Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico

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Abstract: Endophytic fungi were isolated from healthy leaves of *Manilkara bidentata* (Sapotaceae) collected in Puerto Rico. One leaf was collected from each of three trees. Four 5 X 20 mm panels were cut from each leaf, surface sterilized, cut into 50 1 X 2 mm pieces, and plated on malt agar. Petioles were cut into ten 1 mm segments. Fungi were isolated from 90%–95% of the leaf pieces and all of the petiole segments. *Xylaria* spp. were found in 73%–74% of the leaf pieces in two of the three leaves, but only 21% in the third leaf. *Xylaria* cf. *multiplex*, *X. cf. ascendentis*, a member of the *X. mellisii*/*X. arbuscula* complex, and 20 other fungal species were isolated. Nineteen of the 22 species on leaf blades were found on at least two of the three leaves, but half of the 12 species in petioles were unique to one leaf. The 22 species isolated from leaf blades fit a lognormal distribution. An estimated three to six species were not discovered, indicating that the 22 species found on leaf blades represented 79%–88% of the endophytic community.

**Key Words:** *Xylaria*, species diversity, tropical forest

Fungi that live inside healthy plant tissues are known as endophytes. Some endophytic fungi (i.e. latent pathogens and quiescent infections) may induce disease symptoms later when the plant tissues age or become weakened (Viret and Petrini, 1994), but many endophytic fungi are completely benign and may form mutually beneficial relationships with their host plants. For example, studies of temperate grasses (Clay, 1988) have demonstrated that clavicipitaceous endophytes increase plant growth (Clay, 1987) by protecting their host from herbivory (Chelpik and Clay, 1988; Clay, et al., 1985) and probably also afford protection from fungal pathogens (Christensen and Latch, 1991; White and Cole, 1985). The relationship of trees with their endophytes is less clear. Although it is postulated that the secondary fungal chemicals that are antagonistic to insect herbivores may increase fitness of the host (Carroll, 1988, 1991, 1995), such compounds may not be produced in sufficient quantities *in vivo* to be effective, and evidence of antagonism between endophytes and insects are almost nonexistent (Carroll 1995; but see Webber 1981). Regardless of the ecological role of endophytic fungi in forests, endophytes are routinely screened for *in vitro* production of useful compounds (e.g., Polishook, et al. 1993) so their diversity is of economic importance.

Endophytic fungi have been isolated from leaves, stems and roots of woody plants both in temperate regions (Bills and Polishook, 1992; Fisher and Petrini, 1990; Fisher, et al. 1995; Carroll, 1988; Wilson and Carroll, 1994) and the tropics (Dreyfuss and Petrini, 1984; Laessøe and Lodge, 1994; Rodrigues and Samuel, 1990; Rodrigues, et al., 1993; Rodrigues, 1994). Anamorphs of xylariaceous fungi are much more common among endophytes isolated from the few tropical plants that have been studied (Rodrigues and Samuel, 1990; Rodrigues, 1994) than from trees in temperate climates (Carroll and Carroll, 1978; Fisher and Petrini, 1990; Fisher, et al., 1994, 1995). Although most of the tropical xylariaceous endophytes that have been identified because of their characteristic cultures are species with wide host ranges (Rodrigues, 1994; Rodrigues and Samuel, 1990), it is not known if there are host- or substrate-specific strains (Lodge and Cantrell, 1995). Less is known about host-specificity or life-histories of non-xylariaceous tropical endophytic fungi. May (1990) hypothesized that the low frequency and disjunct distributions of plant species characteristic of highly diverse tropical forests could select against host-specificity among the organisms that depend on them.
and tropical forests will thus have proportionately fewer host-specific species as compared to temperate forests that are dominated by a few tree species. Such issues are important in arriving at estimates of global biodiversity (e.g., Hawksworth 1991), which are often calculated based on the distribution of host plants and a constant ratio of fungi:plant species (Hammond 1992). This is the first investigation on fungal endophytes in leaves of a tree growing in Puerto Rico, and one of the first on a dicotyledonous tropical tree. The study by Dreyfuss and Petrini (1984) of endophytes in tropical epiphytes and vines focused on determining patterns of occurrence across a wide host range, while the quantitative studies by Rodrigues (1994) and Rodrigues and Samuels (1990) concerned endophytes of two palm species. In a previous study at our site by Cowley (1970), a great diversity of fungi was cultured from Manilkara bidentata (A. DC.) Chev. (Sapotaceae) leaves collected at different levels of the canopy. However, Cowley (1970) ground and plated unwashed leaves and thus could not distinguish between epiphytic and endophytic fungi. Our study was undertaken to assess the extent and variability of colonization by endophytic fungi in order to evaluate sampling strategies for leaf endophytes in tropical broad-leaved trees, and for focusing future research on the mechanism of infection. Furthermore, we plan a future comparison of endophyte species composition in leaves of M. bidentata with those of another tree species growing next to the trees used in this study in order to investigate the degree of host-specificity among tropical tree endophytes.

**Study site.**—Our study was conducted in tabonuco forest near the El Verde Field Station in the Luquillo Mountains of eastern Puerto Rico (18° N, 65° W, 350 m elev.) in a former shade-coffee plantation that was abandoned about 1940. Manilkara bidentata, Dacryodes excelsa Vahl (tabonuco), and Sloanea berteriana Choisé are the dominant canopy trees that characterize the tabonuco forest, which grows in the subtropical wet forest life-zone according to the Holdridge system (Brown, et al., 1983). El Verde receives ca. 3500 mm of rain annually, with only slight seasonal variation in precipitation. Consequently, the forest is non deciduous and average tree leaf lifespans are thought to be at least 1.3 years (Lodge, et al., 1991).

**Leaf material.**—One healthy, young, fully expanded leaf, measuring approximately 150 × 70 mm, was collected from each of three trees of M. bidentata spaced about 20 m apart along the trail to the Quebrada Sonadora. The leaves were sent to Exeter (U.K.) in paper bags and processed within 5 d. Four panels, each measuring 5 × 20 mm, were cut from each leaf blade, two on either side of the midrib; a 12 mm piece of leaf petiole was also cut from each leaf. The plant material was washed in running water and surface sterilized by sequential immersion in 75% ethanol for 1 min, 0.93–1.3 M solution of sodium hypochlorite (3%–5% available chlorine) for 3 min and 75% ethanol for 0.5 min. After surface sterilization each leaf panel was divided serially into 50 leaf fragments each measuring 1 × 2 mm (10 rows of 5 pieces each) and placed in serial order in petri dishes containing 1.5% Oxoid malt extract agar (MEA) amended with 250 mg/L oxytetracycline to suppress bacterial growth. The petioles were cut into 10 equal fragments (2 mm wide × ca. 1 mm long), surface sterilized and serially plated. All plates were incubated at 20 ± 2°C for 5–14 d. Fungi were isolated by transferring mycelium or conidia to 2% MEA plates, and in selected cases onto oatmeal agar (2% agar with 75 g oatmeal per L) to induce sporulation. In the few cases where two fungi grew from the 1 × 2 mm leaf fragment, each was assumed to occupy half of the area for purposes of mapping and calculations of infection frequencies.

**Endophytes.**—At least one fungus was isolated from 90%–95% of the leaf blade fragments, and all of the petiole segments. This is a much higher frequency of infection than previously found in tropical palms (up to 30%, Rodrigues, 1994; Rodrigues and Samuels, 1990) or for conifers in the northwest coast of the USA (Carroll and Carroll, 1978), and somewhat higher than the 60% mean peak infection rate of Quercus garryana leaves by Discula quercina in Oregon (Wilson and Carroll, 1994). However, Carroll (1995) found similar frequencies of infection in Douglas-fir needles at a scale comparable to this study, but lower frequencies if the needles were cut into pieces measuring 400 × 500 μm.

The log of each species' frequency was plotted in the order of decreasing frequency using Whittaker plots (Krebs 1989, p. 345), and was found to fit a lognormal distribution using the method of Cohen (1959, 1961; Krebs 1989; p < 0.10). The species were then grouped by their frequencies according to Preston's octave scale and plotted (Preston, 1948, 1962; Krebs 1989; equivalent to log_{10} scale; Fig. 1). The octave with the greatest number of species (the mode) contained six species that were isolated 8–15 times. There were three octaves to the left of the mode, indicating that sampling was sufficient to use these data for estimating the number of rare species (2.62) that were not discovered to the left of the veil line, and the expected total number of species present (22 + 2.6 = 25; area beyond the veil line = 0.147; Krebs 1989).
The frequency of species falling into different frequency classes of Preston's octave scale (= log2 scale). Octaves one through eight have species with frequencies of 1, 2–3, 4–7, 8–15, 16–31, 32–63, 64–127, and 128–255, respectively. Rare species that were not found would be represented by the area in the extension of the normal curve to the left of the veil line (1).

The Jackknife method (Heltshe and Forester, 1983a, b; Krebs 1989) was also used to estimate the total number of species on the leaf blades. These estimates are calculated based on the number of samples (n), the number of species observed (s), and the number of species that were unique to a given sample (k) according to the formula:

$$\text{Total number of species} = s + \frac{n - 1}{n} (k)$$

There were three species unique to a given sample if leaf panels were used as the sample units, yielding an estimated total of 25 species (i.e., three species not found). However, six species were unique to a sample if leaves were used as the sample units, yielding an estimated total of 28 species (i.e., six species not found). The first Jackknife estimate matches the estimated total number of species obtained using Preston’s octave method for analyzing lognormal distributions, but the latter is a more conservative estimate because it takes into account the slightly greater diversity of endophytic fungi encountered between leaves than within leaves. Together, the Xylaria species occupied 73% to 74% of the leaf area in leaves 1 and 2, but only 21% of the area in leaf 3. These same three Xylaria species were also commonly isolated from leaves of three other M. bidentata trees located across the Quebrada Sonadora at El Verde during a preliminary study, suggesting that they are present in most trees.

In addition to the xylariaceous fungi, Colletotrichum crassipes (Speg.) Arx and Pestalotiopsis versicolor (Speg.) Steyaert were isolated at low frequencies from all three leaf blades (≤ 5%, except for C. crassipes at 17% in leaf 3), while Fusarium solani (Martius) Sacc., Glomerella cingulata (Stonem.) Spauld. & H. Schrenk, Phyllosticta sapotae Sacc. and sterile mycelium 'X' were isolated at frequencies between 5% and 15% from one of the three leaves. Other species that were rarely isolated, included Arthrinium sacchari M. B. Ellis, Chaetomium sphaerale Chilvers, Coniothyrium fuckelii Sacc., Fusarium avenaceum (Fr.) Sacc., F. decemcellulare Brick, Khuska oryzae H.J. Hudson [Nigrospora oryzae (Berk. & Br.) Petch], Penicillium graminum (Wehmer) Westl., Phomatospora sp., Phomopsis manilkae R. K. Rajak & A. A. K. Chatterjee, Trichoderma koningii Oudem., Zygosporium echinosporum Bunting & Mason, and sterile mycelia 2, and 4. These nonxylariaceous species are known from a wide host range, except perhaps for P. manilkae and P. sapotae.

Although only one of the fungi isolated was unique to the petioles (C. sphaerale), the fungal abundance patterns in petioles were very different from the leaf blades. The frequencies of endophytes from the petioles were therefore not combined with those from the leaf blades for estimating species richness. Samples from different habitats that are combined often deviate from a lognormal distribution, and are thus more difficult to analyze (Coddington, et al. 1991). The high frequency of species that were unique to a given petiole (six of the 12 spp.) suggests that this substratum was undersampled, and that further data analyses would therefore be inappropriate. Arthrinium sacchari and P. versicolor were especially prevalent while Xylaria species were rare or absent in petioles (Fig. 2). This may suggest that the xylariaceous fungi do not colonize the leaves of M. bidentata by growing from the twigs through the petioles. Similarly, the
FIG. 2. Maps of fungi cultured from leaf blade panels and petiole segments of *Manilkara bidentata* in Puerto Rico. Four panels, measuring $5 \times 20$ mm were cut from each leaf, surface sterilized, cut into 50 $1 \times 2$ mm pieces, and cultured on agar. Petioles were cut into 15 segments 1 mm long. Each leaf was from a different tree. The number of endophytic species isolated from each leaf blade were 12, 12, and 17 for leaves 1–3, respectively. One additional species was found in the petiole of leaf 1.
nonxylariaceous species also appear to have originated from airborne or water-borne propagules based on their scattered, isolated colonization patterns.

_Xylaria cf. multiplex_ and _X. arbuscula_ were previously reported as common endophytes of an Amazonian palm (Rodrigues, 1994; Rodrigues, et al. 1993), while a member of the _X. arbuscula_ complex was frequently isolated from green petioles of _Schefflera morototoni_ (Aubl.) McGuire at El Verde (Laessoe and Lodge, 1994). Furthermore, ascomata of _X. melissii_ and _X. multiplex_ are frequently encountered on branches and twigs of _M. bidentata_ at El Verde, and both species were also isolated from leaf litter of _M. bidentata_ at our site (Polishook, et al., in press) so their presence as leaf endophytes was not unexpected. Their biology is puzzling, however, because neither of these _Xylaria_ species fruits on leaves. The cultures of endophytes belonging to the _X. arbuscula_ complex isolated from _S. morototoni_ petioles (Laessoe and Lodge 1994), and epiphytic orchids (Bayman, et al., 1995) closely resemble those from _M. bidentata_ leaves, so they may belong to the same population. However, the cultural characteristics for endophytic isolates of both _Xylaria cf. multiplex_ and the _X. arbuscula_ complex differ slightly from those obtained from ascomata, particularly in the number of stromatal initials. Population level studies are needed to confirm these similarities and differences. The presence of _X. cf. adscendens_ was somewhat surprising because the ascomata of this species are uncommon at El Verde, but Rodrigues, et al. (1993) had similarly found it as an endophyte of an Amazonian palm. Perhaps another endophytic species produces cultures resembling those of _X. adscendens_. The constancy with which _Xylaria_ species were isolated, and their high frequency relative to other fungi in the leaf blades, is consistent with previous studies of leaf endophytes in the tropics (Rodrigues and Samuels, 1990, Rodrigues, 1994). The incidence of two fungi emerging from the same leaf fragment was low (1%-10%). This could have resulted from fast growing fungi (e.g., _Pestalotiopsis_, and _Xylaria_ spp.) outgrowing slower growing coelomycetes and hyphomycetes.

Preston's octave method for analyzing lognormal distributions and the Jackknife both estimate yielded similar estimates of total species richness for leaf blade endophytes. These estimates indicate that we found most of the leaf blade endophytes that were present in the sampled stand of _M. bidentata_ by isolating from 200 pieces of leaf measuring 1 × 2 mm from each of three trees. Future sampling could be made more efficient, however, by reducing the number of isolations per leaf and increasing the number of trees that are sampled. Data from the petioles suggest that many more than three petioles need to be sampled to characterize their endophytic fungal community.

**ACKNOWLEDGMENTS**

We thank J. Martin for assistance in making leaf maps and photographing cultures, G. Bills, T. Laessoe, G. Samuels and G. Carroll for reviewing the manuscript, and M.R. Willig for reviewing the statistical analyses.

**LITERATURE CITED**


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