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Isolation and identification of beneficial orchid mycorrhizal fungi in *Paphiopedilum barbigerum* (*Orchidaceae*)

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ABSTRACT

Seed germination and seedling development in nearly all orchid species rely on a symbiotic relationship with mycorrhizal fungi; however, this is not the case with all mycorrhizal fungi. This study aims to provide an understanding about the important role of mycorrhiza in seed germination and growth of *Paphiopedilum barbigerum*. Therefore, we isolated and identified endophytic fungi from the roots of wild *P. barbigerum*. The beneficial mycorrhizal fungi *Epulorhiza* sp. FQXY019 and *Tulasnella calospora* FQXY017 were screened by seed symbiotic germination tests and found to promote seed germination. However, only the seeds inoculated with FQXY019 progressed from the seed germination to rooting stage. This shows that mycorrhizal fungi and *P. barbigerum* have a specific relation at different growth phases. In addition, we selected FQXY019 and inoculated it into MS medium, B5 medium, OMA medium, and PDA medium. The results showed that FQXY019 co-cultured on PDA significantly promoted the increase in seedling fresh weight, leaf length, and root length ($p < .01$). Furthermore, it significantly promoted the root number and leaf number of seedlings compared with those co-cultured on MS, B5, and OMA media and control ($p < .05$). Thus, this study demonstrated the promoting effect of *Epulorhiza* sp. FQXY019 on seed germination and seedling development, making it an alternative method for the artificial propagation of *P. barbigerum*.

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Introduction

Under natural conditions, orchids rely on mycorrhizal fungi early in their life cycle.[1]The seeds of the orchids are small, so a single fruit encloses millions of them.[2] Orchid seed lack a developed endosperm and reserves are limited to a small store of complex carbohydrates. Thus, nearly all orchid species rely on a symbiotic relationship with fungi for seed germination and protocorm development.[3] Depending on the presence of functional fungi in cortical cells, orchid fungi are classified as mycorrhizal and nonmycorrhizal fungi.[4] Mycorrhizal fungi (soil saprotrophs),[5] such as *Ceratobasidiaceae, Tulasnellaceae*, and *Sebacinaceae*, are collectively referred to as *Rhizoctonia*; they increase seed germination as well as the development rate of protocorm and seedlings of orchids.[6–12] However, not all mycorrhizal fungi can promote seed germination, seedling growth, and development of orchids.[13,14] Therefore, it is essential to screen and identify beneficial mycorrhizal fungi in orchids.

Orchidaceae is a species-rich and diverse family of flowering plants comprising approximately 28,000 species in 880 genera.[15,16] *Paphiopedilum* Pfitzer are famous worldwide because of their unique flower shape, attractive colors, and long flower lifespan. They are also known as slipper orchids; they have a high ornamental value and are one of the most popular genera of family Orchidaceae.[17,18] *Paphiopedilum barbigerum* is native to southwest China and northern Vietnam[19] and is one of China's state-protected one-grade rare and endangered plants. It is an endangered species that is critically threatened by excessive harvesting, climate change, and severe habitat destruction.[20] The only wild population have been discovered in northern and western Guangxi and Guizhou provinces, China.[21] Therefore, we must understand the establishment and maintenance of the mutualistic interactions between this orchid species and its corresponding mycorrhizal fungi to conserve wild populations of the threatened species and reintroduce the plant into declining populations. Unfortunately, there are no reports on the isolation, identification, and screening of the mycorrhizal associates of *P. barbigerum*.

Artificial propagation technology can be used to realize the mass reproduction of *Paphiopedilum*,[22]while the use of mycorrhizal fungi can help overcome the problems in the culture process, such as low survival rate, growing slowly after transplanting and poor resistance.This study aimed to isolate and identify mycorrhizal fungi from the roots of *P. barbigerum*. Additionally, the effects of the mycorrhizal fungi on seed germination and seedling growth of *P. barbigerum* were evaluated. The isolation, identification, and screening of mycorrhizal fungi are important to determine the specific and beneficial endophytic fungi species in *P. barbigerum* that will

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increase *in vitro* germination and seedling growth and provide fundamental guidance for the symbiotic techniques for *P. barbigerum* artificial propagation and conservation.

Materials and methods

As part of *in vitro* propagation studies, root samples of *P. barbigerum* were collected from their native habitat from a mountain near Fuquan County in the Guizhou Province, China, in February 2021 and the fungi were isolated from these samples.

Isolation of mycorrhizal fungi from the root of P. barbigerum*: Zhu et al.'s method*[23]

First, wild and healthy *P. barbigerum* root samples were chosen, rinsed under tap water for 4–6 h and cut into 4–6-cm long segments. The root segments were placed in a Petri dish filled with sterile distilled water, and root hairs, epidermis, velamen, and other attachments were gently scraped off using a scalpel on an ultra-clean workbench. Additionally, brownish zones containing pelotons were selected after microscopic examination, isolated, and rinsed three times with sterile distilled water. Then, the root segments were surface-sterilized using 70% ethanol for 30 s, 0.1% mercuric chloride for 4 min, and finally rinsed with sterile distilled water five times. The root segments with pelotons are cut 2-cm long segments. This released individual pelotons from the cortex cells, which were then placed in a 60-mm sterile Petri dish containing 10 ml sterile distilled water. Pelotons from all roots were teased three times from the exodermis to the endodermis. These were then incubated in the dark at 24°C and microscopically examined after 24 h.Second, the pelotons in sterile distilled water were placed under an optical microscope at low power. A 50-µl solution containing germinated mycelium was transferred to 1 cm^2 PDA disks using a 1-ml Eppendorf micropipette and the samples were cultured at 24°C until hyphae growth was observed.

Finally, the germinated pelotons and emerging hyphae on agar disks are observed under an optical microscope. These are then cut out from the agar disks, transferred onto fresh PDA plates, and cultured at 24°C.This was repeated until pure cultures were obtained and stored at 4°C for further observations and identification.

Morphological and molecular identification of mycorrhizal fungi

The purified fungal strains were inoculated on PDA plates and incubated at 24°C. The colony characteristics of each strain were observed and recorded daily, including colony shape and color.

Purified genomic DNA of the fungal strains was extracted using the cetyltrimethylammonium bromide (CTAB) method. Take an appropriate amount of fungal mycelium and 800 µl CTAB was then added to the 2 ml centrifuge tube,ground until it was melted, incubated at 65°C for 30–40 min using a water bath.800 µl of Chloroform/Isoamyl alcohol (24:1) was added, homogenized, and centrifuged at 12,000 rpm for 10 min. Supernatant was taken and transferred to a tube, and 1 ml of 95% cold ethanol was added, shaked gently for 2 min, and incubated at −20°C for at least 30 min. It was centrifuged at 12,000 rpm for 10 min. The supernatant was removed, 1 ml of 70% ethanol was added and centrifuged at 12,000 rpm for 10 min. The supernatant was then discarded, and dried on the super clean workbench. After drying, the tube was then added 50 µl TE buffer.DNA integrity was analyzed by electrophoresis on 1% agarose gels.

The primers for ITS sequencing of the fungi were ITS1 (5′-TCCGTAGGTGAAC

CACCTGCGG −3′) and ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′). Polymerase chain reaction (PCR) was performed in a 30-µL reaction mixture solution containing 1 µL of DNA, 1 µL each of the downstream and upstream primers, 12 µL of double-distilled water, and 15 µL of 2× Power Taq PCR Master Mix (Beijing Baitaike Biotechnology Co., Ltd). The amplification program comprised initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min by 35 cycles, and a final extension for 5 min at 72°C. The amplified fragments were purified and sequenced by an external service provider (Tianyihuiyuan Inc., China). The sequences were used to perform a BLAST search against the National Center for Biotechnology Information (NCBI) sequence database (GenBank) to compare the sequences with the most similarity to identify each endophytic fungus.

Phylogenetic analysis

The sequences of FQXY019 and FQXY017 were subjected to BLAST in the NCBI BLAST similarity search tool to find the closest match from the GenBank database. Phylogenetic trees were constructed using the neighbor-joining method on the basis of the available DNA data obtained during this study.

Symbiotic germination

The mycelium of each isolated endophytic fungi was inoculated onto 90-mm Petri dishes containing 30 mL of PDA medium (200 g potato, 10 g agar, 20 g glucose, and 1 L distilled water). This was repeated three times. After culturing for 2 weeks, it will be used for symbiotic seed germination tests.

Symbiotic germination tests were performed using the green fruit capsules of *P. barbigerum*. First, the fruit capsules were surface-sterilized using 70% ethanol for 30 s, followed by 0.1% mercuric chloride for 6 min, and rinsed with sterile distilled water five times. Then, the seeds were scooped out of the sterilized pods using a sterile scalpel and forceps. Approximately 100 seeds were sown in each fungus-PDA medium as described above. The plates were sealed and cultured in a 18-h light and 6-h dark cycle at 22°C-24°C and a light intensity of 1200 lux. After inoculation and culture for 2 weeks, seed germination, protocorm formation, and seedling development were monitored. The average of the three replicates of

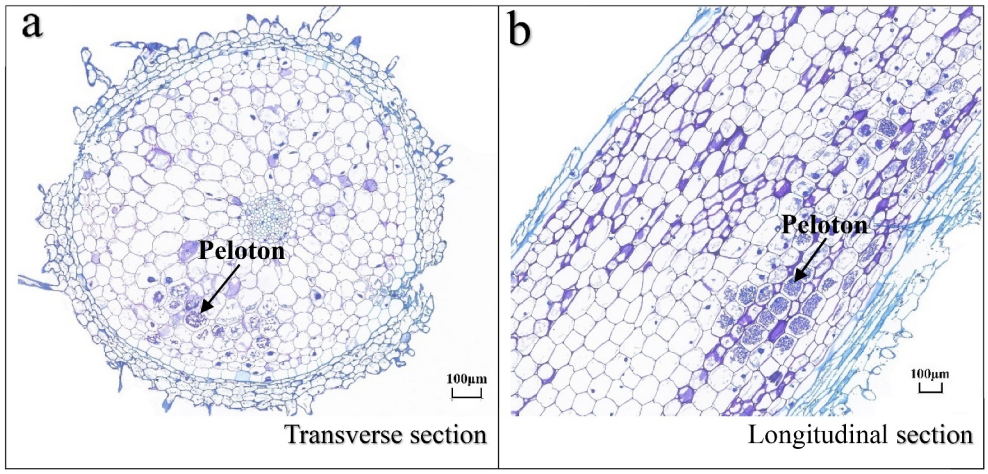

Figure 1. Longitudinal and transverse profiles of the roots of *Paphiopedilum barbigerum* grown from wild seedlings. Arrows represents peloton (a,b).

plate cultures was calculated in subsequent analyses. The germination rate was calculated as follows: the number of germinated seeds/number of inoculated seeds × 100%.

Effects of mycorrhizal fungus co-cultured with different media on seedling growth of P. barbigerum

The test seedlings were obtained from the symbiotic germination of seeds and mycorrhizal fungi. The mycorrhizal fungi and seedlings were co-cultured on six replicate plates of each treatment (I.e., Murashige and Skoog medium, Gamborg's B5 medium, OMA medium, and PDA medium).First, the four abovementioned media were inoculated with a 0.5 × 1 cm^2 block of FQXY019 into each tissue culture bottle,cultured in the dark at 28°C. Media without treatment (without the treatment of FQXY019) were considered as the control (CK).After 2 weeks of culture, the test seedlings were inoculated into tissue culture bottles. Finally, the bottles were sealed and incubated in 18-h light and 6-h dark cycle at 22°C–24°C at a light intensity of 1200 lux.

Statistical analysis

The SPSS (Statistical Package for the Social Sciences v.21.0) software was used for statistical analysis. Data were statistically analyzed using the single-factor analysis of variance method; significant differences between the treatments were determined using multiple comparisons using LSD ($p< 0.05$, $p< 0.01$).

Results

Isolation and identification of mycorrhizal fungi from P. barbigerum

Observation under an optical microscope revealed that the pelotons were unevenly distributed in the root cortex cells of wild *P. barbigerum* seedlings (Figure 1a, 1b). According to Bernard,[24] peloton formation in the culture that characterized *Rhizoctonia*-like orchid mycorrhizae was also produced by *Fusarium* isolated from the orchid.[25] Endophytic fungi were isolated from the root at different frequencies; 127 strains of endophytic fungi isolates were obtained in pure culture (Table 1), mostly belonging to the Sordariomycetes, Agaricomycetes, and Leotiomycetes class. These are *Fusarium nematophilum, Fusarium* sp., *Fusarium solani, Fusarium redolens, Epulorhiza* sp., *Pezicula ericae, Dactylonectria novozelandica, Chaetomium nigricolor*, and *Tulasnella calospora* (Table 2). *Fusarium* sp., *Epulorhiza* sp., and *Tulasnella* sp. accounted for 56.7%, 28.3%, and 11.8% of the total strains, respectively.

Symbiotic seed germination of *P. barbigerum*

This experiment evaluated the effects of endophytic fungi on promoting *in vitro* symbiotic seed germination. The results showed that the seeds inoculated with FQXY019 after 15 d of culture on PDA plates turned light brownish green. The seed coat ruptured due to enlarged embryos and protocorm-like bodies were formed after 30 d of culture (Figure 2a). Further, the first leaf emerged after 50 d of culture (Figure 2b) and further development and rooting occurred after 80 d of culture (Figure 2c). Seeds inoculated with FQXY017 turned light brownish green after 40 d of culture on PDA, and the seed coat ruptured due to the enlarged embryo (Figure 2d). Further, protocorm-like bodies formed after 55 d of culture (Figure 2e) and the coleoptile emerged after 75 d of culture (Figure 2f). Additionally, further development and browning after 90 d of culture could not be achieved (Figure 2g). However, seeds inoculated with FQXF001, FQXF002, FQXF005, FQXF006, FQXF008, FQXF016, FQXF015, FQXF042, FQXF045, and FQXF047 could absorb water and expand but not break through the testa on PDA (Figure 2h, 2i). Few seeds germinated and broke through the seed coat without inoculation but could not further develop CK (Figure 2j) (Table 3). As a results, FQXY019 and FQXY017 could promote seed germination. However, seeds inoculated with FQXY019 germinated and formed protocorms 25 days earlier than FQXY017, respectively.Notably, only seeds inoculated with FQXY019 on PDA plates could promote seedling formation and rooting, whereas FQXY017 could not.

Table 1. Occurrence and frequency of the endophytic fungi isolates.

Fugal Isolate	Number of Strains	Isolation Frequency
FQXY001	21	0.16
FQXY002	17	0.13
FQXY005	16	0.13
FQXY006	7	0.06
FQXY008	9	0.07
FQXY016	2	0.02
FQXY015	1	0.01
FQXY019	36	0.28
FQXY042	1	0.01
FQXY045	1	0.01
FQXY047	1	0.01
FQXY017	15	0.12

Normal sterilized *P. barbigerum* seeds were used as control (CK)

Effects of mycorrhizal fungus co-cultured with different media on seedling growth of *P. barbigerum*

We selected the beneficial mycorrhizal fungus FQXY019 to be inoculated into four co-culturing media to determine the best medium for co-culturing mycorrhizal fungus and seedlings. After 90 d of culturing, FQXY019 co-cultured with PDA medium significantly increased seedling fresh weight, leaf length, and root length compared with MS, B5, OMA medium, and CK ($p < .01$). Furthermore, PDA significantly promoted root and leaf numbers compared with MS, B5, OMA medium, and CK ($p < .05$). Remarkably, the root length increments of *P. barbigerum* seedlings were significantly inhibited by MS and B5 media ($p < .01$ and $p < .05$, respectively) (Figures 3 and 4). Thus, the best co-culture medium was the PDA medium. The co-culture of different media and mycorrhizal fungi had different effects on the growth of *P. barbigerum* seedlings.

*represents significant differences ($p< .05$), ** represents extremely significant differences ($p < .01$)

Morphological and molecular identification of beneficial mycorrhizal fungi FQXY019 and FQXY017

We screened out the beneficial mycorrhizal fungi FQXY019 and FQXY017 that promote seed germination of *P. barbigerum* by symbiotic seed germination test. Therefore, this study focuses on the morphological and molecular identification of the two strains on the PDA medium observed for FQXY019. Results showed that colonies were creamy-yellow and center-ring milky-white with irregular and serrated edges; hyphae grew close to the medium without aerial hyphae in its early stage (Figure 5a). Later, colonies became waxy and brown, had center-ring depression and small spherical bulges (Figure 5b). Hyphal branching occurred in the right- or near-right-angle (5.39–10.04 μm diameter); monilioid cells were oval, branched monilioid cell chains (16.79–23.82 × 34.35–45.38 μm) composed of more than 6–13 monilioid cells, which clustered to develop compact mass clusters of monilioid cell chains that form tufts (Figure 5c). PDA medium was observed for FQXY017, and colonies were light-yellow and a milky-white center-ring with velvet and even edges (Figure 5d). Later, colonies became creamy-yellow and waxy (Figure 5e). Hyphal branching occurred in the right- or near-right-angle (5.58–8.54 μm diameter); monilioid cells were long-cylinders with branched monilioid cells chains (5.48–9.76 × 13.21–28.31 μm) composed of more than 4–9 monilioid cells, which clustered and developed loose mass clusters (Figure 5f).

The ITS sequence of the mycorrhizal fungi FQXY019 and FQXY017 exhibited the highest similarity to *Epulorhiza* sp. (*Epulorhiza* sp., GU166409.1) and *Tulasnella calospora* (*Tulasnella calospora*, GU166406.1), respectively. Furthermore, a phylogenetic tree was constructed using the ITS sequence of mycorrhizal fungi in *Epulorhiza* sp. and *T. calospora*. Finally, the best phylogenetic tree was constructed using the neighbor-joining method (Figure 6).

Discussion

P. barbigerum is a wild plant species with an extremely small population in China. It only grows in the limestone hills in northern Guangxi and Guizhou, China. This study collected the root segments of *P. barbigerum* epiphytic on limestone in Guizhou with a shallow soil layer and poor habitat. Altogether, 127 endophytic fungi were isolated from *P. barbigerum* roots. Among them, the dominant genera were *Epulorhiza, Fusarium*, and *Tulasnella. Tulasnella and Epulorhiza* were considered universal orchid symbionts,[26] i.e., they can form mycorrhizal fungi with most orchids.[27–37] Studies have reported that *Fusarium* is the dominant species of *P. armeniacum, P. micranthum*, and *Bletilla striata*.[19,28,38] Additionally, *Fusarium* sp. has been recorded as an endophytic fungus.[39,40] Such studies have reported that *Epulorhiza, Fusarium*, and *Tulasnella* are the mycorrhizal fungi of orchids,[11,41–43] consistent with the results obtained in our study.

Table 2. Identification of ITS sequences of endophytic fungi isolates from *Paphiopedilum barbigerum* roots.

Fugal Isolate	Assigned identity based on most similar GenBank accession	Identity (%)	Query Coverage (%)	Class	GenBank Accession Number
FQXY001	*Fusarium nematophilum*	96.83	100	Sordariomycetes	MN540302.1
FQXY002	*Fusarium* sp.	99.43	97	Sordariomycetes	KT269843.1
FQXY005	*Fusarium* sp.	99.59	92	Sordariomycetes	MT649908.1
FQXY006	*Fusarium solani*	100	100	Sordariomycetes	KY283799.1
FQXY008	*Fusarium* sp.	99.62	98	Sordariomycetes	KT269843.1
FQXY016	*Fusarium redolens*	100	100	Sordariomycetes	MT563396.1
FQXY015	Fungal endophyte	97.21	100	–	KY765176.1
FQXY019	*Epulorhiza* sp.	97.93	94	Agaricomycetes	GU166409.1
FQXY042	*Pezicula ericae*	99.62	100	Leotiomycetes	KX000263.1
FQXY045	*Dactylonectria novozelandica*	100	100	Sordariomycetes	MN817697.1
FQXY047	*Chaetomium nigricolor*	99.81	100	Sordariomycetes	JF439467.1
FQXY017	*Tulasnella calospora*	96.56	100	Agaricomycetes	GU166406.1

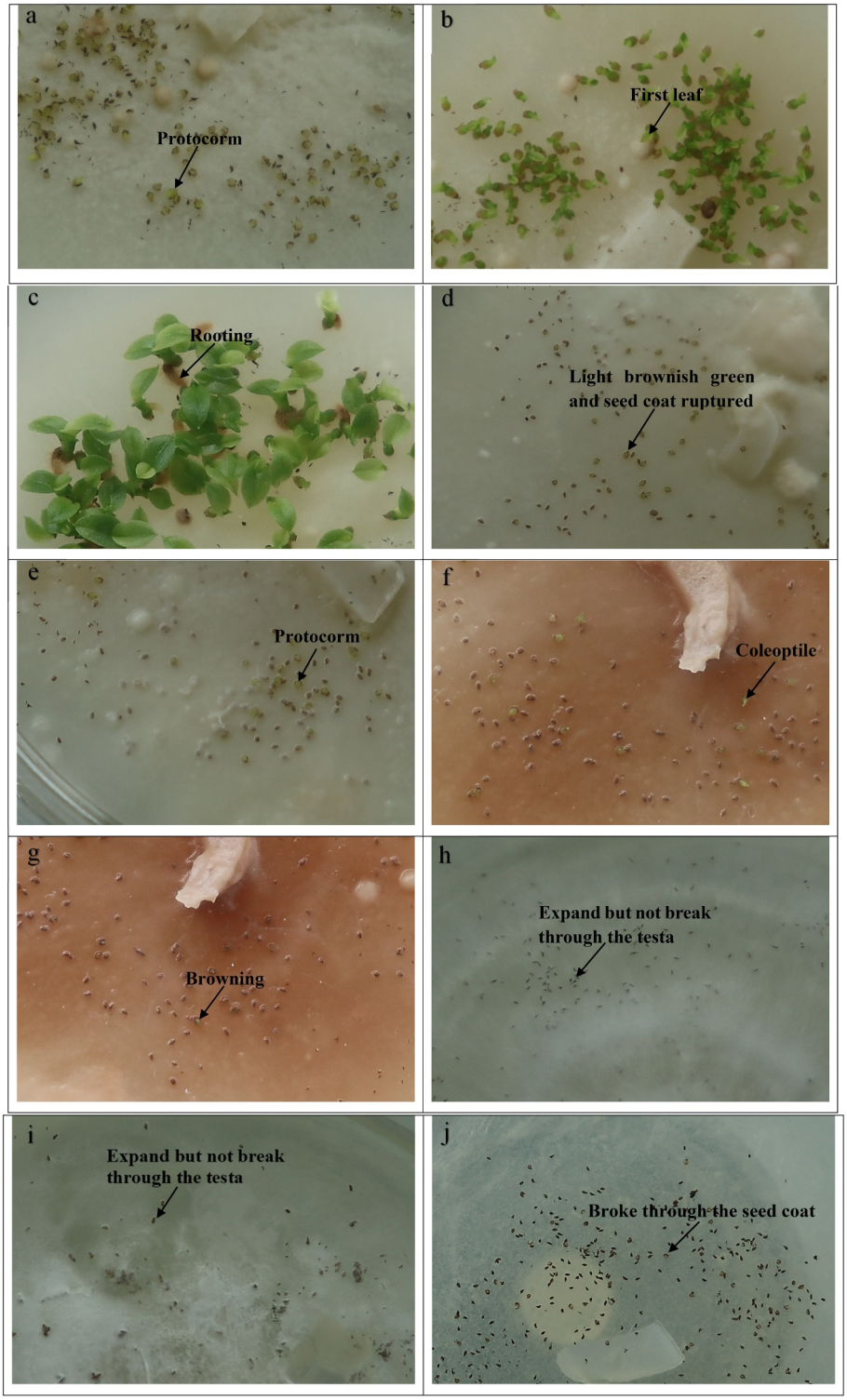

Figure 2. Protocorm-like bodies were formed after 30 d with FQXY019 (a). The first leaf emerged after 50 d with FQXY019 (b). Rooting occurred after 80 d with FQXY019 (c).Turned light brownish green and seed coat ruptured after 40 d with FQXY017 (d). Protocorm-like bodies formed after 55 d with FQXY017 (e). The coleoptile emerged after 75 d with FQXY017 (f). Browning after 90 d with FQXY017 (g). Seeds expand but not break through the testa after 90 d with FQXY001 (h). Seeds expand but not break through the testa after 90 d with FQXY005 (i). Seeds germinated and broke through the seed coat but could not further develop after 90 d (CK) (j).

Recognition processes are an essential part of most symbiotic associations,[44]but orchids with broad webs of fungi lack these recognition processes, which only germinate and grow in the presence of a suitable fungus.[45] According to this, our study analyzes and tests their roles in seed germination. Furthermore, The results of our in vitro study indicate that *Epulorhiza* sp. FQXY019 and *T. calospora* FQXY017 could promote seed germination and protocorm formation. However, only FQXY019 could promote seedling formation and rooting, whereas FQXY017 could not. The seeds germinate in the presence of fungus, but many of the esulting protocorms abort because of physiological imbalances with these fungus.[45]

Table 3. Germination of *Paphiopedilum barbigerum* seeds inoculated with endophytic fungi cultured on PDA plates.

	Germination and growth stage (germination and growth rate)			
Fugal Isolate	Seed coat ruptured by enlarged embryo (%)	Formation protocorm-like bodies (%)	Emergence of first leaf (%)	Further development and rooting (%)
FQXY019	76	69	67	65
FQXY017	52	41	0	0
FQXY001	0	0	0	0
FQXY002	0	0	0	0
FQXY005	0	0	0	0
FQXY006	0	0	0	0
FQXY008	0	0	0	0
FQXY016	0	0	0	0
FQXY015	0	0	0	0
FQXY042	0	0	0	0
FQXY045	0	0	0	0
FQXY047	0	0	0	0
CK	27	12	0	0

Because only fully compatible fungus resulted in orchid seedling growth.This result agrees with the reported findings that strains M2, M4, and HR1-1 stimulate seed germination and protocorm formation but do not promote seedling growth in *B. striata*.[11] Although *Epulorhiza* isolated from *P. integra* promoted seed germination of *P. integra in vitro*, its development was blocked when the protocorms grew with these mycorrhizal fungi.[46] These conclusions may be explained because different mycorrhizal fungi play different roles in seed germination and subsequent seedling growth stages of *P. barbigerum*.

Other isolated endophytic fungi besides Rhizoctonia, such as *F.nematophilum, F. solani, F. redolens, Fusarium sp., P. ericae, D. novozelandica, C. nigricolor* could not promote seed germination on PDA medium. The seed germination rate was lower than that of CK. Peschke and Volz defined *Fusarium* as one of the most ubiquitous and interesting orchid

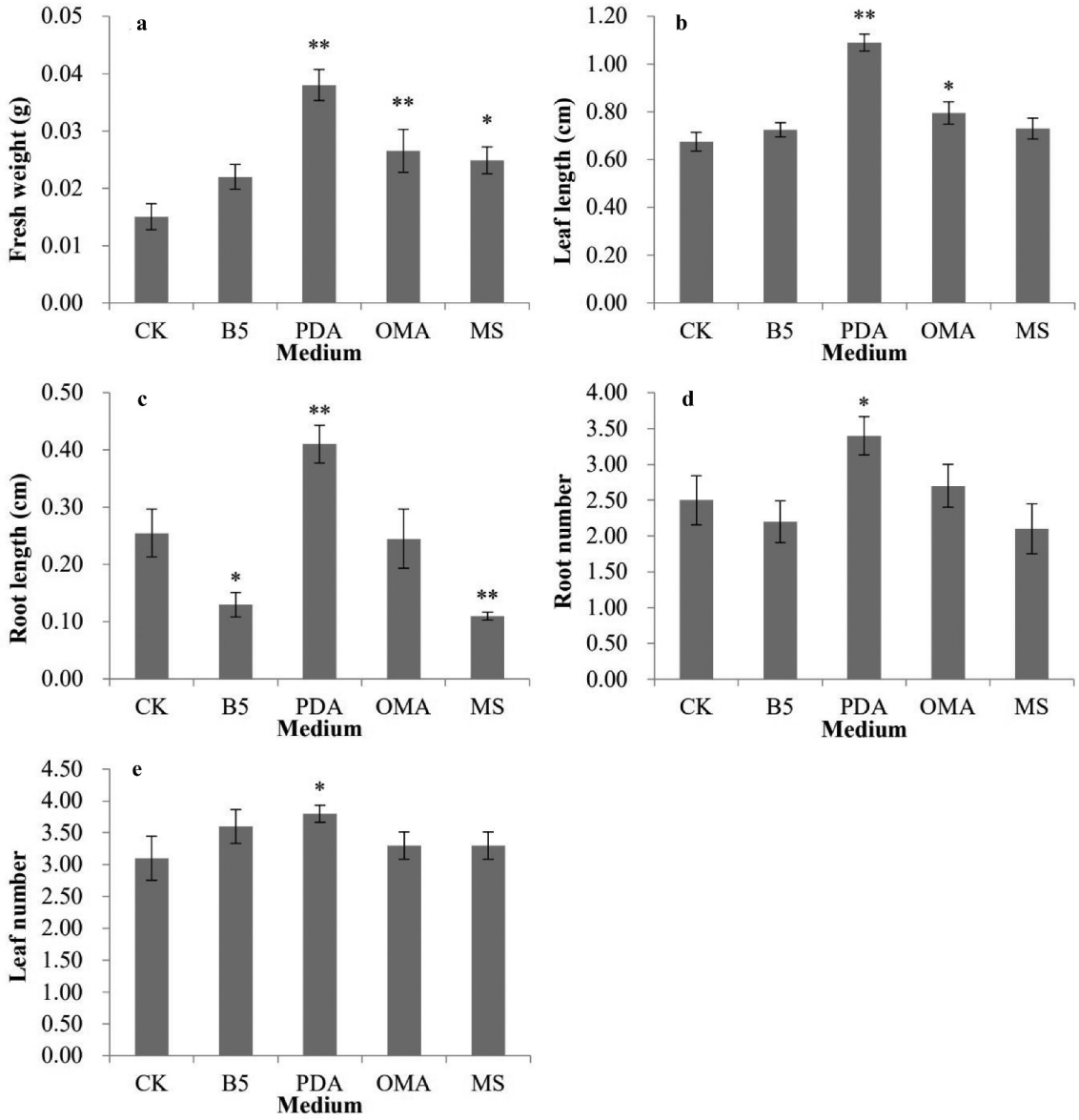

Figure 3. Effects of the mycorrhizal fungus FQXY019, co-cultured with different media on seedling growth of *Paphiopedilum barbigerum*.

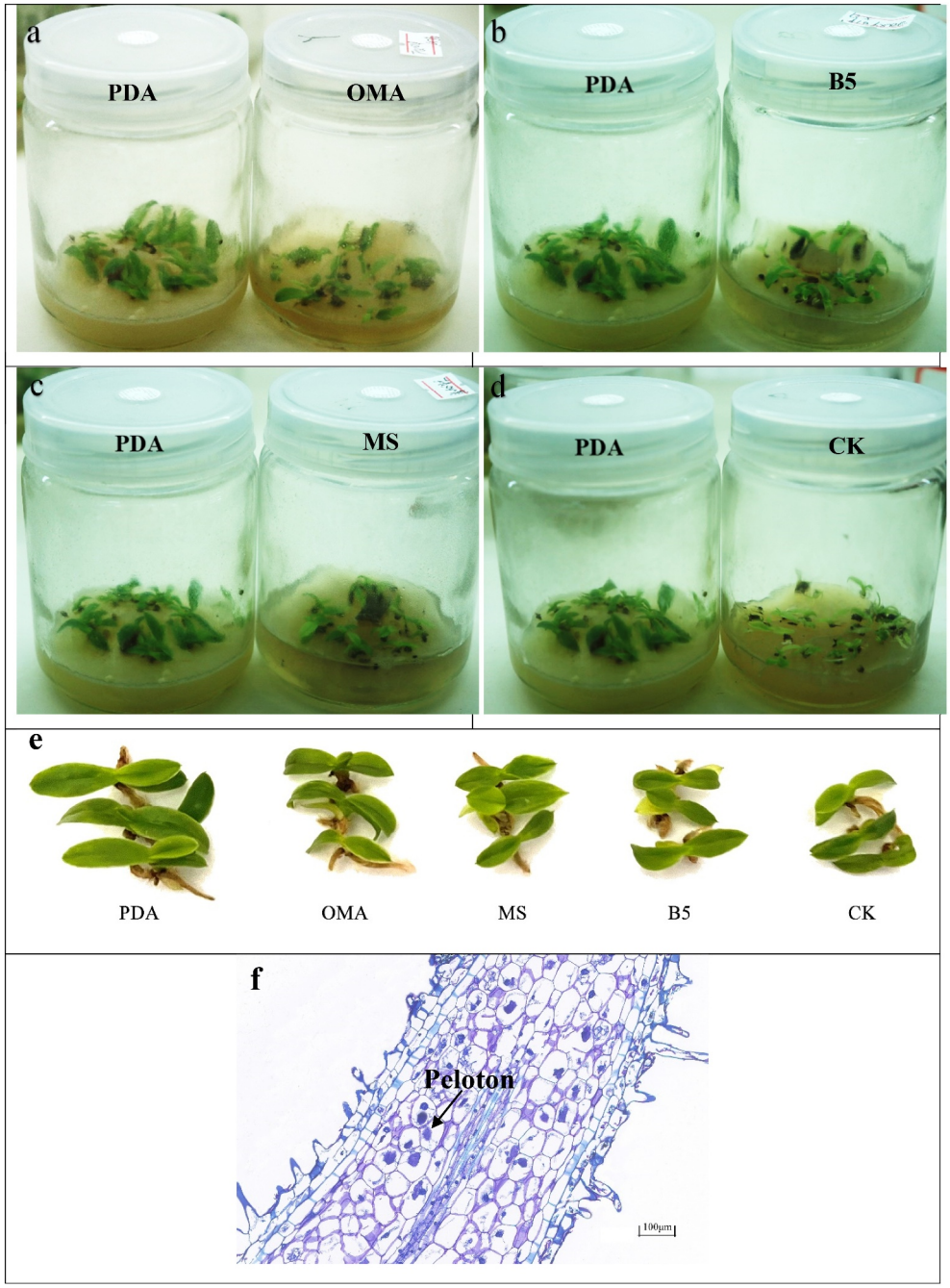

Figure 4. The growth state of *Paphiopedilum barbigerum* seedlings inoculated with FQXY019.Symbiotic mycorrhizal fungal and seedlings cultured on PDA, OMA, B5,MS and CK (a,b,c,d). Comparison of seedlings growth of five treatments (e). Longitudinal section of seedling root inoculated with FQXY019 on PDA (f).

endophytes,[47] since they could promote seed germination and several species could cause diseases. Previous studies have identified that several *Fusarium* species do not make suitable symbionts for orchids, and some are pathogenic.[48] *F. solani, F. oxysporum* and *F. proliferatum* cause the some disease in orchids.[49] Under optimal growth conditions, *F. solani* as a virulent species, tend to asymptomatic endophyte rather than pathogens.[50] However, *Fusarium* species enhance seed germination and protocorm development in some orchids.[40,42,51] Although this effect of promoting germination and growth was relatively minor compared with that of specific *Rhizoctonia*-like orchid mycorrhiza.[25] In a recent study, *F. oxysporum* KB-3 significantly promoted the vegetative growth of *Bletilla striata* and *Dendrobium candidum*, typical orchid mycorrhizal structures were observed in their roots.[52] Endophytic *Fusarium* promoted seed germination in *Cypripedium* and *Platanthera*.[53] As reported, fungus-plant interactions can easily shift from mutualism to parasitism depending on the plant's physiology and environmental conditions.[54] These studies showed that it is necessary to

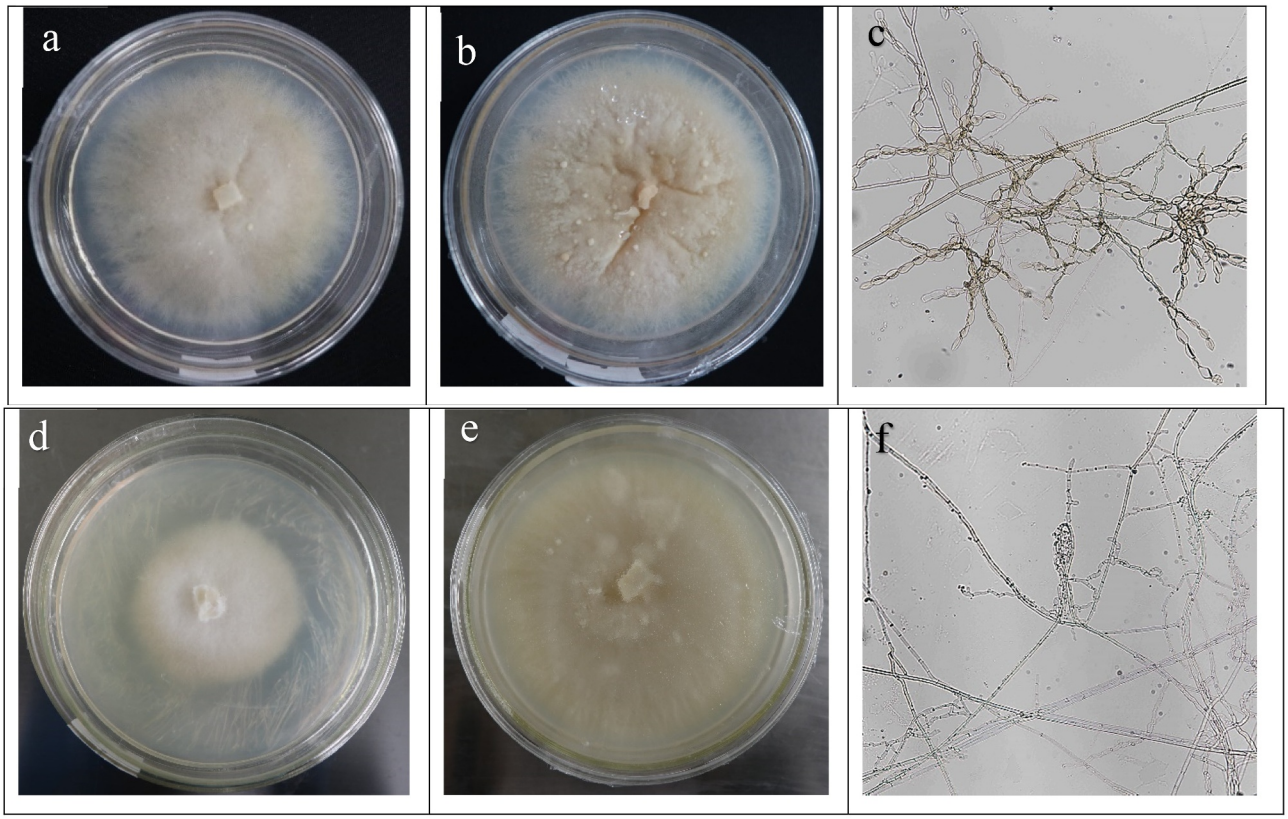

Figure 5. The morphology of the colony and mycelium of the mycorrhizal fungi FQXY019 and FQXY017. FQXY019 colonies were at the early stage (a); FQXY019 colonies were at the later period (b); mycelium of FQXY019 (c); FQXY017 colonies were at the early stage (d); FQXY017 colonies were at the later period (e); mycelium of FQXY017 (f).

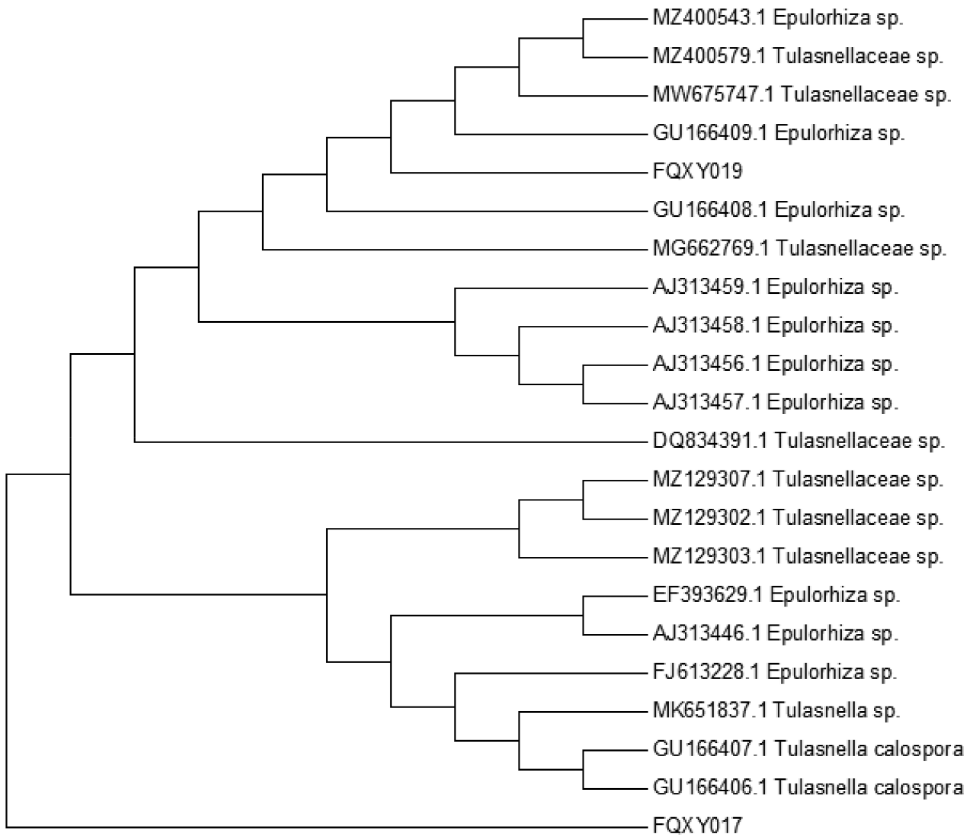

Figure 6. Phylogenetic tree based on ITS-rDNA sequence of mycorrhizal fungi isolated from *Paphiopedilum barbigerum* and the closest identified relatives from GenBank.

explore the role of endophytic and nonpathogenic *Fusarium* strains in controlling pathogens and promoting germination and development of orchid species.

Furthermore, this study compared the effectiveness of four media co-cultured with the compatible mycorrhizal fungus *Epulorhiza* sp. FQXY019 on seedling growth and development. The promoting effects of different media on seedling development of *P. barbigerum* were unequal. The seedlings were inoculated on PDA, and those co-cultured with FQXY019 developed best compared with those inoculated on MS, B5, OMA, and CK media. The average fresh weight, leaf length, root length, root number, and leaf number of seedlings among these treatments had significant or extremely significant differences. PDA medium has low carbon and nitrogen content and poor nutrition compared with the other three media, which agrees with the characteristics of the shallow soil layer and poor nutrition in the field habitat of *P. barbigerum*. Additionally, mycorrhizal fungi can promote the growth of *Paphiopedilum* in nutrient-poor media, which agrees with the findings of previous studies.[55] It is suggested that symbiosis with mycorrhizal fungi may be a reason for *P. barbigerum* to adapt to harsh habitats. Furthermore, it was observed from the experiments that the mycelium of the fungus FQXY019 on PDA medium grew vigorously and had dense colonies. Further, the hyphae were observed under the microscope with many monilioid cells, while the colony surfaces of MS, B5, and OMA media were sparse. This is probably because the FQXY019 exhibits a high diversity of morphological and growth characteristics on the PDA medium[56] and that the fungus supplies carbohydrates, vitamins, or other growth factors to seedlings. These results indicate that FQXY019 is a beneficial orchid mycorrhiza in seed germination and subsequent seedling growth stages of *P. barbigerum*.

Symbiotic (co-culture with mycorrhizal fungi) seed germination and seedling development methods require a relatively simple medium formula, which is cost-effective and time-saving. Furthermore, symbiotic plantlets may have more advantages than asymbiotic plantlets because seedlings inoculated with mycorrhizal fungi grow better and have stronger resistance.[57,58] Research shows that *Epulorhiza* sp. increased nutrient intake in plants and increased seed germination on *Dendrobium nobile* and *Dendrobium chrysanthum*.[16] *Epulorhiza repens* ML01 can successfully establish and survive in pot orchids under greenhouse conditions, and reduce the occurrence of diseases in the *Cymbidium hybridum* roots.[59] This study demonstrated the promoting effect of *Epulorhiza* sp. FQXY019 on seed germination and seedling development. Results showed that FQXY019 forms a good symbiotic system with *P. barbigerum* seed and seedlings and can be used as symbiotic mycorrhizal fungi for the growth and reproduction of *P. barbigerum* culture seedlings. Therefore, this this can be used as an alternative method for the artificial propagation of *P. barbigerum*.

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