by *R. occidentalis*). The concentrations at which half the seedlings were colonized appeared to be about the same for both species, but were difficult to estimate accurately due to variation in *R. salebrosus* colonization.

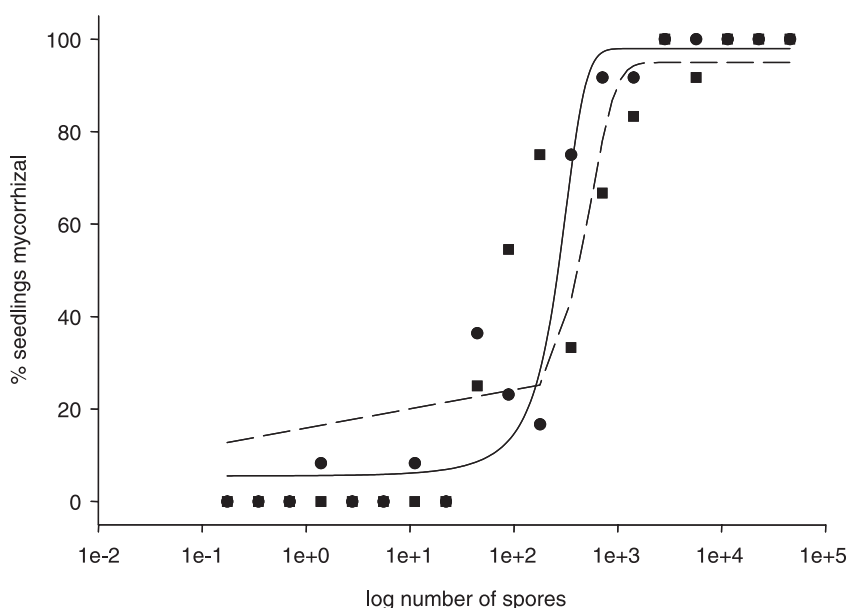
In the microcosm experiment, both the percentage colonization and the biomass of *R. salebrosus* began very low, but increased through time, while the percentage colonization and the biomass of *R. occidentalis* stayed relatively constant through time (% colonization species  $\times$  time interaction:  $F_{2,40} = 4.87$ ,  $P = 0.013$  (Fig. 2); biomass species  $\times$  time interaction:  $F_{2,40} = 4.211$ ,  $P = 0.022$  (Fig. 3)). *R. occidentalis* had no significant differences in percentage colonization or biomass between the single- and two-species treatment, while *R. salebrosus* had significantly lower percentage colonization and biomass in the two-species treatment than in the single-species treatment (% colonization species  $\times$  treatment interaction:  $F_{1,40} = 4.740$ ,  $P = 0.035$ ; biomass species  $\times$  treatment

interaction:  $F_{1,40} = 7.000$ ,  $P = 0.012$ ). There was also a significant increase in biomass of the single-species treatment through time (which was caused mostly by changes in *R. salebrosus*), while biomass in the two-species treatment stayed relatively constant (biomass treatment  $\times$  time interaction:  $F_{2,40} = 3.361$ ,  $P = 0.045$ ). None of the other higher-order interactions were significant.

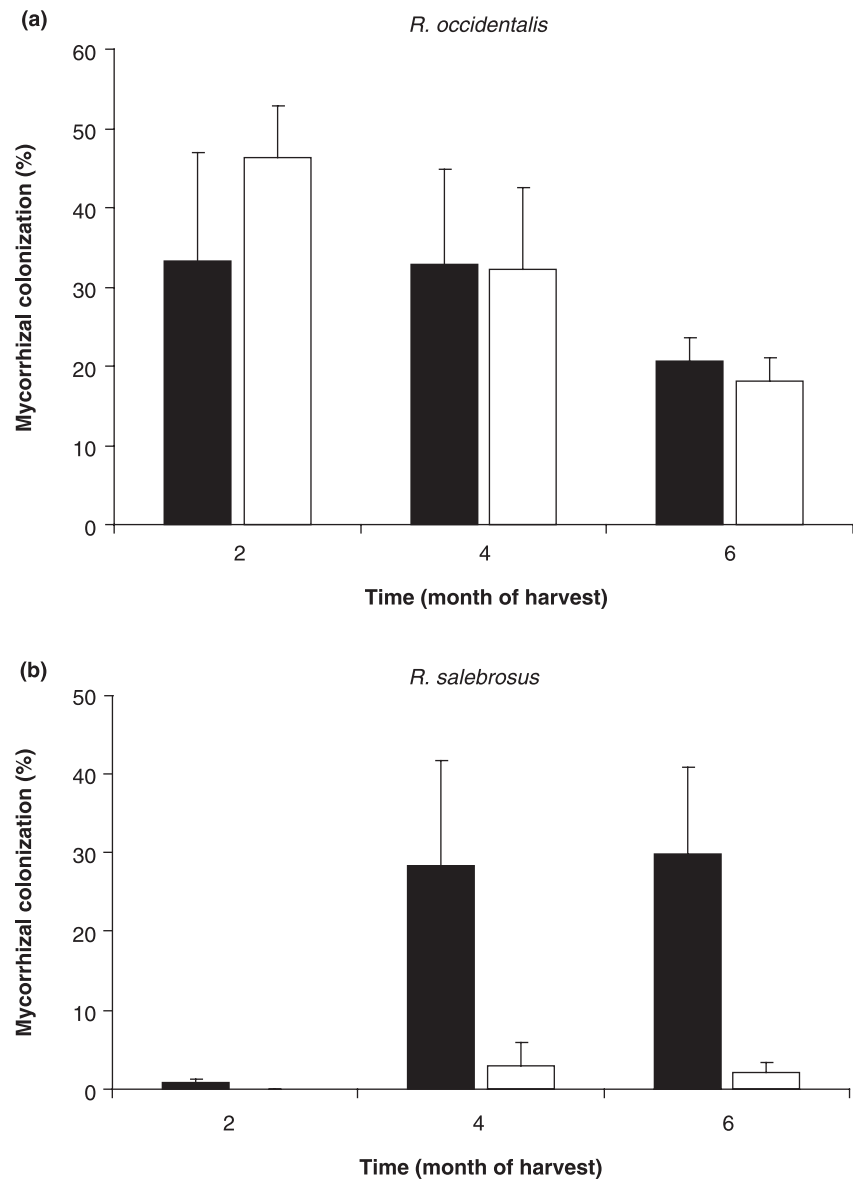
## Discussion

We found *R. occidentalis* quickly colonized seedlings in both the single- and two-species treatments. By contrast, *R. salebrosus* colonized seedlings more slowly in the single-species treatment and had significantly lower biomass and percentage colonization than *R. occidentalis* in the two-species treatment. These results suggest that the earlier colonization of *R. occidentalis* gave it a significant competitive advantage. The reason why *R. salebrosus* had a slower colonization response is not clear. *R. occidentalis* is the more ruderal of the two species and part of its success in disturbed settings may be due to the faster response of its spores. Different effective spore concentrations, however, are not a likely explanation, as the spore preparation of both species behaved similarly across a broad range of concentrations (Fig. 1). In addition, the high initial spore concentration used in the microcosm experiment was well into the upper plateau region of the colonization curves, and should therefore have insured high inoculum potential for both species. The fact that seedlings were well colonized in each of the single-species treatments also reinforces this view.

Competitive priority effects have been clearly documented among other sessile organisms including other types of fungi (Harper, 1961; Platt & Weis, 1985; Shorrocks & Bingley,



**Fig. 1** The percent mycorrhizal colonization of *Rhizopogon occidentalis* (circular points, solid curve) and *R. salebrosus* (square points, dashed curve) across a range of spore inoculum concentrations. The range represents a serial dilution initially containing  $9.1 \times 10^4$  spores  $\text{ml}^{-1}$  with two-fold steps to a final concentration of 0.17 spore  $\text{ml}^{-1}$ . Points represent the proportion of 12 seedlings colonized at each of the 19 dilutions.

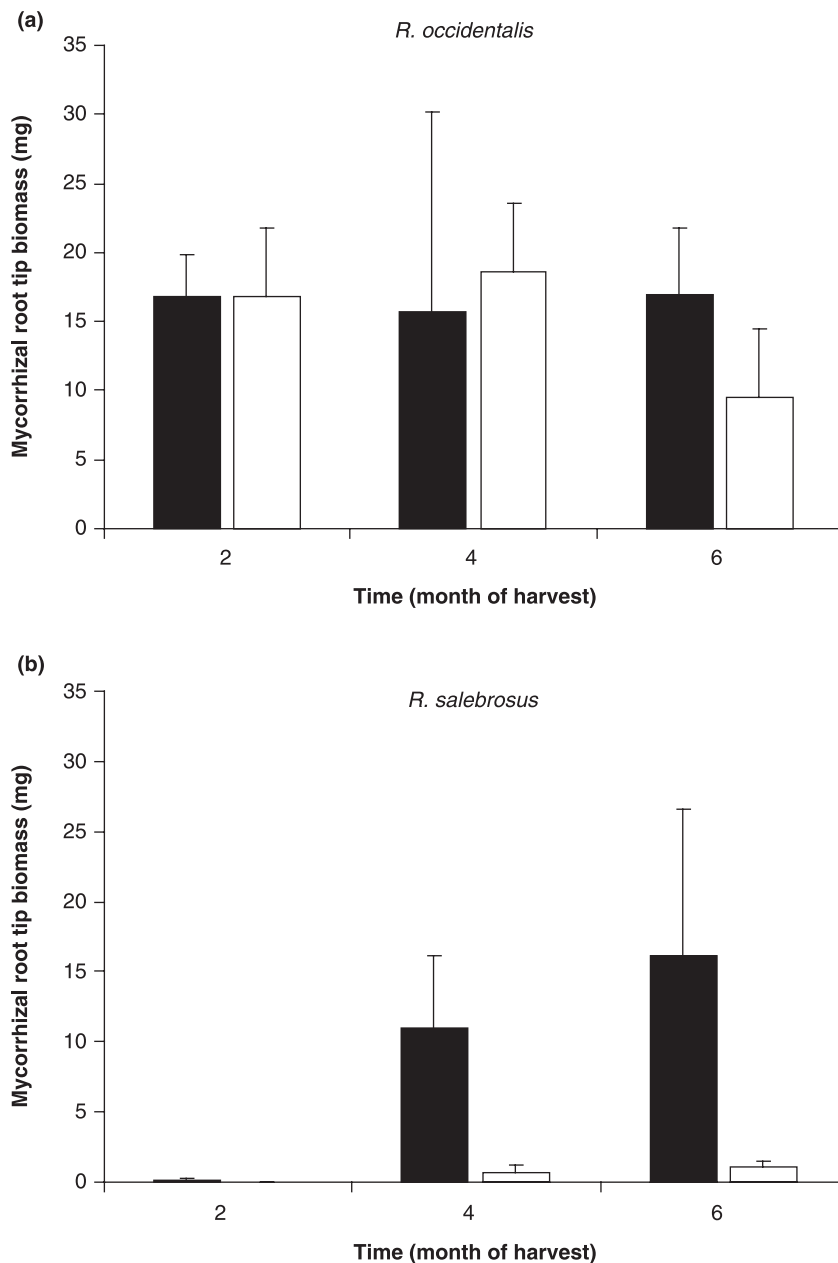


**Fig. 2** Mycorrhizal colonization (%) of (a) *Rhizopogon occidentalis* and (b) *R. salebrosus* in the single-species (filled bars) and two-species (open bars) treatments over the 6 months of the experiment. Bars represent the mean  $\pm$  1 SE.

1994, see Dix & Webster, 1995 for fungal examples), but their general role in EM competition is not yet known. The recent study by Lilleskov & Bruns (2003) found that 7 months after coinoculation, *Tomentella subulilacina* was the dominant species on *Pinus muricata* seedlings, despite high initial colonization by *Rhizopogon occidentalis*. Their results contradict what we observed because *T. subulilacina* displaced *R. occidentalis* over a similar time period to our experiment, although without a single-species treatment it is difficult to determine whether the change in *R. occidentalis* abundance was driven by competitive replacement or a decrease independent of the increase in *T. subulilacina*. Although we observed a strong priority effect, the mechanism by which it occurred is not yet clear. Suppression of *R. salebrosus* in the two-species treatment could have been caused by either direct antagonistic

interactions from *R. occidentalis*, such as sporophagy (Fries & Swedjemark, 1985) or mycelial overgrowth (Wu *et al.*, 1999), or indirect antagonistic interactions mediated by the plant (see Fig. 4 and below). Alternatively, differences in the timing of colonization of *R. occidentalis* and *R. salebrosus* could also have been driven by past competition (i.e. 'the ghost of competition past' *sensu* Connell, 1980), which would minimize the amount of present-day competition.

Despite the strong competitive effects of *R. occidentalis* on *R. salebrosus*, the resource for which they were putatively competing, that is seedling root tips did not appear to be limited in availability. Based on both the percent EM colonization and visual observation of seedlings in the two-species treatment, half or more of the root tips always appeared to be unoccupied and therefore available for colonization by the

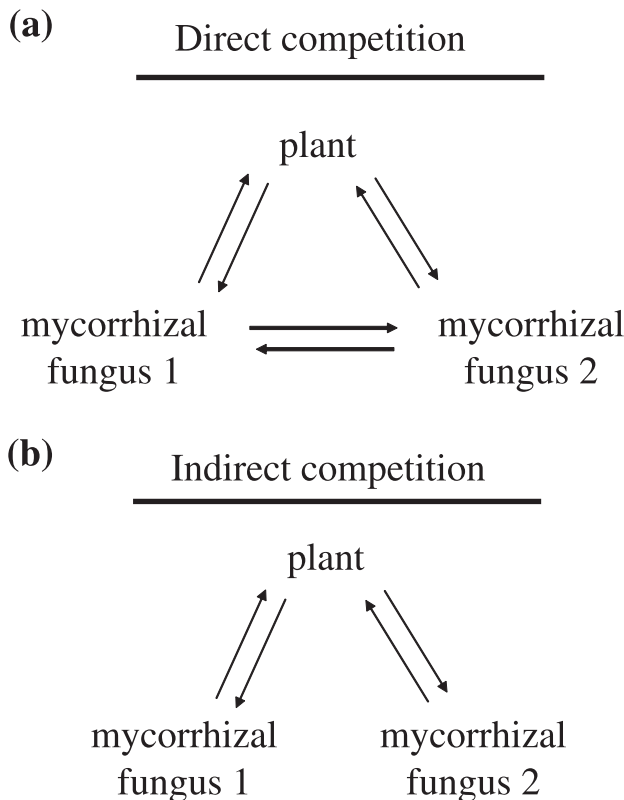


**Fig. 3** Mycorrhizal biomass of (a) *Rhizopogon occidentalis* and (b) *R. salebrosus* in the single-species (filled bars) and two-species (open bars) treatments over the 6 months of the experiment. Bars represent the mean  $\pm$  1 SE.

competitively inferior species (Fig. 2). Reasons for this lack of colonization are unclear but may be related to the type of competition occurring between EM fungi. Plants can vary their carbon input depending on the benefit provided by the fungi (Johnson *et al.*, 1997; Jones & Smith, 2004), which leads to two different types of competition that can occur between the obligately symbiotic fungi (Fig. 4). The first type is direct competition, where fungi compete for plant carbon via mechanisms such as alleopathy, sporophagy, or mycelial overgrowth (Marks & Foster, 1967; Fries & Swedjemark, 1985; Danielson & Visser, 1989; Wu *et al.*, 1999). Alternatively, fungi may compete indirectly, such that they only interact

through plant carbon allocation. The colonization pattern we observed suggests seedlings may be able to decrease the mycorrhizal receptivity of other roots to minimize below-ground carbon loss if they are getting sufficient nutrients from a portion of the root system that is colonized. While there have been no direct studies of this phenomenon, the ability of plants to decrease colonization in high nutrient settings indicates that they may have substantial control over root tip colonization and hence EM competitive dynamics.

If EM competition occurs primarily through indirect interactions and plants can differentially reward the fungi, differences in nutrient uptake and transfer to plants among



**Fig. 4** A conceptual diagram of the two types of competition among mycorrhizal fungi. Direct competition (a) involves antagonistic interactions through both the plant and with other fungi. Indirect competition (b) involves antagonistic interactions only through the plant. Currently there is no experimental evidence that differentiate these two types of competition.

EM species should be a major factor determining the winner of competition. If plants reward the most beneficial fungi with more carbon, one would predict that the best fungal competitors are the species that provide the greatest benefit to the plant. Alternatively, fungi may be able evade the plant's reward system or competition could occur directly between fungi and therefore not strongly mediated by plant feedbacks. In these cases, the plant would be forced to associate with the best fungal competitor, independent of its nutritional benefit to the plant (see Hoeksema & Kummel, 2003) for theoretical discussion of this scenario). We did not see any clear differences in percentage shoot nitrogen of seedlings grown in the different treatments (data not presented), but this similarity was not too surprising given our comparison of EM congeners that may have similar nutrient foraging strategies and uptake abilities (Agerer, 2001). The seedlings were also putatively not nutrient-limited, which one would expect would weaken the feedback between choice of fungal partner and the nutrients they provide the plant. Future studies examining competitive interactions among EM species with different nutrient uptake abilities (of either the same or different nutrients) will help

resolve the ways in which plants affect, and are in turn affected by, EM competitive interactions.

Competition may be one of the most important, yet poorly studied, processes in EM assemblages judging from the pivotal role of competitive interactions in structuring many other organismal assemblages and from the growing evidence of the existence of competition among EM fungi. We recognize that in diverse and patchily distributed assemblages such as those of EM fungi (Dahlberg, 2001; Horton & Bruns, 2001), factors operating at small spatial scales (e.g. interspecific interactions on root tips) may not have the same impact on assemblage structure at larger spatial scales (e.g. forest stands and above). At these larger scales, spatially and temporally variable rates of colonization and resource renewal (e.g. host seedling recruitment) may strongly influence species coexistence (e.g. Sousa, 1990). However, our study provides a clearer understanding of the nature of EM competition at the small scale, which is an essential step towards assessing the general role of competition in the structuring of natural EM assemblages.

### Acknowledgements

The authors would like to thank M. Bitartondo for assistance with microcosm set-up and maintenance, K. Shamieh for assistance with molecular analyses, J. Kennedy, M. Palomino, K. Peay, A. Pringle, members of the labs of T.D. Bruns and W.P. Sousa, UC Berkeley, and three anonymous reviewers for constructive comments on previous versions of this manuscript. Support for this work was provided by a National Science Foundation (NSF) graduate research fellowship to P.G. Kennedy, a NSF doctoral dissertation improvement grant (DEB 0309152) to P.G. Kennedy, T.D. Bruns, and W.P. Sousa, and NSF grant DEB 0236096 to T.D. Bruns.

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