

# *In Situ* Seedling Baiting to Isolate Plant Growth-Promoting Fungi From *Dendrobium Officinale*, An Over-Collected Medicinal Orchid In China

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## Research article

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# Abstract

**Background:** Orchids are highly dependent on mycorrhizae for survival and growth. Traditionally, to obtain orchid mycorrhizal fungi (OMFs) for plant growth, fungi have been isolated from the roots of mature wild plants; however, the species of OMF may change as the plants undergo different developmental stages. In this study, we developed the idea of using *in situ* seedling baiting to capture seedling growth-promoting fungi from *Dendrobium officinale*, an overcollected medicinal orchid in China.

**Results:** *In vitro*-produced seedlings of *D. officinale* were transplanted into their original habitats, and newly established roots of well-growing seedlings were sampled for fungal isolation. Roots were sampled at 6 different times over one year, and five *Tulasnella* species and one *Fusarium* species were obtained and identified morphologically and molecularly. The ability to promote seedling growth was tested in three *Tulasnella* species TPYD-1, TPYD-2 and TPYD-3, with high isolation frequencies by inoculation onto *in vitro*-produced *D. officinale* seedlings. Although the three fungi were closely related species and clustered together in the phylogenetic tree, they showed different efficiencies in promoting *D. officinale* seedling growth. *Tulasnella* TPYD-2 showed a strong ability to promote seedling growth and could be selected for use in restoration plantings of *D. officinale*.

**Conclusions:** Our results suggest that using an *in situ* seedling baiting technique could be an efficient way to obtain seedling growth-promoting fungi, and this approach could have broad applications in orchid mycorrhiza studies and orchid conservation.

## Background

The genus *Dendrobium* Swartz is one of the largest genera in Orchidaceae, comprising approximately 1,100 species distributed in the tropical and subtropical areas of Asia and Australia, and approximately 78 species, including 14 endemic species, are native to China [1]. Of these, more than 40 *Dendrobium* species have a very long history of use in traditional Chinese medicine (TCM) and are known as Shi-Hu in Chinese [2]. Consequently, most of these medicinal *Dendrobium* species have been exploited to the point of local extirpation, especially in some “hotspot” areas [3, 4].

Increasing supplies is considered an important method to reduce harvesting pressures of overexploited wild species [5]. For medicinal *Dendrobium* species, the Shi-Hu industry has developed rapidly in southern China since the 1990s as a result of massive commercial cultivation; however, due to doubts about the quality and efficacy of the products obtained with this cultivation method, the lack of product standards and the low quantities of products, the utilization of these products in TCM is limited [6]. Moreover, such massive commercial cultivation has not alleviated pressure on wild populations, according to recent surveys on the orchid trade in China [7]. This reflects a consumer preference for wild-collected materials and the fact that wild collection maintains the livelihoods of local suppliers who do not have the capital to invest in rearing facilities. Preference for wild-collected medicines by Chinese consumers reflects the belief that these materials are more effective and contaminant-free than cultivated materials [8]. To

balance the conservation of threatened *Dendrobium* species and the continuing demand for wild-grown materials, a restoration-friendly cultivation model for medicinal orchids, especially *Dendrobium* species, has been proposed, in which orchids are planted in natural settings [3, 8]; this method is considered promising for linking the commercial TCM industry with biodiversity conservation initiatives in China [6].

In the context of restoration-friendly cultivation, future scientific research on *Dendrobium* species, including mycorrhizal technology and microbial fertilizers, is urgently needed [6]. In our previous studies focused on the conservation of over-collected *Dendrobium* species, we obtained germination-enhancing fungi for different *Dendrobium* species through an *in situ/ex situ* seed baiting technique [9–12], and the fungi have also been successfully used in restoration plantings of medicinal *Dendrobium* species [13]. However, the dynamics of fungal associations at the seedling stage remain largely unknown. In practice, we found that after seedling establishment, seedling growth varied greatly even in a small-scale environment [13], indicating that new fungal partners are established with seedlings and play an important role in plant growth. Orchids have complex symbioses with fungi throughout their lifespan, and the species of orchid mycorrhizal fungi (OMFs) may change in different plant developmental stages [14, 15]. Under natural conditions, the broadening and/or changing of a mycorrhizal association may enable orchids to adapt to the varied physiological changes during seedling development, including the switch to partial or full autotrophy, an increase in transpiration, or environmental fluctuations [16].

Motivated by the success of *in situ/ex situ* seed baiting, in this study, we developed the idea of using *in situ* seedling baiting to obtain seedling growth-promoting fungi, in which *in vitro*-produced seedlings of *D. officinale* were transplanted into their original habitats, and newly established roots of well-growing plants were sampled for fungal isolation. The aim of this study was to understand fungal diversity at the seedling stage and obtain OMFs for seedling growth to support restoration plantings of *D. officinale*. Here, we present our results, addressing three principal questions: (1) Does this method work to obtain OMFs associated with seedlings of *D. officinale*? (2) What are the effects of the dominant OMFs on seedling growth in *D. officinale*? (3) Have the OMFs obtained in the current study also been obtained in other studies in which OMFs were isolated from roots of mature wild plants, and do these fungi have positive effects on seedling growth in *D. officinale*?

## Results

### Fungal isolation and identification

In total, 64 purified fungal strains were successfully obtained from 600 root fragments of *D. officinale* that were sampled at 6 different times. All fungal strains were morphologically and molecularly identified as six fungal species, including five *Tulasnella* species and one *Fusarium* species (Additional file 1; Table 1). Among the 5 *Tulasnella* species, TPYD-1, TPYD-2, and TPYD-3 were isolated 15, 12 and 7 times and were present in 6, 5 and 3 samples, respectively (Fig. 1D-F), while TPYD-4 and TPYD-5 were isolated three and one times, respectively, and were only present in one sample each. *Fusarium oxysporum* TPYD-6 was isolated from all 6 samples with a high isolation frequency of 26 out of 64 times (Additional file 1).

Table 1

Molecular identification of six fungal species isolated from roots of *Dendrobium officinale* seedlings.

Fungus	Total isolation times	GenBank accession number	Close relative	Accession number	Ident	References
TPYD-1	15	MN545675	<i>Tulasnella</i> sp.	EF127682.1	98.82%	–
TPYD-2	12	MN545849	<i>Tulasnella</i> sp.	KP050605.1	97.42%	–
TPYD-3	7	MN545858	<i>Tulasnella deliquescens</i> T326	LC175331.1	97.67%	[62]
TPYD-4	3	MN545859	uncultured <i>Tulasnellaceae</i>	GQ241817.1	99.83%	[63]
TPYD-5	1	MN545860	<i>Tulasnella</i> sp. DerV	MK555332.1	99.33%	[11]
TPYD-6	26	MN545304	<i>Fusarium oxysporum</i> MC-26F	KU527806.1	99.23%	[64]

## Phylogenetic analysis

A total of 28 fungal species/strains from 11 studies were reported to have positive effects on seedling growth in *D. officinale* (Additional file 2). Among them, 12 fungi were isolated from the protocorms or roots of *D. officinale*, 10 fungi were isolated from the roots of other *Dendrobium* species, and 6 fungi were obtained from the roots of non-*Dendrobium* orchid species. Interestingly, 17 fungi were OMFs, and the other 11 fungi were non-OMFs (Additional file 2). The ITS-rDNA sequences of 21 out of 28 fungi were downloaded from the GenBank database and used to generate the phylogenetic tree with the fungal sequences of five OMFs obtained in the current study. In the phylogenetic tree, the fungi TPYD-1, TPYD-2 and TPYD-3 were clustered together, while TPYD-4 and TPYD-5 were scattered with other species of *Tulasnella* and *Epulorhiza* (Fig. 2).

## Testing the capacity of fungi to support seedling growth

In the incubation experiments, the three fungi TPYD-1, TPYD-2 and TPYD-3 with high isolation frequencies were examined in terms of their capacity to support seedling growth in *D. officinale*. At 30 and 60 d after incubation, no pelotons were observed in the roots under all treatments (Fig. 1G), and only scarce pelotons could be found in the roots under the TPYD-1 and TPYD-2 treatments at 90 d. All three fungi clearly started to colonize the roots from 120 d after incubation, and the numbers of pelotons in the roots under the three fungal treatments increased greatly at 150 and 180 d (Fig. 1H).

### 120 days after incubation

All three fungi showed positive effects on seedling growth (Fig. 3A-E). The lengths of the longest roots in all three fungal treatment groups were significantly longer than that in the control group ( $F = 4.970$ ,  $P = 0.003$ ) with the highest growth rate in the TPYD-2 treatment ( $R = 29.78\%$ ; Fig. 3A). TPYD-1 and TPYD-2 significantly increased the biomass of seedlings as the fresh and dry weights in the two treatments were significantly higher than those in the control treatment (Fig. 3D & E). However, there were no significant differences in the longest leaf length ( $F = 1.676$ ,  $P = 0.174$ ; Fig. 3B) or plant height ( $F = 3.212$ ,  $P = 0.205$ ; Fig. 3C) among all treatments.

**150 days after incubation:** At this stage, all three fungi significantly increased the root length and dry weight compared with the control treatment (Fig. 3F & J), and the lengths of the longest leaf in the TPYD-2 and TPYD-3 treatment groups were significantly longer than in the control group (Fig. 3G). Moreover, TPYD-2 showed very positive effects on seedling growth among all treatments, as all five measured indices were significantly higher in the TPYD-2 treatment than in the control treatment, and the plant height (growth rate:  $H = 36.44\%$ ) and fresh weight (growth rate:  $F = 73.27\%$ ) in the TPYD-2 treatment were significantly higher than in other two fungal treatments (all  $P < 0.001$ ; Fig. 3H & I).

**180 days after incubation:** Clearly, TPYD-2 promoted seedling growth more effectively than TPYD-1 and TPYD-3 (Fig. 1I; Fig. 3K & O). Except for the longest leaf length, the longest root length, plant height, fresh weight and dry weight were significantly longer or higher in the TPYD-2 treatment than in the TPYD-1 and TPYD-3 treatments and the control treatment (all  $P < 0.001$ ). For the longest leaf length, there were no significant differences between the TPYD-2 and TPYD-3 treatments ( $P = 0.204$ ), but the leaves were significantly longer than those in the TPYD-1 and control groups (all  $P < 0.05$ ; Fig. 3L). The biomasses of seedlings were also significantly higher under the TPYD-3 treatment than under the control (fresh weight:  $P < 0.001$ ; dry weight:  $P = 0.018$ ; Fig. 3N & O).

## Discussion

Orchid mycorrhizae are symbiotic associations between orchid plant roots and fungi, in which fungal hyphae grow within living plant cells and form intracellular pelotons [17, 18]. Orchid mycorrhizal fungi (OMFs) are considered to belong to the so-called rhizoctonia aggregate, a polyphyletic group of fungi belonging to Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae [19]. Orchids are highly dependent on mycorrhizae for survival and growth: OMFs not only supply carbohydrates to facilitate the growth of nonphotosynthetic protocorms during the seed germination stage but also provide nutrients and growth factors to plants, conferring plant metal tolerance and inhibiting the development of pathogens [17, 20–23].

Mycorrhizal technologies and microbial fertilizers could simplify orchid seedling production and promote plant growth and have been considered important aids to the sustainable development of the *Dendrobium* industry in China [6, 24, 25]. As the most popular medicinal plant species in the genus *Dendrobium*, *D. officinale* has received much research attention regarding its mycorrhizal symbionts [25]. Traditionally, to obtain the optimal source of fungal mycobionts for symbiotic germination or plant

growth, fungi have been isolated and screened from the roots of mature wild plants [26, 27]. Among the 28 fungal species reported in 11 *D. officinale* studies, only five fungal species in one study were obtained from protocorms via *ex situ* seed baiting [28], while the other 23 fungi in the remaining 10 studies were isolated from the roots of wild mature plants, including *D. officinale* and other orchid species (Additional file 2). The 28 fungal species/strains, originally obtained from different orchid species and belonging to a wide range of taxonomic groups (17 OMFs and 11 non-OMFs), were found to have positive effects on the growth of *D. officinale* seedlings (Additional file 2). Seemingly, a randomly obtained fungus could promote seedling growth in *D. officinale*; however, all of the above fungi were tested *in vitro*, and there have been no reports on the successful application of these fungi in practice so far. Theoretically, a high diversity of fungi may associate with the roots of an adult orchid plant, an orchid will utilize different OMFs at different life history stages, and the mycorrhizal symbionts may change in different developmental stages [14, 15, 29, 30]. It is unclear whether the fungi involved in the seedling stage remain until the plants reach adulthood in *D. officinale*.

The *in situ/ex situ* seed baiting technique has been suggested as an effective and easy way to obtain seed germination-enhancing fungi [31, 32]. Using this method, we successfully obtained efficient germination-enhancing fungi for different *Dendrobium* species [9–12], as well as other terrestrial orchids [33, 34]. This led to the development of the idea of using seedling-trap experiments to capture seedling growth-promoting fungi in the current study. After *in vitro*-produced seedlings of *D. officinale* were transplanted for more than one year in their original habitats, newly established roots of well-growing plants were sampled six times across one year in different seasons (Additional file 1). In total, five OMFs and one non-OMFs were obtained and identified (Table 1). *Tulasnella* species TPYD-1 and TPYD-2 were the dominant OMFs with a higher frequency of isolation than other OMFs, and TPYD-1 was present in all six samples, while TPYD-2 was present in five samples (Additional file 1). The three fungi TPYD-1, TPYD-2 and TPYD-3 with high isolation frequencies were closely related species, as they were clustered together in the phylogenetic tree (Fig. 2). *Fusarium oxysporum* TPYD-6 was also dominant, with a high isolation frequency and presence among the samples (Additional file 1).

In this study, to test and screen the fungi that could be used in real practice for growth of *D. officinale* seedlings, *in vitro*-produced seedlings were moved to mixed cultivation substrates in open environments, in which seedlings normally undergo an acclimatization stage. This could be a possible reason why the three fungi TPYD-1, TPYD-2 and TPYD-3 started to colonize seedling roots after a long period of 120 d. However, the corresponding increases in pelotons in the roots and in the five measured indices in all three fungal treatments at 150 and 180 d suggested that all three fungi could promote seedling growth in *D. officinale* but showed different efficiencies (Fig. 3). At 180 d after incubation, the longest root length, plant height, fresh weight and dry weight of seedlings in the TPYD-2 treatment were significantly longer or higher than those in the other two fungal treatments and the control treatment (Fig. 3). TPYD-2 could be selected as an ideal OMF for seedling growth in the restoration-friendly cultivation of *D. officinale*.

Tulasnellaceae is regarded as one of the main fungal families of orchid mycorrhizae, and many of these species are found to support seed germination and seedling growth in *Dendrobium* species [9, 10, 19, 24].

The other two *Tulasnella* species, TPYD-1 and TPYD-3, were closely related to TPYD-2 but showed less pronounced effects on seedling growth in *D. officinale*. Seedlings of *D. officinale* might need a longer time to acclimatize to fungal infection [35], or the two fungi might have other ecological functions [21, 22]. In the current study, we focused on the effects of fungi obtained from seedling baiting on seedling growth, but it would also be worthwhile to explore whether these fungi could effectively promote seed germination in *D. officinale*. In another study, we conducted comparisons of symbiotic germination of seeds inoculated with the TPYD-2 obtained in this study and six other fungal strains isolated from protocorms of *D. officinale* via *in situ* seed baiting. At 90 d after incubation, the percentage of seedlings in the LQ treatment was  $70.09 \pm 3.2\%$ , while no protocorms or seedlings were found in the TPYD-2 treatment (Wang et al. unpublished data). The results also suggested that *D. officinale* could associate with different fungi in different life stages.

In addition to OMFs, other root-associated nonmycorrhizal endophytes have also been recorded and identified from a wide range of orchid species [36, 37]. In this study, *Fusarium oxysporum* TPYD-6 was isolated from all 6 samples with high isolation rates. *Fusarium* species have been reported to be associated with different orchid species [33, 38]. Interestingly, although *Fusarium* species have been reported as pathogens in many orchid species [39], especially *F. oxysporum*, which was reported to cause wilt disease in *D. officinale* [40], other studies have suggested that *Fusarium* species can stimulate seed germination in the terrestrial orchid *Cypripedium reginae* [41] and enhance resistance to pathogens and promote plant growth in *Dendrobium* species [42, 43]. In a recent study, *F. oxysporum* KB-3, obtained from the roots of *Bletilla striata*, was considered an OMF because it could promote seed germination of *B. striata*, establish colonization and produce coiled hyphal structures within the cortical cells in the roots of *B. striata* and *Dendrobium candidum* [44]. As noted in many studies, the border between endophytic and mycorrhizal fungi could be difficult to define, and some fungus-plant interactions can easily shift from mutualism to parasitism depending on the plant's physiology and environmental conditions [45, 46, 47]. For the current study, it is worth exploring the effect of *F. oxysporum* TPYD-6 alone as well as the possible synergistic effect of TPYD-6 with other OMFs on the growth of *D. officinale* seedlings.

## Conclusions

In this study, *in situ* seedling baiting was used to capture orchid seedling growth-promoting fungi for the first time. Five *Tulasnella* species and *Fusarium oxysporum* TPYD-6 were successfully isolated from seedlings of *D. officinale* that were produced *in vitro* and transplanted for more than one year into their original habitats. *Tulasnella* sp. TPYD-2 showed a great ability to promote plant growth in *D. officinale* and could be selected for practical use in restoration plantings of *D. officinale*. For any given orchid species, it is still uncertain whether the fungi involved in the seedling stage remain until plant adulthood. Our results suggest that using an *in situ/ex situ* seedling baiting technique could be an efficient approach to obtain plant growth-promoting fungi. This method might be more reliable for overcollected orchids for which mature plants are hard to find in the wild but their original habitats are known. A similar idea has been used in studies on the interactions between orchids and fungi [48] and suggests that this approach could have broad applications in orchid mycorrhiza studies and orchid conservation.

# Materials And Methods

## Study species and study site

*Dendrobium officinale* Kimura & Migo (synonym of *D. catenatum* and *D. candidum*) is a lithophytic orchid widely distributed in subtropical areas, especially karst regions at altitudes from 500 to 1600 m in China [1]. *D. officinale* is one of the most popular TCM herbs and is known as Tie-Pi-Shi-Hu in Chinese. The wild populations of *D. officinale* are very small and sparsely distributed due to severe overharvesting over the past 30 years [3], and this species is listed as critically endangered on the IUCN Red List [49]. The study site was located at a limestone mountain near Lengdong village (24°54'13"N; 104°58'43"E; alt. 1100 m; Fig. 1A) in Xingyi, Guizhou Province, China. This region is a typical karst landform, and more than 20 species of *Dendrobium* including *D. officinale* have been reported in the area but are seriously threatened due to habitat loss and overcollection [50]. According to local residents, wild plants of *D. officinale* could be found in the mountains of the study site several years ago.

### In situ seedling baiting and sampling

An *in situ* seedling baiting experiment was conducted using *in vitro*-produced seedlings of *D. officinale*, in which mature seeds were obtained via hand-outcross pollination of flowers between different individuals of *D. officinale* and asymbiotically germinated on MS medium. The seedlings were removed from the flasks, and the roots were cleaned and then transplanted to the cracks and crevices of calcareous rocks at the study site during May and June at the start of the rainy season in 2016 (Fig. 1B). After more than one year of natural growth, root samples were taken six times at the study site during August 2017 and May 2018 (Additional file 1). For each sampling time, 10 clusters of seedlings containing 3–5 individuals with robust growth and newly established roots were randomly selected. Ten roots from each cluster were randomly sampled. Collected root samples were placed in an icebox and transferred to the laboratory for fungal isolation on the same day.

## Fungal isolation and identification

The sampled roots were washed with running water, surface sterilized with 75% ethanol for 2 min and 2% sodium hypochlorite for 3 min, and then washed three times with distilled water. Mycorrhizal fungi were isolated from roots of *D. officinale* following the method described by Bayman et al. [51]. The superficial uncolonized root tissue was removed using a thin sterile blade, and root fragments containing intracellular pellets were obtained by scraping the roots using needles and forceps. The root fragments were scraped as small as possible with an anatomical needle under a 10 × 20 magnification microscope (XS-A, Shanghai Pudan Co., Ltd.), transferred to PDA medium (200 g/L potato, 20 g/L dextrose and 20 g/L agar) with 0.05 g/L penicillin and 0.05 g/L streptomycin and incubated at 25 ± 2 °C. The root fragments were monitored daily during incubation on PDA. When hyphal growth from the root fragments exceeded 0.5 cm in length, tips of the hyphae were cut and transferred to new PDA medium for purification. After repeating this purification step 4–5 times, purified strains were obtained.

All obtained fungal strains were morphologically identified and then grouped based on culture characteristics such as colony morphology, hyphal characteristics, hyphal colour, and the colours associated with secondary metabolites [52]. For each group, a strain obtained from each sampling time was randomly selected for molecular identification. The rDNA region containing two ITS regions and the 5.8S gene was amplified using the ITS1 and ITS4 primers [53], and PCR was performed following Selosse et al. [54]. All ITS-rDNA sequences obtained were compared with those deposited in the GenBank database (National Center for Biotechnology Information, NCBI 2012) using the Basic Local Alignment Search Tool (BLAST), which allows the identification of isolates at the genus or species level when the ITS sequence similarity exceeds 95% or 97%, respectively [55]. Later, one sequence per species was deposited in NCBI GenBank under the accession numbers listed in Table 1.

## Phylogenetic analysis

A phylogenetic tree was generated to visualize the phylogenetic positions of the OMFs obtained in the current study among other known fungal species associated with *Dendrobium officinale*. By searching scientific databases (e.g., Web of Science; Google Scholar; Weipu Chinese journals database) with the key words "*Dendrobium officinale*", "*Dendrobium catenatum*", "*Dendrobium candidum*" and "mycorrhizal fungi", we collected all the available information on fungi that are considered to have positive effects on *D. officinale* seedling growth, including original resources, host orchid species and their effects on seedling growth in *D. officinale* (Additional file 2). Then, the available ITS-rDNA sequences of all related representative fungi were downloaded from the GenBank database (<https://www.ncbi.nlm.nih.gov/>) and used to generate a phylogenetic tree with the fungal sequences obtained in the current study. The ITS sequence of *Armillaria sinapina* As93 (FJ495039) was set as an outgroup based on previous studies [24]. Alignment of nucleotide sequences was performed by Clustal X version 1.81 [56] followed by manual adjustments using BioEdit [57] (Hall 1999). Maximum likelihood (ML) phylogenetic tree searches and ML bootstrapping were conducted using the web server RAxML-HPC2 on TG ver. 7.2.8 [58, 59] with 1000 rapid bootstrap analyses followed by a search for the best-scoring tree in a single run [58]. Then, FigTree V1.4.3 was used to analyse the resulting RAxML-Bipartitions file. Bayesian inference (BI) was conducted using MrBayes 3.1.2 on Cipres [59, 60, 61], and the MAX time was set from 3 to 4 hours. The resulting file (infile.nex.con.tre) was downloaded, and FigTree v1.4.3 was used for analysis. Then, Figtree v1.4.3 was used to organize the basic form of the phylogenetic tree and export it as a PDF file. Finally, Adobe Illustrator CC 2018 was used for the final modification.

## Testing the capacity of fungi to support seedling growth

The three OMFs with the highest isolation rates were selected, and their capacity to support seedling growth in *D. officinale* was tested. *In vitro*-produced seedlings of *D. officinale* were transplanted from flasks to circular plastic pots (height \* diameter = 8 cm \* 10 cm) after the medium on roots was washed away (Fig. 1C). For each pot, 5 seedlings were planted together in cultivation substrate with the same proportion of volcanic stone, peat and bark fragments. Once seedling transfer was completed, each pot was inoculated with one cubic centimetre of the three fungal inoculants, which were placed in the centres of the pots, to establish three fungal incubation treatments, and a sterile control without fungal

inoculation was used as a control treatment (CK). Each treatment was replicated in 24 pots, and to avoid possible fungal infection among treatments, all pots from a given treatment were placed in separate germination chambers at  $25 \pm 2$  °C with a 12/12-h light/dark cycle.

To determine if and when mycorrhizal symbiosis actually became established, three roots from each treatment were randomly selected and examined for the presence of pelotons at 30, 60, 90, 120, 150 and 180 d after incubation. Pelotons were cleared using 10% KOH solution, washed with 1% HCl solution, stained with 0.05% (w/v) trypan blue in lactic acid glycerol solution overnight [11], and then destained in acetic glycerol solution before observation under a microscope (DM2000, Leica Microsystems GmbH, Wetzlar, Germany).

## Data collection and statistical analysis

At the same time as seedling transplanting, 40 seedlings were randomly selected to measure the lengths of the longest root and leaf, plant height, and fresh and dry weights. Later, 40 seedlings from each treatment were randomly selected, and the same measurements were made at 120, 150 and 180 d after incubation. Moreover, to assess the effects of different fungi on seedling growth, the growth parameters including the longest root length ( $R$ ), longest leaf length ( $S$ ), plant height ( $H$ ), fresh weight ( $F$ ) and dry weight ( $D$ ) under different fungal treatments at different times were also calculated according to the following formula:  $R = [(R_{ex} - R_{CK}) / R_{CK}] 100\%$ ,  $S = [(S_{ex} - S_{CK}) / S_{CK}] 100\%$ ,  $H = [(H_{ex} - H_{CK}) / H_{CK}] 100\%$ ,  $F = [(F_{ex} - F_{CK}) / F_{CK}] 100\%$  and  $D = [(D_{ex} - D_{CK}) / D_{CK}] 100\%$ . The subscript  $ex$  represents different numerical values measured in the different fungal treatments at different times, while the subscript CK represents the numerical value of the control treatment.

The effects of different OMFs on seedling growth at different time points were studied. The lengths of the longest root and leaf as well as plant height, and fresh and dry weights among the fungal inoculation treatments and control at 120, 150 and 180 d were recorded. Comparisons were made with one-way ANOVA and the least significant difference ( $LSD$ ) method when the data showed a normal distribution or the generalized linear model ( $GLM$ ) when the data were not normally distributed. All statistical analyses were performed using SPSS software (version 25.0).

## Declarations

## Authors' contributions

DYC: performed experiments and data analysis; XJW: data collection and phylogenetic analysis; TQL: data analysis; NQL: samples collection and performed experiments; JYG: project design, data analysis and manuscript writing. All authors have read and approved the manuscript.

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## Availability of data and materials

All data analyzed during this study are included in this published article and its supplementary information files. Additional raw data from sequencing and quality control datasets are available from the corresponding author upon reasonable request.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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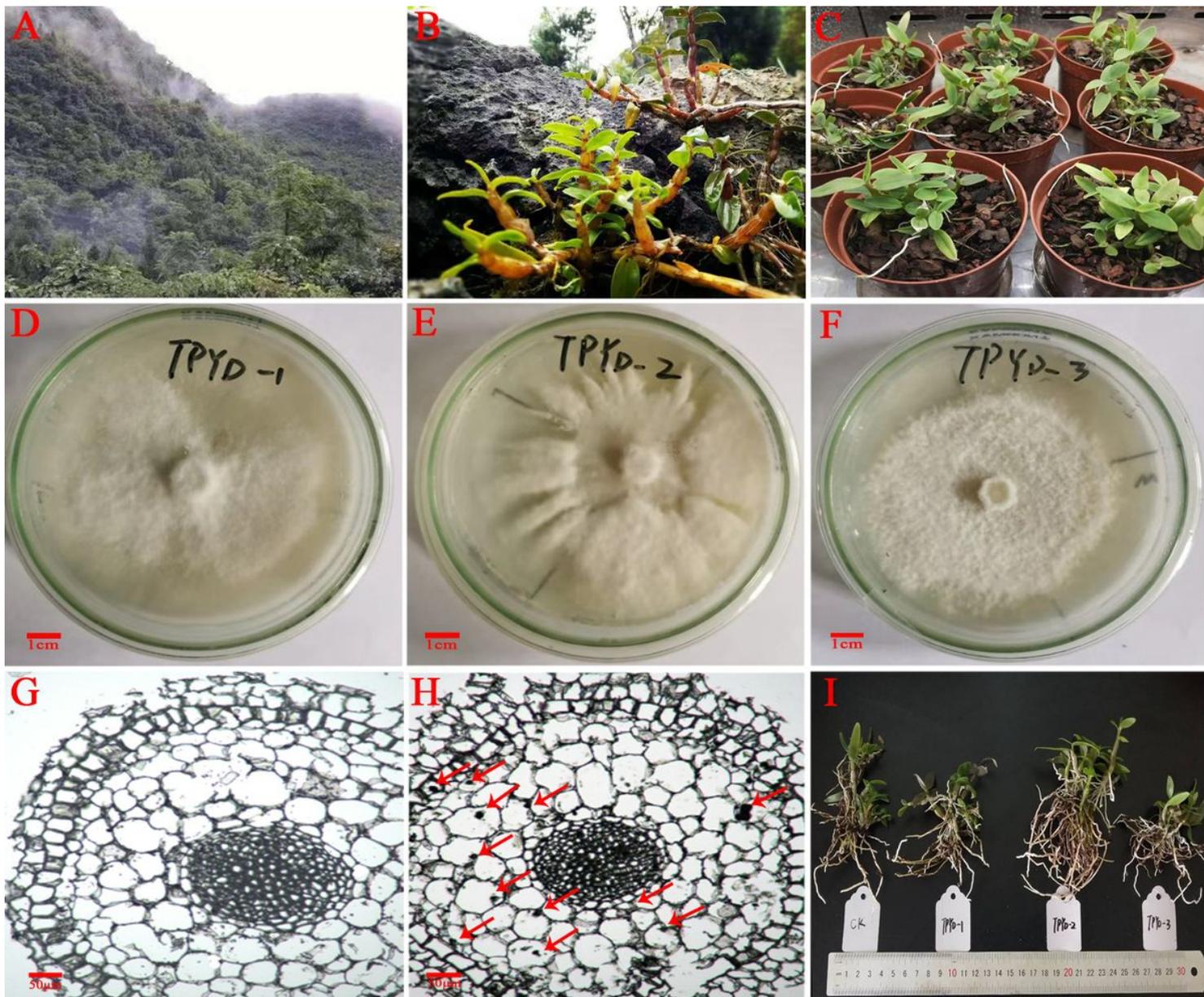
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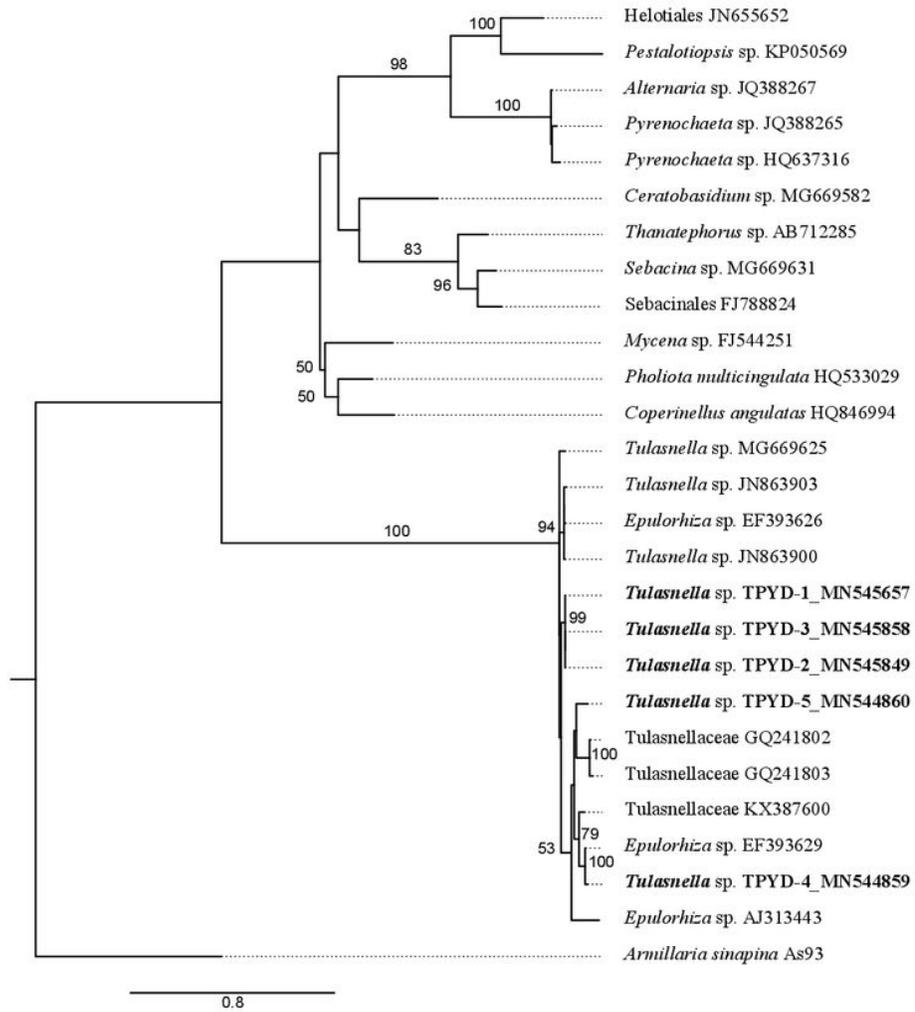
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## Figures



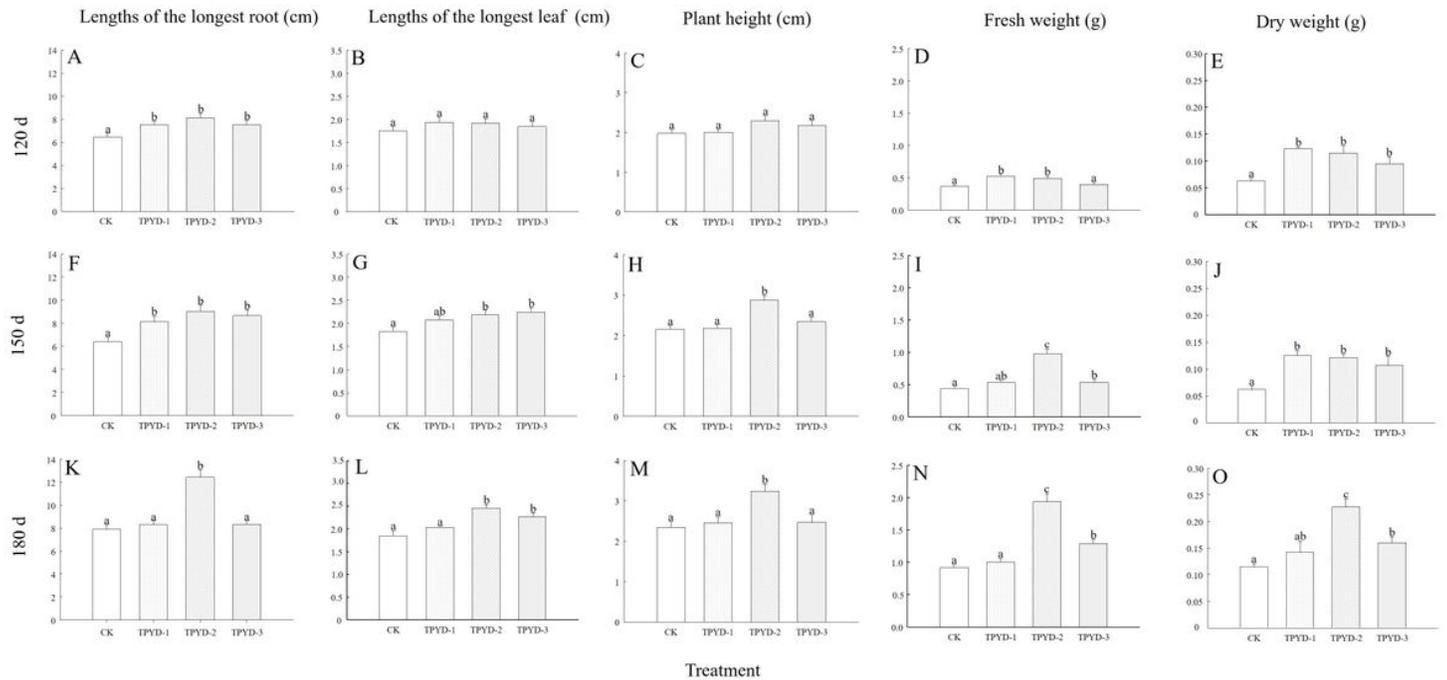
**Figure 1**

A, The study site, which is a limestone mountain and part of the original habitat of *Dendrobium officinale*. B, In vitro-produced seedlings of *D. officinale* transplanted on rocks at the study site. C, Potted in vitro-produced seedlings of *D. officinale* used to test the abilities to fungi to promote seedling growth by inoculation with different fungal strains. D, A colony of fungal strain TPYD-1. E, A colony of fungal strain TPYD-2. F, A colony of fungal strain TPYD-3. G, Cross sections of *D. officinale* seedling roots at 60 d after incubation showing no pelotons established in the roots under any treatment. H, Cross section of seedling roots of *D. officinale* at 180 d after incubation showing pelotons (red arrows) established in the roots under the three fungal treatments. I, Seedlings under different fungal treatments and the control treatment at 180 d after incubation showing that the fungus TPYD-2 can effectively promote growth of *D. officinale* seedlings.



**Figure 2**

Maximum-likelihood tree constructed using ITS sequences of five *Tulasnella* species (in bold) obtained from in situ baiting of *Dendrobium officinale* seedlings in this study and of other fungi reported to have positive effects on the growth of *D. officinale* seedlings. Numbers above branches are bootstrap probabilities (whenever  $\geq 50\%$ ) out of 1000 replicates.



**Figure 3**

Effects on the length of the longest root, length of the longest leaf, plant height, fresh weight and dry weight from different treatments (control on MS medium or OMA with addition of one of the three *Tulasnella* isolates - TPYD-1, TPYD-2 or TPYD-3) at 120, 150 and 180 d after incubation. In each panel, different letters indicate significant differences based on one-way ANOVA and the least significant difference (LSD) method where the data show a normal distribution and the generalized linear model (GLM) where the data are not normally distributed.

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