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MYCORRHIZAL FUNGI AND *FUSARIUM* SPECIES ASSOCIATED WITH VANILLA IN TRADITIONAL MANAGEMENT SYSTEMS IN PAPANTLA, VERACRUZ, MEXICO

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Abstract

Background: The Totonacapan region is recognized as the origin of vanilla cultivation (*Vanilla planifolia* Andrews), an orchid used for its gastronomic and medicinal values in rituals from pre-Hispanic times. Since that time, locals have cultivated vanilla using several traditional systems with different management practices. This plant forms symbiotic associations with mycorrhizal fungi inside its roots, which serve as a source of nutrients.

Question: The objective of this study was to isolate and identify mycorrhizal fungi from traditional systems to search potential bioinoculants that enhance the organic and traditional cultivation among local communities.

Studied species: *Vanilla planifolia* Andrews.

Study site and dates: We sampled four management systems: Acahual (Traditional management, forest composed mainly of secondary vegetation), shade house (semi-technified cultivation system), under orange (*Citrus sinensi*s L.) and *pichoco* (*Erythrina* spp.) trees in the region of Papantla, Veracruz, Mexico in 2022-2023.

Methods: The isolation was made from terrestrial roots, after the morphological and molecular characteristics of isolates by amplifying the ITS region.

Results: A total of ten strains were obtained from the four systems, which all correspond to the genus *Tulasnella*. At the same time, isolates of the genus *Fusarium* (*F. falciforme* and *F. oxysporum* f. sp. *vanillae*) were identified as endophytes and pathogenic species of vanilla.

Conclusions: The genus *Tulasnella* is the main mycorrhizal partner associated to vanilla in the Totonacapan cultivars; according to the phylogenetic analysis, seven of the isolated strains form a separate clade, suggesting that these strains are from the region of Papantla, Veracruz.

Keywords: agroforestry, bioinoculants, *Epulorhiza*, *Fusarium*, *Tulasnella*.

Resumen

Introducción: La región del Totonacapan es reconocida como el origen del cultivo de la vainilla (*Vanilla planifolia* Andrews), orquídea utilizada por sus valores gastronómicos y medicinales desde la época prehispánica. Los lugareños han cultivado la vainilla utilizando sistemas tradicionales con diferentes prácticas de manejo. Esta planta forma asociaciones simbióticas con hongos micorrícicos en el interior de sus raíces que le sirven como fuente de nutrientes.

Objetivo: aislar e identificar hongos micorrícicos de estos sistemas para buscar posibles bioinoculantes que potencien el cultivo orgánico y tradicional.

Especie de estudio: *Vanilla planifolia* Andrews.

Sitios y fechas de estudio: Se muestrearon cuatro sistemas de manejo: Acahual (manejo tradicional, bosque compuesto principalmente por vegetación secundaria), casa sombra (sistema de cultivo semitecnificado), bajo árboles de naranja (*Citrus sinensis* L.) y pichoco (*Erythrina* spp.) en Papantla, Veracruz, México.

Métodos: El aislamiento se realizó en raíces terrestres, previas caracterízación morfológicas y moleculares mediante la amplificación de la región ITS.

Resultados: Se obtuvieron un total de diez cepas de los cuatro sistemas, las cuales corresponden al género *Tulasnella*. Al mismo tiempo, se identificaron aislados del género *Fusarium* (*F. falciforme* y *F. oxysporum* f. sp. *vanillae*) como especies endófitas y patógenas de la vainilla.

Conclusiones. El género *Tulasnella* es el principal socio micorrízico asociado a la vainilla en los cultivares del Totonacapan; de acuerdo con el análisis filogenético, siete de las cepas aisladas forman un clado separado, lo que sugiere que estas cepas son originarias de la región de Papantla, Veracruz.

Palabras clave: agroforestería, bioinoculantes, *Epulorhiza*, *Fusarium*, *Tulasnella*.

Mexico has been recognized as the center of origin and diversification of vanilla, specifically in the north of Veracruz, where it was domesticated by the Totonac people in pre-Hispanic times (Lubinsky *et al.* 2008, Shinga *et al.* 2008). Mexico is one of the top three producers of vanilla worldwide, contributing nearly 8 % of the world's supply (543 t), surpassed only by Madagascar (44 %, 3,000 t) and Indonesia (23 %, 1,600 t). In Mexico, the state of Veracruz- specifically in the regions of Papantla, Tecolutla, and Gutiérrez Zamora- accounts for 76.4 % of the country's production, followed by Puebla (12 %) and Oaxaca (10 %) (SADR 2024). The primary export countries are the USA (1,807 t), France (1,081 t), and Germany (711 t).

In the Totonacapan region, vanilla is still cultivated using the traditional system, which involves using the disturbed tropical forest "acahual", where plant communities regenerate. This cultivation system involves a variety of native trees that act as "tutors" for the vanilla vines. This preserves the biocultural diversity of the Totonac people's cultivation practices (Velázquez-Rosas *et al.* 2024). However, due to land use changes and landscape alteration, vanilla cultivation has evolved into several agroforestry systems: a) citrus-associated system: this is one of the most common practices, were vanilla is cultivated alongside orange trees (*Citrus sinensis* L.), offeringthe dual benefit of both vanilla and citrus production; b) pichoco tree (*Erythrina* spp.) and/or cocuite tree (*Gliricidia sepium*): in this method, the vanilla vines are supported by these trees, which provide good shade and organic matter; however, both are susceptible to pests and diseases; c) shade-house: this semi-technified cultivation system uses a mesh that provides 50 % darkness to the plants, with live tutors (such as *Erythrina* spp.), or inert supports. This system is increasingly popular due to the lower cost of materials (Barrera-Rodríguez *et al.* 2011, Sánchez-Ramírez 2022) (Table 1).

Table 1. Crop systems for vanilla culture in Papantla, Veracruz, México.

	ACAHUAL (traditional)	**UNDER ORANGE TREE**	**SHADE HOUSE**	**PICHOCO TREE**
Type of cultivation	Agroforestry	Policulture	Technified monoculture	Monoculture
Support	*Erythrina* spp., *Litsea glaucescens, Tabernaemontana alba, Bauhinia forficata, Glyricidia sepium* Jacq.	*Citrus sinensis* L.	*Erythrina* spp., *Saurauia yasicae, Tabernae montana alba*	*Erythrina* spp.
Pests and diseases	Fungi and red bug Stem rot and root	Pest in support post	Fungi stem rot and root	Fungi stem rot and root
Pest and disease control method	Organic	Organic and Foley^{R}*	Organic and Foley^{R}*	Organic
Irrigation	No	No	Yes	No
Fertilizer	Yes	Yes	Yes	Yes
Yield (Kg/ ha)	50-500**, >50***	500-2,500**, >500***	5,140**, 3,400***	1,000- 4,000**, >1,000***
Advantages	-Conservation of native species -Sustainable production methods -Promotes the quality of the fruit in its aromatic content	Increases at double the economic value	Better yield production	Soils with more organic content
Disadvantages	-Susceptibility to fungal diseases -Low yield -More vulnerability to environmental damages	-Large input of agrochemicals -Increased frequency of citrus pests	-Unavailable for all producers	-Plagues in phorophytes. -More vulnerability to environmental damages

*Clorpiriphos (Foley^{R}), organophosphate insecticide. **Barrera-Rodríguez *et al.* 2009, Hernández-Hernández *et al.* 2010b, Bautista 2014, Trinidad García 2014, Sánchez- Ramírez 2022, Alejo-Viderique, unpublished. ACAHUAL (Traditional management): forest composed mainly of secondary vegetation, UNDER ORANGE (*Citrus sinensis* L.) and PICHOCO (*Erythrina* spp.) trees both tutors for vanilla cultivation, SHADE HOUSE: semi-technified cultivation system with shadow mesh.

Nowadays, vanilla production is critical in the region due to the low yields in recent years, which reached the minimal values reported for the region at the beginning of the century (Hernández-Hernández 2010a, Barrera-Rodríguez *et al*. 2011, SAGARPA 2019). However, production has been declining hastily due to several factors, including the abandonment of the crops due to a fragile organization of producers (Barrera-Rodríguez *et al.* 2014), organized crime (Jaramillo *et al*. 2012), management problems (Barrera-Rodríguez *et al.* 2011, Alejo-Viderique *et al.* datos no publicados), migration causing the loss of ancestral cultivation knowledge, and environmental factors that lead to diseases and pests exacerbated by climate change (Santillán-Fernández *et al.* 2018). In its natural habitat, *V. planifolia* requires pollinators for fecundation. However, for its cultivation, manual fertilization is needed, a technique developed years ago to allow vanilla to be grown outside of its native distribution region (Bory *et al*. 2008). This technique, together with clonal vegetative propagation (by cuttings), has resulted in low genetic diversity among vanilla cultivars worldwide, which has made it difficult to cope with environmental conditions, diseases, and climate change (Bory *et al*. 2008, Chambers *et al*. 2021, Hu *et al*. 2019, Lubinsky *et al*. 2008). It is important to note that in addition to very limited genetic variability in this crop, since its propagation is clonal by cuttings; therefore, the production of seedlings from the germination of seeds that come from manual pollination is inexistent (Schlüter *et al.* 2007). As a result, vanilla crops are very susceptible to the attack of pests and diseases (Menchaca *et al.* 2011, Ramos-Castellá & Iglesias-Andreu 2022).

Mexico should strive to produce better-quality vanilla rather than competing in quantity for its industrialization and technification with countries like Madagascar, the world's leading producer of this crop (Lubinsky *et al.* 2008, Barrera-Rodríguez *et al.* 2014). To achieve this, it is necessary to analyze how traditional (ancestral) cultivation promotes sustainable vanilla cultivation, as it must be consistent not only with local resources and traditional knowledge, but also with low input and local possibilities. To this end, solutions must be sought to the current problems that most affect vanilla production concerning its *per se* cultivation, among them, diseases caused by fungi such as *F. oxysporum* f. sp. *vanillae* (Hernández-Hernández 2010b, Adame-García *et al.* 2015, Casillas-Isiordia *et al.* 2017, González-Reyes *et al.* 2020). This is a saprophytic fungus of cosmopolitan distribution that infects the plant through lesions on the root's surface and causes a disease known as stem and root rot. While this disease can occur year-round, it is more severe during the rainy season when the increased humidity favors fungal growth and can cause crop losses of up to 67 % (González-Reyes *et al*. 2020). Improper crop management includes removing diseased plant tissues and then throwing them into the same cultivar, thus reinoculating the fungus in the soil, which could survive in it (Adame-García *et al*. 2015, Hernández-Martínez *et al*. 2020, Alejo-Viderique *et al.* unpublished). Yet, the soil also harbors beneficial microorganisms that can be used to improve the health of vanilla plants (Chen *et al.* 2023), such as mycorrhizal fungi (mainly *Tulasnella* spp. and *Ceratobasidium* spp.) and bacteria of the genera *Bacillus*, *Rhizobium*, *Mesorhizobium*, and *Pseudomonas*, which help fix nitrogen, improve nutrient uptake, and promote plant growth (Wanjofu *et al*. 2022, Díaz-Urbano *et al*. 2023). Some of these microorganisms are the consortium of mycorrhizal fungi, for the better plant nutrition, resistance to water stress, and act as a protective biological barrier to the attack of pathogenic fungi. In fact, it could possibly make the fruit quality higher (Gamboa-Gaitán 2014, Bleša *et al.* 2021, Manrique-Barros *et al.* 2023). Global research on the use of these microorganisms for biofertilizers has been limited because foreign producers prioritize investments in biotechnology for inorganic fertilizers and advanced agricultural systems, driven by the high demand for these products, rather than traditional methods (Egamberdieva *et al*. 2015, Maitra *et al*. 2021, Gamage *et al*. 2022). For these reasons, the search for beneficial microorganisms that provide nutrients to plants, promote plant growth and resistance to biotic and abiotic factors -known as bioinoculants- is crucial. Bioinoculants are urgently needed to develop highly competitive certified organic crops as local resources to fully or partially replace the use of chemical fertilizers (Armenta-Bojórquez *et al.* 2010, Abhilash *et al.* 2016, Maitra *et al.* 2021). This, in turn, creates more sustainable agricultural systems to increase production without compromising human and animal health, and preserve essential resources such as soil, water, and diversity (Armenta-Montero *et al*. 2022, Díaz-Urbano *et al*. 2023). *V. planifolia* crops, specific studies have been conducted with several microorganisms like *Trichoderma harzianum*, *Bacillus megatherium*, and *Pseudomonas fluorescences* used alone or in consortia as bioinoculants to evaluate the effect of rooting and growth of cuttings, showing positive effects (Murthy *et al.* 2010). Moreover, mycorrhizal fungi of the genera *Ceratobasidium* and *Tulasnella* have also been used for biocontrol against *Fusarium* (spp.). All these studies contribute to a better understanding of interactions between tropical orchids and their microbiota (Manrique-Barros *et al*. 2023). The search for bioinoculants is urgent to develop highly competitive certified organic crops as local resources; however, this must start by the knowledge of the *in situ* mycorrhizal partners.

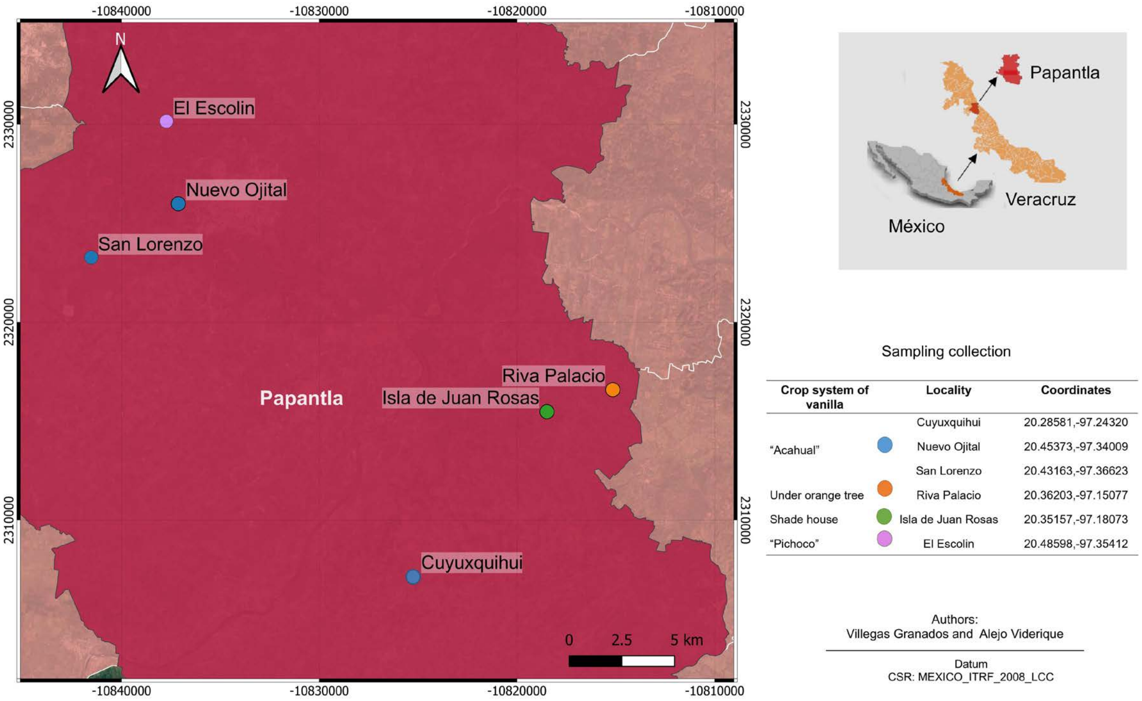
Most orchids are known to associate primarily with mycorrhizal fungi of the anamorphic genus *Rhizoctonia* (Basidiomycota) (Kristiansen *et al.* 2001, Rasmussen 2002, Rasmussen & Rasmussen 2014). This group of fungi is characterized by the absence of reproductive structures in the sexual stage (anamorph), the presence of bifurcations that generate right angles with respect to the main hypha, and a constriction at the point of bifurcation with an adjacent septum, which makes these key criteria for identification (González García *et al*. 2006, Porras-Alfaro & Bayman 2007). Additionally, monilioid cell formation, hyphal width and length, and number of nuclei per cell are also used for characterization (Andersen 1996, Rachanarin *et al*. 2018). The association with mycorrhizal fungi is decisive for their establishment, survival, distribution, and development of orchids (Li *et al*. 2021, Rasmussen & Rasmussen 2014), as these fungi play an important role in nutrient and water uptake in terrestrial as well in epiphytes species (Zotz & Hietz 2001, Yoder *et al*. 2010, Martos *et al.* 2012). Additionally, it is worth noting that mycorrhizal fungi form masses of intracellular hyphae within the root, known as pelotons (Currah *et al*. 1997). Unlike orchids from temperate regions, the mycorrhizal relationships of terrestrial orchids from tropical zones are largely unexplored (Pereira-Liparin *et al.* 2005). In vanilla, there is little information on mycorrhizal fungal communities in association with roots of native cultivars (Porras-Alfaro & Bayman 2003, 2007, Bayman *et al.* 2011, González-Chávez *et al.* 2018, Johnson *et al.* 2021). *Ceratobasidium* (anamorph *Ceratorhiza*), *Thanatephorus* (anamorph *Rhizoctonia*), and *Tulasnella* (anamorph *Epulorhiza*) have been reported as common mycorrhizal fungi of vanilla roots (Porras-Alfaro & Bayman 2007), with the latter genus being the most abundant in vanilla crops (Petrolli *et al*. 2024, Hellequin *et al*. 2024). This highlights the ability of *V. planifolia* to associate with different types of fungi depending on its environment (Rasmussen & Rasmussen 2014, Hellequin *et al*. 2024). The diversity in vanilla cropping systems where different phorophytes (support tree for epiphytes) are growing, as well as differential management practices (Table 1), can provide distinct nutrients and microenvironmental niches for both plants and fungi. This, in turn, promotes the diversity of the microbial community (Johnson *et al*. 2021, Petrolli *et al*. 2024). The mycorrhizal association with *V. planifolia* is facultative during the photosynthetically active stage, and the plant's nutritional status is favored through the carbon exchange that occurs when it degrades the fungal hyphae (Porras-Alfaro & Bayman 2007). The objective of this study was to identify mycorrhizal fungi from the four cultivation systems of *V. planifolia* used by Totonac producers utilizing a combination of molecular and morphological techniques. In addition, the phylogenetic relationship of the mycorrhizal fungi isolated from vanilla grown in these traditional systems was analyzed with those reported in the literature for other cultivars worldwide.

Materials and methods

Study area. Four different vanilla management systems were sampled in the locality of Papantla Veracruz, Mexico (Figure 1). The characteristics of each management system are summarized in Table 1.

Root sampling. Prior diagnosis of the mycorrhizal status of vanilla plants in the study systems; it was found that the roots with the highest colonization percentages were the terrestrial secondary roots, while the aerial roots showed very little to no colonization. Consequently, the sampling consisted of taking 10 secondary terrestrial roots from different plants distributed along each cropping system collected during the rainy season of August 2022-2023 (Figure 2 A-F). Colonization characterization was performed and segments with abundant mycorrhiza and without peloton degradation were selected for isolation.

Mycorrhizal isolation and morphometric characterization. The mycorrhizal segments were disinfected with 10 % sodium hypochlorite for 10 min, followed by a wash with sterile distilled water, a wash with 70 % ethanol for one



Sampling collection

Crop system of vanilla		Locality	Coordinates
		Cuyuxquihui	20.28581,-97.24320
"Acahual"	●	Nuevo Ojital	20.45373,-97.34009
		San Lorenzo	20.43163,-97.36623
Under orange tree	●	Riva Palacio	20.36203,-97.15077
Shade house	●	Isla de Juan Rosas	20.35157,-97.18073
"Pichoco"	●	El Escolin	20.48598,-97.35412

Authors:
Villegas Granados and Alejo Viderique

Datum
CSR: MEXICO_ITRF_2008_LCC

Figure 1. Locations in Papantla, Veracruz, Mexico, where the four cropping systems of *Vanilla planifolia* were sampled.

min, and yet another wash with sterile distilled water. The pelotons were removed from the root tissues of each segment and seeded on Fungal Isolation Medium (FIM) (Clements *et al.* 1986), as well as on water-agar. Plates were observed daily for growth and pelotons free of contamination and with emerging hyphae were subcultured onto Potato-Dextrose-Agar medium (PDA 39 g, pH 6.8; DIBICO®, Mexico) (Figure 2 G-H). In this medium, the colony was allowed to grow, and they were subcultured in the same medium in case it expressed characters of fungi associated with mycorrhizae formers (*Rhizoctonia* anamorphs). Next, their morphometry was characterized by quantifying the growth rate on PDA and malt extract agar (MEA 15 g, pH 5.0; DIBICO®, Mexico) (Tom & Raper 1945), appearance of colonies, meristics of hyphae and monilioid cells, nuclei staining (Meinhardt *et al.* 2001), and reaction to polyphenoloxidase in tannic acid medium which allows differentiation between families such as Ceratobasidiaceae and Tulasnellaceae (Davidson *et al.* 1938, Zelmer *et al.* 1996).

Phylogenetic analysis. Once the *Rhizoctonia* anamorphic characters were confirmed, the cultures were selected for sowing in liquid medium Potato-Dextrose-Broth (PDB, 24 g, pH 6.8, Difco™, USA) for massing. From there, subsamples were taken for DNA extraction with the Promega Wizard® kit, according to the manufacturer's characteristics. DNA was also extracted from root pelotons to identify mycorrhizal fungi that could not be isolated. Samples of mycorrhizal root explants -each approximately 0.5 cm thick- were also extracted. The extracted DNA was used for amplification of the ITS region with the ITS1-4, ITS1-4 Tul oligos (Gardes & Bruns 1993, Taylor & Mccormick 2008). PCR conditions were 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min elongation time 72 °C. For ITS1-4 Tul, they were 96 °C for 2 min, 35 cycles of 94 °C for 30 s, 54 °C for 40 s and 72 °C for one min, elongation time 72 °C. PCR products were sent for sequencing by capillary electrophoresis to Macrogen, Inc. The sequences obtained were visualized and edited in the program Geneious® version 11.1.5. Alignment was carried out in the program SeaView Version 4 (Gouy *et al.* 2010). The construction of the phylogenetic tree based on the ITS1-4 regions was performed by Maximum Likelihood trees (Felsenstein 1981) and Tamura Nei (Tamura & Nei 1993), using the program MEGA X version 10.2.6 (Kumar *et al.* 2018). Some of the sequences obtained corresponded to endophytes of *Fusarium* spp.; therefore, we proceeded to perform their phylogeny and associate it with cultivar management.

Figure 2. *Vainilla planifolia* cultivation systems in Papantla, Veracruz. (A) *Acahual*, (B) under orange tree, (C) shade house, (D) associated with pichoco tree, (E) sampling of terrestrial roots, (F) presence of mycorrhizal pelotons in terrestrial root (red arrow) and aerenchyma around the vascular cylinder (black arrows), (G and H) hyphal growth from the pelotons for isolation. Bar 100 µm.

Results

Morphological and physiological characterization of isolates. During the isolation, pelotons with oval and spherical morphotypes were observed in the system under orange trees (Figure 2 G-H). A total of 74 isolates were obtained, of which 10 were mycorrhizal fungi (isolation efficiency 13 %). The cultivar systems that presented the greatest difficulty in isolation were the shade house and the *acahual* (traditional management system) in the Cuyuxqui community (Figure 1). In the latter, no isolates could be obtained (the resulting sequences were directly obtained from the pelotons in the roots). This may be due to the presence of fungi, such as *Fusarium oxysporum,* in the soil of the cultivation systems, particularly in the acahual and shade house systems; additionally, plants with lesions such as stem and root rots were observed. The description of the 10 strains with characteristics of the anamorph *Rhizoctonia* is presented in Table 2. The appearance of the colonies (Figure 3) was of strains with slow growth (< 4 mm per day), submerged mycelium, some were slightly waxy (Uo-01, A-3, A-12, P-02 and P-06) with soft aerial mycelia without forming rings like strains A-V1, A-V5 and Sh-01. The hyphae measured on average 3.6 µm, and some strains presented monilioid cells in digitiform form (A-3, A-V1, Uo-05 and Uo-09) (Figure 3A-B) with other characteristics (Figure 3C-D). All of them presented binucleate hyphae (Figure 3E-H) and were negative to tannin degradation. During the isolation process, there were mostly strains corresponding to morphological characteristics of *Fusarium*. These isolates presented a white cottony appearance and –as they aged– they turned purple or brown with pink-brown pigmentation on the reverse side. Microconidia and chlamydospores were also observed in the morphological structure, so some were selected for molecular identification (Figure 4).

Phylogenetic analysis. The phylogenetic analysis is presented in Figure 5, which shows the distribution of the 16 sequences obtained, of which 6 were directly from mycorrhizal root explants (identified with *), and 10 sequences were obtained from isolates. It was observed that the sequences are grouped in two main clades associated with fungi identified for *Vanilla planifolia* and *V. poitaei* from Puerto Rico (Gamboa-Gaitán 2014). These sequences show a 99 % similarity, indicating that they are likely the same species obtained from the system under orange tree (strains Uo-01 and Uo-03*). Strains P-06, U0-09 and A-10* in this same clade are associated with a 99 % similarity to a *Tulasnella* identified from a terrestrial orchid from Reunion Island (Kumar *et al.* 2018). Sequence Sh-04* is found in the clade of a *Tulasnella* isolated from *V. planifolia* from Puerto Rico, and sequence Sh-07* is found in the clade of a *Tulasnella* isolated from *V. poitaei* also from Puerto Rico (Porras-Alfaro & Bayman, 2003) with the same similarity as the previous ones. The remaining isolates form a distinct clade supported by a bootstrap value of 96.0. This clade consists of sequences from the four culture systems, seven isolates, and two tissues (Sh-03 and A-07). The percent similarity to *Tulasnella* AP-27, isolated from *V. poitaei* from Puerto Rico, ranged from 94.8 to 97.4 representing the closest clade.

Figure 6 shows the phylogenetic analysis of seven selected *Fusarium* strains, where five of the strains correspond to *F. falciforme*, and strains Sh-04 (shade house) and A-01 (acahual) correspond to *F. oxysporum* f. sp. *vanillae* (99.99% similarity) -the main pathogen associated with stem and root rot disease (Pinaria *et al.* 2010, Adame-García *et al*. 2015).

Discussion

In this study, only mycorrhizal fungi of the genus *Tulasnella* were isolated, identified by morphology (hyphae smaller than 4 microns, no polyphenol oxidase activity, non-digitiform monilioid cells, slow growth rates, binucleate cells, submerged growth) and confirmed by molecular identification, in the four cropping systems. Besides observing pelotons with different morphologies, no other genus associated with vanilla such as *Ceratobasidium*, *Thanatephorus*, *Sebacina* and *Scleroderma* were found, -not even from direct tissue amplifications (Otero *et al.* 2002, Porras-Alfaro & Bayman 2003, 2007, González-Chávez *et al*. 2018, Carbajal-Valenzuela *et al.* 2021).

Table 2. Morphological characters of *Tulasnella* spp. isolates from *Vanilla planifolia* from Papantla, Veracruz, Mexico.

Type of cultivation	Isolates	Colony	Color	Growth rate at 25 °C (mm/d) on:		Basal septum	Hyphal width	Monilioid cells (µm)	
				PDA	EMA	(µm)	(µm)	Width	Length
Acahual	A-3	Submerged flat	Cream to brown	3.8 ± 1.54^{bc}	2.51 ± 1.08^{bc}	3.76 ± 0.55^{abc}	3.43 ± 0.41^{abc}	$7.78\pm1.06^{*a}$	39.04 ± 9.35^{a}
	A-12	Flat	Cream to white	4.02 ± 1.04^{abc}	3.35 ± 0.55^{abc}	3.44 ± 0.56^{bc}	3.99 ± 0.47^{ab}	G	
	A-V1	Flat	Cream to white	$3.29\pm0.65^{*c}$	$2.91\pm0.43^{*c}$	3.33 ± 0.91^{bc}	3.70 ± 0.54^{ab}	G	
	A-V5	Flat	Cream to white	3.2 ± 0.93^{abc}	4.13 ± 0.86^{abc}	$3.12\pm0.55^{*c}$	3.8 ± 0.45^{ab}	NO	
Under orange tree	Uo-01	Sumerged flat	Cream to white	3.12 ± 1.1^{bc}	3.02 ± 1.12^{bc}	3.34 ± 0.85^{bc}	$4.20\pm1.18^{*a}$	NO	
	Uo-05	Velvety flat	Cream to white	$3.97\pm0.83^{*a}$	$4.5\pm1.58^{*a}$	3.35 ± 0.61^{bc}	3.18 ± 0.65^{bc}	3.18 ± 0.65^{b}	$28.99\pm3.69^{*b}$
	Uo-09	Flat	Cream to white	3.43 ± 0.52^{bc}	2.56 ± 1.5^{bc}	3.83 ± 0.99^{bc}	$4.11\pm0.81^{*a}$	$8.95\pm1.28^{*a}$	$26.75\pm6.97^{*b}$
Shade house	Sh-01	Sumerged flat	Cream to white	$1\pm0.25^{*d}$	$0.97\pm0.24^{*d}$	$5.96\pm1.24^{*a}$	3.99 ± 0.48^{ab}	NO	
Pichoco tree	P-02	Sumerged flat	Cream to white	4.05 ± 0.84^{ab}	3.7 ± 1.07^{ab}	3.18 ± 0.39^{bc}	3.47 ± 0.32^{abc}	G	
	P-06	Flat	Cream to white	3.43 ± 1.04^{abc}	3.42 ± 0.95^{abc}	$4.36\pm1.07^{*b}$	$2.73\pm0.42^{*c}$	NO	

Asterisks represent significant differences $p \leq 0.05$; letters show pairwise comparisons according to Tukey's method with confidence value of 95%; NO= not observed, G, growing. Acahual (Traditional management): forest composed mainly of secondary vegetation, Under orang (*Citrus sinensis* L.) and Pichoco (*Erythrina* spp.) trees both tutors for vanilla, Shade house: semi-technified cultivation system with shadow mesh.

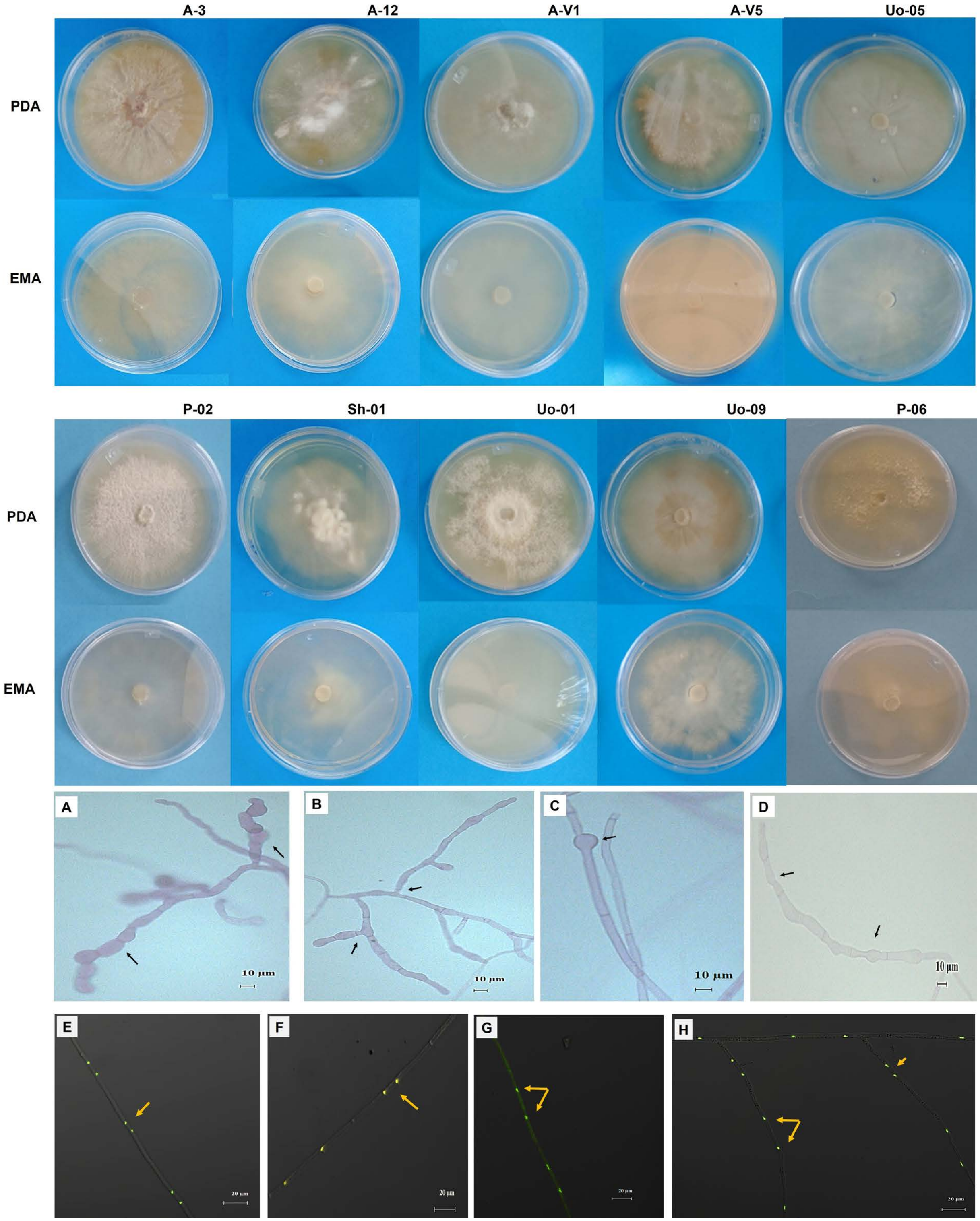


Figure 3. Morphological characteristics in culture medium PDA and EMA, and morphometric characteristics of *Tulasnella* spp. strains isolated from *Vanilla planifolia* of four cultivars from Papantla, Veracruz, Mexico. A-*acahual*; U-under orange tree; Sh-shade house; P-pichoco. (A) Digitiform monilia of isolated *Tulasnella* A-12; (B) Monilioid cells and 90° angle of growth of *Tulasnella* A-V1; (C) intercalary spherical structure observed in hyphae of *Tulasnella* Sh-01; (D) Digitiform monilia with a "waist" of *Tulasnella* Uo-09. Bar 10 µm; (e-i) Binucleate hyphae of *Tulasnella* strains Uo-01, A-3, Sh-01, Uo-01 and Uo-09. Bar 20 µm.

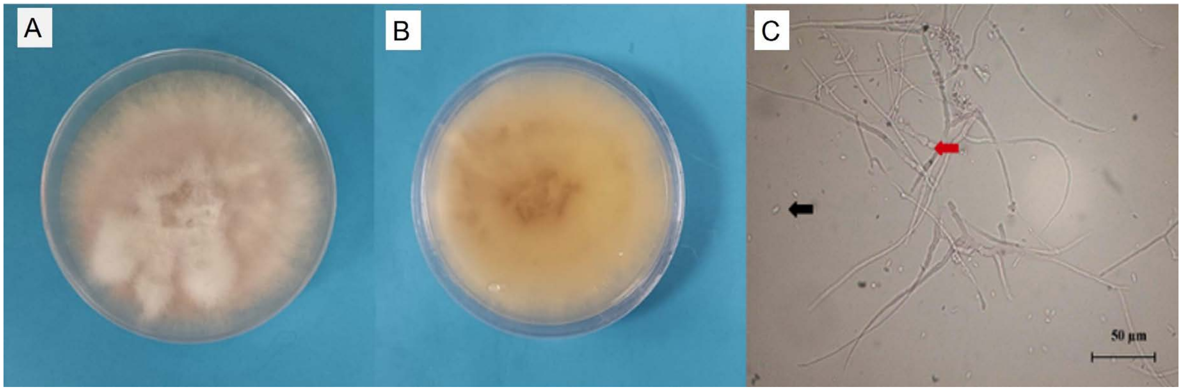


Figure 4. Colonial growth of *Fusarium oxysporum* f. sp. *vanillae* Sh-04 isolated from *Vanilla planifolia* roots of the shade house system in Papantla, Veracruz, Mexico. (A) Front, (B) back, (C) microconidia (black arrow), chlamydospores (red arrow). Bar 50 µm.

Conversely, no consistent morphological differences of the colonies grouped in the different clades were found. Except for two isolates (A-V5 and Uo-05), all strains grew faster on PDA than on MEA. Among the strains forming a separate clade, only Sh-01 differs from all isolates in basal septum and hyphal width, and it does not cluster with the rest of isolates in the phylogenetic tree. Hyphal width is a distinguishable feature to separate *Epulorhiza* anamorphs, with most measuring less than 4 microns or slightly above. Isolate P-06 was significantly smaller than the others, but it did not affect its growth rate or cause it to separate and form a distinct clade with fine hyphal strains. In the case of monilia, only four isolates generated these asexual cells, making it difficult to associate their appearance to phylogeny. Nonetheless, all exhibited characteristics of highly elongated cells, which further differentiate the anamorph *Ceratorhiza* (Fujimori *et al*. 2019). This is even though each cultivation system has different management conditions, such as the incorporation of distinct organic matter sources, which could be reflected in the diversity of saprobes in the soil, as mycorrhizal fungi are. A study conducted in three localities in Veracruz, at distances between 0.8, 10, and 32 km from the sites of this research, investigated three different traditional management systems using other tutors (trunks of *Gliricidia sepium* and *Pachira macrocarpa*); however, this study did not report any *Tulasnella* sequences (Johnson *et al.* 2021). One reason for this could be that they do not isolate the fungi from mycorrhizal structures despite the higher diversity of endophytes sequenced directly from the roots; indeed, most of them are not assigned as mycorrhizal. It is common for the number of molecular species identified in orchids to be several times higher than the number of isolated endophytes, likely because some fungi of the *Rhizoctonia* genus do not grow in culture media (Bayman & Otero 2006). The genus *Tulasnella* has also been found to be the most abundant genus in vanilla crops on Reunion Island (Petrolli *et al*. 2024, Hellequin *et al*. 2024). However, many orchid genera are known to be associated with specific families of mycorrhizal fungi, such as the terrestrial orchid *Cypripedium*, which is associated with the family Tulasnellaceae (Moreno-Camarena & Ortega-Larrocea 2022).

This demonstrates that the management system, phorophyte species, and soil nutrients influence the symbionts associated with vanilla (Johnson *et al*. 2021, Hellequin *et al*. 2024, Petrolli *et al*. 2024). It is possible that some fungi coming from crops outside the harvesting site are inoculated already in the soil, as many producers usually buy rooted cuttings from different locations. Instead, fungi can also originate from transplanted or natural soils, depending on the host or phorophyte. In citrus cultivations, plants are introduced to the soil, whereas in traditional management systems, *acahual* and *pichoco* tree, most phorophytes are naturally occurring trees from the site. Additionally, many of the mycorrhizal fungi associated with vanilla are genetically similar to those found in tropical orchids worldwide (Nontachaiyapoom *et al.* 2010).

Vanilla crops are highly susceptible to attacks from fungi of the genus *Fusarium* because the temperature and humidity that promotes growth also create the ideal conditions for these pathogens' development, which are among the most abundant soil and root endophytes. However, not all species are necessarily pathogenic or cause symptoms

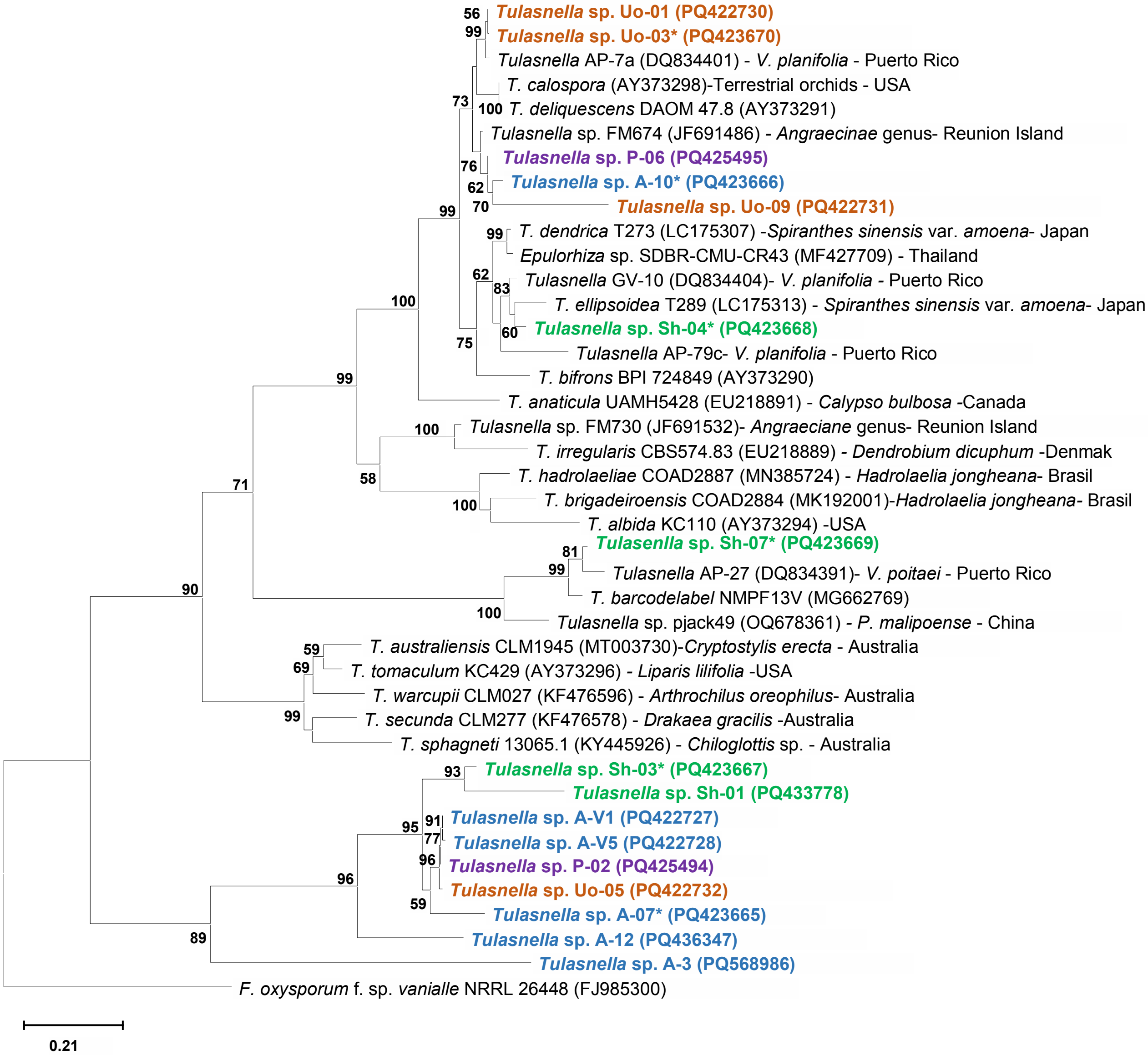


Figure 5. Phylogenetic tree based on the ITS regions of strains of the genus *Tulasnella* isolated from various cropping systems of *Vanilla planifolia* from Papantla, Veracruz. The color indicates the cropping system from which they were isolated: blue is acahual (secondary vegetation of tropical forest); orange is under orange tree (*Citrus sinensis*); green is shade house and purple is pichoco (*Erythrina* spp.); * indicates that the sequences were obtained directly from mycorrhizal roots. The analysis was performed by maximum likelihood with bootstrap replicates = 1,000.

when vanilla plants are healthy (Hernández-Hernández *et al.* 2010b, Adame-García *et al.* 2015). In Mexico, *F. oxysporum* has been reported on vanilla plants in the state of Veracruz (Adame-García *et al.* 2015, Gónzalez-Reyes *et al.* 2020), Nayarit (Casillas-Isiordia *et al.* 2017), and the Huasteca Potosina (Hernández-Martínez *et al.* 2020, Franco-Galindo & Mosquera-Espinosa 2023). This work identifies at least two species -*F. falciforme* and *F. oxysporum* f. sp. *vanilla*- across different cropping systems. The latter was identified in the *acahual* (Cuyuxquihui site) and shade house systems, where plants exhibited symptoms of chlorosis, as well as stem and root rot. In the *acahual* system, the prevalence of the pathogen is linked to inadequate management practices for the control of this disease. This issue is particularly critical in agricultural crops, where management strategies aimed at enhancing plant growth and yield can inadvertently create environmental conditions that favor the emergence and proliferation of pathogenic microorganisms (Dearnaley *et al*. 2012, Hernández-Hernández *et al.* 2010b). Control of fungal diseases is therefore carried out by applying different nonspecific fungicides that also affect mycorrhizal fungi populations.

The mycorrhizal genus *Tulasnella* is the predominant symbiotic genus in four vanilla cropping systems in the totonacapan region, indicating that this plant specifically associates with several species, both globally distributed as well as local to Papantla, Veracruz. This highlights the need for molecular tools to distinguish between potential different species based on their percentage of similarity, even though their mycorrhizal potential does not correlate with their mycorrhization efficiency. The diversity of mycorrhizal fungi associated with orchids is still underexplored, leading to the discovery of more new molecular species encrypted by somatic mycelia (Freitas *et al*. 2020, Arifin *et al*. 2022), which have insufficient structural features to identify them (Vilmar *et al*. 2013, Li *et al*. 2021). The results offer valuable insights into potential fungal strains adapted to local management conditions, which could be of particular interest in vanilla cultivation and could serve as future research to produce bioinoculants that help increase nutrient availability in commercial vanilla plantations. According to Álvarez-López *et al*. (2014), rhizospheric microorganisms isolated from vanilla plants have the potential to increase biomass, height, and phosphorus (P) content in vanilla plants (Osorio *et al*. 2012, Mahadeo *et al*. 2022). Furthermore, it would be of utmost importance to evaluate their capacity as biological control agents against pathogens such as *Fusarium*. Although cosmopolitan species were

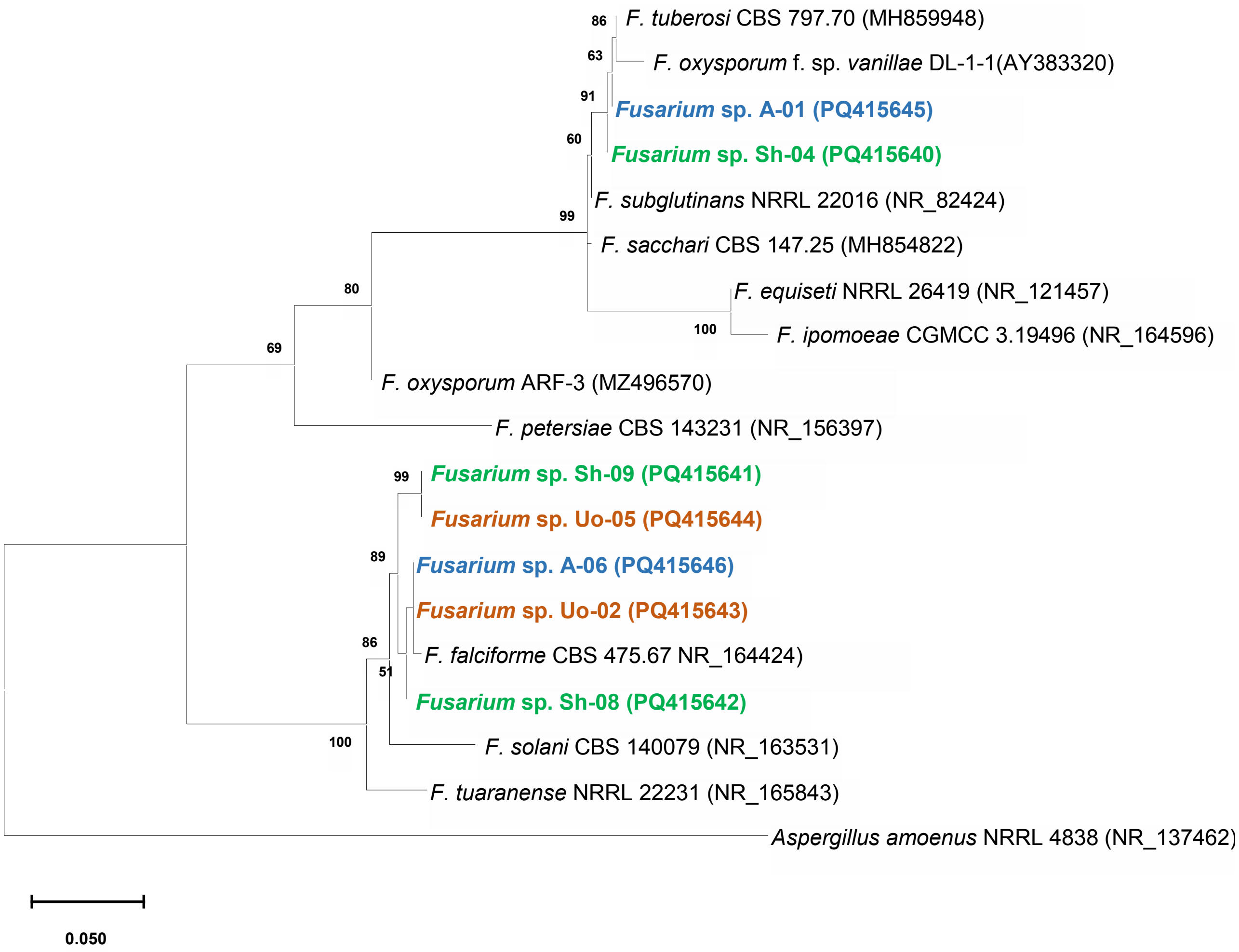


Figure 6. Phylogenetic tree based on the ITS regions of strains of the genus *Fusarium* and strains isolated from three cropping systems of *Vanilla planifolia* from Papantla, Veracruz. The color indicates the cultivation system from which they were isolated: blue is Acahual (secondary vegetation of tropical forest); orange is under orange tree (*Citrus sinensis* L.); green is shade house. The analysis was performed by maximum likelihood with bootstrap replicates = 1,000.

found, both genotypes could be explored as inoculants either prior to transplanting cuttings or directly inoculated into the substrates after mass inoculation. It is necessary to begin this type of research because vanilla cultivation in Mexico is highly significant, not only for its commercial value but also for its importance in its cultural representation (Kourí 2013, Amenta-Montero *et al*. 2022, Karremans 2022). Consequently, it is crucial to explore ecological alternatives that facilitate the cultivation of organic vanilla.

Given the scenario of climate change, deforestation, loss of traditional knowledge and social issues related to organized crime, vanilla seems to be destined to extinction (Villanueva-Viramontes *et al.* 2017), so local vanilla producers of vainilla should seek alternatives together with the denomination of origin (DOF 2009) (ancestral connotation) and move towards organic cultivation. This would enable them to have a slogan to face up to its decreased production (Barrera-Rodríguez *et al.* 2014) as the "Organic SAGARPA Mexico" seal (SENASICA 2025) or the Green Seal, that promotes agro-ecological production (SEDEMA 2025).

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