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Mycorrhizal Compatibility and Germination-Promoting Activity of *Tulasnella* Species in Two Species of Orchid (*Cymbidium mannii* and *Epidendrum radicans*)

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Abstract: In nature, Orchidaceae seeds establish a relationship with orchid mycorrhizal fungi to obtain essential nutrients for germination. The orchids, *Cymbidium mannii* and *Epidendrum radicans*, have significant ornamental and economic value. We isolated and cultured mycorrhizal fungi from *C. mannii*, *E. radicans*, and *C. goeringii* roots. Three strains of fungi, *Tulasnella calospora* (Tca), *T. asymmetrica* (Tas), and *T. bifrons* (Tbi), were identified using ITS-rDNA sequencing. Their mycorrhizal compatibility, germination-promoting effects, and symbiosis with the seeds of *C. mannii* and *E. radicans* were studied in vitro using various concentrations of oatmeal agar (OA) medium. Tca exhibited significant seed-germination-promoting effects on *C. mannii* (92.1%) and *E. radicans* (84.7%) on 2.0 and 4.0 g/L OA, respectively. For Tbi and Tas, the highest germination percentages were observed on 4.0 g/L OA in *E. radicans* (73.60% and 76.49%, respectively). Seed germination in *C. mannii* was enhanced by high oatmeal concentrations (8.0 and 12.0 g/L) during symbiosis with Tas, whereas Tbi had no effect regardless of OA concentration. Tca exhibited high compatibility with *C. mannii* and *E. radicans*, and the oatmeal concentration of the medium affected this compatibility. The findings of this study will aid in the propagation of endangered orchid species for conservation and commercial purposes using mycorrhizal technology.

Keywords: *Cymbidium mannii*; *Epidendrum radicans*; *Tulasnella* spp.; mycorrhizal fungi; symbiosis

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1. Introduction

Orchidaceae is widely regarded as the largest and most diverse family of angiosperms, consisting of 17,000–35,000 species and encompassing 8% of all vascular plants [1,2]. Orchid seeds tend to be small and dust-like, and lack an endosperm [3]. Low seed germination and seedling survival rates are the main factors limiting the protection of endangered orchids [4]. Consequently, germination and protocorm development depend on compatible mycorrhizal fungi to obtain carbon, water, and nutrients under natural conditions [5–7]. Given this obligate requirement, at least in the early developmental stages, obtaining optimal sources of mycorrhizal fungi and understanding the patterns of orchid–mycorrhizal relationships may facilitate the conservation of threatened orchid species and are, therefore, important steps for seed-based orchid conservation [8–10].

Compatibility between mycorrhizal fungi and orchids has an important effect on successful symbiotic seed germination [11]. Almost all known orchid mycorrhizal

symbionts belong to two orders of Basidiomycetes, Cantharellales and Sebacinales [2,12]. Species of *Tulasnella* (Basidiomycota) are the most commonly reported mycorrhiza-forming fungi in orchids in both temperate and tropical zones, and can promote seed germination in many orchid species [13–15]. Moreover, it has been found that several orchid species may exhibit a certain degree of specificity with mycorrhizal fungi during seed germination and growth [10,16–20]. For example, there is a low degree of specificity toward orchid species and nine different fungal partners [21], and the mycorrhizae associated with *Dendrobium* seedlings are host-specific.

Symbiotic seed germination is a popular tool for studying orchid–fungus specificity; it plays a role in the production of mycobiont-infected healthy seedlings, which are valuable for both horticultural and conservation purposes [22,23]. This technique is largely performed by co-cultivating fungi and orchid seeds on oatmeal agar (OA) medium under laboratory conditions [23,24]. Many experiments have demonstrated that mycorrhizal symbiosis can be established in vitro on OA medium including 2.5, 3.0, 4.0, and 10.0 g/L oatmeal [8,24–27]. A previous study compared the effect of different oatmeal concentrations in OA on in vitro symbiotic seed germination, and it was found that the three OA concentrations tested (1.0, 5.0, and 10.0 g/L) exhibited similar germination percentages [28]. However, in our previous work, the symbiotic seed germination of orchid–*Tulasnella* differed under different OA concentrations (0.25, 2.5, and 5.0 g/L); 2.5 g/L OA was found to be the most effective symbiotic medium [29]. Based on this, adjusting the concentration of the orchid–fungal co-culture medium will help us to screen and establish a good symbiotic environment.

Species belonging to the genus *Cymbidium* have diverse life forms; about 70% are epiphytic, whereas a minority are terrestrial or lithophytic [30,31]. However, in recent years, *Cymbidium* species have been excessively exploited by humans. Their habitat has been seriously damaged, and their distribution range has been sharply reduced. As their wild resources gradually decrease, these *Cymbidium* species are in danger of extinction [4]. *Cymbidium mannii* is an epiphytic orchid with important ornamental and medicinal value [31,32]. *Epidendrum* is widely distributed, contains both terrestrial and epiphytic species, and has great potential for commercialization. *Epidendrum radicans* is one of the most highly valued flowers, potentially exhibiting the longest vase life in the cut-flower industry. It thrives in the tropics and subtropics [33]. At present, all wild orchids worldwide are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and it is essential to safeguard these resources for the future. In recent years, most studies on orchid–fungi symbiotic seed germination have been conducted in *Dendrobium* [10,34–36]. Relatively few studies are available on the promotion of seed germination in symbiotic *Tulasnella*–*C. mannii* [4] and *Tulasnella*–*E. radicans.* In this study, we established symbiotic relationships between three *Tulasnella* species (isolated from three orchid species, two epiphytic and one terrestrial) and two epiphytic orchids, *C. mannii* and *E. radicans*, under different OA concentrations. We aimed to evaluate (1) whether *Tulasnella* species are compatible with various orchid plants and can promote the seed germination; and (2) whether the concentration of oatmeal in the medium affects their compatibility and germination-promoting activity. The results of this study may provide more clues for exploring the relationship between orchids and mycorrhizal fungi and will provide information on the propagation of endangered orchid species for conservation [11].

2. Materials and Methods

2.1. Fungal Isolation and Tulasnella Identification

The original sources of the plant samples are listed in Table 1. *Cymbidium mannii* was collected from Xinglong tropical botanical garden, Wanning, Hainan Province, where the cultivation matrix was a proportioned mix of chopped bark, moss, and Portland stone with a 2:1:1 mixing ratio. *Epidendrum radicans* was collected from the South China

Botanical Garden, Guangzhou, Guangdong Province, where the cultivation matrix was a proportioned mix of chopped bark, moss, and brick fragments with a 3:1:1 mixing ratio. *Cymbidium goeringii* is a terrestrial orchid, and was collected from Shaoxing, Zhejiang Province, China (120°49′ E, 29°92′ N).

Three roots were collected from each plant and three plants were collected from each species. The roots were packed in zip-lock bags and transferred to the Laboratory of the Research Institute of Forestry, Chinese Academy of Forestry. Hand-cut sections of the roots were examined under a light microscope (Olympus BX41, Tokyo, Japan) to observe the presence of pelotons, and uncolonized tissue was removed. Colonized roots were washed under running tap water for 4–6 h, followed by immersion in 70% ethanol for 30 s. Further, the roots were immersed in 5.0% sodium hypochlorite solution for 4–5 min. The samples were rinsed with sterile distilled water 3–4 times. The roots were cut into slices using a sterile blade, and the sections were placed onto potato dextrose agar (PDA, BD Difco 213400, NewYork, NY, USA) with 50 μg/mL streptomycin to inhibit bacterial growth. At the same time, after undergoing the same treatment, uncut roots were placed directly on the Petri dish without cutting to test the surface-sterilization effectiveness. Petri dishes were incubated at 25 °C until colonies were observed. Fungi that grew from the cut wounds in the plant roots were selected and transferred to new PDA medium. Fungi colonies from the cultures of the original isolates were re-cultured on new PDA medium an additional 3 times (or more) until purified fungal strains were obtained [5,25,37].

Table 1. Sources of the orchid species from which the samples were collected.

Isolates	Species	NCBI Accession Number of ITS Sequence	Deposit Number	Original Plant
Tca	*Tulasnella calospora*	OK413294	CPCC 401,220	*Cymbidium mannii* (epiphytic)
Tas	*Tulasnella asymmetrica*	OK413349	CPCC 401,221	*Epidendrum radicans* (epiphytic)
Tbi	*Tulasnella bifrons*	OK413293	CGMCC 14,794	*Cymbidium goeringii* (terrestrial)

The fungal mycelia were frozen using liquid nitrogen, and DNA was extracted using an E.Z.N.A™ Fungal DNA Miniprep Kit (D3390-01, Omega, Guangzhou, China) according to the manufacturer's instructions. Amplification of the ITS sequence was performed using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). ITS sequences were compared to those in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST), and the ITS sequences of the three strains were registered in GenBank (Table 1).

Well-characterized and closely related taxa identified in the BLAST searches were included in the phylogenetic analyses. Multiple sequence alignments of each ITS sequence were made using ClustalW packaged with the BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic trees were generated using maximum likelihood analysis (MEGA X) [38], and 1000 bootstrap replicates were performed to estimate the node reliability of the resulting trees.

The strains were deposited in the China General Microbiological Culture Collection Center (CGMCC) and China Pharmaceutical Culture Collection (CPCC).

2.2. Seed Harvest

The seeds of the orchids *C. mannii* and *E. radicans* were used in this study. Plants were maintained in a greenhouse at the Chinese Academy of Forestry (Beijing, China) under day/night temperatures of 24 °C/18 °C, 65–75% relative humidity, and an 8-h day/16-h night photoperiod. The flowers of the plants were pollinated by hand in the greenhouse. Mature capsules of *C. mannii* and *E. radicans* (about four capsules from each species) were collected 4–6 months after pollination, just prior to dehiscence (Figure 1).

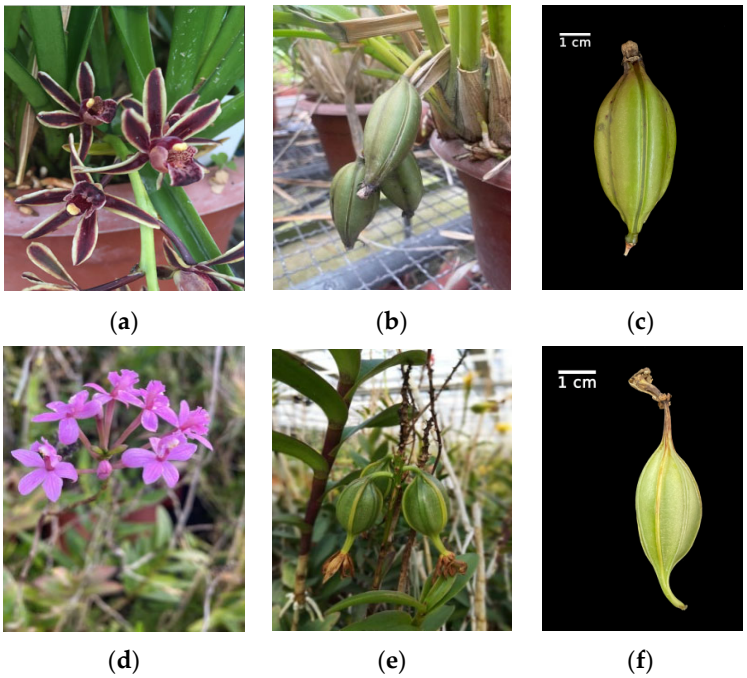


Figure 1. Flowers and capsules of *Cymbidium mannii* and *Epidendrum radicans*. (**a**) The flowers of *C. mannii*. (**b**,**c**) The capsules of *C. mannii*. (**d**) The flowers of *E. radicans*. (**e**,**f**) The capsules of *E. radicans*.

2.3. Symbiotic Germination

Orchid seed capsules were collected and then scrubbed with a soft brush under running tap water to remove any debris. Each capsule was further swabbed thoroughly thrice with 75% ethanol, disinfected with 5% NaClO for 5–10 min, and rinsed thrice in sterile distilled water. The capsules were then incised longitudinally, and the dusty mature seeds picked out of them. Approximately 100–150 seeds were added to plates containing OA medium [39,40]. The concentrations of OA medium used were 0.5, 1.0, 2.0, 4.0, 8.0, and 12.0 g/L. Cubes of about 1 cm^3 of each fungal culture were transferred onto the surface of the OA medium.

The control treatment (CK group) contained no fungal inoculates. The plates were sealed with Parafilm (Bemis, Neenah, WI, USA) and maintained at day/night temperatures of 24 °C/18 °C and 65–75% relative humidity. Each treatment consisted of 15 replicates.

2.4. Assessment of the Fungal Capacity to Promote Seed Germination

The seed germination and protocorm developmental stages were observed daily under a stereomicroscope (Nikon HFX, Tokyo, Japan). The toluidine blue colorimetric assay was performed to examine fungal colonization [34], and the timing of peloton-forming in the orchids was measured. Using the identified colonization times, data were subsequently collected 60 days after sowing to determine the growth index (GI) and percentage of seed germination. Seed germination status was assessed 60 days after incubation, and the standardized GI was calculated according to the criteria described by Otero [41]. The appearance of the promeristem was used as the primary criterion for defining germination [42].

The GI value was determined as follows: $(N_1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4 + N_5 \times 5 + N_6 \times 6)/(N_0 + N_1 + N_2 + N_3 + N_4 + N_5 + N_6)$. The germination rate (%) was determined as follows: $(N_3 + N_4 + N_5 + N_6)/(N_0 + N_1 + N_2 + N_3 + N_4 + N_5 + N_6) \times 100$, where N_0 is the number of seeds at stage 0, N_1 is the number of seeds at stage 1, and so on (Figure 2).

Seeds can begin germinating, but then be unable to proceed beyond N_3 to complete germination and successfully develop into seedlings. Therefore, even if the GI value is higher than zero, the germination rate could still be zero.

Figure 2. Stages in the development of *E. radicans* protocorms. (**a**) Stage N_0, no germination. (**b**) Stage N_1, production of rhizoids by the embryo. (**c**) Stage N_2, rupture of testa by the enlarged embryo. (**d**) Stage N_3, appearance of the promeristem. (**e**) Stage N_4, appearance of the first true leaf. (**f**) Stage N_5, elongation of the true leaf. (**g**) Stage N_6, appearance of the second true leaf.

2.5. Statistical Analysis

All the experiments followed a completely randomized design. One-way ANOVA followed by Tukey's multiple comparison tests were performed to compare the germination rates and GI values among different treatments and to determine statistical significance ($p < 0.05$). All statistical analyses were performed using R Studio software (version 1.4.1717).

3. Results

3.1. Tulasnella Identification and Phylogenetic Analyses

Mycorrhizal fungi were isolated from three orchid plants (Table 1). The ITS-5.8s rDNA sequences of the three isolates were found to be highly homologous with those of *Tulasnella*-like isolates, with 94–99% similarity in the NCBI gene database. Meanwhile, in

the phylogenetic tree based on ITS-5.8s rDNA gene sequences using the maximum-likelihood method (Figure 3), the three strains were clustered with the clade comprising *Tulasnella* species. Strains Tca and Tbi were clustered with *T. calospora* strain MAFF P305802 (DQ388042) and *T. bifrons* specimen voucher BPI 724,849 (AY373290), respectively. Tas formed a phylogenetic lineage within the clade comprising the strain *T. asymmetrica* AL. PC10.6 (MH134557).

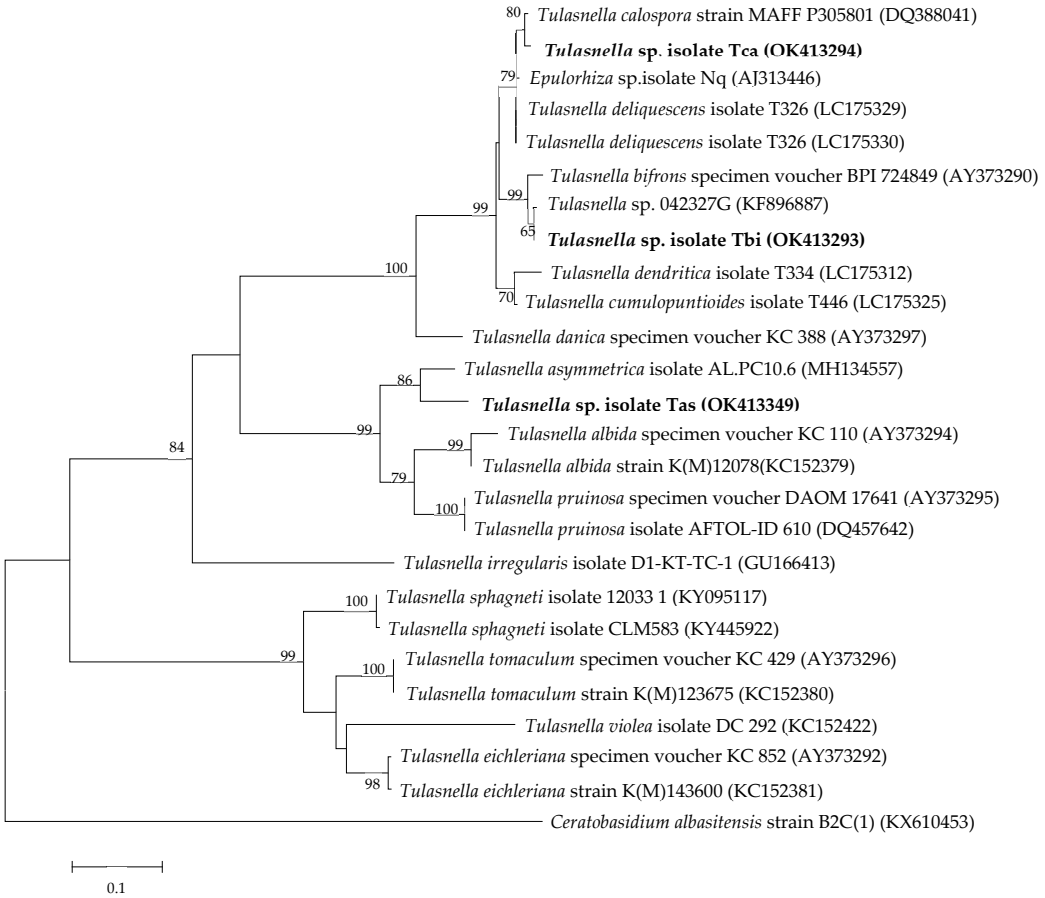


Figure 3. Maximum likelihood trees of ITS sequences of *Tulasnella* spp. The sequence of *Ceratobasidium albasitensis* strain B2C (1) was used as an outgroup. Bootstrap percentages (based on 1000 replications) >50% are shown at the branching points. Scale, 0.1 nt substitutions per site.

3.2. Colonization of Mycorrhizal Fungi

The seeds of the two orchid species were co-cultivated with the three fungal strains under different concentrations of OA, and germination was monitored. Those showing protocorm formation were observed for fungal colonization under a stereomicroscope (Figure 4). The times of colonization of the three fungi were detected in orchid protocorms under different OA concentrations (Table 2).

Table 2. The time of mycorrhizal fungi peloton formation was detected in orchid protocorms under different oatmeal agar (OA) concentrations (days ± s.d.).

OA Concentrations (g/L)	*C. mannii*			*E. radicans*		
	Tbi	Tas	Tca	Tbi	Tas	Tca
0.5	n	n	34.0 ± 1.0	n	n	25.0 ± 0.0
1.0	n	n	23.0 ± 0.0	n	n	24.0 ± 1.0
2.0	n	n	20.3 ± 0.6	18.3 ± 0.6	23.0 ± 0.3	20.0 ± 1.0
4.0	n	n	37.0 ± 0.0	19.0 ± 0.0	22.3 ± 0.6	19.3 ± 0.6
8.0	n	56.0 ± 0.0	55.3 ± 0.6	n	24.0 ± 0.0	22.0 ± 0.3
12.0	n	56.0 ± 0.0	56.3 ± 0.6	n	25.0 ± 0.0	22.0 ± 1.0

n: No symbiosis observed.

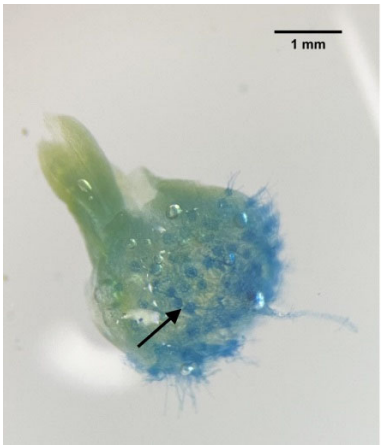
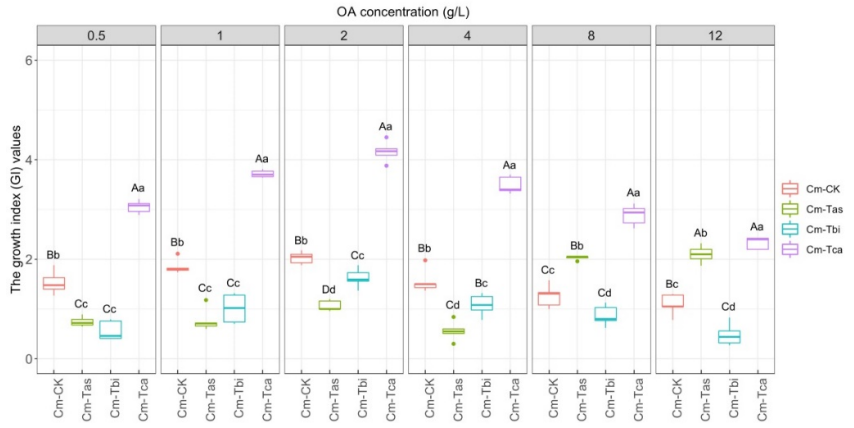


Figure 4. Fungal strain Tca colonizing and forming pelotons in a protocorm of *E. radicans.*

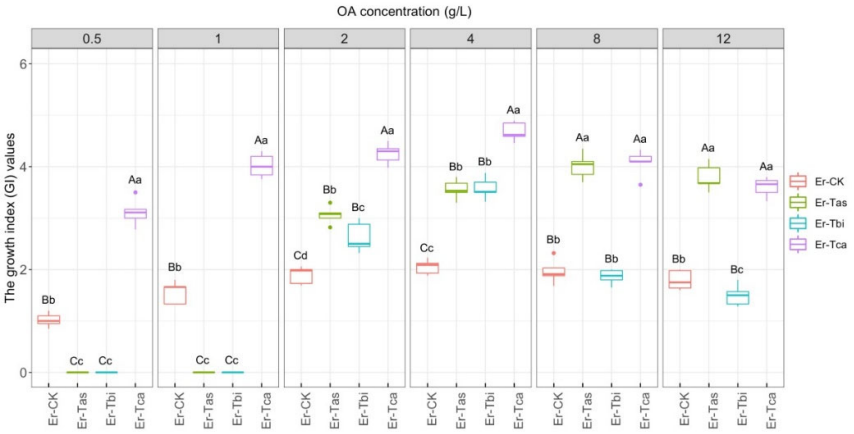
For *C. mannii*, Tbi could not form pelotons at any of the OA concentrations. The time of detection of Tca pelotons varied from 20.3 ± 0.6 (2.0 g/L OA) to 56.3 ± 0.6 days (12.0 g/L OA), whereas that of Tas was 56.0 ± 0.0 days at 8.0 and 12.0 g/L OA. The peloton-forming times of the strains Tbi, Tas, and Tca in *E. radicans* varied depending on the concentrations of OA. The shortest time was for Tbi (18 days) at 2.0 g/L OA, and the longest time was for Tas (25 days) at 12.0 g/L OA. Tca and Tas could colonize both orchid hosts, whereas Tbi only formed pelotons in *E. radicans*.

3.3. Effects of Different Fungal Strains on Seed Germination

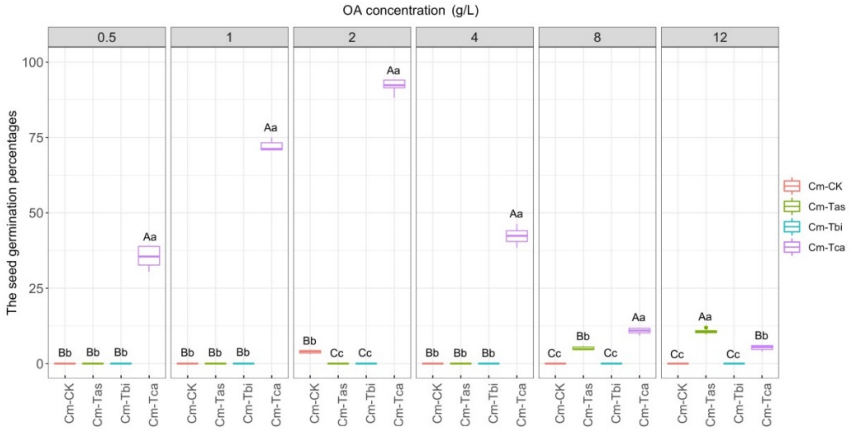
After 60 days of incubation, the *C. mannii* seeds co-cultivated with Tca had the highest GI value (4.16 ± 0.21; Figure 5a) and germination percentage (92.05 ± 2.45%; Figure 5c). The effect of Tas on *C. mannii* seed germination was only higher than on the CK at 8.0 and 12.0 g/L OA, and only a small proportion of seeds germinated at these OA concentrations (5.06 ± 0.56 and 10.67 ± 0.84, respectively; Figure 5c). Meanwhile, the GI values (Figure 5a) and seed germination percentages (Figure 5c) revealed that Tbi exhibited no evidence of promoting seed germination or seedling formation. The GI values and germination percentages of *C. mannii* seeds co-cultivated with Tca were significantly ($p < 0.05$) higher than those of the other three groups (Cm-Tas, Cm-Tbi and Cm-CK) (Figure 5a,c). In terms of the successful promotion of seed germination and seedling formation, Tca exhibited a stronger effect than Tas, whereas Tbi exhibited no effect (Figure 5a,c).


OA concentration (g/L)
0.5
1
2
4
8
12
The growth index (GI) values
Cm-CK
Cm-Tas
Cm-Tbi
Cm-Tca

(**a**)


OA concentration (g/L)
0.5
1
2
4
8
12
The growth index (GI) values
Er-CK
Er-Tas
Er-Tbi
Er-Tca

(**b**)


OA concentration (g/L)
0.5
1
2
4
8
12
The seed germination percentages
Cm-CK
Cm-Tas
Cm-Tbi
Cm-Tca

(**c**)

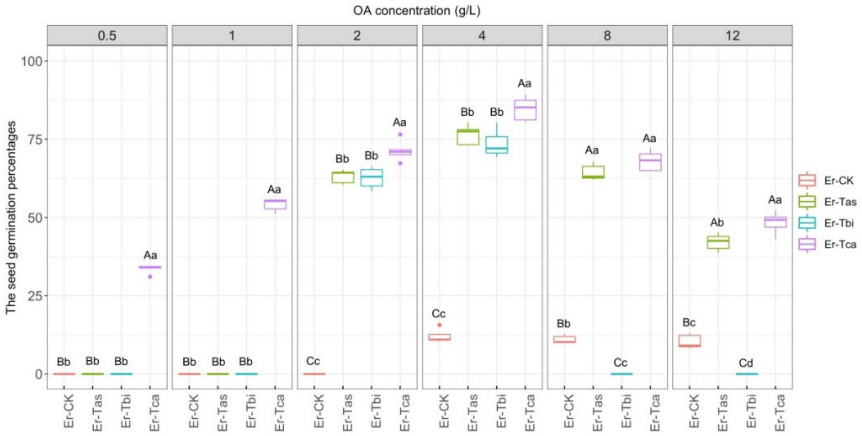


Figure 5. The effect of different fungal strains on seed germination in orchids 60 days after cultivation. (**a**) The growth index (GI) values of *C. mannii* with Tca (Cm-Tca), Tas (Cm-Tas), Tbi (Cm-Tbi), and CK (Cm-CK). (**b**) The GI values of *E. radicans* with Tca (Er-Tca), Tas (Er-Tas), Tbi (Er-Tbi), and CK (Er-CK). (**c**) The germination percentages of *C. mannii* with Tca (Cm-Tca), Tas (Cm-Tas), Tbi (Cm-Tbi), and CK (Cm-CK). (**d**) The germination percentages *of E. radicans* with Tca (Er-Tca), Tas (Er-Tas), Tbi (Er-Tbi), and CK (Er-CK). In each panel, strains with same letters are not significant; strains with different capital letters are extremely significantly different ($p < 0.001$) and lowercase letters are significantly different ($p < 0.05$) based on Tukey tests.

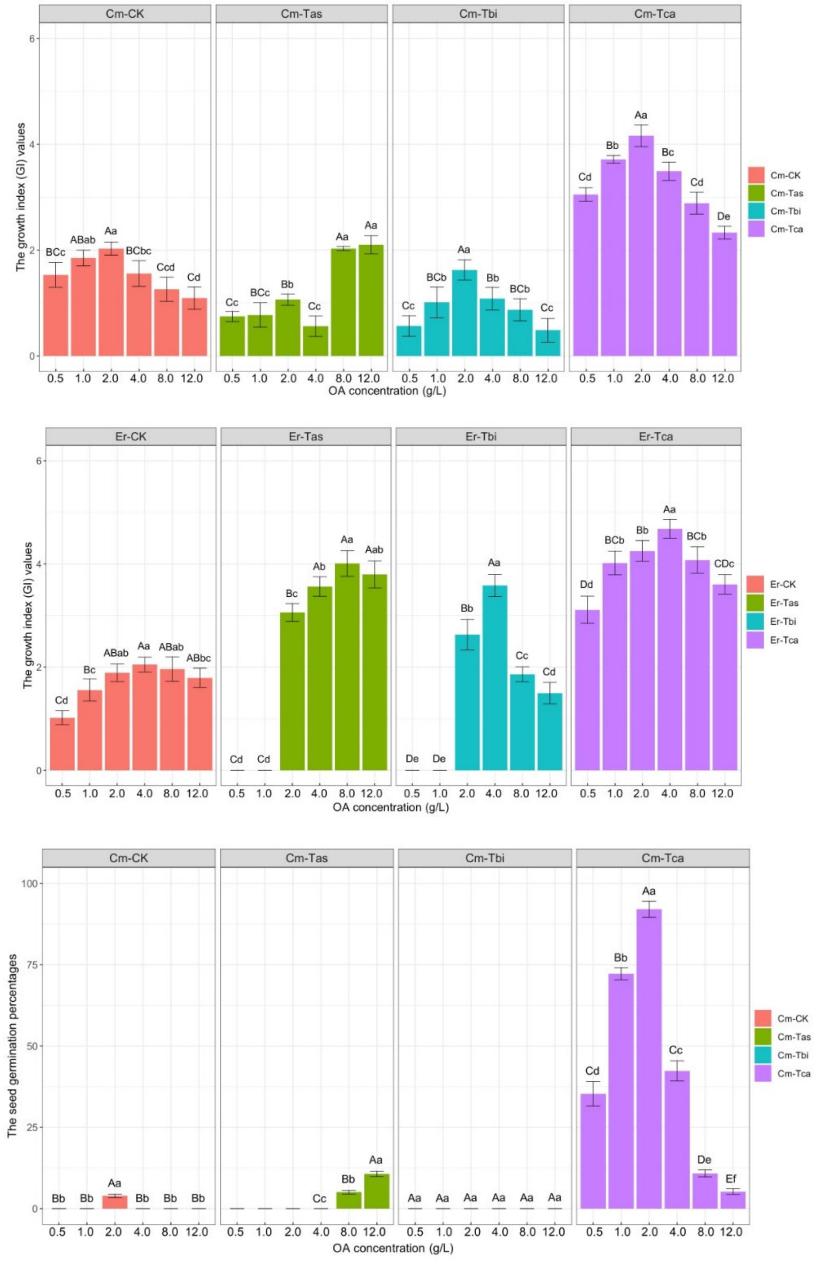
In *E. radicans*, Tca exhibited the most obvious promotional effect on seed germination, with the highest GI value (4.68 ± 0.18; Figure 5b) and the highest proportion of seeds germinating (84.74 ± 3.83%; Figure 5d). Tas had a better germination-promoting effect; it promoted seed germination at four OA concentrations (2.0, 4.0, 8.0, and 12.0 g/L), with the highest value being 76.49 ± 3.14% on 4.0 g/L OA; (Figure 5d). Tbi caused seed expansion and germination at 2.0–12.0 g/L OA; however, this strain could not support the seeds in completing subsequent stages of germination and becoming seedlings at the two higher OA concentrations (8.0 and 12.0 g/L); its GI value was lower than that of the CK group and the germination percentage was zero. The highest GI values of Tca were significantly ($p < 0.05$) higher than those of the other three groups at four concentrations of OA (0.5, 1.0, 2.0, and 4.0 g/L); however, they were not significantly higher than those of Tas on 8.0 and 12.0 g/L OA (Figure 5b). Considering the seed germination percentages, Tca performed significantly ($p < 0.05$) better than the other three strains, but the result was not significantly different from that of Tas on 8.0 g/L OA. No significant differences were observed between the seed germination percentages of Tas and Tbi on 0.5, 1.0, 2.0, or 4.0 g/L OA; however, Tas performed significantly better ($p < 0.001$) than Tbi on 8.0 and 12.0 g/L OA (Figure 5d).

3.4. Effects of Different Concentrations of OA on Seed Germination

The concentration of the co-culture medium played an important role in determining symbiosis and its effects. For *C. mannii*, Tca could promote seed germination at all six OA concentrations; the germination percentage varied from 5.23 ± 0.86% (12.0 g/L OA) to 92.05 ± 2.45% (2.0 g/L OA; Figure 6b), and the GI values varied from 2.33 ± 0.12 (12.0 g/L OA) to 4.16 ± 0.21 (2.0 g/L OA; Figure 6a). The GI values and germination percentages of Tca in *C. mannii* were significantly ($p < 0.001$) higher on 2.0 g/L OA than at other concentrations (Figure 6a,b). The GI values of the symbiotic Tas groups at two OA concentrations, 8.0 g/L and 12.0 g/L, did not differ significantly, but both values were significantly ($p < 0.001$) higher than those at the other four OA concentrations (Figure 6a).

When the three fungal strains were grown symbiotically with *E. radicans*, the seed germination rates on 4.0 g/L OA (84.74 ± 3.83% for Tca, 73.60 ± 4.42% for Tbi, and 76.49 ± 3.14% for Tas) were significantly ($p < 0.001$) higher than those at other OA concentrations.

For Tas and Tbi, the seeds did not germinate at certain concentrations of OA (Tas: 0.5 and 1.0 g/L; Tbi: 0.5, 1.0, 8.0, and 12.0 g/L OA; Figure 6c,d).



(**a**)

(**b**)

(**c**)

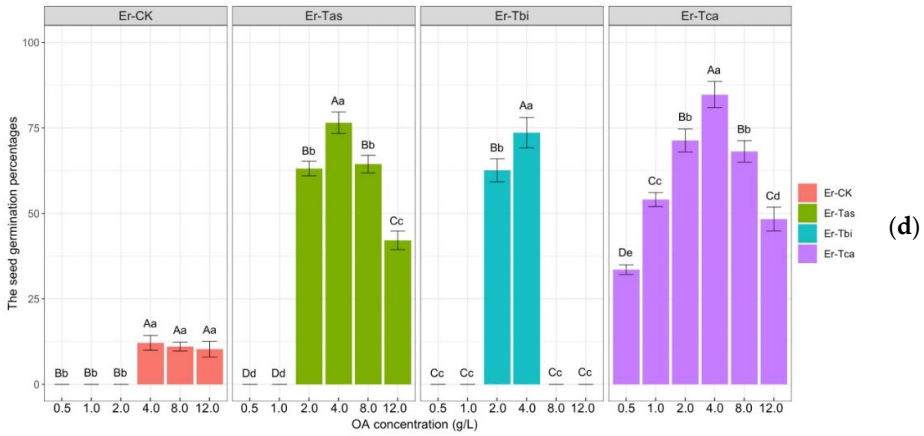


Figure 6. The effects of different concentrations of OA on seed germination 60 days after cultivation. (**a**) The GI values of *C. mannii* with Tca (Cm-Tca), Tas (Cm-Tas), Tbi (Cm-Tbi), and CK (Cm-CK). (**b**) The GI values of *E. radicans* with Tca (Er-Tca), Tas (Er-Tas), Tbi (Er-Tbi), and CK (Er-CK). (**c**) The germination percentages of *C. mannii* with Tca (Cm-Tca), Tas (Cm-Tas), Tbi (Cm-Tbi), and CK (Cm-CK). (**d**) The germination percentages of *E. radicans* with Tca (Er-Tca), Tas (Er-Tas), Tbi (Er-Tbi), and CK (Er-CK). In each panel, concentrations with same letters are not significant; concentrations with different capital letters are extremely significantly different ($p < 0.001$) and lowercase letters are significantly different ($p < 0.05$) based on Tukey tests.

4. Discussion

Orchid growth depends on various environmental factors, including temperature, soil type, competition, pollination, and the presence of mycorrhizal fungi [9,43–45]. Mycorrhizal fungi are essential for the successful symbiotic development of orchids under natural conditions, upon reintroduction of endangered orchid species to their natural environment, and in the cultivation of ornamental and medicinal species [27,44,46,47].

Most symbiotic fungi found in orchids are within the 'rhizoctonia' species complex [6,48], which includes the Ceratobasidiaceae and Tulasnellaceae in the Cantharellales and the Serendipitaceae in the Sebacinales [49–52]. Despite a lack of references concerning the teleomorphic states of *Tulasnella*, molecular tools can be effectively applied to identify the strains; phylogeny based on ITS sequences was found to be better resolved than *mtLSU* phylogeny for identifying the *Tulasnella* genus [53]. In our study, three fungal isolates were obtained from three species of orchids. They were identified as *Tulasnella* using ITS-5.8S rDNA sequence analysis, and the phylogenetic trees showed that the three strains belonged to three clusters. Based on the results of the molecular analysis, we proceeded to establish mycorrhizal symbiosis with orchids using the three mycorrhizal strains and attempted to understand the relationships between *Tulasnella* and orchids. Under suitable external conditions, fungi recognize plants and subsequently establish symbiosis. The compatibility between orchids and their mycorrhizal fungi is variable and influenced by the surrounding plant species and environmental conditions [20,44,53]. It is generally believed that orchid mycorrhizal fungi show a certain degree of compatibility with species other than their hosts [54]. Some orchid species can maintain contact with a single fungal strain, while others can establish contact with different strains at different growth stages [55]. The evolutionary mechanisms of the interaction relationships of species involved in such broad interactions is likely to be "apparent generalism", which is defined as "one species is specific to one or several host species that provide unique resources, but it is also related to other host species that provide functional redundant resources, and the phylogeny of orchid plants may be an important determinant of fungal association" [55]. In this study, the Tca strain isolated from the roots of *C. mannii* successfully established symbiosis and promoted seed germination in both *C. mannii* and *E. radicans*. In other words, strain Tca was compatible with the orchid species examined in this study. Strain

Tas, isolated from *E. radicans* (an epiphytic orchid), also could establish symbiosis and promote seed germination in the above two species of orchids; however, compared with Tca, its colonization took longer and even failed at certain OA concentrations. Strain Tbi, isolated from *C. goeringii* (a terrestrial orchid), could promote seed germination in *E. radicans* only on 2.0 and 4.0 g/L OA; however, it had no germination-promoting effects in *C. mannii*. These results suggest that fungal strains present a certain degree of compatibility with various orchids, and are also affected by the environment.

High compatibility contributes to beneficial associations between fungi and orchids, promoting seed germination and protocorm development. However, these beneficial reactions are easily affected by several ecological factors, such as nutrients, photoperiod, temperature, and plant hormones [24]. Nutrient concentration plays an important role in mycorrhizal fungi–induced promotion of germination and its symbiotic relationship with orchid seeds. It has a long been understood that orchids receive not only mineral nutrients, such as N and P, but also organic C from orchid mycorrhizal fungi without rewards during germination [56,57]. Studies have examined the symbiotic promotion of orchid seed germination by mycorrhizal fungi using OA [28].

Because OA has the advantages of a controllable nutrient concentration and simple operation, we used mediums with different oatmeal concentrations to illustrate their effects on seed germination. The results show that germination percentages varied with oatmeal concentration under the same strain conditions. Tas promoted the seed germination of *C. mannii* at 8.0 and 12.0 g/L OA but not on other OA concentrations. However, Tca promoted seed germination in both *C. mannii* and *E. radicans* under all OA concentrations used in this study.

Fungi compatible with orchid plants may colonize orchid protocorms to form pelotons, after which, the protocorms may develop into photosynthetic seedlings. However, the orchid protocorms may also be overgrown by the fungal mycelium, and this can eventually lead to rot and death [52,57]. It is speculated that when nutrients are abundant or too scarce to meet the needs of seed germination and growth, fungi tend to live in saprophytic rather than symbiotic conditions with plants [52,57]. Recent work has shown that orchid mycorrhizal fungi may have more genes involved in the degradation of plant cell wall components than other mycorrhizal types [58]. The expression of CAZyme genes, which were identified in the process of establishing symbiotic relationships between *T. calospora* and two orchid species (*Serapias vomeracea* and *Cattleya purpurata*), showed that the degradative potential of *T. calospora* could possibly participate in regulating a switch between saprotrophic growth and symbiosis [52]. However, the mechanisms leading to mycorrhizal fungus transition between the mycorrhizal and saprotrophic stages are complex and need to be further elucidated [52].

In this study, the optimal fungal strains and culture media for efficient symbiosis were determined in two orchid plants. The results provide a technical reference for further studies on the molecular mechanism of orchid symbiosis in vitro and can be applied to the protection of orchid plant resources.

5. Conclusions

In this study, three fungal strains were isolated from the roots of different orchid plants and were identified as *Tulasnella* based on their ITS-rDNA sequences. The compatibility and germination-promoting activities of these fungi were investigated at different OA concentrations. In germination trials under laboratory conditions, the strain Tca exhibited the best compatibility and germination-promoting effects on both *C. mannii* and *E. radicans*, and all three fungal strains used in this study could promote seed germination in *E. radicans*. Moreover, the concentration of oatmeal in the medium affected the compatibility and germination-promoting activity of the fungi. These findings indicate that orchid mycorrhizal fungi present a certain degree of compatibility with various orchid plants, and are affected by the environment. These results may pave the

way for further research on mycorrhizal technology in orchid propagation for conservation and commercial purposes.

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