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MYCORRHIZAL FUNGI OF *VANILLA*: ROOT COLONIZATION PATTERNS AND FUNGAL IDENTIFICATION

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Orchid mycorrhizal fungi are characterized by the presence of complex, globose masses of hyphe, called pelotons, in the cortex of the root (Hadley *et al.* 1989, Esnault *et al.* 1994). Extent and patterns of infections are very variable, and this variation is observed in many epiphytic orchids and some terrestrial tropical orchids (Hadley *et al.* 1989, Allen 1991).

Symbiotic germination of many orchid seeds with mycorrhizal fungi has been described (Hadley *et al.* 1989, Rasmussen *et al.* 1993, Johnson *et al.* 1994, Van Der Kinderen 1995, Zelmer *et al.* 1997, Tan *et al.* 1998, Bruns *et al.* 2000, Zettler *et al.* 2001, Kristiansen *et al.* 2001, McKendrick *et al.* 2002) but much less is known about the distribution, role and specificity of mycorrhizal fungi in tropical adult orchids.

Vanilla is an orchid known for its economic value. About 50 species have been described but only 3 are important commercially; *V. planifolia* G. Jackson is one of them (Childers *et al.* 1959). However, there are very few reports of mycorrhizal fungi in *Vanilla*. Mycorrhizal fungi in *Vanilla* were described by Decordenoy (1904) and Tonnier (1954), and in Puerto Rico by Alconero (1968 and references therein).

Here we present data on root mycorrhizal infection in *Vanilla*. The hypotheses were: 1) The level and patterns of mycorrhizal infection will differ between *V. planifolia* and *V. poitaei* Rchb.f.; 2) The level and patterns of mycorrhizal infection will differ among substrates; 3) Species of mycorrhizal fungi from *Vanilla* roots in soil will be different from those of epiphytic orchids. A new method to quantify the mycorrhizal distribution in *Vanilla* roots is described.

Sampling of roots. Roots of *Vanilla* were collected (20-40 cm) from two forests in Puerto Rico (Cambalache and El Yunque). They were separated according to substrate: soil, bark and rock. Soil roots of *V. planifolia* were also separated by age.

Preliminary analysis of the degree of mycorrhizal infection was done by sectioning 80 roots at 2 cm intervals. Transverse sections were fixed with Triton X-100 + ethanol 25% and stained with toludine blue.

To distinguish zones of mycorrhizal infection, velamen tissue was removed and mycorrhizal zones were identified as brown zones along the length of the root. Roots were kept in water to avoid desiccation and to facilitate the identification of light mycorrhizal zones.

Methodology validation was done to demonstrate that brown zones had pelotons and white zones did not. Sixteen 10 cm samples of mature roots were analyzed. For each root, five transverse sections of brown zones and five sections without brown zones were stained as described for the preliminary analysis.

Vanilla planifolia and *V. poitaei* roots were compared with the methodology described above. Number of roots with mycorrhizal zones were compared with Fisher's exact test and the number of mycorrhizal zones per root were compared with Mann-Whitney rank sum test.

Isolation of fungi. Roots were washed with water, ethanol 70% for 1 min, sodium hypochlorite 1% for 5 min, and distilled water 2x. Four pieces of the root (5 mm) or pelotons collected with a needle were inoculated on each of three media: Potato Dextrose Agar (PDA), Water Agar (WA) and Malt Extract Agar (MEA). Each medium contained 50 ppm of streptomycin, tetracycline and penicillin (Otero *et al.* 2002) to avoid growth of bacteria. Plates were incubated at 23ºC. About 600 root pieces were evaluated. Colonies of *Rhizoctonia*-like fungi were transferred to PDA.

DNA isolation and ITS amplification. DNA was extracted following the procedure of Lee and Taylor (1990). The polymerase chain reaction (PCR) was performed using primers ITS 1 and ITS 4 (Otero *et al.* 2002). PCR products were sequenced and aligned with sequences published in GenBank.

RESULTS AND DISCUSSION

Vanilla planifolia and *V. poitaei* differed in root development. *Vanilla planifolia* had a more abundant root system, larger roots, and more obvious differentiation between young and mature roots.

Preliminary analysis showed that mycorrhizal fungi were present in *Vanilla* roots. Root sections showed that 63 of 86 roots of *Vanilla* had at least one section in which pelotons were present. In *V. poitaei*, 26 of a total of 139 sections had pelotons. In *V. planifolia*, 123 of 507 sections analyzed had pelotons. *Vanilla planifolia* and *V. poitaei* did not differ significantly in frequency of the sections with pelotons. Some areas with mycorrhizal zones were missed between the sectioning intervals, showing limitations of this methodology.

A new method for the analysis of mycorrhizal fungi in *Vanilla* roots was developed. This allowed the identification of mycorrhizal zones without sectioning. Mycorrhizal zones were localized by removing the velamen tissue and looking for brown zones. Methodology validation was done in two *Vanilla* species: *V. poitaei* and *V. planifolia*. In soil roots of *V. poitaei*, 100 % of 80 brown sections had pelotons. In contrast none of 80 white zones had pelotons. Forty sections were observed in *V. planifolia* and the same results were obtained, but in white zones four sections had less than ten cells with pelotons.

These data showed that brown zones could be used as a new method for the study of the distribution patterns of mycorrhizal infection, in contrast with sectioning of the root where areas with pelotons could be missed in the section intervals. Katiyar *et al.* (1986) reported sectioning intervals of 1 cm for 12 species of terrestrial orchids, but in *Vanilla* roots were observed mycorrhizal zones less of 1 cm . Also this method facilitates identification of mycorrhizal zones in roots longer than 50 cm, like *Vanilla* roots. Few studies have quantified mycorrhizal distribution patterns in orchids (Katiyar *et al.* 1986, Goh *et al.* 1992, Richardson *et al.* 1993, Bayman *et al.* 1997, 2002), and ability to recognize mycorrhizal zones could facilitate large scale studies.

Table 1. Comparison of number of mycorrhizal zones among roots in *Vanilla* (T:Mann-Whitney Rank Sum Statistic. N: number of roots).

	Average (s.d.)	N	T	P
V. planifolia* vs *V. poitaei				
Total roots				
V.poitaei	2.59 (2.51)	22		
V.planifolia	2.45 (2.89)	22	474.5	0.639
Substrates				
V. poitaei				
Soil	2.22 (2.39)	14		
Rock	2.89 (2.80)	9	116.0	0.636
V. poitaei				
Soil and rock	2.48 (2.52)	22		
Bark	0	7	45.5	0.002
V.planifolia				
Soil	2.75 (2.90)	22		
Bark	2.29 (4.27)	7	79.0	0.305
Age				
V. planifolia				
Young	0.91 (1.3)	11		
Mature	4.00 (3.25)	11	162.5	0.020

Comparison among roots were done with the mycorrhizal zones method. *Vanilla planifolia* and *V. poitaei* did not differ significantly in the number of roots with mycorrhizal zones (Fisher's exact test, P=0.72) or in the number of mycorrhizal zones per root (Table 1). High variability was observed in the number of mycorrhizal zones in all samples.

Number of roots with mycorrhizal zones was much lower in bark roots than in soil roots. Differences between bark and soil roots were significant in *V. poitaei* (Fisher's exact test, P=0.007 and Table 1), but were not significant in *V. planifolia* (P=0.30 and Table 1), probably because distribution of mycorrhizae was highly variable. Roots collected from trees with rough bark showed the highest infection frequency. Mycorrhizal zones were found only in the side of the root that was in direct contact with the bark. Roots growing in soil and on rocks in *V. poitaei*

did not differ significantly in the number of mycorrhizal zones per root (Table 1).

Mature soil roots had a higher density of root hairs than young roots in *V. planifolia*. These roots had significant differences in the number of mycorrhizal zones per root (Table 1). Mycorrhizal zones were not found within 10 cm of the root apex.

Color of the mycorrhizal zones varied independently of origin of the root. Dark brown mycorrhizal zones were infrequent and were sometimes associated with diseased areas, where infection percentage was 100%.

Two transverse sections for each mycorrhizal zone were analyzed in *V. planifolia* and *V. poitaei* roots. Most pelotons were highly degraded. Rapid digestion of mycorrhizal fungi in orchids has been reported by Hadley (1971, 1989).

In most transverse sections of mycorrhizal zones, 25-50% of cells contained pelotons. However infection levels in soil roots differed in *V. poitaei* and *V. planifolia*. *Vanilla poitaei* roots were more densely infected than those of *V. planifolia*: 55% of sections had 100% infection, compared to 11% in *V. planifolia.*

Indian terrestrial orchids studied by Katiyar *et al*. (1986) had infection levels of 10% in transverse sections. *Vanilla* roots analyzed showed variable infection levels (25% to 50%) but higher than the Indian orchids. Goh *et al.* (1992) reported heavier infection in tropical terrestrial orchids than in temperate orchids.

Isolation of *Rhizoctonia*-like fungi was sporadic: only four *Rhizoctonia*-like fungi of soil roots were isolated from about 600 root pieces inoculated in the different media. Morphology was studied in PDA plates following description of Warcup *et al.* (1971) and Andersen (1990), and monilioid cells and upright hyphae were observed. *Rhizoctonia* from *Vanilla* showed less hyphal production and slower growth in culture than mycorrhizal fungi isolated from epiphytic orchids in Puerto Rico (Otero *et al.* 2002).

Sequences showed the isolates of *V. poitaei* soil roots were *Tulasnella* fungi. Warcup (1971, 1981) and Taylor *et al.* (1999, 2002) reported the genus *Tulasnella* associated with terrestrial orchids. Richardson *et al.* (1993) and Otero *et al.* (2002) reported that Costa Rican and Puerto Rican epiphytic orchids, respectively, were associated with *Ceratobasidium* spp. Terrestrial and epiphytic tropical orchids may have different mycorrhizal fungi.

Our data showed that frequency of mycorrhizal infection in roots of *Vanilla* is highly variable. This variability makes patterns harder to recognize. The new method described here is rapid, efficient, and quantitative, and will facilitate study of spatial, temporal, and taxonomic patterns of orchid mycorrhizae.

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