Stayin’ alive: survival of mycorrhizal fungal propagules from 6-yr-old forest soil

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Abstract

Spores and sclerotia are the main propagules that allow fungi to persist through unfavorable conditions and disperse into new environments. Despite their importance, very little is known about their longevity and dormancy, especially in ectomycorrhizal fungi. To assess the viability of ectomycorrhizal fungal spores in forest soil, we collected and buried non-sterile forest soil, in pots, in the field distant from an inoculum source. After 6 yr, a subset of this soil was assayed for viable spores by baiting the fungi with Bishop pine (Pinus muricata) seedlings. Our results show that the three most frequent colonizers in year 1 continued to colonize significant percentages of seedlings in year 6: Wilcoxina mikolae (77 %), Rhizopogon vulgaris (13 %) and Suillus brevipes (9 %). While three species that colonized low percentages of seedlings in year 1, Suillus pungens (1 %), Rhizopogon salebrosus (2 %), and Thelephora terrestris (5 %) were not recovered in year 6. Laccaria proxima, a species not seen in year 1, was recovered on a single seedling in year 6. This is the first report of long-term survival of S. brevipes and W. mikolae. Our results reveal a more complete picture of ectomycorrhizal fungal spore longevity in soil spore banks.

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Introduction

Propagules such as spores and sclerotia are a means for fungi to escape their current environment, overcome dispersal barriers, and establish in a new favorable habitat. Dispersal may involve these propagules being carried by the air or through the guts of various animals (Claridge et al. 1992; Lilleskov & Bruns 2005). Aerial dispersal may involve the risk of spore dehydration (Ashkannejhad & Horton 2006) or UV damage (Ulevicius et al. 2004) that can lead to a loss of viability. Even if the spores are able to survive these barriers, reaching new substrata does not guarantee favorable conditions for germination, particularly in ectomycorrhizal fungi (Massicotte et al. 1994; Rusca et al. 2006). To counter this problem, it was suggested that some fungi produce resistant propagules that can form a spore bank and potentially wait for decades before germination (Miller et al. 1994; Bruns et al. 2009). Most reports of spore banks are based on anecdotal evidence (Putnam & Sindermann 1994) and few experiments have attempted to address the question of spore survival through an extended time (Bruns et al. 2009; Nara 2009). This is especially true for ectomycorrhizal fungi (EMF).

Longevity and dormancy of EMF spores have been studied using various microscopy techniques (Lamb & Richards 1974; Miller et al. 1993, 1994; Torres & Honrubia 1994) and host seedling bioassays (Castellano & Molina 1989; Ashkannejhad & Horton 2006; Ishida et al. 2008; Bruns et al. 2009). Some EMF spores remained viable from 1 month and up to 4 yr in various storage conditions. Bruns et al. (2009) showed that spores of several Rhizopogon species remained viable for at

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http://dx.doi.org/10.1016/j.functal.2012.05.006

Please cite this article in press as: Nguyen NH, et al., Stayin’ alive: survival of mycorrhizal fungal propagules from 6-yr-old forest soil, Fungal Ecology (2012), http://dx.doi.org/10.1016/j.functal.2012.05.006
least 4 yr, and surprisingly, the inoculum potential of the soil mixture increased during this time. This pattern of increased inoculum potential by ectomycorrhizal spores over time has so far only been observed in Rhizopogon species, which largely depend on mammals for spore dispersal through the ingestion of basidiocarps. The genus Suillus, an epigeous relative of Rhizopogon, usually relies on air for dispersal and apparently showed an opposing pattern when dispersed in deer feces, where its spores lose viability within a year after being desiccated in deer fecal pellets (Ashkannejhad & Horton 2006).

As a complement to the Rhizopogon spore longevity experiments described in Bruns et al. (2009), we tested the long-term viability of a diversity of fungal spores present in the spore bank of a natural Pinus muricata stand. Here, we report the fungi that survived after 6 yr of field incubation using pine seedling bioassays to capture the viable and receptive fungal spore community.

**Material and methods**

**Soil collection and incubation**

In 2003, 88 l of soil was collected underneath P. muricata trees from Muddy Hollow Knoll (MHK) in Point Reyes National Seashore, CA (38°.2736′N, 122°.52.136′W, WGS84 datum), brought back to the lab and homogenized with 8 l of sterile horticultural sand (1:1 ratio). Sixteen terracotta pots, measuring 16.5 cm in diameter with a volume of 1.6 l, were filled to the top with 1.6 l of the homogenized soil. 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 pot to allow unequivocal identification. The tops of the pots were covered with 19 cm diameter terracotta saucers and these were secured with plastic cable binders. In the field, we observed basidiocarps of Rhizopogon vulgaris, Roccidentalis occidentalis, Suillus pungenus, and Laccaria proxima in the immediate area from which the soils were collected, and we assume that spores of these species would be in the soils. However, the starting quantities and age of the inocula were not known and we assumed that all spores were deposited immediately prior to collection.

The pots were buried under 15 cm of grassland soil in situ (38°.11.7983′N, 122°.57.7517′W, WGS84 datum) in an area where little or no EMF inoculum is present (Bruns et al. 2009). The burial site was about 1.25 km upslope from a small number of native, local P. muricata, the same species that grows from native, local P. muricata, the same species that grows where the test soil was originally collected. Mature cones were collected along Limantour 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, soaked overnight, and allowed to germinate on moist, sterile filter paper in sterile Petri dishes for 10 d. Two germinated seeds were planted in each prepared soil tube and watered with the outside soil. It also allows the spores to interact with soil microinvertebrates and the microbial community therein.

**Bioassays**

Year 0 data were not collected due to unanticipated problems with the hydrophobic nature of the autoclaved MHK soil used for dilutions that created high seedling mortality. Instead, we began sampling in 2004, which marks year 1. In year 1, we bioassayed the soils following the methods detailed in Bruns et al. (2009). Briefly, grassland soil with low EMF inoculum from the site where the pots were buried was collected in Sep. 2003 prior to the beginning of seasonal rain. This soil was taken back to the lab and mixed 11:1 with coarse sand, autoclaved for 45 min, mixed again and then autoclaved for another 45 min for complete sterilization under standard autoclaving conditions. Non-sterile MHK soil from 1 of the 16 buried pots was used as the bioassay soil for year 1. This non-sterile soil was diluted 19 times in two-fold serial dilutions with the sterilized soil-sand mix. A total of 55 ml of each dilution was placed into a 115 ml Cone-tainer tube (Steuwe & Sons Inc., OR, USA) plugged with synthetic cotton to prevent soil from leaking out, and a seasonal dry summer with no precipitation, it does get heavy rain to prevent soil and seeds from sticking to the cotton. For each dilution, 12 replicates were planted, and tubes were kept moist. Each bioassay tube was planted with soil microinvertebrates and the microbial community therein.

Year 6 of our soil burial experiment was in 2009. One of the MHK soil pots was dug from the field at that time and carefully cleaned with 70% ethanol before the plastic cable binders and lid were removed. The original 20-step, two-fold dilution series used in year 1 was designed to quantify loss of inoculum with time. This worked well with single-species inoculum (e.g., Bruns et al. 2009), but the complex multispecies inoculum present in the MHK forest soil prevented quantification due to competitive interactions (Kennedy et al. 2009). For that reason, in year 6, we replaced this dilution series with a simpler one that allowed us to assay for species present, but we abandoned the attempts to quantify abundance of individual species as compared to year 1. The new method was as follows: soil from the pot was diluted to 1:2, 1:9, 1:49, and 1:99 with autoclaved soil-sand mixture as described above. A total of 12 tubes per dilution were prepared in addition to seven control tubes filled with sterilized soil-sand mix. The total number of bioassay tubes for year 1 was 12 replicates × 20 dilutions + 12 controls = 252 tubes.

For both years 1 and 6, seed source for the bioassays was from native, local P. muricata, the same species that grows along Limantour 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, soaked overnight, and allowed to germinate on moist, sterile filter paper in sterile Petri dishes for 10 d. Two germinated seeds were planted in each prepared soil tube and topped with a 1 cm layer of sand to prevent soil and seeds from sticking to the cotton.
from being dislodged during watering. After 1 month of growth, seedlings were thinned to one per tube. Seedlings were watered daily only with distilled water and grown in a greenhouse under filtered natural light at 65–70 °C for 6 months.

**Molecular identification of EMF**

After 6 months, seedlings were removed from the bioassay tubes, soil was washed from the roots, and the root systems were examined under a dissecting microscope (magnification ~10×). Morphologically different root-tips were collected from each seedling. In year 1, root-tips of each distinct morphotype per soil dilution were collected among the 12 replicate tubes and sequenced, whereas in year 6, all distinct morphotypes from each of the six replicate tubes across all soil dilutions were collected and sequenced. DNA was extracted using the standard ethanol precipitation method for year 1 and a modified (10 µl rxn) XNAP RedExtract-N-Amp kit (Sigma–Aldrich, Inc.) was used for year 6. The fungal internal transcribed spacer region primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) were used for both PCR and sequencing. Sequence identity was established initially by BLAST then assigned to bins based on 97% divergence, followed by phylogenetic reconstruction to confirm their taxonomic placement.

**Results**

In year 1, 57 root-tips were collected from 240 seedlings. In year 6, 116 root-tips were collected from 24 seedlings, with an average of 4.8 root-tips per seedling. No colonized root-tips were found in sterile soil control tubes in either year 1 or year 6. From colonized root-tips, we successfully sequenced 44 root-tips from year 1 and 93 root-tips from year 6. All sequences grouped within 97% ITS similarity to sequenced voucher basidiocarp specimens and nested within a phylogenetic clade for that species. Representative sequences of each species in both year 1 and year 6 were deposited in GenBank under accession numbers JQ310816–JQ310826. To account for the differences in sampling and dilution series between year 1 and year 6, we binned the dilution series into dilution groups (dg), which allowed us to compare between these 2 yr. The 20 two-fold dilution series in year 1 were binned into 8 dg, where dg 1–4 are comparable to the four dilutions (1:2, 1:4, 1:8, 1:16) from year 6. The average dilution rates for the dilution groups were dg 1 = 1:2, dg 2 = 1:11, dg 3 = 1:47, dg 4 = 1:191, dg 5 = 1:194, dg 6 = 1:9557, dg 7 = 1:76 458, and dg 8 = 1:305 835. In year 1, we recovered seven species, and in year 6, we recovered four species (Table 1). Suillus brevipes, R. vulgaris, and Wilcoxina mikolae were found in both years. S. pungens, Rhizopogon salebrosus, R. occidentalis, and Telephora terrestris were found in year 1 but not found again in year 6. Conversely, L. proxima was found once in year 6 but not in year 1 (Fig 1). The communities recovered in both years were near saturation based on rarefaction analysis (Supplementary Fig 1).

Because of inconsistent sampling between the years, the abundance data from year 1 and year 6 cannot be compared directly. However, data from year 6 is useful for comparisons with future sampling points and can be found in Table 1. Based on the frequency of occurrence within year 6 samples, W. mikolae (77%) was the most abundant taxon, followed by R. vulgaris (13%), S. brevipes (9%) and L. proxima (1%).

**Discussion**

We were not able to directly compare the frequency of occurrence between the two sampling years due to experimental design and sampling differences between these 2 yr. Half of our seedlings in year 6 died, probably due to infection with soil pathogens. As a result, there was a large overall discrepancy between the amounts of soil in which roots (144 bioassays in year 1 vs. 24 bioassays in year 6) could explore for fungal spores. However, despite root sampling intensity, sampling differences between the 2 yr and high seedling mortality in year 6, we nearly saturated the species accumulation curve for year 1 and saturated it for year 6 (Supplementary Fig 1).

Despite the difficulty in comparing frequency of occurrence between the 2 yr, our results clearly showed that spores of at least four taxa, W. mikolae, S. brevipes, R. vulgaris and L. proxima, remain viable in natural field conditions for at least 6 yr. However, this is a minimum estimate of prolonged viability because it assumes that all the spores were deposited in year 0.

W. mikolae associates with conifer seedlings of the genera Pinus, Larix, Picea, Abies and Tsuga in early successional habitats and disturbed mineral soil such as those of nurseries (Mikola 1988; Taylor & Bruns 1999; Smith & Read 2008). The resistant propagules of W. mikolae are either ascospores or chlamydospores (Mikola 1988; Ivory & Pearce 1991; Taylor & Bruns 1999). However, because we do not know which type of propagule was present in the soil, from here on we will refer to them simply as spores. In both sampling years, we found W. mikolae in moderately dilute soils (dg 4–5), indicating that perhaps the number of viable spores in the forest soil was less numerous than other species such as R. vulgaris, S. brevipes and T. terrestris. Colonization by spores of W. mikolae after short-term air drying had been observed (Taylor & Bruns 1999) but this is the first report of long-term viability.
S. brevipes is a widespread pioneer species in both Western and Eastern North America and associates with a wide range of two and three-needle pines (Smith & Thiers 1964). It can show up in young forest settings (Ashkannejhad & Horton 2006; Peay et al. 2007), but can also persist in more mature forests (TDB pers. observ.). Although the spores of this species can remain in dry deer pellets for at least 1 yr, they appear to be much less viable or less responsive than when freshly deposited (Ashkannejhad & Horton 2006). Here, we provide the first report of any Suillus species in which spores can remain viable for at least 6 yr.

R. vulgaris is a pioneer species in early successional habitats. It was found at both of our sampling time points. Bruns et al. (2009) showed that the spores of this species lasted at least 4 yr in the soil. Our results here corroborated that study and added two additional years of longevity. In Point Reyes, it is not a very common species, but at the site from which the soil was collected, it is the most abundant species of Rhizopogon based on observed fruiting (TDB pers. observ.).

Much like the species described above, R. salebrosus, R. occidentalis, T. terrestris and S. pungens are pioneer species that colonize young pines in early successional habitats at Point Reyes (Peay et al. 2007), but because all of these species were rare in our sample in year 1, it is not possible to draw conclusions about their long-term viability based on their apparent absence in year 6 (Fig 1). The fact that R. salebrosus is known to have long-lived spores (Bruns et al. 2009), and is common in the spore bank in many other areas of California (Kjeller & Bruns 2003; Rusca et al. 2006; Izzo et al. 2006), shows that its absence here at year 6 may not be very meaningful. T. terrestris is often found as a greenhouse contaminant (Castellano & Molina 1989; NHN pers. observ.). Its longevity and importance in the spore bank is unclear in the literature. Ashkannejhad & Horton (2006) showed that a related species, Thelephora americana, could survive up to 1 yr in dry deer feces.

L. proxima is another example of an early successional species that was rarely observed in our soil bioassays, but in this case, the reverse pattern was found. It was absent from our sample in year 1 but occurred on a single seedling in year 6. Similar to Thelephora species, Laccaria laccata has been reported as a greenhouse contaminant (Castellano & Molina 1989); however, we have never seen Laccaria behave as one in the greenhouse in which we grow our seedlings. In addition, we did not observe any colonization in our negative control tubes. For these reasons, we interpret its presence in year 6 as evidence that it can survive multiple years in the soil. Furthermore, its apparent absence in year 1 and the fact that it was only found in the lowest soil dilution (dilution group 1 in Fig 1 and Table 1) supports the idea that it is rare in at least the reactive component of the spore bank, or perhaps that it is a poor competitor with other spore bank species. Ishida et al. (2008) showed that spores of L. laccata (same species complex as L. proxima) and Laccaria amethystina do germinate at observable frequencies and remained viable after 1 yr, although germination was reduced by 60%. Thus, a small percentage of Laccaria spores with extended viability is not too surprising.

The near absence of S. pungens in year 1 was surprising because this species is much more common than S. brevipes at Point Reyes and it was an abundant fruiter in the area where the non-sterile soil was collected. It is an effective spore colonizer both of laboratory seedlings (NHN unpublished results) and in the field. In the field, it is the most common species found under 10-yr-old pines that have established in areas that were not previously forested and are distant (~1 km) from a forest (Peay et al. 2007). In previously forested areas affected by stand-replacing fires, S. pungens was a common fruiter prior to a fire (Bonello et al. 1998) but was either rare or absent on roots of regenerating seedlings (Horton et al. 1998; Baar et al. 1999; Grogan et al. 2000). These authors found that despite being present on seedling roots the first year after a fire, it only became common again 5 yr after the fire. Genetic evidence demonstrated that its presence at this site was likely due to establishment by spore rather than by hyphal survival, but it did not address the issue of whether the spores involved recent dispersal or presence of a spore...
bank (Bruns et al. 2002). From this assembled evidence, we speculate that S. pungens is an able disperser and spore colonizer, but one need not hypothesize a long-lived spore bank to explain the observed pattern.

Our experiments were based on a single sampling time point (6 months) of seedling bioassays. From prior studies, we have determined that this time period is enough for spores of many Rhizophogon and Suillus species to become active and colonize seedlings (Bruns et al. 2009), although longer sampling points may allow more recalcitrant spores to colonize seedlings. We aimed to dilute the forest soil so that the less abundant and better competitive species would not outcompete and mask the presence of other species (Rusca et al. 2006; Kennedy et al. 2009). However, this is an imperfect system because if the most competitive species are also abundant, or if a large number of poor competitors are low in abundance, many species would still be missed. We saturated the species accumulation curve in year 6 but only approached saturation in year 1 (Supplementary Fig 1). Because of this, it is likely that we missed additional species.

It is becoming increasingly clear that a spore bank is often important for the establishment of EMF host plants into new habitats (Baar et al. 1999; Ashkannejhad & Horton 2006; Ishida et al. 2008; Collier & Bidartondo 2009). Therefore, traits such as long-term spore survival and quick response to disturbance are likely to be common in pioneer or early successional EMF. For example, spores of pioneer species (Inocybe, Laccaria, Rhizophogon, some Suillus spp. and Wilcoxina) appear to survive longer than mature forest species (Tricholoma, Lactarius, Russula, Cortinarius spp.) as determined by bioassays with live plants (Ishida et al. 2008) or a variety of staining techniques (Torres & Honrubia 1994). However, it is important to note that the long-term viability of mature forest species, including the species listed above, has yet to be determined due in part to the difficulty of establishing these species with seedlings in the laboratory.

Interestingly, based on the relatively few studies currently published, spore longevity does not appear to be a phylogenetically conserved trait within the Basidiozyma or Ascomycomycota. Thus, the ability of some fungi to form long-lived spore banks is likely an example of convergent evolution. However, future studies are necessary to test this empirically. Morphologically, all of the spore bank species from this study, except L. proxima, have dark spores that could protect them from UV radiation and thus increase their longevity (Ulevicius et al. 2004). However, besides this one characteristic, it is unclear what other traits common spore bank fungi may share, or conversely, what unique spore survival mechanisms specific fungal lineages have evolved. Future investigations on the morphological and biochemical traits that enhance spore survival are necessary to understand early successional ectomycorrhizal community assemblies.

Acknowledgments

We thank Jennifer Kerekes, Valerie Wong, and two anonymous reviewers for useful commentaries on this manuscript. Funding was provided by NSF GRFP and the Mycological Society of America to NHN, and NSF grants DEB 236096 and DEB 0742696 to TDB.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.funeco.2012.05.006.

References


523.

using two hosts that exhibit different seedling establishment
Kennedy PG, Peay KG, Bruns TD, 2009. Root-tip competition
among ectomycorrhizal fungi: are priority effects a rule or an
Kjøller R, Bruns TD, 2003. Rhizopogon spore bank communities within
and among Californian pine forests. Mycologia 95: 603–613.
Lamb RJ, Richards BN, 1974. Survival potential of sexual and
asexual spores of ectomycorrhizal fungi. Transactions of the
Lilleskov EA, Bruns TD, 2005. Spore dispersal of a resupinate
ectomycorrhizal fungus, Tomentella subbilacina, via soil food
ectomycorrhizal genus, Rhizopogon. II. Patterns of host-fungus
specificity following spore inoculation of diverse hosts grown in
19–27.
Miller LS, Torres P, McClean TM, 1993. Basidiospore viability and
germination in ectomycorrhizal and saprotrophic
basidiomycetes. Mycological Research 97: 141–149.
Miller SL, Torres P, McClean TM, 1994. Persistence of
basidiospores and sclerotia of ectomycorrhizal fungi and
Peay KG, Bruns TD, Kennedy PG, Bergemann SE, Garbelloto M,
2007. A strong species-area relationship for eukaryotic soil
microbes: island size matters for ectomycorrhizal fungi. Ecology
Putnam ML, Sindermann AB, 1994. Eradication of potato wart
Rusca TA, Kennedy PG, Bruns TD, 2006. The effect of different
pine hosts on the sampling of Rhizopogon spore banks in five
Smith AH, Thiers HD, 1964. A Contribution Toward a Monograph of
the Genus Suillus. Privately printed, Ann Arbor, Michigan.
Taylor DL, Bruns TD, 1999. Community structure of
ectomycorrhizal fungi in a Pinus muricata forest: minimal
overlap between the mature forest and resistant propagule
study on changes in viability of airborne fungal propagules
exposed to UV radiation. Environmental Toxicology 19: 437–441.
sequencing of fungal ribosomal RNA genes for phylogenetics.
In: Innis M, Gelfand D, Sninsky D, White T (eds), PCR Protocols –
a guide to methods and applications. Academic Press, New York,
pp. 315–322.