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Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae)

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Abstract: Fungal endophytes inhabit healthy tissues of all terrestrial plant taxa studied to date and are diverse and abundant in leaves of tropical woody angiosperms. Studies have demonstrated that plant location and leaf age influence density of endophyte infection in leaves of tropical forest trees. However, ecological factors underlying these observations have not been explored in detail. Here, we establish that foliar endophytes of a tropical tree (*Theobroma cacao*, Malvaceae) are transmitted horizontally and that endophyte-free seedlings can be produced for experimental manipulation by protecting aerial tissues from surface wetting. At Barro Colorado Island, Panama, we used transects of endophyte-free seedlings to determine the importance of several factors (canopy cover, abundance of aerial and epiphytic propagules, leaf age, leaf chemistry, leaf toughness and duration of exposure to viable air spora) in shaping colonization by endophytic fungi. Endophytes colonized leaves of *T. cacao* more rapidly beneath the forest canopy than in cleared sites, reflecting local abundance of aerial and epiphytic propagules. The duration of exposure, rather than absolute leaf age, influenced endophyte infection, whereas leaf toughness and chemistry had no observed effect. Endophytes isolated from mature *T. cacao* grew more rapidly on media containing leaf extracts of *T. cacao* than on media containing extracts from other co-occurring tree species, suggesting that interspecific differences in leaf chemistry influence endophyte assemblages. Together, these data allow us to identify factors underlying patterns of endophyte colonization within healthy leaves of this tropical tree.

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INTRODUCTION

Endophytes are microorganisms that colonize and cause asymptomatic infections in healthy plant tissues (Wilson 1995). Considered ubiquitous among terrestrial plants, fungal endophytes have been found in healthy tissues of all plant taxa sampled to date (e.g., Petrini et al 1982, Clay 1988, Legault et al 1989, Schulz et al 1993, Rodrigues 1996, Fisher 1996, Lodge et al 1996, Fröhlich and Hyde 1999) and in habitats ranging from coastal mangroves (Kumaresan and Suryanarayanan 2001, Okane et al 2001) to north-temperate evergreen forests (e.g., Espinosa-Garcia and Langenheim 1990, Muller and Hallaksela 1998), temperate pastures and grasslands (e.g., Clay and Holah 1999, Vinton et al 2001), semiarid regions of the southwestern U.S.A. (Faeth and Hammon 1997), and tropical forests (e.g., Rodrigues 1994, Arnold et al 2000, Cannon and Simmons 2002). Most studies have focused on clavicipitaceous fungi that inhabit temperate grasses (e.g., Clay 1991, Saikkonen et al 1998, Malinowski and Belesky 1999); in contrast, endophytic fungi associated with leaves of woody angiosperms, especially in tropical forests, are poorly known.

Factors that have stimulated interest in endophyte ecology in tropical forests is the growing interest in the role played by endophytes in calculating estimates of global fungal diversity (Fröhlich and Hyde 1999, Hawksworth 2001), in shaping plant community dynamics (Clay and Holah 1999, Arnold 2001) as sources of novel bioactive compounds (Bills and Polishook 1992, Strobel and Long 1998) and as biological control agents for use in tropical agroforestry (Arnold et al 2001b). Recent surveys in a lowland forest of central Panama suggest that endophytes inhabit every mature leaf sampled among 24 species of taxonomically diverse, woody angiosperms (Arnold 2001), and that infection densities in leaves of tropical dicotyledonous hosts often approach one endophytic isolate per 2 mm^2 of leaf area (see also Lodge et al. 1996). Moreover, highly diverse endophytes in neotropical forests demonstrate both host prefer-

ence, with respect to tree species, and heterogeneity on small spatial scales (e.g., Rodrigues 1994, Arnold et al 2000; but see Cannon and Simmons 2002), such that endophytes represent a ubiquitous, albeit cryptic, component of tropical forest communities. Despite their ubiquity and abundance, however, ecological interactions of tropical endophytes with their hosts have not been explored in detail.

In contrast to clavicipitaceous endophytes of temperate grasses, which are characterized by vertical transmission of fungal symbionts from maternal parent to offspring via seed (e.g., Clay 1991), several lines of evidence suggest that vertical transmission is not the only mode of transmission in host-endophyte associations. Notably, observations that >10 species of endophytes typically inhabit individual tropical leaves (e.g., Lodge et al 1996, Arnold et al 2000), that endophyte species composition and infection frequency vary with habitat (Petrini 1991, Rodrigues 1994, Bayman et al 1998, Gamboa and Bayman 2001), and that infection density in woody plants tends to increase with leaf age (e.g., Bernstein and Carroll 1977, Petrini et al 1982, Rodrigues 1994) appear consistent with horizontal transmission. Under this scenario, fungal endophytes of woody plants travel among hosts as spores, germinating epiphytically and penetrating leaf cuticles to grow intercellularly within healthy tissues (Arnold et al 2001b, Lebrón et al 2001). Therefore, successful colonization of host tissues by foliar endophytes likely is correlated with factors influencing local abundance of aerial and epiphytic propagules, and with diverse aspects of host plant suitability, including host genotype and leaf characteristics. However, no study has critically assessed the roles of these biotic and abiotic factors in influencing endophyte infection in tropical forest trees.

Here, we explore dynamics of endophyte infection in a lowland, moist, tropical forest in central Panama. We first establish that foliar endophytes of *Theobroma cacao* (Malvaceae), an understory tropical tree, are transmitted horizontally. We then use endophyte-free seedlings of *T. cacao* to assess effects of plant habitat at a small spatial scale (forest versus clearing) on natural colonization by foliar endophytes. To explore short-term, site-specific disparities in endophyte infection, we experimentally assess the relative importance of leaf chemistry and abundance of aerial and epiphytic propagules. We then address the common observation that foliar endophyte infections increase in density with leaf age, assessing relative importance of age-specific leaf chemistry, leaf toughness and duration of exposure to fungal inocula. In so doing, we explicitly identify ecological factors underlying patterns of endophyte colonization within healthy leaves of this tropical tree.

MATERIALS AND METHODS

Study site.—Formerly a hilltop within contiguous tropical forest, Barro Colorado Island, Panama (BCI; ~9°9′N, 79°51′W) was isolated ca 90 yr ago when the Chagres River was dammed for construction of the Panama Canal (Leigh and Wright 1990). BCI has been maintained since 1921 as a field station by the Smithsonian Tropical Research Institute and comprises ca 1500 hectares of moist, semideciduous, lowland tropical forest. Roughly two-thirds of the island is covered by mature forest >400 yr old; the remainder of the island bears late secondary forest approximately 70–100 yr old (Croat 1978, Sagers and Coley 1995). In the younger forest at BCI, where this study was conducted, ca 137 species of woody angiosperms >2.5 cm DBH occur per hectare (Lang and Knight 1983). The island has a mean annual temperature of 27 C and receives ca 2600 mm of precipitation annually, of which 90% falls during the May–December wet season (Leigh et al 1996).

Study species.—*Theobroma cacao* (Malvaceae) is an understory tree native to forests of north-central South America (Young 1994). Pantropically cultivated for cocoa production, *T. cacao* produces seeds (cocoa beans) in oblong fruit borne on stems and branches. In Panama, cacao is cultivated intensively in the western province of Bocas del Toro, but individuals of the species occur incidentally and for research purposes throughout much of the isthmus (Arnold pers obs). At BCI, *T. cacao* occurs at low frequency in both primary and secondary forest (Croat 1978).

Endophyte isolations.—For all surveys of endophyte infection in the present study, healthy leaves were harvested, washed in running tap water and processed within 4 h of collection (Arnold et al 2000). Healthy leaves were defined as leaves undamaged by herbivores and free of overt symptoms of disease. From each leaf, we cut 16 adjacent, 2 mm^2 segments from the middle lamina (midway between the petiole and leaf tip and midway between the midvein and margin). Leaf segments were surface sterilized by sequential washes in 70% ethanol (2 min) and 0.5% NaOCl (2 min), rinsed with sterile water, and allowed to surface-dry under sterile conditions. This method of surface-sterilization eliminates epiphyllous microorganisms from endophyte cultures (Arnold et al 2000; see also Schulz et al 1993).

We placed leaf segments in Petri dishes containing 2% malt-extract agar (MEA), a medium that yields large numbers of diverse endophytic isolates (e.g., Fröhlich and Hyde 1999). Following Bills and Polishook (1992), no antibiotics or growth inhibitors were included in the nutrient medium. We incubated plates at room temperature and with ambient light, assessing each plate for hyphal growth every 3 d for 21 d. Cumulative proportions of leaf segments yielding endophytes were used as a measure of endophyte infection density.

Transmission patterns and production of endophyte-free seedlings.—In April 1999, we harvested ripe, healthy fruit from

mature *T. cacao* at three sites in Panama: Nombre de Dios, Colón, where a three-hectare stand of mature trees grows in scattered shade; Divisa, Herrera, where healthy trees are tended for study at the Instituto Nacional de Agricultura (INA); and near Almirante, Bocas del Toro, where cacao is cultivated on many small farms overseen by the COCABO collective.

From each fruit, we harvested asymptomatic, mature seeds, rinsed them in running tap water, and soaked them for 5 min in 0.5% NaOCl (10% Clorox) to sterilize seed surfaces. Seeds were planted in sterilized forest soil contained in pots that had been washed with 1% NaOCl. Pots were maintained in a screened room with a solid, opaque roof on BCI, where plants experienced ambient temperatures and moderate light but were protected from rain. After germination, we took care not to wet aerial tissues, because spores of many fungi germinate and penetrate leaf cuticles only if water is on leaf surfaces (e.g., Frias et al 1991, Sparace et al 1991).

When plants had produced at least four true leaves (i.e., post-cotyledons), we randomly chose 20 seedlings and harvested one mature leaf from each. Within four hours of harvesting, we sampled those leaves for endophytes using methods described above. After 7 d in culture, <1% of leaf segments showed evidence of infection by endophytic fungi. In contrast, mature leaves of *T. cacao* collected from forest trees at BCI during the same period bore evidence of endophytic fungi in >95% of leaf segments (Arnold et al 2001a). Based on this disparity, we considered seedlings endophyte-free, randomized them with respect to maternal origin and placed them in experimental arrays as described below.

The near absence of culturable endophytes in mature leaves of these seedlings suggested that endophytes of *T. cacao* are horizontally transmitted. To investigate further the possibility of vertical transmission, we assessed whether seeds contained culturable endophytes. From each of 20 surface-sterilized seeds of *T. cacao*, we cut 10 tissue segments (each <2 mm^3) and plated them on MEA. After 21 d, no seed segments showed evidence of endophyte infection. We concluded that culturable endophytes of *T. cacao* are not transmitted vertically from maternal plants to offspring via seed but instead are acquired as propagules transmitted horizontally among hosts. This finding corroborates previous work on temperate and tropical hosts (e.g., Rodrigues 1994, Faeth and Hammon 1997, Bayman et al 1998, Lebrón et al 2001), confirming that horizontal transmission of foliar endophytes is typical for woody angiosperms in a variety of habitats. For this reason, we relied on natural, horizontal transmission of endophytes to experimentally assess endophyte colonization in seedlings of *T. cacao*.

Endophyte colonization: plant habitat.—To assess effects of plant habitat on colonization by endophytes, we placed experimental arrays of endophyte-free seedlings in the secondary forest and in the BCI laboratory clearing in late June 1999 and monitored endophyte infection. Forest arrays included four groups of six seedlings placed at 10 m intervals along a transect in the secondary forest starting 10 m from the forest edge (N = 24 seedlings). Clearing arrays were similar but began ca 30 m beyond the forest edge and extended into the laboratory clearing (N = 24 seedlings).

After 7 d, we harvested one mature leaf from each of 45 seedlings distributed equitably across forest and clearing arrays. After 15 d, we harvested one additional mature leaf from each of 16 forest- and 16 clearing-grown seedlings. In each case, we collected only leaves that had been mature when placed in the field. For each leaf, we quantified endophyte infection, as described above, and abundance of epiphytic propagules (see below).

Endophyte colonization: leaf age.—In the forest understory, leaves of different ages inherently differ in duration of exposure to ambient fungal inocula, such that it is difficult to separate effects of exposure time from other age-specific leaf characteristics (e.g., leaf chemistry and toughness). For this reason, we used endophyte-free seedlings in forest arrays to compare endophyte colonization in young and mature leaves exposed to inocula for equal time. At the outset of experimental arrays, we marked each young leaf (ca 5 d past budbreak; they were characterized by pinkish or pale green coloration, soft, pliable texture and were small relative to mature leaves) with a ring of colored wire around the petiole. Young leaves matured fully during exposure in forest arrays, as evidenced by laminar greening, cuticular toughening and expansion to full size. After 15 d, we harvested one mature leaf (>20 days past budbreak) from 16 forest-grown seedlings, collecting only leaves that had matured in the field. Harvesting of leaves that matured in the field was concurrent with harvesting of leaves that were mature at the outset (above). We then compared endophyte infection densities for leaves of each type.

Abundance of aerial inoculum and epiphytic fungi.—To assess abundance of aerial inoculum, we measured sporefall on four rain-free days during the experimental period (1, 5, 7, 8 July 1999). For each measurement, we placed two sterile Petri dishes containing 2% MEA at ground level ca 1 m from each plant array. On each sampling date, we exposed plates for 30 min between 11 a.m. and 2 p.m. After exposure, plates were sealed, incubated at room temperature 3 d, and scored for the number of fungal colony-forming units (CFU). Because CFU were expected to include representatives of diverse guilds of fungi, including pathogens, saprophytes, epiphytes and endophytes, measurements were used as an index reflecting total abundance of viable fungi in the air column. For analysis, we standardized counts of CFU to include only the central 2 cm^2 of each plate to avoid areas close to walls of the culture dish, which might have influenced propagule deposition.

To assess abundance of epiphytic propagules, the top of each leaf harvested for endophyte analysis was pressed for 10 s against 2% MEA in a sterile Petri plate. We then sealed plates, incubated them at room temperature for 3 d, and categorically scored each for the number of fungal CFU (0, 1–24, 25–49, 50–74, ≥75 CFU) within the central 2 cm^2 of each leaf impression, which corresponded to the central portion of the lamina of each leaf.

Endophyte growth.—To assess roles of site- and age-specific leaf chemistry in influencing endophyte colonization, we examined growth rates of endophytes on water agar containing leaf extracts of young or mature leaves of forest- or clearing-grown *T. cacao*. To determine further the sensitivity of endophytes to variation in leaf chemistry, we then assessed endophyte growth on media containing extracts of mature leaves of *Laetia thamnia* (Flacourtiaceae) or *Trichilia tuberculata* (Meliaceae), two co-occurring tree species known to differ markedly in quantities and components of leaf chemical defense (Coley 1983, Coley pers comm). Several authors have used plant secondary metabolites in media for similar purposes (e.g., Guiraud et al 1995, Vega et al 1997), such that this method is considered useful for detecting sensitivity to leaf chemistry among fungal endophytes.

For extracts, healthy leaves were harvested from >3 host individuals of each species. For extracts of *T. cacao* used to assess site- and age-specific leaf chemistry, leaves were obtained from randomly chosen individuals in experimental arrays after exposure to natural inocula (mature leaves) or shortly after budbreak, but after short exposure to inocula (young leaves). For extracts of *T. cacao*, *Laetia thamnia* and *Trichilia tuberculata* used to assess effects of species-specific leaf chemistry, mature leaves were collected from individuals occurring naturally in the understory, near plant arrays. Within 1 h of collection, leaves were washed in running tap water, surface-dried and randomized with respect to individual of origin. For each extract, 3.6 g of leaf tissue was cut into ca 1 cm^2 pieces and ground for 5 min in a clean mortar and pestle with 36 mL of distilled water and a pinch of fine sand. We removed large pieces of leaf tissue from extracts, decanted 25 mL of each liquid extract into media bottles, added 225 mL of distilled water and 3.75 g agar, and autoclaved the mixture (20 min, 121 C).

Endophyte isolates used in growth trials represent three distinct morphospecies (sensu Arnold et al 2000) drawn at random from a living collection of sterile endophytes frequently isolated from healthy leaves of adult *T. cacao* at BCI. For growth trials, endophytes were subcultured from agar slants onto 2% MEA, allowed to grow until colony diameter exceeded 1 cm, and transferred as 12.5 mm^2 plugs of hyphae and agar to three replicate plates for each extract treatment. We used calipers to measure colony diameter every 2 d until growth neared the edge of the Petri dish.

Leaf toughness.—Examination of germinating spores on leaf surfaces has demonstrated that diverse endophytes of *T. cacao* enter leaves via cuticular penetration (Mejía pers comm). Yet, the relationship of leaf toughness and successful colonization by endophytic fungi is not known. We therefore assessed leaf toughness for one young (5–10 d old) and one mature (>20 d old) leaf on 10 seedlings of *T. cacao* grown under protected conditions. To measure leaf toughness, we used a spring-loaded Newton scale, which measures force needed for a rod with diameter of 3 mm to penetrate leaf tissue (penetrometer, as described by Coley 1983). Three replicate measures were made for each leaf. Because rod diameter influences the scale of leaf toughness measurements, values obtained here are not directly comparable to those presented in previous studies of diverse tropical trees (e.g., Coley 1983). However, when scaled according to the rod diameter used in that study (5 mm), values obtained for *T. cacao* in this study are consistent with ranges of values presented therein.

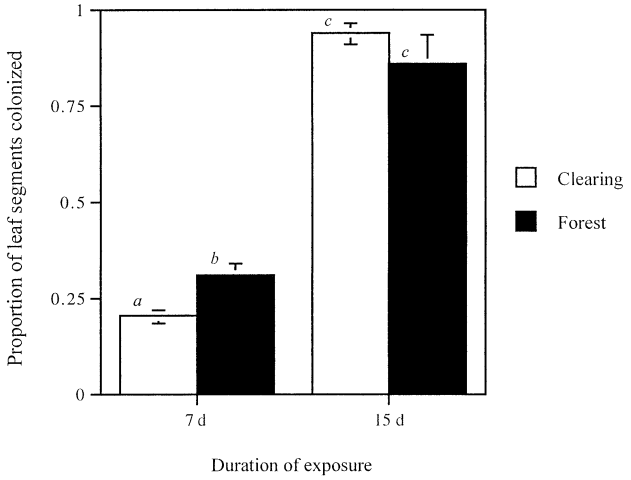

FIG. 1. Proportions of leaf segments colonized by endophytes for forest- and clearing-grown seedlings of *T. cacao* exposed to natural endophyte colonization under field conditions for 7 d and 15 d at Barro Colorado Island, Panama. Differing superscripts indicate significant differences among means ($\alpha = 0.05$). As in subsequent figures, error bars represent ±1 SE.

Data analysis.—Endophyte infection densities and leaf toughness data were not normally distributed and were analyzed with Kruskal-Wallis rank-sums tests. Sporefall data were logit-transformed ($\ln [y/(1 - y)]$) for normality and compared with ANOVA. Because epiphyte data were categorical, frequency distributions of CFU were compared with χ^2 tests. Growth data were normally distributed and were compared with ANOVA. All analyses were carried out using the statistical package JMP (Sall and Lehman 1996).

RESULTS

Endophyte colonization: plant habitat.—Regardless of plant location, all leaves harvested after 7 d and 15 d of exposure contained fungal endophytes. Density of endophyte colonization differed significantly between seedlings in the forest and clearing after 7 d of exposure, but after 15 d, endophyte densities converged for seedlings at each site (FIG. 1). After 7 d, endophyte infection density in leaves of forest seedlings exceeded that of seedlings in the clearing by a factor of 1.5 ($\chi^2_1 = 9.12$, $P = 0.0025$). After 15 d, density of endophyte infection had increased significantly for leaves of seedlings in the forest ($\chi^2_1 = 13.51$, $P = 0.0002$) and in the clearing ($\chi^2_1 = 30.37$, $P < 0.0001$), but mean infection densities no longer differed with location ($\chi^2_1 = 0.34$, $P = 0.5627$).

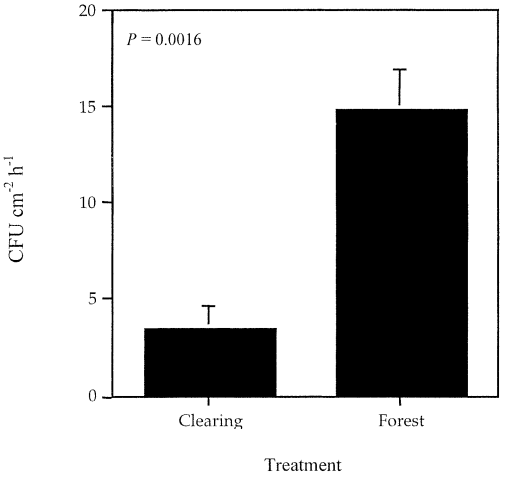

FIG. 2. Colony-forming units (CFU cm^{-2} h^{-1}) were deposited by natural sporefall in clearing and forest sites at BCI, Panama. Data reflect only CFU that grew on 2% MEA.

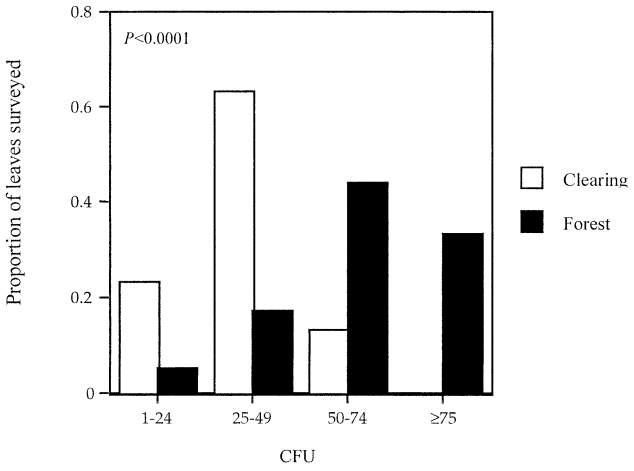

FIG. 3. Frequency distribution of abundance classes for viable fungal propagules occurring on leaf surfaces of forest- and clearing-grown seedlings of *T. cacao* at BCI. Measures represent CFU occurring within the central 2 cm^2 of the lamina of each sampled leaf, and reflect only CFU that grew on 2% MEA.

Endophyte colonization: leaf age.—Regardless of age at outset, all mature leaves of forest seedlings contained fungal endophytes. We observed no qualitative differences in morphology between leaves that matured in the forest and leaves that were mature at outset. Among seedlings in the forest exposed for 15 d, infection densities for leaves that matured in the forest (94.5% ± 1.8) did not differ from leaves that were mature at the outset of the experiment (85.2% ± 5.6; $\chi^2_1 = 1.02$, $P = 0.3116$).

Aerial inoculum and epiphytic propagules.—CFU obtained on four survey dates and at four sites within each habitat type (forest, clearing) did not differ significantly, with respect to sampling date or site, and were combined to generate mean abundances of aerial inoculum. Colonies generated by sporefall were ca fivefold more numerous for forest samples than clearing samples ($F_{1,6} = 29.89$, $P = 0.0016$; FIG. 2). Similarly, viable epiphytic propagules were significantly more abundant on leaves of forest seedlings than on leaves of clearing seedlings ($\chi^2_3 = 24.68$ for frequency distributions, $P < 0.0001$; FIG. 3).

Leaf chemistry.—Growth rates of endophytes on media containing extracts from seedlings of *T. cacao* are shown in TABLE I. Because growth rates of endophytes did not vary among isolates, data were combined to generate mean values. In vitro growth rates did not differ on media infused with leaf extracts from seedlings in the forest or clearing ($F_{1,17} = 0.13$, $P = 0.7233$) or on extracts from young or mature leaves cultivated beneath the canopy ($F_{1,17} = 0.66$, $P = 0.4270$). Furthermore, growth rates on extracts of mature leaves from experimental seedlings did not differ significantly from those on extracts of mature leaves from trees occurring naturally in the forest understory ($F_{1,52} = 2.00$, $P = 0.1630$). However, growth rates differed significantly on media containing mature leaf extracts from different host species, with growth rates on *T. cacao* exceeding those on both *Trichilia tuberculata* and *Laetia thamnia* ($F_{2,65} = 10.61$, $P < 0.0001$; FIG. 4).

Leaf toughness.—Results of leaf toughness measures are shown in FIG. 5. For seedlings of *T. cacao*, mean leaf toughness of mature leaves exceeded that of young leaves by a factor of ca 2.2 ($\chi^2_1 = 34.21$, $P < 0.0001$).

DISCUSSION

By subjecting endophyte-free plants to natural inoculation under field conditions, we found that col-

TABLE I. Growth rates of endophytes on media containing extracts from young or mature leaves of clearing- or forest-grown seedlings of *Theobroma cacao*. Growth rate is defined as the mean increase in colony diameter (mm d^{-1}) during three consecutive days. Means (±1 SE) represent combined data for isolates of three endophyte morphospecies. No significant differences were observed in growth rates on any media containing leaves of *T. cacao*

	Growth rate (mm d^{-1})	
Leaf age	Clearing	Forest
Mature	8.23 ± 0.81	8.67 ± 0.89
Young	8.48 ± 0.57	9.47 ± 0.47

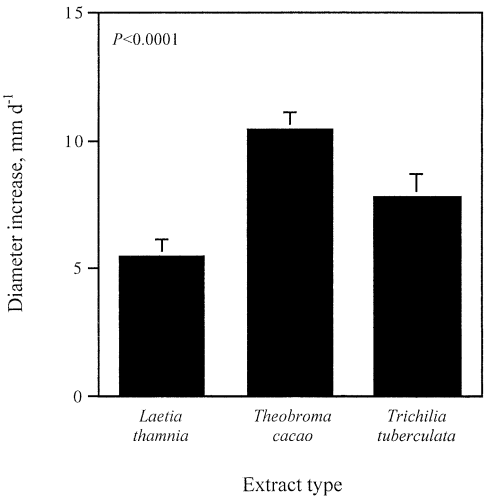

FIG. 4. Growth rates are illustrated for isolates of three endophyte morphospecies, which were cultured on media containing mature leaf extracts from *Laetia thamnia* (Flacourtiaceae), *Theobroma cacao* (Sterculiaceae), or *Trichilia tuberculata* (Meliaceae).

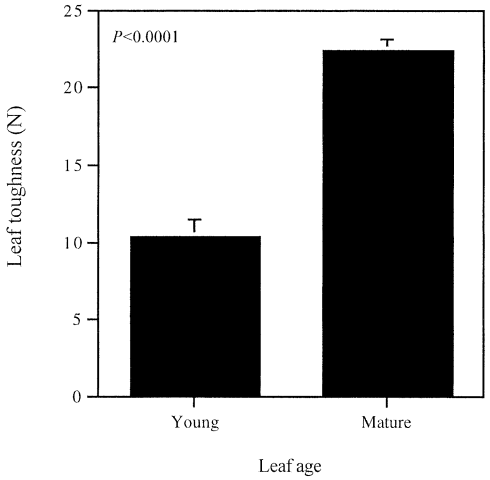

FIG. 5. Leaf toughness of mature and young leaves of *T. cacao*.

onization of leaves by endophytes in the short term (7 d) was positively related to local abundance of aerial and epiphytic propagules. With greater duration of exposure, there was no observed effect of inoculum abundance on density of endophyte colonization. Absolute leaf age, leaf chemistry and leaf toughness apparently were unrelated to density of colonization by endophytic fungi.

Patterns and processes external to the leaf.—Habitat-specific differences in endophyte colonization during the first 7 d of exposure paralleled differences in abundance of aerial and epiphytic inocula between forest and clearing sites. Sporefall rates in this study are consistent with those determined by Gilbert (2002) for forested sites at BCI, suggesting that deposition of aerial propagules during wet seasons might be relatively consistent across years. Similarly, our data coincide with those of Gilbert (2002) to suggest that leaves receive large quantities of aerial propagules throughout their lifetimes: Deposition of ca 15 CFU cm^{-2} h^{-1} (FIG. 2: mean deposition rate at forest sites in this study) suggests that beneath the canopy, mature leaves of *T. cacao*, which average >100 cm^2 in leaf area at BCI (Arnold unpubl data), might receive >36 000 aerial propagules d^{-1}. In contrast, our data suggest that leaves in the clearing receive ca 30-fold fewer propagules on a daily basis (ca 1100 propagules d^{-1}; FIG. 2). This site-specific disparity might be attributed to abiotic conditions, such as higher humidity (see Croat 1978 for data specific to the laboratory clearing and forest at BCI) and lower UV radiation (Braga et al 2001), which might enhance viability of fungal propagules beneath the canopy. Furthermore, both plant diversity and vertical structure are greater within the forest, such that total abundance and diversity of fungal propagules should be greater beneath the canopy than in clearings (see Lodge and Cantrell 1995). Because our data reflect only viable propagules that are capable of germinating and growing on MEA, 15 CFU cm^{-2} h^{-1} likely underestimates total deposition of air spora and preferentially might underestimate abundance of fungal propagules in the clearing if, as expected, unfavorable environmental conditions reduce propagule viability there. However, our data provide a useful working estimate of propagule deposition within the forest and clearing at BCI and corroborate previous work indicating that tropical plants are subject to deposition of large quantities of fungal inoculum (Rodrigues et al 1995).

Because abundance of epiphytic propagules depends in part on deposition of aerial inoculum, it is likely that abundances of aerial and epiphytic propagules are positively correlated. However, our study design precludes distinguishing between these as causal factors influencing endophyte abundance. Moreover, we did not assess species composition of inocula, such that underlying spatial or temporal differences in the prevalence of taxa capable of growing as endophytes might have influenced our results. Because boundaries among guilds of tropical fungi are poorly defined, because many endophytes fail to sporulate in culture and because diversity of tropical fungi has not been quantified thoroughly, inference of life history for aerial and epiphytic propagules will prove difficult in the absence of molecular and/or inoculation studies.

On and within tropical leaves.—We have shown that canopy cover is related to density of endophyte infection over the short term, reflecting patterns of total inoculum abundance. However, in this study, endophyte infection densities in forest- and clearing-grown seedlings equalized over time. In contrast to other plant-fungus interactions in which primacy of infection might reduce the probability of later infection by other strains or species (e.g., Norman et al 1996), these data suggest that leaves remain receptive to endophyte infection after initial infections occur. Furthermore, that age-specific leaf toughness and chemistry were not associated with colonization or growth further suggests that young and mature leaves are equally receptive to infection by foliar endophytes.

Infection densities observed in the present study were similar to values for mature leaves of woody angiosperms developing naturally in the forest understory (ca 90–95%: see Lodge et al 1996, Gamboa and Bayman 2001, Arnold et al 2001a). That experimental seedlings achieved high rates of infection after only 15 d suggests that infection densities in living leaves rise quickly after budbreak to levels that remain relatively constant throughout leaf lifetimes. Whether such asymptotes reflect filling of intercellular spaces by immigration or growth, limitations in quantities of apoplastic nutrients, biochemical antagonism among endophytes, or long-term activation of antifungal defenses remains to be elucidated. To discriminate among these hypotheses, in vitro assessments of endophyte interactions could assess potential for endophytes to interact directly via hyphal contact, inter- and intraspecific antagonism and nutrient competition. Although in vitro trials represent simplified interactions in artificial environments, studies using leaf extracts in media and large numbers of interacting species could more closely approximate conditions in host leaves. Similarly, exploring age-, diversity- and abundance-dependent leaf chemistry would shed light on activation of host defenses over time.

Leaf chemistry: Is it a determinant of endophyte species composition?—Many tropical microfungi are thought to exhibit some degree of substrate specificity (Bills and Polishook 1994, Lodge 1997) mediated, at least in part, by substrate chemistry. Among foliar endophytes, frequent association between particular fungal species and hosts (Arnold et al 2000), and variable growth rates of endophytes on media containing leaf extracts of various host species (see FIG. 4) suggest that endophytic fungi are sensitive to gross leaf chemistry.

Prevalence of antifungal secondary compounds in young leaves, and in leaves that develop under high light conditions, has been documented for many tree species in tropical forests (e.g., Coley 1983, Coley and Barone 1996). Phenolic compounds have been especially well studied (e.g., Denslow et al 1990, Coley and Barone 1996) and are known to have antifungal effects both in vivo (Klepzig et al 1996, Souto et al 2000) and in vitro (Guiraud et al 1995, Ejechi 2001). Concentrations of simple phenolics and condensed tannins might be twofold greater in young leaves than in mature leaves for a variety of tropical taxa (Coley and Kursar 1996) and tend to be greater in sun-grown than in shade-grown foliage (Ganzhorn 1995). Thus, it is likely that quantities of leaf phenolics varied with leaf age and plant habitat in this study. However, we found that extracts from forest- and clearing-grown leaves, and from young and mature leaves, did not affect growth of endophytes isolated from mature leaves of *T. cacao*. These data suggest that neither phenolics, nor other chemical defenses that vary with leaf age or illumination (e.g., anthocyanins, Coley and Aide 1989; terpenes, Crankshaw and Langenheim 1981), differed sufficiently to directly influence in vitro hyphal growth for endophytes surveyed here.

Leaf chemistry might have been altered during autoclaving in such a way that some compounds might have been liberated or destroyed; however, inclusion of nonvolatile compounds in molten media has induced differential fungal growth in vitro in numerous studies (e.g., Vega et al 1997, Lacey and Mercadier 1998). Moreover, the method used here induced differential endophyte growth when extracts were drawn from host species that differ quantitatively in phenolics and other defenses (Coley 1983). These data suggest that endophytic fungi isolated with frequency from a given host species might be relatively insensitive to chemical conditions in leaves of that host at different ages or illuminations, whereas interspecific differences in chemistry are sufficient to yield a strong growth response. Whether leaf defenses affect life stages other than hyphal growth, including epiphytic germination, cuticular penetration and persistence in living leaves, remains to be assessed for tropical endophytes. However, given that endophytes appear to demonstrate host preference in vivo (Arnold et al 2000, but see Cannon and Simmons 2002), we hypothesize that leaf chemistry might play an important role in shaping endophyte assemblages in tropical forest trees.

Other aspects of host suitability.—Extensive literature suggests that host genotype (Morrison 1996, Yates et al 1996, Elamo et al 1999) and factors related to host environment, such as carbon-nitrogen ratio (Crone

and Jones 1999, Hoffland et al 1999), water stress (Biggs 1993, Ragazzi et al 1995, Hong and Michailides 2001, McElrone et al 2001, Ma et al 2001), and wetness duration after precipitation (Vloutoglou et al 1996, Turechek and Stevenson 1998) might influence both the penetrability of aerial tissues by fungal hyphae and the probability of fungal survival within host tissues. In this study, host seedlings were randomized with respect to maternal origin, such that host genotype should not have systematically biased our results. Moreover, soil used for all seedlings was drawn from a shared source. Carbon-nitrogen ratios were not assessed, but a lack of differences across treatments in terms of endophyte infection density and growth suggests that endophytes might not be sensitive to the range of ratios present among our plants. However, duration of wetness did differ between clearing and forest treatments, with forest-grown seedlings maintaining water on leaf surfaces for several hours after rainfall (Arnold pers obs). This might have influenced spore germination and survival on leaf surfaces and is worthy of further exploration.

Similarly, water stress has been implicated in plant susceptibility to fungal infection (e.g., Luo et al 2001), such that disparities in water relations across treatments might have influenced penetrability of host tissues over the short term. Plants grown in clearings were notably stressed during this study, appearing chlorotic and wilted after several days of exposure. Thus, it is possible that water stress might have influenced endophyte infection. However, because leaves of clearing-grown plants contained similar densities of endophyte infections relative to leaves of forest-grown seedlings after 15 d, it appears that water stress, or other factors influencing site-specific suitability of host plants, did not have long-term consequences for endophyte colonization or persistence.

Implications for studies and applications of tropical fungal endophytes.—Preliminary data have led some authors to speculate that as many as 1.3 million species of fungal endophytes may exist (Dreyfuss and Chapela 1994), and it is expected that the greatest diversity of endophytes will occur in tropical forests (e.g., Fröhlich and Hyde 1999, Arnold et al 2000, Hawksworth 2001). However, quantification of endophyte diversity is inhibited by inconsistent methods, which prevent effective comparisons among studies in disparate sites and with different host taxa. By noting leaf age and exposure, researchers could reduce artifactual variation in endophyte abundance and thereby could improve comparability among studies of endophyte diversity. Similarly, our data suggest that foliar endophytes of tropical trees are sensitive to leaf chemistry. We suggest that studies of endophyte biodiversity might benefit by considering broad patterns of leaf chemistry in choosing host taxa (see Coley and Barone 1996). Finally, our results suggest that applications of endophytes in various human endeavors (e.g., biological control) will benefit from an understanding of endophyte ecology. This study indicates that high inoculum volume, beneficial conditions for survival of epiphytic propagules and multiple applications throughout leaf and plant lifetimes should increase colonization of healthy tissues by foliar endophytes under field conditions.

Tropical endophytes constitute a diverse but poorly known group. Further study of endophyte ecology in natural systems promises to elucidate both potential applications of endophytic fungi for human use and ecological roles of these ubiquitous associates of healthy plant tissues. Such efforts will shape our understanding of the scale of fungal biodiversity, the nature of plant-fungus interactions in tropical forests and the ecological importance of cryptic symbionts in diverse plant communities.

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