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Variation in endophytic fungi from roots and leaves of *Lepanthes* (Orchidaceae)

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SUMMARY

Little is known about non-mycorrhizal endophytic fungi in tropical orchids; still less is known about how endophytes vary within and between individual orchid plants. Fungal endophytes were isolated from roots and leaves of epiphytic and lithophytic orchids in the genus *Lepanthes*; seven species, from rainforests in Puerto Rico, were sampled. The endophytes observed most frequently were *Xylaria* species and *Rhizoctonia*-like fungi, found in 29% of roots and 19% of leaves, and 45% of roots and 31% of leaves, respectively. Five deuteromycete genera were also isolated, occurring in 19% of roots and 43% of leaves (combined). At least nine species of *Xylaria* were found, with several species sometimes occurring in a single plant. Differences between roots and leaves in frequency of *Xylaria* and *Rhizoctonia* isolates were not significant, although differences among orchid species in number and types of endophytes were. Heterogeneity of endophytes in single plants and plant organs was greater than differences between species. Many *Lepanthes* species are very restricted in distribution, and knowledge of their interactions with endophytes might be useful in species management.

Key words: Endophyte, mycorrhiza, orchid, *Rhizoctonia*, *Xylaria*.

INTRODUCTION

Most work on orchid–fungus relationships has dealt with mycorrhizas of terrestrial, temperate orchids. However, most orchids are epiphytic and tropical or subtropical, and much less is known about their associations with fungi (Richardson, Currah & Hambleton, 1993). Although mycorrhizas are found less consistently in epiphytic orchids than in terrestrial, temperate orchids (Hadley & Williamson, 1972), they appear to be an important component of the epiphytic habit (Benzing & Friedman, 1981).

Furthermore, many of the fungi that grow in plants are not mycorrhizal. The term 'endophyte' includes all organisms that grow inside plant tissues without causing disease symptoms (Petrini, 1991; Chanway, 1996). Endophytic fungi can be latent pathogens, mutualists, and/or saprobes (Carroll, 1991; Fisher & Petrini, 1992). An example of latent pathogenicity is *Fusarium moniliforme*, which causes serious diseases of maize but can also be isolated from most healthy maize plants (Leslie *et al.*, 1990). Examples of mutualism include endophytes of grasses that make secondary metabolites which are unpleasant or toxic to herbivores, causing enormous losses of livestock (Clay, 1988).

Apart from mycorrhizas, little is known about endophytic fungi in orchids. Published studies are scarce, although the Orchidaceae is large (Petrini & Dreyfuss, 1981; Dreyfuss & Petrini, 1984; Richardson *et al.*, 1993; Richardson & Currah, 1995). Based on knowlege of endophytes in other plants, it is likely that all orchids contain fungal endophytes, and that these fungi are a largely overlooked component of fungal biodiversity. It is unclear what adaptive significance, if any, endophytic fungi have for the orchid host. The main purpose of this study was to compare diversity of fungal endophytes in different species of *Lepanthes* orchids in Puerto Rico. Additional goals were to compare endophyte flora from roots and leaves of the same plants, and to look at consistency of occurrence

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Table 1. *Numbers of isolates of* Xylaria *species made from roots and leaves of* Lepanthes *spp.**

	Host species											
	L. woodburyana		*L. rupestris*		*L. dodiana*		*L. selenitepala*		*L. caritensis*		*L. sanguinea*		Total	
Xylaria species	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf
X. cf. *arbuscula*	10	—	3	—	2	—	1	—	4	4	—	—	20	4
X. cf. *hypoxylon*	1	4	4	4	—	—	4	—	—	—	—	—	9	8
X. ovobata	3	—	3	1	—	—	2	1	—	—	—	—	8	2
X. corniformis	—	—	—	—	—	—	—	—	7	—	—	—	7	0
X. aff. *cubensis*	1	—	—	—	—	—	—	—	—	1	—	—	1	1
X. multiplex	—	1	—	1	—	—	—	—	1	1	—	—	1	3
X. cf. *curta*	—	—	—	—	—	—	—	—	—	1	—	—	0	1
X. polymorpha complex	1	—	—	—	—	—	—	—	—	—	—	—	1	0
X. enteroleuca ?	1	—	—	—	—	—	—	—	—	—	—	—	1	0
X. unknown	7	3	1	—	5	2	6	2	1		3	1	23	8
Total	24	8	11	6	7	2	13	3	13	7	13	7	71	27

* This table includes isolations from additional plants not shown in Table 2. Number of plants and organs sampled varied among *Lepanthes* species. *L. veleziana* is not included because no *Xylaria* strains were isolated.

of endophytes within different organs of a single host. This study is a preliminary step towards determining functional relationships between orchids and their endophytes, and towards a more comprehensive picture of what orchid endophytes do in nature.

Lepanthes Swartz is large genus of pleurothallid orchids (Luer, 1986). Puerto Rico has nine species, of which eight are endemic and represent half its endemic orchid species (Ackerman, 1995). Most species are restricted to small areas (Tremblay & Ackerman, 1993). *Lepanthes* is an interesting group for endophyte studies for a number of reasons. Ranges of many species overlap, allowing testing for host specificity in endophytes of related species which live in the same environment. Limited ranges and small population sizes put some of these species at risk of extinction. Relationships with mycorrhizal or endophytic fungi could be factors that affect their distribution and survival.

MATERIALS AND METHODS

Plants were collected in the Caribbean National Forest and Carite State Forest, Puerto Rico. All plants were epiphytic or lithophytic and appeared healthy. Species studied were *Lepanthes rupestris* Stimson, *L. woodburyana* Stimson, *L. sanguinea* Hooker, *L. dodiana* Stimson, *L. veleziana* Stimson, *L. selenitepala* Rchb.f., and *L. caritensis* Tremblay & Ackerman. Plants were identified according to Tremblay & Ackerman (1993) and Ackerman (1995).

Roots and leaves were washed under running water to remove dirt, surface-sterilized in a sequence of 75 % ethanol for 1 min, 65 % Clorox® (≡ 3·4 % NaOCl) for 10 min and 75 % ethanol for 30 s, and rinsed in sterile distilled water (Rodrigues, 1994). Six 2–5 mm pieces were cut from each root; the rest of each root was saved for microscopic examination. Three 4-mm diameter discs were cut from each leaf, from distal, central, and proximal parts of the blade, and cut in half. Three pieces of each root and half of each leaf disc were plated on each isolation medium. Media used were half-strength malt extract agar (MEA, Difco Inc.) with 35 μg ml^{-1} rose bengal added after autoclaving, and a *Rhizoctonia* isolation medium (CM1, Andersen, 1990) with 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} chloramphenicol added after autoclaving. The antibiotics were used to limit bacterial growth. Cultures were incubated at 22 °C with 12 h light/day. Fungal colonies were transferred to MEA or V8 juice® agar.

For identification, putative *Xylaria* colonies were also transferred to oatmeal agar (60 g ground oatmeal, 15 g agar l^{-1}) and incubated for 2 months to allow stromatal initials to develop. *Xylaria* cultures were identified to species level by colony colour (both front and reverse), location and shape of stromatal initials, and conidial morphology. Identi-

fications were based on published descriptions (Callan & Rogers, 1990, 1993) and photographs of cultures obtained from teleomorphs in the CFMR collection. *Rhizoctonia*-like isolates were identified by hyphal morphology, colour, and number of nuclei per cell (Sneh, Burpee & Ogoshi, 1991).

For microscopy, pieces of orchid roots were stained by immersion in 50 % ethanol for 5 min, 50 % toluidine blue for 5 min, and 50 % ethanol for 5 min. Pieces were then teased apart and mounted on slides in 50 % glycerol for observation. To count nuclei in *Rhizoctonia* cultures, mycelia were stained with the fluorochrome mithramycin (Bayman & Collins, 1990).

Statistical analyses were performed using a Monte Carlo simulation for categorical data with programs Fisher 6 and Monte Carlo (Engels, 1988). These tests were preferred because expected values of some cells were below five, causing a high probability of Type 1 error (Sokal & Rohlf, 1981). All simulations used at least 10000 trials; we present the probability of rejecting the null hypothesis and the standard error on the estimated probability.

Some data on *L. caritensis* came from a separate study on causes of rarity in orchids (Tremblay *et al.*, unpublished) and are included here for comparison. Sampling of *L. caritensis* was not replicated in the same manner as for the other species. Thus data on *L. caritensis* are not included in statistical tests.

RESULTS

Diversity of endophytic fungi

Of 98 *Xylaria* isolates, 68 % could be classified in nine species or species complexes on the basis of culture morphology (Table 1); 32 % could not be placed. Since each organ was sampled three times and several *Xylaria* colonies were isolated from some sections, Table 1 probably includes multiple isolations of the same individual colony.

Mixed infections of individual plants were common; as many as four species of *Xylaria* were found in a single plant. The *X. arbuscula* Sacc./*X. mellisii* Berk. complex and *X. hypoxylon* (L.:Fr.) Grev. were the most common species, with 24 and 17 isolates, respectively. All species represented by more than one isolate were found in more than one species of orchid, except for *X. corniformis* (Fr.) Fr. which was found only in *L. caritensis*.

Rhizoctonia-like isolates were similar to, but distinct from, R. *solani* Kuhn, based on morphology in culture (Sneh *et al.*, 1991). All cultures examined were multinucleate. All produced constricted, right-angled branches typical of *Rhizoctonia*, but the branches were usually not delimited by septa at the branch point. Colonies had slower growth rates on PDA and narrower runner hyphae than expected for R. *solani*. Dark, irregularly shaped sclerotia were formed in some older cultures.

Six other genera of fungi were found in 19 % of roots and 43 % of leaves (combined). In roots, they were (in order of decreasing frequency): *Colletotrichum*, *Aspergillus*, *Penicillium* and *Pestalotia*. In leaves they were (in decreasing frequency): *Colletotrichum*, *Penicillium*, *Trichoderma*, *Pestalotia* and *Phoma*.

Interactions between fungi

Xylaria and *Rhizoctonia*-like fungi were frequently isolated together from the same piece of root or leaf. However, there was no evidence of significant association between them, either positive or negative (Fisher 6 program, Engels, 1988), ($P > 0{\cdot}19$, df = 1). Similarly, presence of *Xylaria* or *Rhizoctonia* was not correlated with the presence of 'other fungi' ($P > 0{\cdot}14$ and $P > 0{\cdot}50$, respectively; df = 1).

Differences among Lepanthes *species*

Among *Lepanthes* species, there were significant differences in the frequency of isolation of *Xylaria* ($P < 0{\cdot}03$, df = 5). Similarly, differences among orchid species in the presence of *Rhizoctonia*-like fungi were significant ($P < 0{\cdot}001$, df = 5). *Lepanthes woodburyana* had the highest incidence of both *Xylaria* and *Rhizoctonia*. When data for the class 'other fungi' were combined, they also differed significantly between *Lepanthes* species ($P < 0{\cdot}001$, df = 5).

Differences between roots and leaves, and within-plant variation

In most species of *Lepanthes*, *Xylaria* and *Rhizoctonia* were found in both roots and leaves (Table 2). When data from all *Lepanthes* species were pooled, frequency of *Xylaria* did not differ significantly between roots and leaves (Fisher 6; $P > 0{\cdot}22$, df = 1). All *Xylaria* species represented by more than one isolate were found in both leaves and roots, except *X. corniformis*. However, *X.* cf. *arbuscula/mellisii*, the most common group, was found in roots 20 out of 24 times. Frequency of *Rhizoctonia*-like fungi did not differ significantly between roots and leaves (Fisher 6; $P > 0{\cdot}26$, df = 1). The other fungi (combined) were significantly more common in leaves than in roots (Fisher 6; $P = 0{\cdot}004$, df = 1).

There was heterogeneity in endophytes among replicate pieces from a single plant and even a single organ. Of the roots and leaves from which *Xylaria* or *Rhizoctonia*-like fungi were isolated, less than 12 % had the endophyte in all three sections, whereas in

Table 2. *Number of* Xylaria *and* Rhizoctonia*-like fungi isolated from roots and leaves of* Lepanthes

	Xylaria	*Rhizoctonia*	Both	Other fungi
L. rupestris (3)*				
Roots infected (9)	1	4	0	3
Leaves infected (9)	4	5	3	3
L. woodburyana (3)				
Roots infected (9)	6	9	6	2
Leaves infected (9)	2	4	1	8
L. sanguinea (3)				
Roots infected (9)	3	2	1	0
Leaves infected (9)	1	1	0	1
L. dodiana (3)				
Roots infected (9)	1	1	0	2
Leaves infected (9)	0	1	0	4
L. selenitepetala (1)				
Roots infected (3)	1	0	0	0
Leaves infected (3)	1	0	0	1
L. veleziana (1)				
Roots infected (3)	0	3	0	1
Leaves infected (3)	0	2	0	1

* Number of plants or organs samples are given in parentheses.

Table 3. *Consistency of isolation of endophytes from replicated pieces of a single* Lepanthes *root or leaf*

		No. of replicate pieces of each organ that contain fungus				
Fungus	Organ	0	1	2	3	Total
Xylaria	Root	30	9	1	2	42
Xylaria	Leaf	34	6	2	0	42
Rhizoctonia	Root	24	10	6	2	42
Rhizoctonia	Leaf	28	10	2	2	42

67% the endophyte was only isolated from one section (Table 3).

Microscopic observations of roots

Stained root pieces contained several distinct types of hyphae. Many sections showed proliferations of hyphae inside single cells. These hyphae were septate and had constricted, right-angled branches characteristic of *Rhizoctonia* (Sneh *et al*., 1991). However, proliferations of hyphae were less organized than the peletons reported for *Rhizoctonia* in orchid mycorrhizas (Alconero, 1969; Richardson *et al*., 1993), and these proliferations were seen in roots from which neither *Xylaria* nor *Rhizoctonia*-like fungi were isolated. Thus it was difficult to assign these hyphae to taxa.

DISCUSSION

The similarity of root and leaf endophytes was surprising. Endophytic fungi (including *Xylaria*) often differ in frequency among organs or even parts of organs (Fisher & Petrini, 1992; Rodrigues, 1994; Carroll, 1995). Orchid mycorrhizal fungi such as *Rhizoctonia* are generally believed to be limited to roots only; orchid shoots are thought to contain defensive compounds that exclude mycorrhizal fungi (Hadley, 1982). Similarly, most studies that use the term 'endophyte' sample shoots but not roots; in some cases, the term endophyte is restricted to fungi in aerial parts of the plant (Carroll, 1986; Chanway, 1996). Yet there was no significant difference between roots and leaves of *Lepanthes* in frequency of *Rhizoctonia*-like fungi, considered mycorrhizal symbionts and root pathogens of orchids (Alconero, 1969; Hadley, 1982), or of *Xylaria*, usually regarded as an endophyte (Table 2).

Similarity of root and leaf endophytes might reflect the epiphytic and epilithic habit of the orchids, in which roots and leaves are exposed to similar environments. In terrestrial plants, environmental differences might contribute to differences between organs in endophyte flora (Fisher, Petrini & Petrini, 1991). For example, *Alnus* roots in soil were shown to have different endophytes from those in water (Fisher, Petrini & Webster, 1991).

Given the limited amount of published data on endophytes of tropical plants, it is difficult to say which patterns of distribution are normal and which are peculiar to the material sampled. Expectations about where different endophytic fungi will be found in the plant might influence sampling protocols, which in turn produce results that reinforce these expectations.

A single *Lepanthes* plant, and even a single root or

leaf, has a very diverse endophytic flora; a plant only 5 cm in size contained up to four separate *Xylaria* species, in addition to *Rhizoctonia*-like fungi and other genera.

Furthermore, less than 12% of roots and leaves from which *Xylaria* or *Rhizoctonia* was isolated contained the fungi in all three sections examined (Table 3). This suggests a fine-scale spatial heterogeneity in endophyte populations. Other studies have shown that the diversity of endophytic fungi increases with the number of samples and decreases with the size of samples (Carroll, 1995; Lodge, Fisher & Sutton, 1996). Studies of orchid mycorrhizas and endophytes have rarely reported sampling more than a single root per plant. These data suggest that this underestimates fungal diversity.

Two isolation media were used here: half-strength malt extract agar with rose bengal, and a *Rhizoctonia* isolation medium (Andersen, 1990). *Xylaria* grew only on the first medium, and *Rhizoctonia* only on the second. The use of either medium alone would thus have underestimated the diversity present. Also, there might have been endophytes present that did not grow on either medium.

Endophyte diversity within a plant or leaf also exists at the intraspecific level, a level of resolution not dealt with here. Mixed populations of genetically distinct individuals of *Xylaria* have been found in single leaves of the palm *Euterpe oleracea* (Rodrigues, Leuchtmann & Petrini, 1993). Genetic variation among individuals in a single plant or leaf has also shown for other endophytes (Clay, 1991; McCutcheon, Carroll & Schwab, 1993; Carroll, 1995).

Xylaria species are common endophytes, especially in the tropics, and have been previously reported from orchid roots and leaves (Dreyfuss & Petrini, 1984; Petrini & Petrini, 1985; Richardson & Currah, 1995). *Xylaria* is also a common saprotroph, and some of the same species isolated from *Lepanthes* occur as saprotrophs in Puerto Rico (Laessoe & Lodge, 1994; Lodge & Cantrell, 1995; Lodge *et al*., 1996). It is not clear whether the same strains are both endophytes and saprotrophs, or whether they are specialized for one or the other lifestyle. More detailed knowledge of how and where the endophytic strains live would be useful for understanding how they are dispersed from plant to plant (Lodge & Cantrell, 1995).

The endophytic existence of *Xylaria* is sometimes viewed as opportunistic, i.e. presence in the living plant gives the fungus a head-start over other saprotrophs after the plant dies (Fisher & Petrini, 1992; Whalley, 1993). However, the *Xylaria* endophytes in *Lepanthes* fit at least three of Carroll's (1988) five criteria for assuming that an endophyte is a mutualist: they are widespread and cause no apparent damages, they grow throughout host tissues, and based on reports of other *Xylaria* species (Whalley & Edwards, 1987), they might produce toxic secondary metabolites. More information is needed to determine whether xylariaceous endophytes are mutualists, and whether they produce significant quantities of secondary compounds *in planta* (Clay, 1988; Carroll, 1995; Rodrigues, 1996).

Although *R. solani* is a serious plant pathogen, it can be both mycorrhizal and pathogenic on *Vanilla* orchids in Puerto Rico (Alconero, 1969). It has been reported as a mycorrhizal associate of other orchids (Sneh *et al*., 1991). Knowledge of host specificity of *Rhizoctonia* and *Rhizoctonia*-like associates of orchids, and of other roles these fungi might play in nature, has been impeded by difficulty in identification (Andersen, 1990). The *Rhizoctonia* group of fungi is notoriously difficult to identify and rarely produces sexual stages in culture (Andersen, 1990; Sneh *et al*., 1991); we are attempting to identify them from restriction patterns of ribosomal DNA (Vilgalys & Cubeta, 1994). Details of this work will be published separately.

There was no apparent antagonism between *Xylaria* and *Rhizoctonia* in orchid roots and leaves (Table 2). On eight occasions both fungi were isolated from the same small piece of root or leaf. In other plants, however, presence of one endophyte has been shown to reduce plant infection by other fungi. In two grass species, plants with endophytes had lower levels of leaf diseases than endophyte-free plants (Clay, 1991). Leaves of the weed *Cyperus rotundus* contain either *Rhizoctonia solani* or the epiphyte *Balansia cyperi*, but not both (Stovall & Clay, 1991). Extracts of *Balansia*-infected leaves and extracts of pure cultures of *B. cyperi* inhibited growth of *Rhizoctonia*.

Many aspects of the behaviour of a fungus–for example, secondary metabolite production–might be substantially modified by encounters with other fungi (Rayner, 1991). The complexity and variablity of the endophyte flora of each leaf and root imply equally complex and variable interactions among the endophytes (Carroll, 1995; Lodge *et al*., 1996). These interactions are likely to affect not only the host plant but also the plant's interactions with herbivores and other organisms.

Species of *Lepanthes* differed significantly in the frequency of *Xylaria* and *Rhizoctonia* endophytes, and in the combined frequency of the other fungi isolated. Habitat and microclimate did not appear to be the cause of these differences, since several of the *Lepanthes* species are sympatric. For example, populations of *L. woodburyana* were mixed with populations of *L. veleziana*, in which fewer endophytes were found.

However, differences between host species in frequency of endophytes might not be biologically significant, despite being statistically significant. Given the small number of plants we sampled (one to three per species) and the high variability within

individual plants, it is hard to estimate the total endophytic diversity of each individual and each species, let alone make comparisons between species. This problem, and host specificity of endophytic fungi in nature, have been reviewed recently (Carroll, 1995). There is no published record of intensive sampling of fungi from a single species of any epiphytic orchid.

One orchid species we studied, *L. caritensis*, is known to be highly host-specific: it occurs only in one forest and only on trunks of *Micropholis guyanensis* A. DC. (Sapotaceae) (Tremblay *et al*., unpublished). This species had a *Xylaria* in its roots that was not found in other *Lepanthes* (Table 1) (leaves of *L. caritensis* were not sampled). In this case, it is not clear if the apparent host specificity of the fungus is related to the biology of the host orchid, or to environmental factors determined by the orchid's specialized habitat.

What are the implications of this study for orchid biology? Most of the *Lepanthes* species we studied have small population sizes and very limited distributions; several are at risk of extinction. It is possible that availability of endophytic fungi is one of the limiting factors for establishment of new plants and populations (Tremblay *et al*., unpublished). Life-history studies of *Lepanthes* species might reveal whether or not parameters such as survivorship and reproduction correlate with endophyte frequency.

Our results suggest that the endophyte flora of *Lepanthes* roots and leaves is heterogeneous on several levels: within single organs, among organs of a single plant, and possibly among host species. If the endophytes affect plant health or survival, these effects might differ greatly within and among plants.

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