



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.

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1. Introduction

Seeds are the primary mode of reproduction for most plants, including the majority on which human sustainability depends (Kozłowski 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.

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