Received: 13 July 2023 | Accepted: 27 October 2023

DOI: 10.1111/1365-2435.14476

Functional Ecology BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

The Influence of Beneficial Fungi on Plant–Enemy Interactions and Plant Community Structure

# Decadal survival of tropical pioneer seeds in the soil seed bank is accompanied by fungal infection and dormancy release

Paul-Camilo Zalamea[1,2] | Carolina Sarmiento[1,2] | A. Elizabeth Arnold[3,4] | Venus Kuo[5] | Carolyn Delevich[2] | Adam S. Davis[6] | Thomas A. Brown[7] | James W. Dalling[2,5]

[1]Department of Integrative Biology, University of South Florida, Tampa, Florida, USA; [2]Smithsonian Tropical Research Institute, Panama City, Republic of Panama; [3]School of Plant Sciences, The University of Arizona, Tucson, Arizona, USA; [4]Department of Ecology and Evolutionary Biology, The University of Arizona, Tucson, Arizona, USA; [5]Department of Plant Biology, University of Illinois, Urbana, Illinois, USA; [6]Department of Crop Sciences, University of Illinois, Urbana, Illinois, USA and [7]Lawrence Livermore National Laboratory, Center for Accelerator Mass Spectrometry, Livermore, California, USA

**Correspondence**
Paul-Camilo Zalamea
Email: paulcamiloz@usf.edu; camilozalamea@gmail.com

**Funding information**
Division of Environmental Biology, Grant/Award Number: NSF DEB-1119758, NSF DEB-1120205 and NSF DEB-BIO-2231760; Simons Foundation, Grant/Award Number: STRI-429440; U.S. Department of Energy by Lawrence Livermore National Laboratory, Grant/Award Number: DE-AC52-07NA27344

**Handling Editor:** Sunshine Van Bael

## Abstract

1. Pioneer trees require high-light environments for successful seedling establishment. Consequently, seeds of these species often persist in the soil seed bank (SSB) for periods ranging from several weeks to decades. How they survive despite extensive pressure from seed predators and soil-borne pathogens remains an intriguing question.
2. This study aims to test the hypotheses that decades-old seeds collected from the SSB in a lowland tropical forest remain viable by (i) escaping infection by fungi, which are major drivers of seed mortality in tropical soils, and/or (ii) maintaining high levels of seed dormancy and seed coat integrity when compared to inviable seeds.
3. We collected seeds of *Trema micrantha* and *Zanthoxylum ekmanii* at Barro Colorado Island, Panama, from sites where adult trees previously occurred in the past 30 years. We used carbon dating to measure seed age and characterized seed coat integrity, seed dormancy and fungal communities.
4. Viable seeds from the SSB ranged in age from 9 to 30 years for *T. micrantha*, and 5 to 33 years for *Z. ekmanii*. We found no evidence that decades-old seeds maintain high levels of seed dormancy or seed coat integrity. Fungi were rarely detected in fresh seeds (no soil contact), but phylogenetically diverse fungi were detected often in seeds from the SSB. Although fungal infections were more commonly detected in inviable seeds than in viable seeds, a lack of differences in fungal diversity and community composition between viable and inviable seeds suggested that viable seeds are not simply excluding fungal species to survive long periods in the SSB.

---

Paul-Camilo Zalamea and Carolina Sarmiento contributed equally to this work.

5. *Synthesis*. Our findings reveal the importance of a previously understudied aspect of seed survival, where the impact of seed–microbial interactions may be critical to understand long-term persistence in the SSB.

KEYWORDS
Seed coat integrity, seed dormancy, seed endophytes, seed germination, seed long-term persistence, seed–fungal interactions, soil seed bank, tropical pioneer trees

## 1 | INTRODUCTION

Tropical pioneer trees colonize and establish in treefall gaps and other forest disturbances. In old-growth forests, treefall gaps typically result from seasonal blowdowns and lightning strikes (Gora et al., 2021) and are rare at the landscape level (Lobo & Dalling, 2013). Their rarity limits recruitment opportunities for seeds of pioneer trees in space and time. Therefore, some species of pioneer trees compensate for infrequent recruitment opportunities by prolonging seed persistence in the soil.

In the seed ecology literature, it is often stated that seed dormancy, defined as physical or physiological characteristics of seeds that prevent germination when environmental conditions are appropriate for germination, improves the chances of survival by delaying germination until conditions are favourable for seedling establishment and growth, thus reducing the risk of reproductive failure (e.g. Long et al., 2015). Mechanisms of dormancy differ among species: for example, physically dormant seeds are enclosed inside a water-impermeable layer that prevents germination, whereas physiologically dormant seeds are permeable but can only germinate if dormancy is broken, often as a response to specific chemical or environmental cues (Thompson, 2000). Seed persistence, defined as the time between soil arrival and germination, ranges from a few weeks to a few decades for tropical pioneer trees (Dalling & Brown, 2009; Long et al., 2015). Burial experiments and carbon dating of seed coats of pioneer species from the lowland forests in Panama suggest that decadal seed persistence may be common among larger seeded pioneers that have either physical or physiological seed dormancy (Dalling & Brown, 2009; Zalamea et al., 2018).

After reaching the soil, seeds can be attacked by granivores or infected by microbes, including soil-borne fungi that are significant causes of seed mortality in tropical forest soils (Dalling et al., 1997, 1998). Thus, seeds have a range of defensive strategies, with the production of hard and fibrous external layers or investment in chemical defences fine-tuned to limit predation and pathogen infection (Dalling et al., 2020). Recent work on tropical seeds has revealed how investments in physical and chemical seed defences vary with seed dormancy class, reflecting the importance of seed permeability as a key trait (Zalamea et al., 2018). Seed mortality due to fungi is widespread across pioneer species (Dalling et al., 1998; Gallery et al., 2010). However, recent evidence also suggests that non-dormant (i.e. quiescent) seeds of *Cecropia* species may be particularly dependent on protection from certain soil-borne, seed-infecting fungi (Dalling et al., 2020; Gallery et al., 2007; Zalamea et al., 2021). Thus, fungal infections may have positive or negative effects, with the outcome of interactions between seeds and fungi (i.e. functional specificity, Sarmiento et al., 2017) highly dependent on host species identity.

Seed burial experiments often are used to study seed fate in the soil seed bank (SSB) (Long et al., 2015). However, they are usually short in time and therefore do not address mechanisms of long-term seed persistence. Census data collected from long-term forest dynamics plots contain fine-scale data on the past locations of trees, which allows sampling of potentially decades-old SSBs. Using this approach in the 50-ha forest dynamics plot on Barro Colorado Island (BCI), Panama, Dalling and Brown (2009) found that physiologically dormant seeds of *Croton billbergianus*, *Trema micrantha* and *Zanthoxylum ekmanii* collected at or near the soil surface (<3 cm soil depth) can survive for at least 38, 31 and 18 years respectively. Seed burial experiments at BCI that excluded seed predators but allowed microbial colonization also showed that seeds of *T. micrantha* and *Z. ekmanii* did not lose viability after 30 months of burial (Zalamea et al., 2018). However, little is known about the effect of dormancy status and microbial colonization on the fate of decades-old seeds with seed dormancy from the natural SSB.

The aim of this study was to test the hypotheses that decades-old seeds collected from the SSB in a lowland tropical forest remain viable by (i) escaping infection by fungi and/or (ii) maintaining high levels of seed dormancy and seed coat integrity when compared to inviable seeds. We focused on *Trema micrantha* and *Zanthoxylum ekmanii*, which are pioneer species that have seeds that can persist in the SSB for decades, produce physiologically dormant seeds and differ in their investment in chemical defences (Dalling & Brown, 2009; Zalamea et al., 2018). We collected seeds of each species from sites at BCI where conspecific reproductive trees had occurred over the previous 30 years. We then used carbon dating to measure seed age and characterized seed coat integrity, seed dormancy and fungal communities to test three predictions. First, if dead seeds accumulate saprobic infections, we predicted that infection rates of old seeds would be higher for dead seeds than live seeds. Second, if dead seeds can be colonized by opportunistic fungi, but viable seeds are mainly colonized by a narrower pool of endophytes or pathogens that can impact seed survival, inviable seeds should have higher fungal diversity when compared to viable seeds. Third, as long-term seed persistence can be achieved by having permeable seeds that are well-defended chemically or by having impermeable seeds that are well-defended physically (Zalamea et al., 2018), we predicted that viable old seeds of *T. micrantha* and *Z. ekmanii* should maintain high levels of seed dormancy and seed coat integrity. Finally, we explored if the focal plant species harbour distinct seed-associated fungal communities as previous work suggested for other pioneer seed species (Sarmiento et al., 2017; Zalamea et al., 2021).

## 2 | METHODS

### 2.1 | Study site and species selection

This study was conducted in a seasonally moist lowland tropical forest on BCI, Republic of Panama (9°10′ N, 79°51′ W). Rainfall on BCI averages 2600 mm/year, with a pronounced dry season from January to April (Windsor, 1990). The BCI 50-ha forest dynamics plot on the central plateau of the island was established in 1980 with all trees >1 cm diameter at breast height (DBH) identified, mapped, and censused every 5 years.

*Trema micrantha* (Cannabaceae) and *Zanthoxylum ekmanii* (Rutaceae) are pioneer species that retain high seed viability in burial experiments (Dalling et al., 1997; Zalamea et al., 2018). *Trema micrantha* is a rare canopy tree in old-growth forest at BCI. Its fruits are produced year-round and seeds, with an average seed mass of 3.2 mg, are dispersed by birds. *Zanthoxylum ekmanii* is a relatively common dioecious canopy tree in old-growth forest at BCI. Its fruits are produced annually and seeds, with an average mass of 16.2 mg, are dispersed primarily by primates and secondarily by ants (Dalling & Brown, 2009; Ruzi et al., 2017, 2021; Zalamea et al., 2018).

### 2.2 | Seed sampling

#### 2.2.1 | Replication statement

| Scale of inference | Scale at which the factor of interest is applied | Number of replicates at the appropriate scale |
|---|---|---|
| Species | Species | Two species, five individuals for *Trema micrantha* and six individuals for *Zanthoxylum ekmannii*, two levels of seed viability (i.e. viable and inviable).<br>Number of seeds of *Trema micrantha* and *Zanthoxylum ekmannii* used for viability and ID of seed-associated fungi (692 and 593 respectively); germination (236 and 196 respectively); carbon dating (9 and 9 respectively); and fracture resistance (224 and 142 respectively). |

We used the BCI 50-ha plot census data to locate sites occupied in the past by reproductive-sized individuals of *T. micrantha* and *Z. ekmanii*. Sites were selected to ensure that at least one reproductive-sized individual of each tree species died at least 5 years before the seed sampling, with no subsequent recruitment, mature conspecifics in the immediate area.

We collected soil cores between July and December 2014 from five individual localities where *T. micrantha* occurred previously, and six localities where *Z. ekmanii* occurred. We collected 0.5 L cores consisting only of surface soil layers (0–3 cm depth) to ensure that seeds were collected at depths from which emergence can successfully occur (Pearson et al., 2002; Ruzi & Suarez, 2022). For each sampled site, soil cores were collected from at least three haphazardly chosen areas in a 5 m by 5 m quadrat. No Panamanian permits are required for collecting soil cores at BCI and permission to sample in the 50-ha plot was granted by the Smithsonian Tropical Research Institute Scientific Committee.

### 2.3 | Seed germination, seed viability, isolation of seed-associated fungi and seed fracture resistance

Seeds were extracted from soil cores by rinsing the soil with tap water under a series of sieves, with a minimum pore diameter of 2 mm for *Z. ekmanii* and 1 mm for *T. micrantha*. Seeds were identified to species and then partitioned for tests of (i) seed viability measured using the tetrazolium (TZ) test and identification of seed-associated fungi (692 and 593 seeds of *T. micrantha* and *Z. ekmanii* respectively), (ii) germination and carbon dating (a total of 239 and 196 seeds of *T. micrantha* and *Z. ekmanii*, respectively, where nine viable seeds of each species were carbon dated) and (iii) seed fracture resistance as a measure of seed coat integrity (224 and 142 seeds of *T. micrantha* and *Z. ekmanii* respectively).

Fungal isolation and seed viability assessment followed Sarmiento et al. (2017) and Zalamea et al. (2021). About 60% of seeds recovered from each soil core were haphazardly selected, surface sterilized (95% ethanol, 10 s; 0.7% sodium hypochlorite, 2 min; 70% ethanol, 2 min), and allowed to surface-dry under sterile conditions. Surface sterilization allows the isolation of fungi that had colonized the interior of the seed while eliminating fungi that were restricted to the seed surface. After surface sterilization, the seeds were cut in half under sterile conditions. One half was used to score seed viability using TZ (2, 3, 5-triphenyl tetrazolium chloride), and the other half was placed on 2% malt extract agar (MEA) in an individual 1.5 mL microcentrifuge tube for fungal isolation. With this approach both fungal infection and seed viability status were scored for each seed. For viability testing, seed halves were placed in Petri dishes lined with filter paper, saturated with 0.5% TZ immediately after cutting, and kept at room temperature (ca. 22°C) in the dark for 24 h prior to scoring as viable or inviable. To avoid seed desiccation throughout the incubation time, dishes were sealed with Parafilm®. For fungal isolation, tubes were incubated at room temperature with natural light–dark cycles and checked for fungal growth after a few months of incubation (often 2–4 months). We also made sure that every tube was checked at least once after 12 months of incubation, so slow-growing fungi were able to grow. If fungal growth was observed, we excised a small piece of fresh mycelium from each emergent culture under sterile conditions. Fungi were isolated into pure culture, vouchered as living mycelium in sterile water, and processed for DNA extraction and Sanger sequencing (see below). All vouchers have been archived at the Robert L. Gilbertson Mycological Herbarium, University of Arizona (MYCO-ARIZ).

Seed germination experiments followed Zalamea et al. (2015). Briefly, to score seed germination we placed seeds in groups of ~10 into a Petri dish lined with paper towel, moistened with sterile distilled water and sealed with two layers of Parafilm®. Petri dishes were incubated in a shade house on BCI under ca. 30% sunlight, high red: far-red irradiance ca. 1.4, and ambient temperature (23–30°C). Germination was defined as radicle protrusion and scored weekly for 6 weeks.

Seed fracture resistance, defined as the minimum force required to initiate seed rupture, was measured as a proxy of physical protection. Seed fracture resistance was measured by an Instron Single Column Testing System Model 3342 (Instron Company, USA) and followed Zalamea et al. (2018). Briefly, each seed was loaded between the anvil and the compression probe and then compressed until the seed coat ruptured. The seed coat rupture creates a sudden drop in force, such that the instrument can precisely record the force causing the fracture. After measuring seed fracture resistance, we scored seed viability via TZ staining in old seeds, so that seed viability and seed fracture resistance could be examined in tandem.

To determine baseline values of initial fungal infection rates, fungal diversity, dormancy levels, and seed fracture resistance we also collected fresh seeds (i.e. seeds that did not have soil contact) of *T. micrantha* and *Z. ekmannii* from at least five different maternal sources per species. For each species we evaluated 200 seeds for fungal infection rates and fungal diversity, 200 seeds for germination and 35 seeds for seed fracture resistance.

## 2.4 | Age determination of seeds

To determine seed ages we followed Dalling and Brown (2009). Seed coat samples from germinated seeds or seed halves from viable seeds used for fungal isolation were cleaned and dated by accelerator mass spectrometry (AMS) at the Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory. By directly counting $^{14}$C ions AMS yields high-precision $^{14}$C/$^{13}$C measurements from very small amounts of sample carbon. The measured $^{14}$C/$^{13}$C ratios were converted to F$^{14}$C values following standard conventions (Reimer et al., 2004; Stuiver & Polach, 1977). The calendar year dates of carbon fixation for seed coats were determined through comparison of the sample F$^{14}$C values to a long-term record for Northern Hemisphere atmospheric $^{14}$C. Seed ages obtained with this technique are conservative because we assume that carbon fixation occurred during the period of declining $^{14}$C/$^{13}$C (post-1963) rather than ascending $^{14}$C/$^{13}$C (1953–1963). Because of the cost of AMS, we evaluated 18 individual seeds: we selected three sites per species representing the highest number of seeds recovered from soil cores and dated three individual seeds per site (Table 1). We averaged dates for sites that included three seeds if the ages differed. For the other two sites for *T. micrantha* and three for *Z. ekmannii*, we selected the census year when the tree under which we collected the soil was first reported dead. Carbon-dated seeds were consistently older than expected from the date of tree death recorded in the 50-ha census data, suggesting that this is a conservative age for the seeds that were not carbon dated.

## 2.5 | Seed-associated fungal communities

We used the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich, Saint Louis, MO, USA) to extract total genomic DNA from ground mycelium of each pure culture. The polymerase chain reaction (PCR) was performed using the primers ITS1F and LR3 to amplify the nuclear ribosomal internal transcribed spacers and 5.8S gene (ITS rDNA) and an adjacent portion (ca. 600 bp) of the large subunit (LSU rDNA). If amplification failed, PCR was repeated with primers ITS5 and LR3. PCR reaction mixture and cycling reactions followed Sarmiento et al. (2017). PCR products were verified by staining with SYBR Green I (Molecular Probes, Invitrogen, Carlsbad, CA, USA) followed by electrophoresis on a 1% agarose gel. Following Sarmiento et al. (2017) and Zalamea et al. (2021), all PCR products that yielded single bands were cleaned using 1 μL of ExoSAP-IT reagent (Affymetrix, Inc., Cleveland, OH, USA), quantified, normalized and sequenced bidirectionally at the University of Arizona Genetics Core with the original sequencing primers. We assembled contigs and verified base calls from chromatograms following Sarmiento et al. (2017).

Isolation frequency was calculated as the number of isolates divided by the total number of examined seeds. Following Zalamea et al. (2021), we defined operational taxonomic units (OTUs) by 97% similarity in the Sanger clustering workflow of the Mobyle SNAP Workbench (Monacell & Carbone, 2014). We measured fungal diversity as Fisher's alpha, because this index is robust to variation in sample size and it is appropriate given the relative abundance of OTUs (Sarmiento et al., 2017).

In total, 692 and 593 seeds of *T. micrantha* and *Z. ekmanii* were screened for fungal growth. Overall, 174 and 220 fungal strains were isolated from each species, and high-quality sequence data were obtained from 100% of all strains (Table 1; Table S1). A total of 129 OTUs were found, all of which were included in measures of fungal diversity.

We defined the seed-associated fungal communities as the group of fungal isolates coming from seeds of a given plant species, tree site and seed viability status. OTUs that are rare in the database could not be present in all plant species or all communities; thus, we excluded singletons and fungal communities with fewer than three

**TABLE 1** Seed ages, number of viable and inviable seeds processed, number of cultivable fungi, number of sequences, number of operational taxonomic units (OTUs) and fungal diversity obtained from old seeds of *Trema micrantha* and *Zanthoxylum ekmanii* collected from the soil seed bank in lowland tropical forest on Barro Colorado Island.

| Species | C-dated seed age (years) | 95% CI | Viability | Number of seeds | Number of cultivable fungi | Number of sequences | Total number of OTU | Fisher's alpha |
|---|---|---|---|---|---|---|---|---|
| *Trema micrantha* | 9 | ±2 | Inviable | 66 | 32 | 32 | 14 | 9.5 |
| | | | Viable | 154 | 47 | 47 | 22 | 16.1 |
| | 18 | ±2 | Inviable | 37 | 14 | 14 | 9 | 10.9 |
| | | | Viable | 156 | 33 | 33 | 16 | 12.2 |
| | 25 | * | Inviable | 2 | 1 | 1 | 1 | NA |
| | | | Viable | 2 | 0 | NA | NA | NA |
| | 27 | ±2 | Inviable | 79 | 17 | 17 | 10 | 10.2 |
| | | | Viable | 167 | 26 | 26 | 19 | 31.8 |
| | 30 | * | Inviable | 20 | 2 | 2 | 2 | NA |
| | | | Viable | 9 | 2 | 2 | 1 | NA |
| *Zanthoxylum ekmannii* | 5 | * | Inviable | 20 | 10 | 10 | 8 | 18.6 |
| | | | Viable | 2 | 1 | 1 | 1 | NA |
| | 18 | ±2 | Inviable | 38 | 22 | 22 | 13 | 13.3 |
| | | | Viable | 33 | 12 | 12 | 12 | NA |
| | 21 | ±1.5 | Inviable | 233 | 115 | 115 | 56 | 43.1 |
| | | | Viable | 94 | 34 | 34 | 23 | 31.2 |
| | 25 | * | Inviable | 5 | 1 | 1 | 1 | NA |
| | | | Viable | 1 | 0 | NA | NA | NA |
| | 30 | * | Inviable | 60 | 15 | 15 | 12 | 27.9 |
| | | | Viable | 4 | 1 | 1 | 1 | NA |
| | 33 | ±1.5 | Inviable | 81 | 8 | 8 | 8 | NA |
| | | | Viable | 22 | 1 | 1 | 1 | NA |

*These seeds were not C dated. We selected the census year when the tree under which we collected the soil was first reported to be dead.

isolates from analyses of community structure. For analyses, we ended with a pool of 55 OTUs and 13 communities.

### 2.6 | Data analysis: Linking isolation frequency, fungal diversity, seed germination, physical protection, seed age and seed-associated fungal communities

We used linear mixed effect models to test whether isolation frequency, fungal diversity, proportion of dormant/germinating seeds and seed fracture resistance varied by plant species, seed viability status and their interaction. For each response variable, we coded each tree site as a random effect and plant species, seed viability status and their interaction as fixed effects. We also used linear regression analysis to determine if fungal isolation frequency and fungal diversity were associated with seed age. Data analyses were done in R version 4.0.4 (R Development Core Team, 2021) using the package *nlme* (Pinheiro et al., 2023).

We used a multivariate generalized linear model (GLM) to test the extent to which each factor (i.e. plant species, tree site or seed viability) was relevant to fungal community structure. A negative-binomial GLM based on sequence count data was performed with the R package *mvabund* (Wang et al., 2012). To complement the results from the multivariate GLM, non-metric multidimensional scaling (NMDS) and variance-partitioning analyses were implemented with the R package *vegan* (Oksanen et al., 2019). The NMDS was performed using the function metaMDS with the dissimilarity matrix calculated using the Morisita–Horn index and the parameter *k* set to three dimensions. The Morisita–Horn index was used because it has an advantage over the most commonly used Bray–Curtis index in that it is not strongly sensitive to sample size (Chao et al., 2006). For graphical representations of the NMDS, only the first two dimensions were included. We used the function *varpart* to perform variance-partitioning analyses, with plant species, tree site and seed viability coded as explanatory factors. Finally, taxonomic annotation of the sequences was based on T-BAS v. 2.1 (Carbone et al., 2019).

## 3 | RESULTS

Viable seeds from the SSB ranged in age from 9 to 30 years for *T. micrantha*, and 5 to 33 years for *Z. ekmanii* (Table 1). Fresh unburied

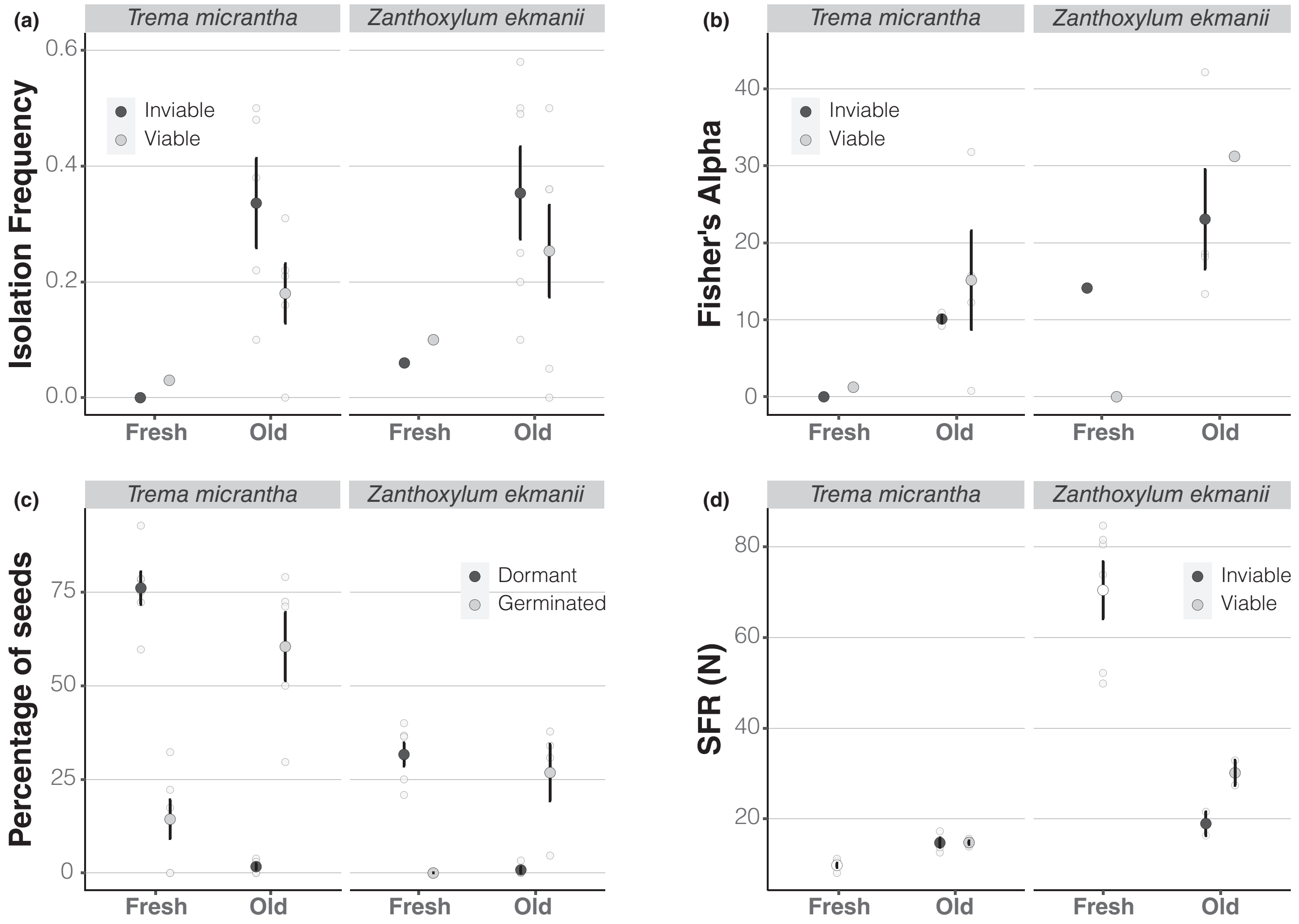

FIGURE 1 (a) Isolation frequency of fungi for fresh unburied and decades-old seeds of *Trema micrantha* and *Zanthoxylum ekmanii*. Old seeds were collected from the soil seed bank in lowland tropical forest on Barro Colorado Island. Isolation frequency was calculated as the number of fungal isolates divided by the number of seeds processed in each collection point and seed viability class. (b) Diversity of fungi isolated from fresh unburied and old seeds. Fisher's alpha was calculated with all sequenced isolates from each plant species and seed viability class. (c) Percentage of seeds that were dormant and germinable from fresh and old seeds. (d) Seed fracture resistance (SFR; Newtons (N)) of fresh and old seeds. SFR was calculated by viability class in old seeds. Error bars represent the standard error.

seeds had low levels of fungal infection and fungal diversity, suggesting that most of the fungi colonized the seeds in the soil (Figure 1a,b).

We predicted that fungal isolation frequency in decades-old seeds would be higher on dead (inviable) seeds than on live (viable) seeds. In agreement with this prediction, we found that the frequency of fungal infections was higher in inviable seeds than in viable seeds collected from the SSB ($F_{1,11}=5.21$, $p=0.043$), a trend that is consistent for both species (species, $F_{1,11}=0.39$, $p=0.542$; species x seed viability, $F_{1,11}=0.26$, $p=0.622$; Figure 1a). When fungal isolation frequency was regressed against seed age there was a significant negative association for both viable and inviable seeds (inviable, $r^2=0.51$, $n=11$, $p=0.008$; viable, $r^2=0.43$, $n=11$, $p=0.017$; Figure 2a,b).

We also predicted that among old seeds, inviable seeds would have higher fungal diversity when compared to viable seeds. Contrary to this prediction, we did not find differences in fungal diversity between viable versus inviable seeds collected from the SSB ($F_{1,3}=0.67$, $p=0.471$). Diversity of seed-associated fungi also did not differ between plant species ($F_{1,3}=2.68$, $p=0.200$), and there was no evidence for a meaningful interaction between seed viability and seed species ($F_{1,3}=0.27$, $p=0.640$; Figure 1b). Fungal diversity and seed age were not associated for inviable or viable seeds (inviable, $r^2=0.02$, $n=7$, $p=0.79$; viable, $r^2=0$, $n=5$, $p=0.92$; Figure 2c,d).

Seed viability differed between *T. micrantha* and *Z. ekmanii* ($F_{1,7}=6.95$, $p=0.033$; Figure 1c). Overall, 62% of *T. micrantha* and 28% of *Z. ekmanii* seeds recovered from soil cores were viable. We found no evidence that decades-old seeds maintain high levels of seed dormancy and seed coat integrity. Instead, dormancy levels of old seeds were less than 2% for *T. micrantha* and 1% for *Z. ekmanii*, whereas they were over 75% and 30% in fresh unburied seeds respectively (Figure 1c).

Our expectation of high seed coat integrity received only partial support: viable seeds were harder than inviable seeds ($F_{1,4}=8.02$, $p=0.047$; Figure 1d), but this pattern was not observed in both species (species × seed viability; $F_{1,4}=15.52$, $p=0.017$; Figure 1d).

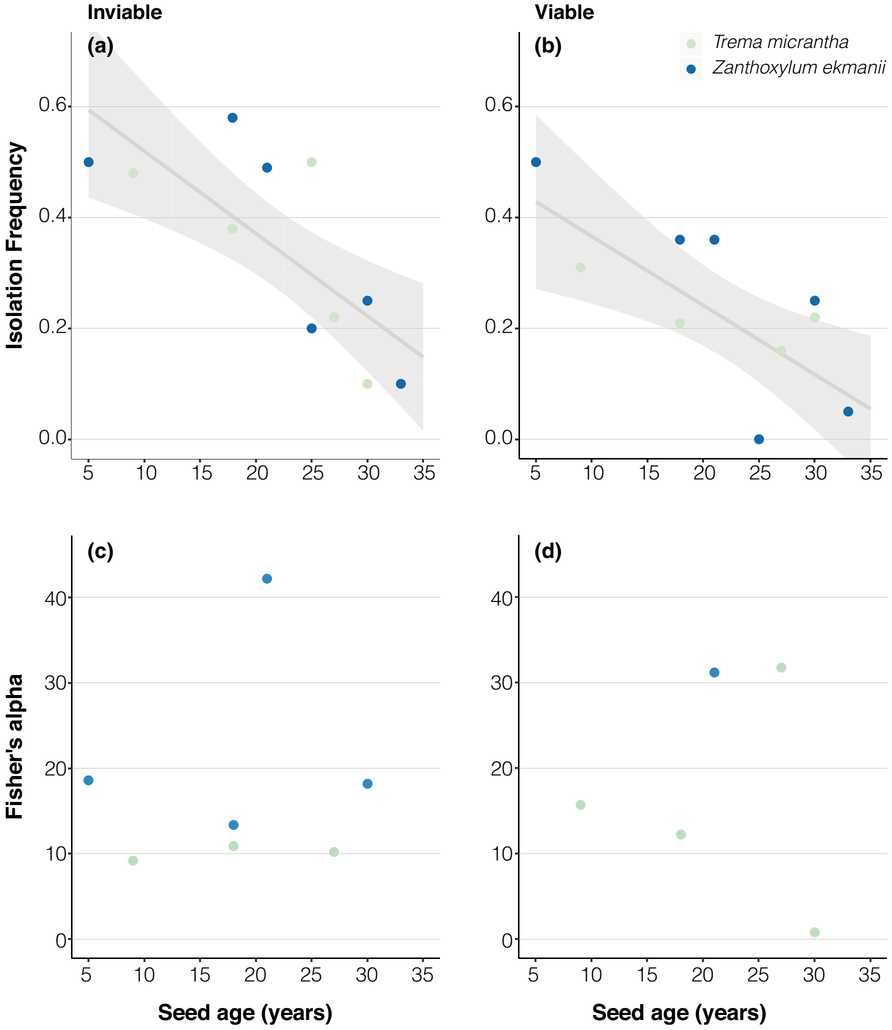

**FIGURE 2** Relationship between isolation frequency of fungi recovered from seeds and seed age for (a) inviable and (b) viable seeds. Relationship between the diversity of fungi isolated from seeds and seed age for (c) inviable and (d) viable seeds. The lines represent significant relationships and shaded areas represent the 95% confidence interval around the lines.

When seed coat integrity values for old seeds were compared to fresh unburied seeds we found mixed results: old seeds were harder than fresh seeds in *T. micrantha*, but softer in *Z. ekmanii* (Figure 1d).

We found that seed-associated fungal communities differed between the focal plant species (negative-binomial GLM; $df_{1,11}$, Dev = 161.1, $p < 0.01$; Figure 3a). However, seed-associated fungal communities did not differ among localities ($df_{1,10}$, Dev = 70.3, $p = 0.23$) or as a function of seed viability ($df_{1,9}$, Dev = 87.3, $p = 0.16$). Plant species alone explained 25% of the total variance in OTU abundance while tree locality only explained 5% and seed viability 3% of the total variance (Figure 3b).

Fungi isolated from old seeds represented a broad range of phylogenetic lineages (Figure 4; Table S1). The three most abundant classes of Ascomycota isolated from *T. micrantha* and *Z. ekmanii* seeds were the same, but the ranking and abundances were different between species (Figure 4a). Sordariomycetes was the most abundant class isolated from both species, followed by Dothideomycetes in *T. micrantha*, and Eurotiomycetes in *Z. ekmanii* (Figure 4a). Hypocreales and Xylariales were the most abundant orders isolated from both species (Figure 4b).

## 4 | DISCUSSION

Soil-borne fungal pathogens (including the fungus-like Oomycota) are a major source of seedling and seed mortality in tropical forests (Augspurger, 1984; Bagchi et al., 2014; Dalling et al., 1998). This study explored how seeds of pioneer trees can survive in the SSB for decades in a lowland tropical forest in Panama. Working with decades-old seeds collected from the SSB, we tested the hypotheses that viable seeds escape infection by fungi and/or maintain high levels of seed dormancy and seed coat integrity. We found support for neither hypothesis. Instead, seeds of *Trema micrantha* and *Zanthoxylum ekmanii* associate with different fungi, including in seeds that are viable after many years of soil contact. Thus, our results highlight the importance of a previously understudied aspect of seed survival, where the impact of seed–microbial interactions that may not necessarily be negative, may be critical to understanding long-term persistence in the SSB.

We used AMS to carbon-date seeds of *T. micrantha* and *Z. ekmanii* that were extracted from undisturbed SSBs in the 50-ha forest dynamics plot on BCI. Seed ages reported in this study were consistent with seed ages reported by Dalling and Brown (2009)

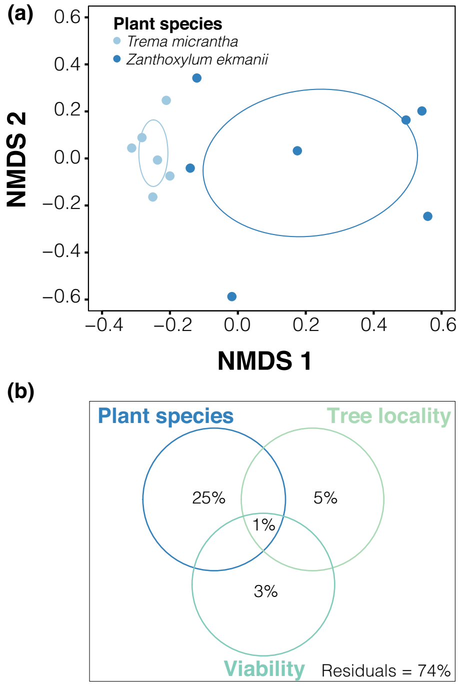

FIGURE 3 Structure of seed-associated fungal communities isolated from old seeds. (a) Non-metric multidimensional scaling (NMDS) analysis representing Morisita–Horn dissimilarity among fungal communities (points) isolated from old seeds of *Trema micrantha* and *Zanthoxylum ekmanii*. Fungal communities are colour-coded to represent plant species; stress = 0.084. Ellipses represent a standard deviation of point scores relative to their centroid. (b) Venn diagram represents the percent of variation in fungal community composition explained by plant species, tree locality (collection site) and seed viability. If the percent of variation is zero, values are not represented in the figure.

for *T. micrantha*, but extended the measured duration of seed persistence from 18 to 33 years for *Z. ekmanii*. Decades-long persistence has been hypothesized as a common trait among large-seeded tropical pioneers that produce physiologically or physically dormant seeds (Zalamea et al., 2018). Our results are consistent with this hypothesis and suggest that at least some large-seeded tropical pioneer species overcome enemy-mediated and environmental pressures by selecting trait combinations that permit long-term persistence (Dalling et al., 2020; Zalamea et al., 2018).

Treefall gaps in tropical lowland forests are rare, resulting in limited recruitment opportunities for pioneer species. In the seed ecology literature, dormancy has often been proposed as an essential mechanism for long-term persistence by which plants can decouple the timing of seed dispersal from the availability of conditions conducive to successful germination and recruitment (Baskin & Baskin, 1998). Over 80% of viable fresh seeds of *T. micrantha* and *Z. ekmanii* were dormant at the time of dispersal, but contrary to our prediction, we found that decades-old viable seeds of both species do not maintain high levels of seed dormancy. These results suggest that in addition to seed dormancy, long-term persistence is achieved through other mechanisms.

The maintenance of seed dormancy plays a key role in seed persistence in weed species in agricultural fields, desert and grassland ecosystems, where opportunities for successful seed germination are frequent, but where post-germination conditions are unpredictable (Long et al., 2015). In a lowland tropical rainforest in Panama, Zalamea et al. (2021) used a 30-month seed burial experiment in the forest understory to show that non-dormant seeds of three species of *Cecropia* and *Jacaranda copaia* can persist in the SSB without being dormant. For pioneer trees in lowland tropical rainforest, the long lifespan of canopy trees (and therefore time interval between treefall gaps), means that seeds likely only experience suitable germination conditions a single time during their lifetime. The avoidance of germination through seed dormancy is therefore unlikely to increase fitness.

We used seed fracture resistance as a proxy for seed coat integrity and found opposing results for seeds of *T. micrantha* and *Z. ekmanii*. Fracture resistance was similar between viable and inviable old seeds of *T. micrantha*, but higher in viable seeds of *Z. ekmanii*. Compared to freshly collected seeds, seed fracture resistance was higher in old seeds of *T. micrantha*, but lower in old seeds of *Z. ekmanii*. The primary barrier isolating the seed interior from the physical environment is the seed coat. It has often been stated that hard seed coats protect the seed from mechanical stresses and pathogen infections (e.g. Mohamed-Yasseen et al., 1994). Previous research found that freshly collected seeds of *T. micrantha* and *Z. ekmanii* are permeable (Zalamea et al., 2018). Collectively, results from this contribution and previous findings suggest that maintaining hard and impermeable seeds is not necessarily critical to long-term persistence.

Previous studies hypothesized that microbially induced defences could range from associations with microbial mutualists that exclude potential pathogens to induction of biochemical defences consisting of latent enzymes that are activated in the presence of pathogens (Dalling et al., 2020; Zalamea, 2024). Thus, we suggest that studying microbially induced defences and the degree to which microbial defences are carried over time could be a fruitful inquiry in future seed ecological research. Germination data acquired in a 30-month burial experiment on BCI, including *T. micrantha* and *Z. ekmanii*, showed that the proportion of dormant seeds decreased through time, while seed viability remained unchanged (Zalamea et al., 2018). The observation that decades-old viable seeds in this study harbour fungal infections and have low levels of seed dormancy highlights the need for further exploration of seed–fungal interactions in achieving long-term persistence in seeds of *T. micrantha* and *Z. ekmanii*, and possibly other species too.

Seeds from tropical pioneers often associate with a wide variety of soil-borne fungi (Gallery et al., 2010; Sarmiento et al., 2017). Sarmiento et al. (2017) found that the composition of fungal communities that infect seeds of nine species of pioneer trees is strongly determined by plant species rather than by forest characteristics, soil type or time seeds spent in the SSB. Zalamea et al. (2021) found that seed-associated fungal communities were also strongly structured by plant species, even among three congeneric species of *Cecropia*. Here, we found that decades-old seeds of *T. micrantha* and *Z. ekmanii* harbour distinctive fungal communities, adding empirical support to previous research findings.

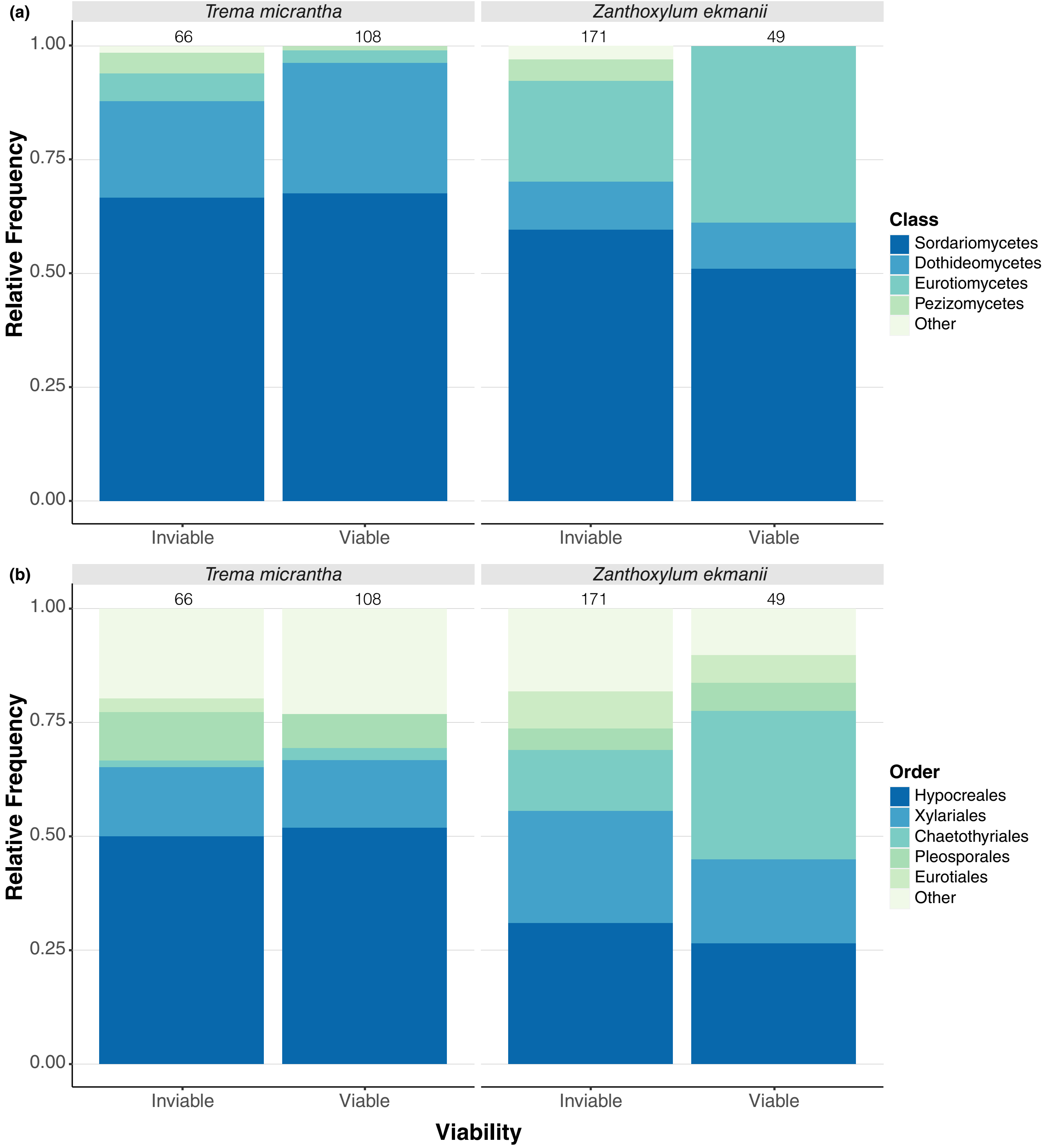

FIGURE 4 Relative frequency and taxonomic composition of seed-associated fungi isolated from old viable and inviable seeds at the (a) class and (b) order levels. Numbers on top of each bar represent the number of isolates for which we obtained sequences.

Previous studies have shown that seed endophytes are primarily filamentous Ascomycota (mainly Pezizomycotina), including many species of Sordariomycetes (Nelson, 2018; Simonin et al., 2022; Zalamea et al., 2021). Although the relative abundance of fungal classes varied considerably among plant species, seed microbiome studies consistently find that seed-infecting fungi of crop and tropical tree species are often represented by many species within a few fungal classes (Simonin et al., 2022; Zalamea et al., 2021). We found that major fungal groups at the class and order levels isolated from decades-old seeds of *T. micrantha* and *Z. ekmanii* are similar between these species and previous seed microbiome studies (Nelson, 2018; Sarmiento et al., 2017; Simonin et al., 2022; Zalamea et al., 2021). Thus, our results suggest that future studies might focus on understanding the functional impact of a few fungal classes on seed survival and persistence.

## 5 | CONCLUSIONS

Seeds are often colonized by soil-borne fungi in both wild and agricultural systems (e.g., Nelson, 2018; Sarmiento et al., 2017; Simonin et al., 2022). Seed microbial community assembly is the result of complex interactions (Bergmann & Leveau, 2022). Previous studies suggest that the diversity of seed-infecting fungi is comparatively low to other plant tissues such as leaves (Arnold & Herre, 2003; Newcombe et al., 2018). The low microbial richness of seeds compared to other plant tissues makes individual seed–microbial interactions potentially more meaningful to study the impact of these interactions on survival and persistence. Contrary to our expectations, long-term persistent seeds of two tropical pioneer trees did not maintain high levels of seed dormancy, high levels of seed coat integrity or avoid fungal colonization. Instead, we documented that a broad range of fungal species colonizes old, viable seeds. This finding supports previous studies that showed that seed infections by non-lethal fungi are common in tropical ecosystems (Sarmiento et al., 2017; Zalamea et al., 2021). Microbially induced defences in seeds remain a topic of great potential in the ecological literature. Still, we consider that with the improvement in methodological approaches to studying plant–microbial interactions, future research inquiries can benefit from a closer look at the impact of these interactions on seed survival and persistence.

## AUTHOR CONTRIBUTIONS

James W. Dalling and Paul-Camilo Zalamea conceived and designed the study; Venus Kuo, Carolyn Delevich, Carolina Sarmiento, Thomas A. Brown, Adam S. Davis and Paul-Camilo Zalamea collected the data; Carolina Sarmiento and Paul-Camilo Zalamea analysed the data; Paul-Camilo Zalamea wrote the manuscript with contributions from James W. Dalling, A. Elizabeth Arnold, Adam S. Davis and Carolina Sarmiento. All authors contributed substantially to revisions and gave final approval for publication.

## ACKNOWLEDGEMENTS

This research was funded by NSF DEB-1120205 (to JWD and ASD) and NSF DEB-1119758 (to AEA). PCZ was supported by NSF DEB-BIO-2231760, a grant from the Simons Foundation to the Smithsonian Tropical Research Institute (429440), and the University of South Florida. This work was performed, in part, under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. We thank the Smithsonian Tropical Research Institute for providing facilities, logistical support and permission to conduct the project. We especially thank A. Ndobegang and A. Pérez for essential assistance in the field and laboratory.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Sequences used in this study are deposited in NCBI GenBank under accession numbers: KY776240–KY776354 and OR658438-OR658846. Data deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.7d7wm381z (Zalamea et al., 2023).

## ORCID

*Paul-Camilo Zalamea* https://orcid.org/0000-0002-0987-4164
*Carolina Sarmiento* https://orcid.org/0000-0002-8575-7170
*A. Elizabeth Arnold* https://orcid.org/0000-0002-7013-4026
*Carolyn Delevich* https://orcid.org/0000-0003-3981-299X
*Adam S. Davis* https://orcid.org/0000-0002-7196-1197
*Thomas A. Brown* https://orcid.org/0000-0001-9984-7647
*James W. Dalling* https://orcid.org/0000-0002-6488-9895

## REFERENCES

Arnold, A. E., & Herre, E. A. (2003). Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia*, *95*(3), 388–398. https://doi.org/10.1080/15572536.2004.11833083

Augspurger, C. K. (1984). Seedling survival of tropical tree species: Interactions of dispersal distance, light-gaps, and pathogens. *Ecology*, *65*(6), 1705–1712. https://doi.org/10.2307/1937766

Bagchi, R., Gallery, R. E., Gripenberg, S., Gurr, S. J., Narayan, L., Addis, C. E., Freckleton, R. P., & Lewis, O. T. (2014). Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature*, *506*(7486), 85–88. https://doi.org/10.1038/nature12911

Baskin, C. C., & Baskin, J. M. (1998). *Seeds: Ecology, biogeography, and, evolution of dormancy and germination*. Elsevier.

Bergmann, G. E., & Leveau, J. H. (2022). A metacommunity ecology approach to understanding microbial community assembly in developing plant seeds. *Frontiers in Microbiology*, *13*, 877519. https://doi.org/10.3389/fmicb.2022.877519

Carbone, I., White, J. B., Miadlikowska, J., Arnold, A. E., Miller, M. A., Magain, N., U'Ren, J. M., & Lutzoni, F. (2019). T-BAS version 2.1: Tree-based alignment selector toolkit for evolutionary placement of DNA sequences and viewing alignments and specimen metadata on curated and custom trees. *Microbiology Resource Announcements*, *8*, 1160–1165. https://doi.org/10.1128/MRA.00328-19

Chao, A., Chazdon, R. L., Colwell, R. K., & Shen, T. J. (2006). Abundance-based similarity indices and their estimation when there are unseen species in samples. *Biometrics*, *62*, 361–371. https://doi.org/10.1111/j.1541-0420.2005.00489.x

Dalling, J. W., Arnold, A. E., Davis, A. S., Sarmiento, C., & Zalamea, P. C. (2020). Extending plant defense theory to seeds. *Annual Review of Ecology, Evolution, and Systematics*, *51*, 123–141. https://doi.org/10.1146/annurev-ecolsys-012120-115156

Dalling, J. W., & Brown, T. A. (2009). Long-term persistence of pioneer species in tropical rain forest soil seed banks. *The American Naturalist*, *173*, 531–535. https://doi.org/10.1086/597221

Dalling, J. W., Swaine, M. D., & Garwood, N. C. (1997). Soil seed bank community dynamics in seasonally moist lowland tropical forest, Panama. *Journal of Tropical Ecology*, *13*, 659–680. https://doi.org/10.1017/S0266467400010853

Dalling, J. W., Swaine, M. D., & Garwood, N. C. (1998). Dispersal patterns and seed bank dynamics of pioneer trees in moist tropical forest. *Ecology*, *79*, 564–578. https://doi.org/10.1890/0012-9658(1998)079[0564:DPASBD]2.0.CO;2

Gallery, R. E., Dalling, J. W., & Arnold, A. E. (2007). Diversity, host affinity, and distribution of seed-infecting fungi: A case study with *Cecropia*. *Ecology*, *88*, 582–588. https://doi.org/10.1890/05-1207

Gallery, R. E., Moore, D. J. P., & Dalling, J. W. (2010). Interspecific variation in susceptibility to fungal pathogens in seeds of 10 tree species in the neotropical genus *Cecropia*. *Journal of Ecology*, *98*, 147–155. https://doi.org/10.1111/j.1365-2745.2009.01589.x

Gora, E. M., Bitzer, P. M., Burchfield, J. C., Gutierrez, C., & Yanoviak, S. P. (2021). The contributions of lightning to biomass turnover, gap formation and plant mortality in a tropical forest. *Ecology*, *102*, e03541. https://doi.org/10.1002/ecy.3541

Lobo, E., & Dalling, J. W. (2013). Effects of topography, soil type and forest age on the frequency and size distribution of canopy gap disturbances in a tropical forest. *Biogeosciences*, *10*(11), 6769–6781. https://doi.org/10.5194/bg-10-6769-2013

Long, R. L., Gorecki, M. J., Renton, M., Scott, J. K., Colville, L., Goggin, D. E., Commander, L. E., Westcott, D. A., Cherry, H., & Finch-Savage, W. E. (2015). The ecophysiology of seed persistence: A mechanistic view of the journey to germination or demise. *Biological Reviews*, *90*(1), 31–59. https://doi.org/10.1111/brv.12095

Mohamed-Yasseen, Y., Barringer, S. A., Splittstoesser, W. E., & Costanza, S. (1994). The role of seed coats in seed viability. *The Botanical Review*, *60*, 426–439. https://doi.org/10.1007/BF02857926

Monacell, J. T., & Carbone, I. (2014). Mobyle SNAP Workbench: A web-based analysis portal for population genetics and evolutionary genomics. *Bioinformatics*, *30*, 1488–1490. https://doi.org/10.1093/bioinformatics/btu055

Nelson, E. B. (2018). The seed microbiome: Origins, interactions, and impacts. *Plant and Soil*, *422*, 7–34. https://doi.org/10.1007/s11104-017-3289-7

Newcombe, G., Harding, A., Ridout, M., & Busby, P. E. (2018). A hypothetical bottleneck in the plant microbiome. *Frontiers in Microbiology*, *9*, 1645. https://doi.org/10.3389/fmicb.2018.01645

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). *vegan: Community ecology package*. R package version 2.3-4. https://CRAN.R-project.org/package=vegan

Pearson, T. R. H., Burslem, D. F. R. P., Mullins, C. E., & Dalling, J. W. (2002). Germination ecology of neotropical pioneers: Interacting effects of environmental conditions and seed size. *Ecology*, *83*(10), 2798–2807. https://doi.org/10.1890/0012-9658(2002)083[2798:GEONPI]2.0.CO;2

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. (2023). *nlme: Linear and nonlinear mixed effects models*. R package version 3.1-162. https://CRAN.R-project.org/package=nlme

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Reimer, P. J., Brown, T. A., & Reimer, R. W. (2004). Discussion: Reporting and calibration of post-bomb $^{14}$C data. *Radiocarbon*, *46*(3), 1299–1304. https://doi.org/10.1017/S0033822200033154

Ruzi, S., Roche, D., Zalamea, P. C., Robison, A., & Dalling, J. W. (2017). Species identity influences secondary removal of seeds of Neotropical pioneer tree species. *Plant Ecology*, *218*, 983–995. https://doi.org/10.1007/s11258-017-0745-7

Ruzi, S. A., & Suarez, A. V. (2022). Seed fate in ant-mediated dispersal: Seed dispersal effectiveness in the Ectatomma ruidum (Formicidae)—*Zanthoxylum ekmanii* (Rutaceae) system. *Biotropica*, *54*(3), 764–775. https://doi.org/10.1111/btp.13098

Ruzi, S. A., Zalamea, P. C., Roche, D. P., Achury, R., Dalling, J. W., & Suarez, A. V. (2021). Can variation in seed removal patterns of Neotropical pioneer tree species be explained by local ant community composition? *Biotropica*, *53*(2), 619–631. https://doi.org/10.1111/btp.12904

Sarmiento, C., Zalamea, P. C., Dalling, J. W., Davis, A. S., Stump, S. M., U'Ren, J. M., & Arnold, A. E. (2017). Soilborne fungi have host affinity and host-specific effects on seed germination and survival in a lowland tropical forest. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 11458–11463. https://doi.org/10.1073/pnas.1706324114

Simonin, M., Briand, M., Chesneau, G., Rochefort, A., Marais, C., Sarniguet, A., & Barret, M. (2022). Seed microbiota revealed by a large-scale meta-analysis including 50 plant species. *New Phytologist*, *234*(4), 1448–1463. https://doi.org/10.1111/nph.18037

Stuiver, M., & Polach, H. A. (1977). Discussion: Reporting of $^{14}$C data. *Radiocarbon*, *19*(3), 355–363. https://doi.org/10.1017/S0033822200003672

Thompson, K. (2000). The functional ecology of soil seed banks. In M. Fenner (Ed.), *Seeds: The ecology of regeneration in plant communities* (2nd ed., pp. 215–235). CABI.

Wang, Y., Naumann, U., Wright, S. T., & Warton, D. I. (2012). mvabund—An R package for model-based analysis of multivariate abundance data. *Methods in Ecology and Evolution*, *3*, 471–474. https://doi.org/10.1111/j.2041-210X.2012.00190.x

Windsor, D. M. (1990). Climate and moisture variability in a tropical forest: Long-term records from Barro Colorado Island, Panama. *Smithsonian Contributions to the Earth Sciences*, *29*, 1–145. https://doi.org/10.5479/si.00810274.29.1

Zalamea, P.-C. (2024). Insights on seed defenses and seed dormancy from the trees of Barro Colorado Island. In H. C. Muller-Landau & S. J. Wright (Eds.), *The first 100 years of research on Barro Colorado Island: Plant and ecosystem science*. Smithsonian Institution Scholarly Press.

Zalamea, P. C., Dalling, J. W., Sarmiento, C., Arnold, A. E., Delevich, C., Berhow, M. A., Ndobegang, A., Gripenberg, S., & Davis, A. S. (2018). Dormancy-defense syndromes and tradeoffs between physical and chemical defenses in seeds of pioneer species. *Ecology*, *99*, 1988–1998. https://doi.org/10.1002/ecy.2419

Zalamea, P. C., Sarmiento, C., Arnold, A. E., Davis, A. S., & Dalling, J. W. (2015). Do soil microbes and abrasion by soil particles influence persistence and loss of physical dormancy in seeds of tropical pioneers? *Frontiers in Plant Science*, *5*, 799. https://doi.org/10.3389/fpls.2014.00799

Zalamea, P. C., Sarmiento, C., Arnold, A. E., Davis, A. S., Ferrer, A., & Dalling, J. W. (2021). Closely related tree species support distinct communities of seed-associated fungi in a lowland tropical forest. *Journal of Ecology*, *109*(4), 1858–1872. https://doi.org/10.1111/1365-2745.13611

Zalamea, P.-C., Sarmiento, C., Arnold, A. E., Kuo, V., Delevich, C., Davis, A. S., Brown, T. A., & Dalling, J. W. (2023). Data from: Decadal survival of tropical pioneer seeds in the soil seedbank is accompanied by fungal infection and dormancy release. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.7d7wm381z

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1:** Sequence accession numbers (GenBank) and taxonomy of fungi isolated from decades-old seeds. Fungal isolates were obtained from seeds of *Trema micrantha* and *Zanthoxylum ekmanii* extracted from soil cores collected from five and six independent localities on Barro Colorado Island respectively. Operational taxonomic units (OTUs) were defined at 97% sequence similarity. For each sequence we provide the GenBank accession number.

**How to cite this article:** Zalamea, P.-C., Sarmiento, C., Arnold, A. E., Kuo, V., Delevich, C., Davis, A. S., Brown, T. A., & Dalling, J. W. (2023). Decadal survival of tropical pioneer seeds in the soil seed bank is accompanied by fungal infection and dormancy release. *Functional Ecology*, *00*, 1–11. https://doi.org/10.1111/1365-2435.14476