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# Context-dependent and variable effects of endohyphal bacteria on interactions between fungi and seeds

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ABSTRACT

Plant-associated fungi often harbor endohyphal bacteria (EHB) that modulate fungal phenotypes. We quantified the effects of EHB on interactions between fungi and seeds of neotropical pioneer trees, which fungi colonize naturally in forest soil. Seeds were exposed to six fungal isolates that harbored EHB, and to clones of those fungi from which EHB were removed by antibiotic treatment. Seed colonization by fungi was evaluated for five tree species, and germination and viability were evaluated for three tree species. EHB influenced seed colonization by fungi in 5 of 30 fungus-tree species combinations, but the magnitude of their effects was small and the direction of effects depended upon fungal isolate-tree species pairs. EHB had rare and context-dependent effects on seed germination and viability, but their effects were strong when observed. Rare but powerful effects of EHB on fungal interactions with seeds highlight important and context-dependent aspects of plant and fungal ecology.


## 1. Introduction

Seeds are the primary mode of reproduction for most plants, including the majority on which human sustainability depends (Kozlowski and Gunn, 1972). Their interactions with soilborne fungi are key to the success of agroecosystems and are important in shaping plant demography and community structure in natural systems (Dalling et al., 1998; Gallery et al., 2007). Soilborne fungi are especially important in tropical forests, where they are the dominant cause of seed mortality in the soil (Baker, 1972; Dalling et al., 1998; Gilbert, 2002; Sarmiento et al., 2017).

Diverse soilborne fungi colonize seeds of tropical forest trees after seeds are dispersed to the soil (Gallery et al., 2007; Kluger et al., 2008; Zalamea et al., 2015). These fungi are particularly important in the demography of species that form seed banks, such as pioneer trees (i.e., species that require high irradiance to establish and mature, and thus are important in early phases of colonizing forest gaps, edges, and cut areas; Swaine and Whitmore, 1988). Soilborne fungi that recruit to seeds (i.e., seed-associated fungi) can affect seed survival and germination in a host-specific manner, with the potential to alter seed bank composition, plant demography, and forest dynamics (Gilbert and Hubbell, 1996; Gallery et al., 2007, 2010; Sarmiento et al., 2017). Seed-associated fungi often are close relatives of foliar endophytes (Shaffer et al., 2016), which can influence plant physiology in early stages of

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seedling emergence and growth (Redman et al., 2002; Arnold and Engelbrecht, 2007). Many seed-associated fungi are generalists in terms of infecting multiple plant species, but each isolate can have distinctive impacts on survival and germination of seeds from different tree species (Sarmiento et al., 2017).

Interactions between fungi and seeds reflect diverse factors, including the genomic architecture of both the fungus and plant, and in some cases, the abiotic context of their associations (e.g., environmental stress, under which some nonpathogenic interactions transition to pathogenicity; Bever, 2015). Such interactions also can be influenced by the biotic context, primarily due to the action of microbes that occur near, on, or within fungal cells (e.g., viruses and bacteria; see Márquez et al., 2007; Partida-Martínez et al., 2007a; Anca et al., 2009; Bonfante and Anca, 2009). For example, many fungi harbor endosymbiotic bacteria (endohyphal bacteria, EHB), which can alter fungal traits relevant to interactions with plants (e.g., Partida-Martínez et al., 2007a; Hoffman et al., 2013; Desirò et al., 2015; Shaffer et al., 2017). A recent survey detected diverse EHB in tropical seed-associated fungi (Shaffer et al., 2016), but their functional roles have not been explored previously.

Functional roles of EHB are best known in the context of associations with diverse Mucoromycota and Basidiomycota that interact with roots and other tissues of plants after germination (reviewed by Araldi-Brondolo et al., 2017). These EHB can influence virulence of fungi, the establishment and function of mutualistic associations, and other fungal traits (Partida-Martínez and Hertweck, 2005; Lumini et al., 2007; Salvioli et al., 2010). For example, the EHB *Paraburkholderia rhizoxinica* (Betaproteobacteria) produces a virulence factor that allows *Rhizopus microsporus* (Mucoromycotina, Mucoromycota) to be pathogenic on rice (Partida-Martínez and Hertweck, 2005; Partida-Martínez et al., 2007a). Without the EHB, *R. microsporus* is no longer pathogenic and ceases to reproduce asexually (Partida-Martínez and Hertweck, 2005; Partida-Martínez et al., 2007b). Similarly, *Candidatus* Glomeribacter gigasporarum (Betaproteobacteria) enhances detection of root-associated strigolactones important for host recognition and the establishment of mycorrhizas by *Gigaspora margarita* (Glomeromycotina, Mucoromycota) (Bianciotto et al., 1996, 2003, 2004; Lumini et al., 2007; Anca et al., 2009). *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*, Alphaproteobacteria) in the endophyte *Piriformospora indica* (Sebacinales, Basidiomycota) promotes growth and resistance to pathogens in barley (Sharma et al., 2008). An endohyphal *Bacillus* sp. (Firmicutes) associated with *Ustilago maydis* (Ustilaginomycotina, Basidiomycota) fixes atmospheric nitrogen, making it available for its host fungus (Ruiz-Herrera et al., 2015).

The majority of seed-associated fungi are members of the Ascomycota, the most species-rich phylum of fungi (Spatafora et al., 2006; Arnold et al., 2009; Schoch et al., 2009; U'Ren et al., 2009). Screening of diverse filamentous Ascomycota (Pezizomycotina) indicates that EHB are common among Pezizomycetes, Eurotiomycetes, Dothideomycetes, and Sordariomycetes that associate with plants (e.g., Hoffman and Arnold, 2010; Hoffman et al., 2013; Arendt et al., 2016; Shaffer et al., 2016, 2017; Araldi-Brondolo et al., 2017). They often form facultative associations, and many EHB can be removed by antibiotic treatments (Hoffman et al., 2013; Arendt et al., 2016; Shaffer et al., 2017). In some cases these EHB can be cultured axenically (but see Shaffer et al., 2017).

Although associations between EHB and Ascomycota are numerous, only two have been explored in detail with regard to functional effects relevant to plant-fungal interactions. Hoffman et al. (2013) described an association between a leaf-endophytic strain of *Pestalotiopsis neglecta* and its endohyphal *Luteibacter* sp. (Gammaproteobacteria). *Luteibacter* sp. enhances production of indole-3-acetic acid when it associates with the fungus (vs. the axenic fungus; Hoffman et al., 2013) and may influence the capacity of the fungus to degrade lignin (Arendt, 2015). More recently, Shaffer et al. (2017) described the importance of an endohyphal *Chitinophaga* sp. (Bacteroidetes) in a seed-associated isolate of *Fusarium keratoplasticum*. *Chitinophaga* sp. enhances hyphal growth on many substrates, including several relevant to seeds (e.g., D-trehalose, *myo*-inositol, sucrose) (Shaffer et al., 2017). Although EHB are common in seed-associated Ascomycota from tropical forests (Shaffer et al., 2016), to date no experiments have evaluated the effects of EHB on seed-fungus interactions.

The aim of this study was to quantify the effects of EHB on the interactions of fungi and seeds. Specifically, we examined how the presence or absence of EHB can influence colonization of seeds by fungi, with a focus on five species of neotropical pioneer trees. We then quantified the impacts of those fungi on seed germination and viability, focusing on three tree species. Together, the focal tree species represent three families and distinctive functional traits. Fungi used in our experiments were isolated directly from seeds or as foliar endophytes that are placed phylogenetically in clades with seed-associated strains (Shaffer et al., 2016). Our experiments centered on six fungal isolates that naturally harbored EHB, which we removed via antibiotic treatment for our study.

## 2. Materials and methods

We selected fungi from the living culture collection at the Robert L. Gilbertson Mycological Herbarium, University of Arizona, Tucson, Arizona, USA (ARIZ). All fungi were isolated originally from seasonally moist tropical forest at Barro Colorado Island, Panama (BCI: 9° 10′N, 79° 51′W; 86 m a.s.l.; for a site description and details of the flora see Croat (1978) and Leigh (1999)). We selected three isolates of seed-associated fungi and three isolates of foliar endophytic fungi found previously to harbor EHB (Shaffer et al., 2016) (Table 1), focusing on two of the most prevalent families of Ascomycota found in seeds and leaves at BCI: Nectriaceae (Hypocreales) and Xylariaceae (Xylariales) (Arnold and Lutzoni, 2007; U'Ren et al., 2009; Sarmiento et al., 2017). Previous research showed that clades within each family typically contain both seed-associated and foliar endophytic isolates, and that EHB are naturally common in these lineages (Shaffer et al., 2016). Multilocus phylotyping previously showed that two of the seed-associated fungal isolates selected here represent the same putative species (Shaffer et al., 2016), although they harbor unique EHB partners (Table 1). Seed-associated fungi were isolated from surface-sterilized seeds of pioneer trees following burial for 1–6 months in the forest understory (Zalamea et al., 2015, 2018; Sarmiento et al., 2017; Table 1). Foliar endophytic fungi were isolated from surface-sterilized, asymptomatic leaves of diverse vascular plants (see Del Olmo-Ruiz and Arnold (2014) for isolation methods; Table 1).

### 2.1. Preparation of axenic fungal strains

Tissue segments from living fungal vouchers were plated under sterile conditions on 2% malt extract agar (MEA) (Amresco, Solon, OH, USA) and incubated at room temperature (*ca.* 22 °C). These isolates harbored EHB, and cultures derived from them are referred to hereafter as EHB+ strains. We removed EHB by culturing subsamples of hyphae from each isolate onto 2% MEA amended with four antibiotics: tetracycline (10 μg/mL), ampicillin (100 μg/mL), ciprofloxacin (40 μg/mL), and kanamycin (50 μg/mL) (Hoffman et al., 2013; Arendt et al., 2016; Shaffer et al., 2017), incubated as above. We refer to these axenic fungi as EHB− strains.

**Table 1**
Fungi used to evaluate the effect of endohyphal bacteria (EHB) on seed-fungus interactions. Fungal and EHB phylotypes and operational taxonomic units (OTU) are from Shaffer et al. (2016). Fungal phylotypes are based on phylogenetic analysis of the nuclear internal transcribed spacers and 5.8S ribosomal RNA (rRNA) gene (ITS rDNA) and the first *ca.* 600 base pairs of the large subunit rRNA gene (partial LSU rDNA) for *Fusarium concolor*, Nectriaceae sp. 1, and *Xylaria cubensis*, ITS-partial LSU rDNA, *RPB2*, and *TEF* for *Fusarium keratoplasticum* and the *Fusarium solani* species complex (FSSC), and ITS-partial LSU rDNA, *RPB1*, and *TEF* for *Gliocladiopsis* sp. 1. Fungal OTU are based on 95% similarity of the ITS-partial LSU rDNA. Phylotypes of EHB are based on phylogenetic analysis of the 16S rRNA gene. Each EHB phylotype indicates the lowest taxon represented by a well-supported clade in which EHB sequences were placed with named, reference bacterial 16S rRNA sequences. Bacterial OTU are based on 97% similarity of 16S rRNA.

| Fungal isolate | Phylotype | Family | Isolation source | Host species | ITS GenBank accession no. | ITS OTU | EHB phylotype | EHB 16S GenBank accession no. | 16S OTU |
|---|---|---|---|---|---|---|---|---|---|
| PS0362A | *Fusarium keratoplasticum* | Nectriaceae | seed | *Cecropia insignis* | **KU977740** | A | *Chitinophaga* | **KU978322** | 5 |
| PS0768 | *Gliocladiopsis* sp. 1[b] | Nectriaceae | seed | *Trema micrantha* "black"[c] | **KU977909** | K | *Enterobacter* | **KU978353** | 4 |
| PS0772[a] | *Gliocladiopsis* sp. 1[b] | Nectriaceae | seed | *Trema micrantha* "black"[c] | **KU977912** | K | *Enterobacter* | **KU978356** | 4 |
| | | | | | | | *Variovorax* | **KU978357** | 18 |
| | | | | | | | Sphingomonadaceae | **KU978359** | s69 |
| P0265[a] | *Fusarium concolor* | Nectriaceae | leaf | *Hybanthus prunifolius* | **KU978419** | J | *Streptococcus* | **KU978236** | s36 |
| | | | | | | | *Cutibacterium* | **KU978237** | 6 |
| | | | | | | | *Rothia* | **KU978238** | s37 |
| P0277[a] | Nectriaceae sp. 1[b] | Nectriaceae | leaf | *Garcinia intermedia* | **KU978420** | V | Oxalobacteriaceae | **KU978239** | 34 |
| | | | | | | | *Curvibacter* | **KU978240** | s38 |
| | | | | | | | *Bradyrhizobium* | **KU978241** | 9 |
| | | | | | | | *Stenotrophomonas* | **KU978242** | 41 |
| | | | | | | | Pseudomonadales | **KU978243** | s39 |
| | | | | | | | *Pelomonas* | **KU978244** | s40 |
| | | | | | | | *Tatumella* | **KU978245** | 23 |
| P0540 | *Xylaria cubensis* | Xylariaceae | leaf | *Xylopia macrantha* | **KU978436** | W | *Ralstonia* | **KU978295** | 3 |

[a] Fungal isolate was found to harbor more than one endohyphal bacterium (Shaffer et al., 2016).
[b] Refers to currently undescribed species (Shaffer et al., 2016).
[c] Refers to the small-seeded morphotype *sensu* Silvera et al. (2003).

### 2.2. Confirmation of EHB status

We confirmed the presence or absence of EHB by light microscopy, molecular analysis, and fluorescence microscopy following Hoffman and Arnold (2010) and Shaffer et al. (2017). We first confirmed the absence of extrahyphal bacteria (i.e., contaminants in the medium or on hyphal surfaces) by examining five preparations of hyphae per fungal strain at 400× and 1,000× on a Leica DM400B compound microscope (Shaffer et al., 2017). We did not observe extrahyphal bacteria in any EHB+ or EHB− strains used in this study.

We then extracted total genomic DNA from the growing edge of fresh cultures (3–10 d old) of all EHB+ and EHB− strains and used the polymerase chain reaction (PCR) to amplify a *ca.* 1,400 base pair (bp) fragment of the 16S ribosomal RNA (rRNA) gene (forward primer 27F, reverse primer 1492R; 10 μM; Lane, 1991). Methods followed Shaffer et al. (2016). Negative controls with water in place of template failed to amplify as expected in all reactions. Positive controls consisting of bacterial DNA known to amplify with these primers (i.e., *Luteibacter* sp. isolate 9143, Gammaproteobacteria; Hoffman et al., 2013) amplified as expected. We cleaned positive products with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. We diluted the cleaned products 1:1 with molecular grade water prior to bidirectional sequencing on an AB3730XL (Applied Biosystems, Foster City, CA, USA) with PCR primers (5 μM) at the University of Arizona Genetics Core (for details see Shaffer et al., 2016). We called bases and assembled bidirectional reads into contigs using the pipeline described in Shaffer et al. (2016). We verified base calls by manual inspection of chromatograms in Sequencher v.5.1 (Gene Codes Corp., Ann Arbor, MI, USA). We consistently detected 16S rRNA of EHB in the EHB+ strains of their respective fungal hosts (see Table 1). We did not observe positive amplification of 16S rRNA in any EHB− strains. No other bacteria were observed in cultures.

We confirmed that EHB were viable and that they occurred within viable hyphae by treating living hyphae with the Live/Dead BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA). Methods followed Shaffer et al. (2017). We consistently observed fluorescence of nucleic acids distinct from fungal mitochondria or nuclear DNA in EHB+ strains (see Fig. 1A and B), but not in EHB− strains (see Fig. 1C and D).

Together, the absence of extrahyphal bacteria and successful amplification of EHB 16S rRNA genes from fungal genomic DNA served as evidence of EHB+ status (Hoffman and Arnold, 2010; Arendt et al., 2016; Shaffer et al., 2016). Similarly, the lack of PCR amplification of 16S rRNA genes and fluorescence confirmed EHB− status (Hoffman and Arnold, 2010; Arendt et al., 2016; Shaffer et al., 2016). We verified the EHB+ and EHB− status of fungi at the outset of the experiments described below, and before and after seed germination assays.

### 2.3. Seed collection and preparation

Seeds were collected from ripe fruits of five species of tropical pioneer trees in lowland tropical forests in Panama during the natural fruiting seasons of 2013–2014 (BCI: 9° 10′N, 79° 51′W; Gamboa: 9° 6′N, 79° 41′W). We collected ripe fruits from the canopy or freshly fallen fruits on the ground beneath crowns of at least three maternal trees of each species. We selected three pioneer tree species with quiescent seeds (i.e., seeds that germinate without need for breaking dormancy: *Cecropia longipes, Cecropia peltata* [Urticaceae], and *Trema micrantha* "brown" [Cannabaceae]), and two with physically dormant seeds (i.e., seeds with a water-impermeable coat; *Apeiba tibourbou* and *Ochroma pyramidale* [Malvaceae]) (see Sautu et al., 2007; Zalamea et al., 2018; Table 2). All are common and occur naturally in the study area, with intermediate-to-wide distributions throughout the neotropics (Croat, 1978).

We removed seeds from fruits and cleaned them manually by i) removing cottony filaments or ii) washing fruit pulp with tap water. We then allowed seeds to surface-dry in a darkroom at room temperature (*ca.* 22 °C). Prior to use in inoculation experiments, we surface-sterilized seeds by sequential immersion in 95% ethanol

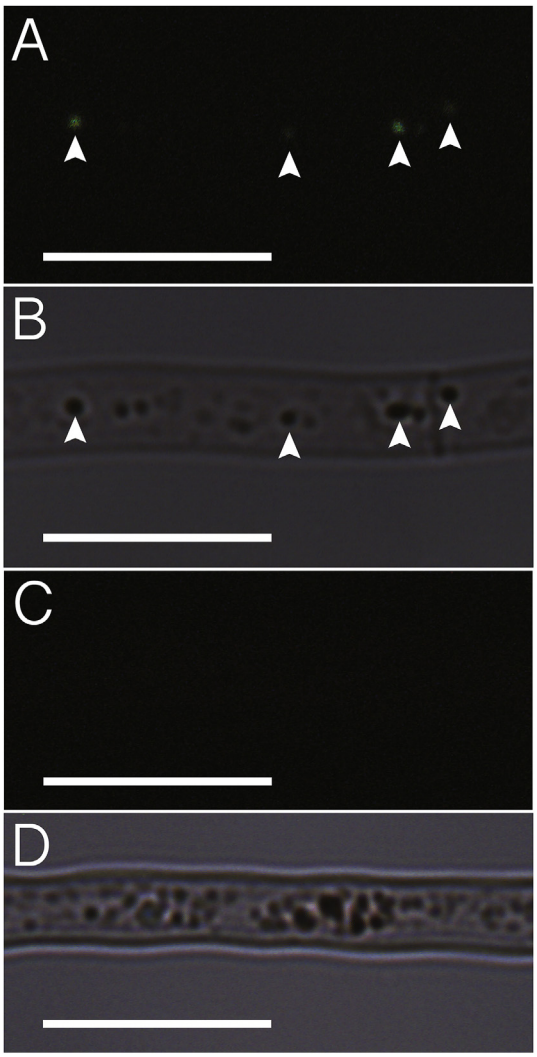


**Fig. 1.** Live/Dead staining of fungi. (A) Fluorescence micrograph highlighting the presence of endohyphal bacteria (EHB) in the naturally infected strain (EHB+) of seed-associated *Gliocladiopsis* sp. isolate PS0772. White arrows indicate viable bacteria tagged in green. (B) Same frame as in (A) viewed with differential interference contrast (DIC). (C) Fluorescence micrograph highlighting the absence of endohyphal bacteria in the cured strain (EHB−) of the same fungal isolate. (D) Same frame as in (C) viewed with DIC. Scale bars = 10 μm.

(10 s), 0.7% sodium hypochlorite (NaClO; 2 min), and 70% ethanol (2 min), and allowed seeds to surface-dry under sterile conditions in the dark (see Gallery et al., 2007; Zalamea et al., 2015).

### 2.4. Seed inoculation and incubation

Fungal cultures were grown in the dark on 2% MEA in 60-mm Petri plates at room temperature (*ca.* 22 °C). After 14 d, the fungi had formed lawns of mycelial growth across the surface of the growth medium. At that time, colony diameter was *ca.* 5 mm from the plate edge for all strains except EHB+ and EHB− strains of P0277 (Nectriaceae), for which the colony diameter was *ca.* 10 mm from the plate edge.

For inoculation, we placed seeds of each tree species onto the surface of these actively growing mycelia. In total, five sets of 20 seeds per tree species were placed into contact with each EHB+ and EHB− fungal strain, for a total of 200 inoculated seeds per tree species per fungal isolate (Table 1). Control seeds were surface-sterilized and plated as above, with ten sets of 20 seeds per tree species placed into Petri plates containing 2% MEA and no fungal growth.

All plates were wrapped with Parafilm M® (Bemis NA, Neenah, WI, USA) and incubated for 21 d in the dark in an outdoor location to mimic natural conditions (average temperature at 1 m above soil for June 2014 = 26 °C). The time needed for seeds to be colonized by fungi was determined in a preliminary trial and was supported by other studies (see Schafer and Kotanen, 2004; Sarmiento et al., 2017). To reverse germination cues that may have been induced by exposure to red light during plate set-up, we exposed plates containing seeds of *C. longipes* or *C. peltata* to far-red light for one hour prior to incubation outdoors (Finch-Savage and Leubner-Metzger, 2006). Overall, 7,000 seeds were used (seeds exposed to fungi: 20 seeds of five plant species, exposed to EHB+ and EHB− strains of each of six fungal isolates, replicated five times; controls: 20 seeds of five plant species, replicated ten times).

### 2.5. Evaluation of seed colonization

After 21 d, the surfaces of seeds of all species exposed to fungi were visibly colonized by those fungi. We scored the degree of colonization using an index similar to those used for assessing disease severity (Horsfall and Barratt, 1945; Agrios, 1997) or percent cover (Daubenmire, 1959) in plant communities. The index is based on an ordinal scale of four classes of increasing mycelial growth on seeds, as evaluated with a stereomicroscope: (1) sparse hyphal growth only on the lower seed half, in contact with the mycelial lawn; (2) sparse growth on both the lower and upper seed halves; seed clearly visible through the mycelium; (3) substantial growth on the whole seed, but the seed remained visible through the mycelium; and (4) substantial growth on the whole seed, such that the seed was no longer visible through the mycelium (Fig. 2A). For analysis we defined the seed colonization index (SCI) as follows:

$$\mathrm{SCI} = \frac{\sum_{i=1}^{n}(0.25 \times i \times C_i)}{n} \quad (1)$$

In Equation (1), $C_i$ represents the number of seeds scored as colonization class $i$, and $n$ is the total number of seeds per Petri dish (e.g., all 20 seeds scored as 4: $[0.25 \times 4 \times 20]/20 = 1$; all 20 seeds scored as 1: $[0.25 \times 1 \times 20]/20 = 0.25$; 10 seeds scored as 1 and 10 seeds scored as 2: $[0.25 \times 1 \times 10] + [0.25 \times 2 \times 10] = 7.5/$

**Table 2**
Tree species used in seed trials. All species co-occur naturally in central Panama.

| Tree species | Family | Dormancy type | Geographic distribution | Seed mass (mg) |
|---|---|---|---|---|
| *Apeiba tibourbou* | Malvaceae | physical[b] | Mexico-Brazil[e] | 6.10[i] |
| *Cecropia longipes* | Urticaceae | quiescent[c] | Panama-Colombia[f] | 0.9 ± 0.07[j] |
| *Cecropia peltata* | Urticaceae | quiescent[d] | West Indies, Venezuela-Brazil[f] | 0.79 ± 0.11[k] |
| *Ochroma pyramidale* | Malvaceae | physical[b] | Mexico-Brazil[g] | 5.73 ± 0.29[j] |
| *Trema micrantha* "brown"[a] | Cannabaceae | quiescent[c] | Florida-Argentina[h] | 1.71 ± 0.1[j] |

[a]Refers to the small-seeded morphotype *sensu* Silvera et al. (2003); [b] Sautu et al. (2007); [c] Zalamea et al. (2018); [d] Tiansawat and Dalling (2013); [e] Tropicos.org (2017); [f] Berg et al. (2005); [g] Zalamea et al. (2015); [h] Silvera et al. (2003); [i] Pearson et al. (2002); [j] Ruzi et al. (2017); [k]Gallery et al. (2010).

20 = 0.375). The values of our SCI therefore range from 0 to 1. We defined contamination as growth inconsistent with the morphology of the original fungal isolate on seeds exposed to fungi, or growth on control seeds.

### 2.6. Evaluation of seed germination

After scoring colonization we transferred seeds to new, sterile Petri plates (60-mm) containing sterile filter paper moistened with sterile water. Plates were sealed with Parafilm M® and incubated in an outdoor shade house at BCI with 30% full sun, high red: far-red irradiance (*ca.* 0.8), and ambient temperature. The same shade house was used in previous studies, such that conditions are appropriate for germinating seeds of many pioneer tree species (see Gallery et al., 2010; Zalamea et al., 2015; Sarmiento et al., 2017). Plates were incubated for 14 d. We then assessed germination every 7 d until plates had been incubated for a total of 49 d, at which point we ended the experiment. We scored seeds with visible radicles and cotyledons as germinated (Fig. 3A). Seeds that swelled when imbibed but lacked visible radicles and/or cotyledons were scored as inviable (dead). After incubation we used the tetrazolium test (TZ; 2, 3, 5-triphenyl tetrazolium chloride; Peters, 2000) to determine the viability of unimbibed, ungerminated seeds. No seeds of *O. pyramidale* and *T. micrantha* germinated, but seeds of those species remained viable (Fig. 4A). This indicates that shade house conditions did not meet germination requirements for these species (Garwood, 1983; Pearson et al., 2002). Therefore, analyses of germination and viability focused on *A. tibourbou, C. longipes,* and *C. peltata.*

### 2.7. Statistical analyses

We used generalized linear models and their extensions to evaluate differences in seed colonization, seed germination, and viability of ungerminated seeds as a function of tree species (Table 2), identity of the fungal isolate, (Table 1), and EHB status of the fungal strain (EHB+ vs. EHB−). We conducted statistical analyses in R (R Core Team, 2018). As the response variable for seed colonization (SCI) ranged from 0 to 1 but represented an ordinal scale of four classes, we avoided fitting a model that assumes a binomial error distribution. Instead, we used beta regression with a logit link function (Ferrari and Cribari-Neto, 2004) implemented with R package *betareg* (Cribari-Neto and Zeileis, 2010). Beta regression is robust to heteroscedasticity and unevenness, can model continuous response variables in the interval (0, 1) (i.e., proportion data), and assumes the data are beta distributed (Ferrari

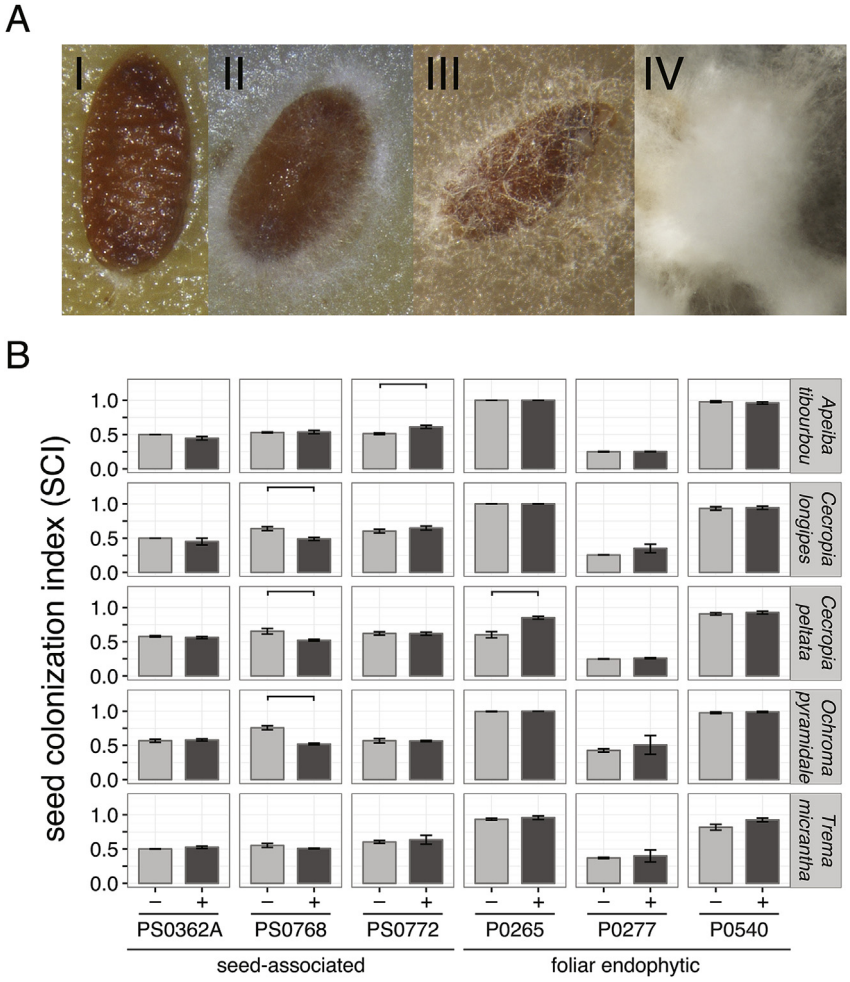


**Fig. 2.** Colonization of seeds by fungi. (A) Seeds of *C. peltata* illustrating the four colonization classes used in the seed colonization index (SCI, see Materials and methods). I and II, infected by Nectriaceae sp. 1 isolate P0277; III, infected by *Gliocladiopsis* sp. 1 isolate PS0772; IV, infected by *Fusarium concolor* isolate P0265. All seeds shown were infected by EHB+ strains (B) Proportion of seeds colonized by fungi (i.e., SCI). following incubation for 21 d in the dark: *Apeiba tibourbou*, *Cecropia longipes*, *C. peltata*, *Ochroma pyramidale*, and *Trema micrantha* "brown". Bars show means and standard errors from five replicate plates of 20 seeds each for EHB− (−) and EHB+ (+) strains of each fungal isolate. For each tree species, solid brackets indicate significant differences in seed colonization between EHB+ and EHB− strains for individual fungal isolates ($p$-value $\leq 0.05$; Supplementary Table 1).

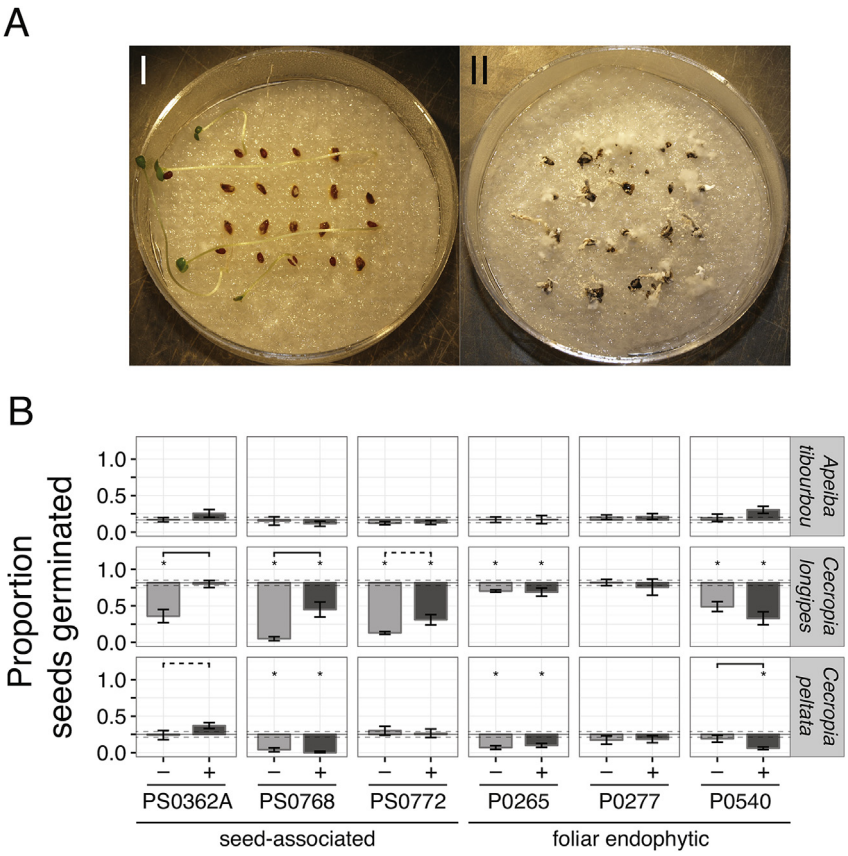


**Fig. 3.** Germination of infected seeds. (A) Seeds of *Cecropia peltata* illustrating differences in seed germination. I, seeds infected by *Gliocladiopsis* sp. 1 isolate PS0772; II, seeds infected by *Fusarium concolor* isolate P0265. All seeds shown were infected by EHB+ strains. (B) Proportion of infected seeds that germinated following incubation for 49 d in the shade house: *Apeiba tibourbou*, *C. longipes*, and *C. peltata*. For each tree species, dark grey horizontal lines represent the proportion of control seeds that germinated, the solid line represents the mean, dotted lines represent ± one standard error, and values are from ten replicate plates of 20 seeds each. Bars highlight deviations from the germination rate of uninoculated control seeds, and indicate the proportion of infected seeds that germinated (mean ± one standard error from five replicate plates of 20 seeds each) for EHB− (−) and EHB+ (+) strains of each fungal isolate. For each tree species, asterisks represent significant differences in germination between seeds infected by fungal strains and uninoculated controls (Supplementary Table 3), solid brackets indicate significant differences in germination after treatment with EHB+ and EHB− strains of individual fungal isolates ($p \leq 0.05$), and dashed brackets indicate trends in the same regard ($p$-value $\leq 0.1$; Supplementary Table 2). No seeds of *Ochroma pyramidale* and *Trema micrantha* "brown" germinated, such that these species were excluded from analyses of seed germination (see Materials and methods).

and Cribari-Neto, 2004). To avoid zeroes and ones, which are not possible in the beta distribution, we compressed the range of SCI measurements as recommended by Smithson and Verkuilen (2006) by taking $y^{adj} = [y\ (N - 1) + 0.25]/N$, where $y^{adj}$ is the adjusted measurement, $y$ is the original measurement, and $N$ is the total number of seeds in the experiment. In turn, as seed germination and viability measurements represented a proportion of seeds at the plate level that responded in one of two ways (i.e., success or failure), we modeled each response using logistic regression with a binomial error distribution (Crawley, 2007).

For each response variable, we included the identities of fungi in models because we expected variation in functional traits among isolates even if they belong to the same phylotype (i.e., genus- or species-level phylogenetic placement and/or operational taxonomic unit [OTU] based on 95% sequence similarity, *sensu* Shaffer et al., 2016; see also Stump, 2015). Similarly, isolates within a given phylotype or OTU often have phylogenetically distinct EHB (see Table 1), which may result in unique host responses (Arendt, 2015). Thus we anticipated interactions between tree species and fungal identity, and between fungal identity and EHB infection status.

For seed colonization, we included all three explanatory variables and their interactions as factors, and tested for their significance using hierarchical fitting of all possible models. We assessed the relative influence and significance of each explanatory variable by calculating likelihood ratios via the R package *lmtest* (Zeileis and Hothorn, 2002), and compared all models by considering the corrected Akaike Information Criterion (AICc) (Sigiura, 1978; Hurvich and Tsai, 1989; Cavanaugh, 1997).

For the generalized linear models explaining germination and viability of seeds, respectively, we examined the effects of tree species, fungal identity, EHB infection status, and their interactions using $\chi^2$ tests in analyses of deviance. For all models, the proportion of variance explained was interpreted using McFadden's pseudo $R^2$ (McFadden, 1973, 1978).

We excluded controls from the above analyses in order to focus comparisons on seeds exposed to fungi with and without EHB. We used two-sided Dunnett's tests (Dunnett, 1955) on separate models that included controls to compare germination and viability of seeds exposed to fungal strains vs. control seeds for each tree species. Models for each response within tree species were fitted as above. Data for each response within tree species were normally distributed. For Dunnett's tests, we controlled for the rate of Type I error via the false discovery rate-controlling method developed by Benjamini and Hochberg (1995), implemented in the R package *multcomp* (Hothorn et al., 2008). We used effect sizes from Dunnett's tests to test for correlations in the effects of fungal strains on seed germination or the viability of ungerminated seeds, with seed

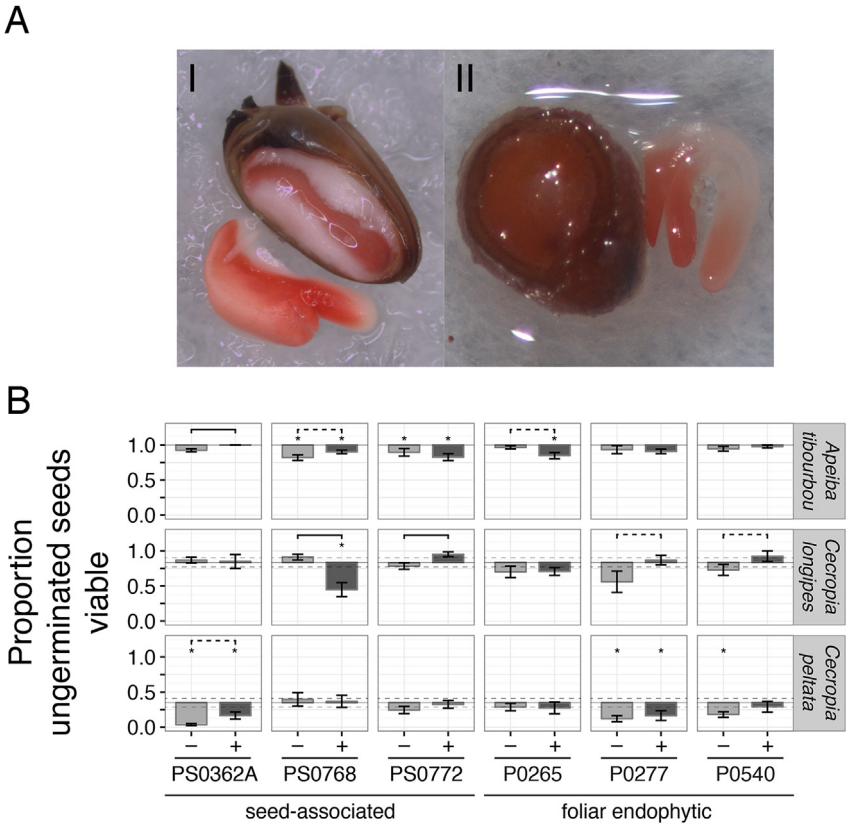


**Fig. 4.** Viability of seeds that did not germinate following incubation in the shade house. (A) Seeds found to be viable following TZ testing (see Materials and methods). The red staining on the cotyledons and embryo is a result of a redox reaction indicating cellular respiration. I, viable seed of *Ochroma pyramidale*; II, viable seed of *Trema micrantha* "brown". (B) Proportion of infected seeds that did not germinate but were viable following incubation for 49 d for *Apeiba tibourbou*, *Cecropia longipes*, and *C. peltata*. For each tree species, the dark grey horizontal lines represent the proportion of control seeds that did not germinate but were viable, where the solid line represents the mean, dotted lines represent ± one standard error, and values are from ten replicate plates of 20 seeds each. Bars highlight deviations from uninoculated control seeds, and indicate the proportion of infected seeds that remained viable (means ± one standard error from five replicate plates of 20 seeds each) for EHB− (−) and EHB+ (+) strains of each fungal isolate. For each tree species, asterisks represent significant differences in the proportion of ungerminated seeds that remained viable between seeds infected by fungal strains and uninoculated controls (Supplementary Table 5), solid brackets indicate significant differences in the proportion of ungerminated seeds that remained viable after treatment with EHB+ and EHB− strains of individual fungal isolates ($p \leq 0.05$), and dashed brackets indicate trends in the same regard ($p$-value $\leq 0.1$; Supplementary Table 4).

colonization (i.e., SCI).

Finally, we explored differences in all response variables between seeds of the same tree species infected by EHB+ vs. EHB− strains of each fungal isolate using Welch two-sample *t*-tests (Welch, 1947), which were robust to unequal variances and Type I error (Welch, 1947; Derrick et al., 2016). Data and code used in analyses are available online (Shaffer, 2018).

## 3. Results

All fungi colonized the surfaces of seeds of all tree species (Fig. 2). The degree to which fungi colonized seeds reflected an interaction of tree species, fungal identity, and EHB infection status (Table 3). However, significant effects of EHB on colonization of seeds by fungi were observed in only 5 of 30 (17%) fungus-tree species pairs (Fig. 2B; Supplementary Table 1). When observed, the magnitude of their effects was moderate (generally less than twofold), and the direction of effects depended upon the fungus-tree species pairs (Fig. 2B; Supplementary Table 1). For example, the presence of EHB decreased the extent of seed colonization by *Gliocladiopsis* sp. 1 isolate PS0768 on three tree species (*C. longipes, C. peltata,* and *O. pyramidale*), and a similar but non-significant trend was observed in a fourth species (*T. micrantha*) (Fig. 2B; Supplementary Table 1). In contrast, the presence of EHB in *Gliocladiopsis* sp. 1 isolate PS0772 and *Fusarium concolor* isolate P0265 increased the extent of colonization of seeds of one tree species each (respectively, *A. tibourbou* and *C. peltata*; Fig. 2B; Supplementary Table 1).

### 3.1. Seed germination

For *A. tibourbou, C. longipes,* and *C. peltata,* the proportion of seeds that germinated reflected an interaction of tree species, fungal identity, and EHB infection status (Fig. 3, Table 4). Significant differences in germination after seeds were treated with EHB+ vs. EHB− strains were observed in only 3 of 18 (17%) fungus-tree species pairs (two additional pairs showed relatively large but non-significant effects; Fig. 3B; Supplementary Tables 2 and 3). However, the magnitude of their effects was relatively large when observed (i.e., more than twofold differences in seed germination; Fig. 3B; Supplementary Tables 2 and 3). The direction of the effects of EHB depended upon the fungus-tree species pairs (Fig. 3B; Supplementary Tables 2 and 3).

Relative to controls, exposure to fungi changed the proportion of seeds germinating in 14 of 36 (39%) fungal strain-tree species pairs (Fig. 3B; Supplementary Table 3). All 14 cases were observed in *C. longipes* and *C. peltata* (not in *A. tibourbou;* Fig. 3B; Supplementary Table 3). Among those cases, EHB− strains reduced seed germination significantly relative to controls, but EHB+ strains did not always do so (Fig. 3B; Supplementary Tables 2 and 3). For example, seeds of *C. longipes* germinated less frequently than controls when exposed to the EHB− strain of *F. keratoplasticum*

**Table 3**
Effects of tree species, fungal identity, EHB infection status, and their interactions on seed colonization by fungi. The corrected Akaike information criterion ($AIC_C$) is reported for each model. Hierarchical model fitting and comparison of log-likelihoods via likelihood ratios produced the *p*-values describing the factors influencing seed colonization. Significant *p*-values (i.e., ≤ 0.05) are bolded.

| Factor | AICc | Pseudo $R^2$ | *DF* | log *L* | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|---|
| tree species | −169.3 | 0.05 | 6 | 90.8 | 745.7 | **<0.00001** |
| fungal identity | −649.6 | 0.71 | 7 | 332.0 | 482.4 | **<0.00001** |
| EHB status | −158.5 | 0.001 | 3 | 82.3 | 499.4 | **<0.00001** |
| tree species x fungal identity | −765.9 | 0.87 | 31 | 417.6 | 670.7 | **<0.00001** |
| fungal identity x EHB status | −651.2 | 0.72 | 13 | 339.2 | 156.8 | **<0.00001** |
| full model | −773.5 | 0.92 | 61 | 463.7 | – | – |

isolate PS0362A ($t = 7.4$, *p*-value < 0.00001), but germination was similar to controls when seeds were exposed to the EHB+ strain ($t = 0.3$, *p*-value = 0.8) (Fig. 3B; Supplementary Table 3). For *C. peltata,* a significant effect of EHB was observed for only one fungal isolate: seeds germinated less frequently than controls when exposed to the EHB+ strain of *Xylaria cubensis* isolate P0540, but not the EHB− strain (Fig. 3B; Supplementary Tables 2 and 3). Overall, the effects of fungi on seed germination were not correlated with SCI (adjusted $R^2 = -0.006$; *p*-value = 0.4).

### 3.2. Viability of ungerminated seeds

Some seeds of *A. tibourbou, C. longipes,* and *C. peltata* that did not germinate remained viable at the end of the experiment (Fig. 4). The proportion of ungerminated seeds that remained viable reflected an interaction of tree species, fungal identity, and EHB infection status (Table 5). Significant differences in viability after seeds were treated with EHB+ vs. EHB− strains were observed in 3 of 18 (17%) fungus-tree species pairs (five additional pairs showed relatively large but non-significant effects; Fig. 4B; Supplementary Tables 4 and 5). When observed, the magnitude of their effects was relatively large (i.e., more than twofold differences in seed viability). The direction of the effects of EHB depended upon the fungus-tree species pairs (Fig. 4B; Supplementary Tables 4 and 5).

Relative to controls, exposure to fungi changed the proportion of ungerminated seeds that remained viable in 11 of 36 (31%) of fungal strain-tree species pairs (Fig. 4B; Supplementary Table 5). For *A. tibourbou* and *C. peltata*, half of fungal isolates had at least one strain associated with a change in viability relative to controls, although for each tree species a different set of fungi was relevant (Fig. 4B; Supplementary Table 5). For *C. longipes*, one fungal strain altered seed viability relative to controls (Fig. 4B; Supplementary Table 5).

In seeds of *A. tibourbou* and *C. longipes,* EHB+ strains consistently reduced viability relative to controls, but EHB− strains did not always do so (Fig. 4B; Supplementary Tables 4 and 5). For example, viability of ungerminated seeds of *C. longipes* decreased relative to controls after exposure to the EHB+ strain of *Gliocladiopsis* sp. 1 isolate PS0768 ($t = 3.3$, *p*-value = 0.01), but was similar to controls after exposure to the EHB− strain ($t = 1.3$, *p*-value = 0.4) (Fig. 4B; Supplementary Table 5). The opposite trend was observed for *C. peltata* infected by *X. cubensis* isolate P0540 (Fig. 4B; Supplementary Tables 4 and 5). Overall, the effects of fungi on viability of ungerminated seeds were not correlated with SCI (adjusted $R^2 = -0.007$; *p*-value = 0.4).

## 4. Discussion

Plant-fungus interactions are major drivers of tree demography, population structure, and community dynamics in tropical forests (Gilbert and Hubbell, 1996; Gallery et al., 2007; Mangan et al., 2010; Bagchi et al., 2014). Fungi are the major causes of seed mortality in the soil, particularly for light-demanding species such as pioneers (Dalling et al., 1998; O'Hanlon-Manners and Kotanen, 2006; Kotanen, 2007). Fungi associated with seeds can influence seed germination and the viability of ungerminated seeds, and thus impact seed bank structure and forest dynamics (Dalling et al., 1998; Gallery et al., 2007; Kotanen, 2007; Sarmiento et al., 2017). We used inoculation experiments to explore the potential for EHB to influence the outcomes of such seed-fungus interactions *in vitro*.

We observed relatively mild impacts of EHB on seed colonization by fungi, but relatively powerful impacts of EHB in shaping fungal effects on seed germination and viability. Such impacts overall were rare among the tree species tested here, but documenting them for the first time provides a new perspective on seed-fungal interactions: that is, a demonstration that EHB can impact the outcomes of seed-fungus associations. More generally, the emergent theme from this study is one of context-dependency: the magnitude and direction of the responses measured here reflected three-way interactions of tree species, fungal identity, and EHB infection status. This argues against a canonical influence of EHB on the effects of these fungi on seeds, and instead suggests context-dependency that mirrors and extends two previous observations.

First, Sarmiento et al. (2017) showed that the effects of particular fungi on seed fate varied among tree species. Specifically, they

**Table 4**
Analysis of deviance for the generalized linear model explaining the proportion of seeds that germinated as a function of tree species, fungal identity, and EHB infection status. The model has an $AIC_C = 825.7$ and a pseudo $R^2 = 0.54$. For each row, a $\chi^2$ test was used to assess the reduction in deviance to the residuals as compared to the null model. Significant *p*-values (i.e., ≤ 0.05) are bolded.

| | *DF* | Deviance | Residual *DF* | Residual deviance | $\chi^2$ *p*-value |
|---|---|---|---|---|---|
| null model | | | 179 | 1110.05 | |
| fungal identity | 5 | 149.26 | 174 | 960.78 | **<0.00001** |
| EHB status | 1 | 12.6 | 173 | 948.18 | **0.0004** |
| tree species | 2 | 404.41 | 171 | 543.77 | **<0.00001** |
| fungal identity x EHB status | 5 | 45.88 | 166 | 497.89 | **<0.00001** |
| tree species x fungal identity | 10 | 177.65 | 156 | 320.24 | **<0.00001** |
| tree species x EHB status | 2 | 11.6 | 154 | 308.64 | **0.003** |
| tree species x fungal identity x EHB status | 10 | 56.19 | 144 | 252.45 | **<0.00001** |

**Table 5**
Analysis of deviance for the generalized linear model explaining the proportion of infected seeds that did not germinate but remained viable as a function of tree species, fungal identity, and EHB infection status. The model has an $AIC_C = 703.7$ and a pseudo $R^2 = 0.67$. For each row, a $\chi^2$ test was used to assess the reduction in deviance to the residuals as compared to the null model. Significant *p*-values (i.e., $\leq 0.05$) are bolded.

| | *DF* | Deviance | Residual *DF* | Residual deviance | $\chi^2$ *p*-value |
|---|---|---|---|---|---|
| null model | | | 179 | 1538.34 | |
| fungal identity | 5 | 18.08 | 174 | 1520.26 | **0.003** |
| EHB status | 1 | 0.3 | 173 | 1519.96 | 0.6 |
| tree species | 2 | 1090.26 | 171 | 429.7 | **<0.00001** |
| fungal identity x EHB status | 5 | 27.92 | 166 | 401.78 | **<0.00004** |
| tree species x fungal identity | 10 | 78.89 | 156 | 322.89 | **<0.00001** |
| tree species x EHB status | 2 | 2.33 | 154 | 320.56 | 0.3 |
| tree species x fungal identity x EHB status | 10 | 48.29 | 144 | 272.27 | **<0.00001** |

documented a fungal isolate x tree species interaction in trials measuring germination and seed viability (Sarmiento et al., 2017). Here, we extend that finding by showing that EHB contribute to effects of fungi on seeds of particular tree species. Previous analyses suggest that EHB can differentially influence the growth and nutrient use of particular fungi (Shaffer et al., 2017), consistent with the broad concept of flexible phenotypic modulation of fungal traits in the context of particular EHB-fungus partnerships. Importantly, we did not observe that EHB simply enhanced or decreased the growth rate of their fungal hosts, suggesting more subtle interactions that should be evaluated in future work.

Second, a growing body of literature suggests that EHB in plant-associated Ascomycota generally are facultative symbionts with ecologically flexible life modes (Araldi-Brondolo et al., 2017). Here, we extend that perspective by showing that, in some cases, the presence or absence of EHB in a fungal isolate can influence how that fungus interacts with seeds. Thus, the present study provides an additional and complementary perspective on the two emerging model systems used as examples of phenotypic modulation of Ascomycota by EHB (Hoffman et al., 2013; Shaffer et al., 2017), and links EHB for the first time to the potential scaling-up of impacts on seeds, the most important sexual propagules of most land plants. Field experiments represent an important next step for linking these observations robustly to fungal and plant ecology.

Conditions used here closely mimicked natural conditions favorable for the germination of seeds of many tropical pioneers (see Pearson et al., 2002), such that we considered any reduction in seed germination or viability relative to controls to be evidence of detrimental interactions. We observed that some EHB can mitigate the detrimental effects of certain fungi on seeds, raising the question of potential mechanisms for such interactions. Previous work showed that the presence of EHB increased the capacity of a fungal host (*Fusarium keratoplasticum* isolate PS0362A) to use simple carbon sources, such as glucose (Shaffer et al., 2017). We speculate that some EHB provide a means for their fungal hosts to obtain extra resources, enhancing their ability to live asymptomatically in association with seeds and reducing the frequency with which they cause seed mortality.

In our experiment, seed-associated and foliar endophytic fungal strains successfully colonized seeds of all tree species. However, effects of EHB were observed more often for seeds infected with seed-associated fungi compared to those infected with fungi isolated originally as foliar endophytes (Figs. 2B, 3B and 4B). That EHB more greatly influenced the effects on seeds of those fungi originally recovered from seeds compared to those from leaves suggests a degree of specificity regarding the context in which they may influence fungal hosts. Previous work has shown that closely related seed-associated and foliar endophytic fungi can harbor distinct EHB communities (Shaffer et al., 2016), perhaps indicating differences in EHB function. However, our sample size is limited with regard to the number of seed-associated and foliar endophytic fungi examined here, precluding a general interpretation of this result.

Our experiment included two fungal isolates, PS0768 and PS0772, that belong to the same putative species (i.e., based on multilocus phylotyping and OTU clustering; see Shaffer et al., 2016). These fungal isolates differ in their EHB (Table 1). Previous studies indicate that even closely related (conspecific) fungi can have different effects on seeds of the same plant species (Gallery et al., 2007; Stump, 2015; Sarmiento et al., 2017). If EHB are the sole driver of variation in the responses of otherwise identical seeds infected by otherwise identical fungi, we would expect seeds infected by EHB− strains of these two isolates to respond similarly. Indeed, that is what we found. For example, seeds of *C. longipes* experienced reduced germination compared to controls, and seeds of *A. tibourbou* experienced reduced viability compared to controls, when infected by EHB− strains of each fungus (Figs. 3B and 4B). However, there was one case in which EHB− strains of these fungi had unique effects. Seeds of *C. peltata* experienced reduced germination compared to controls when infected by the EHB− strain of one isolate but not the other (Fig. 3B), perhaps indicating that relevant genotypic differences exist between these two fungi (i.e., beyond the loci used in phylotyping; Shaffer et al., 2016). Further studies taking into account such differences may shed additional light on the nature of these context-dependent interactions.

Here we aimed to quantify the influence of EHB on the abilities of fungi to colonize and influence the germination and viability of seeds of representative neotropical pioneer trees. We found that even though they have relatively little effect on the ability of fungi to colonize seeds, EHB have the potential to influence the effects of fungi on seeds with respect to germination and viability in a context-dependent manner. Future work addressing the ecological impacts of these interactions is needed to determine the ecological importance of EHB in shaping these important plant-fungus interactions. In turn, context-dependency in the tripartite symbioses of seeds, fungi, and EHB underlines the importance of cryptic plant-microbe interactions in the recruitment processes central to ecological dynamics in tropical forests.

## Author contributions

JS conducted all experimental work and analyzed all data; PCZ and CS led isolation of fungi from seeds, collection of ripe fruits, and recovery of seeds from fruits with assistance from JS and collaboration from AD, JD, and AA; JS and AA led the development of the manuscript, with contributions from all authors.

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## Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.08.008.

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