

Determining the outcome of field-based competition between two *Rhizopogon* species using real-time PCR

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Abstract

Interest in the ecology of ectomycorrhizal (ECM) fungi has increased considerably, but little is known about interspecific interactions among ECM species. We examined competitive interactions between *Rhizopogon occidentalis* and *R. salebrosus* at Point Reyes National Seashore, California, USA. At three field sites, species abundances were compared in single- and two-species treatments on *Pinus muricata* seedlings inoculated with spores. Competition for root tips was assessed using real-time polymerase chain reaction (PCR) of internal transcribed spacer rDNA. In general, we found strong competitive exclusion of *R. salebrosus* by *R. occidentalis*, with $\geq 75\%$ of the seedlings in the two-species treatment colonized exclusively by *R. occidentalis* after 5 and 10 months. However, on the seedlings that were co-colonized, we observed no significant difference in the abundances of *R. salebrosus* and *R. occidentalis*, suggesting that once *R. salebrosus* was established, it was no longer competitively inferior. There were no significant differences in survival, growth, or percentage leaf nitrogen of seedlings colonized with either *Rhizopogon* species, but both growth and percentage leaf nitrogen were significantly higher for ECM than non-ECM seedlings. We also observed strong positive correlations between actual ECM root tip weight and that inferred from real-time PCR for both species, indicating that this method provided an accurate assessment of root tip occupation and hence ECM competitive dynamics. In conjunction with a previous experiment, our results indicate that competition between these two *Rhizopogon* species occurs similarly in both field and laboratory settings and that when colonizing from spore, timing largely determines the outcome of initial competitive interactions.

Keywords: ectomycorrhizal fungi, interspecific interactions, spore, TaqMan

Received 1 July 2006; revision accepted 7 October 2006

Introduction

Ectomycorrhizal (ECM) fungi play an important role in the ecology of many ecosystems through their influence on carbon and nutrient cycling, soil formation and structure, below-ground food webs, and plant community dynamics (Van der Heijden & Sanders 2002 and references therein). Interest in ECM ecology has increased tremendously over the past 20 years, much of which can be attributed to a greater recognition of the importance of ECM fungi by plant ecologists and the widespread implementation of

molecular techniques that have facilitated identification of the below-ground component of the ECM symbiosis (Horton & Bruns 2001). The latter factor in particular has allowed researchers to characterize many ECM assemblages, which have consistently been shown to be highly diverse, with many rare species and few dominants (Dahlberg 2001; Horton & Bruns 2001).

Although our glimpse in the 'black box' that ECM fungi have long occupied has revealed a number of striking patterns, the field of ECM ecology has long lagged behind its counterparts in plant and animal ecology. Arguably, the largest reason for this discrepancy is the difficulty in manipulating ECM fungi in ways that allow for the direct examination of species interactions. Unlike plant and animal

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assemblages, ECM assemblages cannot be selectively weeded or fenced because fungi grow cryptically below ground and often lack species-distinguishing features in their hyphal or root tip morphologies. Additionally, spores of most ECM fungi are notoriously difficult to germinate (Miller *et al.* 1993 and references therein), making artificial inoculations of most species unfeasible. A third factor that has hampered fungal ecologists is that when seedlings inoculated with one ECM species are out-planted in natural settings, native ECM species typically quickly replace the inoculated species on seedling roots, making field-based studies of specific host-fungal combinations difficult (Bledsoe *et al.* 1982; Villeneuve *et al.* 1991).

As a result of these factors, much of the work to date on ECM species interactions has been done in greenhouse or growth chamber settings. In these artificial environments, ECM species can be inoculated onto plants by culture (or in some cases by spore slurry) and different aspects of the symbiosis can be studied. These studies have collectively provided considerable insight into the nature of ECM interactions, particularly in how different ECM species affect host plant performance (Smith & Read 1997). For many years, however, both fungal and plant ecologists have questioned the applicability of laboratory studies for ECM ecology in the field. The call for manipulative field experiments (e.g. Read 2002) has resulted in a growing number of studies examining ECM ecology outside the laboratory. The recent work of Nara (2006), which examined the effects of common mycorrhizal networks on early seedling performance, provides an excellent example of this new approach.

In this study, we had two goals. The first was to determine if the results we obtained in a laboratory-based study of competition between ECM fungi would be similar when conducted in a field setting. In the laboratory study, we found that two species of *Rhizopogon*, *R. occidentalis* and *R. salebrosus*, exhibited strong competition when colonizing the roots of young *Pinus muricata* D. Don (Bishop Pine) seedlings (Kennedy & Bruns 2005). *R. occidentalis*, which colonized the *P. muricata* seedlings before *R. salebrosus*, was able to competitively exclude *R. salebrosus* from subsequent colonization in the two-species treatments. Our results suggested that priority effects may be an important component of ECM species interactions, but their importance in a field setting, where additional variables such as fluctuating abiotic conditions, presence of fungal herbivores, and greater host plant stress (all of which could potentially alter the outcome of ECM competition) was not clear.

Our second goal was to test the use of real-time TaqMan polymerase chain reaction (PCR) to assess the outcome of ECM competition. Real-time PCR has been used to study many microbial systems (Mackay 2004), but only recently has it also been used to study ECM fungi (Landeweert *et al.* 2003; Schubert *et al.* 2003; Raidl *et al.* 2005; Parlade *et al.* 2006). This technique offers these two primary advantages

over other molecular methods: (i) the direct quantification of DNA template from unknown samples, and (ii) the ability to independently quantify multiple DNA templates from mixed samples through species-specific amplification. The latter factor is particularly significant in experiments where more than one species is present in the same sample. For example, Landeweert *et al.* (2003) used real-time PCR on mixed species soil samples to demonstrate that *Suillus bovinus* had a negative competitive effect on *Paxillus involutus*, when both species were co-inoculated onto *Pinus sylvestris* seedlings in a growth chamber environment.

To address our objectives, we took advantage of a study area in which the presence and composition of ECM fungi could be naturally manipulated. At Point Reyes National Seashore, California, the *P. muricata* forests are located directly adjacent to coastal scrub/grasslands (Fig. 1). No plants in the scrub/grassland matrix associate with ECM fungi, so scrub/grassland soils are generally free of ECM inoculum at distances greater than 200 m from mature *P. muricata* forest (T. Bruns, unpublished data). By planting *P. muricata* seedlings in distant scrub/grassland sites, we could naturally manipulate the presence of ECM fungi. We chose to use two ECM species, *R. occidentalis* and *R. salebrosus*, that normally colonize seedlings by spores following disturbance and therefore could be experimentally added to seedlings via spore slurries. Both of these species are among the first and most common ECM fungi-colonizing seedlings in the scrub/grassland and are often codispersed by small rodents (Horton *et al.* 1998; T. Bruns, unpublished data), indicating that they are likely to be natural competitors when colonizing seedling roots from spores in coastal *P. muricata* forests.

Materials and methods

Study system

Point Reyes National Seashore is located in Marin County, California, USA (38°04'N, 122°50'W). The area has a Mediterranean climate, with approximately 80% of the annual precipitation (range 80–160 cm per year) falling from November to March. Annual average air temperatures are between 11 °C and 14 °C, with average summer temperatures around 18 °C. Coastal soils are predominately in the Pablo-Bayview complex, an inceptisol formed from siliceous shale or sandstone. In October 1995, a wildfire burned ~5000 ha of forest, scrub, and grassland at Point Reyes, which has led to a major encroachment of *Pinus muricata* into the coastal scrub/grassland matrix (Fig. 1).

Experimental design

In November 2004, three coastal scrub/grassland sites were located in the vicinity of Limantour beach (38°02.695'N,

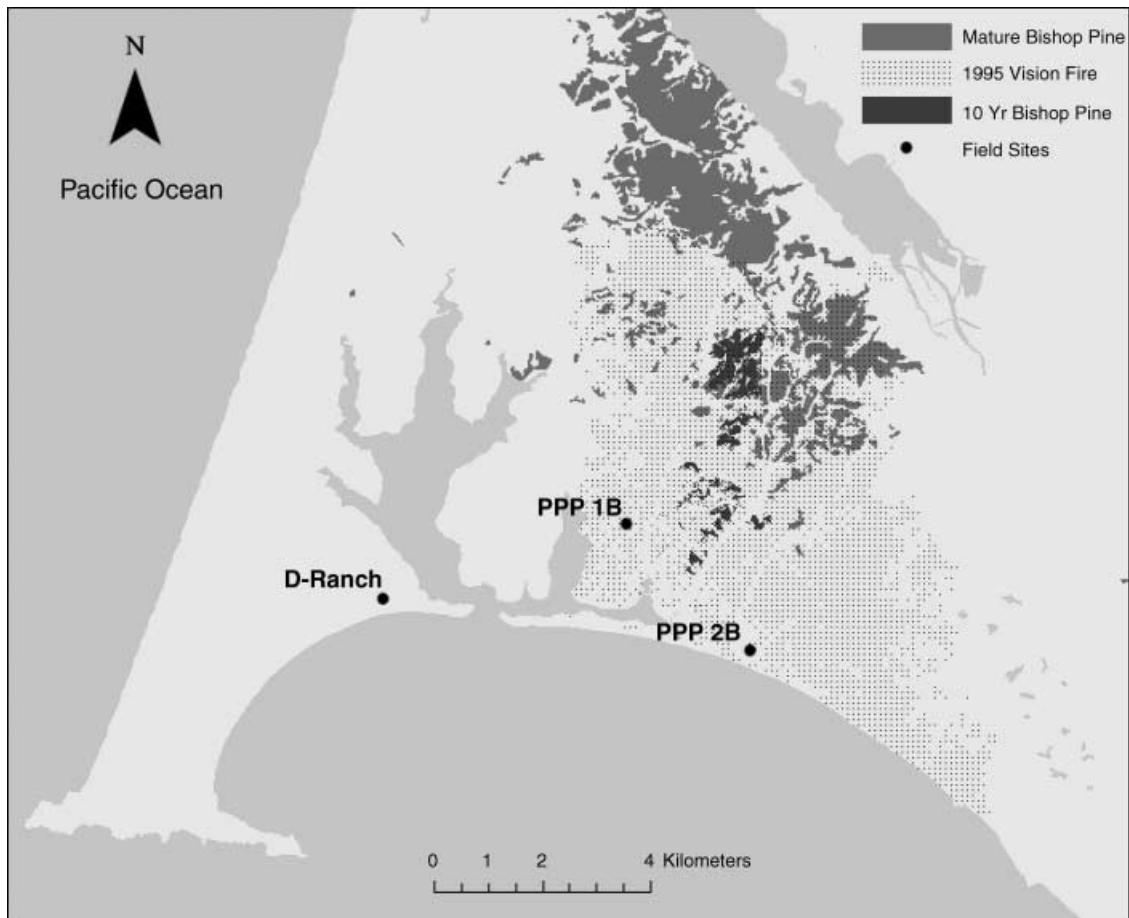


Fig. 1 Study area at Point Reyes National Seashore, Marin County, California (38°02'N, 122°53'W).

122°53.905'W) (Fig. 1). The sites were separated by 3–5 km, located > 2 km from the previous mature forest, and beyond the area of current forest encroachment (at least 75 m from the nearest *P. muricata* sapling). Soil samples from all three sites were previously bio-assayed for ECM inoculum following the methods of Rusca *et al.* 2006, but without diluting the sample. No detectable ECM inoculum was found at any of the three sites. At each site, an 80 m² area was established and 80 seed plots were marked at 1 m intervals. The 80 plots were randomly assigned to one of four treatments: no ECM inoculum, *Rhizopogon occidentalis* inoculum, *R. salebrosus* inoculum, and inoculum of both ECM species ($n = 20$, $N = 240$). In each plot, we planted 20 *P. muricata* seeds, which were surrounded by a 15-cm diameter cage constructed of 1.25 cm diameter metal mesh (Pacific Steel and Supply) to prevent herbivory and trampling by large mammals. In February 2005, spore slurries containing 5×10^8 spores for each species were added to the plots (the two-species plots received 1×10^9 spores total). The slurries were made from multiple sporocarp collections of each species at Point Reyes using

methods previously described, with all sporocarps typed by restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region before inclusion (Kennedy & Bruns 2005). Plots were periodically checked for seed germination and then weeded to one *P. muricata* individual in early April 2005. Five months after spore addition in July 2005, five seedlings in each treatment were harvested from all three sites ($n = 60$). Survival of the remaining 15 seedlings was censused monthly for the duration of the experiment and all surviving seedlings were harvested in December 2005.

Seedling sampling and ECM identification

We attempted to harvest the maximum amount of the root system of the seedlings. To do so, seedlings were removed from each plot with 235 cm³ of the surrounding soil and brought back to the laboratory within 4–6 h. Seedlings were soaked in distilled water for 24–48 h at 4 °C and then the root systems were gently washed over a 0.355-mm sieve. All of the harvested root system was examined under a 10×

dissecting microscope and all ECM fine roots were removed (range: 5 to > 200 tips per seedling). To obtain dry weights, ECM root tips were flash-frozen in liquid nitrogen and then lyophilized while seedling roots and shoots were dried at 55 °C for 3 days.

We observed that a small proportion of the control seedlings (i.e. those that received no inoculum) were colonized by ECM fungi at both sampling dates. ECM fungi were present on four of the 15 noninoculated seedlings at the first sampling and eight of the 34 seedlings at the second sampling, respectively. To identify the fungi that had colonized those seedlings, two representative ECM root tips from each seedling were removed (all were colonized by only one morphotype) and DNA was extracted using the methods described in Kennedy *et al.* (2003). ECM DNA was amplified and sequenced using the primer pair ITS 1f and ITS4 in conditions previously described (Gardes & Bruns 1996) and visualized on an ABI 3100 Genetic Analyser (Applied Biosystems). Sequences for all 12 colonized seedlings had 98–99% GenBank BLAST affinities to *Suillus pungens*. To test whether *S. pungens* had colonized any of the seedlings that had been inoculated with *Rhizopogon*, we analysed DNA of the ECM root tips of all of the seedlings in the three *Rhizopogon* treatments with terminal restriction length polymorphism (T-RFLP) analysis. All of ECM roots of each seedling were bulk-extracted using an UltraClean Soil DNA kit following the manufacturer's instructions (MoBio Laboratories). Samples were PCR-amplified using the same ITS primer pair, with a 6-FAM fluorophore attached to the ITS 1f primer. The PCR conditions were identical to the nonfluorescent PCR above, except primer concentrations were 25 µM instead of 50 µM. One microlitre of each PCR product was then digested using 0.25 µL of *Hinf*I, 1.0 µL of 10X buffer, and 7.75 µL of dH₂O for 3 hours at 37 °C. From prior analyses, we knew that a single enzyme digest was sufficient to distinguish all test species and other common species in our system (T. Bruns, unpublished data). Digested PCR product was cleaned and then analysed on an ABI 3100 Genetic Analyser with a GS500 ROX ladder (Applied Biosystems). Fragment lengths were estimated using GENESCAN 3.1.2 and GENOTYPER 2.5 software (Applied Biosystems). The fragment lengths of each sample were compared to those of sporocarp samples of *R. occidentalis*, *R. salebrosus*, and *S. pungens*. For all of the positively amplified samples in the three *Rhizopogon* treatments, no T-RFLP peaks matching *S. pungens* were present. Expected fragment lengths of both *Rhizopogon* species were observed in the appropriate samples from each treatment and there were occasional additional peaks, but none matching *S. pungens*.

Real-time PCR assays

To assess the abundance of *R. occidentalis* and *R. salebrosus* in the three mycorrhizal treatments, we used real-time PCR

Taqman assays. This approach was necessary because ECM root tips of the two species are not readily distinguished, and although each tip could be individually typed, the quantitative PCR approach allowed us to estimate species composition for a bulked sample from each seedling. The primers and probes were based on ITS rDNA sequences from the *Rhizopogon* database presented in Rusca *et al.* (2006). We used the ITS region because of the high among-species variation and much lower within-species variation in sequence, which allowed us to design the species-specific primers and probes needed for real-time PCR assays (Landeweert *et al.* 2003; Schubert *et al.* 2003; Raidl *et al.* 2005). Forward and reverse primers as well as a fluorescently labelled probe for each species were designed using PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi), with the following specifications: product size 50–90, optimal 60; primer size 18–27, optimal 20; primer Tm 58–60, optimal 59; Hyb oligo size 21–30, optimal 24; Hyb oligo 67–70, optimal 68. For *R. occidentalis*, the forward and reverse primer and probe sequences were (forward: 5'-AGGTCTGCATGGTTTTGGTA-3', reverse: 5'-AGACATAGGTACTTTGTCCTTTCC-3', probe: 5'-6FAM-CAGAACTGTGCTAGAGCCTCTCTCTCTGC-BHQ1-3'), and for *R. salebrosus* (forward: 5'-ACGTCCCCTGCCTCTAA-TAC-3', reverse: 5'-AAAGTCAATAAGGAAGACCCCTGTT-3', probe: 5'-JOE TCGACCGCTTATTATCTCTCCGGAGAG-BHQ1-3).

PCRs were run on an ABI PRISM 7300 detection system (Applied Biosystems). All reactions were conducted in multiplex reactions (i.e. primers and probes of both species were present), which were previously determined not to differ from simplex (i.e. single-species) reactions in DNA yield (P. Kennedy, unpublished data). We also observed no fluorescence of nontarget template in all samples of the single-species treatments, indicating no cross reactivity between the primers and probes of the two species. Each 15 µL multiplex reaction contained 1 × TaqMan IQ Supermix (Bio-Rad), each primer at 400 nM, and each probe at 200 nM. For the environmental samples, reactions also contained 1 µL of 1:1000 diluted ECM root tip DNA and 5.96 µL of sterile de-ionized water. For the standard samples, reactions contained 1 µL of serially diluted ECM root tip DNA for each species (see below) and 4.96 µL of sterile deionized water. PCR conditions were 10 min at 95 °C, 40 cycles at 95 °C for 15 s and 1 minute at 58 °C, and then a hold step of 30 s at 4 °C. The 58 °C annealing temperature was experimentally optimized prior to analysing the samples. On each 96 well plate, all samples and standards were run in three independent reactions. The data were analysed with the baseline set from cycles 3–15 and the threshold manually placed in the exponential phase of the reaction. Due to differences in fluorophore intensity, the thresholds were placed at 10 000 in the log Δ fluorescence per cycle plot for *R. occidentalis* and 4000 for *R. salebrosus* on each plate.

Table 1 Standard curve data from the five real-time PCR plates used to assess the outcome of competition between *Rhizopogon occidentalis* (RO) and *Rhizopogon salebrosus* (RS). Slope is calculated from C_t values at each of the six tenfold dilutions. PCR efficiency was calculated according to Smith *et al.* (2006) as $[(10^{1/[\text{slope}]} - 1)] \times 100$

Plate	Species	Slope	Intercept	R-square	PCR efficiency (%)
1	RO	-3.35	19.17	0.999	98.88
1	RS	-3.46	20.47	0.998	94.51
2	RO	-3.38	19.07	0.998	97.71
2	RS	-3.44	20.47	0.994	95.41
3	RO	-3.40	18.95	0.996	96.72
3	RS	-3.31	20.77	0.996	100.61
4	RO	-3.45	18.99	0.999	95.15
4	RS	-3.40	20.73	0.996	96.80
5	RO	-3.43	19.02	0.994	95.56
5	RS	-3.50	20.63	0.998	93.07

To infer the quantity of roots belonging to each species, we developed independent regressions between known ECM root weight and C_t values for both species (C_t is the cycle number at which the fluorescence value exceeds the background fluorescence and crosses the threshold) similar to the approach of Parlade *et al.* (2006). We produced the regressions using bulked tips from one sample of each of the single-species treatments as a standard. We chose the two standards based on their intermediate and nearly identical bulked ECM root tip weights (0.206 g for *R. occidentalis* and 0.207 g for *R. salebrosus*). Each of the standard samples was diluted at tenfold intervals from 10^{-1} to 10^{-6} and the C_t values for each dilution were obtained. The relationships between the C_t values and the log of the corresponding ECM root tip weights were then used as standard curves (Table 1) from which the C_t values of the environmental samples could be converted into units of ECM root tip weight.

Although there were strong correlations between the predicted and actual root tip weights in both of the single-species treatments (Fig. 2), the predicted weights were consistently lower than the actual weights. Furthermore, the underestimation also varied between species, with the slope of the regression for *R. occidentalis* being two times steeper than that of *R. salebrosus*. To correct for this difference, we divided the predicted root weights by the slopes of the single-species regressions. We confirmed that this corrected the underestimation by summing the new weights (referred to as inferred root weight) of each species on the co-colonized seedlings and comparing them with the actual ECM root tip weights. The slope of the regression was 1.05, indicating inferred weights and actual weights matched very closely. Therefore, we divided all of the predicted root weights in

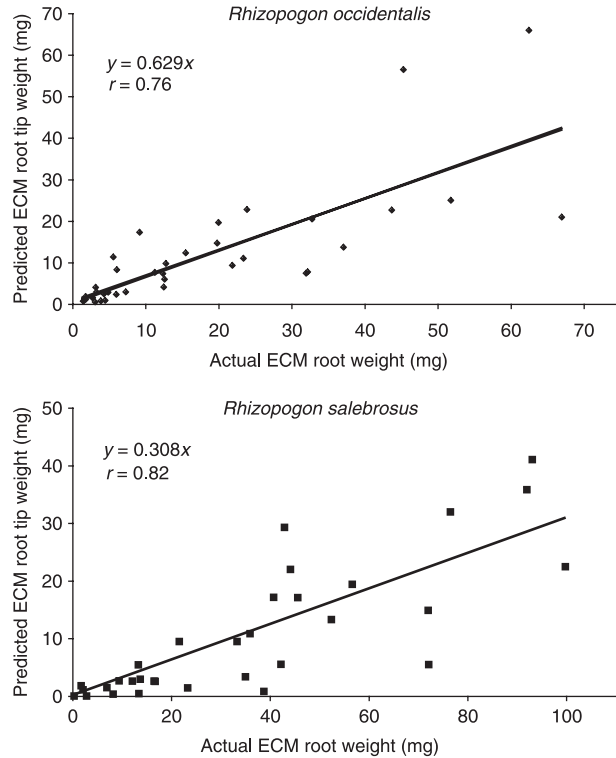


Fig. 2 Relationships between predicted and actual ectomycorrhizal (ECM) root tip weight based on real-time PCR of ITS rDNA for *Rhizopogon occidentalis* and *Rhizopogon salebrosus*. Predicted root weights were calculated by converting the C_t value of each sample to root weight based on standardized regressions between C_t values and the log of known root tip weights (see methods and Table 1 for further details). Inferred weights for each species are calculated by dividing the predicted weights of by the species-specific slopes of the regressions, show above.

each treatment by the species-specific slopes and used inferred root weights for all analyses.

Other paired weight samples could have been used as standards, but we were only interested in the relative differences between species, so absolute DNA concentration was not important. More significantly, using these root tip samples as standards meant that the standards and environmental samples had identical PCR conditions (i.e. both contained both fungal and plant material), so the reaction efficiencies and therefore quantification should be very similar. Visual assessment of the fluorescence-cycle curves of the standards and samples indicated that they were indeed very similar in terms of shape and height. Alternatively, aliquots of pure mycelial cultures in combination with nonmycorrhizal plant root DNA could also have been used as standards, but the ratio of fungal to plant DNA in ECM root tips is not well defined, so we felt colonized root tips provided the best option in terms of representing the PCR reaction conditions of our samples.

Statistical analyses

We used an analysis of covariance (ANCOVA) to determine if the slopes of the standard curves across all five plates were similar for both species ($n = 10$). PCR efficiencies were compared between plates and species using a two-way fixed-factor analysis of variance (ANOVA). For all analyses of ECM root weight, we multiplied all the inferred weight estimates by 1000 to account for the 1:1000 dilution and used the average weight of the three independent reactions run for each sample. The relationship between predicted and actual ECM root weight for each of the single-species treatments was quantified using Pearson's correlation coefficient. Due to differences in sample size and ANOVA model design, we analysed the outcome of ECM competition at each sampling date separately. For the July data, we pooled seedlings across sites (to account for the low number harvested per site) and compared inferred root weights using a two-way fixed-factor ANOVA, with treatment (single- and two-species) and species (*R. occidentalis* and *R. salebrosus*) as the predictor variables. For the December data, we used a three-way mixed-model ANOVA, with treatment (fixed), species (fixed), and site (D Ranch, PPP 1B, PPP 2B) (random) as the predictor variables. We also compared the inferred root weights of each species on the 10 co-colonized seedlings in the two-species treatment using a paired *t*-test. A two-way log-linear model was used to analyse seedling survival, with site and competition treatment as the predictor variables. Total seedling weight was analysed using a two-way mixed-model ANOVA, with site (random) and treatment (fixed) as the predictor variables. Seedling percentage leaf nitrogen was analysed with a Friedman's test (the nonparametric equivalent of a two-way ANOVA), with site and competition as the predictor variables. Because of their mycorrhizal status, the 12 noninoculated seedlings colonized with *S. pungens* were excluded from all analyses. Variances were checked prior to all analyses using Cochran's *C*-test and transformed when necessary to meet the assumptions of homogeneity. If variances still remained heterogeneous after transformation, a nonparametric test was used. Tukey

HSD tests were used for all a *posteriori* comparisons of means. Analyses were conducted using JMP 5.0 (SAS Institute) or SYSTAT (SYSTAT Software), and considered significant at $P < 0.05$.

Results

The slopes of all of the standard curves were very similar between species ($t = 0.107$, d.f. = 60, $P > 0.50$), with the r^2 of all fits > 0.99 (Table 1). PCR efficiencies were also very similar between plates and species, ranging from 93 to 100% (Plate: $F = 1.089$, d.f. = 1, $P = 0.331$; Species: $F = 0.249$, d.f. = 1, $P = 0.633$) (Table 1). There was however, a significant difference in the intercepts of the standard curves, with *Rhizopogon salebrosus* having a higher intercept than *Rhizopogon occidentalis* ($t = -6.309$, d.f. = 57, $P < 0.001$) (Table 1).

Strong competitive interactions were observed in the two-species treatment at both sampling dates. In July, only 3 of the 15 seedlings (20%) were colonized by both species, with the other 12 (80%) being colonized exclusively by *R. occidentalis*. Similarly, in December, 7 of the 31 (23%) were colonized by both species, with the other 24 (77%) colonized exclusively by *R. occidentalis*. At neither sampling date were any of the two-species treatment seedlings colonized exclusively by *R. salebrosus*. In July, *R. occidentalis* showed no significant differences in inferred root weight between treatments, but *R. salebrosus* had significantly lower weights in the two-species treatment than in the single-species treatment (species \times treatment interaction: $F = 4.465$, d.f. = 1,42, $P = 0.041$) (Fig. 3). Similarly, in December, *R. salebrosus* had significantly lower inferred root weights in the two-species treatment at two of the three sites, while *R. occidentalis* had no significant difference in weights between treatments at all sites (species \times site \times treatment interaction: $F = 4.331$, d.f. = 1,110, $P = 0.015$). However, when comparing only the 10 seedlings co-colonized in the two-species treatment, the inferred root weights were not significantly different between species ($t = 0.24$, d.f. = 9, $P = 0.815$) and five of the 10 seedlings had a greater abundance of *R. salebrosus* (Table 2). This indicates that although *R. salebrosus* was typically

Sample	RO inferred weight (mg)	RS inferred weight (mg)	Proportion RO	Proportion RS	Dominant
3862	0.17	0.26	0.40	0.60	RS
4719	1.27	4.09	0.24	0.76	RS
3547	1.24	2.21	0.36	0.64	RS
3880	5.20	3.86	0.57	0.43	RO
4770	4.85	4.09	0.54	0.46	RO
3869	16.69	26.79	0.38	0.62	RS
3893	53.07	5.84	0.90	0.10	RO
3872	68.81	2.60	0.96	0.04	RO
3535	19.52	13.57	0.59	0.41	RO
3954	4.94	83.60	0.06	0.94	RS

Table 2 Ectomycorrhizal (ECM) weights on the co-colonized *Pinus muricata* seedlings in the two-species treatment. RO represents *Rhizopogon occidentalis* and RS represents *Rhizopogon salebrosus*. See methods for the details about calculating inferred root tip weights

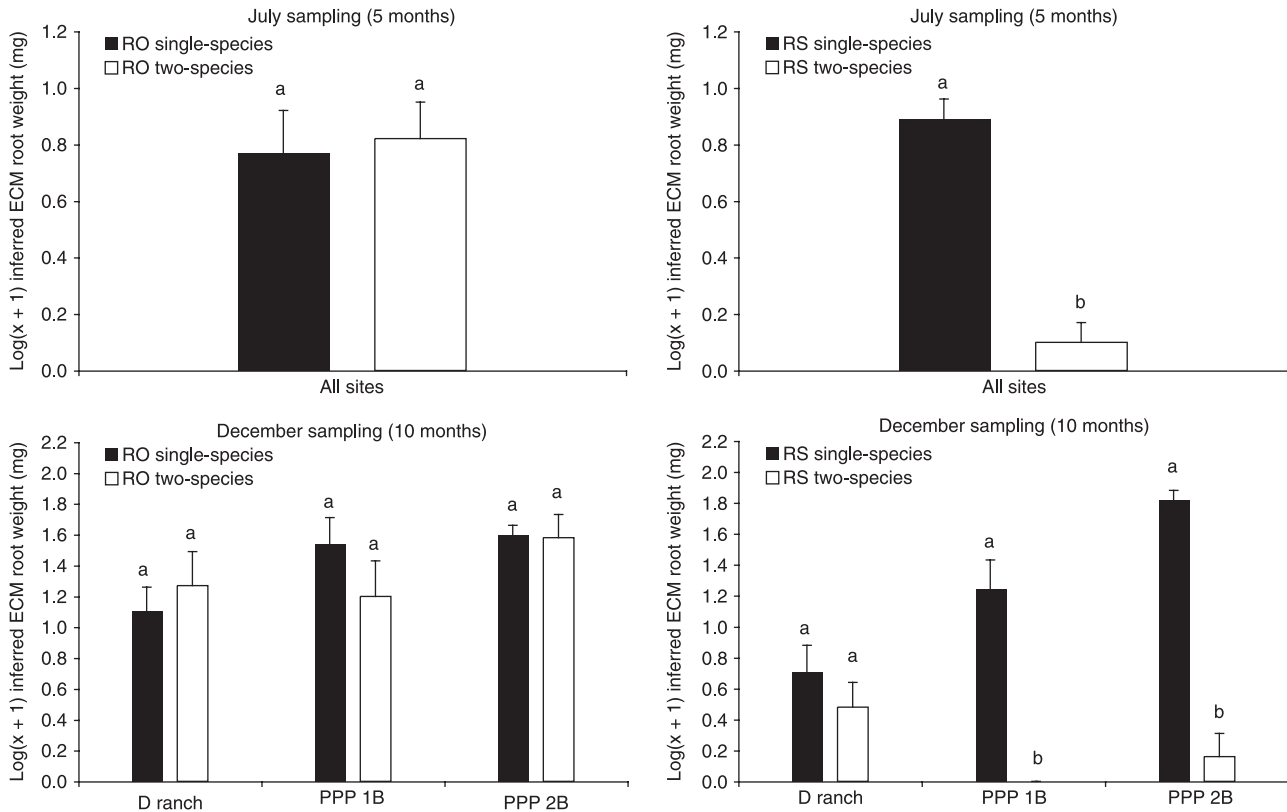


Fig. 3 Inferred ectomycorrhizal (ECM) root tip weights of *Rhizopogon occidentalis* and *Rhizopogon salebrosus* in the single- and two-species treatments in July and December 2005. See methods for details about calculating inferred weight. Bars represent means plus one standard error. Different letters indicate significant differences at $P < 0.05$. Note that the significant decreases in the weights of *R. salebrosus* in the two-species treatments at both dates indicate the negative effects of competition with *R. occidentalis*

Table 3 Performance of *Pinus muricata* seedlings in the coastal scrub/grassland at Point Reyes National Seashore, California. Treatments are noninoculated (NM), single-species *Rhizopogon occidentalis* (RO), single-species *Rhizopogon salebrosus* (RS), and two-species (RO/RS). Values are means with one standard error in parentheses. Different letters indicate significant differences at $P < 0.05$

Parameter	Site			Treatment			
	D Ranch	PPP 1B	PPP 2B	NM	RO	RS	RO/RS
Survival (alive/total)	0.94(0.02)	0.80(0.08)	0.72(0.07)	0.72(0.14) a	0.86(0.04) a	0.86(0.10) a	0.84(0.11) a
Total weight (g)	0.17(0.02) a	0.18(0.02) a	0.28(0.03) b	0.14(0.03) a	0.254(0.03) b	0.212(0.02) b	0.206(0.02) ab
Leaf nitrogen (%)	1.63(0.09) a	1.16(0.11) a	1.57(0.07) a	0.88(0.18) a	1.87(0.09) b	1.87(0.09) b	1.90(0.08) b

competitively excluded from colonizing seedlings by *R. occidentalis*, on those seedlings that it did colonize, it was not necessarily the competitively inferior species.

Pinus muricata seedling survival did not vary significantly across the four treatments ($\chi^2 = 2.645$, d.f. = 3,163, $P = 0.449$), although it was on average 10% lower in the no-inoculum treatment than the three ECM treatments. Survival did differ significantly among sites ($\chi^2 = 2.645$, d.f. = 2,163, $P = 0.007$), being highest at D ranch, intermediate at PPP 1B, and lowest at PPP 2B (Table 3). In July, seedling weights were equivalent among the four treatments, but by Decem-

ber, seedlings were significantly larger in the two single-species treatments than the no-inoculum treatment, with the two-species treatment being intermediate ($F = 4.099$, d.f. = 3,122, $P = 0.008$) (Table 3). In December, seedling weights also varied by site, being significantly larger at PPP 2B than D ranch and PPP 1B ($F = 5.355$, d.f. = 2,6, $P = 0.046$), but there was no site \times treatment interaction ($F = 1.280$, d.f. = 6,122, $P = 0.271$). Finally, percentage leaf nitrogen was significantly different by treatment but not site, being significantly lower in the no-inoculum treatment than the three ECM treatments (D ranch: $F = 16.733$,

d.f. = 3, $P = 0.001$; PPP 1B: $F = 10.650$, d.f. = 1, $P = 0.014$; PPP 2B: $F = 9.30$, d.f. = 3, $P = 0.026$ (Table 3).

Discussion

We found *Rhizopogon occidentalis* typically excluded *Rhizopogon salebrosus* from colonizing *Pinus muricata*, as evidenced by the complete absence of *R. salebrosus* on greater than 75% of the two-species treatment seedlings at both sampling dates. In contrast, competition had little negative effect on *R. occidentalis* throughout the duration of the experiment. These results match closely with those of our previous laboratory experiment (Kennedy & Bruns 2005). In that experiment, we observed that *R. occidentalis* spores germinated faster than *R. salebrosus*, which resulted in earlier colonization by *R. occidentalis* on the two-species treatment seedlings and subsequent competitive exclusion of *R. salebrosus*. The differences in timing of colonization occurred after 2 months in the laboratory experiment, which we did not capture here due to the longer intervals of sampling. However, the legacy of this timing effect was present at both sampling dates and our results indicate that timing of colonization can significantly affect ECM composition on seedlings for at least 10 months. This duration of time is important because the first-year seedling stage is often critical in terms of growth and survival and different ECM fungi have been shown to differentially affect seedling performance (Bledsoe 1992; Nara 2006).

Surprisingly, the competitive dynamics we observed here and in our previous laboratory experiment contrast with the natural patterns of *Rhizopogon* abundance in the mature *P. muricata* forests at Point Reyes. In those forests, *R. salebrosus* is present but not *R. occidentalis* (Gardes & Bruns 1996). So how does this shift in composition occur if *R. salebrosus* is the competitive inferior when colonizing seedling root tips? The data from the co-colonized seedlings show that when *R. salebrosus* establishes on *P. muricata* roots with *R. occidentalis*, it can occupy similar or greater amounts of ECM root tip weight than *R. occidentalis* (Table 2). This suggests that once *R. salebrosus* colonizes a portion of the seedling root system, it is not necessarily competitively inferior to *R. occidentalis*. In ongoing laboratory studies, we have observed that *R. salebrosus* produces more rhizomorphs than *R. occidentalis*, which may make *R. salebrosus* a more effective mycelial competitor (P. Kennedy, unpublished data). If *R. salebrosus* can reach uncolonized tips faster via mycelial spread and/or directly replace *R. occidentalis* on already colonized tips (see Wu *et al.* 1999 for examples of both with other ECM species), then mycelial-based competition may result in a different outcome than competition based on spores. This is similar to the patterns seen in classic successional studies of ECM fungi (Deacon & Fleming 1992). We are currently investigating the differences between spore and mycelial competition in two experiments, one where

the timing of colonization is reversed between *R. salebrosus* and *R. occidentalis* (i.e. spores of *R. occidentalis* are added to pots with seedlings already colonized by *R. salebrosus* to see if they can also colonize), and a second experiment where seedlings are precolonized with each species individually and then combined into the same microcosm to examine direct mycelial interactions.

The difference in competitive outcomes between spores and mycelia is likely related to the types of competition occurring, with that from spores being driven by exploitation competition and that from mycelia being dominated by interference competition (Begon *et al.* 1996). This distinction may help explain why *R. occidentalis* is such a good competitor with *R. salebrosus* from spores. On young seedlings, there are fewer available tips. Consequently, faster germination of *R. occidentalis* leads to its colonization of almost all the receptive root tips. When the resource (i.e. root tips) is limiting, the competitive effect of *R. occidentalis* is very strong, since there are few if any resources left for *R. salebrosus*. However, as the seedling root system increases in size, many root tips are likely to encounter areas where *R. occidentalis* spores are absent. If *R. salebrosus* spores are present in these areas, they could colonize the available root tips and the strong competitive effect from *R. occidentalis* would be eliminated (since the resource would no longer be limited by *R. occidentalis*).

An important caveat to this scenario is that available uncolonized root tips are still receptive to colonization. In our previous laboratory experiment, we observed that many two-species treatment seedlings that were colonized with *R. occidentalis* had additional uncolonized root tips, but those tips did not become colonized by *R. salebrosus*. In high nutrient settings, ECM colonization usually decreases, suggesting that plants may be able to prevent fungi from colonizing when they are not cost-effective (Wallenda & Kottke 1998). In our system, if the resources received from *R. occidentalis* are sufficient for *P. muricata* seedlings, then *R. occidentalis* may still exert a significant competitive effect on *R. salebrosus* even when additional root tips appear to be available for colonization, by limiting access to plant carbon and effectively making uncolonized root tips unavailable (see Kimmel & Salant (2006) for a related discussion of plant choice of ECM fungi).

We observed no significant differences in seedling growth, survival, or percentage leaf nitrogen among the three ECM treatments, suggesting that *R. occidentalis* and *R. salebrosus* provided *P. muricata* with similar resources. This result supports the idea that the timing of colonization can have a major effect on competitive outcome when symbionts are functionally similar. Many studies have, however, shown that different ECM species are not equivalent in terms of nutrient allocation to their hosts (Bledsoe 1992; Nara 2006); thus future studies examining a wider range of species may find that priority effects are less significant than we

observed. In fact, the studies of Lilleskov & Bruns (2003) and Landeweert *et al.* (2003) both indicate that timing of colonization does not always determine the outcome of ECM competition. Lilleskov & Bruns (2003) showed that despite high initial colonization by *R. occidentalis*, *Tomentella subtilacina* became the dominant species on the same *P. muricata* seedlings after 7 months. Similarly, Landeweert *et al.* (2003) found that after equal initial colonization of *Suillus bovinus* and *Paxillus involutus* on *P. sylvestris* seedlings, their abundances changed in contrasting directions through time.

In general, we found that real-time PCR provided a reliable and robust method to assess ECM competition. We did, however, observe significant differences between species in the C_t values of the standard dilutions. A number of factors such as variation in PCR kinetics, DNA template quality or quantity, and ITS copy number between species could have caused the differences. Nonetheless, we believe inferred root weights were still directly comparable between species because the weights are calculated as a relative measure for each species based on its own standard curve and then adjusted for species-specific differences in the relationship between predicted and actual ECM root tip weight. *R. occidentalis* and *R. salebrosus* have very similar root tip morphologies, but this approach to calculating inferred root tip weight should also allow for comparisons between ECM species with very different root tip morphologies or hyphal exploration types as well (see Agerer 2001). However, the applicability of real-time PCR to studying ECM competition in other systems will require further testing to determine its broad-scale suitability.

We conclude that competition can be a significant factor affecting the ECM assemblage patterns observed on seedlings in both field and laboratory studies. Our study adds to a growing body of empirical literature supporting this assertion (Wu *et al.* 1999; Landeweert *et al.* 2003; Lilleskov & Bruns 2003; Kennedy & Bruns 2005; Koide *et al.* 2005) and suggests that a greater understanding of the mechanisms by which competition occurs will help identify the factors that determine ECM assemblage structure.

Acknowledgements

We thank P. Boynton, E. L. Bruns, N. Hynson, M. Palomino, N. Rosenstock, and A. Simonin for field and laboratory assistance; Point Reyes National Seashore for use of the land; and K. Peay for assistance with the production of Fig. 1. J. Parlade is greatly acknowledged for his help in facilitating the visit of S. Hortal to UC Berkeley. A. Izzo, K. Peay, M. Palomino and three anonymous reviewers provided constructive comments on an earlier draft of this manuscript. Financial support for this project was provided by a grant from the Andrew W. Mellon Foundation to the National Parks Ecological Research Fellowship, a partnership between the National Park Service, the Ecological Society of America and the National Park Foundation (P.G. Kennedy), the Gordon and Betty Moore Foundation (awarded to Matteo Garbelotto in support of

S.E. Bergemann), Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya and the European Social Fund (S. Hortal) and NSF grant DEB 0236096 (T.D. Bruns).

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