

Fungi diversity in roots of *Guarianthe skinneri* in urban condition: velamen *versus* cortex

Ochoa-Bonilla, Jorge José¹ ; Bertolini, Vincenzo^{1*} ; Zarza, Eugenia^{1,2*} 

¹ El Colegio de la Frontera Sur unidad Tapachula. Carretera Antiguo Aeropuerto km. 2.5, Tapachula de Córdova y Ordoñez, Chiapas, Mexico, C.P. 30700.

² Investigadora CONHACyT—El Colegio de la Frontera Sur. Av. Insurgentes Sur 1582, Mexico City 03940, Mexico.

* Correspondence: vin.bertolini@gmail.com, eugenia.zarza@ecosur.mx

ABSTRACT

Objective: To Identify the endophytic fungal community of the *G. skinneri* root in urban populations, showing differences in composition between velamen and cortex to better understand the fungus-root interaction in these orchids.

Design/Methodology/Approach: We collected tissues from velamen and root from five specimens of *G. skinneri* growing on urban trees in Tapachula, Chiapas. We extracted DNA, PCR amplified the ITS marker, sequenced on the Illumina platform followed by diversity analyses and taxonomic assignment.

Results: We detected 845 OTUs that were assigned to the Fungi kingdom. Velamen and cortex share 403 OTUs, 402 were found exclusively in velamen and 40 in cortex. Besides orchid mycorrhiza forming fungi, we detected other species (e.g. *Alternaria* sp., *Beauveria* sp., *Fusarium* sp., *Glomus* sp. and *Tricoderma* sp.) that could be involved in root physiology during development and defense against pathogens and predators.

Study Limitations/Implications: Metagenomic studies provide substantial amounts of data that go beyond conventional studies. However, the information generated is still limited regarding the role of each endophyte.

Findings/Conclusions: Despite these limitations, our work fills a knowledge gap because we detected endophytes that were previously unknown for *G. skinneri*, leading to new research questions about root-endophyte relationships.

Keywords: non-mycorrhizal endophytic fungi, orchid mycorrhiza fungi, metagenomics, NOM-059-2010.

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INTRODUCTION

Orchidaceae is one of the most species rich plant families, it includes around 31,000 to 35,000 species (Śliwiński *et al.*, 2022). Seventy percent of orchids are epiphytes (Emeterio-Lara *et al.*, 2021) and they are distributed in tropical and subtropical regions (Zarate-García *et al.*, 2020). There are approximately 1,250 orchid species in México; in Chiapas State inhabit 723 species (Martínez-Meléndez *et al.*, 2017), with 325 species found in the Soconusco region, representing 26% of the national orchid richness (Solano-Gómez *et al.*, 2016). *Guarianthe skinneri* (Bateman) Dressler & W.E. Higgins (Orchidaceae), is an epiphytic orchid distributed naturally from Southern Mexico to Panama (Bertolini *et al.*, 2016). It is used in religious celebrations and as an ornamental plant in Soconusco, Chiapas (Coutiño-Cortés *et al.*, 2018). Currently, *G. skinneri* is listed in the Mexican Norm for species protection (Norma Oficial Mexicana 059-2010; SEMARNAT, 2010), under the ‘threatened’ category due to strong anthropogenic pressure caused by illegal extraction and trade, as well as land use change and biodiversity loss.

Roots are an important feature for epiphytic orchids. Their anatomy is composed of several layers, they are, in order from the exterior to the interior: the velamen, parenchyma

(cortex), endodermis and vascular cylinder (Deepthi and Ray, 2018; Joca *et al.*, 2017; Moreira and Isaias, 2008). Roots serve for anchorage on the phorophyte. The velamen, a dead and spongy tissue, absorb water and nutrients, reduces water loss (Joca *et al.*, 2017; Zotz and Winkler, 2013), provides mechanical protection for the living cortex layers, protects from UV-B radiation and is an ecological niche for diverse microorganisms, including bacteria and endophytic fungi, that would colonize the cortex as mycorrhizal symbionts (Chomicki *et al.*, 2015; Bhargava *et al.*, 2019; Tedersoo *et al.*, 2020). Orchid seeds necessarily depend on orchid mycorrhizal fungi (OMF) for successful germination. OMF supplement the lack of nutrients in the seed endosperm and cotyledons (Bidartondo *et al.*, 2004; Hossain *et al.*, 2013; Tian *et al.*, 2021). OMF penetrate the seed parenchyma cells during germination, a stage known as protocorm, they also penetrate root tissues of seedlings and adult plants. Once OMF are inside the plant cells, intracellular packages of supercoiled hyphae (*i.e.* pelotons) are formed (Dearnaley *et al.*, 2016).

Symbiotic germination processes in orchids have been studied extensively (Alghamdi, 2019; Dearnaley, 2007; Izuddin *et al.*, 2019; López-Chávez *et al.*, 2016), showing that the *phylum* Basidiomycota form relationships with different epiphytic orchid species (Petrolli *et al.*, 2021; Rammitsu *et al.*, 2021). However, there is a knowledge gap regarding the velamen role as microorganism filter (Chomicki *et al.*, 2015; Joca *et al.*, 2017; Zotz and Winkler, 2013) and the fungal diversity that it harbors. Herrera *et al.* (2010) reported that members of the *phylum* Ascomycota colonize this structure. To better understand the ecological complexity and evolution of the mutualistic relationship between orchids and fungi, it is important to uncover the fungal diversity present in the velamen and cortex. Most of the endophytic fungi of orchids are not mycorrhizal and their function is not well studied. However, understanding its diversity, ecological and physiological roles could help in the conservation of the orchid family (Yuan *et al.*, 2009).

To generate a knowledge baseline regarding the adaptation of *G. skinneri* in an urban environment, we aim to determine the fungal diversity of roots in both velamen and cortex. Understanding biotic interactions with root endophytic fungi is essential for the conservation and ecological restoration of endangered species (Ortega-Larrocea and Rangel-Villafranco, 2007).

MATERIALS AND METHODS

Biological material collection

We selected three main roots from plants growing in the streets of Tapachula, Chiapas, Mexico in July 2021. Samples were transported in plastic bags in a cooler to the laboratory of Ecology and Sustainable culture of Orchids in El Soconusco in El Colegio de la Frontera Sur, Tapachula, for DNA extraction.

Sample processing

Roots were rinsed with sterile water to wash away dirt from the surface. Velamen was removed entirely with a scalpel. The cortex was cut into 0.5 cm fragments. Then, velamen and cortex fragments were transferred separately to 1.5 ml tubes for DNA extraction.

DNA extraction

DNA was extracted from the velamen and cortex fragments separately with the Soil Microbe DNA MiniPrep™ Kit (Zymo Research Cat. No. 6001). DNA quality was verified running a 1% agarose gel electrophoresis.

DNA sequencing and Bioinformatic analysis

Amplicon library preparation for the 18S-ITS1-5.8S-ITS2 region, Illumina 250 bp pair-end sequencing and bioinformatic analysis was outsourced to CD-GENOMICS (Shirley, NY_USA).

Raw reads were demultiplexed according to their corresponding index. Demultiplexed reads were trimmed to remove low quality nucleotides, index and primer sequences. Sequences were paired with the software FLASH (V1.2.7) to produce clean reads for analysis with QIIME (V1.9.1). Chimera sequences were removed by comparing sequences to the reference database using the UCHIME algorithm to obtain effective tags (Nilsson *et al.*, 2018). The resulting sequences were grouped at 97% similarity with the algorithm UCLUST in QIIME (versión 1.8.0) to obtain Operational Taxonomic Units (OTUs). OTUs were then taxonomically annotated with the database UNITE. The FUNGuild platform was used to assign an ecological function to the taxonomically identified OTUs. A Venn diagram was drawn to know the number of shared OTUs between velamen and cortex.

Histograms were created to show the distribution of taxa at different taxonomic levels (phylum, class, order, family and genus). Three indices for alpha diversity were calculated: Shannon, Simpson, Chao 1 and ACE (*abundance coverage estimator*). To compare indices among samples, sequence number was standardized. A rarefaction plot was created to explore if sequencing was deep enough to comprehend diversity in cortex and velamen samples. Data were selected randomly from the cortex and velamen samples separately, then the number of species in each sub-sample was determined. A curve rank-abundance was plotted to show species relative abundance, richness and evenness. Finally, an accumulation curve was plotted to show a relationship between sample number and number of genera assigned.

RESULTS AND DISCUSSION

845 OTUs were taxonomically assigned with UNITE (Nilsson *et al.*, 2018). The Venn diagram shows that there are 403 OTUs shared between the cortex and velamen samples, 402 OTUs were found exclusively in velamen, whereas 40 OTUs were exclusive to cortex (Figure 1).

Most of the taxonomically assigned OTUs belonged to the *phylum* Ascomycota (Figura 2 y 3).

Among the OTUs shared between velamen and cortex, 3 phyla, 21 classes, 62 orders, 115 families, 169 genera and 198 species were assigned (Table 1a, supplementary material). Five phyla, 24 classes, 59 orders, 106 families, 168 genera and 187 species were among the OTUs exclusively found in velamen (Table 1b, supplementary material). In cortex, 2 phyla, 9 classes, 19 orders, 25 families, 29 genera and 29 species were identified (Table 1c, supplementary material).

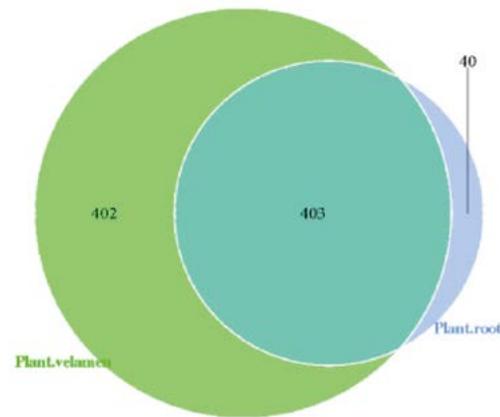


Figure 1. Venn diagram showing number of OTUs detected in velamen and cortex samples of *G. skinneri*.

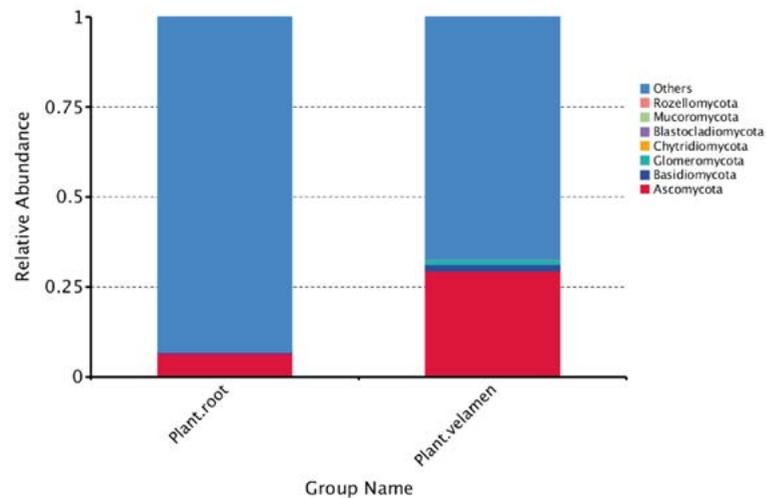


Figure 2. Relative abundance of phyla found in cortex and velamen. 'Others' includes sequences that could not be assigned to any phylum.

Ascomycota phylum shows higher abundance in both sample types. At the class level, Dothideomycetes is the most abundant in both samples, whereas the order Pleosporales is the most abundant in cortex samples (root) and Capnodiales in velamen. The families unidentified *Capnodiales* sp., and Didymellaceae were the most prevalent in cortex and velamen, respectively. At the genus level, *Ectophoma* in cortex and unidentified *Capnodiales* sp. in velamen were the most abundant. The species *Ectophoma pomi* and *Capnodiales* sp. were the most abundant in cortex and velamen, respectively.

Three biodiversity indices were evaluated, all showed that velamen has more fungal species richness than the cortex (Table 1).

According to beta diversity, there is more diversity among the velamen samples than among the cortex samples. The PCoA analysis based on Weighted Unifrac metric, also indicates that the composition of cortex and velamen fungal communities are different (Figure 4).

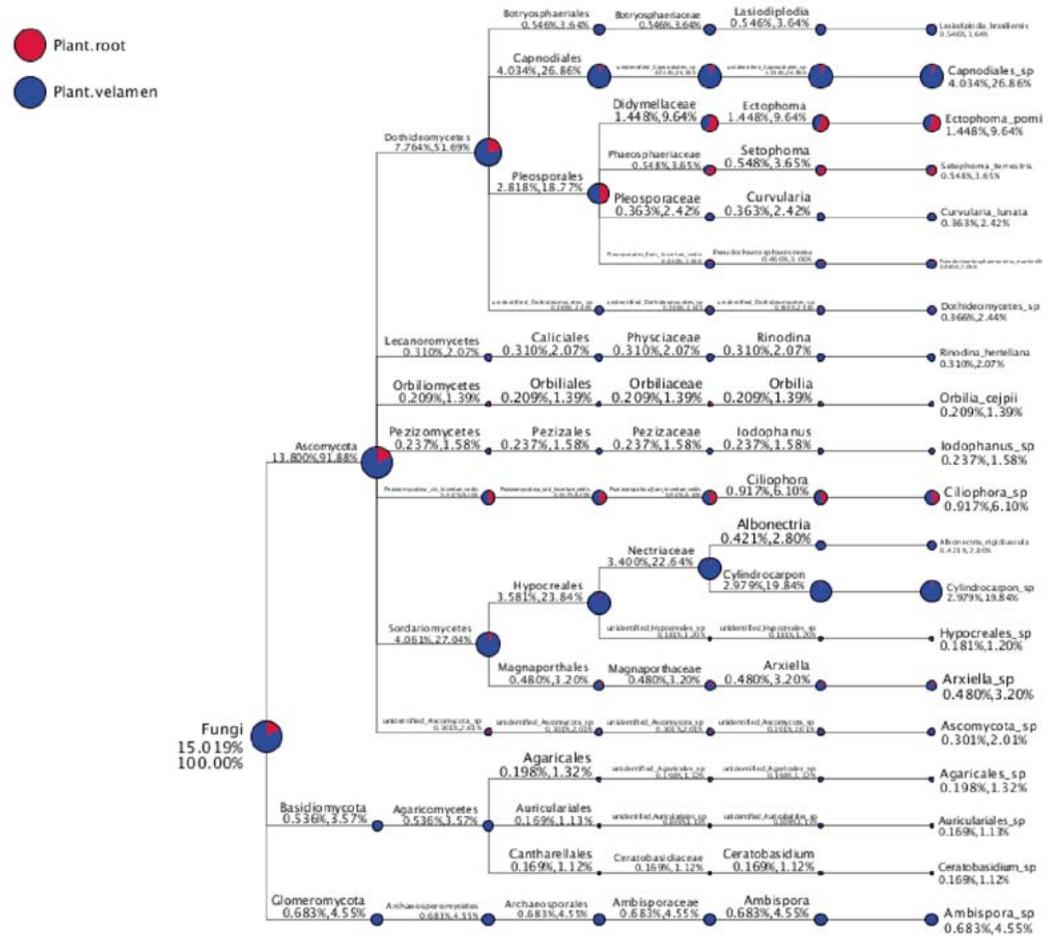


Figure 3. Phylogenetic tree showing total number of OTUs (cortex+velamen) and their relative percentage in each shown in color.

Table 1. Shannon, Simpson, Chao1 and ACE indices calculated based on OTUs detected in cortex and velamen samples.

Sample	Species observed	Shannon	Simpson	Chao1	ACE
Cortex	177	0.658	0.132	197.501	201.888
Velamen	424	2.952	0.581	452.078	458.034

The rarefaction curve (Figure 6) shows that sequencing depth was enough to sample the diversity harbored by cortex and velamen tissues. Indeed, the sampling effort is representative of the fungal diversity found in the *G. skinneri* roots in an urban environment.

The cortex slope shows that with 1,000 sequences around 100 species were observed, and with 6,000 sequences, 150 species were observed. The velamen slope shows that with 1,000 sequences, approximately 250 species were observed. Whereas with 6,000 sequences 420 species can be observed approximately. The abundance rank curve demonstrates that cortex samples (root) harbor less species diversity than the velamen samples, with a species rank of around 150 species in cortex and 420 species in velamen (Figure 7).

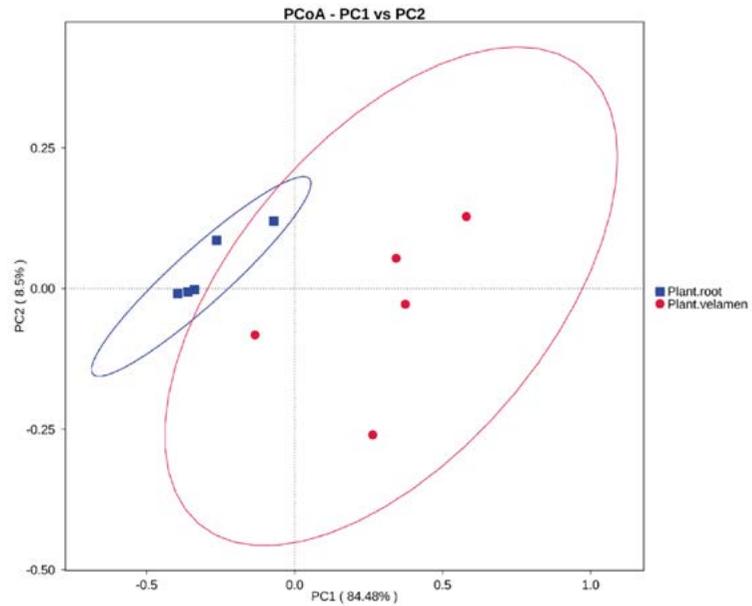


Figure 4. PCoA analysis based on Weighted Unifrac metric.

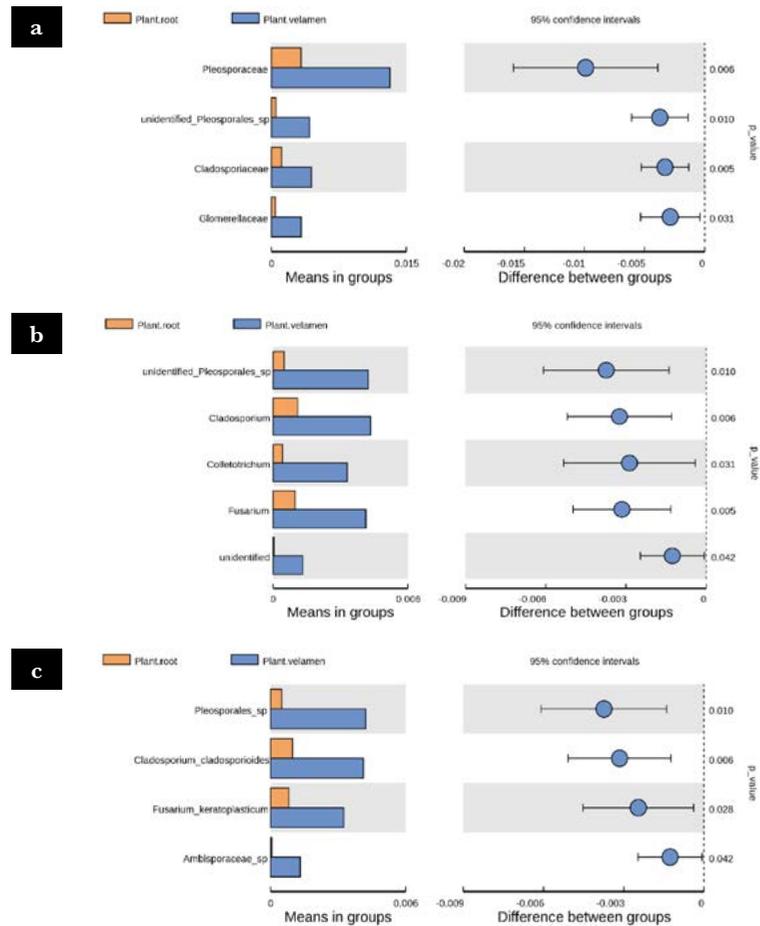


Figure 5. T test analysis comparing relative abundances of OTUs in root and velamen samples at a) family, b) genus and c) species level, showing taxa with significantly different values for each condition.

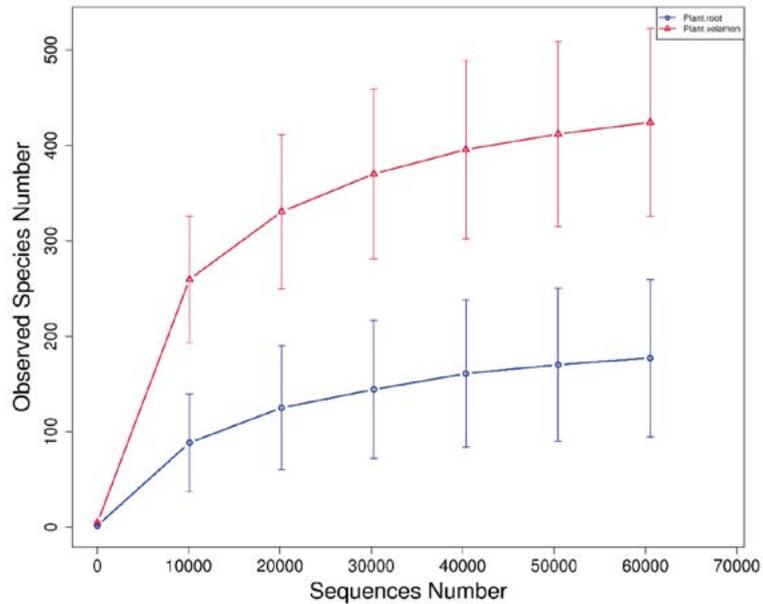


Figure 6. Rarefaction curve showing sequences number *vs.* observed species number for (a) velamen, (b) cortex.

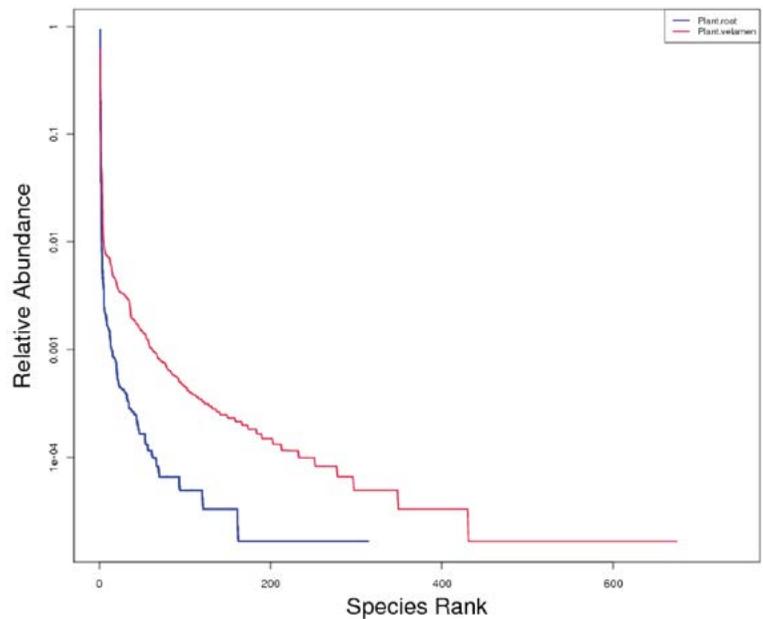


Figure 7. Rank curve of abundance for all (a) velamen and (b) cortex samples. Each curve represents each of the samples plotted as relative abundance on Y axis and abundance rank on X axis.

Velamen is the abiotic interphase between the root and the phorophyte bark (Herrera *et al.*, 2010). It is a sponge composed of dead cells that provide a microhabitat for microscopic life, serving as an abiotic filter for endophytes. It is followed by the exodermis that together with the cortex, select biotically the microorganisms that can enter to become endophytes. Thus, it is logical to think that the numerous taxa that can inhabit the abiotic velamen would reduce their frequency when colonizing

living tissue that applies defense and interaction mechanisms. Although this study is fungi-centered it is necessary to consider that there are root interactions with bacteria (Aguilar *et al.*, 2018), meaning that there are complex interactions. There are statistically significant differences between the two sample groups, showing that velamen has more fungal diversity than the cortex. Interestingly, there are 40 fungi that were found in the cortex but not in velamen. This may be linked to mycorrhizal colonization of epiphytic orchids occurring at a certain time in the year: rain season, when environmental conditions lead to fungi proliferation and root growth, favoring the contact between root surface and the fungi that would enter the velamen and then the cortex (Bertolini *et al.*, 2014). These 40 OTUs likely belong to fungi that could access the cortex prior to sample collection and that disappeared from the velamen due to unfavorable environmental conditions but could survive within the cortex. Our study shows that orchids living in urban environments can still establish mutualistic relationships with orchid mycorrhizal fungi.

Interestingly, we detected a high number of non-mycorrhizal endophytic fungi in velamen and cortex. Beltrán-Nambo *et al.* (2018) reported 17 genera belonging to Ascomycota with four *Trichoderma* species and two *Fusarium* species among other in orchids from southeast Mexico. Although *Fusarium* is a pathogen, previous studies show that it can aid seed germination (Sisti *et al.*, 2019). Similarly, Xue *et al.* (2022) recorded *Trichoderma*, *Fusarium*, *Alternaria* among the non-mycorrhizal fungi in terrestrial orchids in China. There is no doubt that our results showing the presence of *Glomeromycota* sp., *Trichoderma* sp., *Beauveria* sp., *Fusarium* sp., *Alternaria* sp., *Glomus* sp., among others, are novel for scientific advancement and lead to future research to explain the root-microorganism relationships to better understand ecological processes occurring in these interactions and in turn provide efficient conservation protocols.

CONCLUSIONS

Our results are pioneers in providing evidence for statistically significant differences between the fungal composition of velamen and cortex of *G. skinneri*, confirming that velamen harbors a richer community than the cortex. At the same time, we identify beneficial fungi *Beauveria*, *Glomus* y *Trichoderma*, that deserve more specific studies to understand their role in root physiology, development and health of *G. skinneri*.

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SUPPLEMENTARY MATERIAL

Supplementary material can be found in https://www.dropbox.com/scl/fi/m21z8kkn96nqmxnbut98l/material_suplementario_Ochoaetal2024.xlsx?rlkey=m4zkvi8yeepybwt1i54qk380e&dl=0

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