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Measuring diversity of endophytic fungi in leaf fragments: Does size matter?

Miguel A. Gamboa[1,3*], Sugei Laureano[1] & Paul Bayman[1,2]
[1]*Departamento de Biología, Universidad de Puerto Rico - Río Piedras, PO Box 23360, San Juan PR 00931, USA;* [2]*USDA ARS Western Regional Research Laboratory, 800 Buchanan St., Albany, CA 94710, USA;* [3]*Current Address: Departamento de Biología, Universidad Javeriana, Cra. 7 # 40-62, Santafé de Bogotá, Colombia*

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Abstract

Endophytic fungi inhabit living plant tissues without causing disease symptoms. Although abundant, the extent of their contribution to fungal biodiversity remains unclear. Since endophytic fungi are poorly known, especially in the tropics, current estimates of fungal species are probably conservative. Here we tested strategies for sampling endophytic fungi in tropical plants. We compared the number of fungi isolated from 400 mm^2 leaf pieces that were divided into increasingly small fragments. Leaf pieces were surface-sterilized, cut into fragments and plated on culture media. For a given area, cutting leaf pieces into smaller fragments significantly increased the number of fungal morphospecies recovered. There was a strong linear relationship between size of fragments and number of fungi isolated. By extrapolation, an estimated 16 ± 3 fungi could be recovered from a 2 × 2 cm leaf piece, using infinitely small fragments. This represents a large part of the fungal diversity estimated to exist in leaf endophytes in a population. We conclude that reducing the size and increasing the number of leaf fragments will increase the number of fungal species isolated. This strategy will help to estimate real values of endophytic fungal diversity.

Keywords: biodiversity, endophyte, fungal diversity, phyllosphere

Introduction

Measurement of microbial diversity is one of the greatest challenges in modern microbiology, given the astonishing number of microbial species believed to exist. In fungi 1,500,000 species are estimated to exist, based on a 6 : 1 ratio of fungal: plant species [13].

Estimates of the number of fungal species are mainly based on temperate floras and described species. Described fungi are generally the conspicuous ones, i.e., species producing large fruiting bodies and those pathogenic to animals and plants. Two important gaps in this sample are the tropics and the nonpathogenic fungi, of which endophytes are an important group.

Endophytes are microorganisms that live asymptomatically within plant tissues [17, 22]. Every plant studied so far has an array of endophytes within it, and a fine-scale mosaic of fungal individuals and species has been observed in tropical plants [4, 10, 18]. Additionally, the 6 : 1 ratio is probably inaccurate for the tropics. A ratio of 33 : 1 was proposed recently; there are arguments for both higher and lower estimates [2, 8, 13, 14].

No tropical plant or fungal flora is entirely known, and since tropical fungi are so diverse and resources to explore them are limited, we need more efficient methods to sample them. Techniques used for measuring microbial diversity are subject to practical constraints [9]. Current isolation and culturing protocols are empirical and probably far from optimal [6].

In a previous study on endophytes in leaves of the tropical timber tree *Guarea guidonia* (Meliaceae) the number of fungal species isolated was higher when leaf fragments comprising a constant area were subdivided [10, 11]. In that study, 20 mm^2 leaf fragments were placed on culture media and an average of 1.4

* Author for correspondence.

endophytic species per fragment were obtained (N = 20). When 20 mm^2 fragments were subdivided into five 4 mm^2 pieces, an average of 2.9 species were isolated (N = 12). According to these data, the former methodology underestimated by 50% the endophytic species in a given leaf tissue area.

The question we address here is: what is the optimal size of sampled plant fragments for approaching real diversity values of endophytic fungi?

Materials and methods

Endophytic fungi were isolated from five tropical plant species representing five families and four superorders (Table 1) [16]. Leaves were collected at the Río Piedras campus, Botanical Garden, and El Verde Field Station of the University of Puerto Rico. Healthy leaves less than one year old were collected from two individuals of each species. *Guarea guidonia*, *Tabebuia rosea* and *Manilkara bidentata* are perennial trees; individuals sampled were >10 years old. *Coffea arabica* is a perennial shrub, and individuals sampled were escaped plants that grew within a protected forest reserve. *Renealmia jamaicensis* var. *puberula* is a exotic, perennial herb found in shady, wet sites in forests.

Leaves were washed in 0.01% Tween20 and surface-sterilized in ethanol 75% (1 min), commercial bleach 10% (3 min; 0.5% sodium hypoclorite), and ethanol 75% (30 s) [11]. Four 2 × 2 cm leaf pieces were cut and plated on potato dextrose agar (PDA; 12 g Difco potato dextrose broth, 20 g agar/L, with 50 ppm each penicillin, streptomycin and tetracycline added after autoclaving). Treatments were: (1) one piece was plated whole, (2) another was divided in four fragments, (3) another was divided in 16 fragments, (4) another was divided in 64 fragments (Figure 1). Each fragment was individually plated (85 plates per leaf).

Endophytic fungal morphospecies were recorded after 15 days' growth. For identification, fungi were transferred to PDA, malt extract agar (Difco) and V8 agar (5% V8 vegetable juice, 2% agar, pH = 5.2). Identification was based on colony morphology, hyphal morphology, and reproductive structures when present. Many of the fungi did not produce reproductive structures or distinctive features and could not be identified, but were still grouped into morphospecies based on hyphal and colony characteristics on the three media. This is a common problem when dealing with tropical endophytic fungi [2, 11]; a recent study showed a strong relationship between number of morphospecies (as determined by colony morphology) and probable number of species (as estimated by comparison of DNA sequences) [2].

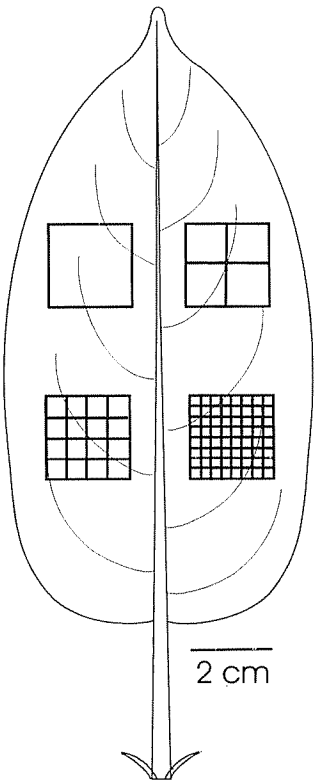

Figure 1. Schematic representation of leaf fragment sampling strategy. The total leaf area samples was the same in all treatments.

Differences among treatments in number of fungal morphospecies isolated were tested for significance by ANOVA. Because of unequal variance among fragment sizes, data were natural log-transformed prior to analysis. A linear regression was used to test the relationship between fragment size and number of fungal morphospecies, and to extrapolate numbers of yielded by even smaller fragments.

Results and discussion

The predominant endophyte genera found in all plants were *Xylaria*, *Colletotrichum*, and *Phomopsis* (Table 1). These genera include common endophytes of tropical plants [4, 11, 18, 20]. Some *Xylaria*, *Colletotrichum* and *Phomopsis* endophytes comprise morphospecies that are morphologically distinct but

Table 1. Number of morphospecies of endophytic fungi isolated from leaf fragments.

Plant species (family)	Mean No. of morphospecies isolated per treatment				Dominant genera of endophytic fungi
	1	4	16	64	
	Leaf fragments				
Coffea arabica (Rubiaceae)	2	4	4.5	13	*Xylaria, Colletotrichum*
Guarea guidonia (Meliaceae)	2.5	2.5	4.5	12	*Phomopsis, Colletotrichum*
Manilkara bidentata (Sapotaceae)	2	4.5	6	12.5	*Xylaria, Colletotrichum, Glomerella*
Renealmia jamaicensis var. *puberula* (Zingiberaceae)	1.5	3.5	7	10.5	*Xylaria*
Tabebuia rosea (Bignoniaceae)	2	3	6	10.5	*Xylaria, Phomopsis*
Mean	2 ± 0.7	3.5 ± 1.0	5.6 ± 1.4	11.7 ± 3.4	

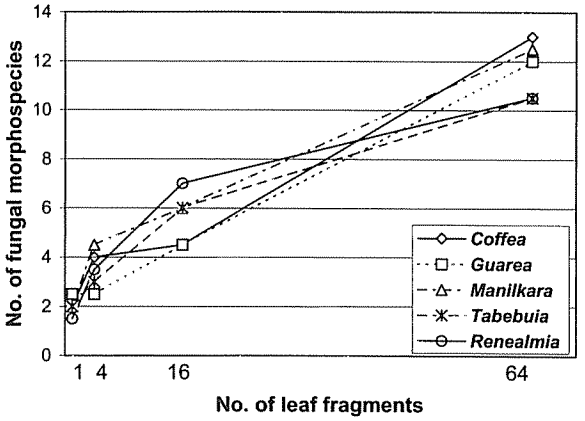

Figure 2. Species abundance curves showing number of fungal morphospecies isolated from each plant species.

are difficult to assign to known species [2, 4, 10, 18, 19, 20]. *Glomerella* was common in *Manilkara bidentata*.

In each plant sampled, reducing the fragment size increased the total number of fungal morphospecies isolated (Table 1, Figure 2). Species saturation curves showed a linear increase of fungal morphospecies as fragment size decreased (Figure 2). The similarity of the curves for each species suggests that the effect of decreasing leaf fragment size is general, and not restricted to certain host plants or endophytes.

When data from all species were pooled, a ln-ln plot of number of endophyte morphospecies vs. leaf fragment area was linear ($r^2 = 0.80$; Figure 3). A regression of number of morphospecies recovered onto fragment size was statistically significant (ANOVA, $F = 156.8$, d.f.=1,38, $P < 0.0001$). The 95% confidence intervals shown in Figure 3 suggest cutting a 2×2 cm leaf piece into infinitely small pieces would yield 16 ± 3 morphospecies.

This estimate can be compared to the number of fungal endophytes proposed for tropical tree leaves using jackknife analyses: 25–28 for *Manilkara bidentata* [18] and 26–33 for *Guarea guidonia* [11]. Comparing these estimates with the results of the present study suggests that about half the leaf endophyte diversity in a population may be present in a 2×2 cm piece of a single leaf. Similar observations have been made on other fungi in other niches and substrates: fungal populations may be highly variable on a very limited spatial scale [3, 4].

Figure 3 implies that further subdivision of the fragments of our treatment 4 (2.5×2.5 mm) would permit recovery of even more species. Given that a single hypha or spore may serve as inoculum, the optimal fragment size for sampling endophytic fungi may be very small indeed. Vertical sectioning of leaves may also increase the number of species covered, since different tissues might contain different endophytes [5, 7, 21]. However, if the optimal size of the plant fragment is much smaller than 2.5×2.5 mm, manual cutting of fragments will be difficult without destroying the leaf tissues.

There are two likely mechanisms by which decreasing fragment size yielded more endophytic

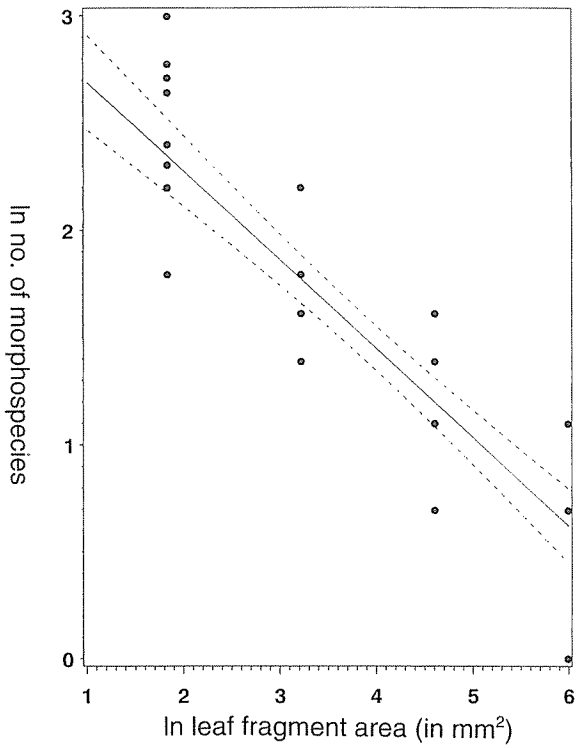

Figure 3. ln-ln plot of linear regression of number of morphospecies isolated on leaf fragment area. Data from all five species are combined. Dotted lines show 94% confidence intervals.

morphospecies. First, reducing fragment size may reduce competition among fungi. The first fungus to grow out of a fragment may inhibit growth of other fungi [6]. Since growth rates *in vitro* do not necessarily reflect growth rates *in planta*, the most important endophytes may be overlooked if they are repressed by more allocthanous neighbors. This mechanism should apply to epiphyllous fungi as well.

Second, smaller pieces have higher edge : area ratios and thus more "escape" routes for the fungi within to reach the medium. The total perimeter of the first treatment was 8 cm, as opposed to 16, 32, and 64 cm for the second, third and fourth treatments. Comparing treatments one and four, the average number of endophytes increased 6.36 times, which is a 1 : 1.25 correspondence with the increase in perimeter.

In a study of endophytes in Douglas fir, Carroll cut individual needles into 200 or more fragments [6]. Twenty-eight percent of the fragments yielded fungi, and a total of five species were isolated from four needles. Carroll extrapolated that if the needles had been cut into only four fragments, infection frequency would have been 100% rather than 28%, and three species would have been found instead of five. Carroll concluded that when the larger fragments were used, some fragments contained multiple infections in which some fungi outcompeted others. He suggested that fragment size should be reduced to minimize the chances of multiple infections, a point that should be reached when 10–20% of fragments yield endophytes.

Our results support Carroll's conclusion: subdividing leaf fragments increases the number of endophyte species found. However, tropical plants and temperate plants differ in two important respects. First, the number of endophytic fungal species in a single leaf tends to be higher: estimates include 25–28 species/leaf for *Manilkara bidentata* [18]. Second, distribution patterns of fungi appears to be different: in temperate plants, some endophyte infections are restricted to single cells [7, 21], and only 28% of the small leaf fragments examined by Carroll yielded fungi [6]. In tropical plants, the size of individual endophyte colonies has never been determined. However, 90–99% of leaf fragments yielded endophytes in *M. bidentata* and *G. guidonia* [11, 18]. This suggests that individual colonies may occupy larger areas of leaf tissue than the conifer endophytes studied by Carroll [6] and Stone [21].

How can we determine the total number of species of endophytic fungi in a leaf? The answer probably lies in a combined use of molecular and culture-based methods, using a proper sample size. Since many microorganisms do not growth in artificial culture media [9], and are difficult to identify in culture [2], DNA- and RNA-based methods may detect some organisms overlooked by culturing [1, 10].

However, new organisms would be revealed if methods based on culturing were optimized. From our data it is clear that plant fragment size has an important effect on number of species isolated. Most studies of endophytic fungi have used larger tissue pieces for isolations. If some of these studies were repeated using a much smaller fragment size, many new species might be revealed. In a study of endophytes of *Manilkara bidentata* in Puerto Rico, Lodge et al. [18] concluded: "Future sampling could be made more efficient ... by reducing the number of isolations per leaf and increasing the number of trees that are sampled". We recommend additionally that leaf fragments should be as small as possible. For efficiency, several fragments can be sown on a single petri plate.

Endophytic fungi have potential as a model system in studies of biodiversity [1, 10, 11]. Although cultural techniques will not reveal all fungi, it is clear that their

potential has not been fully realized. By optimizing such methods we can significantly improve our current understanding of microbial diversity.

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Address for correspondence: Miguel A. Gamboa, Departamento de Biología, Universidad de Puerto Rico - Río Piedras, PO Box 23360, San Juan PR 00931-3360, USA
Phone: 787.764.0000 x4880; Fax: 787.764.3875
E-mail: mgamboa@upracd.upr.clu.edu