

Inoculum potential of *Rhizopogon* spores increases with time over the first 4 yr of a 99-yr spore burial experiment

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Summary

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- In disturbed or pioneer settings, spores and sclerotia of ectomycorrhizal fungi serve as the necessary inoculum for establishment of ectomycorrhizal-dependent trees. Yet, little is known about the persistence of these propagules through time.
- Here, live field soil was inoculated with known quantities of basidiospores from four pine-associated species of *Rhizopogon*; these samples were then buried in retrievable containers, and pine seedling bioassays of serially diluted spore samples were used to measure spore viability.
- In the first 4 yr, no evidence of loss of spore viability was found in the four *Rhizopogon* species tested, but all four species exhibited dormancy in which a maximum of 1–8% of their spores were initially receptive to pine roots. There were some differences between species in overall inoculum potential of their spores, but all species broke dormancy at a statistically similar rate.
- This result provides evidence for spore dormancy in a common ectomycorrhizal genus, but it also precludes our ability to estimate the longevity of the spores accurately. Nevertheless these results, coupled with the observed patterns of *Rhizopogon* spore banks, suggest that at least decade-long durations are likely. As this experiment progresses, the true longevity of the spores will eventually be revealed.

Introduction

Spores are the primary means by which fungi disperse across barriers to their vegetative growth and establish new individuals. Fungi have evolved many ways to disperse their spores through the air or water, or through the use of vectors such as insects, or vertebrates (Ingold, 1971; Maser *et al.*, 1978). However, barriers to continued growth occur in time as well as space. For example, as a resource is exhausted, or as better competitors move in, a habitat may become unavailable to the fungus that first occupied it. If this resource recurs over time, then the ability to wait as a resistant propagule may be the most effective way to rapidly colonize a newly available resource.

Plants, faced with the same problem of dispersal through time, have evolved resistant seeds that can spend decades in the soil seed bank until conditions favor germination and re-establishment (Thompson, 2000; Telewski & Zeevaert, 2002). Similarly, spore-forming organisms, such as bacteria (Henis, 1987), myxomycetes (Elliot, 1948), oomycetes (Henis *et al.*, 1987), bryophytes (Jonsson, 1993; Sundberg & Rydin, 2000), ferns (Dyer, 1994; Ranai, 2003; del Ramirez-Trejo *et al.*, 2004), and fungi (Henis *et al.*, 1987), all include species with spores that retain viability for lengthy periods and germinate when conditions once again become suitable. Among the true fungi, the best-studied examples of long-lived propagules come from the plant pathology literature. For example, teliospores of *Urocystis cepulae* and resting sporangia

of *Synchytrium endobioticum* are thought to last > 25 yr in soil (Thaxter, 1890; Putnam & Sindermann, 1994), but propagules of most other pathogenic fungi have shorter recorded durations (Henis *et al.*, 1987). However, these reports, and others like them, tend to be based on anecdotal evidence and constitute minimum estimates because they are based on observations of disease recurrence in agricultural fields that have not been planted to a particular crop for some period of time, rather than experimental determination of an end point to spore viability. Thus, quantitative parameters, such as half-lives or average spore viability rates, have not been rigorously estimated for fungal spores.

Among mycorrhizal fungi, the longevity of spores is not well known, but it is likely to have a direct impact on the resilience of mycorrhizal dependent plants. For example, many important tree species require mycorrhizal inoculum for successful growth and establishment. Because mycorrhizal fungi are also obligate mutualists, this sets up a difficult 'chicken and egg' problem for colonization of mycorrhizal dependent plants into a system that lacks active mycorrhizal symbiosis (Terwilliger & Pastor, 1999; Weber *et al.*, 2005). In such systems spore longevity would be one way to broaden the effective time window for rare spore dispersal events or to ensure that the symbiosis can be re-established even if all active partners have previously died out. There are reasons to suspect that at least some species of ectomycorrhizal fungi may produce propagules that rival or exceed the longevity of pathogenic fungal species. The first work on mycorrhizal succession showed that 'early stage' fungi had spores that were capable of colonizing seedlings under experimental conditions (Fox, 1983), and that their spores were apparently present in soil, as disturbances that severed roots and mycelia selected for this set of species (Fleming, 1984). Subsequent work by Ishida *et al.* (2008) showed that early successional fungi in the willow-dominated Mt Fuji system tend to have spores that germinate readily when stimulated by host roots. In western North America, spores of some *Rhizopogon* species exhibit similar behavior, but they differ in that their spores are mammal-dispersed, while those species examined by Fox (1983), Fleming (1984) and Ishida *et al.* (2008) are wind-dispersed. Miller *et al.* (1994) showed that spores of *Rhizopogon* can be recovered from the soil and appear to be viable after a year in soil, while Ashkannejhad & Horton (2006) showed that *Rhizopogon* spores retain viability in fecal pellets for at least a year.

There is good evidence that *Rhizopogon* spore banks are widespread in pine-dominated California forests (Taylor & Bruns, 1999; Kjølner & Bruns, 2003; Izzo *et al.*, 2005; Rusca *et al.*, 2006) and are important sources of inoculum for seedlings following stand-replacing fire (Baar *et al.*, 1999). Furthermore, species of *Rhizopogon*, such as *R. occidentalis* and *R. olivaceotinctus*, that are rare or absent as mycorrhizas or as fruiting bodies in old-growth forests are nonetheless common in spore banks in these same settings (Izzo *et al.*, 2005). These same two species were shown to colonize equal

or greater numbers of seedlings after soil was heated to 75°C. This was in contrast to propagules of several other genera that were reduced or eliminated after heat treatment (Izzo *et al.*, 2006b). From these studies, we hypothesize that at least a subset of *Rhizopogon* species have spores that retain their viability for decades. However, all of the existing data are based on natural spore banks in which the age and density of spores are not known.

To test the longevity of *Rhizopogon* directly, we initiated a long-term spore burial experiment similar to long-term seed experiments (Telewski & Zeevaert, 2002). We inoculated known concentrations of spores into live soils that lacked them, and buried these inoculated soils in terracotta flowerpots, so that they could be retrieved and assayed over time. Here we report the first 5-yearly intervals of this experiment.

Materials and Methods

Spore collections

Spore slurries were made on 4 February 2003 for *R. occidentalis*, *R. vulgaris* and *R. salebrosus*, using six collections of each. Unused portions of these collections were deposited as voucher collections at the University of California Herbarium (UC): UC1860234–UC1860242, UC1860244–UC1860253. The portions of six collections used all had mature spores as judged by gleba color, were collected from areas separated by at least 20 m, and were typed by RFLP analysis with restriction enzymes *HinfI* and *CfoI* to confirm species identities (Methods S1). For each species, several mature fruitbodies were selected from each collection, pooled, pulse-ground in a blender with *c.* 500 ml of distilled water for *c.* 1 min, filtered through multiple layers of cheesecloth, and then diluted to 1 l. Spore concentrations were measured with a hemacytometer, and the resulting slurries were stored at 5°C until used 2–3 wk later.

Site selection and description

To assay longevity of *Rhizopogon* spores, we placed them under natural soil conditions in a form that could be retrieved over time. Live field soil was necessary because soil microflora and fauna are likely agents for turnover of the soil spore banks, and we wanted them to be active during the assay. However, because *Rhizopogon* spore banks are so abundant and common in California (Kjølner & Bruns, 2003; Izzo *et al.*, 2006a; Rusca *et al.*, 2006), we needed to identify a source of soil that was naturally devoid of *Rhizopogon* spores, but was similar to soils that harbor them. To this end, live field soil from Tomales Point, a peninsula in Point Reyes National Seashore, was sampled in April 2002 at five locations: 38°11.800'N, 122°57.737'W; 38°12.462'N, 122°58.003'W; 38°12.963'N, 122°58.461'W; 38°13.478'N, 122°58.939'W; and 38°14.051'N, 122°59.220'W. The soils are classified as

coarse sandy loams with typical depths to the quartz-diorite parent material of 100–150 cm. They are very similar to soils found under nearby native pine forests, from which they differ primarily by slope (Anonymous, 1985). Naturally occurring inoculum of *Rhizopogon* was not expected to be present at these sites, because they are grasslands, located on a narrow peninsula bordered by the Pacific Ocean and Tomales Bay. The first, and closest, location sampled was c. 9 km from the nearest single pine tree and several kilometers further to the nearest forest. For these reasons, both small and large mammal dispersal from the pines was deemed unlikely. To test for naturally occurring inoculum, soil was sampled at each site using a surface-sterilized trowel to extract five cores c. 10 cm deep \times 10 cm in diameter. The five cores were taken along a single line with approx. 1 m spacing. We combined soils from all five cores of a single plot. One hundred seedlings (20/site \times five sites) were planted in 55 ml soil samples in individual 'conetainer' pots (RLC-4 Super 'Stubby' Cell Cone-tainer from Stuewe & Sons Inc., Corvallis, OR, USA) and grown for 6 months in a glasshouse. Three of the 100 seedlings were found to have some ectomycorrhizal colonization. Root tips from these positive samples were collected, extracted for DNA, the internal transcribed spacer region was amplified with primers ITS 1f and 4B (Gardes & Bruns, 1993), and the amplicons were directly sequenced using previously described protocols (Kjøller & Bruns, 2003). BLASTn searches revealed that all sequences belonged to an undescribed *Suillus* species. This species has been found fruiting elsewhere and is being described (T. D. Bruns & E. C. Vellinga, unpublished). As no *Rhizopogon* inoculum was detected, and the only detected inoculum was low, the closest site, which we refer to as TP1 (Tomales Point 1, 38°11.800'N, 122°57.737'W), was selected as the source of all soil for our subsequent experiments, and for the burial location for inoculated test samples.

Preparation of spore samples

A total of 88 l of soil was collected from the TP1 site and mixed with 8 l of autoclaved coarse sand to improve drainage. For each of the three initial test species of *Rhizopogon* – *R. occidentalis*, *R. vulgaris*, *R. salebrosus* – 2.5×10^8 spores were sprayed onto 28 l portions of soil/sand and thoroughly mixed in separate batches. Samples of 1.6 l of these single-species treatments were placed into 16 terracotta flowerpots, 6.5" in diameter. Before filling the pots, the bottom holes were covered with glass microscope slides to prevent soil from leaking out, and a numbered metal tag was added to each to allow unequivocal identification. The tops of the pots were covered with 7.5" terracotta saucers and these were secured with plastic cable binders. In addition to single-species pots, live soil from Muddy Hollow Knoll (38°2.736'N, 122°52.136'W) was added to another 16 pot series. This soil was known to contain spores of *Rhizopogon* along with many

several other taxa of mycorrhizal fungi, but the starting quantities and age of this inoculum were not known. Results from this part of the experiment will be discussed in a separate paper, as the complexity of the community is greater and the identification of the fungi involved is more complex.

The 64 pots containing the three single-species treatments and the forest soil treatment were buried to a depth of 15 cm at the top of the pot lids at the TP1 site. One pot from each treatment was buried at the corners of a meter square; this was our sample unit for a given year. The order of the pots was randomized across the 16 units, and sampling order for these units was also randomized (Supporting information, Fig. S1). The experiment was set up to provide flexible sampling over time. For example, if spore longevity was found to be decreasing rapidly then the 16 blocks could be sampled for 16 consecutive years. If, instead, little or no decrease in viability was observed then the sampling could be stretched out for up to 99 yr by sampling five consecutive years, followed by 15 yr of sampling every 5 yr, and 80 yr of sampling every decade, as shown (Fig. S1).

Bioassays

Seed source for the bioassays was from native, local *Pinus muricata*, the natural host for these species and isolates of *Rhizopogon*. Mature cones were collected from native trees along Limatour road in Point Reyes National Seashore and opened by placing them on a small food dryer overnight. Seeds released from the cones were de-winged, surface-sterilized with 30% H₂O₂ for 20 min, rinsed in distilled water, and three seeds were planted in each test pot. After successful germination, seedlings were thinned to one per pot.

We assayed spore viability at each time point with pine seedling bioassays. Because it was unclear at what spore concentration differences in viability would be detectable, we used twofold, serially diluted soil samples to conduct the bioassays. Each twofold soil dilution was assayed for *Rhizopogon* spores with 12 pine seedlings planted in separate conetainer pots with 55 ml of test soil. Because this volume was less than that necessary to fill the pots, a few centimeters of coarse sand were layered in the bottom and top of the pots so that the 55 ml of test soil provided a continuous layer in the central three-quarters of each pot. To perform these assays, soil was collected from the TP1 site, mixed with coarse sand 11 : 1, autoclaved for 45 min, mixed thoroughly and autoclaved for another 45 min, cooled to ambient temperature, and used in the above serial dilutions of the inoculated test soil.

Test soil retrieved from the buried pots was twofold serially diluted with the sterilized soil-sand mix by placing 55 ml of spore-containing test soil and 55 ml of sterile soil-sand mix into a quart Mason jar and shaking vigorously until the soils were well mixed. A sample of 55 ml of this mixed soil was added into the conetainer pot, and the volume of soil in the Mason jar was brought back up to 110 ml with addition of

sterile soil. The process was repeated for a total of 20 twofold dilutions spanning the range of 8.9×10^4 to 1.7×10^{-1} spores ml^{-1} . In addition, 20 control seedlings were planted in the sterile soil mix for each series for a total of 260 seedlings (20 dilutions \times 12 replicates + 20 control seedlings) per time point per species. Seedlings were grown in the glasshouse with watering but no fertilization. After 6 months, seedlings were removed from the containers, soil was washed from the roots, and the root systems were examined under a dissecting microscope (magnification $\times 10$). Seedlings were scored as colonized or not by *Rhizopogon*; this was easy to determine because root tips colonized by *Rhizopogon* are white, often coralloid or densely branched, fluffy, and with many rhizomorphs. Samples with questionable identities and up to 20 putative *Rhizopogon* tips selected from each species series were preserved in CTAB buffer. RFLP analysis was conducted on these samples as described earlier for the fruiting bodies, and all were confirmed to be correctly identified.

Similar analyses of *Rhizopogon olivaceotinctus* were conducted, but we did not produce inoculated soil until 2006 because that was the first year the species was found fruiting at Point Reyes. The *R. olivaceotinctus* samples differed in two other ways. Unlike the more common species, we were unable to find multiple collections, so the spore slurries were produced from fruitbodies that were all collected in close proximity to one another (i.e. from a single collection), and could have been derived from a single individual. In addition a power of 10 error was made when measuring the spore slurry; this resulted in 100-fold lower concentration of the spores in the buried soil samples. We identified our error before the first dilution series and cut back on the number of dilutions so that they spanned a reduced but similar range of concentrations, from 8.9×10^2 to 2.2×10^{-1} spores ml^{-1} . Colonized tips of *R. olivaceotinctus* are much darker and not always as clustered as the other *Rhizopogon* species, and so we had to confirm many more samples by RFLP analyses in both years, all of which were correctly identified.

Statistical analysis

We used a two-step approach to test for the effects of burial time and species identity on spore viability. Because serial dilutions from a single pot are not independent experimental units, we first used multiple logistic regression to characterize the relationship between spore concentration and seedling colonization and to extract from this a single estimate of spore viability for each pot harvested. Logistic regression is appropriate for presence/absence data when the goal is to predict the number of successes in a given number of trials (e.g. the number of seedlings colonized at a given spore concentration). For each *Rhizopogon* species, we set up an independent logistic regression model to predict the number of seedlings colonized at each level of the dilution series and at each time point. The number of spores in the dilution series

was included as a quantitative predictor variable and the effect of years buried estimated as a factor (no interaction was included in the model). For each species, we then used the estimated year effect from the logistic regression as an integrated measure of inoculum potential for each time point (the year effects are equivalent to the intercept of the logistic regression for each year). The second step of our analysis was to use multiple linear regression to test for the effects of burial time, species identity and their interaction on changes in inoculum potential. This approach allowed us to incorporate all of the data from the dilution series into our estimate of spore viability for each pot without pseudoreplication. We also conducted the same analysis using the predicted number of spores required to colonize 50% of the bioassay seedlings for each dilution series as an alternative measure of inoculum potential. The logistic regression models were fitted with maximum-likelihood techniques using the glm procedure in R, version 2.6.1 (R Development Core Team, 2007), with a logit link function and binomial errors. Spore concentrations were natural log-transformed to reduce model overdispersion. Multiple regression models were fitted using the lm procedure in R. Effects were considered significant at $P < 0.05$.

Results

We found the test samples to be easy to retrieve and assay by the procedures outlined earlier. The terracotta flowerpots held up well for the first 4 yr, and the loose-fitting lids and bottom hole allowed mesofauna and grass roots to access the contents, yet contained the inoculated soil. However, by year 4 the pots were beginning to become brittle. As a result we will likely replace them with containers made from polyvinyl chloride pipe in the near future.

Rhizopogon species colonized bioassay seedlings from all the retrieved sample pots in all test years (Fig. 1). Molecular identification of colonized root tips showed no cross-contamination of *Rhizopogon* species and no control seedlings colonized by *Rhizopogon*. Some sterile soil controls and test seedlings were colonized by the glasshouse contaminant *Thelephora*. However, colonization by other nontarget mycorrhizal fungi was never observed and the *Thelephora* colonization is morphologically distinct from *Rhizopogon*, occurred much later than *Rhizopogon*, and did not appear to interfere with the assay.

Spore concentration had a strong, positive effect on colonization for all species (Table S1). The pattern of pine colonization for *R. occidentalis*, *R. vulgaris*, and *R. salebrosus* in all four years yielded sigmoid curves (Fig. 1). At the highest concentrations, all test seedlings were colonized, a log-linear relationship occurred at intermediate concentrations, and a threshold was reached at the lowest concentrations below which no colonization was observed, even though spores were present. This same pattern can be seen in *R. olivaceotinctus* but the upper plateau was not sampled because the initial concentration of the spores was 100-fold lower.

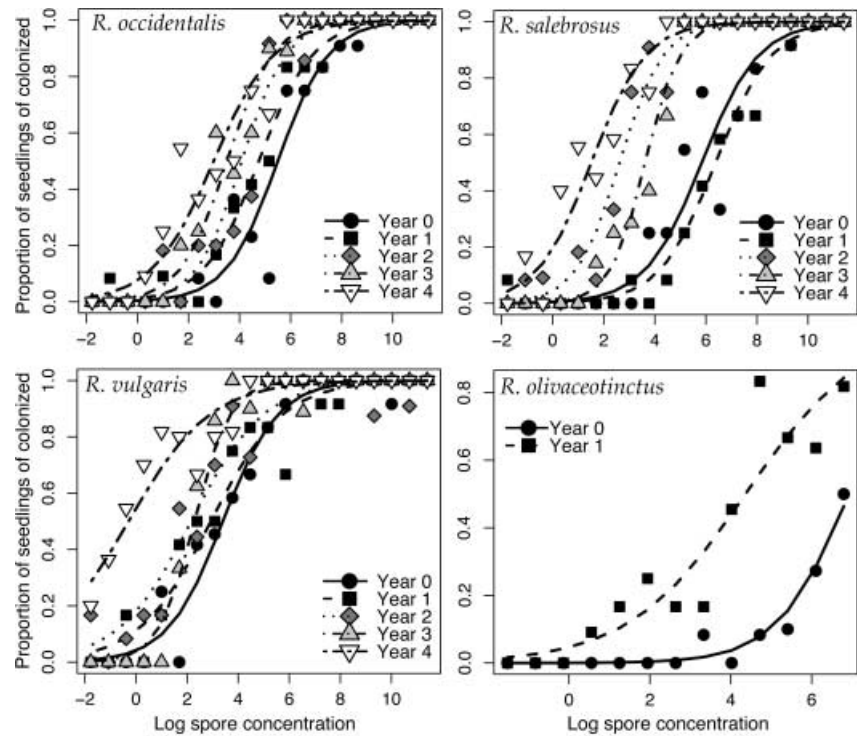


Fig. 1 Proportion of seedlings colonized vs spore concentration over 4 yr. Spore concentrations were derived from twofold serial dilutions. Curves were fitted by multiple logistic regression for each species using the natural log-transformed spore concentrations. The shift of all curves to the left with age is an indicator that inoculum potential increases with time (see also Figs 2, S2). The *Rhizopogon olivaceotinctus* time series was started 3 yr later and so has only two time points (see the Materials and Methods section).

Table 1 Effect tests for predictor variables included in the multiple regression on burial time and species for four *Rhizopogon* species

Variable	df	Sum of squares	Mean square	F	P > F
Year	1	23.16	23.16	40.30	0.0001*
Species	3	19.98	6.66	11.59	0.0019*
Year × species	3	3.29	1.10	0.20	0.20
Residuals	9	5.17	0.57		

*Significant effects of year and species are indicated.

The multiple regression model predicting inoculum potential of each pot from species identity and time buried was highly significant ($R^2 = 0.90$, $F_{7,9} = 11.54$, $P = 0.0007$). Increasing burial time had a strong, positive effect on inoculum potential for all species (Tables 1, S2, Fig. S2), indicating a tendency towards greater numbers of seedlings colonized for a given number of spores. Results were identical when we used the number of spores necessary to achieve 50% colonization of bioassay seedlings as an alternative metric of inoculum potential (Table S3). This is shown in Fig. 2 because the y-axis is more amenable to direct interpretation. The trend is also apparent visually with the leftward shift of the colonization curves over time in Fig. 1. There was some variation in the slope of the relationship between inoculum potential and burial time across species, being steepest in *R. olivaceotinctus* > *R. salebrosus* > *R. occidentalis* > *R. vulgaris* (Fig. 2, Table S2).

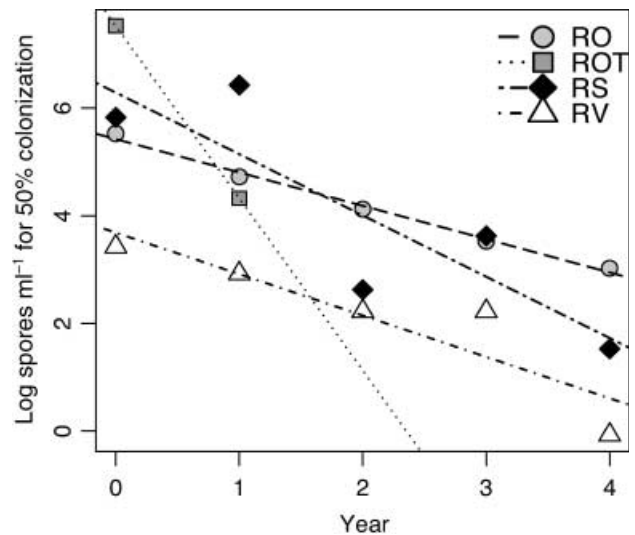


Fig. 2 Inoculum potential of spores vs time. Spore concentrations needed to colonize 50% of the pine seedlings under bioassay conditions are shown vs years of spore burial for *Rhizopogon occidentalis* (RO), *R. olivaceotinctus* (ROT), *R. salebrosus* (RS), and *R. vulgaris* (RV). Spore concentration is predicted from multiple logistic regression as described in the Materials and Methods section. The downward trend shows that fewer spores are needed for colonization in later years.

However, the year × species interaction was not significant (Table 1), suggesting that all species broke dormancy at a statistically indistinguishable rate. Raw data are provided to allow direct comparison with future results (Table S4).

The significant species effect (Tables 1, S3) indicates some differences between *Rhizopogon* species in the behavior of their spores. However, because these are single pooled spore samples, these differences could be indicative of the particular samples rather than the species. Nevertheless, the mean percentage of seedlings colonized differed significantly among species samples, as indicated by the significant species effect (Table 1). This was driven by *R. vulgaris*, which had significantly higher overall seedling colonization compared with the other three species, which all had similar amounts of colonization (Fig. 2, Table S2).

Discussion

Four years into a 99 yr spore viability experiment, the spores of all four *Rhizopogon* species exhibit greater inoculum potential than at year 0 (Figs 1, 2, Table 1). We had expected to observe lower percentages of seedlings colonized by year 4 as a result of loss in individual spore viability with age and reduction in total spore numbers through fungivory in the soil. Instead we observed that equivalent percentages of colonized seedlings were achieved with spore concentrations that were 11- to 70-fold more dilute in year 4 than in year 0. The most reasonable interpretation of this observation is that a proportion of the spores are initially dormant and not receptive to germination cues, and over the 4 yr period of the study they have become receptive. There is a precedent in other fungi in which spores have been observed to become more receptive over the span of several months of storage (Tommerup, 1983; Aime & Miller, 2002), but we know of no published accounts of fungal spores increasing receptivity over the span of several years.

We can make a rough estimate of the maximum percentage of spores that are receptive in year 0 if we make the simplifying assumption that all spores are receptive by year 4, and then compare the concentration of spores needed to colonize 50% of the seedlings in year 4 with that in year 0. Using this approach, we estimate that 8, 2, and 1%, respectively, of the spores were initially receptive for *R. occidentalis*, *R. vulgaris*, and *R. salebrosus*. We consider these maximum estimates, because if all spores are not receptive by year 4 and the colonization rate continues to increase in later years, these estimates would drop. Colgan & Claridge (2002) found a remarkably similar range of 2.4–7.1% of *Rhizopogon vinicolor* spores that stained with Flouricene-diacetate, and they interpreted the nonstaining spores as dormant. Their study included spore samples that were passed through rodent digestive tracts, and they suggest that some stimulation may have occurred during the process, as their two highest estimates (7.0 and 7.1%) were obtained from such spores. However, their results were not consistent across all three samples and would translate to a two- to threefold increase vs 11- to 70-fold increase in our study. The dormancy we observe in *Rhizopogon* spores is similar to that observed in plants and bacteria, in that the entire propagule bank is not

activated simultaneously (Henis, 1987; Thompson, 2000); instead portions of it become more receptive as time accrues. This likely functions to spread the propagule bank over a greater time window, assuming that there is a greater metabolic cost to being receptive than to being dormant.

The high inoculum potential of *Rhizopogon* spores over a 4 yr period contrasts with the results of Torres & Honrubia (1994), who found that viability of *Rhizopogon roseolus* spores stored in water at 3–4°C dropped from 89 to 2% in 180 d. We think their findings were likely the result of the water storage, as we have also seen refrigerated spore slurries drop in inoculum potential within similar time frames, particularly if air space is left in the flask or bottle. However, Castellano & Molina (1989), who used water slurries of *Rhizopogon* spores, cite personal observations that they can last up to 3 yr without loss in viability. These differences between reported results and observations are worth examining more closely, particularly because water slurries are the way that *Rhizopogon* is used in commercial inoculation programs (Castellano & Molina, 1989). However, even if water slurries do lose their viability quickly, it is now clear from our results that this is not what happens with spores in the soil.

The sigmoid shape of the inoculation curves makes it appear as though there is a threshold concentration of spores necessary for colonization. However, as Van der Plank (1975) showed, supposed thresholds are artifacts of the way colonization is assayed and a carefully constructed inoculation curve should be linear and pass through the origin. There are several reasons our apparent thresholds are likely to be artifactual. First, our threshold is essentially a detection amount rather than a colonization amount (Van der Plank, 1975). As such, it is affected by the number of seedlings (in our case 12) used for each dilution. With larger sample replication, the apparent threshold would likely diminish. Second, we don't actually apply spores to the seedlings and observe their behavior; instead, pine roots explore the test soil and encounter spores. We have crudely estimated the volume of roots in uncolonized control seedlings to be *c.* 3–5 ml based on the volume of water that such roots displace. Assuming that roots need nearly direct contact with these small spores (< 8 µm) to be colonized, < 10% of the 55 ml of test soil is actually sampled for spores of these seedlings. Therefore, at low spore concentrations, most seedlings will not encounter spores even though some exist in the sample. Furthermore, not all parts of the root system may be receptive to colonization. If true, this would further lessen the sampling efficiency of the roots. Finally, dormancy must contribute to the apparent threshold effect, as only the concentration of receptive spores contributes to the colonization. Thus, the true origin (i.e. zero receptive spores) is unavoidably shifted to the right of the origin shown; this interpretation is consistent with the apparent shift of the threshold to the left with time (Fig. 1).

According to Van der Plank (1975), a linear relationship between spore concentration and colonization is expected

with the following exceptions. First, the curvature and eventual plateau at the top of the curve (Fig. 1) are primarily the result of the fact that percentage of colonized seedlings was measured instead of the total number of colonization events. Under these conditions, curvature to a plateau occurs (Van der Plank, 1975). Second, curved relationships at lower amounts of inoculum can occur if more than a single spore is needed for colonization. Given that *Rhizopogon* spores are uninucleate (Horton, 2006), and presumably haploid, it is possible that this curved shape results from a requirement for a second compatible spore to form a dikaryon (Kawai *et al.*, 2008).

Although the spore concentrations we used in the current study may seem high, they appear to be well within the range observed in California pine soils. For example, the 19 *Rhizopogon* positive soil samples studied by Kjølner & Bruns (2003) were diluted 50-fold into sterile soil, yet the average number of seedlings colonized was 65%, with a range of 5–100%. Similarly, the five soil samples studied by Rusca *et al.* (2006) were diluted 134-fold, yet the average number of seedlings colonized was 49% with a range of 20–90%. Thus the concentration of spores in all of these highly diluted soils were in the log-linear portion of the inoculation curves (Fig. 1). If we extrapolate back to the concentration in undiluted soils, most would end up somewhere in the plateaued region of spore concentrations (i.e. ≥ 150 spores ml⁻¹ of soil). It is difficult to imagine these high concentrations across all tested soils being assembled in a single year, whereas with high longevity, such spore loads could easily accumulate.

Although the dormancy we found in *Rhizopogon* species is interesting, it has temporarily confounded our efforts to predict the longevity of spores accurately, as any loss in viability that has occurred was masked by a gain in the proportion of spores that have become receptive. However, the inability to see any evidence of lost inoculum potential after 4 yr, coupled with the fact that spore concentrations seen in nature are equally high, and that spores of ruderal species are common in mature forests soils (Taylor & Bruns, 1999; Izzo *et al.*, 2005), all suggest that *Rhizopogon* spores are likely to last decades. This prediction will be tested as longer time points are accumulated from this ongoing experiment. Whether the longevity of *Rhizopogon* spores is unique, or at least limited to other taxa with mammal-dispersed spores, remains unanswered. However, it is likely that the longevity of *Rhizopogon* spores is a major part of the reason that the genus is one of the most common early successional associates of pine in settings where they expand from a forest edge into previously unforested areas (Ashkannejhad & Horton, 2006; Peay *et al.*, 2007).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 A 99 yr spore burial experiment.

Fig. S2 Multiple regression showing the effects of burial time and species identity on the inoculum potential of spores from four *Rhizopogon* species.

Table S1 Logistic regression models used to estimate the relationship between spore concentration and seedling colonization for individual *Rhizopogon* species as well as to generate overall estimates of inoculum potential for each pot

Table S2 Coefficients from a multiple regression predicting the effects of burial time and species identity on the inoculum potential of soil pots containing ectomycorrhizal spores from various *Rhizopogon* species

Table S3 Effect tests for predictor variables included in a multiple regression on the effects of burial time and species identity on the number of spores necessary to colonize 50% of seedlings in a dilution series

Table S4 Raw data from bioassays

Methods S1 Methodological note for future researchers on molecular confirmation of *Rhizopogon* species in the burial experiment.

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