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Research Article

RESEARCH ARTICLE

Soil P reduces mycorrhizal colonization while favors fungal pathogens: observational and experimental evidence in Bipinnula (Orchidaceae)

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One sentence summary: *In situ* P addition reduces mycorrhizal colonization in *Bipinnula fimbriata* in the short term, and this is coupled with an increase in pathogen richness.

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ABSTRACT

Little is known about the soil factors influencing root-associated fungal communities in Orchidaceae. Limited evidence suggests that soil nutrients may modulate the association with orchid mycorrhizal fungi (OMF), but their influence on non-mycorrhizal fungi remains unexplored. To study how nutrient availability affects mycorrhizal and non-mycorrhizal fungi associated with the orchid *Bipinnula fimbriata*, we conducted a metagenomic investigation within a large population with variable soil conditions. Additionally, we tested the effect of phosphorus (P) addition on fungal communities and mycorrhizal colonization. Soil P negatively correlated with the abundance of OMF, but not with the abundance of non-mycorrhizal fungi. After fertilization, increments in soil P negatively affected mycorrhizal colonization; however, they had no effect on OMF richness or composition. The abundance and richness of pathotrophs were negatively related to mycorrhizal colonization and then, after fertilization, the decrease in mycorrhizal colonization correlated with an increase in pathogen richness. Our results suggest that OMF are affected by soil conditions differently from non-mycorrhizal fungi. *Bipinnula fimbriata* responds to fertilization by altering mycorrhizal colonization rather than by switching OMF partners in the short term, and the influence of nutrients on OMF is coupled with indirect effects on the whole fungal community and potentially on plant's health.

Keywords: orchid mycorrhiza; fungal root community; fungal guilds; orchid fertilization; soil nutrients; *Bipinnula*

INTRODUCTION

Almost all plant species have fungal root associates in all their tissues (Rodriguez *et al.* 2009; Hardoim *et al.* 2015) and fungal communities that colonize plant roots can be highly diverse (Vandenkoornhuyse *et al.* 2002). Great focus has been placed on mycorrhizal fungi, which increase plant nutrient uptake (van

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der Heijden *et al*. 2015). They have been crucial for the evolution of plants (Selosse and Le Tacon 1998; Strullu-Derrien *et al*. 2018) and greatly influence plant fitness (Brundrett 2007; Smith and Read 2008), plant interactions and long-term plant dominance (Van Der Heijden 2002; van der Heijden *et al*. 2015), as well as tolerance to fluctuating environmental conditions (McCormick *et al*. 2006). Yet, endophytes, i.e. fungi colonizing roots in a loose pattern, without visible damage nor true mycorrhizal morphogenesis (Wilson 1995), also can confer fitness benefits on the host plants, including tolerance to heat, disease, and drought (Rodriguez *et al*. 2009). The functions of fungal root communities are highly influenced by the diversity level and fungal species involved (Alzarhani *et al*. 2019), so that a main issue is to understand how environmental factors determine the diversity and composition of root fungal communities, including mycorrhizal and endophytic fungi.

Root fungal communities are greatly influenced by abiotic and biotic factors (David, Seabloom and May 2016), including host plants (Roy *et al*. 2013), climate (Tedersoo *et al*. 2012) and soil conditions (Blaalid *et al*. 2012; Huggins *et al*. 2014; Van Geel *et al*. 2016; Boeraeve, Honnay and Jacquemyn 2018). Particularly, soil nutrients play a major role in regulating diversity and taxonomic composition of fungal root associates (Peay, Garbelotto and Bruns 2009; Põlme *et al*. 2013; Yao *et al*. 2013). For example, fungal diversity declined significantly under increasing P addition (Liu *et al*. 2012) and short-term P fertilization produced shifts in root-associated fungal communities (Fabiańska *et al*. 2019). In addition, nutrient addition can also affect mycorrhizal colonization, which tends to decrease under nitrogen (N) and phosphorus (P) fertilization (Treseder 2004; Bechem and Alexander 2012; Balzergue *et al*. 2013).

Little is known about the effect of soil nutrients on fungal root associates in Orchidaceae. Orchids commonly form mycorrhizal associations with fungi belonging to at least three families of basidiomycetes (Tulasnellaceae, Serendipitaceae, and Ceratobasidiaceae; Dearnaley, Martos and Selosse 2012); and in addition to these orchid mycorrhizal fungi (OMF), a large diversity of endophytic fungi associate with orchid roots, some of which may be pathogenic (*e.g*. Julou *et al*. 2005; Bayman and Otero 2006; Shah *et al*. 2019). Limited evidence suggests that soil nutrients play a key role in orchid mycorrhizas. Bunch *et al*. (2013), for example, showed that the composition of mycorrhizal fungi varies among populations of the orchid *Cypripedium acaule* in association with soil pH and percentage of organic matter, C and N, whereas Mujica *et al*. (2016) found a significant correlation between soil phosphorus (P) and nitrogen (N) and the composition and diversity of mycorrhizal fungi in two *Bipinnula* species. However, the effect of soil nutrients on the diversity and composition of orchid non-mycorrhizal fungal associates remains unknown. It has been suggested that the soil environment may impact mycorrhizal associations and other types of fungal associations in similar ways (Bunch *et al*. 2013), since soil conditions may act as an environmental filter for the whole fungal community. Conversely, soil nutrients may differentially affect the mycorrhizal and non-mycorrhizal fungi, which could result from different nutrient requirements among different fungal functional groups (Schappe *et al*. 2019), or from a modulation of selection of partners by the plant depending on soil nutrients (Werner and Kiers 2015).

We previously conducted a correlative field study in *Bipinnula fimbriata* and *B. plumosa*, two terrestrial orchids endemic to Central Chile (Mujica *et al*. 2016). In the case of *B. fimbriata*, we included seven populations of its 600-km-wide distribution range and observed that soil P availability positively correlated with mycorrhizal colonization and OMF species richness, while soil nitrate negatively correlated with OMF phylogenetic diversity. However, given that it was a correlational approach, where in addition only OMF were analyzed, experimental studies combined with assessment of the total fungal diversity are needed to draw conclusions regarding the impact of soil nutrients on OMF communities and to elucidate their effect on non-mycorrhizal endophytic and pathogenic associates. This study is a closer experimental investigation of a large population of *Bipinnula fimbriata* using next-generation sequencing to assess whether (i) nutrient availability affects the mycorrhizal fungi of *B. fimbriata* differently, (ii) nutrient addition influences OMF communities and mycorrhizal colonization as expected from our previous regional correlation study and (iii) nutrient addition influences the non-mycorrhizal fungi associated with *B. fimbriata*. To investigate this, we (i) evaluated the relationships between soil nutrients and the diversity of the whole fungal community associated with the *Bipinnula fimbriata* population before any treatment, and (ii) conducted a P fertilization to evaluate how experimental nutrient addition affects the fungal root community associated with *Bipinnula fimbriata*.

MATERIALS AND METHODS

Orchid species and study site

Bipinnula Comm. ex Juss. (subtribe Chloraeinae, Orchidoideae) is a genus of terrestrial, photosynthetic orchids endemic to southern South America, comprising a separate group of five species endemic to Chile (Cisternas *et al*. ; Novoa *et al*. 2015). *Bipinnula fimbriata* (Poepp.) Johnst. is the most frequent of these five species; it is distributed in lowland (< 500 m) coastal areas from 29 to 35°S (Novoa *et al*. 2015), preferably on sandy stabilized soils, in open sites exposed to sunlight and marine breezes (Elórtegui and Novoa 2009). It has fasciculate fleshy roots and it flowers from July to November. According to previous studies, this orchid forms mycorrhizal associations with members of Ceratobasidiaceae and Tulasnellaceae (Steinfort *et al*. 2010; Mujica *et al*. 2016; Herrera *et al*. 2019).

The study was conducted in a large *B. fimbriata* population located at the 'La Cruz' hill, near the town of Zapallar, on the coast of central Chile (32°33 S, 71°28 W; 30 m above sea level; Fig. 1). The climate is Mediterranean with a mean annual temperature of 14.2° and mean annual precipitation of 384 mm (Luebert and Pliscoff) and the soil type is alfisol, whereas the geological substrate is marine terraces (Casanova *et al*. 2013). The vegetation of the hill is coastal Mediterranean and consists mainly of sclerophyllous shrubs such as *Baccharis macraei* and *Puya chilensis*, perennial herbs as *Bahia ambrosioides* and *Happlopapus foliosus* and annual species such as *Pasithea coerulea* and *Alstroemeria pulchra*. *Bipinnula fimbriata* is the only orchid species in this site, and its population covers the entire hill, with four main subpopulations in four zones differing in slope orientation: North, South, East and West (Fig. 1D).

Sampling and fertilization experiment

Twenty plots of 50 × 50 cm were established in the study site with a distance of at least 1 m between them (the minimum distance was 1m and the maximum was 229 m). The plots had between three and five adult plants of *Bipinnula fimbriata*, and covered the entire orchid population, including the four main zones (Fig. 1D; Fig. S1, Supporting Information). During the 2015 flowering season, three plants were sampled from each plot (60

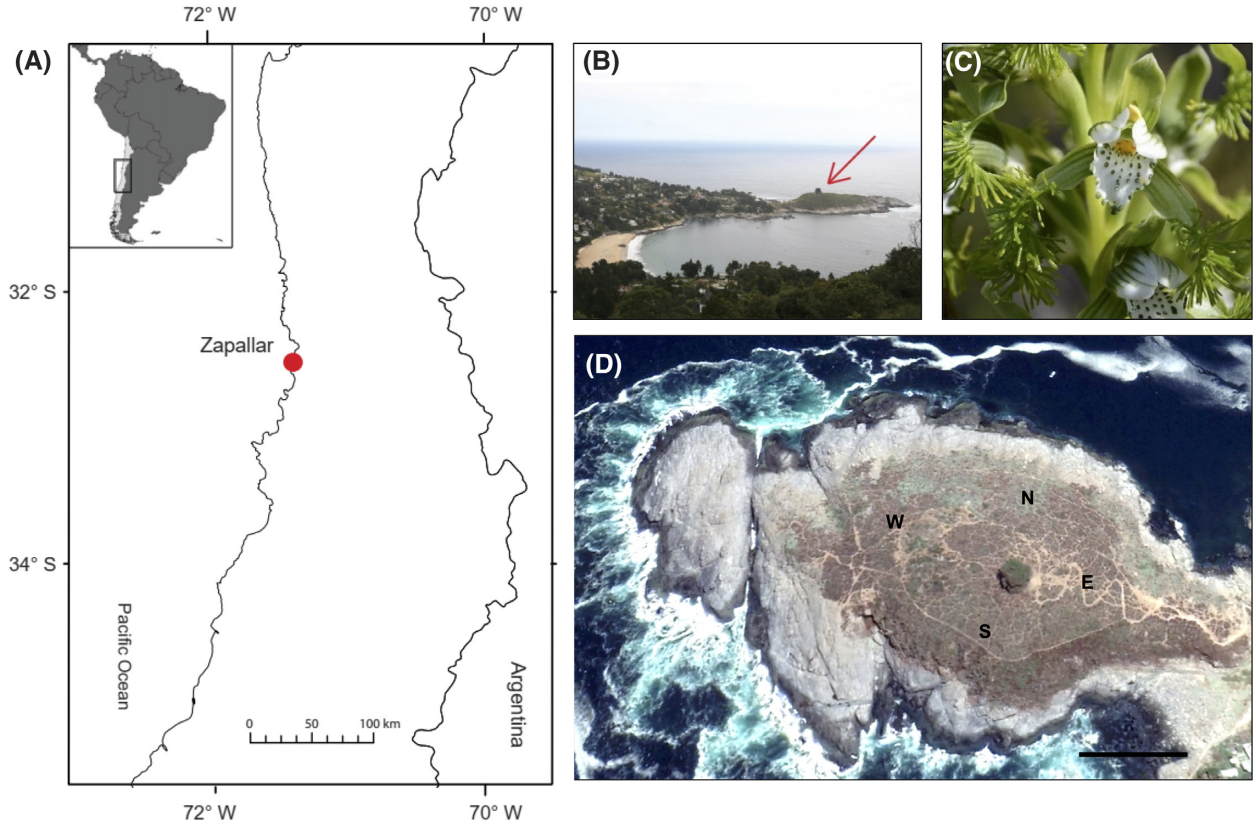

Figure 1. The geographic location of the study site 'Cerro La Cruz'. **(A)**, Regional map with the town Zapallar, where Cerro la Cruz is situated. **(B)**, Red arrow indicates the Cerro La Cruz. **(C)**, *Bipinnula fimbriata* growing at the study site. **(D)**, The four zones of the *B. fimbriata* population in Cerro La Cruz; N = North, S = South, E = East, W = West; scale bar: 100 m.

individual samples in total). To avoid unnecessary plant damage, each sample consisted of three to six root pieces per plant, leaving the rhizome intact, so that the plant could survive (no death was observed over the 3 years after sampling). Collected roots were individually labeled and kept cold during transport to the laboratory, where they were processed. For soil nutrient analyses, one mixed soil sample (1 kg of soil) was extracted in each plot, obtained from the soil that surrounded the sampled orchid roots. Soil samples were dried at 60°C for 24 hrs, sieved and then analyzed for % total N and % total C in the Laboratory of Biogeochemistry at the Pontificia Universidad Católica de Chile (LABASI-PUC, following methods described in Rebolledo *et al.* 2018); while Olsen P and pH were analyzed in the Soil Laboratory at the Pontificia Universidad Católica de Chile. After the 2015 sampling, a P addition treatment was established, where half of the plots received the fertilization treatment while the rest served as control. The treatments were randomly assigned to plots, but both treatments were replicated in each zone (Fig. S1, Supporting Information). The P addition (P) consisted of 10 gr of Triplesuperphosphate ($Ca(H_2PO_4)_2 \bullet H_2O$) in 0.5 L of distilled water while the control (C) received only 0.5 L of distilled water, without nutrient addition. Fertilization or control solutions were applied with a wash bottle covering all the surface of each plot. The same root and soil sampling procedure conducted the first year (2015) was again conducted one year after fertilization during the flowering season (spring 2016).

Sample preparation

Root pieces (of 5 to 10 cm length) were washed under tap water to remove soil and dirt and sterilized as follows: samples were placed for 1 min in 1% sodium hypochlorite and then three times consecutively for 3 min in sterile distilled water. OMF form pelotons in root cortex cells and in *Bipinnula*, as in many other orchid species, groups of pelotons can be viewed on the washed root surfaces as light yellow to dark brown spots under the stereomicroscope. For each root piece, the level of colonization by mycorrhizal fungi was quantified as the fraction of the root surface covered by spots based on coloration, after calibration tests using microscopy proved that coloration successfully identified the colonized and uncolonized root sectors. We used three to six root pieces per plant to quantify colonization. From these pieces, we obtained three root sections 3 mm-wide, that were placed together in one sterile 2mL tubes and stored at −20°C until DNA extraction (three colonized root sections per plant). DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1990).

Metabarcoding analysis

To investigate the fungi associated with roots of the studied plants we used two pairs of primers for each sample to amplify the ITS2 region of the ribosomal DNA, namely ITS86F-ITS4 and ITS3-ITS4OF (White *et al.* 1990; Turenne *et al.* 1999; Taylor and McCormick 2008; Table S1). These ITS primers were used to amplify DNA samples in a first PCR. Each DNA sample was amplified separately with each ITS pair of primers using the following profile: 95°C for 7 min, followed by 30 cycles at 94°C for 30 s, 59.2°C for 40 s, 72°C for 60 s, and a final extension at 72°C for 10 min. The ITS primers had a supplementary sequence at the beginning, which is recognized by the IonXpress barcode primers in a second PCR. After the first PCR, PCR products obtained from each pair of primers were pooled for each sample. Then, these pooled PCR products were amplified in a second

PCR with a unique adaptor (IonXpress barcode primers) for identification after sequencing. Second PCR was run with similar conditions except for the annealing temperature of 60.4°C. The samples obtained were purified with NucleoMag© NGS Clean-up and Size Select (Macherey-Nagel) and subsequently, as one pooled amplicon library, were sequenced with an Ion Torrent PGM (Life Technologies, Carlsbad, USA). The sequenced reads were processed following the procedure described in Schneider-Maunoury *et al.* (2018). Briefly, reads were first demultiplexed and split based on the unique tags, screened for the presence of the primers using Cutadapt version 1.15 (Martin 2011) and trimmed. Next, we generated a reference database of OTUs (operational taxonomic units) using the QIIME pipeline, version 1.9.1 (Caporaso *et al.* 2010). First, we clustered the sequenced reads into OTUs with the SWARM algorithm (Mahé *et al.* 2014), using a threshold of 97% of sequence similarity. After removing singletons, we selected one representative sequence for each chosen OTU, and removed chimeras incorporating the UCHIME software (Edgar *et al.* 2011) to map against the reference dataset for ITS chimera detection (Nilsson *et al.* 2015) version 7.2 (release 2017–06-28) obtained from the UNITE database (Nilsson *et al.* 2019b). Next, we filtered and trimmed the original sequencing reads based on the presence of ITS86F or ITS4 primers using Cutadapt software (the primer pair ITS86F-ITS4 is included in the region amplified by the pair ITS3-ITS4OF). Using BLASTN (Altschul *et al.* 1990), we clustered the filtered reads into OTUs by searching against the OTU reference database generated in the previous steps. Assignment of taxonomy for each OTU was finally recovered by comparing their representative sequences against the reference fungal database UNITE version 7.2 (release 2017–12-01) (Nilsson *et al.* 2019b) using the BLAST algorithm. A final filtering step was to remove non-fungal OTUs (n = 2) and OTUs that corresponded to potential laboratory contamination: fungi from the genera *Wallemia* (n = 5) *Candida* (n = 8) and *Malassezia* (n = 6; Findley *et al.* 2013; Zajc and Gunde-Cimerman 2018; Nilsson *et al.* 2019a). After removing these OTUs, relative abundances of OTUs were recalculated and the reads from the three plants per plot were merged. Then, we considered a threshold of 10 000 sequences per plot.

Fungal guilds

Fungal OTUs were classified into the nutritional guilds: OMF were assigned following (Dearnaley, Martos and Selosse (2012) and the non-mycorrhizal fungal OTUs were classified into three different trophic modes following the classification of FunGuild v1.0 (http://www.stbates.org/guilds/app.php). The guilds rely on the nutrient acquisition mode: (i) pathotroph acquiring nutrients by harming host cells; (ii) symbiotroph exchanging resources with living host cells in a likely mutualistic way and (iii) saprotroph breaking down dead host tissues (Nguyen *et al.* 2016).

Statistical analyses

We merged the reads from three plants per plot. Then, we first analyzed the data of the pre-fertilization samples (2015 season) and tested the effects of zone (of the site, see Fig. 1D; Fig. S1, Supporting Information) and soil nutrients on composition, relative abundance (measured as the proportion of reads in the sample) and richness of each fungal guild and on the level of colonization by OMF. To assess the effects on the composition of the whole root fungal community, fungal guild composition was calculated as the relative abundance of each fungal guild in each plot. The dissimilarity between plots was calculated using Bray–Curtis distances (Bray and Curtis 1957) and then we performed a variance analysis of these distances, using a multivariate permutation test (Adonis test) implemented in the Vegan package of R (Oksanen *et al.* 2013). We included zone, soil nutrients and the interactions between these factors. The effect of zone and nutrients on plot ordination was illustrated by a non-metric multidimensional scaling (NMDS) on Bray–Curtis distances. To further explore the effect of the same factors on the composition of each fungal guild, we calculated the composition of each guild separately. Additionally, the composition of the whole fungal community and of each fungal guild was calculated using OTUs presence and absence rather than relative abundances. For this, the similarity between plots was calculated using Raup–Crick method and then the same Adonis analyses described above were implemented. To assess the effect of zone and soil nutrients on the relative abundance and OTU richness of each fungal guild, we used generalized linear models (GLMs). Each response variable was modeled separately, testing the following components: zone, soil nutrients and the interactions between these factors. In this way, we tested whether the effect of soil nutrients on mycorrhizal fungi differed among zones. Models were built using a bidirectional stepwise selection procedure, starting with a full model and alternately omitting and re-introducing one model component at each step (Pearce and Ferrier 2000). Models were selected according to the lowest values of the Akaike and Bayesian information criteria (AIC and BIC, respectively). The GLM deviance was estimated as the goodness of fit. Significant relationships among variables were described using partial residual plots of the most likely model as judged by the AIC. To explore the relationship between OMF and non-mycorrhizal fungi with an independent measure of OMF abundance (rather than relative abundances), we evaluated the relationship between non-mycorrhizal guilds and mycorrhizal colonization. In addition, to further assess the spatial structuring of fungal guilds, we built a dissimilarity matrix based on Bray–Curtis distances of composition for each fungal guild. Then, we performed a Mantel test between these composition dissimilarity matrices and the distance matrix (built with Euclidian distance among plots). Finally, to test the effect of fertilization, the same analyses on relative abundance, diversity and composition of each fungal guild were performed (linear models and multivariate permutation test), but with samples obtained in the year after the fertilization (2016) and using fertilization treatment and changes in soil P as explanatory variables.

RESULTS

Fungal community found in *Bipinnula fimbriata* roots

Sequencing yielded a total of 8445134 sequences from 120 plants sampled (60 plants each year); from these, 4294 121 sequences passed quality filtering and then after removing non-fungal sequences and laboratory contamination 2843472 fungal sequences remained. We obtained an average of 71 086 sequences per plot, ranging from 12 548 to 232 500 sequences. Sequences were assigned to 668 OTUs based on a 97% similarity threshold, which belonged to 33 classes in 10 phyla (Table S2, Supporting Information). The orders with the highest number of OTUs were Pleosporales (66), Chaetothyriales (40), Hypocreales (38) and Agaricales (35). Regarding fungal guild assignment (OMF, pathotroph, saprotroph or symbiotroph, Fig. S2, Supporting Information), 10 OTUs could be considered as putative

Table 1. Effects of zone and soil nutrients on fungal guild composition associated with roots of *Bipinnula fimbriata*.

Factor	d.f.	F	R^2	P-value
Zone	3	6.59	0.54	0.00
P	1	3.07	0.08	0.05
N	1	1.30	0.04	0.29
%C	1	0.32	0.01	0.79
pH	1	2.02	0.06	0.15

species of OMF (773054 reads): three were related to Tulasnellaceae (two species of *Tulasnella*, *T. calospora* and *T. asymmetrica,* and one unidentified Tulasnellaceae); three to the Ceratobasidiaceae (one unidentified *Ceratobasidium*, *Thanatephorus cucumeris*, and one unidentified Ceratobasidiaceae) and four to the Sebacinales (*Serendipita vermifera*, two unidentified species of the families Sebacinaceae and Serendipitaceae, respectively and one unidentified Sebacinales). Most of the plants had OMF in their roots (86.4%), and the OTU Ceratobasidiaceae sp. was the most frequently present (83%, Fig. 2). However, some plants did not display OMF. The Pezizaceae OTU, were found in 85.5% of plants, including some plants where typical OMF were not detected. Since the mycorrhizal status of Pezizaceae remains unclear, because formation of typical mycorrhizal pelotons is hitherto undemonstrated, it was considered as a separate guild for composition analyses.

From the rest of the non-mycorrhizal species (658 OTUs), a putative trophic mode was found for 523 OTUs using the FunGuild database (Fig. S2, Supporting Information). They were assigned to pathotroph (118 OTUs; 180638 reads), saprotroph (187 OTUs; 260034 reads) or symbiotroph (51; 63421 reads) guilds, or to the mixed guilds saprotroph-symbiotroph (23 OTUs; 337705 reads), pathotroph-saprotroph (70 OTUs; 173397 reads), pathotroph-symbiotroph (25 OTUs; 209687 reads), pathotroph-saprotroph-symbiotroph (49 OTUs; 341135 reads). Among the most frequent OTUs in each guild were the pathotrophs *Ilyonectria mors-panacis* and *Rhexocercosporidium panacis*, the saprotrophs *Filobasidium stepposum* and *Cladophialophora* sp, and the symbiotrophs *Rhodotorula glutinis* and *Cladosporium sphaerospermum* (Fig. 2). Finally, a surprisingly large and phylogenetically diverse number of ectomycorrhizal OTUs were found, including *Scleroderma*, *Cortinarius*, *Cenococcum*, and *Tomentellopsis* species.

Fungal guild composition (abundances)

Fungal guild composition was significantly affected by zone (of the site, see Fig. 1D) and soil P, but not by the other soil nutrients (Fig. 3A), as shown by the Adonis analysis (Table 1). The Mantel test also showed a spatial effect on composition, since a significant correlation between geographic distance matrix and the fungal guild composition dissimilarity was observed ($R^2 = 0.132$, $P = 0.05$). While North and East zones displayed more abundant OMF, the West Zone showed equal abundance of OMF and Pezizaceae, and the South was more abundant in pathotrophs with a very low abundance of OMF (Fig. 3C). Accordingly, GLM analyses on the relative abundance of each guild showed that some guilds were correlated with soil P, and that each fungal guild responded differentially to nutrients and zone. OMF relative abundance was influenced by zone, soil P and soil %C (AIC null model = 1.5, AIC best model = −30.3, $R^2 = 0.85$), showing a negative relationship with soil P and a positive relationship with soil %C (Fig. 4A and B). However, the negative relationship with soil P was not maintained if we remove the two highest values of this variable. The relative abundance of pathotrophs was significantly explained by zone, soil P and soil pH (AIC null model = −12.35, AIC best model = −24.8, $R^2 = 0.6$), showing a positive relationship with pH, and unlike OMF, a positive relationship with soil P. The relative abundance of saprotrophs was also affected by zone and soil pH (AIC null model = −50, AIC best model = −57.69, $R^2 = 0.51$), while both the relative abundance of symbiotrophs and Pezizaceae spp. showed no differences among zones and no effect of soil nutrients. When the composition of each guild was analyzed separately, it also showed a zone effect. For OMF composition, NMDS showed a clear differentiation between zones (Fig. 3B), statistically supported by Adonis analysis (zone effect: $F = 3.12$, $P = 0.002$). Composition of the other three guilds also showed a zone effect, which according to the Adonis analysis was significant in the symbiotrophs but nearly significant in the other two guilds (zone effect in symbiotrophs $F = 3.7$, $P = 0.02$; pathotrophs $F = 1.2$, $P = 0.06$; saprotrophs $F = 1.8$, $P = 0.08$). Results were similar when composition was assessed using presence/absence data, except for the OMF guild, for which no effect of zone was observed (Table S3, Supporting Information). The Mantel test showed no significant correlation between geographic distance matrix and the separate composition dissimilarity matrix of each of these fungal groups.

Fungal richness

OMF richness varied considerably among plants, from zero to 6 OTUs. The best model explaining this variation included only zone (AIC null model = 76.9, AIC best model = 67.4, $R^2 = 0.45$), with East and West having the greatest richness (Fig. 4D). There was no effect of soil nutrients or zone on the richness of the other three fungal guilds.

Mycorrhizal colonization and non-mycorrhizal fungi

The opposite relationship that showed pathotrophs and OMF relative abundances with soil P, suggested a possible negative correlation between both guilds. To further explore this with an independent measure of OMF abundance (instead of relative abundances), we evaluated the relationship between non-mycorrhizal guilds and mycorrhizal colonization as evaluated on the roots. The relative abundance and the richness of pathotrophs were both significantly affected by the interaction between zone and mycorrhizal colonization (abundance: $R^2 = 0.77$; richness: $R^2 = 0.56$). Therefore, these relationships were analyzed separately by zone. Mycorrhizal colonization displayed a negative relationship with pathotroph relative abundance, which was significant in the South zone ($R^2 = 0.79$, $P = 0.01$) and almost significant in the West zone ($R^2 = 0.6$, $P = 0.07$), while no significant relationship emerged in the North and East zones (Fig. 5A). Regarding richness, the relationship between pathotroph richness and mycorrhizal colonization was strong in the South zone ($R^2 = 0.85$, $P = 0.005$) and East zone ($R^2 = 0.85$, $P = 0.05$), nearly significant in the North zone ($R^2 = 0.61$, $P = 0.07$) and non-significant in the West zone (Fig. 5B). The richness of saprotrophs also showed a significant but lower negative relationship with mycorrhizal colonization (AIC null model = 142.2, AIC best model = 136, $R^2 = 0.3$), and there was no effect of mycorrhizal colonization on symbiotrophs.

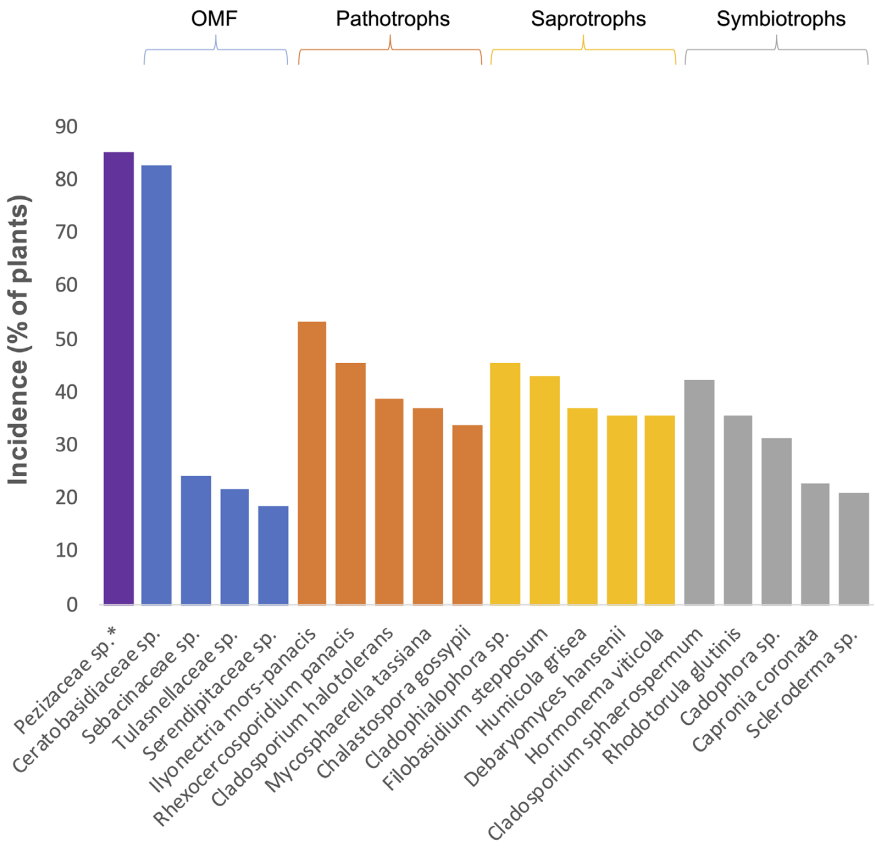

Figure 2. Incidence of the five most frequent OTUs in each fungal guild found in the roots of *Bipinnula fimbriata*. *The role of Pezizaceae in orchid roots is still unclear (see text), but the OTU Pezizaceae sp. is displayed to show its high incidence in *B. fimbriata* plants.

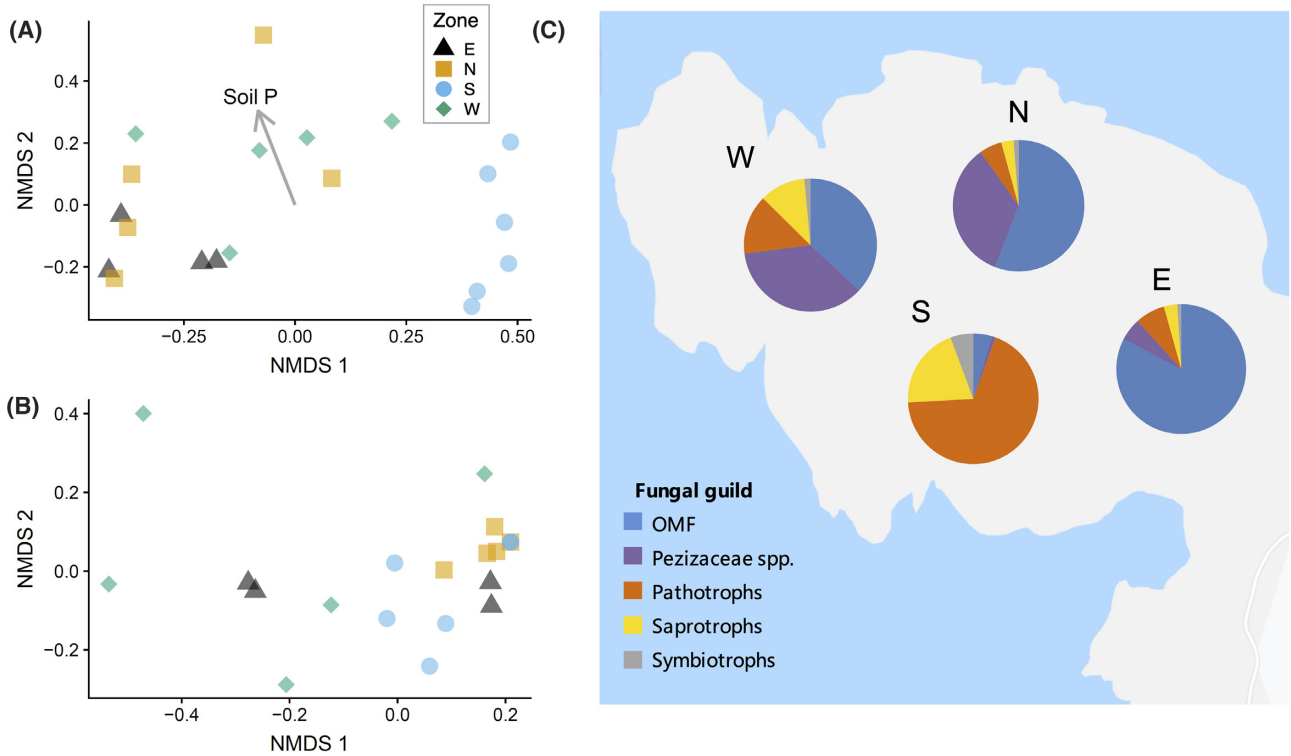

Figure 3. Non-metric multidimensional scaling (NMDS) ordination plots based on the composition of **(A)**, the whole fungal community associated with *Bipinnula fimbriata* and **(B)**, putative mycorrhizal fungal species (OMF). Explanatory variables found to be significant ($P > 0.05$) in the Adonis test are represented as a vector (soil P) or by color (zone). **(C)**, Pie charts showing the abundance of each fungal guild in the four zones of the *B. fimbriata* population in Cerro La Cruz.

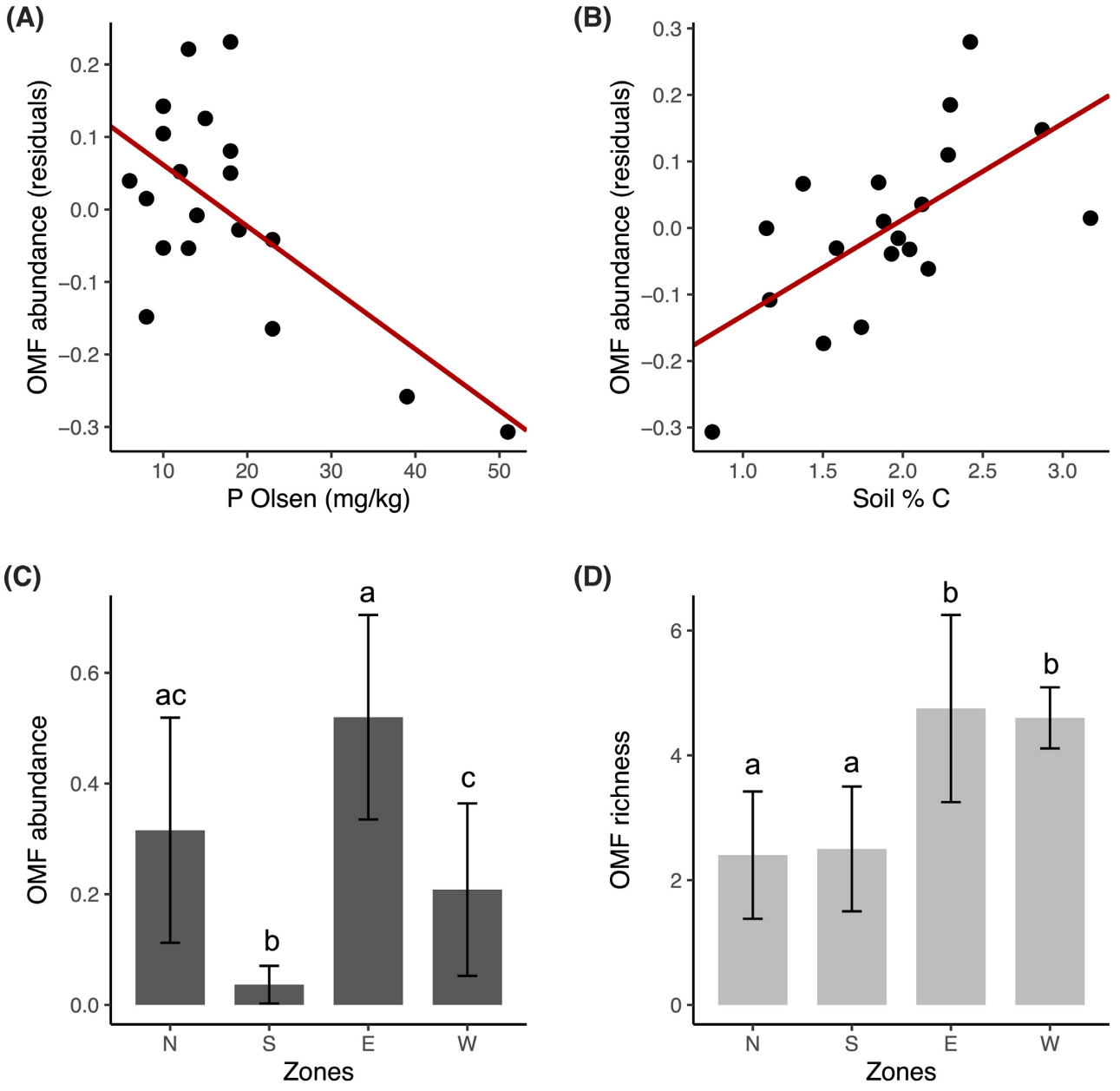

Figure 4. Relationships between abundance and richness of OMF and investigated parameters: OMF abundance as a function of P Olsen **(A)**, soil % C **(B)** and zone **(C)** (AIC null model = 1.5, AIC best model = −30.3, $R^2 = 0.85$), and OMF richness as a function of zone **(D)** (AIC null model = 76.9, AIC best model = 67.4, $R^2 = 0.45$).

Effects of experimental fertilization

Soil P Olsen was significantly higher in P-fertilized plots than in control ones ($F = 42.7$ $P = 3.8e\text{-}6$). However, there was considerable variation in δ P (difference in P between after and before treatment; i.e. value after minus value before) within each treatment (fertilized plots $\delta P = 10.2$ (mean) ±17.53 (SD) mg/kg); control plots $\delta P = 0 \pm 5.25$ mg/kg) and when all plots were analyzed together (control and P treatment), 52% of them showed a significant increase in soil P. There was no significant effect of P fertilization on OMF relative abundance, richness and composition. Also, there was no direct effect of P fertilization on mycorrhizal colonization; however, when considering only the plots that presented an actual increment of soil P after fertilization, the δ P significantly and negatively correlated with δ %mycorrhizal colonization ($R^2 = 0.55$, $P = 0.02$; Fig. 5C). There was no direct effect of P fertilization on the richness and relative abundance of pathotrophs, saprotrophs, Pezizaceae and symbiotrophs. However, given that we observed a negative relationship between mycorrhizal colonization and pathotroph richness and abundance in the first year, we examined whether there was an effect of decrease in mycorrhizal colonization on pathotrophs. Then, when considering the plots where mycorrhizal colonization decreased, the δ pathotroph richness significantly and negatively correlated with δ %mycorrhizal colonization ($R^2 = 0.21$, $P = 0.05$; Fig. 5D).

DISCUSSION

Diversity of fungal community in *Bipinnula fimbriata* roots

By using metagenomics to assess root fungal community, this study reveals a high diversity of mycorrhizal and non-

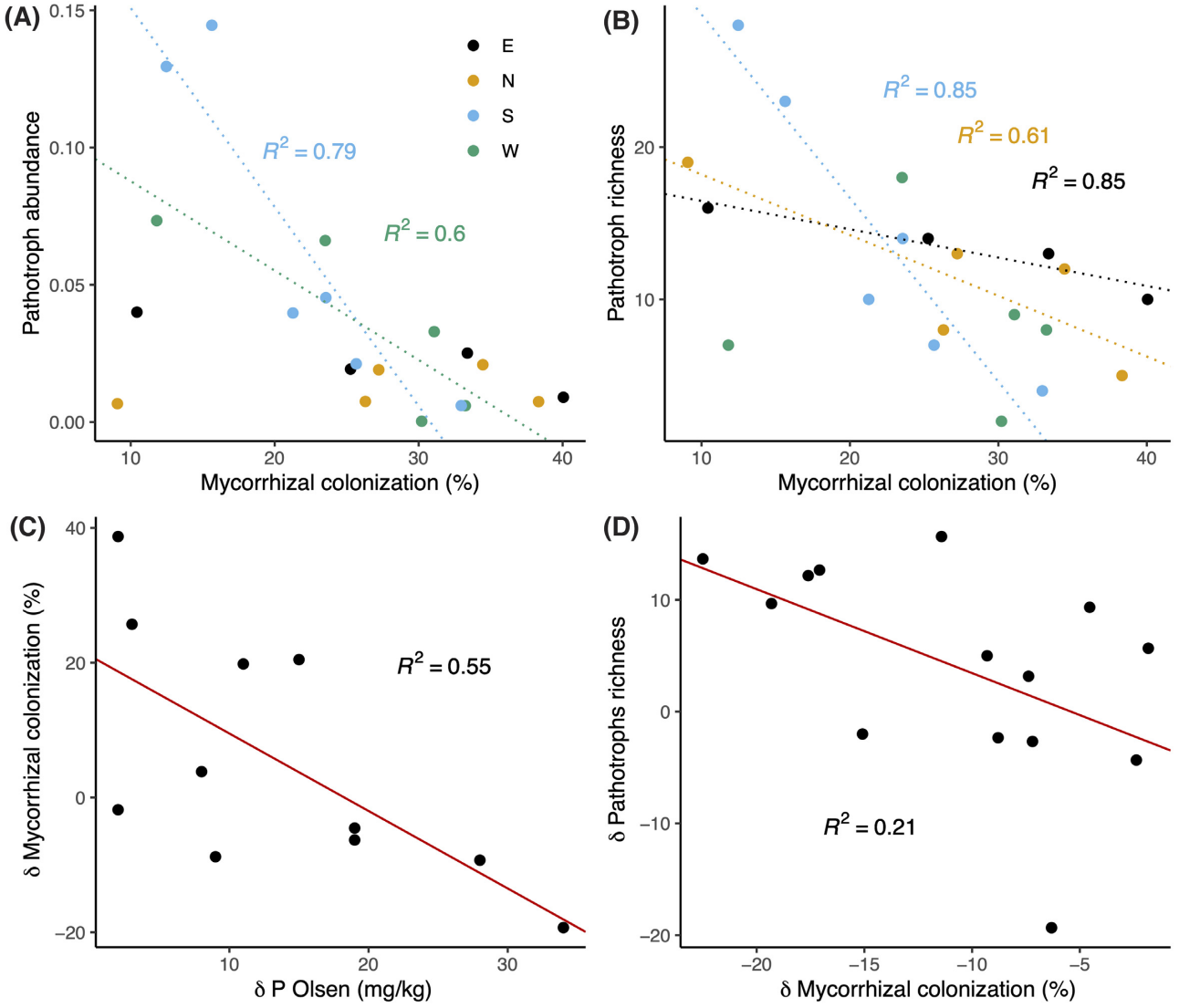

Figure 5. Relationship between mycorrhizal colonization (%) and **(A)**, relative abundance and **(B)**, richness of pathotrophs. Since there was a significant effect of the interaction between zone and mycorrhizal colonization, relationships are plotted separately by zone. Dotted lines show significant relationships. **(C)**, Relationship between δ P Olsen (difference between after and before P fertilization, i.e. value after minus value before) and δ %Mycorrhizal colonization, and **(D)** relationship between δ %Mycorrhizal colonization and δ pathotroph richness.

mycorrhizal fungi and fills a gap in the knowledge of fungal root-associates in terrestrial orchids from South America (Jacquemyn, Duffy and Selosse 2017). Across all samples, we found 10 OMF OTUs belonging to the Tulasnellaceae, Ceratobasidiaceae, and Sebacinales, i.e. the whole taxonomic range of typical OMF (Dearnaley, Martos and Selosse 2012). The same families of OMF have been reported for *B. fimbriata* across different populations using culturing techniques (Steinfort *et al.* 2010; Mujica *et al.* 2016; Herrera *et al.* 2019), with the exception of Serendipitaceae, which was not detected by those studies. Lack of Serendipitaceae has been reported in studies by culturing techniques of other terrestrial orchid species from southern South America (Herrera *et al.* 2019; Silva-Flores *et al.* 2019).

Plants displayed from zero to 6 OMF OTUs, but interestingly 11 of the 16 plants without OMF presented OTUs from Pezizaceae, similar to the observations of Waterman and Bidartondo (2008) in Mediterranean orchid species from the Cape Region, with a high frequency of the OTU 'Pezizaceae sp.' in the roots of *Bipinnula fimbriata* (98 of the 118 plants sampled). Putative Pezizaceae spp. are also associated in other populations of *B. fimbriata* and *B. plumosa* (Mujica *et al.* 2016). Indeed, Pezizomycetes are usually associated with terrestrial and epiphytic orchid roots (e.g. Stark, Babik and Durka 2009; Jiang *et al.* 2011; Ma *et al.* 2015; Těšitelová *et al.* 2015; Jacquemyn *et al.* 2017) and has been suggested as a possible mycorrhizal partner of orchids (Dearnaley, Martos and Selosse 2012); indeed some *Epipactis* species are mycorrhizal with *Tuber* ssp. (Selosse *et al.* 2004). Further microscopic studies to assess the interaction morphology or *in vitro* tests of their interaction with orchids are now needed.

The finding of ectomycorrhizal fungi from diverse taxa was partially unexpected: on the one hand, we did not identify ectomycorrhizal host plants that could support such fungi in the study area; on the other hand, some ectomycorrhizal species have an ability to colonize non-ectomycorrhizal plants as root endophytes (Schneider-Maunoury *et al.* 2018; Selosse *et al.* 2018) and their presence is already reported in other orchid roots

(Selosse *et al.* 2010; Jacquemyn *et al.* 2017) in addition to OMF, raising the possibility as for Pezizaceae of a mycorrhizal interaction with photosynthetic orchids, for which again more studies are needed.

We also report a high diversity of non-OMF, non-ectomycorrhizal fungi. Although the ecological roles of such fungi in orchids are largely unknown (Jiang *et al.* 2011) and likely diverse, the assignment of trophic guilds can give clues to their functions in the roots of *B. fimbriata*. We detected pathotrophic fungi such as *Ilyonectria mors-panacis, Rhexocercosporidium panacis* and *Fusarium oxysporum*. *I. mors-panacis* and *R. panacis* are highly pathogenic in ginseng (*Panax quinquefolius*; Reedeler, Roy and Capell 2002; Farh *et al.* 2017), but we did not find any report of these species infecting orchid roots. On the one hand, there may be true endophytism (i.e. symptomless, commensal to mutualistic; sensu Wilson 1995) in species otherwise reported as pathotrophs, as demonstrated for some *Fusarium* spp. (Lofgren *et al.* 2018). On the other hand, *F. oxysporum* has been demonstrated to cause root rot and other symptoms in several commercial orchid species including vanilla (Kim *et al.* 2002; Swett and Uchida 2015; Koyyappurath *et al.* 2016) and has been recently described as mycorrhizal in the orchid *Bletilla striata* (Jiang *et al.* 2019).

Among the saprophytic taxa observed, some such as *Humicola* sp., *Acremonium* spp., and *Trichoderma*, have already been reported in other orchid species (Bayman and Otero 2006). Similarly, some of the symbiotroph taxa that we detected, such as *Hypoxylon* and *Colletotrichum*, promote growth in plantlets of the orchid *Rhynchostylis retusa* (Shah *et al.* 2019). Nevertheless, the ecological role of most orchid root endophytes remains mostly unknown (Jiang *et al.* 2011) and is likely multiple. Although some contaminations, from soil or during harvesting, cannot be fully ruled out, our observations illustrate our limited and often too restrictive view of fungal ecological niches (Selosse, Schneider-Maunoury and Martos 2018), which deserve further functional investigations in orchids. Moreover, the distinction between mycorrhizal and non-mycorrhizal fungi in orchids is challenging, because mycorrhizal taxa are continuously discovered (Zhang *et al.* 2012; Jiang *et al.* 2019).

Finally, it is important to notice that because our focus was on mycorrhizal and non-mycorrhizal fungi, we chose the primers ITS86F-ITS4 and ITS3-ITS4OF, a complementary combination that amplifies significant sequence diversity and a variety of orchid-associating mycorrhizal families (Waud *et al.* 2014; Jacquemyn *et al.* 2017). However, these pairs of primers might undersample the core-Tulasnellaceae fungi, since this group might require the use of more specific primers. Indeed, Waud et al (2014) recommended to complement the use of ITS86F-ITS4 and ITS3-ITS4OF with specific Tulasnellaceae primers, and suggested the use of a targeting reverse primer developed within the 5.8S rRNA region. Recently, Vogt-Schilb *et al.* (2020) developed a new primer (5.8S-OF) and showed that almost 70% of core Tulasnellaceae OTUs were amplified only by the primer combination 5.8S-OF/ITS4Tul, confirming the need for specific primers to assess the diversity of Tulasnellaceae. Therefore, although here we did detect core-Tulasnella OTUs (OTU T1 in Mujica *et al.* 2016), we cannot exclude that the abundance and diversity of Tulasnellaceae was overlooked. Indeed, in our previous study (Mujica *et al.* 2016) in the same study site, we detected more Tulasnellaceae OTUs by culturing colonized roots and using specific Tulasnella primers (ITS1/ITS4-Tul) than currently. Nevertheless, used in a comparative way, the primers used in this study usefully reveal the effects of soil conditions on the orchid root fungal community.

Effect of zone and nutrients on root fungal communities

Fungal guild composition varied highly among the four zones of the *B. fimbriata* population and the Mantel test showed a correlation between geographic and fungal composition distances. Both results reflect a spatial structure of fungal root communities, a spatial turnover well characterized in the fungal community, e.g. for ectomycorrhizal fungi (Bahram *et al.* 2013). Composition also correlated with soil P and across zones, and we found a negative relationship between soil P and relative abundance of OMF (Fig. 4A). At lower levels of soil nutrients (N or P), plants tend to allocate more carbon to mycorrhizal partners (Johnson *et al.* 2003; Treseder 2004) to maximize the nutrient uptake, thus possibly increasing abundance of mycorrhizal fungi within roots. Changes in C allocation as a function of nutrient availability has not yet been studied in orchid mycorrhiza, but it is likely to occur, as there is evidence of C supply from photosynthetic orchid to mycorrhizal fungi (Cameron, Leake and Read 2006) and obtaining inorganic P from them (Cameron *et al.* 2007). In contrast, the negative relationship between soil nutrients and root fungal abundance is not expected to occur in non-mycorrhizal fungi, given that plants do not obtain mineral nutrients from them. Accordingly, there was no negative correlation between the relative abundance of non-mycorrhizal fungi and soil P.

The tendency of plants to allocate less C to their fungal partners under higher nutrient availability may also explain the observed negative effect of P addition on mycorrhizal colonization one year after treatment. This effect has been reported in fertilization experiments on arbuscular and ectomycorrhizal plants (e.g. Baum and Makeschin 2000; Treseder and Vitousek 2001). Remarkably, the changes in mycorrhizal colonization were not related to significant changes in mycorrhizal richness or composition, suggesting that, at this temporal scale (i.e. one year after P addition), plants reacted by controlling colonization level without any effect on the filtering of the associated fungal diversity. Switching fungal partner under environmental changes has been observed in the orchid *Goodyera pubescens* (McCormick *et al.* 2006), where plants switched fungal partners from one year to the next. However, this occurred under extreme conditions (death of the initial fungus and substantial host mortality; (McCormick *et al.* 2006), so that switching fungi appeared to be the last recourse to face extreme conditions rather than a response to minor environmental fluctuations such as our P fertilization.

Finally, while P addition apparently did not affect non-mycorrhizal fungal diversity, our results suggest that it may have an indirect effect on pathotrophs, either by negatively affecting mycorrhizal colonization and its protection against pathogens or more directly by affecting plant metabolism, with consequent positive effects on pathogen food supply (Walters and Bingham 2007). Given that mycorrhizal colonization negatively correlated with abundance and richness of root pathotrophs (Fig. 5), we hypothesized that a decrease in mycorrhizal colonization could cause the increase in abundance of these fungi. Thus, P fertilization can influence other guilds of the root fungal community, perhaps by way of the level of mycorrhizal colonization. This observation agrees with other studies that have found important shifts in root-associated fungal community under short-term P fertilization (Nielsen *et al.* 2015; Fabiańska *et al.* 2019).

In addition to soil P, the soil C also appears to affect the relative abundance of OMF. The positive relationship between soil C and the relative abundance of OMF (Fig. 4B) could be explained by the saprophytic nutritional mode of OMF fungi (Dearnaley,

Martos and Selosse 2012; Kohler *et al.* 2015), even if their ecological niche may be larger than only saprophytism (including endophytism in non-orchid plants; Selosse and Martos 2014). Although soil C includes total organic and inorganic carbon, given that carbonates are unlikely to be abundant in this site, it mostly encompasses organic C, which may correlate with available nutritional resources for OMF. In a similar way, Bunch *et al.* (2013) found that orchid populations growing in environments with moderate to higher levels of C, N and organic matter had a higher diversity of OMF.

Interaction between mycorrhizal and pathotrophic fungi

Mycorrhizal colonization was negatively related with richness and relative abundance of pathotrophs, which we suggest being related to a potential role of OMF in defense against pathogens since the alternative explanation (pathotrophs excluding OMF) looks less likely. Indeed, although the relationship between pathogen abundance and mycorrhizal colonization has not been reported before in orchid mycorrhiza, it has long and frequently been reported in other mycorrhizal types (*e.g.* Marx 1972; Newsham, Fitter and Watkinson 1994; Azcón-Aguilar and Barea 1997). Recently, Herrera *et al.* (2018) found that mycorrhizal root segments of *Bipinnula fimbriata* stimulated protein synthesis related to pathogen control, which could partially explain our field observations. Future research, especially experimental, is required to test the role of mycorrhizal colonization against pathotrophs in orchids, and to understand the underlying mechanisms. This may be of crucial interest for biocontrol in commercial orchid species (i.e. vanilla cultivation; Koyyappurath *et al.* 2016), where a large provisioning of nutrients may, by reducing OMF colonization, challenge the ability of these fungi to protect the orchid.

CONCLUSIONS

We found a high diversity of fungal associates in roots of *Bipinnula fimbriata* in which there is strong spatial structuring over a relatively small area. Our results showed that soil P was negatively related to the abundance of OMF, but not with the non-mycorrhizal endophytes and that experimental P fertilization negative affected mycorrhizal colonization. We also observed a negative relationship between mycorrhizal colonization and richness and abundance of pathotrophs. P fertilization caused an increment in pathotroph richness in *B. fimbriata* roots, perhaps due to reduced mycorrhizal colonization. These observations indirectly suggest that in addition to seed germination, development and nutrient mycorrhizal exchange, OMF may contribute to orchid defense against pathotrophs. Finally, we observed that mycorrhizal and non-mycorrhizal fungi respond differentially to soil nutrient availabilities. Our results support the idea that soil nutrients play a key role in structuring fungal communities on orchid roots. This information will contribute to *ex situ* work on conservation and adaptation to substrate nutrient level and may also be of interest for commercial orchid production.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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