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SHORT COMMUNICATION

Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species

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Drought strongly influences plant phenology, growth and mortality in tropical forests, thereby shaping plant performance, population dynamics and community structure (Bunker & Carson 2005, Condit *et al.* 1995). Microbial symbionts of plants profoundly influence host water relations (Lösch & Gansert 2002), but are rarely considered in studies of tropical plant physiology. In particular, plant–fungus associations, which are ubiquitous in plant communities and especially common in tropical forests, play important and varied roles in plant water status. Fungal pathogens associated with roots, vascular tissue and foliage may interfere with water uptake and transport, increase rates of foliar transpiration, and induce xylem embolism and tissue death (Agrios 1997). In contrast, rhizosphere mutualists such as ecto- and arbuscular mycorrhizal fungi may benefit hosts by increasing surface area for water uptake, enhancing stomatal regulation of water loss, and increasing root hydraulic conductivity (Auge 2001, Lösch & Gansert 2002).

While the effects of root-associated mutualists and pathogens on plant water status are becoming better understood, little is known regarding the effects of foliar symbionts such as endophytic fungi. Foliar endophytes – fungi inhabiting leaf tissues without causing visible symptoms of disease – have been recovered from living, apparently symptomless leaves in many terrestrial ecosystems and are especially abundant and diverse in lowland tropical forests (Arnold & Lutzoni 2007, Fröhlich & Hyde 1999). Mature, healthy leaves of tropical trees and shrubs typically contain >10 culturable species of endophytes, and individual hosts may harbour dozens to hundreds of endophyte species (Arnold *et al.* 2003). In seasonally moist and everwet forests and agro-ecosystems in Panama, endophytes have been recovered from every mature leaf of every tree species examined thus far (Arnold 2002). Infection densities in mature leaves often approach one isolate per each 2 mm^2 of leaf area (Lodge *et al.* 1996), with numerous, localized infections accumulating rapidly as ambient fungi colonize leaves after emergence (Arnold & Herre 2003, Van Bael *et al.* 2005).

The costs and benefits of endophytes associated with leaves of tropical trees are poorly known. Arnold *et al.* (2003) showed that common endophytes of *Theobroma cacao* (Malvaceae) can protect seedlings against a virulent foliar pathogen, and Costa Pinto *et al.* (2000) showed that symptomless infections by two endophyte species (*Colletotrichum musae* and *Fusarium moniliforme*) led to a reduction in photosynthetic capacity in banana. Herre *et al.* (2005) cite unpublished data indicating that endophyte-infected seedlings of *T. cacao* have lower stomatal conductance and water-use efficiency than do endophyte-free seedlings, suggesting that endophytes may profoundly affect water relations of their hosts.

Here, we use an experimental approach to show for the first time that endophyte colonization increases minimum leaf conductance, a measure of leaf water loss after maximal stomatal closure under drought stress. We focused on *Theobroma cacao* (Malvaceae), an understorey tree native to forests of north-central South America (Young 1994) that is now cultivated for cocoa production throughout the tropics, especially in areas with *c.* 1400–2000 mm of annual rainfall and 3–5 mo of dry season (Alvim 1977). Because of its use in agroforestry, *T. cacao* is one of relatively few tropical tree species for which aspects of plant physiology and the endophyte community have

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been examined in some detail (Arnold *et al.* 2003, Mohd *et al.* 1992, Thomas & Balasimha 1992). This study was conducted in seasonally moist forest at Barro Colorado Island, Panama (BCI), where drought is a pervasive selective agent that influences the mortality of seedlings and mature plants (Bunker & Carson 2005, Condit *et al.* 1995).

To assess effects of endophytes on leaf water status, we generated endophyte-free seedlings, inoculated a subset of leaves with natural complements of endophytic fungi, and then assessed water loss from leaves under drought conditions using leaf drying curves. Seeds of *T. cacao* were collected from sites in the provinces of Nombre de Dios, Bocas del Toro, and Panama, randomized with regard to maternal origin, surface-sterilized, and planted in sterile soil. After germination, aerial tissues were carefully protected from surface wetting in a shade house to prevent colonization by ambient fungi (Arnold & Herre 2003). When plants had produced at least four true leaves, one mature leaf per seedling ($n = 30$ leaves) was sampled for endophytes (see methods below). After 7 d in culture, < 1% of leaf segments yielded endophytic fungi, such that seedlings were considered endophyte-free.

Beneath the forest canopy at BCI, leaves are colonized rapidly by endophytes upon exposure to precipitation and airborne fungal propagules (Arnold & Herre 2003). Deposition rates average *c.* 10–15 colony-forming units (CFU) $cm^{-2}\,h^{-1}$ during the wet season (Arnold 2002, Gilbert 2000), with peaks in sporefall occurring immediately after rainfall events. We recreated the natural inoculum conditions to which seedlings in the forest understorey are exposed by collecting spore-laden throughfall during and immediately following rainfall events. Throughfall was collected for *c.* 1 h beginning 30 min after the onset of three significant rainfall events in the late wet season by placing three 2-litre basins at 10-m intervals along a 30-m transect in late secondary forest. Contents were combined, agitated and placed in sterile spray bottles for application to seedlings. To confirm that inocula contained sufficient propagules to allow natural levels of colonization, we misted two sterile Petri dishes containing 2% malt extract agar (MEA) with inoculum spray on each inoculation day, incubated plates at ambient temperature for 48 h, and then counted fungal CFU in the central 2 cm^2 of each dish. In all cases, inoculum sprays yielded > 50 CFU cm^{-2} and contained fungi that were morphologically consistent with endophytic taxa.

We assigned endophyte-inoculated (E+) or endophyte-free (E−, or control) treatments at random to mature leaves of similar ages on 30 seedlings. E+ leaves were misted with inoculum (*c.* 5 ml per application) at 6–8-h intervals over 72 h. Plants were then maintained for 7 d under protected conditions before examination of epiphyte and endophyte communities and measurements of minimum leaf conductance. We thus ensured that endophytes had sufficient time to colonize E+ leaves (Arnold *et al.* 2003) and avoided any transient effects of leaf wetting on cuticular permeability.

To disassociate effects of epiphytic (surface-inhabiting) fungi from those of endophytic fungi, we surveyed epiphytic fungi by pressing the upper surface of each leaf against 2% MEA in a sterile Petri dish for 30 s. Plates were sealed, incubated at ambient temperatures for 48 h, and assessed for CFU in the central 2 cm^2 of each leaf impression.

To confirm the quality of inoculations, we assessed E+ and control leaves for endophyte infection ($n = 9$ leaves per treatment). When possible, E+ leaves were paired with E− leaves on the same plants; however, pairing was imperfect due to variation in leaf number and development times. Following Arnold *et al.* (2000), healthy leaves were harvested, washed in running water, and processed within 4 h. From each leaf, 16 1 × 2-mm segments were surface-sterilized by sequential washes in 70% ethanol (2 min) and 0.5% NaOCl (2 min) and allowed to surface-dry under sterile conditions before plating on 2% MEA (Arnold *et al.* 2000). Plates were incubated at room temperature and assessed daily for hyphal growth over 21 d.

Throughout the experiment, all E+ and control leaves remained asymptomatic. Viable epiphytic fungi were abundant on leaf surfaces of both endophyte-inoculated (E+) and control leaves, which consistently bore > 25 CFU cm^{-2}. Abundance of epiphytic fungi did not differ with treatment, and no overt morphological differences were observed among epiphyte assemblages from E+ and control leaves (data not shown).

In contrast, proportions of leaves colonized by endophytic fungi differed significantly with treatment. Endophytes were present in 77.7% of E+ leaves, but only 11.1% of control leaves (i.e. one of nine leaves; Fisher's exact test, $df = 1, 15$, $P = 0.0152$). The single E− leaf that contained endophytes was heavily infected by a single fungal morphospecies that occupied 9 of 16 leaf segments (56%). Overall, the proportion of leaf segments infected by endophytes for E+ leaves (31.9%) significantly exceeded that for control leaves (6.25%; Kruskal–Wallis test, $\chi^2_1 = 5.68$, $P = 0.0172$). Infection densities in E+ leaves were similar to those observed in leaves shortly after budbreak under natural conditions in the forest understorey (Arnold *et al.* 2003). Two to seven morphospecies were isolated from each infected E+ leaf, equivalent to similarly aged leaves in the forest at BCI (Arnold *et al.* 2003). Emergent fungi were morphologically consistent with common endophytes isolated from *T. cacao* in Panama under field conditions, including *Xylaria*, *Colletotrichum*, *Phomopsis*, *Guignardia* and *Botryosphaeria* (Arnold & Lutzoni 2007).

To assess the effects of endophytic fungi on host water relations, we assessed specific leaf traits and minimum leaf conductance for E+ and control (E−) leaves ($n = 12$ leaves per treatment). Plants were watered

thoroughly 12 h before pre-dawn collection. Harvested leaves were immediately assessed for fresh weight (FW; to 0.0001 g), and projected leaf area (A_{proj}; LI-3000, Li-Cor Inc. Lincoln, Nebraska, USA). We then followed the decrease in leaf weight over time by weighing leaves at intervals of 45 min to 3 h over a period of 72 h. Between weighing, leaves rested on wire mesh *c.* 10 cm above the laboratory bench and were exposed to slight air movement from air conditioning to minimize boundary layer resistance. Concurrent with each weighing, we determined air and wet bulb temperature with a sling psychrometer (Bacharach Instruments, Pittsburgh, PA, USA). Room temperature during the experiment averaged 24.3 ± 0.6 °C (mean ± SD). Leaf-to-air water vapour deficit, calculated under the assumptions that air is water-vapour saturated within the leaf (von Caemmerer & Farquhar 1981), and that leaf temperature equals air temperature, was 6.3 ± 0.5 kPa. Values were converted to the difference in water vapour concentration (g m^{-3}) according to von Willert *et al.* (1995). The minimum rate of leaf water loss (*F*, the slope of the linear phase of the leaf drying curve, Kerstiens 1996a) was used to calculate minimum leaf conductance: $G_{min} = F/(A \times dw)$, where *F* is the flow rate of water (g s^{-1}) through the leaf surface area *A* (m^2: twice the projected leaf area (A_{proj}) of the flat leaf) at the concentration gradient *dw* (g m^{-3}; see Schreiber & Riederer 1996). With known temperature and pressure, G_{min} (m s^{-1}) can be converted to units usually used for stomatal conductance (mol m^{-2} s^{-1}; Pearcy *et al.* 1989). If all stomata are completely closed in an intact leaf, G_{min} equals cuticular permeability. However, a fraction of stomata usually remains partly open, such that G_{min} represents the sum of cuticular permeability and residual stomatal conductance (Kerstiens 1996a). Leaf conductance that includes residual stomatal conductance, rather than the conductance of astomatous cuticles alone, represents the minimum leaf conductance attainable for living leaves under drought stress, and is therefore a meaningful ecological parameter.

At the end of the weighing cycles, we dried leaves for 24 h at 70 °C and assessed leaf dry weight (DW). We then calculated leaf water content ($(FW - DW)/A_{proj}$) and specific leaf weight (DW/A_{proj}) for each leaf.

Leaf area, fresh weight, dry weight, water content and specific leaf weight did not differ with regard to endophyte treatment (data not shown). In contrast, mean minimum leaf conductance was significantly higher for endophyte-infected leaves (E+, $10.2 \times 10^{-5} \pm 0.9 \times 10^{-5}$ m s^{-1}) than for control leaves (E−, $5.7 \times 10^{-5} \pm 0.3 \times 10^{-5}$ m s^{-1}; $F_{1,22} = 10.9$, $P = 0.0032$), with E+ leaves demonstrating nearly twofold greater minimum leaf conductance. Because we found no significant differences in epiphytic fungal abundance or richness, we conclude that endophyte infection markedly influenced minimum leaf conductance.

These results show that during maximum stomatal closure, leaves infected with a natural density and diversity of endophytes exhibit almost double the rates of water loss relative to uninfected leaves. The observed effect of endophyte infection on minimum leaf conductance in *T. cacao* is equivalent to the difference in minimum leaf conductance between soybean and corn (6×10^{-5} and 12×10^{-5} m s^{-1}, Kerstiens 1996a), or between the tropical understorey shrub *Faramea occidentalis* and the tropical dry forest tree *Cedrela odorata* (6.2×10^{-5} and 10.1×10^{-5} m s^{-1}, Engelbrecht & Herz, unpubl. data).

Plants in moist tropical forests are regularly exposed to seasonal water stress (Walsh & Newbery 1999). Under severe drought, stomata of many plant species are maximally closed (Lambers *et al.* 1998), reducing leaf conductance by 90–99.7 % (Körner 1994, Larcher 1995). Low residual conductance from the cuticle and incompletely closed stomata can be crucial for plant survival under such conditions (Kerstiens 1996b), with increases in minimum leaf conductance accentuating drought stress and threatening to lead to critically low water potentials, xylem embolism, tissue death, leaf abscission and ultimately plant death (Tyree & Ewers 1991). While the degree to which minimum leaf conductance in other hosts is influenced by endophyte infection is not yet known, the ubiquity of endophytes in leaves of tropical trees suggests that these fungi may play cryptic roles in water relations of many plant species.

Elucidating the mechanisms underlying the observed increase in minimum leaf conductance was beyond the scope of this study. However, we suggest that cuticular penetration by endophytes, which germinate epiphytically but colonize internal leaf tissues by haustoria, penetration pegs, or other specialized hyphal structures, may cause cuticular wounding and/or interfere with stomatal closure. Cuticular wounds are characteristic of many fungal pathogens (Agrios 1997), but healthy leaves have not been surveyed for damage incurred by endophyte colonization. Further study is needed to evaluate the importance of effects mediated by the fungi themselves, and plant-mediated or physiological responses to infection.

Foliar endophytes comprise varied life histories and host interactions, and the degree to which individual fungi may have temporally plastic or host-specific interactions with plants is not known. This study provides first evidence that natural endophyte assemblages impose a potentially significant cost on an important tropical tree by nearly doubling rates of water loss when stomata are maximally closed. These data join a growing number of studies highlighting the previously overlooked costs and benefits of endophytic fungi in tropical plants (see Herre *et al.* 2005). Our data also provide a note of caution for tropical plant physiologists: cryptic fungal symbionts should be considered in studies of plant water relations.

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