

CHAPTER NINE

Tropical plants as chimera: some implications of foliar endophytic fungi for the study of host-plant defence, physiology and genetics

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Introduction

Fungal endophytes are defined as those fungi that live inside plant tissues (e.g. roots, stems, leaves) without causing apparent harm to their host (see Wilson 1995). Although we will also mention stem-associated endophytes (see Evans *et al.* 2003) and endophytes associated with roots (mycorrhizae; see Herre *et al.*, this volume), throughout this chapter, we will focus primarily on the implications of recent studies of the endophytic fungi that live inside plant leaf tissue. These foliar endophytes are extremely diverse phylogenetically and have been documented in nearly all plants sampled (e.g. mosses, liverworts, ferns, conifers and angiosperms; Carroll 1988; Clay 1988; Petrini 1991; Schultess & Faeth 1998; Frohlich & Hyde 1999; Stone *et al.* 2000; Arnold *et al.* 2000; Arnold 2002; Arnold *et al.* 2003; Davis *et al.* 2003). Despite the growing recognition of their wide distribution across plant taxa, basic attributes of their biology are still poorly understood. Specifically, endophyte diversity, distributions, life cycles, interactions with hosts and other fungi, and their net chemical, physiological and ecological influences are only beginning to be appreciated and studied. This is particularly true in the extremely diverse tropics.

The best-studied endophytes are ascomycetes belonging to the family Clavicipitaceae. These fungi grow throughout the aboveground tissues of some temperate grass species (e.g. *Festuca arundinacea*, see Clay & Schardl 2002). Typically,

in infected individuals, a single fungal genotype infects a single plant individual. In these grasses, endophytes appear to be predominantly asexual, and are transmitted vertically from maternal plants to their offspring via seeds (but see Schultess & Faeth 1998). The foliar endophytes that are associated with the grasses have often been shown to benefit their hosts through a variety of effects, including tolerance to heavy metals, increased drought resistance, reduced herbivory (due to chemicals, primarily alkaloids, produced by the endophytes), defence against pathogens, and enhanced growth and competitive ability (Carroll 1988; Clay *et al.* 1989; Ford & Kirkpatrick 1989; West *et al.* 1990; Gwinn & Gavin 1992; Welty *et al.* 1993; Saikonen *et al.* 1998; Clay & Schardl 2002; but see Faeth 2002). These beneficial effects are consistent with much evolutionary theory that predicts that vertical transmission will tend to align symbiont interests with those of the host, and that this will tend to promote mutualistic interactions (reviewed in Herre *et al.* 1999).

However, the grass-clavicipitaceous endophyte systems, characterized by vertical transmission of host-specific fungi that show low within-host fungal diversity, appear to present a special case that is not a useful general model for the vast majority of host-endophyte systems (Carroll 1988; Saikonen *et al.* 1998; Faeth 2002). Specifically, most endophytes associated with foliage of woody plants (as well as most monocots; Schultess & Faeth 1998; Frohlich & Hyde 1999) appear to be acquired from the environment. This sort of horizontal transmission is not generally considered to promote mutualistic interactions (Frank 1996; Herre *et al.* 1999), although many cases of horizontally transmitted mutualisms clearly exist (Herre 1999; Wilkinson 2001; Kiers *et al.* 2003; Arnold *et al.* 2003). Further, the community of endophytes even within a single leaf of a given host plant can be extremely diverse (Carroll 1988; Lodge *et al.* 1996; Bayman *et al.* 1998; Frohlich & Hyde 1999; Lebrón *et al.* 2001; Mejia *et al.* 2003; Arnold *et al.* 2003). Moreover, many endophytes of woody plants appear to be closely related to known pathogens (Carroll 1988; Freeman & Rodriguez 1993; Stone *et al.* 2000; Ortiz-Garcia *et al.* 2003). Therefore, it has been suggested that endophytes associated with leaves of woody angiosperms are unlikely to play protective or mutualistic roles in their host plants (Faeth 2002; Faeth & Fagan 2002).

Endophyte research in tropical areas has been primarily descriptive and restricted to dicot host plant species (e.g. Lodge *et al.* 1996; Bayman *et al.* 1998; Rajagopal *et al.* 2000; Gilbert *et al.* 2002b, Arnold & Herre 2003; Evans *et al.* 2003; Suryanarayanan *et al.* 2003). Recent work in Panama has demonstrated that tropical angiosperms host an extraordinary diversity of endophytes, which are horizontally transmitted and occur at very high densities in the leaf tissue of mature and old leaves (e.g. Arnold *et al.* 2000; Arnold *et al.* 2003). Moreover, this work has demonstrated that endophyte-free plants can be grown in the greenhouse and that single endophyte species or combinations can be experimentally reintroduced into plant tissue (Arnold & Herre 2003; Arnold *et al.* 2003; Mejia *et al.* 2003; see Table 9.1, Fig. 9.1). This technique allows for explicit experimental

Table 9.1 Summary of major findings from studies of endophytic fungi (EF) in Panama

Topic	Host plant species	Summary of findings
Diversity	<i>Theobroma cacao</i> (similar results in: <i>Ipomoea philomega</i> <i>I. squamata</i> <i>Merremia umbellata</i> <i>Witheringia solanacea</i>)	<ul style="list-style-type: none"> • A high diversity of endophytic fungi (EF) exists within a single host species. From 400 cm² leaf area ($n = 126$ leaves), 344 morphotaxa were isolated.^c • Most EF morphotaxa are rare.^{a-g} • Within a single leaf, the species diversity of EF increases after initial leaf flush and then decreases following leaf saturation.^{e,f}
Transmission	<i>T. cacao</i> <i>Ipomoea philomega</i> <i>I. squamata</i> <i>Merremia umbellata</i> <i>Witheringia solanacea</i>	<ul style="list-style-type: none"> • EF transmission is horizontal: leaves are flushed free of EF, and EF are acquired from the habitat through time.^{b,c,e,f,g} • Leaves appear to saturate in density of EF after 3–4 weeks.^{e-g}
Spatial structure	<i>T. cacao</i>	<ul style="list-style-type: none"> • EF communities are very similar at small spatial scales (0–50 km).^c; but see a • Similarity of EF communities declines dramatically with distances > 50 km.^{c,e,f}
Host-affinity	<i>T. cacao</i> <i>Faramea occidentalis</i> <i>Heisteria concinna</i> <i>Ouratea lucens</i>	<ul style="list-style-type: none"> • EF exhibit differential host affinity.^{a,c,g} • EF morphotaxa that dominate one host are markedly less common, rare or absent in other hosts.^{a,b,f,g} • EF growth in vitro is affected by the inclusion of host-specific leaf extracts.^{b,c} • EF that commonly occur in a given host tend to grow best in media with extracts of that host.^{c,e,f}
Interactions	<i>T. cacao</i>	<ul style="list-style-type: none"> • In vitro interactions between two EF species range from indifference to overgrowth or active inhibition.^{d,f} • EF species commonly found in a given host tend to dominate interaction trials with rare EF species when trials take place in media with extracts of the host.^{d,e,f}
Experimental manipulation	<i>T. cacao</i>	<ul style="list-style-type: none"> • EF-free leaves can be experimentally produced and EF can be re-introduced into EF-free leaves.^{b,c,d}
Pathogen resistance	<i>T. cacao</i>	<ul style="list-style-type: none"> • EF can enhance host defence against pathogens.^{c,d}

^a Arnold *et al.* (2000)

^b Arnold & Herre (2003)

^c Arnold *et al.* (2003)

^d Mejia *et al.* (2003)

^e E. Rojas *et al.* (unpublished results)

^f E. A. Herre *et al.* (unpublished results)

^g S. A. van Bael *et al.* (unpublished results)

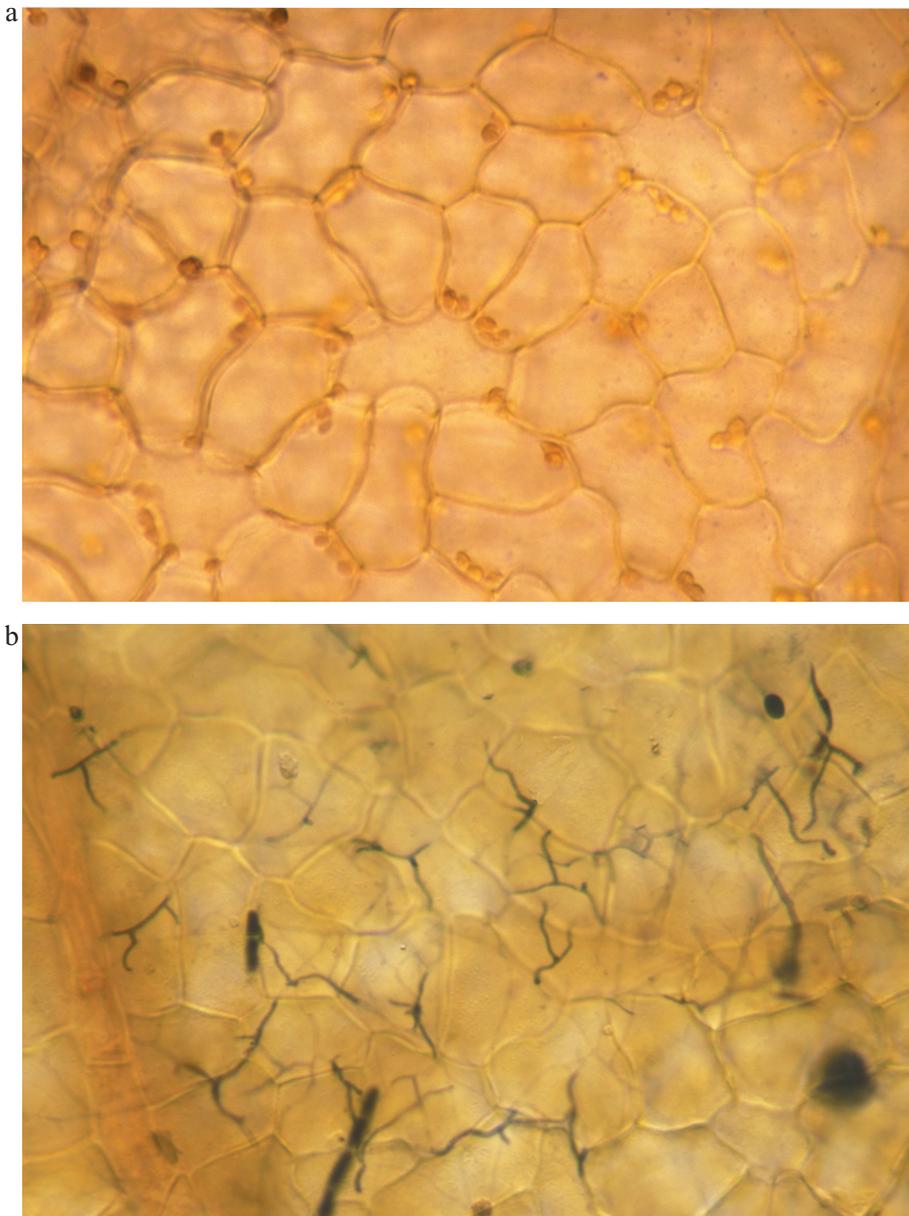


Figure 9.1 High magnification (400 \times) photograph of *Theobroma cacao* leaves: (a) without endophytic fungi, (b) with endophytic fungi (photos by L. C. Mejia).

comparisons of growth, physiology, defence, chemistry and genetic composition between plants (or their tissues) with and without endophytes. Such comparisons open a large number of possibilities that previously were only available in the study of grass-endophyte interactions. For example, experimental studies of plant-endophyte-herbivore interactions in these horizontally transmitted endophyte systems are now poised to complement the pioneering but primarily

correlative work with adult oaks and other temperate trees (Carroll 1988; Wilson & Carroll 1994; Preszler *et al.* 1996; Wilson & Carroll 1997; Wilson & Faeth 2001; Faeth & Fagan 2002; Faeth & Hammon 1997).

In this chapter we discuss the implications of recent studies of the ecology of tropical foliar endophytes that use *Theobroma cacao* (Malvaceae) and other plant species as hosts in Panama (Arnold *et al.* 2000; Arnold & Herre 2003; Mejia *et al.* 2003; Arnold *et al.* 2003; findings are summarized in Table 9.1). Fundamentally, it is becoming increasingly clear that plants are effectively chimera: organisms composed of both plant and fungal tissues. As is the case with most examples in which simpler biological entities combine to form more complex associations (eukaryotes, multicellular organisms, social insects, etc.), the composite possesses emergent properties that neither party possesses separately (Maynard Smith & Szathmari 1995; Margulis & Sagan 2002). Drawing on these and other studies, we will discuss some implications of the documented and potential influences of endophytic fungi for the study of tropical plant biology.

Life cycle and general natural history of endophytes

For many species that occur as endophytes, it appears that the portion of their life cycle that is involved with leaves begins as a taxonomically diverse assemblage of airborne spores that land on leaf surfaces (Carroll 1988). Generally, it appears that most leaves of tropical trees are flushed in a largely endophyte-free condition (Arnold 2002; Arnold & Herre 2003; Arnold *et al.* 2003; Mejia *et al.* 2003). After the wetting of the spore-laden leaf surfaces, a subset of spores germinate and are able to penetrate into the leaf tissue either through the stomata, or more directly through the cuticle (Arnold *et al.* 2003; Mejia *et al.* 2003). After penetration, a subset of the endophytes differentially proliferates within the leaf tissue (also see Deckert *et al.* 2001). During the lifetime of the leaf, there appear to be few, if any, recognizable symptoms of the presence of the endophytes. However, both tissue samples and microphotographs show that the plant tissue is indeed full of fungal hyphae (Fig. 9.1; Fig. 9.2; Arnold *et al.* 2000; Arnold *et al.* 2003; Mejia *et al.* 2003). Finally, many endophyte species appear to complete their life cycle (sporulate) on abscised leaves, effectively as saprophytes (see Fig. 9.2; J. Bischoff, L. C. Mejia and E. Rojas, personal observations).

The net effect from a plant's (or researcher's) perspective is that young leaf tissues (up to roughly 1 week following leaf flush) are relatively free of endophytic fungi. This is the time period during which secondary chemicals or DNA extracted from a leaf are most certain to be of exclusively plant origin (Arnold *et al.* 2003; Mejia *et al.* 2003, see below). During the first few weeks following leaf flush, the density of endophytic fungi in the leaf increases (as measured by the proportion of 2-mm² leaf punches that yield culturable endophytic fungi). At roughly 3–4 weeks the endophyte content of the leaf tissues appears to saturate (Arnold & Herre 2003; Arnold *et al.* 2003; Mejia *et al.* 2003). The trajectory of endophyte diversity within a leaf is less clear. However, it appears that diversity

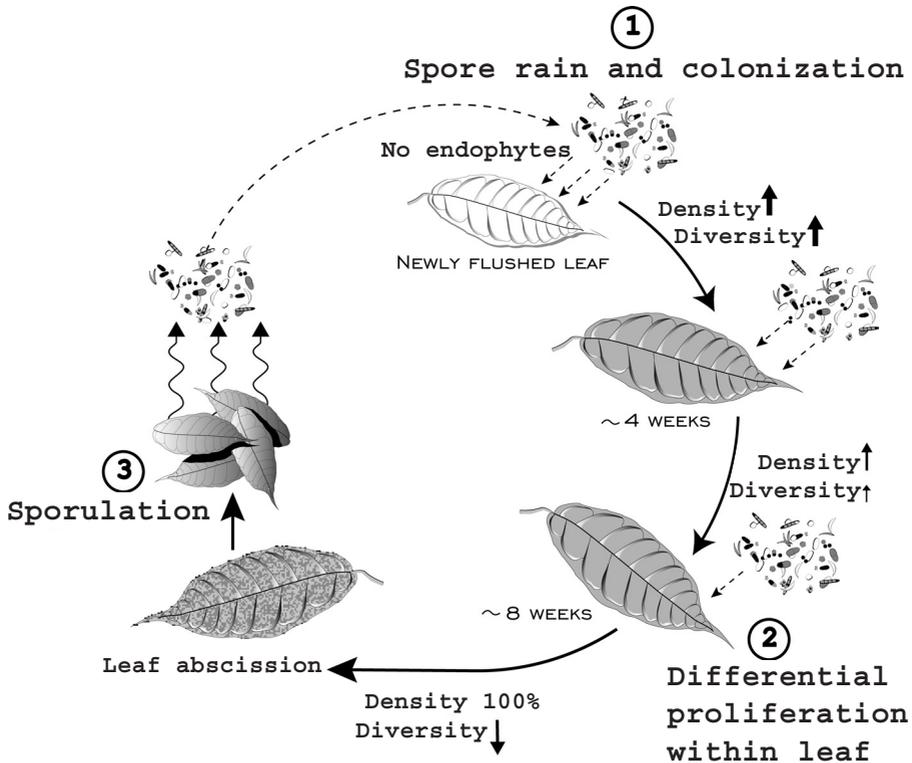


Figure 9.2 Inferred life cycle of many tropical foliar endophytic fungi based on observations made in *Theobroma cacao* (Arnold & Herre 2003; Arnold *et al.* 2003; Mejia *et al.* 2003; E. Rojas, J. Bischoff and A. E. Arnold, unpublished observations, see text; figure by D. A. Kyllo).

at first rises (as more of the leaf tissue becomes colonized by the diverse fungi that land on the leaf surface as spores, see Arnold *et al.* 2003). This phase (4–8 weeks following leaf flush) appears to be followed by a phase characterized by a reduction in overall diversity (as measured by the number of distinct morphospecies encountered per fungal isolate; E. Rojas *et al.* and E. A. Herre *et al.*, unpublished results). We interpret the observed decrease in diversity to reflect differential proliferation (or success in competition) of a subset of the colonizing fungi (see Arnold *et al.* 2003). In any event, whatever effects the endophytic fungi have on the host leaf are likely to become more pronounced with leaf age, as the leaf passes from an endophyte-free to an endophyte-saturated state (see below).

Effects on host-plant defence

By experimentally manipulating the presence or absence of foliar endophytic fungi among leaves within individual *Theobroma cacao* seedlings, Arnold *et al.* (2003) demonstrated that the presence of endophytes substantially reduced leaf

loss and damage due to an oomycete pathogen, *Phytophthora* sp., commonly isolated from necrotic leaves. The endophytic fungi that were introduced into the endophyte-plus (E+) leaves had previously been isolated from healthy *T. cacao* leaves (Arnold *et al.* 2003; Mejia *et al.* 2003). 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 localized. Importantly, the fungal isolates used for inoculation were selected on the basis of a combination of extensive survey work that showed them to occur commonly in the leaves of healthy hosts, and *in vitro* experiments that showed them to possess anti-*Phytophthora* activity (Mejia *et al.* 2003; L. C. Mejia *et al.*, unpublished results). Given the observed *in vitro* activity of the inoculated endophytes against *Phytophthora* (Mejia *et al.* 2003), we suspect that direct inhibition of pathogens by resident endophytes contributes to the observed enhancement of host defence (Arnold *et al.* 2003; Mejia *et al.* 2003; E. A. Herre unpublished results).

That the endophytes would actively inhibit pathogens from invading and/or proliferating within the leaf tissues is also consistent with the observation that in the vast majority of cases, endophytes appear not to harm their host plant, and not to reproduce (sporulate) until after the host leaf has been abscised (J. Bischoff, E. Rojas, L. C. Mejia and A. E. Arnold, personal observation). We suggest that much of the observed host defence results from endophytes essentially 'guarding their turf' from potential usurpers. 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 endophytes associated with grasses (Saikkonen *et al.* 1998; Clay & Schardl 2002). As is clearly true in the case of vertically transmitted endophytes in some grasses, it is in the interest of both the plant and endophyte for leaf tissues not to be lost to herbivores or pathogens. It is also certainly in the interest of the endophyte not to be displaced by other endophytes. Therefore, we can expect that the enhancement of host defences observed in *T. cacao* is not going to present an isolated case, and that endophytes will generally contribute a great deal that has previously been unappreciated to the defence of their hosts against pathogens and herbivores (see below).

Effects on host physiology, growth and the costs of endophytes

Endophytic fungi are obligately heterotrophic organisms. Further, direct observations (see Fig. 9.1) demonstrate abundant endophyte tissue within host leaves (Lodge *et al.* 1996; Bayman *et al.* 1998; Arnold *et al.* 2003). Therefore, we expect that endophytes derive their nutrition largely from their host plant, and that they should present at least a modest drain on host-plant resources. From the perspective of the defensive benefit they have been shown to provide, this hypothetical drain would constitute at least one principal 'cost' to the host plant. Obtaining accurate measurements of the 'cost' is a priority from a number of perspectives. Our efforts to measure such a cost have involved the comparison

of growth rates and physiological parameters (see below) of E⁻ and E⁺ plants. Current estimates suggest that the effects of endophytes on host growth and biomass accumulation are small, at least over a period of 23 weeks under greenhouse conditions.

However, a series of experiments comparing E⁺ and E⁻ plants showed that maximum photosynthetic assimilation (A_{\max}), stomatal conductance (g) and water-use efficiency were all significantly reduced in E⁺ seedlings relative to E⁻ seedlings of *T. cacao* (R. A. Cordero *et al.*, unpublished results). We tentatively interpret these findings as reflecting a net drag on water movement through leaves due to the presence of the endophytes throughout the intercellular spaces (see Fig. 9.1). Given these decreases in water movement and A_{\max} , we suspect that over longer periods than we have thus far examined, the effect of endophytes on reducing host biomass accumulation will become more clearly pronounced. Nonetheless, it is clear that foliar endophytes, like mycorrhizae, have the capacity to alter host physiological properties. A future research priority is to determine the degree to which different endophytic species induce different physiological responses in their hosts.

Effects on host chemistry and genetics

It can be taken as given that fungi are chemically distinct from plants. Therefore, it should not be surprising that E⁺ plant tissues have been found to have different chemical profiles from E⁻ plant tissues (Bacon *et al.* 1977; Weber 1991; Petrini *et al.* 1992; Saikkonen *et al.* 1998; Yue *et al.* 2001; L. C. Mejia *et al.*, unpublished results). Further, *in vitro* studies give clear evidence that some endophyte species commonly encountered in *T. cacao* exude substances that inhibit the growth of other fungal species in the absence of physical contact between colonies (Mejia *et al.* 2003). Combined with the observation that endophytes in grasses are known to produce a suite of chemicals that deter herbivory by large grazing mammals, as well as insect herbivores (Clay & Schardl 2002), it is reasonable to expect that endophytes will contribute to the chemical mix extracted from a 'plant'. It follows that many chemicals that have previously been attributed to plants may actually be produced by the endophytes within them. This probability has a series of implications for studies of the role of 'plant' chemistry in host-plant defence, and in drug discovery programs.

Moreover, there are now several known instances in which researchers who have extracted DNA from a 'plant' have in fact isolated and amplified fungal DNA (Camacho *et al.* 1997; Chiang *et al.* 2001). Researchers conducting genetic studies of plants, particularly those studies using techniques that are not known to be specific for the host plant species (e.g. Rapids, AFLP, etc.), need to be conscious of this fact when collecting and interpreting their data (Chiang *et al.* 2003; C. Woodward *et al.*, unpublished results).

It is no longer a question of *whether* the endophytic fungi imbedded within host plant tissues (in leaves, in stems, or in roots) affect many properties that

Table 9.2 Some observations and implications of the presence of endophytic fungi for the study of defence, physiology, and chemical and genetic composition of host plants

Plant defences	<ul style="list-style-type: none"> ● EF in <i>Theobroma cacao</i> can enhance host defence against pathogens (Arnold <i>et al.</i> 2003) ● EF in <i>Festuca</i> sp. and other species also can enhance host defence against pathogens and herbivores (Carroll 1988; Saikkonen <i>et al.</i> 1998; Clay & Schardl 2002) ➤ We can expect that some portion of host-plant defences is actually due to endophytes, as opposed to being intrinsic to the host plant per se
Physiology	<ul style="list-style-type: none"> ● EF in <i>Festuca</i> sp. and other species can increase drought and heavy-metal tolerance (Saikkonen <i>et al.</i> 1998; Clay & Schardl 2002) ● EF in <i>Theobroma cacao</i> can affect levels of photosynthesis and hydraulic properties (Cordero <i>et al.</i>, unpublished results) ● Mycorrhizae affect many physiological attributes of host plants ➤ We can expect that foliar endophytic fungi will influence at least some aspects of plant physiology
Chemical and genetic composition of leaf extracts	<ul style="list-style-type: none"> ● The comparison of EF⁻ and EF⁺ leaves shows that extracted secondary chemicals and genetic material can have an endophytic origin (Bacon <i>et al.</i> 1977; Weber 1991; Petrini <i>et al.</i> 1992; Saikkonen <i>et al.</i> 1998; Yue <i>et al.</i> 2001; Mejia <i>et al.</i>, unpublished results) ➤ Studies of plant chemistry and genetics must be designed with the likelihood of endophytic contribution/contamination in mind

researchers have long attributed to the plant (defence, growth, physiology, chemistry and genetic content, Table 9.2). Rather, the more appropriate questions are the degree to which those ‘plant’ properties are due to endophytic fungi, and the degree to which the identities of the endophytic fungi in a given host plant influence them. If the 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 appears to be the case with plant defence in *T. cacao*; see Arnold *et al.* (2003), Mejia *et al.* (2003)), then much of how we go about studying and interpreting many seemingly familiar ‘plant’ characteristics may need to be reconsidered.

Acknowledgements

We thank two anonymous reviewers and David Burslem for constructive suggestions. We thank Greg Gilbert, Tom Gianfagna and Prakash Hebbar for essential technical advice and training. We thank the Smithsonian Institution, the Andrew W. Mellon Foundation, National Science Foundation (DEB 9902346 to L. McDade and A.E.A.), the American Cacao Research Institute, the World Cacao Foundation and the John Clapperton Fellowship of Mars Incorporated for financial support. Finally, we thank the Smithsonian Tropical Research Institute for

providing the stimulating intellectual environment, infrastructure and logistical support that made this work possible.

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