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# ECOLOGICAL IMPLICATIONS OF ANTI-PATHOGEN EFFECTS OF TROPICAL FUNGAL ENDOPHYTES AND MYCORRHIZAE

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*Abstract*. We discuss studies of foliar endophytic fungi (FEF) and arbuscular mycorrhizal fungi (AMF) associated with *Theobroma cacao* in Panama. Direct, experimentally controlled comparisons of endophyte free (E–) and endophyte containing (E+) plant tissues in *T. cacao* show that foliar endophytes (FEF) that commonly occur in healthy host leaves enhance host defenses against foliar damage due to the pathogen (*Phytophthora palmivora*). Similarly, root inoculations with commonly occurring AMF also reduce foliar damage due to the same pathogen. These results suggest that endophytic fungi can play a potentially important mutualistic role by augmenting host defensive responses against pathogens. There are two broad classes of potential mechanisms by which endophytes could contribute to host protection: (1) inducing or increasing the expression of intrinsic host defense mechanisms and (2) providing additional sources of defense, extrinsic to those of the host (e.g., endophyte-based chemical antibiosis). The degree to which either of these mechanisms predominates holds distinct consequences for the evolutionary ecology of host–endophyte–pathogen relationships. More generally, the growing recognition that plants are composed of a mosaic of plant and fungal tissues holds a series of implications for the study of plant defense, physiology, and genetics.

*Key words: arbuscular mycorrhizal fungi; endophytic fungi; mutualism; pathogens; plant defense;* Theobroma cacao; *tropical plant ecology*.

Special Feature

## Introduction

Endophytes are commonly defined as fungi or bacteria that live asymptomatically within healthy plant tissue (leaves, stems, roots) for at least a part of their life cycle (Malloch et al. 1980, Petrini 1991, Wilson 1995, Stone et al. 2000, Evans et al. 2003). Fungal endophyte associations with plant aboveground tissues have been generally viewed under two categories: the grass–fungal endophyte (e.g., Clay 1988) and the woody plant–fungal endophyte associations (e.g., Petrini 1991). Belowground plant tissues (roots) also have their own suite of endophytes (e.g., arbuscular mycorrhizae). Despite their widespread occurrence, with both descriptive and experimental work from temperate regions (see reviews in Carroll 1988, Petrini 1991, Saikkonen et al. 1998, Wilson 2000), relatively little is known of the nature of the interactions between woody plants and their foliar endophytes, particularly in tropical regions (see Lodge et al. 1996, Bayman et al. 1998, Frohlich and Hyde 1999, Arnold et al. 2000, Rajagopal and Suryanarayanan 2000, Cannon and Simmons 2002, Gilbert et al. 2002, Van Bael et al. 2005). Similarly, most research on arbuscular mycorrhizal fungi (AMF; i.e., root-associated endophytes) has been conducted in temperate regions. Nonetheless, for both foliar endophytic fungi (FEF) and arbuscular mycorrhizal fungi (AMF) there is accumulating evidence that these fungi provide previously under-appreciated beneficial effects for their hosts. Specifically, recent work has demonstrated that, under some circumstances, both types of endophytes (FEF in leaves and AMF in roots) can enhance host resistance against attack and damage by pathogens (Smith 1988, Smith and Gianinazzi-Pearson 1988, Newsham et al. 1995, Shaul et al. 1999, Borowitz 2001, Arnold et al. 2003, Evans et al. 2003, Garmendia 2004, Holmes et al. 2004, Herre et al. 2005*a*, *b*, Rubini et al. 2005, Van Bael et al. 2005; but see Faeth 2002). Discovering what these effects are, clarifying their proximal mechanisms, and understanding the ultimate selective pressures that influence them are primary goals for the study of the evolutionary ecology of endophyte-host interactions (Carroll 1991, Herre et al. 1999).

Here we outline our current understanding of life cycles and natural history of FEF and AMF in tropical systems. After presenting data demonstrating beneficial anti-pathogen effects in both groups, we discuss the potential mechanisms underlying these observations. We then point out how different mechanisms potentially hold very different consequences for the evolutionary ecology of host–pathogen relationships. Finally, we

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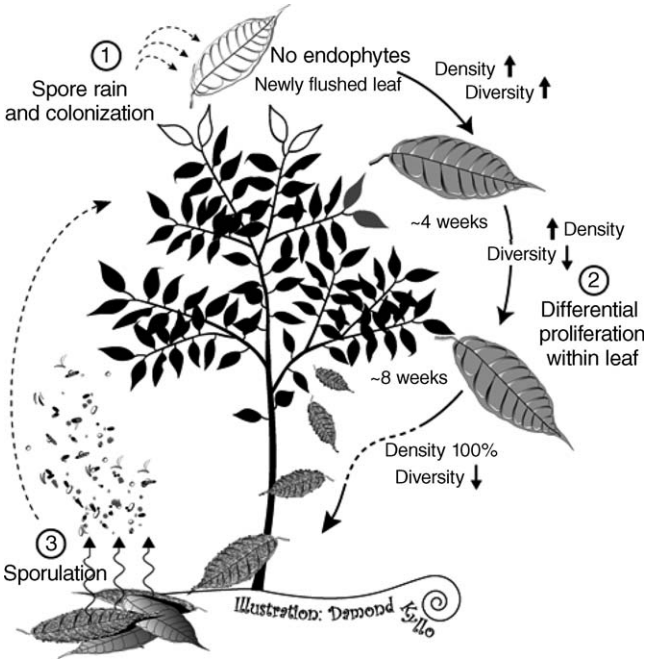


FIG. 1. Proposed life cycle for tropical foliar endophytic fungi (FEF) and their host plants. Leaves are flushed, essentially free of FEF; spores land on the leaf surfaces and, upon wetting, germinate and penetrate the leaf cuticle. After a few weeks, the density of FEF infection within the leaf appears to saturate with a very high FEF diversity. Over several months, FEF diversity usually declines. After leaf senescence and abscission, FEF sporulate and the cycle begins anew.

briefly discuss some implications of endophytic fungi for the study of the chemistry, physiology, and ecology of host plants.

## ENDOPHYTE ECOLOGY

### *Life cycle and general natural history of foliar endophytic fungi (FEF)*

During the lifetime of the leaf, there appear to be few, if any, recognizable symptoms of the presence of the endophytes. Nonetheless, both isolates from tissue samples and microphotographs show that the plant tissue is full of a diversity of fungi (Rodrigues 1994, Lodge et al. 1996, Arnold et al. 2000, 2003). Further, extensive surveys across several host plant species suggest that while many tropical endophytes may be generalists (Cannon and Simmons 2002, Suryanarayanan et al. 2002, 2003), some exhibit clear differential host preferences (Gilbert et al. 2002, Arnold et al. 2003, Herre et al. 2005*b*, Van Bael et al. 2005; also see Petrini et al. 1992, Rollinger and Langenheim 1993, Fisher et al. 1994, Schulz and Boyle 2005).

For many foliar endophytes, the portion of their life cycle involved with leaves begins as part of a taxonomically diverse assemblage of airborne spores that land on leaf surfaces (Carroll 1986, 1988; Fig. 1). In most tropical tree species, the leaves are flushed in a largely endophyte-free (E–) condition (Arnold and Herre 2003, Arnold et al. 2003). After the wetting of the spore-laden leaf surfaces, some spores germinate and are further able to penetrate directly through the cuticle into the leaf tissue, where the hyphae grow between cells (Fail and Langenheim 1990, Deckert et al. 2001, Herre et al. 2005*b*). With time, the initially endophyte-free leaf tissues become saturated with endophytes, with ~100% of sampled leaf fragments (2 × 2 mm) containing culturable fungi (Figs. 1, 2). However, data from cohorts of leaves suggests that the diversity of endophytes (number of species per isolate) usually declines as leaves age (Figs. 1, 2). Within the leaf, the distribution of the diverse fungal species resembles a quilt-like patchwork with different species usually abutting the others (Hata and Futai 1996, Lodge et al. 1996, Gamboa and Bayman 2001), producing an extremely heterogeneous mix of different fungal species and genotypes at very fine scales within the matrix of the plant leaf (Fig. 3).

It is not clear how these fungi subsist for up to several years within a host leaf apparently as semi-dormant hyphae. As heterotrophs, we suspect that they must be consuming some plant product (e.g., intercellular exudates), and there is some evidence that their presence can reduce host growth (Herre et al. 2005*b*; P. Carlsen, *personal communication*). However, our observations suggest that it is only after the leaf has abscised that most endophyte species appear to grow rapidly and sporulate (Herre et al. 2005*b*), as has been proposed by Wilson and Carroll (1994, also see Wilson 2000). Tropical endophytes apparently spend a long time "waiting" and then complete their life cycle essentially as saprotrophs (Herre et al. 2005*b*, Van Bael et al. 2005; see Figs. 1 and 2).

One previous study has emphasized the importance of closed (vs. open) forest canopy on enhancing the rate of initial endophyte colonization of seedlings (Arnold and Herre 2003). However, these experiments confounded intact or open canopy cover with intact or absent leaf litter, respectively. Experiments comparing the rate of endophyte accumulation when endophyte-free seedlings

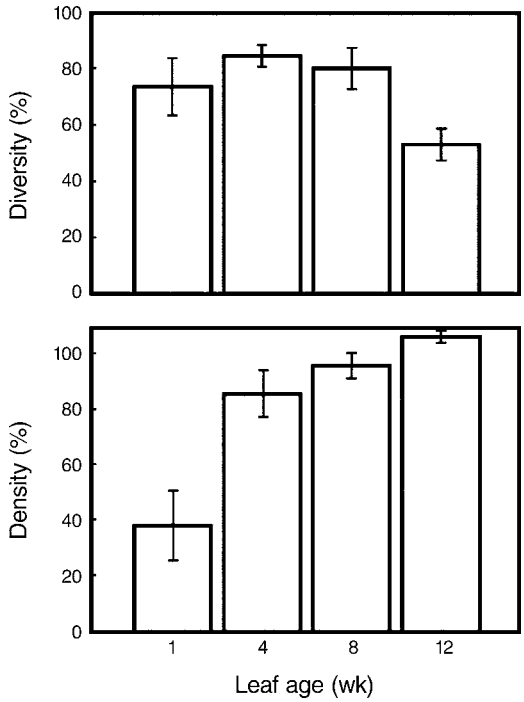


FIG. 2. Progression of diversity and density of endophyte colonization through time. A cohort of concurrently flushed *Theobroma cacao* leaves was sampled at 1, 4, 8, and 12 weeks to examine endophyte presence and identity (see Arnold et al. 2003). At least 16 2 × 2 mm fragments were sampled per leaf ($n$ = 6 leaves at 1, 4, 8, and 12 weeks). Diversity is the mean percentage (±SE) of different morphospecies of endophytes per fungal isolate. Density is the mean percentage (±SE) of leaf fragments from which fungal endophytes grew. Note that values can be higher than 100% because more than one fungus can grow from a single fragment.

are placed under intact forest canopies with intact or removed leaf litter show a much more rapid accumulation of endophytes in the presence of intact leaf litter (Fig. 4). This not only suggests that dead leaves appear to be a primary source of inoculum of FEF, it further suggests that local FEF sources (i.e., the local litter) can dominate the composition of colonizing spores. If true, this would provide one mechanism explaining reports of relatively fine scale local differentiation of FEF communities within the same host plant (Arnold et al. 2000, 2003).

### *Life cycle and general natural history of tropical arbuscular mycorrhizal fungi*

It is well known that AMF can benefit their hosts by providing increased nutrient access, and thereby usually increasing growth rates and general vigor (e.g., Kyllo et al. 2003). In turn, the hosts provide these fungi with carbon-based resources (photosynthates). By the isolation and use of pure cultures of AMF, researchers are recognizing that different species of AMF can differentially affect the physiology and growth of a given host plant. In some cases, a given AMF species can even produce a net loss in growth to the host relative to other AMF species, or non-AMF controls. Similarly, a given AMF may affect two different hosts in different ways. Finally, different hosts can produce different effects on the growth and spore production of any given AMF species. These studies show that different combinations of AMF and host species are functionally different, and these properties of AMF–host plant interactions can contribute to the generation and maintenance of aboveground host diversity (Schneck and Smith 1982, Mosse 1992, Bever et al. 1996, van der Heijden et al. 1998*a*, *b*, Kiers et al. 2000, Klironomos et al. 2000, Bever 2002, Klironomos 2002, Sanders 2002, Kyllo et al. 2003, Herre et al. 2005*a*).

Although in some settings the dominant root associations are with ectomycorrhizae (e.g., Asian Dipterocarp forests and various mono-dominant New World, Australian, and African forests), most hosts in most tropical forests exhibit AMF associations (Malloch et al. 1980). Recent work in Brazil, Costa Rica, Mexico, Panama, and other sites has greatly expanded our knowledge of tropical AMF ecology (Janos 1980, Allen et al. 1998, Siqueira et al. 1998, Guadaramma et al. 1999, Picone 2000, Husband et al. 2002*a*, *b*, Mangan and Adler 2002, Lovelock et al. 2003, Zangaro et al. 2003). As with FEF, survey work suggests that AMF community diversity is higher in wet tropical forests than in temperate grasslands or woodlands (Herre et al. 2005*a*). This basic result has been found in studies based both on descriptions of spore communities and on molecular analyses of AMF in association with roots. Importantly, the same researchers used the same sampling techniques in both regions (Herre et al. 2005*a*).

This work demonstrates non-random associations of AMF species with respect to time, space, and host species (Lovelock et al. 2003, Herre et al. 2005*a*). For example, AMF spore production varies seasonally, with peak spore abundance occurring just before peak seed germination. AMF community composition also varies both with abiotic factors (e.g., nutrients, water) and the aboveground plant community (Mangan et al. 2004). Moreover, several lines of evidence suggest some level of differential AMF-host affinity in tropical systems (Kiers et al. 2000, Husband et al. 2002*a*, *b*, Herre et al. 2005*a*). Finally, molecular analysis of the AMF community directly in the roots of seedling cohorts for two host species showed successional changes in the AMF community (Husband et al. 2002*a*, *b*), analogously to the apparent succession observed for FEF in leaves (Herre et al. 2005*a*, *b*, Van Bael et al. 2005; Fig. 2).

### *Experimental studies of the defensive role of FEF and AMF against pathogens*

With both FEF and AMF, endophyte-free (E–) plants can be grown and then single endophyte species or combinations of them can be experimentally reintroduced into plant tissue (Arnold et al. 2003, Holmes et al. 2004, Herre et al. 2005*a*, *b*, Rubini et al. 2005, Van

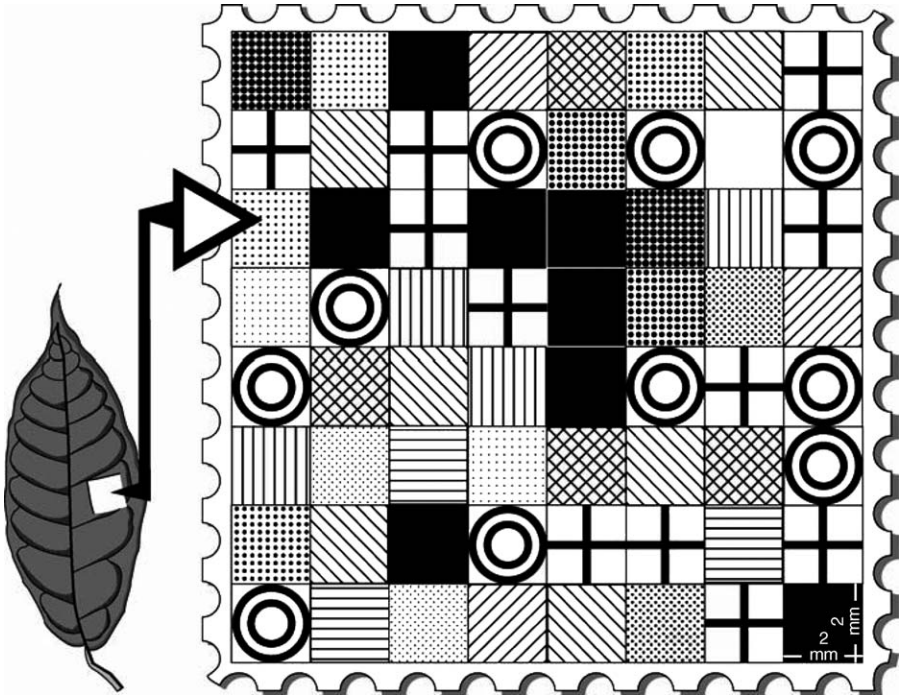


FIG. 3. Physical map of endophyte morphospecies in a postage stamp-sized piece of 12-week-old leaf from *T. cacao* (an 8 × 8 matrix of fungi reared out of 2-mm$^2$ leaf fragments). Symbols indicate different endophyte species: the most common species is indicated by large plus signs; the second most common species is shown by large double circles. Other patterns indicate less common species, solid black indicates morphospecies that occurred only once (singletons) in a sample of 1602 isolates from 1746 samples of 2-mm$^2$ leaf fragments.

Bael et al. 2005; Mejía et al., *in press*; Fig. 5). This technique allows for explicit experimental comparisons of growth, physiology, defense, chemistry, and genetic expression/composition between plants (or their tissues) with and without endophytes (E+/E–). Importantly, experimenters can choose the species of endophytes to be introduced, and base those choices on a variety of ecological and in vitro properties of the particular endophytes (e.g., their relative abundance in the field, or their growth or chemical production *in vitro*. Such comparisons open a large number of research possibilities. For example, Arnold et al. (2003; also see Mejia et al., *in press*) demonstrated that FEF substantially reduced leaf loss and damage due to *Phytophthora palmivora* infection (see Davidson et al. 2000) in *Theobroma cacao* seedlings. Because E– (endophyte-free) and E+ leaves could be produced and compared within individual plants, it is possible to conclude that the benefit to the host of having endophytes was quite localized. Similarly, *T. cacao* seedlings that had received root inoculations of common AMF showed dramatically reduced damage in the leaves due to *Phytophthora* (Fig. 5). However, in this case, the effect is apparently not local (see *Discussion*).

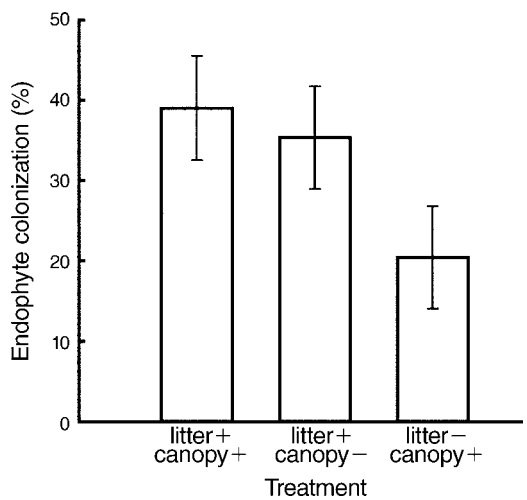


FIG. 4. Local leaf litter is a more important source of foliar endophytic fungal inoculum than intact canopy cover. Mean percentage (±SE) of leaf tissue colonized by endophytes in endophyte-free seedlings of *Theobroma cacao* after a one-week exposure to each of three habitats: (1) intact forest (closed canopy with intact litter, ++; $n = 15$); (2) forest gap (open canopy but with leaf litter intact, −+; $n = 16$); (3) intact forest (closed canopy, with ~90% leaf litter removed within >20 m of the seedlings, +−; $n = 16$) (see Sayer et al. [2006*a*, *b*] for site description). One leaf from each seedling was sampled, with 64 2-mm$^2$ fragments per leaf assayed for endophyte infection. In a Kruskal-Wallis one-way ANOVA, $H = 7.914$, df = 2, $P = 0.019$.

## DISCUSSION

The observation that the presence of endophytes (either FEF or AMF) can limit pathogen damage in host

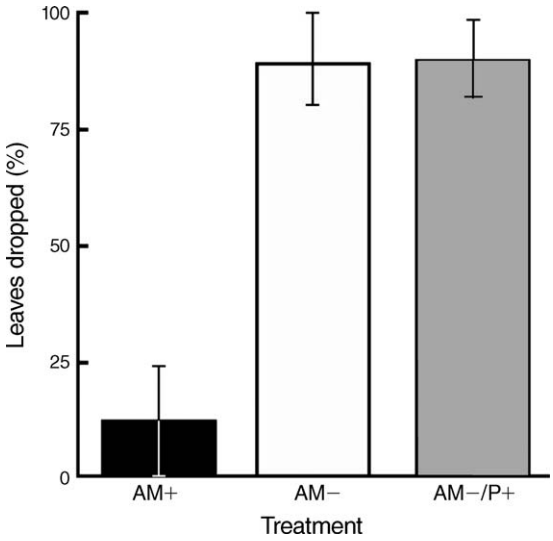


FIG. 5. Percentage (mean ± SD) of *Theobroma cacao* leaves dropped two weeks after inoculation with a foliar pathogen (*Phytophthora palmivora*). Arbuscular mycorrhizal fungi (AMF) colonization of roots can enhance plant defense against foliar pathogens. Seedlings had 3–7 leaves/individual, (mean = 4.5 leaves). Seedlings ($N = 14$ individuals/treatment) were grown for 90 d in sterile soil containing a mix of arbuscular mycorrhizal fungi (AM+), non-mycorrhizal controls (AM−), and controls fertilized with phosphorus (P+). *Phytophthora* zoospores (20 μL at 10 000 zoospores/mL) were applied to the upper leaf surface on both sides of the midvein and covered with a 5-mm agar plug to promote infection. $\chi^2 = 94.1$, df = 0.05, 2, $P \ll 0.001$.

plants clearly indicates that there is more to host-pathogen interactions than just "hosts" and "pathogens." Further, the observation that the identity of the endophytes can make a difference to the outcome of host-pathogen or host-herbivore interactions (Borowitz 2001, Herre et al. 2005*a*, *b*; Mejia et al., *in press*; S. A. Van Bael, *unpublished manuscript*) raises a series of interrelated questions. What influences endophyte community composition within a root or a leaf? What are the potential mechanisms for how endophytes influence the outcome of host-pathogen interactions? What are the implications for the study of host ecology in general and host defense in particular?

### *Community composition*

First, what influences endophyte community composition within a host? For both FEF and AMF available evidence suggests that the colonization of initially endophyte-free host tissues by a diversity of species is followed by processes leading to the relative dominance of one or a relatively few species. Both morphological and molecular studies provide clear evidence that sites differ in the relative abundance of different species for AMF (Husband et al. 2002*a*, *b*, Lovelock et al. 2003, Mangan et al. 2004, Herre et al. 2005*a*). Similar conclusions can be drawn for FEF (Arnold et al. 2000, 2003; Fig. 4). Preliminary evidence suggests that the scale of these site differences roughly correspond to scales at which the soil or leaf litter is likely to be dominated by the roots or leaves of different species of emergent canopy trees (see Mangan et al. 2004). Together, these observations suggest an important effect of local site on the source pool of AMF or FEF. In addition, there is evidence of differential host affinity both for FEF and AMF, with host chemistry implicated in shaping differential affinities of FEF for different hosts (Arnold et al. 2000, 2003, Gilbert et al. 2002, Herre et al. 2005*a*, *b*, Van Bael et al. 2005). Finally in both AMF and FEF, available data suggest that after initial colonization by a diversity of species, overall diversity declines (Husband et al. 2002*a*, *b*; Fig. 2). Thus, both AMF and FEF colonization is followed by a process of differential proliferation and/or competitive exclusion. Research priorities include determining whether endophyte-free plants exposed to different environments acquire different suites of FEF and/or AMF, and whether experimental inoculations with different FEF–AMF mixes reach similar or different within-host endophytic "climax communities."

### *Potential mechanisms for enhanced host defense*

Second, what are the potential mechanisms by which endophytes influence the outcome of host-pathogen interactions? Although there are several possible physiological mechanisms by which fungal endophytes (FEF and AMF) can contribute to defense responses (e.g., simply by occupying space within the host), they fall into two broad categories: indirect or direct effects. Indirect effects are defined here as endophyte-induced increases in the host plant's intrinsic chemical or physiological anti-pathogen defenses (see Aneja and Gianfagna 2001, Durrant and Dong 2004). Direct effects are defined as anti-pathogen defenses that are produced directly by the endophytes themselves, and are thus extrinsic to the host (e.g., endophyte-based antibiosis, see Stovall and Clay 1991, Stahla and Christensen 1992).

*Indirect effects.*—In the case of the AMF–*T. cacao* results (Fig. 4), because the AMF are confined to the roots, there is no possibility for a direct physical interaction of the fungi. Outside the possibility that AMF-derived products are translocated to the leaf, the anti-pathogen effects observed in the leaves are probably due to an indirect effect. This result may simply be based on improved host vigor (increasing a host's capacity to allocate to defense) due to increased access to nutrients (see Smith 1988, Mosse 1992). If so, then we expect that the ability of different AMF species to provide different levels of host protection should be in part based on their different abilities to provide resources to the host, and be reflected in the degree to which they promoted host growth (Herre et al. 2005*a*). In the case of foliar endophytes associated with limiting pathogen damage in *Theobroma cacao* (see Arnold et al. 2003), preliminary evidence from microarray assays of mRNA expressed in E+ and E− seedlings indicates that the inoculation of at

least one species results in the up-regulation of some genes that are part of known defensive pathways (M. Guiltinan and S. Maximova, *personal communication*, *unpublished data*). Thus, different types of endophytes can indirectly influence host defensive status either by increasing overall host vigor and/or by affecting the expression of specific host genes. Research priorities include determining what the specific indirect mechanisms are, and the degree to which the effects are highly localized (e.g., within part or all of a leaf) or systemic, across all host tissues (Durrant and Dong 2004). Also relevant is the degree to which different endophyte species (AMF or FEF) are interchangeable with respect to the form and extent of inducing indirect effects in the hosts.

*Direct effects*.—In the case of FEF–pathogen interactions within *T. cacao*, there are several lines of evidence suggesting that direct, in addition to indirect, effects are influencing host defense. First, there is precedent. Particularly in case of the vertically transmitted endophytic fungi associated with some grasses, endophyte-derived chemicals can provide either anti-pathogen or anti-herbivore protection to the host (see Petrini et al. 1992, Saikkonen et al. 1998, Yue et al. 2000, 2001). Second, in many cases, the FEF associated with *T. cacao* show in vitro chemical activity (antibiosis) against pathogens (Mejía et al., *in press*). Importantly, an endophyte that produces chemicals that inhibit one particular pathogen (or endophyte) might not inhibit another. Finally, nonanoic acid is a compound produced by the endophyte, *Trichoderma hartzium*. It exhibits strong in vitro inhibitory effects against the *T. cacao* pathogens *Crinipellis perniciosa* and *Moniliophthora rorei* (Aneja et al. 2006). Unpublished work by the same authors shows that seedlings inoculated with this endophyte possess this chemical in their tissues while uninoculated seedlings do not (T. Gianfagna, *personal communication*).

### *Conclusions and implications*

We suggest that much of the observed host defense (Arnold et al. 2003) results from foliar endophytes which live as "sit-and-wait saprotrophs" and therefore are strongly selected to "guard their turf" from potential usurpers (i.e., competitive exclusion; see Yodzis 1978, 1986). What we interpret as "turf guarding" with respect to pathogens (and other endophytes), should also be expected to occur with respect to herbivores, as has been observed in the case of the vertically transmitted endophytes associated with grasses (Saikkonen et al. 1998, Clay and Schardl 2002, Omacini et al. 2004). It is clearly in the interest of both the plant and endophyte for leaf or root tissues not to be lost to herbivores (particularly if the herbivores can digest the fungi) or pathogens. It is also certainly in the interest of the endophyte (FEF or AMF) not to be displaced by other endophytes. Research priorities include determining the degree to which AMF and FEF colonization and succession within hosts is determined by direct fungal interactions, what mechanisms determine the outcomes of those interactions, and how hosts mediate those outcomes.

The relative importance of different potential mechanism underlying endophyte-enhanced host defenses—indirect induction of host defenses vs. direct endophyte-pathogen interactions—determines the relative importance of the local environment (i.e., source pool of FEF and AMF) of the host plant on those defenses. If the effects are largely indirect through induction of intrinsic host defenses, then the host–pathogen interactions can be well understood primarily as just that of the particular host and the particular pathogen. That is, if endophytes serve little or no function beyond jumpstarting the host's intrinsic defenses, their identities and possibly even presence can be relatively less important. On the other hand, if even some portion of endophyte effects are direct (e.g., if particular endophytic species directly inhibit particular pathogen species via chemical antagonism at a very local scale within the host tissues), then the identities, diversities, and distributions of endophytes at a very fine scale within the host plant tissues becomes very relevant for understanding host defense. Moreover, the identities, diversities, and distributions of AMF and FEF at the very coarse scale of the environments in which the host plants establish and grow become important considerations for understanding the composition and establishment of this component of host defense.

As we have seen, the diversity of FEF within a leaf can be extremely high, and the resulting distribution very heterogeneous (Fig. 3). Particularly if direct fungal-fungal interactions are important, this observed pattern has implications both for the successful entry and proliferation of any given pathogen strain, as well as for a wider diversity of potential pathogens. Any particular strain of would be pathogen might be able to enter the leaf tissue and displace the endophytic fungus at any one point. However, depending on the identity of the endophyte occupying the adjoining piece of leaf tissue, the pathogen may be unable to proliferate, or even survive. Further, a leaf heterogeneously filled with different fungi provides a much more complex and presumably much more challenging environment for even a diversity of potential pathogens. Moreover, although a given plant host is more or less fixed genetically, the fungi associated with it can change and evolve over the lifetime of the host plant. Importantly, FEF will evolve on timescales that are roughly comparable to those of the pathogens. Particularly to the degree to which endophyte effects are direct, FEF may provide the host with many benefits that are usually associated with vertebrate immune systems. Therefore, the degree to which endophyte-mediated host defense is primarily direct or indirect presents a crucial area for future research.

It is no longer a question of whether the endophytic fungi imbedded within host plant tissues (in leaves, in stems, or in roots) do or do not affect many properties that researchers have long attributed to the plant. It has long been known that different AMF isolates differentially affect a given plant's growth and physiological properties (Mosse 1992, van der Heijden et al. 1998*a*, *b*, Kiers et al. 2000, Klironomos et al. 2000, Kyllo et al. 2003, Herre et al. 2005*a*, *b*). Further, it can be taken as given that fungi are chemically distinct from plants. Therefore, it should not be surprising then that E+ plant tissues have been found to exhibit different chemical profiles from E- plant tissues (Petrini et al. 1992, Saikkonen et al. 1998, Yue et al. 2000, 2001; L. C. Mejia, T. Gianfagna, and E. A. Herre, *unpublished manuscript*). Even genetic content that has been attributed to being of plant origin sometimes turns out to be derived from the fungi (Camacho et al. 1997, Chiang et al. 2001).

The more appropriate questions are the degree to which "plant" properties are due to endophytic fungi, the degree to which the identities of the endophytic fungi influence them, and the mechanisms that underlie those effects. If fungal effects are generally small, then viewing plants as "just plants" is perfectly adequate. However, if the fungal effects on their hosts turn out to be large (as some data suggest), then how we go about studying many seemingly familiar "plant" characteristics may need to be reconsidered.

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