Effects and Benefits of Orchid Mycorrhizal Symbionts on Dendrobium officinale

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Abstract: Dendrobium officinale Kimura et Migo, a highly valued Chinese herbal medicine, is on the verge of extinction in the wild, and is not cultivated efficiently. In this study, we explored the possibility that orchid mycorrhizal fungi (OMF) might improve the growth and cultivation of D. officinale. Serendipita sp., Tulasnella calospora and Tulasnella asymmetrica isolated from three different orchids were co-cultured with sterile seedlings of D. officinale. The seedlings were found to stably coexist with fungi after 60 days of co-culture. The co-culture of T. calospora with plants upregulated the activity of antioxidant enzymes, stimulated the production of osmoregulatory substances and reduced electrical conductivity. Plants with T. calospora had longer roots (141.2%), thicker leaves (58.3%), increased root number (71.4%) and leaf number (11.1%), and increased weight (155.2%) and photosynthetic pigment content (99.6%), relative to controls. The content of total medicinal polysaccharides increased by 42.69 % due to the addition of T. calospora. T. asymmetrica was less effective, followed by Serendipita sp. When T. calospora established a symbiotic relationship with D. officinale, resistance indicators increased. The content of functional components was significantly increased. This study contributes to the protection and commercial reproduction of endangered orchid plants with mycorrhizal technology.

Keywords: Dendrobium officinale; orchid mycorrhizal fungi; symbiosis; growth morphology; resistance; functional components

1. Introduction

Dendrobium officinale Kimura et Migo is a well-known medicinal plant in the orchid family. Its stems have health benefits and accordingly have high economic value [1,2]. It is rich in beneficial components, including polysaccharides and amino acids, and some with anti-tumor and anti-oxidant properties, and others that have been reported to enhance immunity [3,4]. However, the accumulation level of beneficial components in medicinal plants is generally low, insufficient to meet the growing market demand [5]. The production of natural products requires a high biomass yield of medicinal plants to extract the high-value bioactive molecules [6]. Historically, D. officinale was mainly obtained using the exploitation of wild resources. Due to excessive harvesting and habitat destruction, the wild D. officinale resources are on the verge of extinction [7]. Artificial plantings instead of wild resources have become the main source of D. officinale, but artificially planted seedlings based on plant tissue culture technology are characterized by weak seedlings, reduced levels of active substances and a low survival rate outside the bottle [8,9]. Therefore, we need a more ecological method to protect and breed D. officinale and develop a cultivation technique with higher economic value. Some studies have found that there is no significant difference between wild and artificially cultured D. officinale, but wild D. officinale can improve their growth and resistance to adverse
conditions and pathogens by finding suitable endophytic fungi through symbiosis, whereas there is no such good opportunity for tissue-culture seedlings [10,11]. On this basis, there is an urgent need for new and improved cultivation techniques of D. officinale and taking advantage of the symbiosis of orchids and fungi provides one possible solution [12]. The use of beneficial symbiotic fungi to improve the production and accumulation of active metabolites of medicinal plants has already attracted some attention [13].

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Different OMF have different effects on different host plants, depending on the recognition process between the host plants and OMF and the compatibility for symbiosis [41]. When the two are compatible with each other, the interaction can occur smoothly. In this process, there is a lack of nutrients around the plants initially, the hyphae invade the roots of the plants, and the hyphae increase the nutrient absorption area of the roots, fostering stronger growth of the plant [42,43]. Only one of the two strains of Serendipita sp. isolated from D. dentate had a significant effect on its growth and resistance [44]. Among the three fungi isolated from D. nobile, only strain Chaetomium Kunze had an obvious growth-promoting effect on D. officinale [45]. Other studies found that OMF isolated from wild D. officinale also had a good growth-promoting effect on D. officinale and D. bicolor [46].

In summary, there are some OMF that are potentially capable of specific and compatible interactions with the genus Dendrobium. At the beginning of the study design, in order to compare the compatibility between orchid mycorrhizal fungi, we adopted mycorrhizal fungi isolated from D. officinale and other orchid species. This study was conducted to explore the results of co-culturing three OMF with the host plant D. officinale. We wished to determine whether the OMF can promote the growth and levels of functional components of D. officinale after establishment of the symbiosis and compare the effects of different OMF species. The results of the study may provide more evidence for in-depth exploration of the relationship between orchids and OMF and provide a resource for improved conservation efforts and economic value enhancement of D. officinale.
2. Materials and Methods

2.1. Plant Materials and Fungal Strains

In this study, artificially cultured D. officinale seedlings from Shaoxing, Zhejiang Province, China (120°49' E, 29°92' N) were used. Symbiosis experiments were carried out when the seedling height was about 4–5 cm. Three OMF strains were preliminarily selected and were temporarily stored in the Hunan Mid-Subtropical Quality Plant Breeding and Utilization Engineering Technology Research Center, Changsha, China. The original sources of three fungal samples are listed in Table 1.

Table 1. Sources of the fungal samples from which the samples were collected.

| Treatment Group | Species                 | ITS Sequence Accession Number (NCBI) | Plant Source             |
|-----------------|-------------------------|--------------------------------------|--------------------------|
| DSs             | Serendipita sp.          | OM778283                             | Dendrobium officinale    |
| CTc             | Tulasnella calospora     | OM756775                             | Cymbidium goeringii      |
| ETa             | Tulasnella asymmetrica   | OM778264                             | Epidendrum radicans      |

The plant material from D. officinale was cultured on aseptic MS medium for six months. Based on previous research, oatmeal agar (OA) medium was chosen as the medium for co-culture of D. officinale and OMF [47]. OA medium is composed of 8 g/L oatmeal + 8 g/L agar. Four D. officinale seedlings of approximately the same size were selected from each culture bottle and transferred to the symbiotic medium for one week of adaptive culture. At the same time, Serendipita sp., Tulasnella calospora, and Tulasnella asymmetrica were cultured on Potato Dextrose Agar (PDA) medium, 10 plates for each strain. After a week of growth, PDA plates completely covered with mycelium were selected, and a puncher (0.3 cm²) was used to place the activated fungi agar blocks of each strain onto the PDA plate culture medium. They were placed into OA medium equidistant to four seedlings. The three treatment groups were designated DSs, CTc, and Eta. The control group consisted of sterile agar blocks inserted into the tissue-culture seedlings and were designated CK. There were 25 repeats of each treatment group. All seedlings were placed at 25 °C and under a light intensity of 50 μmol·m⁻²·s⁻¹, photoperiod 13 h/d.

2.2. Electron Microscopic Determination of Mycorrhizal Fungi Symbiosis

The roots were manually cut with a blade to prepare slices for cross-sections. Sections were stained on the slide for 15 min with 0.1% trypan blue and rinsed with deionized water. A drop of glycerol lactate was applied to the glass slide, a sample sheet placed, and then the specimen was observed under the electron microscope (Leica D-35578, Wetzlar, Germany). Observations were repeated three times in each group, and the roots were examined every 10 days.

2.3. Determination of Relative Conductivity

The relative conductivity of leaves was determined according to published methods [48]. Three to five mature leaves of the same size were randomly selected from each group as samples. Leaves were washed with tap water, rinsed with deionized water three times and blotted dry with filter paper. The leaves were cut into long strips of suitable length (avoiding the central vein). Three fresh samples were quickly weighed, about 0.1 g each, and each placed in a centrifuge tube to which 10 mL deionized water was added. The tubes were shaken every 5 min, until after 30 min conductivity of the extract at this time was determined with a conductivity meter (DZS-706-C, Inesa Scientific Instruments Co., Ltd., Shanghai, China). This was recorded as R1. The samples were then heated in a boiling water bath for 30 min, cooled to room temperature and shaken evenly. We
2.4. Determination of the Osmoregulatory Substance and Antioxidant Enzyme Activity in *D. officinale*

The content of leaf soluble sugar was determined with the Plant Soluble Sugar Assay Kit (BC0030, Solar-bio company, Beijing, China). The content of soluble protein in leaves was determined with the Protein Quantitative (TP) Assay Kit (A045-2, Jiancheng company, Nanjing, China). We studied three antioxidant enzymes in leaves, including catalase, peroxidase and superoxide dismutase, which were all tested with the Catalase (CAT) Activity Detection Kit, Peroxidase (POD) Activity Detection Kit, and Superoxide Dismutase (SOD) Activity Detection Kit, respectively (BC0200, BC0090, BC0107, Jian Cheng company, Nanjing, China). Every measurement was repeated three times per treatment.

2.5. Measurement of Morphological Indices of *D. officinale*

The extent of fungal colonization in *D. officinale* was identified by staining. Staining was undertaken every 10 days after co-culture. The growth of each group of plants was also observed and photographed. Three plants in each treatment group were selected randomly to measure the fresh and dry weights of stems with an electronic balance. Morphological indices such as stem length, stem diameter, average leaf length, leaf width, leaf thickness, leaf number, root length and root number were determined using vernier calipers.

2.6. Determination of Photosynthetic Pigment Content

Three to five mature leaves of the same size were randomly selected as samples from each group, washed with deionized water and blotted dry with filter paper. The petioles and main leaf veins were removed and discarded. The leaves were cut into pieces, and 0.2 g samples were taken and measured using an electronic balance. The pieces were put in a 10 mL centrifuge tube, 95% ethanol was added as the extraction solution, and the volume fixed to 1 mL. The tubes were sealed and then stored in the dark for 24 h. The solution was mixed thoroughly, and after the broken leaves sink, the supernatant was removed immediately. The supernatant was placed in a cuvette with a light path of 1.0 cm, and the absorbance measured with an ultraviolet spectrophotometer (TSD-599, Shanghai, China). The absorbances of the extracts were recorded at 663, 645 and 470 nm. The absorbances were used to calculate the concentrations of chlorophyll a, chlorophyll b and carotenoids \[49\], with three biological repetitions per treatment. The formulae used to calculate the concentration of the pigments are as follows:

\[
\text{Chl a (mg/g)} = 12.7 \times A_{663} - 2.69 \times A_{645} \times \frac{V}{1000W} \tag{1}
\]

\[
\text{Chl b (mg/g)} = 22.9 \times A_{645} - 4.68 \times A_{663} \times \frac{V}{1000W} \tag{2}
\]

\[
\text{Car (mg/g)} = \frac{1000 \times A_{470} - 3.27 \times \text{Chl a} - 104 \times \text{Chl b}}{229} \times \frac{V}{1000W} \tag{3}
\]

In the above formulae, A663, A645 and A470 represent the absorbances at 663, 645 and 470 nm, respectively; Chl a, Chl b represent the total concentration of chlorophyll a, chlorophyll b, and Car represents the concentration of carotenoids; V is the volume of the extraction solution; and W is the mass of the leaf sample.

2.7. Determination of the Polysaccharide Content of Functional Components in Stems of *D. officinale*

The polysaccharide content of stems was determined with the Plant Polysaccharides Assay Kit (JL-C-B630, Jiang Lai Biological, Shanghai, China). Every measurement was repeated three times per treatment.
2.8. Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and R software (4.2.1) (RStudio 2019.09.0 Build 351; RStudio Team, 2019). Differences were regarded as significant at \( p < 0.05 \). Figures were generated using the SigmaPlot 12.5 software package (Systat Software GmbH, Erkrath, Germany), GraphPad Prism (8.4.3), and Excel software (Microsoft Office Excel 2019).

3. Results

3.1. Colonization of Orchid Mycorrhizal Fungi

In the process of establishing a symbiotic culture, after the sixth test staining (60 days), it was observed that the hyphae in the roots of the co-cultured group were stained with trypan blue. This indicated that the fungi in each group had formed stable symbiotic relationships with \( D. officinale \). The results of trypan blue staining of the four groups are shown in Figure 1. There was only a little floating color, mainly around the root epidermal cells, in CK (Figure 1A), whereas in DSs (Figure 1B), CTc (Figure 1C) and ETa (Figure 1D), the pelotons were plainly visible, mainly formed clumps and were mainly distributed in the parenchyma tissue of roots.

![Figure 1](image1.png)

**Figure 1.** Determination of fungal colonization in the roots of \( D. officinale \) with trypan blue staining under a 20x microscope. (A) represents the control group CK, (B) represents DSs (Serendipita sp. from \( D. officinale \)), (C) represents CTc (\( T. calospora \) from \( C. goeringii \)) and (D) represents ETa (\( T. asymmetrica \) from \( E. radicans \)). The white circle represents partial peloton in the root. VS: vascular bundle sheath; EX: exodermis; PA: parenchyma; VE: velamen.

3.2. Comparison of Relative Conductivity, Osmoregulatory Substance Content and Antioxidant Enzyme Activities between Co-Cultured and Control Groups

After the establishment of stable symbioses between fungi and \( D. officinale \), the relative conductivity of each treatment group was significantly lower than that of CK and there was a significant difference between each group (Figure 2A). The relative conductivity of the CK group (without fungi) was the highest (43%), which was...
significantly higher than that of other treatment groups. DSs was 41%, ETa was 19% and the CTc group was only 12%, which were 2%, 23% and 31% lower than CK, respectively.

The effect of fungal symbiosis on the soluble sugar content in the leaves of *D. officinale* is shown in Figure 2B. The sugar content in DSs, CTc and ETa was significantly higher than that in CK. Among them, the content of soluble sugar in CTc was the highest, and was significantly higher than that of other groups, up to 1.73 mg/g, 181.2% higher than CK. There was no significant difference between DSs (1.53 mg/g) and ETa (1.60 mg/g), 149.5% and 159.9% more than CK, respectively. In contrast, the soluble sugar content was the lowest in the leaves of CK, only 0.62 mg/g.

The soluble protein content of the co-cultured groups was higher than that of CK (Figure 2C). The soluble protein content of CTc and ETa was significantly higher than that of the other two groups; there was no significant difference between the two groups. ETa was the highest, 66.2% higher than that of CK, followed by CTc, which was 60.1% higher than that of CK.

After inoculation with the three strains, the activities of antioxidant enzymes varied under different treatments (Figure 2D–F). The activities of CAT were significantly higher than those of CK, CTc (295.6% higher than CK) > ETa (139.6% higher than CK) > DSs (39.6% higher than CK). The activities of POD and SOD were significantly higher than those of CK under CTc treatment, but in DSs, POD and CAT activities were significantly lower than CK, while in ETa, POD activity was not significantly different from CK, and SOD activity was also significantly lower than CK.

![Figure 2](image)

**Figure 2.** Related resistance indexes of *D. officinale* in CK, DSs, CTc and ETa groups. (A) Relative conductivity, (B) soluble sugar content, (C) soluble protein content, (D–F) CAT, POD and SOD enzymes activities, respectively. Different lowercase letters are significantly different (*p* < 0.05) based on Tukey tests.

3.3. Effects of Orchid Mycorrhizal Fungi on *D. officinale* Morphology and Growth

There were differences in growth morphology between CK and the three groups treated with fungi (Figures 3 and 4). After 60 days of co-culture, the root length and root coefficient of the CTc group were significantly higher than those of the CK group, being 141.2% and 71.4% higher than those of CK, respectively. The presence of the fungi also
significantly promoted an increase in leaf number, leaf length and leaf thickness, being 11.1%, 181.1% and 58.3% higher than those of CK, respectively. In addition, whole plant fresh weight was also significantly increased in the CTc treatment, being 155.2% higher than that of CK. The stem length, leaf width and dry weight of the ETa treatment group were significantly higher than those of CK, being increased by 82.0%, 78.9% and 497.1%, respectively. However, the performance of the DSs treatment group was flat, and while the performance of morphological indicators was better than that of CK, the effect was not significant.

**Figure 3.** Comparison of growth of tissue-culture seedlings of *D. officinale*. Seedlings from the groups CK, DSs, CTc and ETa are shown from left to right.

**Figure 4.** Growth of *D. officinale* after establishment of symbiosis with different fungi. (A) The average root length and root number of the four groups CK, DSs, CTc and ETa. (B) The average
stem thickness and stem length of each group. (C) The average leaf length, leaf width, leaf thickness and leaves of each group. (D) The fresh weight and dry weight of the whole plant of each group. Different lowercase letters are significantly different ($p < 0.05$) based on Tukey tests.

3.4. Orchid Mycorrhizal Fungi Promote an Increase in the Content of Photosynthetic Pigments in Leaves of *D. officinale*

The content of photosynthetic pigments in the leaves of each group are shown in Figure 5. Photosynthetic capacity is positively correlated with photosynthetic pigment content, and to some extent reflects the growth ability of plants. The results showed that chlorophyll a had the highest content in each group, followed by chlorophyll b, and then carotenoids. The co-culture group was found to have increased contents of chlorophyll a, chlorophyll b and carotenoids. Compared with CK (2.27 mg/g), CTc significantly increased the total photosynthetic pigment content (4.53 mg/g), 99.6% higher than CK, followed by the ETA treatment group (4.46 mg/g), and DSs (2.82 mg/g) had the least significant effect. In addition, CTc was the most conducive to increasing levels of chlorophyll a (18.1% more than CK) and chlorophyll b (151.5% more than CK) content, while ETA was the most conducive to increasing carotenoid content (109.1% more than CK).

![Figure 5](image-url)

**Figure 5.** The content of photosynthetic pigments in leaves of the four treatment groups. Color bars of each group from left to right are chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoid (Car). Different lowercase letters are significantly different ($p < 0.05$) based on Tukey tests.

3.5. Effects of Orchid Mycorrhizal Fungi on Stem Polysaccharide Content of *D. officinale*

After establishment of stable symbiosis, the content of polysaccharides in the stem of *D. officinale* treated with OMF was higher than in those of the CK (Figure 6). Among them, the content of polysaccharide in CTc (42.69 mg/g) was significantly higher than that of the other groups. The treatment groups ETA and DSs had 32.53 and 29.93 mg/g, respectively. CTc, ETA and DSs had 1.46, 1.12 and 1.02 times as much polysaccharide as CK, respectively. This result showed that one of the key nutritional components of *D. officinale* was also significantly improved by OMF.
4. Discussion

4.1. Adaptability of D. officinale in a Symbiotic Environment by Resistance Index

The infection rate and infection index of plants vary with mycorrhizal fungi. An invasion by some fungi may be destructive to some plants, while for some it may be beneficial. A few mycorrhal fungi can successfully enhance the growth of the host under compatible conditions [36,50,51]. In this study, we demonstrated using trypan blue staining the stable colonization of D. officinale with fungi. Peloton were mainly localized in the parenchyma tissue of D. officinale roots. When the peloton passed through the cell wall to the parenchyma tissue of the root, the antioxidant enzyme activity and the content of osmoregulatory substances in D. officinale inoculate with T. calospora significantly increased. However, the relative electrical conductivity was lower than in the control group, which suggests that the hyphae disturbed the original electrolyte balance in the cells but perhaps did not cause serious electrolyte leakage in the cells [52,53]. This is a positive measure taken by plants and indicates that colonization by mycorrhizal fungi may trigger host defense responses [54,55]. Increased expression of defense enzymes, changes in cell wall structure and accumulation of metabolites are widely accepted strategies for host resistance to infection [56–59]. The increase in the antioxidant enzyme activity and osmoregulatory substances of T. calospora could stabilize the osmotic balance and help to maintain the normal activities of plants. Inoculation of T. calospora may improve the adaptability of D. officinale to help promote the growth of D. officinale. According to previous studies, fungal symbiosis can promote osmotic balance by increasing the content of osmoregulatory substances and activities of antioxidant enzymes, and these substances may be directly produced by fungi [60] or may be promoted by affecting plant gene expression [61]. Further studies are needed to understand the changes at a molecular level that are caused by plant inoculation with different endophytic fungi [62].

4.2. Effects of Symbiotic Fungi on Growth and Morphology of D. officinale

The pronounced effects of symbiotic fungi in augmenting the growth and yield, including plant height, dry weight, chlorophyll content, plant root length, nodulation, seeds, seed oil contents, and total biomass of several crops is well established [63–66]. The results of this study showed increases in the root number, leaf number, fresh weight and leaf length of D. officinale inoculated with T. calospora, and increases in the leaf width, fresh weight and dry weight of D. officinale inoculated with T. asymmetrica. The growth of D. officinale inoculated with Serendipita sp. was not significantly different from that of the control group that was not inoculated. In general, T. calospora had the best promotion effect on the growth of D. officinale, and the growth of these tissue-culture seedlings was
the best, followed by *T. asymmetrica*. The growth of *Serendipita* sp. was not significantly different from that of the control group (not inoculated). Different OMF strains have different promoting effects. One study found that *Epulorhiza* sp. had the greatest promoting effect on the rooting number, root length, leaf number and plant height of *D. officinale* [67]. Other studies reported that the total fresh weight of the inoculated peloton after 19 months of growth was 2.69 times that of the non-inoculated peloton [68]. In this study, the fresh weight of *D. officinale* inoculated with *T. calospora* was 2.72 times than that of the control group (not inoculated), providing strong support for the use of *T. calospora* in commercial production.

Biomass is determined by a variety of environmental factors. Photosynthesis is also an important factor in determining biomass, and photosynthetic pigment content is one indicator for testing plant photosynthetic capacity [69]. This study showed that the photosynthetic pigment content of *D. officinale* increased to varying degrees after the establishment of symbiosis with fungi. The inoculation with *T. calospora* was the most favorable for the increase in the photosynthetic pigment content of plants, while the inoculation of *Serendipita* sp. was the most unfavorable. This result was consistent with the growth morphology index, which once again proved that the increase in photosynthetic pigment content laid the foundation for plant growth. According to the study of Kang et al. [70], *Epulorhiza* sp. (GDB254) was more conducive to the increase in chlorophyll content in *D. officinale*, but only increased it by 86.5% compared with the control group, and the effect was much lower than that of *T. calospora* (96.8%), selected in this experiment. This reflects that *T. calospora* is more capable of improving the assimilation capacity of *D. officinale*, providing sufficient nutrients for plant growth, thus significantly promoting seedling growth [71].

4.3. Effect of Different Symbiotic Fungi on Total Polysaccharide Accumulation in *D. officinale* Stems

Polysaccharides are the main medicinal component of *D. officinale*, and the content determines the biological activity and economic value of *D. officinale* [72,73]. It could be seen from our study that the symbiosis of OMF and *D. officinale* can increase the accumulation of polysaccharides. The total polysaccharide content was most significantly increased after inoculation with *T. calospora*. Previous studies have shown that the polysaccharide content of *D. officinale* inoculated with *T. calospora* was the highest, 1.01 times higher than that of the non-inoculated group at 19 months [74]. However, we found that the polysaccharide content of *D. officinale* inoculated with *T. calospora* was 1.46 times higher than that of the non-inoculated group at 60 days, while the polysaccharide content of *D. officinale* inoculated with *Serendipita* sp. increased the least, only 1.02 times higher than that of CK, but the effect was still better than that of Chen et al. [74]. It also proved that a different processing time may lead to different results, and long-term treatment may not be better. However, little is known about the mechanisms by which fungi influence plant functional metabolic components, including polysaccharide accumulation in *D. officinale* [75]. A previous study had strongly suggested that the biological functions of glucomannan or galactoglucomannan, the two major medicinal polysaccharides in the stem of *D. officinale*, are related to environmental-stress tolerance [76]. Plant-associated OMF initiate the expression and production of defense-mechanism-related metabolites and other fungi-specific compounds, which provide protection against biotic and abiotic stresses [77]. Thus, the observed increase in polysaccharide content may be due to the fungal infection itself triggering a defensive response in the plantlets, but the mechanism for this process requires further study.

5. Conclusions

In summary, this study evaluated for the first time the effect of three orchid mycorrhizal fungi isolated from *D. officinale*, *C. goeringii* and *E. radicans* on the growth of the host *D. officinale* and the accumulation of functional components. The three fungi (*Serendipita* sp., *T. calospora* and *T. asymmetrica*) played a unique role in the growth and
accumulation of functional components in *D. officinale*. All the three strains could form a symbiotic relationship with *D. officinale* in vitro and had different promotion effects on its growth. *T. calospora*, isolated from *C. goeringii*, had a strong affinity for *D. officinale* and could stimulate the plants to produce more antioxidant enzymes and osmoregulatory substances to adapt to the symbiotic environment. In addition, they had significant effects on promoting the growth of root organs, leaf organs and the whole plant. More importantly, they could significantly increase the content of medicinal polysaccharides in *D. officinale*, thereby increasing their economic value. This study may provide a theoretical basis for the further study of mycorrhizal technology in orchid protection and commercial production.

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