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Colonization of Seeds by Soilborne Fungi: Linking Seed Dormancy-Defense Syndromes, Evolutionary Constraints, and Fungal Traits

22

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Abstract

The diverse soilborne fungi that recruit to seeds after dispersal include some of the most important agents of seed mortality, as well as strains that enhance germination or inhabit seeds without detriment. Ecological factors that influence seed colonization are not well understood yet are fundamental to the interactions between soilborne fungi and seeds that ultimately influence plant demography and community structure. Here we present current perspectives on seed defense syndromes and related frameworks for predicting colonization success of fungi, with a focus on seeds of tropical pioneer trees. We present a case study that tests whether fungal host range can be predicted by field observations of host use, seed defense syndromes, or phylogenetic relatedness of fungi or hosts. We show that phylogenetic relatedness of hosts, but not fungi, is a strong predictor of fungal colonization of seeds. We posit that the impacts of individual fungi and microbial consortia on seed viability and germination may in turn reflect fungal interactions with the suites of plant defenses codified recently under the broad framework of seed dormancy-defense syndromes. Our findings set the stage for experiments that track colonization, germination, and seedling establishment in the field, important for understanding impacts of fungi on the recruitment of tropical trees.

Keywords

Barro Colorado Island · *Clonostachys* · Effective specialization · *Fusarium* · *Lasiodiplodia* · Phylogenetic signal · Pioneer trees · *Trichoderma*

22.1 Introduction

Fungi are important drivers of plant distributions, demography, and fitness, influencing the growth, survival, reproduction, and nutrient uptake of the plants with which they interact (Kirkpatrick and Bazzaz 1979; Harley and Smith 1983; Agrios 2005; Augspurger and Wilkinson 2007). Insights into their ecology and natural history in agricultural and agroforestry systems are important for clarifying the economic aspects of plant-fungal interactions and their response to altered ecosystems (e.g., Parker and Smith 1990; Agrios 2005; Gilbert 2005; Desprez-Loustau et al. 2007; Barrett et al. 2009; Bonfante and Anca 2009). Despite a growing interest in plant-fungal interactions in natural systems, substantial gaps in knowledge remain regarding the diversity, composition, functional traits, and importance of the fungi that affiliate with plants in unmanaged plant communities. These gaps are especially profound for one of the most important but least-studied guilds of fungi: those that interact with seeds in the soil.

Together, the diverse soilborne fungi that recruit to seeds after dispersal include some of the most important agents of seed mortality, as well as fungal strains that enhance germination or coexist with seeds without detriment (see Gallery et al. 2007; Kluger et al. 2008; Zalamea et al. 2015; Sarmiento et al. 2017; Shaffer et al. 2018). These impacts are especially profound in earth's most diverse terrestrial

ecosystems—tropical forests—where soilborne fungi have emerged as major determinants of seed fate (Gallery et al. 2010; Dalling et al. 1998; Sarmiento et al. 2017; Zalamea et al. 2018).

As a prelude to the effects of pathogens and mutualists that interact with seedlings at establishment and in early phases of growth (e.g., Mangan et al. 2010; Bashyal et al. 2014), seed-associated fungi act as a primary filter that determines the capacity of tropical seeds to survive and germinate (Zalamea et al. 2015). Understanding the factors that shape fungal colonization of seeds, and their subsequent impacts on seed germination and viability, is important for developing predictions about host range, host specificity, and the roles of fungi in shaping the dynamics of natural and human-maintained ecosystems from the earliest stages of tree recruitment.

Recent studies suggest that many tropical seed-associated fungi are generalists in terms of their ability to colonize diverse hosts (e.g., Gallery et al. 2007; Kluger et al. 2008). However, host-specific effects on seed viability and germination are common and important (Sarmiento et al. 2017). Such functional or effective specialization plays out in the form of differential impacts of individual fungi on diverse plant species and, in turn, differential responses of individual plant species to diverse fungi (Sarmiento et al. 2017).

To date most of the analyses of interactions between tropical seeds and soilborne fungi have focused on germination and seed viability of species of tropical pioneer trees (e.g., Sarmiento et al. 2017; Shaffer et al. 2018). Pioneer trees are compelling for the study of seed-fungal interactions because the small seeds of such early successional trees frequently persist in soils after dispersal for periods ranging from weeks to decades, only germinating when conditions become appropriate (e.g., when canopy gaps form; Schupp et al. 1989; Dalling et al. 1998). Because the time between gap formation events in a given site can be on the order of many years, many pioneer trees have seed traits that allow them to persist in the soil seed bank for years to decades (Dalling and Brown 2009).

Recent work has shown that seeds of pioneer trees possess suites of defensive traits that are relevant for their interactions with fungi (Zalamea et al. 2018; see also Dalling et al. 2011). These "dormancy-defense syndromes" (DDS) represent constitutive physical and chemical defenses that, in particular combinations, can be linked directly to seed dormancy classes (Zalamea et al. 2018). Broadly, tropical pioneer trees can have seeds that are ephemeral in the soil (i.e., are quiescent, do not display strong physical or chemical defenses, and typically germinate without dormancy when conditions are right). Other species have seeds that are permeable and chemically well-defended (e.g., with phenols), corresponding to physiological dormancy (Zalamea et al. 2018). As a third strategy, some species have seeds that are impermeable and exhibit robust physical defenses, corresponding to physical dormancy (Zalamea et al. 2018). Strikingly, physical and chemical defenses of tropical seeds do not display univariate trade-offs, instead working in concert and linked directly to dormancy classes (Zalamea et al. 2018).

The DDS framework fosters a predictive approach whereby seed dormancy class can be used as a proxy for estimating strategies of seed defense against fungi. Such predictions can be tested experimentally. Sarmiento et al. (2017) and Shaffer et al.

(2018) have shown that seeds of tropical pioneer trees respond differently to particular fungi, with the next step being an explicit linkage of the outcome of such interactions to the DDS model. An important first step is to explore the factors that shape the earliest phases of interactions between soilborne fungi and seeds—that is, the process and dynamics of seed colonization, when fungal hyphae first contact seed surfaces and colonize seed interiors.

Factors that influence colonization of seeds have not been identified for tropical seed-associated fungi but may include seed traits that reflect the evolutionary placement and relatedness of host species or functional traits relevant to DDS that do not necessarily reflect phylogenetic relatedness. Plants that are closely related to one another typically share more fungal associates with one another than with evolutionarily distant plants, suggesting that traits reflecting phylogenetic relatedness are important in determining host ranges of fungi (Webb et al. 2002; Blomberg et al. 2003; Gilbert and Parker 2016). In line with that prediction, common garden experiments with nine species of tropical pioneer trees in a lowland tropical forest in Panama revealed that the communities of fungi that infect seed interiors after burial in soil (mimicking dispersal) are structured much more strongly by host taxon (e.g., host species) than by burial duration, burial location, or seed viability (Sarmiento et al. 2017). This suggests that the early phases of colonization should reflect the evolutionary relatedness of hosts. However, some functional traits of seeds are relatively decoupled from phylogeny, instead appearing to reflect trait convergence. For example, different species in the genus *Trema* produce seeds that represent different dormancy classes (Zalamea et al. 2018). In some cases such functional traits might vary with phylogenetic relatedness, but in tropical pioneer trees, members of different families have converged on particular DDS (Zalamea et al. 2018).

Here we provide a case study in which we examine seed colonization by soilborne fungi. Our aim is to quantify the host range of fungal strains isolated originally from seeds of tropical pioneer trees, which they colonized in soil in the experiments described by Zalamea et al. (2015) and Sarmiento et al. (2017). We evaluate whether host range can be predicted by field observations of host use, seed dormancy-defense syndromes, or phylogenetic relatedness of fungi or hosts. On the basis of previous work, we predicted that host range observed in the field would represent a subset of the potential host range of each strain, that strains would be host-generalists with regard to seed colonization, and that the earliest phase of seed colonization would reflect seed traits in a manner consistent with the DDS framework.

Consistent with our first prediction, we show that each fungus colonized multiple tree species beyond those observed in field surveys. In line with our second prediction, individual strains differed in their capacity to colonize different tree species. In contrast to our third prediction, we found that phylogenetic relatedness of hosts was a stronger predictor of fungal colonization than seed dormancy class alone. This suggests that the ultimate filter of community composition in a given seed may be the host taxon, consistent with the results shown by Sarmiento et al. (2017). In turn, effective specialization, which results in differential impacts of fungi on seed viability and germination in different tree species, may be structured by factors relevant to

dormancy-defense syndromes of host plants and the functional traits of fungi themselves.

22.2 Case Study

Our case study was conducted in conjunction with a common garden experiment in lowland tropical forest on Barro Colorado Island (BCI), Panama, where we are the seed dormancy-defense syndrome hypothesis (Zalamea et al. 2015, 2018; Sarmiento et al. 2017). The study examines the defensive traits and microbial associations of seeds of 18 species of pioneer trees. Briefly, seeds of each species were collected from multiple maternal sources, surface-sterilized, and buried in mesh bags to exclude macroscopic predators in common gardens at five locations on BCI. Bags were retrieved at timepoints ranging from 0 to 30 months, after which seeds were assessed for viability, germinability, seed coat integrity, and microbial infection (Zalamea et al. 2015, 2018; Sarmiento et al. 2017).

The experimental design permits seed traits to be linked to microbial infection at the level of individual seeds, providing an opportunity to estimate the observed host range of each fungus and their effect on seed survival (Sarmiento et al. 2017). Fungi were isolated on 2% malt extract agar (MEA) from surface-sterilized seeds that had been buried in forest soil. The viability of each of those seeds was scored by tetrazolium staining (Peters 2000), such that each fungal isolate could be traced to a given tree species, experimental garden, seed burial duration, and seed viability class. Each strain was vouchered at the University of Arizona Robert L. Gilbertson Mycological Herbarium (ARIZ) and sequenced bidirectionally for a ca. 1000 base pair fragment comprising the nuclear ribosomal internal transcribed spacers and 5.8S gene (ITSrDNA) and ca. 600 base pairs of the nuclear ribosomal large subunit (LSUrDNA) (Sarmiento et al. 2017). These data were used to establish operational taxonomic units (OTUs) at 95%, 97%, 99%, and 100% sequence similarity, and taxonomic analyses were placed strains to the genus level and above (Sarmiento et al. 2017).

For the present case study, eight isolates were selected to represent a phylogenetically diverse pool of strains that contain both distantly and closely related taxa (Table 22.1). Together the focal strains represent four genera and four families of *Ascomycota* and a range of observed abundance, host range, and host effects (Sarmiento et al. 2017). Our selection included two pairs of isolates that are 99% similar in the ITSrDNA-LSUrDNA region, and a pair of isolates that are 95% similar (Table 22.1). We present results that use 99% ITSrDNA-LSUrDNA similarity as our OTU designation, though results with other OTU cutoffs gave qualitatively similar results.

These fungi were used to inoculate seeds of five species of pioneer trees in vitro: *Apeiba membranacea* (Malvaceae, physical dormancy), *Ficus insipida* (Moraceae, quiescent), *Zanthoxylum ekmanii* (Rutaceae, physiological dormancy), *Trema micrantha* "brown" (Cannabaceae, quiescent), and *Trema micrantha* "black" (Cannabaceae, physiological dormancy; see Dalling et al. 1997; Silvera et al. 2003; Pizano et al. 2010). These species co-occur on BCI (Dalling et al. 1997) and are being

Table 22.1 Seed-associated fungi used in case study to assess seed colonization in in vitro trials

OTU identification	Isolate frequency (%)	Viability score	Observed associations	Focal isolate (s)	Original source
Clonostachys sp.	1.6	0.09	AS, LL, TB	PS0504	TB
Fusarium sp. 1	0.06	0.33	AM, AS, CI, CL, CP, CG LS, TB, ZE	PS0018	AM
				PS0943	AS
Fusarium sp. 2	0.02	0	TB	PS0547	TB
Fusarium sp. 3	1.2	0.32	AS, CP, JC, LL, TB, ZE	PS0993	AS
Lasiodiplodia sp.	2.8	0.63	AS, AM, CI, CL, CP, CV, FI, HA, JC	PS0042	AM
			LL, LS, TB, ZE	PS1042	AS
Trichoderma sp.	5.2	0.63	AS, AM, CI, CL, CP, FI, HA, LL, OP, TB, ZE	PS0037	AM

Table lists the genus-level identification of each operational taxonomic units (OTU, based on 99% ITSrDNA-LSUrDNA sequence similarity); the isolation frequency for each OTU (based on the number of isolates among a total of 5323 isolates collected; Sarmiento et al. (2017)); the proportion of seeds from which the OTU was isolated that were viable; the host range (observed associations) for field collections of each OTU; the focal isolates used in these experiments; and the species from which each focal isolate was originally obtained

Plant species names: AS, *Annona spraguei*; AM, *Apeiba membranacea*; CG, *Colubrina glandulosa*; CI, *Cecropia insignis*; CL, *Cecropia longipes*; CP, *Cecropia peltata*; CV, *Cochlospermum vitifolium*; LL, *Lindackeria laurina*; LS, *Luehea seemannii*; HA, *Hieronyma alchorneoides*; FI, *Ficus insipida*; JC, *Jacaranda copaia*; OP, *Ochroma pyramidale*; TB, *Trema micrantha* "black"

studied as part of the larger experiment described above (Sarmiento et al. 2017; Zalamea et al. 2018).

22.2.1 Experimental Procedures

Fresh, mature fruits or recently fallen seeds of each focal species were collected from multiple adult trees per species at BCI. Seeds were removed from the fruits, cleaned, allowed to dry, and stored using standard protocols for each species (Zalamea et al. 2018). Our methods followed Sarmiento et al. (2017) and Shaffer et al. (2018). Briefly, we exposed 5 sets of 20 seeds of each species to each fungal isolate. Prior to inoculation, seeds were surface-sterilized by sequential immersion (95% EtOH, 10 s; 0.7% NaClO, 2 min; 70% EtOH, 2 min). This procedure removes surface microbes, but does not affect germination or viability (Gallery et al. 2007; Sarmiento et al. 2017). Seeds then were placed on a lawn of actively growing fungal mycelium (ca. 11–13 days old) on 2% MEA in 60 mm Petri dishes (20 seeds/dish). Dishes were wrapped with Parafilm and incubated in the dark at ambient temperatures (consistent

with outdoor temperatures; ca. 26 °C) for 5–7 days. Control seeds were surface-sterilized, placed into Petri dishes containing 2% MEA but no fungal growth, and incubated as above. Overall, 4500 seeds were included in the case study.

After incubation, seeds were examined for visible colonization by fungi by scoring the number of seeds per plate with evident hyphal growth on their seed coats. Fungi on seeds were judged to be consistent with the inoculated strains (rather than contaminants) by visual inspection of morphological characteristics.

To test whether colonization was an indication of internal infection, we surface-sterilized a subset of seeds and transferred them to sterile Petri plates lined with sterile filter paper. We moistened the filter paper with sterile water, sealed the plates with Parafilm, and placed them in a shadehouse at ambient outdoor temperature (Gallery et al. 2007). Fungi reappeared in each Petri plate, providing evidence of internal infection.

22.2.2 Data Analyses

Except when otherwise noted, colonization was examined using generalized linear models with a quasibinomial error family, implemented with glm() in R (R Development Core Team 2009). A quasibinomial error family was used because our data showed more between-plate variation than expected under a binomial distribution. The per-dish colonization fraction was used as the response variable. Values of 0% or 100% were amended by adding one success and one failure to each plate, as the logit transformation needed for a binomial or quasibinomial error family is undefined at 0 and 1.

We measured whether fungi had host-specific colonization rates by determining the significance of (seed species) × (fungal OTU) interactions. Generalized linear models require that a particular fungal OTU and seed species be chosen as the null case, against which interactions are judged (Crawley 2007). The no-fungus control was an obvious choice for a fungal null case, as the null hypotheses were that fungi did not colonize seeds differently. However, there was no obvious choice for a null seed species, and the choice altered which interactions were significant. To account for this, we assessed the number of significant interactions taking every seed species as the null case.

We tested two hypotheses about the host range of fungi: (1) fungi cannot infect seeds outside of their observed host range, and (2) fungi are capable of infecting seeds outside of their observed host range, although colonization rates are low. The first hypothesis was tested by observing whether fungi colonized seed species that were not one of their known associations. Known associations were defined by the observation of that OTU in a seed of that species in the field experiments detailed by Sarmiento et al. (2017) (Table 22.1). We tested the second hypothesis by determining if seed colonization was higher on species known previously to be associated with that OTU vs. species for which such associations had not been observed in the field (Table 22.1). We used a generalized linear mixed model with known association as a fixed effect and fungal OTU and seed species as random effects (to account

for fungi being differently able to colonize seeds and seeds being differently protected against fungal colonization). We also included an interaction between fungal OTU and known association, in case some fungi were more able to colonize seeds outside of their known host range.

We tested for evolutionary constraints on host range and host affinity among fungi using three methods. First, we tested for evolutionary constraints over short timescales by testing whether colonization differed within fungal OTU. We did this by testing for significant (seed species) × (fungal isolate) interactions among our 99% similar isolates (i.e., within *Fusarium* sp. 1 and *Lasiodiplodia* sp.) or among those representing our 95% similar species pair (i.e., *Fusarium* sp. 2 and *Fusarium* sp. 3). Second, we tested for evolutionary constraints at intermediate timescales by testing if within-OTU differences in colonization were smaller than between-OTU differences in colonization. The difference in colonization between isolates was quantified using two dissimilarity indices. First, mean dissimilarity (d_{jk}) between two isolates j and k is

$$d_{jk} = \overline{I_j} - \overline{I_k}$$

where $\overline{I_j}$ and $\overline{I_k}$ are the mean colonization fraction of isolates j and k, respectively, across all seed species. Second, relative dissimilarity (r_{jk}) is

$$r_{jk} = \sqrt{\sum_s \left(\left(I_{sj} - \overline{I_j} \right) - \left(I_{sk} - \overline{I_k} \right) \right)^2}$$

where I_{sj} and I_{sk} are the proportion of seeds of seed species s that were colonized by fungal isolate j and k in vitro, and the summation is over all seed species. Thus, if strain j had a higher colonization rate on all seeds than strain k, but each had relatively similar colonization once the mean difference was removed (e.g., both specialized on physically dormant seeds), then d_{jk} would be large and r_{jk} would be small. We tested whether within-OTU dissimilarity was smaller than between-OTU dissimilarity using a one-tailed randomization test ($n = 1{,}000{,}000$), implemented in R. To calculate p-values, we factored out cases where the randomized within-OTU dissimilarities were the same as the actual within-OTU dissimilarities.

Finally, we tested whether host range and affinity are conserved among fungi over longer timescales by testing for phylogenetic constraints in the colonization fraction on seeds of each plant species, the mean colonization fraction across all species, and the relative colonization fraction (i.e., colonization fraction of a given species—mean colonization overall). A phylogenetic tree for the fungi examined here was generated using LSUrDNA data (obtained by Sarmiento et al. 2017). Sequences were aligned using Muscle (Edgar 2004), and a tree was inferred in RAxML (Stamatakis 2006). Phylogenetic constraints were assessed with Blomberg's K (Blomberg et al. 2003) and Pagel's λ (Pagel 1992), as each produces slightly different outcomes (Godoy et al. 2014). Significance was assessed using randomization ($n = 1{,}000{,}000$) and likelihood ratio tests, respectively, implemented using phylosig in R (R Development Core Team 2009).

We tested whether fungal colonization reflects plant relatedness or dormancy-defense syndromes by testing how much variation in fungal colonization could be

explained by clade or dormancy class. There were 40 ways that the 5 plant species could be categorized into 2 or 3 groups (i.e., there were 10 3-1-1 groupings, 10 3-2-0 groupings, 5 4-1-0 groupings, and 15 2-2-1 groupings). One of these groupings was by plant order (*F. insipida*, *T. micrantha* "brown," and *T. micrantha* "black" are Rosales, *A. membranacea* is Malvales, and *Z. ekmanii* is Sapindales). Another was by dormancy class (*F. insipida* and *T. micrantha* "brown" are quiescent, *T. micrantha* "black" and *Z. ekmanii* are physiologically dormant, and *A. membranacea* is physically dormant), as dormancy class can provide insight into the seed defense syndrome (Dalling et al. 2011; Zalamea et al. 2018). We assessed how well each of the 40 groupings fit the data using a generalized linear model with a binomial error family. Our model had fungal OTU, seed species, and group identity as fixed effects, along with the (seed species) × (group identity) interaction. The grouping with the lowest Akaike information criterion (AIC, Akaike 1974) was considered to be the best. We compared the ranking of clade and dormancy class among all possible groupings.

22.2.3 Results

Seeds of five species of tropical pioneer trees were colonized by all focal fungi in in vitro inoculation trials (Fig. 22.1). Fungal colonization success varied as a function of fungal strains and plant species (Fig. 22.1). However, even accounting for this, there were on average 8 significant (seed species) × (fungal OTU) interactions ($p < 0.05$) and 4 highly significant interactions ($p < 0.01$), out of a possible 24. Thus, if one selected two fungal isolates and one seed species at random, there was about a one in three chances that those isolates had significantly different colonization rates on that seed (and one in six chances that they differed at the $p < 0.01$ level). Similarly, if two seed species and one fungal isolate were selected at random, there was about a one in three chances that those that isolate had different colonization rates on each seed. Our results were similar if we

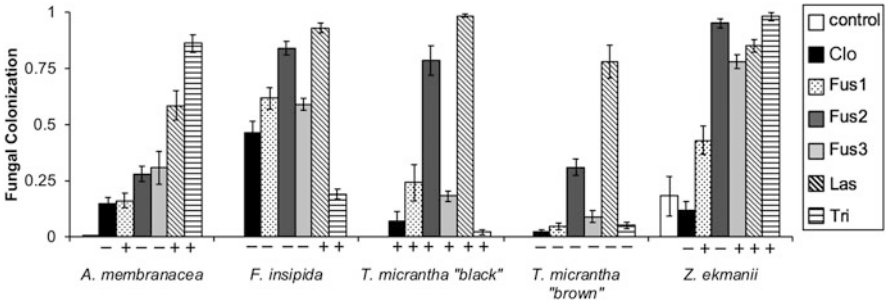

Fig. 22.1 Fraction of seeds colonized by fungi. Each bar represents the fraction of seeds colonized in each OTU-seed species pairing (20 seeds per plate, for a total of 100 seeds). Error bars represent ±1 standard error. Bars marked with "+" indicate OTU that were isolated from seeds of that species in field surveys, and bars marked with "–" have not yet been isolated from seeds of that species. Clo, *Clonostachys* sp.; Fus, *Fusarium* sp; Las, *Lasiodiplodia* sp.; Tri, *Trichoderma* sp.

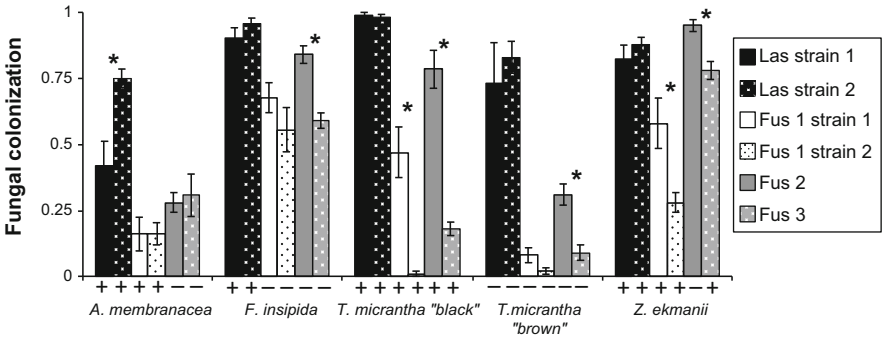

Fig. 22.2 Fraction of seeds colonized by fungi as a function of seed species and fungal isolate. Each bar represents 100 seeds. Error bars represent ±1 standard error. Bars marked with "+" indicate OTU that were isolated from seeds of that species in field surveys, and bars marked with "–" have not yet been isolated from seeds of that species. Any significant ($p < 0.05$) within-OTU differences in colonization or germination are marked with a star. Las strain 1 (PS0042) and Las strain 2 (PS1042) are 99% similar in their ITSrDNA-LSUrDNA. Fus 1 strain 1 (PS0018) and Fus 1 strain 2 (PS0943) are 99% similar in their ITSrDNA-LSUrDNA. Fus 2 and 3 (*Fusarium* sp. 3 and 4) are 95% similar in their ITSrDNA-LSUrDNA

focused on individual isolates instead of OTU: an average of 12.4 interactions were significant ($p < 0.05$), and an average of 8.8 interactions were significant ($p < 0.01$), out of a possible 32.

All fungi were able to colonize seeds outside of their previously observed associations (Fig. 22.1). When average colonization fraction was accounted for, fungi showed no difference in their ability to colonize seeds in their known host range vs. seeds of other species ($p = 0.74$).

Our results suggested a limited amount of phylogenetic constraint on host range among fungi. Isolates within the same OTU (both at 99% and 95% of sequence similarity) differed in their ability to colonize seeds of at least one plant species (Fig. 22.2). Colonization patterns for a given isolate were marginally more similar to that of isolates of the same OTU than isolates of different OTU (relative dissimilarity; $p = 0.06$ for 99% OTU and $p = 0.07$ for 95% OTU). However, mean dissimilarity, d_{jk}, was not significantly lower within-OTU vs. between them ($p = 0.15$ for 99% OTU and $p = 0.13$ for 95% OTU). Mean colonization across all seed species showed phylogenetic constraint using both focal statistics ($p = 0.055$ for K and $p = 0.02$ for λ, Table 22.2). We found evidence for phylogenetic constraint in the relative ability to colonize *Z. ekmanii* (i.e., similar fungi had similar colonization rates on *Z. ekmanii*, $p = 0.048$ for K and $p = 0.061$ λ, Table 22.2). In other cases, the colonization fraction showed a significant constraint for only one of the two statistics.

Plant order was the best grouping (AIC = 1088) for explaining fungal colonization (Fig. 22.3). The next four best groupings also grouped species into monophyletic groups: they placed *Z. ekmanii* and *A. membranacea* in their own group and then contained every possible permutation of *T. micrantha* "brown," *T. micrantha* "black,"

Table 22.2 Phylogenetic conservatism in fungal colonization

Seed species	K estimate	*p*-value	λ estimate	*p*-value
A. membranacea—mean	8.16×10^{-5}	0.307	0.204	0.696
F. insipida—mean	$\mathbf{5.65 \times 10^{-4}}$	**0.032**	0.744	0.110
T. micrantha "black"—mean	1.46×10^{-4}	0.156	**0.614**	**0.066**
T. micrantha "brown"—mean	1.68×10^{-3}	0.090	**0.906**	**0.001**
Z. ekmanii—mean	1.26×10^{-4}	0.196	0.000	1.000
A. membranacea—relative	9.27×10^{-5}	0.245	0.000	1.000
F. insipida—relative	$\mathbf{5.40 \times 10^{-4}}$	**0.051**	0.000	1.000
T. micrantha "black"—relative	7.39×10^{-5}	0.238	0.295	0.434
T. micrantha "brown"—relative	1.55×10^{-4}	0.139	**0.856**	**0.011**
Z. ekmanii—relative	$\mathbf{3.76 \times 10^{-4}}$	**0.048**	**0.959**	**0.061**
Overall mean	$\mathbf{2.46 \times 10^{-4}}$	**0.055**	**0.772**	**0.024**

Phylogenetic constraint was assessed using Blomberg's *K* (Blomberg et al. 2003) and Pagel's λ (Pagel 1992). Mean colonization fraction on a particular seed species is the fraction of seeds colonized across all five replicates. Mean overall colonization is the mean colonization across the entire study (i.e., all five replicates of all five tree species). Relative colonization is the mean colonization on a particular species minus the mean overall colonization and is used to disentangle overall colonization ability from the ability to colonize particular seeds. Significant or marginally significant ($p < 0.07$) values are shown in bold. Significant constraints indicate that related fungi are more likely to have a similar colonization fraction on a given seed species, a similar colonization fraction overall, or a similar relative colonization fraction on a given seed species

and *F. insipida*. Dormancy class was the 11th best grouping, with an AIC of 1250 (Fig. 22.3).

22.2.4 Perspectives

Seed-associated fungi play a critical role in the demography of tropical trees (Kirkpatrick and Bazzaz 1979; Harley and Smith 1983; Arnold et al. 2003; Agrios 2005; Augspurger and Wilkinson 2007; Sarmiento et al. 2017). However, basic details about the host range and specificity of seed-associated fungi are rarely known. Our experiments complement previous observations about seed fate and seed-fungal associations in a lowland tropical forest (Sarmiento et al. 2017; Zalamea et al. 2018). Our results suggest that the fungi we considered had a wide host range in terms of colonization but had isolate-specific colonization rates on different hosts. We found that all fungal OTU were able to colonize species they had not been associated within field surveys, suggesting that potential host range is larger than observed host range. We found that host range was evolutionarily labile. Finally, we found that host taxonomy was a strong predictor of fungal colonization patterns. Because infection persisted among the subset of seeds that was surface-sterilized after allowing for colonization by fungi, our results imply that colonization indicated internal infection. This suggests that the fungi we considered have a wide potential host range, even if their observed host range appears narrow.

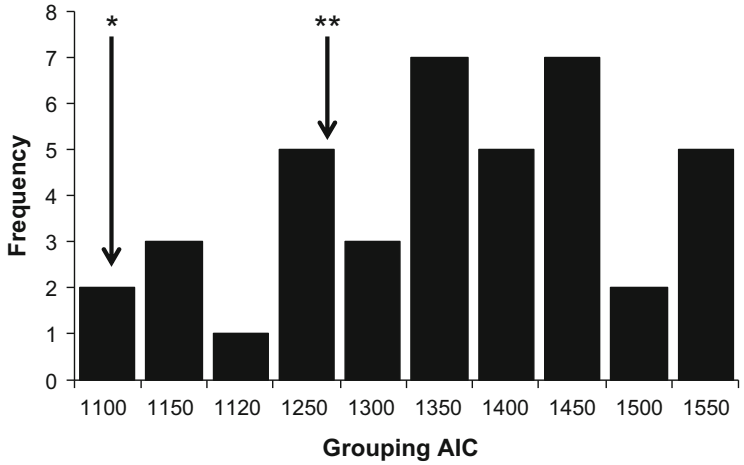

Fig. 22.3 Predictors of fungal colonization on seeds. A histogram displays the AIC values of a generalized linear model of every possible fungal grouping. There are 40 possible ways to group 5 fungi into 2–3 groups. The "plant order" grouping (*Z. ekmanii* by itself, *A. membranacea* by itself, and a group of *T. micrantha* "brown," *T. micrantha* "black," and *F. insipida*) is the best grouping (AIC 1088), indicated by an arrow and one star (*). The dormancy class grouping (*F. insipida* paired with *T. micrantha* "brown," *Z. ekmanii* paired with *T. micrantha* "black," and *A. membranacea* by itself) is the 11th best grouping (AIC 1250), indicated by the arrow and two stars (**). The second, third, fourth, and fifth best grouping paired *Z. ekmanii* with *A. membranacea* and then included every possible permutation of the other species

Potential host range could be wider than the observed host range for several reasons. First, some seed-fungal associations may be rare and thus simply have not yet been catalogued in field surveys. Alternatively, these seed-fungus associations may be short-lived, rare, or absent under natural conditions because of competition with other microbes (Barrett et al. 2009). This could be tested in part by inoculating seeds with fungal consortia and testing if colonization is reduced in some cases. A third alternative is that in vitro colonization may not be indicative of the ability to infect seeds under natural conditions, where factors such as soil chemistry, or remnants of fruit on seed surfaces (here removed by treatment before inoculation), could be important. These questions could be addressed with a colonization experiment performed under more natural conditions. For example, instead of placing seeds on agar that have been colonized by fungus, we could place seeds in soil that has been sterilized and then inoculated with a small amount of fungi.

The observation that the actual host range of plant-associated fungi is often more narrow than their potential host range has been documented previously for a number of pathogenic fungi, including fungi associated with seeds (Beckstead et al. 2014, see also de Vienne et al. 2009). However, while many studies have documented this trend, relatively few have explored for predictors for novel plant-fungus associations (de Vienne et al. 2009). It is becoming clear that phylogenetic relatedness of hosts is a strong factor in determining whether they are likely to share certain pests (reviewed

in Gilbert and Parker 2016). Here, we add merit to the importance of host relatedness in predicting susceptibility to seed-associated fungi by showing that plant order best explained seed colonization by fungi (Fig. 22.3).

Overall, results of our case study suggest that host range and affinity of seed-infecting fungi are evolutionarily labile: there was little evidence of phylogenetic constraint on specialization (Table 22.2), and even fungal strains that were 99% similar at the ITSrDNA-LSUrDNA locus differed in colonization on seeds of different plant species (Fig. 22.2). It is possible that these differences are due to within-OTU variation in loci that code for functional traits. Alternatively, these fungal isolates may differ in other factors, such as infection with endohyphal bacteria (Hoffman and Arnold 2010; Shaffer et al. 2016). Given how quickly microbes adapt to new hosts under experimental conditions (e.g., Little et al. 2006; Wallis et al. 2007; Agudelo-Romero et al. 2008), it seems reasonable that specialization at the species level could change quickly over evolutionary time.

Interestingly, our results suggest that generalized colonization ability was phylogenetically conserved among fungi, even if species-specific colonization rates were not: mean colonization showed phylogenetic constraint among both statistics, whereas the majority of colonization on individual plant species showed no significant constraint (Table 22.2). This result would be unlikely if the ability to colonize each seed species evolved independently. The evolution of host-specific virulence often comes at a cost of virulence on another host (Ebert 1998); it is thus reasonable to suspect that the ability to colonize particular hosts is more labile than the ability to colonize any host. Alternatively, the effect we detected may reflect a phylogenetic signal in the environmental conditions that promote colonization by particular strains or taxa. Infection is partly a function of environmental conditions (Parker and Gilbert 2004; Barrett et al. 2009), and different symbiont species can be differently infectious under different conditions (Whipps 1987; Pažoutová et al. 2000). Perhaps the phylogenetic signal was observed because the fungal isolates with high in vitro colonization were most infectious under the temperature, moisture, and nutrient conditions in our study, and their sister taxa had similar environmental requirements. This could be tested by doing another inoculation experiment under different conditions and testing whether colonization fractions change but phylogenetic signal holds.

Although related fungi did not necessarily colonize the same species of plants, related plants were colonized similarly by fungal isolates: plant taxonomy was the strongest predictor of fungal colonization (Fig. 22.3). We know of only one study that examined both phylogenetic constraint in host defenses and pathogen virulence, and they found that pathogens were far less phylogenetically constrained than their hosts in terms of associations (Mariadassou et al. 2010). This result should perhaps not be surprising, given that the generation time of fungi is far shorter than that of trees (Gilbert and Parker 2016).

Overall, dormancy class (and thus DDS) did not emerge as clear predictor of fungal colonization. We anticipate that DDS may be particularly important at the next phase of seed-fungal interactions: that is, we expect that host taxon is the first major filter that will select communities of seed-associated fungi, as suggested by Sarmiento et al. (2017). Subsequently, those that survive in seeds and function, individually or in consortia, to

influence seed viability and germination may be influenced by the defense and dormancy traits codified as DDS. In that situation we would anticipate that fungi would be differentially sensitive to phenols and other seed chemical defenses, especially those mobilized by seed imbibition and the physiological cascades associated with germination.

22.3 Future Directions

The case study presented here represents an early step in documenting the natural history of seed-associated fungi in tropical soils, with special attention to the earliest phases of fungal contact with and establishment in seeds. In general, our results suggest fungal host range in nature is limited more by competition or environmental conditions, rather than an inability to infect certain seeds. The ability of fungi to colonize seeds was more labile than the ability of seeds to protect themselves from fungal colonization. Our study points to the primary role of host taxonomy in determining colonization success but suggests also the need to learn more about fungal functional traits. Although phylogenetically diverse, the fungi that colonize seeds of tropical trees are especially common among genera such as *Fusarium* and *Xylaria* (see Shaffer et al. 2016), suggesting the potential to evaluate functional traits in robust phylogenetic contexts at the genus level.

In future work we advocate exploring the relevance of DDS for predicting the responses of seeds to infection by individual fungi and consortia and cataloguing fungal traits to understand how growth rate, enzyme production, and nutrient scavenging may influence the host breadth, colonization efficiency, and impacts on seed fate of soilborne fungi. Recent attention to the capacity of some soilborne fungi to harbor endohyphal bacterial symbionts that influence seed colonization by fungi and their subsequent impacts on seed viability and germination (Shaffer et al. 2018) speak to important and under-explored roles of such traits in driving the dynamics of seeds in soil seed banks, in turn relevant to the dynamics of earth's most diverse terrestrial ecosystems.

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