Changing balance between dormancy and mortality determines the trajectory of ectomycorrhizal fungal spore longevity over a 15 year burial experiment

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Brief heading: Fungal spore retain viability after being buried in the soil for 15 years

Summary

Propagule longevity is an important ecological trait enabling organisms to survive through long

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Introduction

Temporal variability, ranging in scale from minutes to decades, imposes both challenges (Roff, 2002) and opportunities (Hutchinson, 1961; Roy &

Chattopadhyay, 2007) for the survival of most organisms (Levin, 1992). In response, organisms have developed a range of adaptations to cope with temporal changes in their environment; these can be coarsely divided into two non-mutually exclusive groups: those that enable them to cope with variability and those that enable them to avoid the variability. Adaptations related to avoidance usually involve the organism either moving in space (Bowler & Benton, 2005) or having the ability to be inactive for long periods of time (Siewert & Tielborger, 2010; Tielbörger *et al.*, 2012). In many organisms, it is the resistant propagules that can be inactive over long time scales. Examples can be found in seeds of desert annuals, which are known to maintain viability for decades (Gutterman, 2012), as well as spores, sclerotia, or other quiescent states of ferns (Smith & Robinson, 1975), bacteria (Seaward *et al.*, 1976; Ulrich *et al.*, 2018) and fungi (Bruns *et al.*, 2009), some of which are thought to last for centuries.

Propagule longevity (i.e. the ability to remain viable for long periods of time), is often combined with some level of dormancy (i.e. the state of metabolic inactivity). In fact, under this broad definition of dormancy, propagule persistence and dormancy are essentially synonymous. However, in physiological or seed bank studies the term dormancy is often restricted to propagules that are self-inhibited from germination under otherwise favorable conditions (Thompson *et al.*, 2003). This type of dormancy has been referred to as constitutive, innate, primary, or endogenous dormancy (Sussman and Douthit 1973) or simply “dormancy” (Thompson *et al.*, 2003). The concept is useful because it is a property of a propagule rather than a missing feature of the environment that stimulates germination. Here we will refer to it as **constitutive dormancy** because it differs from the broader way dormancy is sometimes defined (Lennon & Jones, 2011). Constitutive dormancy has an important ecological role: it minimizes the chances of complete failure due to extreme events (bet-hedging; Cohen, 1966; Seger & Brockmann, 1987; Sadeh *et al.*, 2009), while longevity enables organisms to wait out long periods of unfavorable conditions.

Successional shifts in community composition often result after long periods of unfavorable conditions, particularly for ruderal species that are usually out competed relatively quickly after initial colonization (Chapin *et al.*,

1994). Thus, for long-term survival within the site, these less competitive species must be able to remain inactive over long time periods. While such life-history trade-offs are well established in macroorganisms, adaptations to temporal environmental variability in microorganisms are less studied (but see Jones & Lennon, 2010; Shade *et al.*, 2012 for reviews on the topic). Ectomycorrhizal fungi (EMF), a key group of symbionts on woody plant species world-wide (Smith & Read, 2008), often have temporally variable communities, with initial colonization by ruderal species in many cases being replaced by more competitive EMF as trees mature (Visser, 1995; Twieg *et al.*, 2007). However, the outcompeted EMF species are maintained by a long-lasting spore bank in the soil (Bruns *et al.*, 2009; Nara, 2009; Huang *et al.*, 2015). The multi-decade longevity of most forest trees and the relatively low disturbance return interval in many forests suggests that selection should favor EMF ruderal species with high spore longevity.

To date, most data regarding EMF spore longevity are anecdotal and originate from uncontrolled observations (Pither & Pickles, 2017). In 2003, we started a planned 99 year-long controlled experiment aimed at examining the longevity and constitutive dormancy of spores of early successional EMF species (Bruns et al. 2009). Spores of three *Rhizopogon* species that were derived from multiple mature sporocarps were mixed into EMF-free soil, buried in the field within semi-porous ceramic containers, and sampled once a year during the first four years of the experiment. Within the first four years, the inoculum potential of the soils increased (i.e. fewer spores required for colonization), demonstrating that some of the spores that were initially dormant were becoming active and that this break in constitutive dormancy was happening at a higher rate than spore mortality (Bruns *et al.*, 2009). We present here the results from years 10 and 15 of the experiment, a rare opportunity to accurately measure spore longevity in fungi under field conditions.

Methods

Experimental methods are presented here in brief; for a detailed description see Bruns et al (2009). Spore slurries were made in February 2003 for *R. occidentalis*, *R. vulgaris* and *R. salebrosus*, using six mature collections of each

species. To assay spore longevity, a grassland site lacking *Rhizopogon* spores (live field soil from Tomales Point, a peninsula in Point Reyes National Seashore) was located and was used both as a soil source for the experiment, and for the burial location for inoculated test samples. For each species of *Rhizopogon,* 2.5×10^8 spores were sprayed onto 28 L of soil and thoroughly mixed. Subsamples of 1.6 L of these single species mixtures were placed into 16 terracotta flowerpots, 16.5 cm in diameter. The drainage holes were covered by a glass microscope slide and the tops of the pots were covered with 19 cm terracotta saucers. The 64 pots containing the three single-species treatments were buried to a depth of 15 cm at the top of the pot lids. The pots were then buried at the Tomales Point grassland site. The semiporous nature of these pots maintained a realistic soil environment and allowed for retrieval over the decadal scale of this experiment while the gaps between the glass slide and pot bottom should still allow for microarthropods to enter the pot.

Spore viability was assayed at each time point by inoculating *Pinus muricata* seedlings with spores from buried soil pots and measuring the extent of ectomycorrhizal formation. Two-fold soil dilutions were assayed for *Rhizopogon* colonization with 12 pine seedlings planted in separate RCL4 cone-tainers (Steuwe & Sons, Tangent, OR, USA). A total of 20 two-fold dilutions spanning the range of 8.9×10^4 to 1.7×10^{-1} spores ml^{-1} were conducted. In addition, 20 control seedlings were planted in the sterile soil mix for each series for a total of 260 seedlings (20 dilutions × 12 replicates + 20 control seedlings) per time point per species. Of the 60 seedlings planted in uninoculated soil as controls, 10 were colonized by airborne spores, but none were colonized by *Rhizopogon.* Contamination within the dilution series was only observed in the high dilutions in which *Rhizopogon* had been diluted to near extinction.

Seedlings were grown in the glasshouse with watering but no fertilization. After 6 months, seedlings were removed from the cone-tainers, soil was washed from the roots, and the root systems were examined under a dissecting microscope. 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 and preserved in CTAB buffer. RFLP analysis was conducted on these samples (see Bruns *et al.* 2009 for additional information) and all were confirmed to be correctly identified.

Statistical analysis

A two-step approach was used to test for the effects of burial time and species identity on spore viability. Multiple logistic regression was used to characterize the relationship between spore concentration and seedling colonization and to extract from this a single estimate of spore receptivity for each pot harvested ($C_{50\ (year\ N)}$). $C_{50\ (year\ N)}$ is the estimate of the concentration of the spores initially inoculated into each pot that is required to colonize 50% of the seedlings in the experiment for a given year (see below for further discussion of this term). For each *Rhizopogon* species, an independent logistic regression model was used 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). Then for each species, the estimated C_{50} for each year in the logistic regression was used as an integrated measure of inoculum potential for each time point.

The second step of our analysis was to use multiple linear regression to statistically test for trends in inoculum potential over time. A linear model was used to predict changes in $C_{50\ (year\ N)}$ due to burial time, species identity, and their interaction. A quadratic term (burial time^2) was also included to account for non-monotonic changes in $C_{50\ (year\ N)}$. Non-significant terms (starting with the highest order terms) were eliminated until all terms in the model were significant.

The logistic regression models were fitted with maximum-likelihood techniques using the glm procedure in R, version 4.1.1 (R Core Development Team, 2016), with a logit link function and binomial errors. Spore concentrations were log-transformed to reduce model overdispersion. Multiple regression models were fitted using the lm procedure in R. Effects were considered significant at $p < 0.05$. R commands for this project are available on GitHub (https://github.com/nnguyenlab/rhizopogon-longevity).

Results and Discussion

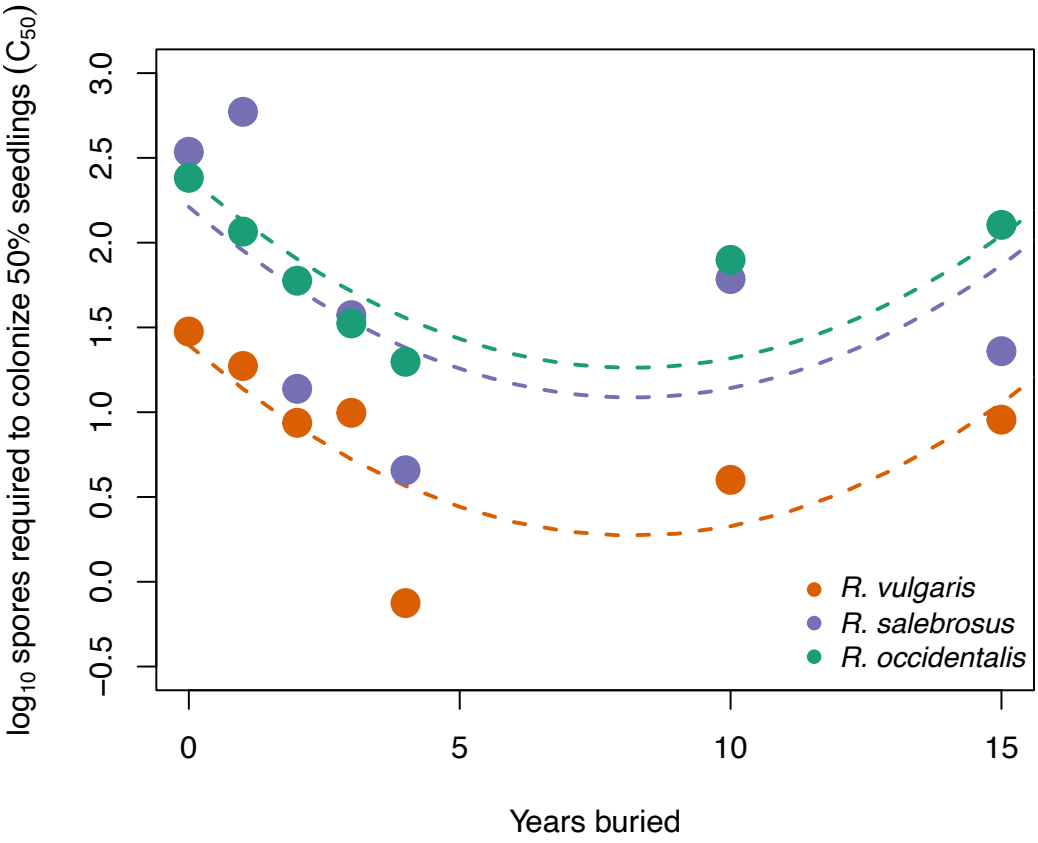

Figure 1. Spore concentration required to colonize 50% of seedlings (C_{50}) for three *Rhizopogon* species over time. C_{50} was estimated from logistic regression for each species and burial time. Lines are from a generalized-linear model fit of a polynomial regression (time $p = 0.004$; time^2 $p = 0.006$).

We found that spores of all three *Rhizopogon* species survived 15 years. After an apparent increase in their viability during the first four years (Bruns et al 2009), they have now almost returned to levels of inoculum potential measured at year zero (Fig. 1). This means that the full longevity of these spores is likely to be much longer than 15 years. However, providing an accurate estimate for how long they will survive involves understanding both the processes of spore mortality and release from constitutive dormancy.

It is possible to define a simple model demonstrating how the processes of spore mortality and release from constitutive dormancy explain the patterns

184 we observe. First, some fraction of the spore pool is currently living, or viable,
185 but some fraction of these may be constitutively dormant and unable to colonize
186 seedlings. Receptive spores are those that are currently capable of colonizing
187 seedling roots, and therefore must be both viable and not constitutively
188 dormant. Here we define $\mathbf{C_{50\text{-}Actual}}$ as the true concentration of receptive spores
189 needed to colonize 50% of the test seedlings. This is an ideal concept and
190 cannot be measured directly since neither viability nor receptiveness are
191 independently measured in our system. In our analyses, $C_{50(\text{year N})}$ then is the
192 total concentration of buried spores from our dilution series that are observed
193 to cause colonization of 50% of the test seedlings in year N. Inoculum potential
194 then is the ability of the spore-laden soil to cause colonization of seedlings, with
195 greater inoculum potential yielding higher percentages of colonized
196 seedlings. In our model we compare changes in inoculum potential by
197 estimating $C_{50(\text{year N})}$ across years. However, inoculum potential and $C_{50(\text{year N})}$
198 are inversely correlated; a rise in $C_{50(\text{year N})}$ corresponds with a decrease in
199 inoculum potential because a greater concentration of spores was needed to
200 cause the same level of colonization.
201 To develop our model, we first assume that the number of receptive
202 spores needed to achieve a given inoculum potential is a constant for a given
203 species as long as environmental conditions are also constant. In the context
204 of the terms we outlined above, this assumption means that $\mathbf{C_{50}}$, our ideal but
205 unmeasurable variable, is constant for each species (Fig. S1). This simply
206 means that if, for example, 100 receptive spores/ml of soil colonize 50% of the
207 seedlings today, then at any later date this same quantity of receptive spores
208 will colonize 50% of the seedlings as long as environmental conditions are
209 constant. However, our measured $C_{50(\text{year N})}$ are not expected to stay constant
210 because they are based on the measured spore concentration from year zero,
211 and the percentage of receptive spores will change with time due to two
212 processes: mortality and release from constitutive dormancy. If no dormancy
213 was present, and only spore mortality occurred, then the observed $C_{50(\text{year N})}$ will
214 necessarily increase over time because a larger concentration of spores added
215 at year zero are needed to achieve the same C_{50} of viable spores. In contrast,
216 if no spore mortality occurs, but a larger proportion of spores become receptive
217 over time through release from dormancy, then our observed $C_{50(\text{year N})}$ would

decrease over time. However, when both mortality and breaking of dormancy occur simultaneously then the stronger of the two processes will dominate.

With this model in mind, the trend and interpretation of the $C_{50(\text{year N})}$) over time (Fig. 1) becomes clear. During the first four years of the experiment, inoculum potential was increasing, as evidenced by the drop in $C_{50(\text{year N})}$ in all three species. This means that during these early years spores were released from constitutive dormancy at a greater rate than they were dying (Bruns et al. 2009). However, sometime after year 4, mortality became the stronger process, and the $C_{50(\text{year N})}$ started to increase in all three species. At year 15, all three species are just beginning to achieve the same inoculum potential seen in year 0. This means that these spores now colonize seedlings at the same concentration that they did when they were first collected from mature fruit bodies. It is also noteworthy that our statistical model indicates that all three species share a similar quadratic curve, which we interpret to mean that the rates at which they break constitutive dormancy and experience mortality is likely to be quite similar. Given that we have a relatively small number of temporal observations (7 per species), it is possible that this may change with more data. While the shape of the C_{50} curve was not distinguishable among species, the starting spore efficacy (variation in y-intercept in Fig. 1) did vary significantly among species. Specifically, *Rhizopogon vulgaris* had a lower C_{50} relative to the other two species (t = -3.99, p = 0.001). This means that fewer spores were needed for this species to colonize seedlings across all measured years compared to the two other congeneric species.

These results show that the spores of *Rhizopogon* have both constitutive dormancy and high longevity. This is similar to results from plant seed banks (Koornneef *et al.*, 2002; Thompson *et al.*, 2003; Finch-Savage & Leubner-Metzger, 2006), and has some precedent in other fungal systems (Sussman & Douthit, 1973). In EMF systems, Pither and Pickles (2017) assembled some suggestive examples of much greater longevity, but the only experimentally measured longevity were previously part of the same study we report here (Bruns *et al.*, 2009; Nguyen *et al.*, 2012). While the spores and seeds of some species of EMF and extreme desert annual plants have at least decadal levels of longevity, they might differ in their need for dormancy for two reasons. First, unlike annual plants that have only one chance to reproduce and are therefore

susceptible to total reproductive failure due to extreme events, EMF are not annual organisms and can produce spores repeatedly (iteroparity). This reduces their chances for complete offspring failure, therefore reducing their need for a bet-hedging reproductive strategy such as dormancy (Cohen, 1967) while not affecting their need for propagule longevity. While our data does not allow us to make any inferences about what predisposes certain spores to enter dormancy or be receptive at the time of burial, it is possible that spore characteristics such as cell wall development (e.g. Nakano *et al.*, 2016) could be used to make *a priori* predictions about the likely trajectory of a population of spores.

The second difference between desert seeds and EMF spores with respect to dormancy, originates from the spatial distribution of their germination cues. Desert seeds germinate in response to rainfall, a cue that can be homogenously distributed over large areas. Therefore, an erroneous cue (early season rains followed by mid-season drought) might produce pseudo-suitable germination conditions that initiate germination of the *entire* seed bank over large areas and result in its exhaustion because of seedling death. Seed dormancy prevents this from happening by restricting germination of some of the seeds even under apparently favorable conditions. In contrast, EMF spores germinate in response to specific host root exudates (Fries, 1990). The spatial distribution of this cue is much patchier because host roots are heterogeneously distributed in the soil and germination requires close proximity between the spore and the roots (Fries, 1990). Therefore, even in the case of an erroneous cue (e.g., host seedling germination and root exudates initiates spore germination that is then terminated by seedling death), only a small fraction of the spore bank will be exhausted.

Establishing seedlings must be colonized either from a persistent spore bank or new dispersal from undisturbed areas. The density of EMF spores in natural settings is poorly known relative to other fungi and microbes, but it has been shown to vary considerably depending on the habitat (Galante *et al.*, 2011; Policelli *et al.*, 2019), the stage of forest development (Jumpponen *et al.*, 2002), depth of soil (Miller *et al.*, 1994) and distance from host trees (Smith *et al.*, 2018). Nevertheless, EMF spore banks are widespread in mature pine

forests (Glassman *et al.*, 2015) and sufficient to colonize the majority of seedlings after natural fire disturbance (Glassman *et al.*, 2016).

For *Rhizopogon*, Miller et al. (1994) found that spores could be recovered at multiple soil depths (0-3, 3-6 cm) at densities ranging from 10^8 to 10^{10} spores per g of soil. However, these high estimates are likely high due to seasonal sampling associated with the production of nearby fruiting bodies. The starting spore densities in our experiment are within the range encountered in field settings (Miller *et al.*, 1994; Jumpponen *et al.*, 2002; Bruns *et al.*, 2009), but given the notably variable patterns of colonization in seedling bioassays (Kjøller & Bruns, 2003; Rusca *et al.*, 2006), it is clear that EMF spores have a more patchy distribution in natural soils.

The key question of this long-term experiment is how long will these spores remain viable? As mentioned above, the interplay between spore mortality and spore dormancy complicates our ability to estimate longevity. To address this, we attempted to extrapolate the shape of these curves beyond the 15 years of data using polynomial models. However, we had no confidence in the estimates we obtained because they varied greatly depending on models used. Nevertheless, as we accumulate more observations from the next time points, the shape of these curves will better constrain the models and enable a more accurate estimate for the spore longevity in these species. When complete, the entire experiment will yield a 99-year record that is relevant on the ecological timescales over which forest disturbance and recovery occur.

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Author Contributions

TDB planned and designed the research. HS, PGK & NHN performed the experiments. HS, TDB and KGP analyzed data. HS wrote the manuscript and all authors made significant contributions to the writing process.

Competing interests

None declared

Data Availability

The data tables and R codes used in this study are available at https://github.com/nnguyenlab/rhizopogon-longevity .

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Supporting information: Figure S1 shows the relationship between spore concentration and proportion of pine seedlings colonized for three *Rhizopogon* species.