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Seasonality and mycorrhizal colonization in three species of epiphytic orchids in southeast Mexico

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ABSTRACT

Orchids establish symbiosis with *Rhizoctonia* mycorrhizal fungi, forming the characteristic pelotons within the cells of the root cortex. Under natural conditions, terrestrial and epiphytic orchids have different levels of dependence upon the fungal symbiont, although various authors have mentioned that once orchid plants reach maturity the interaction becomes weaker and intermittent. Recent evidence shows that in some epiphytic orchid species mycorrhization is constant and systematic. In three species of wild orchids from southeast Mexico, we show that mycorrhization is systematically present in roots of different ages, in the wet and dry seasons. We demonstrate that the volume of the root that is colonized depends upon the quantity of rainfall and the diameter of the root, and that rainfall also determines the presence of fresh, undigested pelotons. In very thin roots, mycorrhizal colonization occupies a considerable proportion of the cortex, whereas in thicker roots the proportion of the volume of the root cortex colonized is lower.

Key words: *Epidendrum stamfordianum*, *Erycina crista-galli*, *Stelis quadrifida*, mycorrhization intensity

Introduction

The nutritional role of mycorrhizae in the Orchidaceae has been well documented for temperate, terrestrial species, and especially those native to North America, Europe and parts of Asia and Australia (Rasmussen 1995), including photosynthetic species with partial mycotrophy (mixotrophy) and obligate mycoheterotrophs that depend upon the symbiosis to complete their life cycle (Leake 1994; Gebauer & Meyer 2003; Julou *et al*. 2005; Girlanda *et al*. 2006). However, since the 1970s, the discussion has focused upon epiphytic species, which represent 80% of the Orchidaceae and dominate neotropical regions (Kottke *et al*. 2006; Otero *et al*. 2007; Martos *et al*. 2012; Valadares *et al*. 2013).

It has been assumed that, for epiphytic orchids, dependence upon mycorrhizae is probably not obligate during the first stages of germination, for three reasons: some orchid seeds contain small drops of lipids and protein in the embryo; the rapid assimilation of simple nutrients in *in vitro* culture media; and after imbibing water, and when exposed to light, the seeds rapidly pass to a photosynthetic state (Arditti 1992). Nevertheless, studies on symbiotic germination *in vitro* demonstrate dependence upon the mycorrhizae for the differentiation and development of each life stage, and that growth ceases and the plant eventually dies in the absence of a mycorrhizal symbiont (Zettler *et al*. 1998; Markovina & McGee 2000; Pereira *et al.* 2005a).

Results of the analysis of canopy "soils" indicate that they are rich in organic material and nutrients, mainly NO_3, NH_4 and PO_4, which are, however, difficult to assimilate due to acidity and the particular nutrient balance of the medium (Lesica & Antibus 1990; Nadkarni & Matelson 1991). In addition, the surface area of the roots of epiphytic orchids is small in proportion to the biomass of the stems and leaves (Benzing & Ott 1981). Therefore, epiphytic orchid roots have evolved a diversity of functions, including an epidermal structure (velamen) to facilitate the rapid absorption and retention of water; the differentiation of anatomic structures (tilosomes) to reduce transpiration; and the photoassimilation of CO_2 via the photosynthetic routes C_3, CAM or both. As mentioned by Benzing & Friedman (1981), it can be assumed that "a combination of phototrophy and mycotrophy should be an advantage for an epiphyte" (Rasmussen & Whigham 2002; Julou *et al*. 2005; Girlanda *et al*. 2006). Nevertheless, studies on mycorrhizal colonization in mature, epiphytic orchids have presented discrepancies, various authors concluding that mycorrhizal colonization is occasional (Hadley & Williamson 1972; Lesica & Antibus, 1990), whereas more recent studies have indicated systematic colonization in species from Florida,

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Singapore, Costa Rica and Brazil (Benzing 1982; Goh *et al.* 1992; Rivas-Rossi *et al.* 1998; Pereira *et al.* 2005b). Despite valuable contributions toward the understanding of the importance of mycotrophy in epiphytic orchids, the intensity of colonization has not been sufficiently studied, and one way to do that is to estimate the volume of the root system colonized (Rasmussen & Whigham 2002). This is particularly relevant for epiphytic species with extreme reduction of stems and leaves wherein the root biomass accounts for 95% of the total (Benzing & Ott 1981). The aim of this study was to characterize the relationship between the intensity of mycorrhizal colonization and the diameter and length of the roots, in the wet and dry seasons, for three species of epiphytic orchids native to southeast Mexico.

Materials and methods

Study site

The root samples were collected from plants within dense populations of each species, at four sites within the Sierra Madre mountain range, in the state of Chiapas, in southeast Mexico (Tab. 1), in a region known as Soconusco. The region is characterized by remnant fragments of tropical moist broadleaf forest at lower elevations and tropical montane cloud forest at higher elevations, now mostly given over to extensive areas of coffee plantations shaded by native species and a diversity of *Inga* sp. (Fabaceae) (Damon & Colín-Mártinez 2005). As shown in Tab. 1, the climate of the region is warm and humid, with abundant rain in summer that increases with elevation (INEGI 2011).

Biological material

Three orchid species were studied: *Epidendrum stamfordianum* Bateman, *Stelis quadrifida* (La Llave & Lex.) R. Solano & Soto Arenas and *Erycina crista-galli* (Rchb.f.) N.H. Williams & M.W. Chase, and the majority were found growing upon mature trees of *Roseodendron donnell-smithii* (Rose) Miranda, *Tabebuia rosea* (Bertol.) Bertero ex A. DC. (Bignoniaceae) and *Enterolobium cyclocarpum* (Jacq.) Griseb. (Fabaceae), in coffee (*Coffea arabica* L.) and cocoa (*Theobroma cacao*) L. plantations.

Epidendrum stamfordianum has large pseudobulbs and few, long, thick roots; *Stelis quadrifida* is a smaller sized plant with no pseudobulbs and roots that are numerous, grouped, short and thin, whereas *Erycina crista-galli* is a miniature twig epiphyte, now mostly found growing upon coffee bushes and has long, thin roots that are few in number. The roots of all three species grow along the surface of the bark, but in the case of *S. quadrifida* the roots were also seen to penetrate slightly into the external, decomposing layer of the bark.

The roots were collected during the dry season in February and March and in the wet season in August and September. Three healthy and complete roots of different sizes were sampled per orchid plant, the shortest (youngest) and longest (oldest) roots were sampled along with a medium sized root. Two plants per site were sampled for each species. The roots were carefully removed from the bark, placed into labeled, plastic bags, packed with ice and taken to the laboratory (Tab. 2).

The length of each root and the diameter of each section were measured with a Vernier caliper. Due to the differences in the size of the roots among the three species (*Epidendrum stamfordianum*, *Erycina crista-galli* and *Stelis quadrifida*, with maximum lengths of 92.0, 6.5 and 11.0 cm and average diameters of 4.0, 0.8 and 0.8 mm, respectively), the number of sections taken from each root was standardized. A series of three sequential, transverse sections were taken within the first 3.0 mm of the apical meristem of each of the roots. Then, from the base to the tip of the root, sections were taken at intervals of approximately 1.5 times the diameter of the root; every 1.2 mm for *S. quadrifida* and *E. crista-galli*; and every 6.0 mm for *E. stamfordianum*.

Mycorrhization was evaluated using a modified version of the methods described by Goh *et al.* (1992) and Rasmussen & Whigham (2002). Thin roots were wrapped in a layer of parafilm for support and rigidity. Sections from each root were mounted onto a slide, in order from tip to base, and then stained with Acid Fuchsin (0.01% w/v in lactic acid-glycerol-distilled water 14:1:1 v/v/v) and mounted in Polyvinyl alcohol-lactic acid-glycerol, for observation with an optic microscope at ×40 magnification. Mycorrhization intensity was obtained for each section, using six scales, representing 0, 12.5, 25.0, 50.0, 75.0 and 100% of the cortical area, according to Rasmussen & Whigham (2002). Undefined pelotons with intact hyphae, visible at ×40 and slightly stained, were classified as live and recently penetrated into the cell. Well-defined, dense, heavily stained

Table 1. Geographic and macroclimatic characteristics of the collection sites.

Colection site	Lat.	Long.	Altitude (m)	Temp. (av./year) (°C)	Precipitation (mm/year)	Dry season (<60mm/year)
Tuzantán	15°06'N	92°24'W	118	28	2668	December - April
Izapa	14°58'N	92°09'W	482	26	4660	December - February
Unión Roja	15°02'N	92°12'W	500	22	4800	December - February
Santo Domingo	15°01'N	92°06'W	900	22	4800	December - February

Table 2. Biological material and collection sites.

Species	Site	Plants/site	Shortest roots /plant	Medium roots /plant	Longest roots /plant	Total roots
Epidendrum stamfordianum	Tuzantán	2	1	1	1	6
	Unión Roja	2	1	1	1	6
Erycina crista-galli	Santo Domingo	2	1	1	1	6
	Unión Roja	2	1	1	1	6
Stelis quadrifida	Unión Roja	2	1	1	1	6
	Izapa	2	1	1	1	6

pelotons without visible hyphae were classified as dead. Cells that showed evidence of recent recolonization were also classified as live.

To analyze the correlation between the percentage of fresh pelotons and colonization, a non-parametric softener was adjusted, considering the factors "species", "season" (wet and dry) and "length of root". The factor "length of root" was classified as L - longest (*Epidendrum stamfordianum*: 50-90 cm; *Erycina crista-galli*: 3.5-7 cm; *Stelis quadrifida*: 5-11 cm), M - medium (*E. stamfordianum*: 15-50 cm; *E. crista-galli*: 1.5-3.5 cm; *S. quadrifida*: 2-5 cm) and S - shortest (*E. stamfordianum*: 5-15 cm; *E. crista-galli*: 0.2-1.5 cm; *S. quadrifida*: 0.5-2 cm).

The relation between mycorrhization intensity and root diameter was also analyzed using a non-parametric softener. The percentage colonization for each species was analyzed using the non-parametric Kruskal-Wallis test. The statistical analysis was carried out with the software R, version 3.0.1 (R Development Core Team 2013).

Results and discussion

Considering the combination of the three types of roots (L, M and S), all three orchid species had a significantly higher percentage of the volume of the root cortex colonized by mycorrhizae in the wet season (Kruskal-Wallis test, *Epidendrum stamfordianum*: W=58421, p<2.2e-16; *Erycina crista-galli* W=8956.5, p<2.2e-16; *Stelis quadrifida*: W=9845.5, p<2.2e-16), as shown in Fig. 1. Analysis of mycorrhization intensity in function of the length of the roots (L, M and S), which is related to root age, also demonstrated significant differences according to the season. The mycorrhization of the shortest/youngest (S) roots was shown to be either particularly dependent upon the humidity of the wet season (Fig. 2 and Tab. 3) or simply reflecting the time necessary for mycorrhizal colonization.

New roots are usually formed toward the end of the dry season, in anticipation of the coming rains, and new root formation marks the end of a rest period typical of epiphytic orchids even in tropical climates. At first, these new roots have little or no mycorrhizal colonization, which was also observed in the growing tips of the oldest roots. In the wet season, roots were seen to have more fresh pelotons than in the dry season. In the dry season, fresh pelotons were found only in *Erycina crista-galli*, and there were far fewer than in the wet season (Fig. 3).

The *Rhizoctonia* group is characterized by dispersion by sclerotia in liquid water (rain). When climate conditions are adverse, the mycelium develops the sclerotium to survive during these periods. Therefore, the water represents a fundamental medium for dispersing these structures, and without rains or adequate humidity the sclerotium does not germinate unless in contact with the host plant. Ogoshi (1996) mentions that *Rhizoctonia solani* mycelium was found in the soil in the autumn and spring, whereas it was almost absent in summer (the hot, dry period in temperate regions). In the same way, in tree branches, if the humidity is too low, the probability of association between orchid roots and mycelium is lower during the dry season in tropical regions. In contrast, the wet season induces the development of orchid roots, which form as well-hydrated vegetal soft tissues, allowing the penetration of mycelium.

Diameter was a determining factor for the intensity of mycorrhizal colonization. The intensity of colonization was greater in thin roots, which could be due to a relatively higher proportion of available root surface for mycorrhizal colonization in relation to volume, and the highest percentage colonization in this study was observed in the thin roots of the twig epiphyte *Erycina crista-galli* (Fig. 4). A further implication could be that orchid species with thin roots absorb less from the surrounding environment and are more dependent upon mycorrhizae. In the case of *E. crista-galli*, the particularly extreme, variable and stressful xerophytic conditions of the microhabitat occupied by this miniature orchid could have resulted in greater dependence upon mycorrhizal fungi.

Stelis quadrifida and *Erycina crista-galli* are medium- and small-sized species, respectively, both having thin roots, which maintain greater contact with the surface of the substrate, whereas a substantial proportion of the thick roots of *Epidendrum stamfordianum* remain in contact with the air. Colonization was seen to approach 100% in sections of the roots of the two species with thin roots, whereas for *E. stamfordianum*, which has thick roots, colonization did not exceed 60% (Fig. 4), further supporting the suggestion

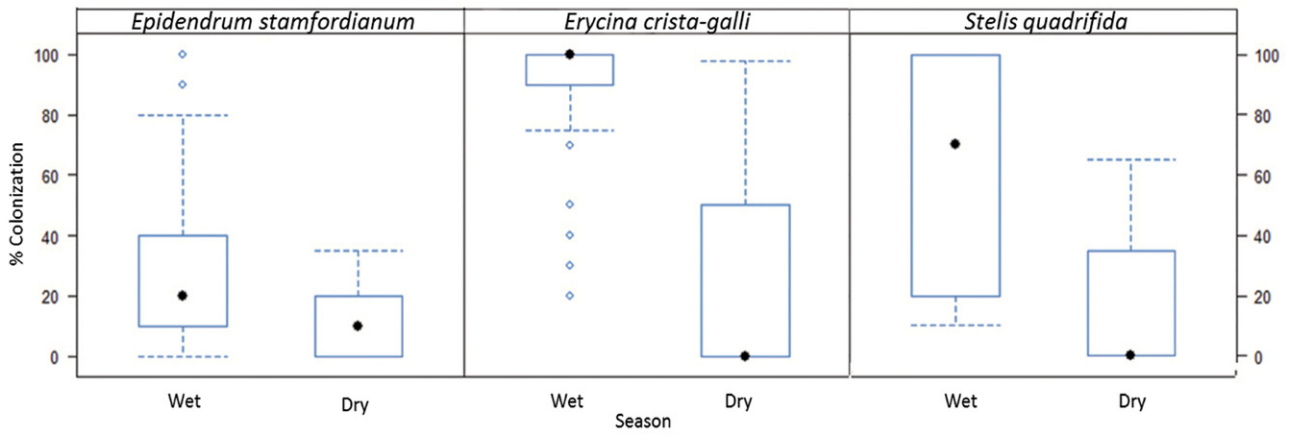

Figure 1. Comparison of the mycorrhizal percentage in the roots of three species of epiphytic orchids, in the wet and dry season, in southeast Mexico.

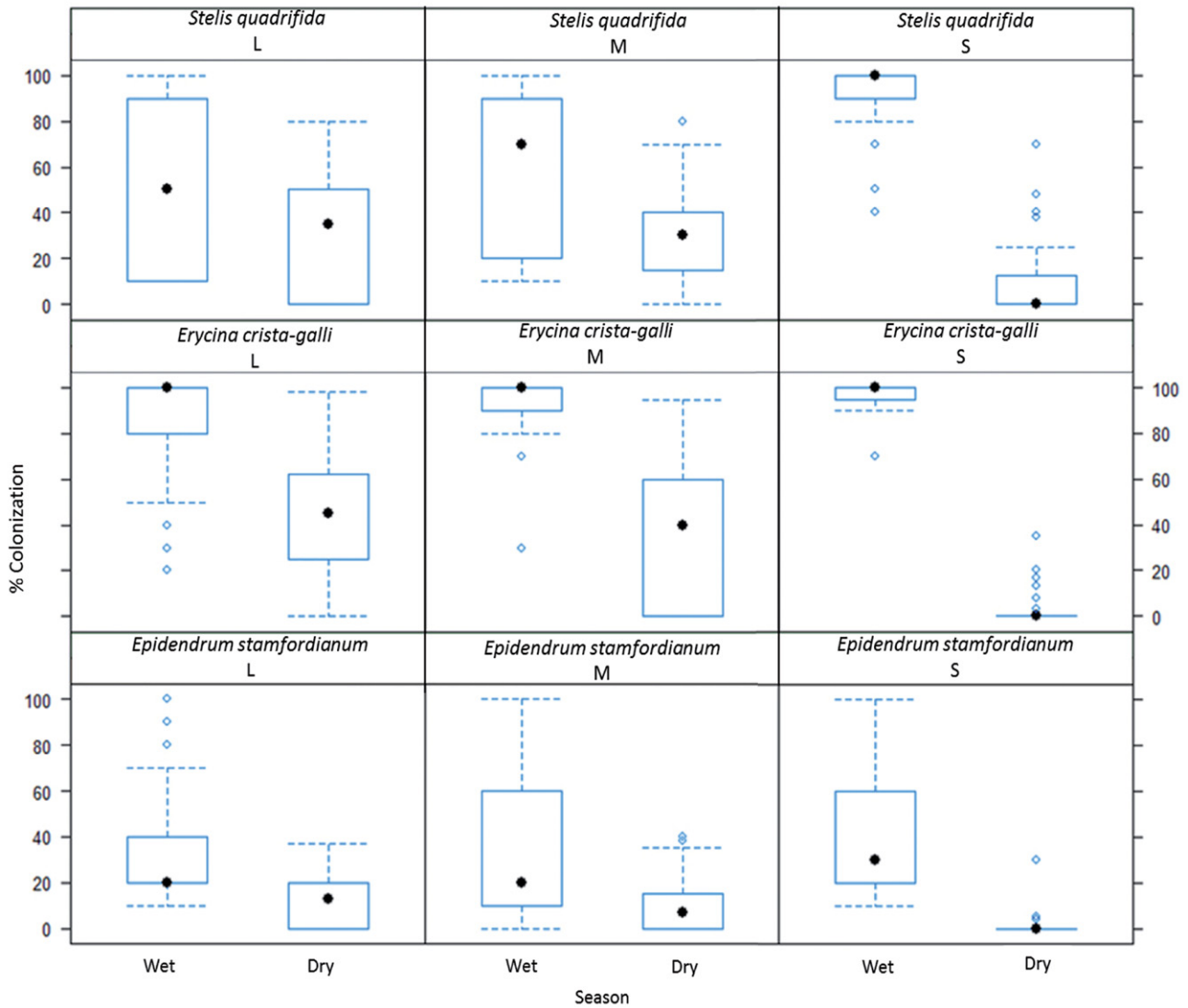

Figure 2. Comparison of the proportional mycorrhizal colonization of three classes of roots (**L**: longest/oldest; **M**: medium; **S**: shortest/youngest) of epiphytic orchids, in the wet and dry season, in southeast Mexico.

Table 3. Comparison of the intensity (%) of mycorrhizal colonization of three classes of roots (**L**: longest/oldest; **M**: medium; **S**: shortest/youngest) of epiphytic orchids, in the wet and dry season, in southeast Mexico, with the non-parametric Kruskal-Wallis test.

Specie		W* value	P< value
Epidendrum stamfordianum	**L**	54258	2.20e-16
	M	12971	2.20e-16
	S	2340	2.20e-16
Erycina crista-galli	**L**	3896	4.09e-14
	M	3253.5	2.20e-16
	S	1219	6.62e-15
Stelis quadrifida	**L**	5050	4.60e-05
	M	3200.5	3.22e-06
	S	795	2.59e-10

*All comparisons have 1 degree of freedom

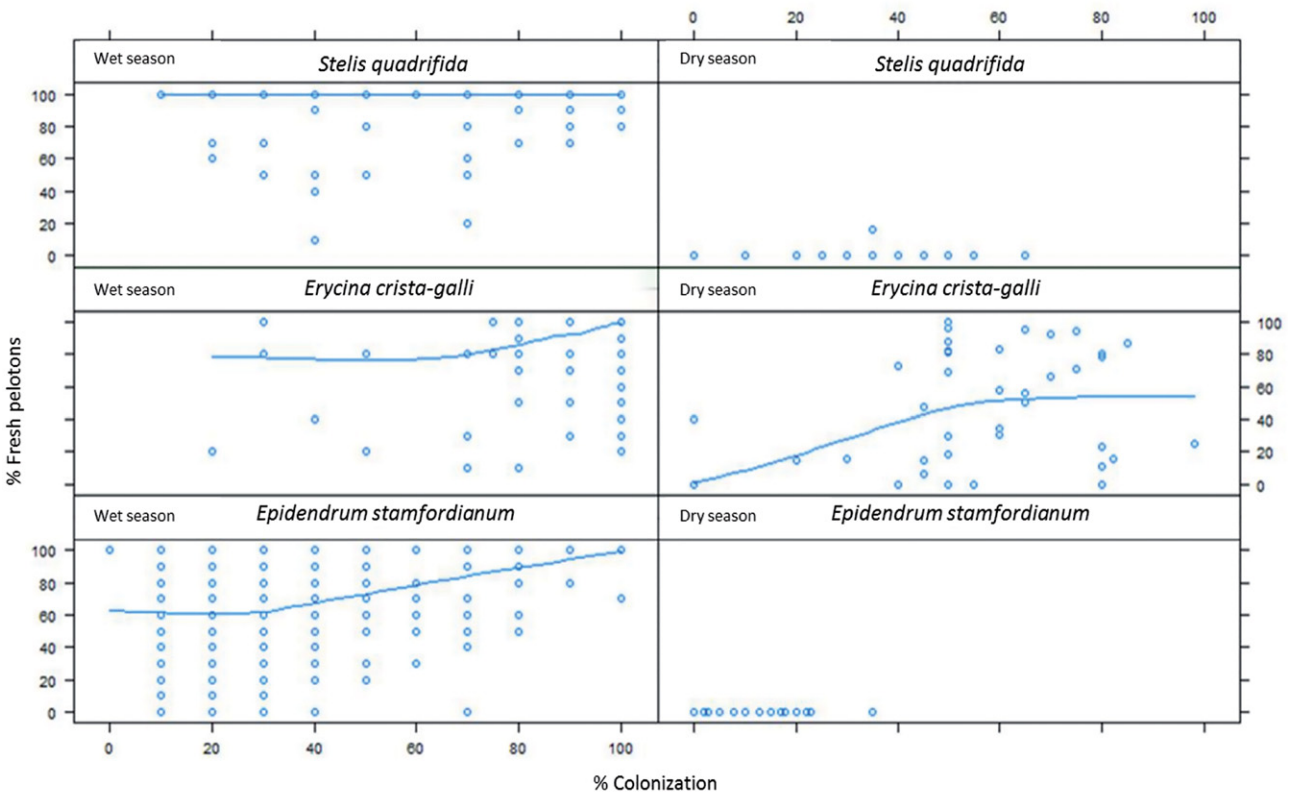

Figure 3. Comparison of the percentage of fresh pelotons in the roots of epiphytic orchids, in the wet and dry season, in southeast Mexico.

that orchid species with thin roots are more dependent upon mycorrhizae. In the two species with thin roots, mycorrhizal colonization was uniform throughout, which was not the case for the thick roots of *E. stamfordianum*, in which colonization was observed mostly in the areas of the root that had been in contact with the substrate.

The only roots areas seen to be free of mycorrhizal fungi were the apical meristem, the basal area of the main root and the ramifications. The new sections of growing roots originate from the vascular cambium, which is not host to mycorrhizal fungi, and mycorrhization occurs as the roots enter in contact with the substrate or other mycorrhizal roots, and in the latter case then acquire the same pattern of colonization. Similarly, the process of colonization is influenced by the extent and distribution of organic detritus and the extension of the roots into these areas, reaching 100%

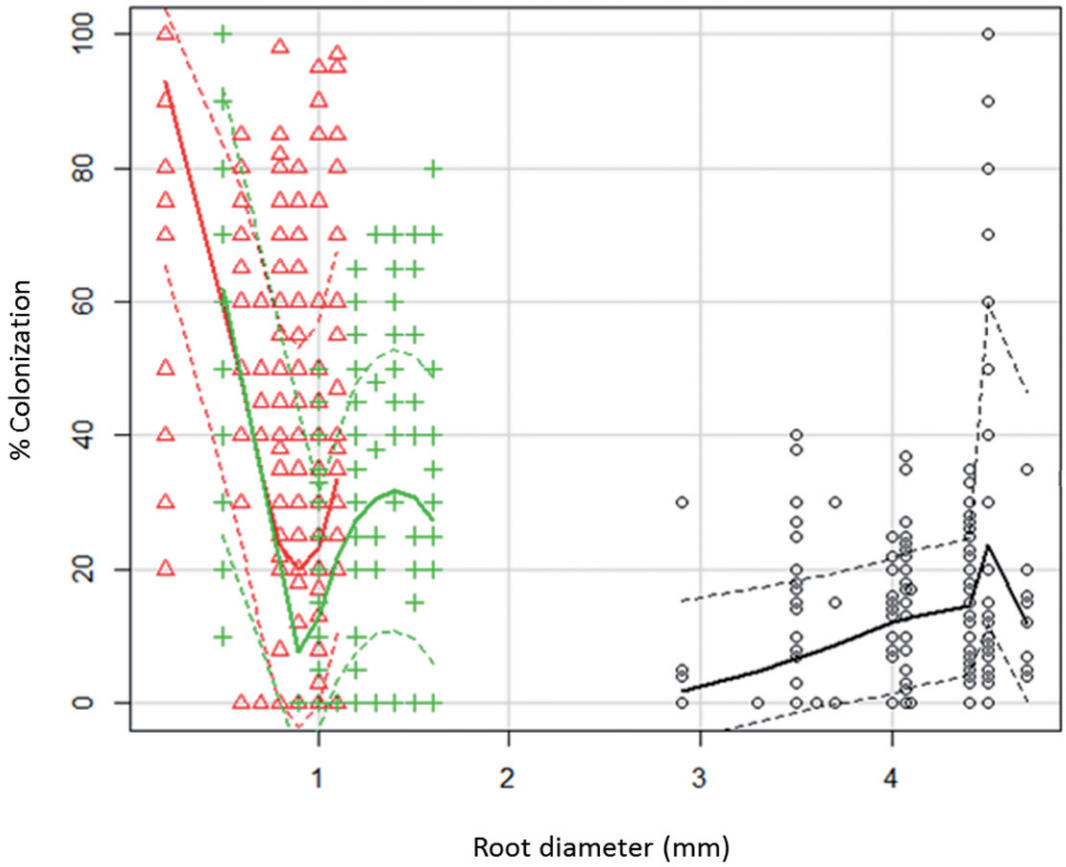

Figure 4. Relationship between mycorrhization intensity and root diameter, for three species of epiphytic orchids in southeast Mexico: *Epidendrum stamfordianum* (°); *Erycina crista-galli* (Δ); and *Stelis quadrifida* (+).

colonization. Further studies should analyze the mechanisms by which orchid cells and fungal hyphae recognize each other, the process of penetration and colonization and the control of fungal development within orchid root cells, as well as why some areas are colonized and others are not.

Mycorrhization was found in the roots of all the plants sampled in this study, although in varied proportions and quantities and with seasonal fluctuations, supporting the results in the literature that consider the mycorrhizal colonization of the roots of epiphytic orchids as systematic (Benzing 1982; Goh *et al.* 1992; Rivas-Rossi *et al.* 1998; Pereira *et al.* 2005b). However, the affirmation of Hadley & Williamson (1972) that "in epiphytic orchids infection is spasmodic and confined to a small proportion of cells in the root cortex" was based on the quantification of the numbers of cells colonized in a few root sections. The results obtained by Goh *et al.* (1992) and in the present study show that the intensity of colonization can vary between 0% and almost 100% along the length of the root. To guarantee an unbiased and realistic picture of mycorrhizal colonization, samples should be taken at regular intervals along the entire length of the root. A better measure of the intensity of colonization is the volume of root colonized, considering the whole root, as demonstrated by Rasmussen & Whigham (2002).

Our results suggest that the diameter of the roots of an epiphytic orchid is one of the factors that determine the percentage colonization of the root cortex by mycorrhizal fungi. However, Goh *et al.* (1992) and Pereira *et al.* (2005b) reported intense colonization, reaching 95%, in the root cortex of the epiphytic orchids *Dendrobium crumenatum* Sw., *Maxillaria marginata* (Lindl.) Fenzl, *Oncidium flexuosum* Lodd and *Oncidium varicosum* Lindl., which have thin roots, as well as in that of *Isochilus linearis* (Jacq.) R. Br., which has thick roots.

Conclusions

The results of the present study show that the intensity of mycorrhizal colonization of the root cortex of three epiphytic orchids in southeast Mexico is continuous and systematic, with higher intensity and greater numbers of undigested, live pelotons in the wet season. Our results also show that, for the species studied, the intensity of mycorrhizal colonization is relatively greater on thin roots and in areas in contact with the substrate.

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