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## RESEARCH ARTICLE

# Mycorrhizal communities of *Vanilla planifolia* in an introduction area (La Réunion) under varying cultivation practices

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## Societal Impact Statement

Vanilla is one of the most valuable spices in the world. In Madagascar and La Réunion, the world's leading producers, vanilla is of great economic and cultural importance. Like all orchids, vanilla plants associate with mycorrhizal fungi in their roots forming mutualistic associations that allow them to grow and thrive. Understanding the diversity of mycorrhizal fungi adapted to vanilla cultivation, particularly in the Indian Ocean islands where they have never previously been studied, is becoming a necessity for maintaining vanilla crops in these regions in the face of climate change and the emergence of new pathogens.

## Summary

- The vanilla orchid (*Vanilla* spp.) is one of the most valuable cultivated plants worldwide. As with all orchids, vanillas form mycorrhizal associations with fungi in their roots, but their fungal partners have not been investigated outside their native geographic range in Central America.
- We investigated the whole fungal and mycorrhizal associations in cultivated vanilla (*Vanilla planifolia*) by sequencing the fungal ITS-2 marker in the terrestrial and aerial roots using a metabarcoding approach. We selected plants cultivated in three conditions (i.e., cultivation under shade house, in openfield, or in the understory) in one locality of La Réunion island (Indian Ocean) and tested for a possible effect of cultivation practices on fungal communities.
- Cultivated vanillas in La Réunion mainly associate with Tulasnellaceae (75 OTUs) and Ceratobasidiaceae (8 OTUs). Among the seven most abundant Tulasnellaceae, six are similar to fungi detected in the roots of cultivated vanillas in Central America or in the roots of native orchids in La Réunion. Cultivation practices impacted both total fungal and mycorrhizal community compositions with no clear effect on fungal richness. Notably, Tulasnellaceae and Ceratobasidiaceae were scarce in aerial roots, except in the traditional cultivation in the forest understory.
- These results shed light on the geographical origins of mycorrhizal fungi of cultivated vanillas in La Réunion and show that they form a pool of both locally and globally distributed fungal partners. These mycorrhizal communities vary

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according to cultivation practices, and their potential roles in plant nutrition and resistance against pathogens await further attention.

#### KEYWORDS

Ceratobasidiaceae, *Colletotrichum*, cultivated vanillas, epiphytic roots, *Fusarium*, orchid mycorrhizal fungi, *Resinicium saccharicola*, Tulasnellaceae

## 1 | INTRODUCTION

Vanillas (*Vanilla* spp.) are herbaceous perennial vines that root both on the ground and on the surface of trees on which they grow vertically until they reach the canopy. The genus *Vanilla* comprises more than 110 described species, all tropical or subtropical (Cameron, 2011).

As orchids belonging to the subfamily Vanilloideae (Cameron & Molina, 2006; Givnish et al., 2015), vanillas develop orchid mycorrhizas characterized by the presence of coiled fungal hyphae (known as pelotons) within the plant cell walls in root tissues (Dearnaley et al., 2012; Smith & Read, 2008). These endomycorrhizal symbioses are obligatory for orchids because their tiny seeds do not have sufficient nutrient reserves to germinate and sustain the embryo until the development of the first leaf and autotrophy (Dearnaley et al., 2012). Following the onset of photosynthesis, these fungi persist in the root cortex of seedlings and adults of orchids, where they supply water and nutrients to the hosts (Cameron et al., 2006; Fochi et al., 2017; Valadares et al., 2021). Additionally, they may also protect the orchid roots against pathogens in the rhizosphere (Jung et al., 2012; Mujica et al., 2020). In vanillas, terrestrial roots are more frequently colonized by mycorrhizal fungi than aerial roots (Porrás-Alfaro & Bayman, 2007), and the former are thus believed to serve for nutrition primarily, whereas the latter, not replaced after death (Fouché & Jouve, 1999), may primarily serve for anchorage.

In its native range in Central America, the cultivated vanilla *Vanilla planifolia* was shown to associate with two families of Basidiomycota fungi, that is, the Tulasnellaceae and Ceratobasidiaceae (both in the Cantharellales), in both terrestrial and aerial roots (Johnson et al., 2021; Porrás-Alfaro & Bayman, 2003, 2007). Additionally, the recent use of high-throughput sequencing techniques has also revealed a broader range of mycorrhizal fungi in the vanilla, including Serendipitaceae and several families of fungi known to form mycorrhizal symbioses with trees, that is, ectomycorrhizal fungi (Johnson et al., 2021). Although some of these fungi could be non-mycorrhizal endophytes in orchids (sensu Wilson, 1995; see Schneider-Maunoury et al., 2018, 2020), their ability to form mycorrhizal pelotons in the roots of vanilla has been demonstrated at least for *Scleroderma* (González-Chávez et al., 2018).

Vanilla was introduced early in other tropical regions, especially in the southwestern islands of the Indian Ocean. Contrary to Madagascar where species of the genus *Vanilla* are native (Andriamihaja et al., 2021), vanilla (*V. planifolia*) was introduced in La Réunion island where there were no native species of this genus nor of its subfamily despite an orchid flora of about 200 species. Historical records indicate

that it was introduced in La Réunion in the 1820s, from plants brought from America and kept in cultivation in Paris (Bory et al., 2008, 2008). No work has been done to date on the fungal partners recruited by vanilla in this geographically isolated island. Yet, vanilla plants in La Réunion could associate either with neotropical strains of mycorrhizal fungi co-introduced with the living vanilla vines or could have developed new mycorrhizal associations (with partial or total turnover) among the fungi already associated with the native orchid flora of the island (e.g., Downing et al., 2020; see also Pringle et al., 2009).

The cultivation of vanillas in La Réunion has diversified and has gone from a traditional culture in the understory (US) of increasingly degraded forests, to a more intensive culture in openfield (OF) or in cultivation shade house (SH). These different cultivation methods, by providing different nutrient sources for both plants and fungi, may influence the mycorrhization of vanillas as already suggested by Johnson et al. (2021) in Mexico. Notably, given the saprotrophic abilities of orchid mycorrhizal fungi (Dearnaley et al., 2012; Miyauchi et al., 2020), the type of substrate in both terrestrial (e.g., dead leaves versus coconut fibers) and aerial (e.g., dead or inorganic stake versus living tree trunk) parts could strongly influence the ability of these fungi to get nutrients and eventually transfer them to their host plant. Additionally, these different cultivation methods, by modulating the balance between beneficial (e.g., mycorrhizal) and detrimental (e.g., pathogens) fungi, may also influence the presence of common pathogens in vanilla (Mujica et al., 2020) such as *Fusarium oxysporum* or *Colletotrichum* spp. (Charron et al., 2018; Koyyappurath et al., 2016). Putative antagonistic effects between mycorrhizal and pathogenic fungi can be assessed by co-occurrence analyses at the plant individual scale, because mycorrhizal fungi may compete with or inhibit the presence of pathogens in the plant roots (Manrique-Barros et al., 2023; Mujica et al., 2020).

In this study, we investigated the diversity of (i) mycorrhizal fungi and (ii) fungal pathogens associated with cultivated vanilla in north-eastern La Réunion island and compared them with those known from studies of vanilla mycorrhizas in Central America (Porrás-Alfaro & Bayman, 2007) and with known vanilla fungal pathogens, respectively. Because the cultivated vanilla is considered to be generalist regarding its mycorrhizal fungi (Porrás-Alfaro & Bayman, 2007), we hypothesized that it could at least partly renew its fungal partners compared with Central America. In addition, (iii) we compared mycorrhizal assemblages associated with the terrestrial and aerial roots of vanilla when grown in the forest US, in the OF, or in the SH within the same geographic area (<2.5 km<sup>2</sup>). Following the pioneering work of Johnson et al. (2021) in Mexico, we hypothesized that mycorrhizal assemblages

should vary, even at small scale, under varying cultivation practices. Finally, (iv) we tested the hypothesis that mycorrhizal fungi could be biocontrol agents for vanilla diseases and assessed the co-occurrence patterns between mycorrhizal and pathogenic fungi in vanilla roots.

## 2 | MATERIALS AND METHODS

### 2.1 | Study sites and root sampling

The study was conducted in Sainte-Suzanne in the northeast of La Réunion Island (Mascarene archipelago, SW Indian Ocean; 20°54'16"S, 55°35'18"E) and focused on four modes of vanilla cultivation: three of them (the SH with two types of substrate and the OF) are located within a few tens of meters of each other on a vanilla farm, and the third (the forest US) is located less than 2.5 km away (Figure 1). On the vanilla farm, three types of experimental crops have been established for public awareness and agricultural research. In the SH, 88 vanilla plants belonging to 22 accessions (including 16 accessions of *V. planifolia*) were planted in trays (c. 1 m<sup>2</sup>) filled with bagasse or dead leaves from a lychee orchard; the supports for the aerial roots of the lianas consist of grade wood stakes. In the OF, plants of *V. planifolia* (unknown accessions) are planted evenly in a coconut mulch, and the aerial roots grow on exotic shrubs of *Jatropha curcas* (Euphorbiaceae) whose leaves provide light shade. In the forest US, plants of *V. planifolia* (unknown accessions) grow on the riverbank in a secondary forest with various exotic trees and shrubs like *Calophyllum* sp., *Cordyline* sp., *Dracaena* sp., *Psidium cattleianum*, and *Mangifera indica*.

The sampling was conducted in August 2019. In SH, 24 individuals belonging to 12 accessions (11 *V. planifolia* et 1 *V. pompona*) were sampled, with two individuals per accession: one fed with bagasse and the other fed with dead leaves (the other 10 accessions in SH had not been planted in the leaf litter substrate, not allowing a direct comparison between substrates). Eight terrestrial roots were sampled in each individual in the four corners of the root system (2 replicates per corner), and for 12 individual representatives of the 12 accessions, two aerial roots per plant (24 in total) were sampled against the inert wood stake (Figure 1). Aerial roots were not sampled on all plants studied in SH, as they did not show any mycorrhizal colonization under the light microscope unlike terrestrial roots (see results). In OF and US, 8 terrestrial roots and 2 aerial roots were sampled on 11 randomly selected vanillas per site (110 root samples per site). This sampling resulted in  $N = 436$  root samples (c. 2 cm) including SH ( $n = 216$ ), OF ( $n = 110$ ), and US ( $n = 110$ ). These samples were observed in optical microscopy for selection of mycorrhizal root sections (c. 5 mm), cleaned on surface, and conserved at 4°C until molecular analyses.

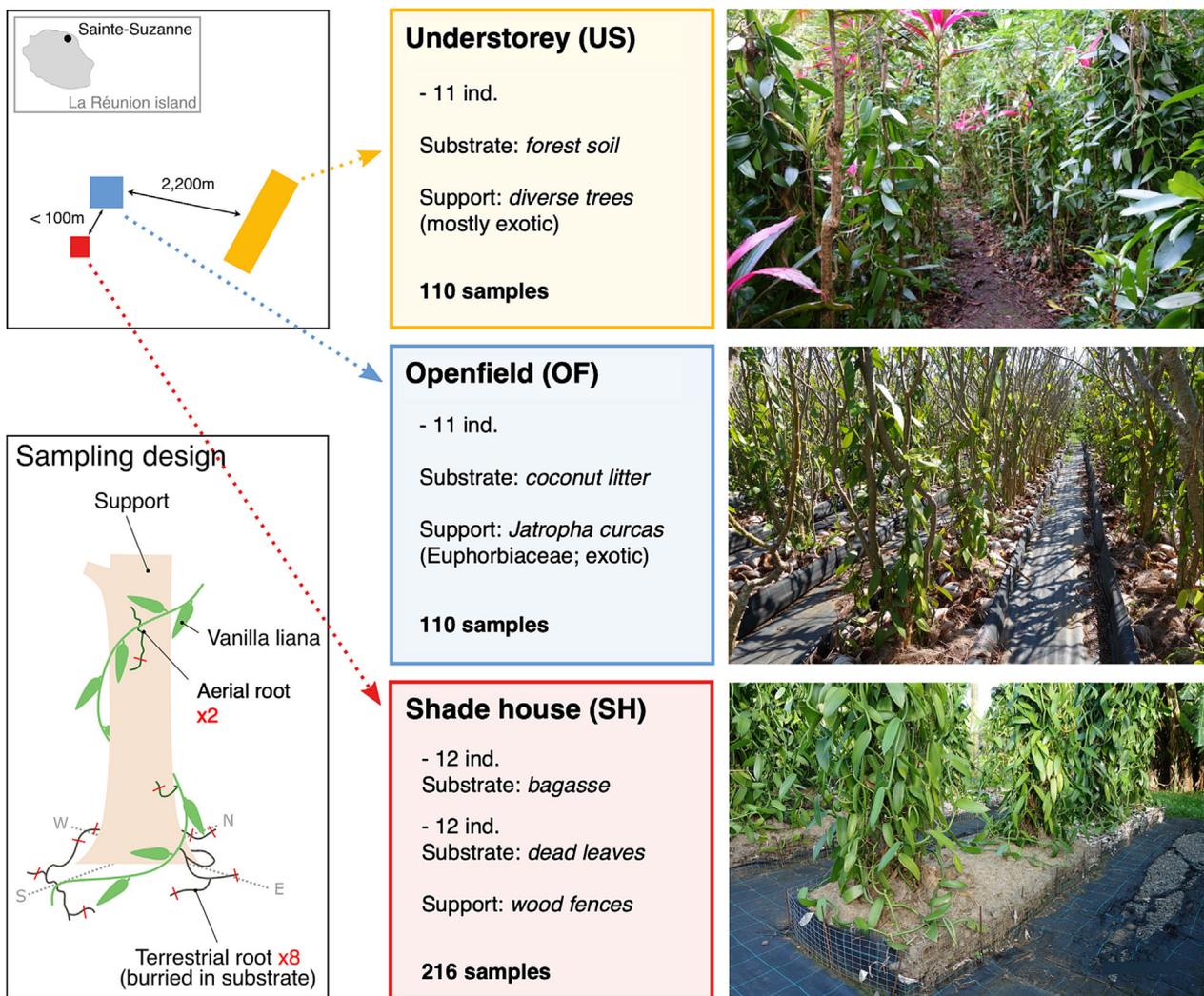
### 2.2 | Molecular analyses of fungi

Each of the 436 root samples were quickly frozen into liquid nitrogen and then grinded using a TissuLyser II (Qiagen) with one sterilized tungsten bead per tube, a pinch of Fontainebleau's sand and 200 µl of

lysis buffer (DNeasy® Plant Mini Kit; Qiagen). Genomic DNA was then extracted using this kit following the standard procedure but eluting the DNA in 50 µl of TE buffer. Each isolate was amplified by polymerase chain reaction (PCR) using two sets of primers targeting the internal transcribed spacer 2 (ITS2) of the ribosomal DNA: the fungal-specific primer set ITS86-F/ITS4 (White et al., 1990) to amplify most of the Asco- and Basidiomycota and the *Tulasnella*-specific primer set 5.8S-OF/ITS4-Tul (Vogt-Schilb et al., 2020) to specifically target the Tulasnellaceae fungi, which are frequently found in orchid mycorrhizas including in vanilla (Porrás-Alfaro & Bayman, 2007) but does not amplify with fungal-specific primers (Johnson et al., 2021; Rammitsu et al., 2021). Each PCR amplification was conducted with both forward and reverse tagged primers using a metabarcoding approach (Petrolli et al., 2021). Positive (homemade mock fungal community as in Petrolli et al., 2022) and negative (ultrapure water) controls were included in every PCR plates, as well as tagging system negative controls to assess cross-contamination during PCR trials, library preparation, or sequencing (Hornung et al., 2019; Zinger et al., 2019). The mock community was a mixture prepared from DNA isolates of six Asco- and four Basidiomycota (including one Tulasnellaceae) strains in pure culture. Each PCR was performed in 25 µl containing 0.2 mM each dNTP, 0.4 µM each primer, 1 unit AmpliTaq Gold® 360 DNA Polymerase (Thermo Fisher Scientific), 1X AmpliTaq Buffer supplied with MgCl<sub>2</sub>, and 1.5-µl template DNA, using the following program: initial denaturation 10 min at 95°C; 35 cycles of denaturation 30 s at 95°C, annealing 30 s at 56.5°C (ITS86-F/ITS4) or 52°C (5.8S-OF/ITS4-tul), and elongation 30 s at 72°C; final elongation 12 min at 72°C. Each PCR product was loaded on agarose gel for visualization of the amplicons, then purified using the NucleoMag® NGS Clean-up and Size Select (Macherey-Nagel) and quantified by fluorescence using the Qubit™ dsDNA High-Sensitivity (Invitrogen™). All amplicons (including the controls and the 436 samples) were then mixed equimolarly in two pools (one for each set of primers) and then sequenced with 2 \* 250-bp MiSeq Illumina sequencing at FASTER (Geneva, Switzerland).

### 2.3 | Raw sequencing data analyses

Paired reads were processed as in Perez-Lamarque et al. (2022) using a customized pipeline based on VSEARCH (Rognes et al., 2016). Briefly, paired-end reads were assembled using the *fastq\_mergepairs* function using default parameters. Assembled reads with more than two errors in the alignment were discarded. The resulting assembled reads were assigned to samples according to their tag + primer sequences using CUTADAPT (Martin, 2011) allowing zero discrepancies. Tag-jumps (Zinger et al., 2019) were estimated at this step and represented less than 0.2% of the reads. Operational taxonomic units (OTUs) were clustered using a 97% similarity threshold with VSEARCH, and chimeras were removed at this step using the *uchime\_denovo* option of VSEARCH. OTUs were taxonomically assigned using the *usearch\_global* option of VSEARCH with default parameters on the UNITE V8.3 (2021-05-10) eukaryotes reference database (Nilsson et al., 2015) and



**FIGURE 1** Study location and sampling design used to investigate root fungal communities of cultivated vanillas in La Réunion island. Sampling was performed in Sainte-Suzanne in the northeast of La Réunion island (top-left panel). For 46 vanilla vines, eight terrestrial roots were sampled at the four corners of the root system (two replicates per corner), and two aerial roots were sampled at different heights on the live or inert stake (bottom-left panel). Because mycorrhizas were not observed in the aerial roots on inert stakes in the shade house, they were only sampled on 12 of the 24 plants investigated in this cultivation practice.

named after their abundance in the global dataset. Singletons and short OTUs (<200 bp) were not considered. Non-fungal OTUs and OTUs abnormally detected in the positive and negative controls were also discarded from the data, as well as OTUs identified as contaminants by the DECONTAM R-package with frequency method (Davis et al., 2018). Finally, based on the previously estimated tag-jumps rate, OTUs that represented less than 0.5% of the sample reads were set to 0, hence minimizing the impact of artefactual sequences and tag jumps. Samples with <1000 reads ( $N = 19$  samples) were not considered in subsequent analyses.

## 2.4 | Blast and phylogenetic analyses

In order to identify the closest fungal relatives of OTUs detected in this study, we first manually blasted the most 10 frequent mycorrhizal

OTUs identified in this study on GenBank (default *blastn* parameters), and for each one of them, we indicated the three best accessions based on their identity and coverages (when >85%; Table 1). We repeated the same operation considering as the reference database the sequences from Martos et al. (2012) and from Porras-Alfaro and Bayman (2007) in order to detect fungal relatives in native orchids of La Réunion or in *Vanilla* spp. in Central America, respectively. When sequence identities were high (i.e., >97%), we aligned the OTU's sequence and the corresponding hit on Geneious and reported the number of mismatches and gaps in the overlapping regions (Table S1).

Additionally, we estimated the phylogenetic proximity between OTUs identified in vanilla in La Réunion (this study) and those identified in vanilla elsewhere in the world (available in GenBank), for orchid mycorrhizal fungi (Tulasnellaceae, Ceratobasidiaceae, and Sebaciniales) and for fungal pathogens of cultivated vanilla (*Fusarium* and *Colletotrichum*). Sequences from Weiß et al. (2016) and Veldre et al. (2013)

**TABLE 1** Best blast hits on GenBank for main mycorrhizal operational taxonomic units (OTUs) detected in the roots of vanilla plants in La Réunion. The cultivation practices in which each OTU has been detected is specified. US, understory; OF, openfield; SHb, shade house with bagasse; SHdl, shade house with dead leaves. Cultivation practices in bold indicate that the OTU was detected in >10% of the samples, whereas those in brackets indicate <10%. The three best hits (ranked by e-values) with >95% identity are given with their corresponding isolation or amplification source and location as provided by the authors. Identities >97% are bolded.

OTU	Cultivation practices		Best hit				Location	
	Terrestrial	Epiphytic	Blast	Accession	Cover	Identity		Source
TUL1	OF, SHb, SHdl, US	US	<i>Tulasnella</i> sp.	KX387593.1 LC175324.1 AB506842.1	100 100 99	100 99,17 99,44	Roots of <i>Geodorum eulophioides</i> (Orchidaceae, Epidendroideae) <i>Spiranthes sinensis</i> (Orchidaceae, Orchidoideae) Roots of <i>Cymbidium goeringii</i> (Orchidaceae, Epidendroideae)	China, Asia Japan, Asia Japan, Asia
TUL3	OF, US (SHb, SHdl)	US	<i>Tulasnella</i> sp.	LC175324.1	99	95,83	<i>S. sinensis</i> (Orchidaceae, Orchidoideae)	Japan, Asia
TUL11	US, OF (SHb, SHdl)	(US)	<i>Tulasnella</i> sp.	AB506842.1 KX387607.1	98 100	96,06 99,73	Roots of <i>C. goeringii</i> (Orchidaceae, Epidendroideae) Roots of <i>Paphiopedilum hirsutissimum</i> (Orchidaceae, Cyripedioideae)	Japan, Asia China, Asia
TUL14	US, SHdl (OF, SHb)	(US)	<i>Tulasnella</i> sp.	MW432188.1 JF691481.1	100 100	99,73 99,45	<i>Dendrobium officinale</i> (Orchidaceae, Epidendroideae) Roots of terrestrial orchids (Epidendroideae, Orchidoideae): <i>Oeceoclades</i> , <i>Habenaria</i> , <i>Eulophia</i>	China, Asia La Réunion, Indian Ocean
TUL19	SHdl (OF, SHb, US)	-	<i>Tulasnella</i> sp.	MT611052.1 HG995559.1 LR994041.1 EU218889.1 JF691532.1	100 100 100 100 100	99,45 99,45 98,9 100 97,16	<i>Eulophia graminea</i> (Orchidaceae, Epidendroideae) Soil DNA Soil DNA Type strain of <i>Tulasnella irregularis</i> Roots of epiphytic orchids (Epidendroideae): <i>Angraecum</i> , <i>Palystachya</i>	Florida, North America Benin, Africa - - La Réunion, Indian Ocean
TUL123	OF, SHb, SHdl (US)	(US)	<i>Tulasnella</i> sp.	GU166413.1 KX387593.1 AB506842.1 LC175324.1	94 98 98 98	99,09 97,74 97,74 97,46	Roots of orchids Roots of <i>G. eulophioides</i> (Orchidaceae, Epidendroideae) Roots of <i>C. goeringii</i> (Orchidaceae, Epidendroideae) <i>S. sinensis</i> (Orchidaceae, Orchidoideae)	Thailand, Asia China, Asia Japan, Asia Japan, Asia
TUL251	OF, SHb, SHdl	-	<i>Tulasnella</i> sp.	KX387593.1 LC175324.1 AB506842.1	100 100 99	96,98 96,7 96,95	Roots of <i>G. eulophioides</i> (Orchidaceae, Epidendroideae) <i>S. sinensis</i> (Orchidaceae, Orchidoideae) Roots of <i>C. goeringii</i> (Orchidaceae, Epidendroideae)	China, Asia Japan, Asia Japan, Asia
CER9	US, OF (SHdl)	US	<i>Ceratobasidium</i> sp.	KF267048.1 MT134840.1 KP715606.1	100 100 100	99,69 99,38 99,38	Roots of <i>Caladenia reticulata</i> (Orchidaceae, Orchidoideae) Roots of <i>Dendrobium</i> sp. (Orchidaceae, Epidendroideae) <i>Dichromanthus cinnabarinus</i> (Orchidaceae, Orchidoideae)	Australia China, Asia Mexico, North America
CER15	SHb, SHdl	-	<i>Ceratobasidium</i> sp.	KM505162.1 HM623631.1 KF907736.1	100 100 100	100 100 99,69	<i>Colocasia esculenta</i> (Araceae) - Strain of <i>Rhizoctonia solani</i> , isolated from <i>Brassica chinensis</i> (Brassicaceae)	China, Asia China, Asia Vietnam, Asia

(Continues)

TABLE 1 (Continued)

OTU	Cultivation practices		Best hit	Accession	Cover	Identity	Source	Location
	Terrestrial	Epiphytic						
RES25	SHb (SHdl)	(SHb)	<i>Resinicium saccharicola</i>	JQ081805.1 KY995328.1 DQ826548.1	100 100 100	100 99,66 99,66	Soil DNA - Soil DNA	Brazil, South America Martinique, Caribbean Puerto Rico, Caribbean

were used to precise the phylogenetic position of OTUs in the Sebaciniales (incl. Serendipitaceae and Sebacinaceae) and in the Ceratobasidiaceae, respectively, in addition with sequences from Martos et al. (2012) and Porras-Alfaro and Bayman (2007). Because no broad phylogenetic analysis of Tulasnellaceae has been conducted so far, only the sequences from these two latter references were used for this family, complemented with few other manually selected sequences from GenBank. For *Fusarium* and *Colletotrichum*, we used sequences from GenBank obtained from various plants including vanilla to determine the phylogenetic position of our OTUs.

As the OTU sequences from this study were shorter (<500 bp) than the full ITS sequences in GenBank, we first aligned the longer sequences using MAFFT (Kato & Standley, 2013) with default parameters and then added our OTU sequences using the --add option. The alignments were then trimmed using trimAl v1.2. (Capella-Gutiérrez et al., 2009) in order to keep only informative regions. Trees were built using IQTREE 2.1.3 (Minh et al., 2020) with standard model selection and using the first alignment as a constraint (-g option) to add our OTU sequences. Bootstrap and sh-aRT values were computed using 1000 replications.

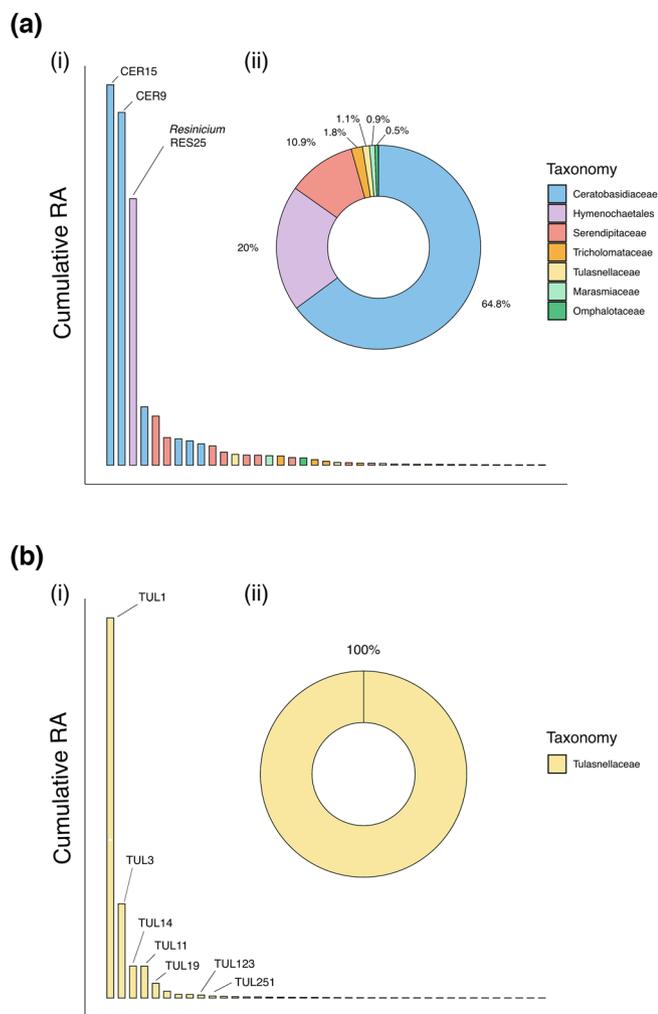
## 2.5 | Statistical analyses

As we used two pairs of primers targeting different fungal taxa ranges (i.e., most Dikarya versus Tulasnellaceae only), we described abundance data (read counts) from both tables of OTUs separately in Figure 2. For the statistical analyses below, we merged the two OTU tables considering presence/absence data (1/0).

Variation in fungal communities between cultivation practices (i.e., SH, OF, and US) and root types (i.e., terrestrial versus aerial) were assessed using beta-diversity analyses. Beta-diversities between pairs of samples were calculated using the Jaccard index with the *vegdist* function in the VEGAN R-package (Oksanen et al., 2013). Differences in fungal composition in the overall dataset were visually assessed through a Non-Metric Multidimensional Scaling (NMDS) using the *metaMDS* function of VEGAN. Statistical differences of fungal communities between the above-cited variables were tested using PERMANOVA (*adonis* function of VEGAN, 999 replications).

In order to further identify mycorrhizal OTUs specific of cultivation practices and/or root types, we performed an analysis of specificity using the *multipatt* algorithm of the INDICSPECIES package, specifying “r.g” method (9999 permutations; Dufrene & Legendre, 1997). This function, based on correlation analyses, allows to identify significant patterns of associations between fungal OTUs and cultivation practices or root types.

Finally, we performed co-occurrence analyses to shed light on positive or negative relationships between mycorrhizal fungi and pathogenic fungi occurring in vanilla roots. To do this, a network of co-occurrences was reconstructed by merging data from the terrestrial or aerial roots of the same vanilla plant. OTUs that occurred in less than 20% of the individual's replicates were discarded at this step. The frequency of each remaining OTU in the individual's replicates



**FIGURE 2** Composition of mycorrhizal communities in the roots of cultivated vanillas in La Réunion island. Composition of mycorrhizal communities amplified with the primers (a) ITS86-F/ITS4 and (b) 5.8S-OE/ITS4-Tul. (i) Cumulative relative abundances (RA) of the 40 most abundant operational taxonomic units (OTUs) in the whole dataset and (ii) cumulative RA at lower taxonomic rank. Main OTUs discussed in text are labeled.

was used to evaluate patterns of co-occurrences using the spearman correlation index. *P* values were computed by comparing each index with 1000 additional calculations for the same OTU–OTU interaction using randomized matrices obtained with the *permutswap* function of VEGAN. Only correlation index with corresponding *p* value  $\leq .001$  was considered as significant.

### 3 | RESULTS

#### 3.1 | Sequencing data

Mycorrhizal pellets were observed in all samples collected in SH, OF, and US, except for aerial roots adhering to inert stakes in SH. The MiSeq Illumina sequencing generated a total of 19,530,449 raw reads.

Bioinformatics analyses and removal of non-fungal OTUs or putative contaminants yielded to a total of 3421 fungal OTUs. After further data filtering (see methods), all PCR replicates of the mock community exhibited only the OTUs expected from the mock community, indicating that no contaminant OTU remained in the data after this step. This yielded to a final set of 1237 OTUs. Despite our dense sampling of vanilla roots, none of the rarefaction curves reached a plateau in the three cultivation practices (Figure S1).

The generalist ITS86-F/ITS4 set of primers yielded to a majority of Ascomycota (51.9%) and Basidiomycota (26.7%). Other OTUs (21.5%) corresponded to unidentified fungi (21.1%) or non-Dikarya fungi (0.4%) (Figure S2A). The 5.8S-OE/ITS4-Tul set of primers only amplified Cantharellales and the Tulasnellaceae family specifically (Figure S2B).

#### 3.2 | Mycorrhizal OTUs of vanilla

A total of 110 OTUs were considered as orchid mycorrhizal fungi based on the taxonomic list of Dearnaley et al. (2012). The richest mycorrhizal families were Tulasnellaceae (75 OTUs), followed by Sebaciales (all Serendipitaceae, 14 OTUs) and Ceratobasidiaceae (8 OTUs; Figure 2). Yet, Serendipitaceae were scarce in samples compared to Tulasnellaceae and Ceratobasidiaceae.

The majority of abundant mycorrhizal OTUs in the Tulasnellaceae or in the Ceratobasidiaceae had close fungal relatives in other parts of the world, mainly in Asia (Table 1). For instance, TUL1 in the Tulasnellaceae was identical to an OTU detected in the roots of *Geodorum eulophioides* in China (KX387593.1) and very close to an OTU detected in the roots of *Spiranthes sinensis* (LC175324.1; cover: 100%, id.: 99.17%) in Japan (Table 1). Among abundant OTUs, only TUL3 and TUL251 did not match any sequence deposited in GenBank at a threshold of 97% (Table 1).

Among the nine most frequent OTUs in these two families (i.e., Tulasnellaceae and Ceratobasidiaceae), five (all Tulasnellaceae such as TUL1 or TUL11) matched sequences retrieved in the roots of vanilla in Puerto Rico Porras-Alfaro & Bayman, 2007 and in other orchid species native from La Réunion (Martos et al., 2012) at a threshold of 97% (including TUL251 that showed 96.99% of identity). Most of them showed high identity (>97%) but low coverage (<90%) with sequences of these two studies (Table S1). At the contrary, one OTU (TUL19) was close to a sequence already detected in the roots of orchids in La Réunion (cover: 100%, id.: 97.16%, JF691532.1) but did not blast any sequence identified in Puerto Rico in vanilla roots at a threshold of 97%. Finally, three OTUs (TUL3 in the Tulasnellaceae and CER9 and CER15 in the Ceratobasidiaceae) did not match any OTU previously identified in the roots of orchids in La Réunion or in Puerto Rico (Table S1).

One OTU of *Resinicium* (RES25) was abundant in the terrestrial vanilla roots in SH (Figures S3 and S4), colonizing 19 root samples, among which 18 were terrestrial, and 17 were in the bagasse substrate, representing up to 67% of these sample's reads. It was identified as *R. saccharicola* (KY995328.1; cover: 100%, id.: 99.66%). Other

putative mycorrhizal OTUs were scarce in samples and not abundant in the overall dataset.

Our phylogenetic analysis of Tulasnellaceae showed that their 20 most abundant OTUs in this study belonged to several different clades including basal and core Tulasnellaceae (Figure S5). The eight OTUs of Ceratobasidiaceae also belonged to different clades (Figure S6), whereas all Sebaciales belonged to the Serendipitaceae family (data not shown). The phylogenetic analysis was in accordance with the blast analysis, with OTUs belonging to the same clades as sequences from both Puerto Rico and La Réunion (e.g., TUL1 and TUL14), from La Réunion only (e.g., TUL19) or from none of them (e.g., CER9 and CER15; Figures S5–S6).

### 3.3 | Fungal pathogens of vanilla

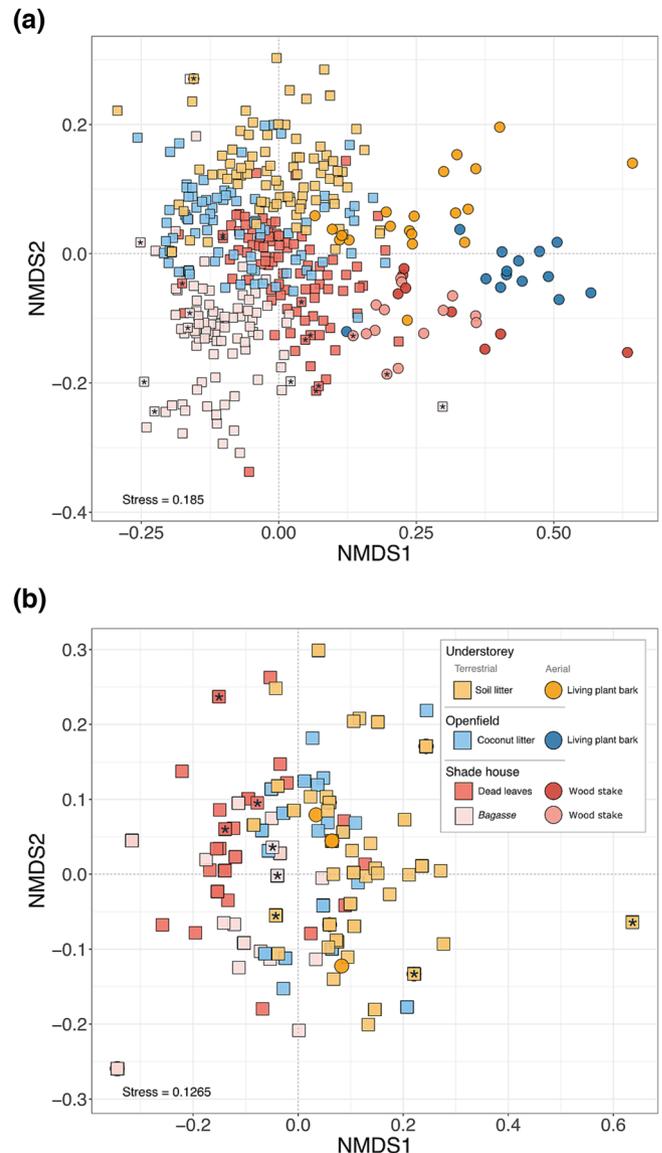
*Fusarium* spp. were abundant in vanilla roots, with one OTU (FUS2) representing 7.0% of the sequences retrieved with the ITS86-F/ITS4 primers and colonizing 38.6% of the root samples. From the phylogenetic analysis, FUS2 was identified as *F. oxysporum* (Figure S7). Other less abundant *Fusarium* OTUs belonged to clades with *F. solani* (4 OTUs), *F. proliferatum* (1 OTU), or unidentified species (4 OTUs). Although 5 OTUs of *Colletotrichum* were detected in this study, they were all rare, and none of them was identified as the vanilla pathogen, *C. orchidophilum* (Figure S7).

### 3.4 | Fungal communities across cultivation practices and root types

Rarefaction curves showed that when considering equal sampling effort between cultivation practices, US harbored the richest overall fungal community, whereas OF or SH showed equal fungal richness (Figure S1). Total fungal communities segregated between cultivation practices (PERMANOVA,  $F = 9.686$ ,  $R^2 = .043$ ,  $p = .001$ ) and root types ( $F = 7.319$ ,  $R^2 = .049$ ,  $p = .001$ ; Figure 3a and Table 2). Notably, roots of *V. pompona* (1 accession) and *V. planifolia* (11 accessions) in SH did not segregate in the NMDS visualization (Figure 3a).

Contrary to the overall fungal communities, vanilla roots in OF harbored more diverse mycorrhizal communities than those in SH or in US at equal sampling depth (Figure S1). Considering mycorrhizal fungi, samples still segregated by cultivation practices ( $F = 18.800$ ,  $R^2 = .103$ ,  $p = .001$ ) but not by root types probably due to low colonization of aerial roots ( $F = 1.515$ ,  $R^2 = .008$ ,  $p = .093$ ; Figure 3b and Table 2). Roots of *V. pompona* harbored the same main mycorrhizal OTUs than nearby *V. planifolia* plants in SH, and samples of the two species did not segregate in the NMDS visualization (Figure 3b).

We observed no clear difference in mycorrhizal composition at the family level between cultivation practices (Figures 4, S3, and S4). At the OTU level, the analysis of specificity between OTUs and cultivation practices showed that the main OTU of Tulasnellaceae (TUL1) was not specific of any cultivation practices whereas TUL3 specifically colonized vanilla roots in US (Figure S8). CER9 and CER15 were



**FIGURE 3** Fungal community variation in the roots of cultivated vanillas under several cultivation practices in La Réunion. Non-Metric Multidimensional Scaling (NMDS) analysis of fungal communities in vanilla roots considering (a) all operational taxonomic units (OTUs) and (b) mycorrhizal fungi only. Symbols are colored according to the cultivation practices, whereas shapes refer to the type of roots (terrestrial or aerial). All roots belonged to *Vanilla planifolia* except those marked with an asterisk (\*), which belonged to *V. pompona*. In panel b, four samples with distant mycorrhizal communities were removed to improve visualization.

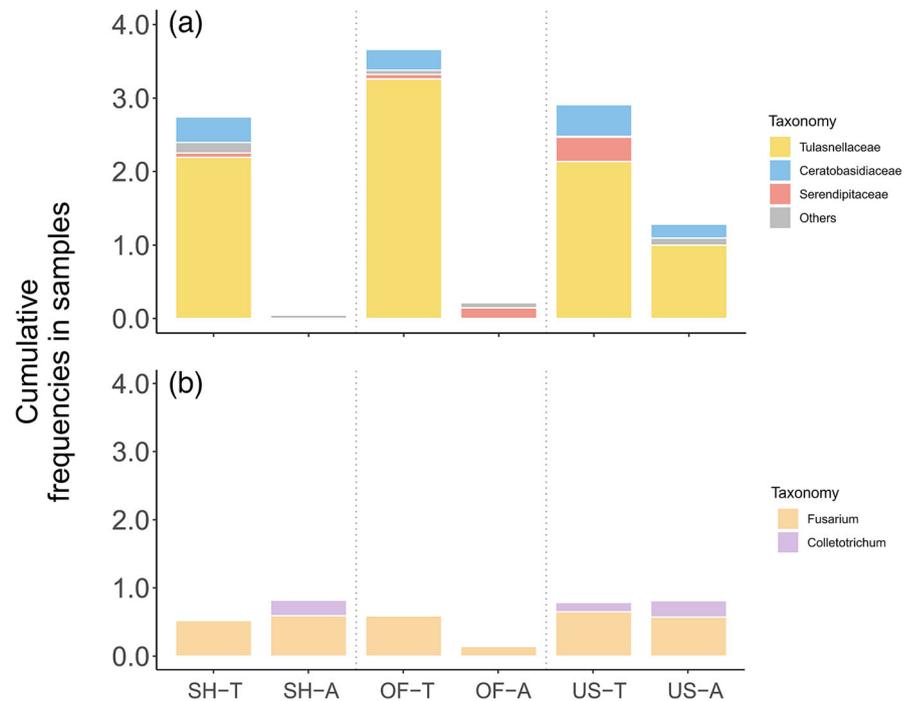
restricted to US and SH, respectively. RES25 was only found in the terrestrial roots in SH especially those fed with bagasse (Figures S4 and S8).

The aerial roots were hardly colonized by mycorrhizal OTUs in SH and OF, whereas they harbored mycorrhizal fungi in US (Figures 4 and S1). Overall, only 5.5% of the mycorrhizal OTUs were specific to aerial roots compared to 21.8% of the fungal OTUs (Chi-squared = 15.631,  $df = 1$ ,  $p < .001$ ; Figure 5). Considering US only, OTUs specific to aerial roots represented 20.0% of the mycorrhizal OTUs (Figure S9).

**TABLE 2** Results from PERMANOVA analysis of total fungal and mycorrhizal community variation between cultivation modes (Cm) and root types (terrestrial versus epiphytic; Rt). The formula used in the models was:  $X \sim Cm + (Cm/Rt)$  where Rt is nested into Cm. Significant  $p$  values are bolded. Df, degree of freedom.

Fungal guild	Variable	Df	F value	R <sup>2</sup>	P value
Total	Cultivation mode	2	9.686	.043	<b>.001</b>
	Cultivation mode: root type	3	7.319	.049	<b>.001</b>
Mycorrhizal	Cultivation mode	2	18.800	.103	<b>.001</b>
	Cultivation mode: root type	2	1.515	.008	.093

**FIGURE 4** Mycorrhizal and fungal pathogens composition of cultivated vanilla roots in La Réunion. The height of each bar represents the cumulative frequencies of each operational taxonomic units (OTUs) in samples, considering (a) mycorrhizal families and (b) known vanilla pathogens. SH, shade house; OF, openfield; US, understory; A, aerial; T, terrestrial.



### 3.5 | Co-occurrence between mycorrhizal and pathogenic fungi in vanilla roots

No apparent sign of antagonism between mycorrhizal fungi and pathogens of vanilla plants was observed at the family level (Figure 4) even when considering relative abundancies (Figure S3). To investigate this tendency at the OTU's level, we analyzed the co-occurrence of OTUs in vanilla terrestrial or aerial roots, showing that mycorrhizal OTUs co-occurred significantly and positively (and to a lesser extent negatively; 14.8% of the interactions) with fungal OTUs but not with *Fusarium* or *Colletotrichum* fungi (Figure S10). The most significant positive co-occurrences were observed between Tulasnellaceae OTUs, whereas no strong negative co-occurrence (i.e.,  $< -0.6$ ) was detected in the whole dataset.

## 4 | DISCUSSION

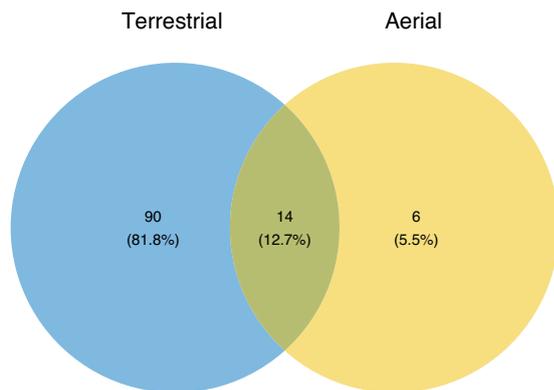
### 4.1 | Mycorrhizal communities of vanilla in La Réunion island

With the use of a high-throughput sequencing approach, we identified a total of 1237 fungal OTUs in vanilla roots in La Réunion island

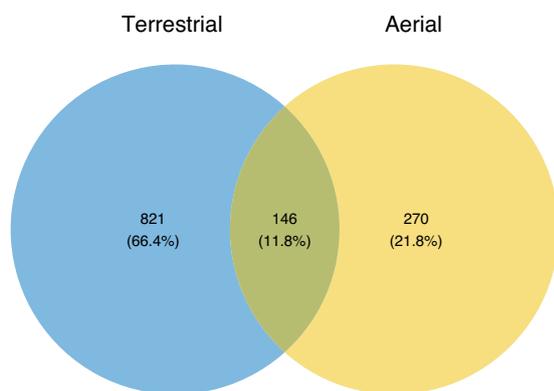
(Sainte-Suzanne). Among them, 110 were identified as orchid mycorrhizal fungi. They mainly belonged to the Tulasnellaceae (75 OTUs, 68%), which may be explained by the use of the specific primers 5.8S-OF/ITS4-Tul. However, specific primers are required to amplify this family (Rammitu et al., 2021), including in vanilla roots (Johnson et al., 2021). The Ceratobasidiaceae were the second most frequent and abundant family of orchid mycorrhizal fungi (8 OTUs, 7%), and other mycorrhizal families were both less frequent and less abundant in the vanilla roots investigated here. These results are in line with previous studies of the mycorrhizal communities of vanilla in Central America (Johnson et al., 2021; Porras-Alfaro & Bayman, 2003, 2007) and suggest that the main mycorrhizal partners of vanilla in La Réunion are also Tulasnellaceae and Ceratobasidiaceae.

In addition with common orchid mycorrhizal fungi (i.e., Tulasnellaceae, Ceratobasidiaceae, and Sebaciniales), orchids can form mycorrhizas with a large range of Asco- and Basidiomycota, which may be saprotrophic or also form ectomycorrhizas in other plants (Dearnaley et al., 2012; Selosse et al., 2022; Wang et al., 2021). Although a recent study conducted in Mexico by González-Chávez et al. (2018) suggested that *Sclerotinia* fungi could also form pelotons in the roots of *V. planifolia*, we did not find any *Sclerotinia* in the investigated roots. In another study, Johnson et al. (2021)

## (a) Mycorrhizal



## (b) All fungi



**FIGURE 5** Fungal sharing between terrestrial and aerial roots of cultivated vanillas in La Réunion. Venn diagrams show the numbers of fungal operational taxonomic units (OTUs) exclusive or shared between terrestrial and aerial roots of *Vanilla* spp. in the whole dataset, considering (a) mycorrhizal families and (b) all fungi. The same tendencies were observed in each culture condition (Figure S9).

detected a large diversity of mycorrhizal fungi in vanilla roots, both saprotrophic (e.g., *Mycena*) and ectomycorrhizal. In our study, we did not detect any ectomycorrhizal fungi according to previously identified ectomycorrhizal fungal genera or clades inside the main mycorrhizal families (Bidartondo et al., 2003; Veldre et al., 2013). Still, we cannot exclude that one or several of the OTUs detected in this study can, at least in certain habitats (e.g., in the forest understory), act as ectomycorrhizal fungi, including for Tulasnellaceae and Ceratobasidiaceae (Suetsugu & Matsubayashi, 2021; Yagame et al., 2012). Further studies are thus needed to assess whether the results of Johnson et al. (2021) can be extended to La Réunion island.

Regarding saprotrophic fungi, we found a very scarce and low colonization of vanilla roots by *Mycena* fungi, which was thus unlikely to form mycorrhiza in the plant cells (Harder et al., 2023). However, we also detected an OTU of *Resinicium* (RES25, Hymenochaetales) identified as *Resinicium saccharicola* Nakasone (Nakasone, 2000), which was abundant in the terrestrial roots of vanilla plants cultivated in SH on

bagasse substrate. *Resinicium* are wood- or leaf litter-decaying fungi, but some members are also known to form mycorrhizal associations in myco-heterotrophic orchids in the genus *Gastrodia*, including in La Réunion (Martos et al., 2009). This is the first occurrence of *R. saccharicola* in La Réunion island, and it has probably been introduced with the bagasse substrate as this species is known to decompose sugarcane leaf litter in Asia (Miura et al., 2015). It will be necessary to verify whether this fungus is able to form pelotons in vanilla roots and transfer nutrients from sugarcane residues to vanilla or whether it is an endophyte of vanilla roots. Hence, future work should investigate *R. saccharicola* in vanilla roots using microscopic technics.

#### 4.2 | What is the origin of mycorrhizal fungi in vanilla plants of La Réunion?

Studies on vanilla mycorrhizas have been mainly conducted in Central America (González-Chávez et al., 2018; Johnson et al., 2021; Porrás-Alfaro & Bayman, 2003, 2007) where the main cultivated species *V. planifolia* originates. In our study conducted in La Réunion island (Indian Ocean), none of the Ceratobasidiaceae were close to fungi of vanilla plants in Central America. However, our most abundant Tulasnellaceae (TUL1) belonged to the same clade as sequences retrieved from Porrás-Alfaro and Bayman (2007) in Central America. Although the coverage between our sequence and those of Porrás-Alfaro and Bayman (2007) was 72% (Table S1), because of the use of a different reverse primer (Tul4 in the latter study, upstream of ITS4-Tul in our study), the sequences only presented 2 mismatches in their overlapping region (263 bp) also suggesting a same species of mycorrhizal fungus. Two other abundant Tulasnellaceae TUL11 and TUL14, phylogenetically distinct from TUL1, were also close to OTUs from Central America. Overall, and even though further studies are needed using full ITS sequences, we propose that the vanilla in La Réunion associates with at least some fungi (or closely relatives) also found in Central America. If these fungi were introduced alongside their vanilla hosts, as was the case for some ectomycorrhizal fungi with their host tree (e.g. Dickie et al., 2010, 2017; Séne et al., 2018), or if they were widespread in the tropics before the introduction of vanilla, deserves further attention.

*V. planifolia* from America may have been introduced only once in La Réunion in the 1820s (Bory et al., 2008). Even though independent introductions of their symbionts (Pringle et al., 2009) cannot be excluded (e.g., alongside with other *Vanilla* species more recently introduced in La Réunion), this single introduction may not have been sufficient to co-introduce the large spectrum of fungal partners retrieved here. Rather, the mycorrhizal fungi identified here could have been present on the island before the introduction of vanillas allowing them to establish and to partly renew their fungal symbionts during their establishment/naturalization (Pringle et al., 2009). This scenario was for example suggested for *Eulophia graminea* native to Asia (Downing et al., 2020) and *Oeceoclades maculata* native to Africa (Bayman et al., 2016), two orchid species that became invasive following their introduction in America.

In our study, the same OTUs cited above (i.e., TUL1, TUL11, and TUL14) were close to sequences identified in native La Réunion orchids by Martos et al. (2012). Given that these OTUs were also close to fungi identified in other orchid species, mainly in Asia, this suggests that these mycorrhizal partners show a worldwide distribution independently of vanilla plants. Large distributions of mycorrhizal OTUs have already been documented in orchids (e.g., Duffy et al., 2019; Phillips et al., 2011; Swarts et al., 2010), but further work is needed to better understand the biogeography of these fungi (Jacquemyn et al., 2017). By contrast, one OTU (TUL3) could not be related to any sequence from Genbank, suggesting that the vanilla in La Réunion is also colonized by so far unknown mycorrhizal fungi. As La Réunion island does not have any species of Vanilloideae in its native flora, future studies could verify whether this fungus is a mycorrhizal associate of vanilloid orchids native in Madagascar and the Comoros (Andriamihaja et al., 2021).

### 4.3 | Impact of cultivation practices on fungal communities of vanilla

In Mexico, Johnson et al. (2021) suggested that cultivation practices could influence the fungal endophytes of vanilla, including mycorrhizal fungi. However, their study was comparing four sites distant of >50 km from each other, and the authors could not exclude that the observed differences were due to spatial effects. In our study conducted in an area of <2.5 km<sup>2</sup>, we confirmed that cultivation practices do influence fungal and mycorrhizal communities of vanilla.

Rarefaction curves showed that whole fungal communities were richer in the US compared with SH and OF. Comparisons of richness between aerial and terrestrial roots were limited by different sampling sizes, but aerial roots in the US seem to host a richer fungal community than terrestrial roots.

When considering mycorrhizal fungi only, aerial roots were hardly colonized except on living trees and shrubs in the US. However, vanillas growing in the SH or in the OF both rooted epiphytically on dead wood stake of Pinaceae and on the exotic trunks of *J. curcas* (Euphorbiaceae) trees, respectively. The chemistry of these tutors (e.g., latex produced by *J. curcas*; e.g., Igbinosa et al., 2009) might be responsible for this low colonization. When considering all sites, the majority of mycorrhizal fungi detected in the aerial roots of vanilla plants (i.e., in the US) were also detected in terrestrial roots (including in the farm), meaning that they may not be specific of the epiphytic environment. Indeed, mycorrhizal fungi might grow from the soil onto the tutors' bark and colonize aerial roots. For instance, CER9 was shared between the terrestrial and aerial vanilla roots in the US. As it was not similarly shared in other cultivation practices, it also suggests that the substrate could limit the growth of some fungi in aerial parts. Yet, the low colonization by mycorrhizal fungi of vanilla aerial roots has already been demonstrated by using microscopy (Porrás-Alfaro & Bayman, 2007) or sequencing (Johnson et al., 2021) and might also be a property of this species.

The mycorrhizal communities also varied between cultivation practices, including between different substrate types in SH, even

though the composition at the family level was similar. For instance, the *Resinicium* RES25 was almost exclusively detected in the roots of vanilla in bagasse (see above). The variation of cultivation practices could then favor OTUs with complementary niches as exemplified in the Ceratobasidiaceae family in which the two main OTUs were specific of the SH and of the OF and US, respectively. By modulating the nutrient sources such as nitrogen, different substrates could promote various mycorrhizal partners (Nurfadilah et al., 2013), which may provide specific services to the plant and sustain the diversification of cultivation systems in vanillas.

### 4.4 | Co-occurrence between mycorrhizal and pathogenic fungi in vanilla roots

Different mycorrhizal fungi could provide different services to plants, for instance by providing different nutrients and/or direct protection against pathogens. In a recent study, Mujica et al. (2020) evidenced that the pathogens in roots of the orchid *Bipinnula fimbriata* negatively correlated with mycorrhizal colonization. Mycorrhizal fungi could therefore inhibit either directly (e.g., through allelopathy) or indirectly (e.g., through competition) other fungi including pathogens, as already documented in other mycorrhizal symbioses (Azcón-Aguilar & Barea, 1997). Notably, members of the Ceratobasidiaceae family have been shown to control the infection by *F. oxysporum* in other crop systems (e.g., Muslim et al., 2003) but have only recently been experimentally tested in vanilla (Manrique-Barros et al., 2023) with contrasting results. It is noteworthy to mention that *F. oxysporum* has been reported as potential mycorrhizal fungi in orchids (Jiang et al., 2019), but additional works are needed to assess its potential as mycorrhizal partner of *Vanilla* spp. Meanwhile, *F. oxysporum* is a very common pathogen of vanillas (Koyyappurath et al., 2016), including in La Réunion island.

In our study, the *Fusarium* FUS2 was frequently found in roots that were asymptomatic, including alongside with mycorrhizal fungi, and we found no pattern of negative co-occurrence between mycorrhizal fungi and pathogens, suggesting that the growth of pathogenic fungi could be limited rather than fully avoided by the mycorrhizal fungi (Mesny et al., 2021). However, co-occurrence analyses solely based on extracted DNA may not reflect true abundances or living organisms, and *Fusarium* might also be colonizing the roots as diffuse hyphae or latent spores. The potential biocontrol of pathogens by mycorrhizal fungi may also depend on external factors such as other co-occurring fungi or bacteria as already described in vanillas (Radjacommaré et al., 2010). Hence, future experimental works (such as co-inoculations) are needed to determine the factors affecting the balance between mycorrhizal and pathogenic fungi in vanillas (Manrique-Barros et al., 2023).

### AUTHOR CONTRIBUTIONS

FM, HK, and MAS designed the study; and FM, BC, and GLT performed the sampling. CB, CG, and RP performed the lab work, and RP performed the bioinformatics and statistical analyses. RP and FM wrote the manuscript, which was approved by all authors.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The raw sequencing data that support the findings of this study are openly available in Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/genbank/>, under the BioProject PRJNA1044433.

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