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Symbiotic Culture of Three Closely Related *Dendrobium* Species Reveals a Growth Bottleneck and Differences in Mycorrhizal Specificity at Early Developmental Stages

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Abstract: Mycorrhizal specificity, i.e., the range of fungi allowing mycorrhizal partnerships, differs among orchid species, but that at early developmental stages is unclear. We investigated whether mycorrhizal specificity during seed germination and seedling development differs among three *Dendrobium* species, *D. officinale*, *D. okinawense* and *D. moniliforme*, in vitro. Nine mycorrhizal fungal strains were obtained from the roots of these species and cultured with a seed of each *Dendrobium* species. Five to eight fungal strains stimulated seed germination, whereas one to four fungal isolates significantly promoted protocorm development in the three species. To evaluate effects on leafy seedling growth, seedlings obtained from asymbiotic culture were cultured with nine fungal isolates. *D. officinale* and *D. okinawense* showed specificity for a single Serendipitaceae or Tulasnellaceae isolate, whereas *D. moniliforme* exhibited specificity for three isolates of Serendipitaceae and Tulasnellaceae. Therefore, the three *Dendrobium* species had a growth bottleneck from seed germination to the protocorm stage, and mycorrhizal specificity of protocorm growth and seedling development in vitro varied among the species. Our findings imply divergent mycorrhizal specificity in *Dendrobium* species at early developmental stages. This study provides insights into the diversity of orchid mycorrhizal specificity, as well as valuable information for conservation of endangered orchids.

Keywords: fungal specificity; orchid mycorrhiza; Orchidaceae; protocorm growth; *Serendipita*; symbiotic culture; *Tulasnella*

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1. Introduction

Orchidaceae is one of the largest plant families with >28,000 species [1]. All orchids need mycorrhizal fungi to provide nutrients for seed germination and early seedling development because orchid seeds are tiny, lack endosperm, and have minimal nutrient reserves [2]. Most orchids establish photosynthesis after leafing but remain reliant on mycorrhizal fungi for nutrients [3]. Most orchids form associations with three basidiomycete families: Tulasnellaceae, Serendipitaceae, and Ceratobasidiaceae [4]. Mycorrhizal specificity, i.e., the range of fungi allowing mycorrhizal partnerships, differs among orchid species from low (generalist; many mycorrhizal fungal taxa) to high (specialist; few taxa) [5]. The mycorrhizal specificity of orchids ontogenetically changes from juvenile to

adult [6]. Although mycorrhizal specificity is diverse in adult-stage orchids [7], in early developmental stages, it is unclear. Orchid seeds germinate and continue growing in the presence of suitable mycorrhizal fungi; therefore, fungi that promote seed germination and seedling growth mediate orchid establishment and survival [8]. Clarifying the mycorrhizal association at early developmental stages would provide insight into the diversity of orchid mycorrhizal specificity and elucidate orchid distributions. In addition, many orchid species are endangered due to habitat destruction and over-exploitation [9]. Understanding the mycorrhizal fungi involved in seed germination and seedling growth can provide valuable information for orchid conservation.

During orchid germination, the swollen embryo develops into a unique globular structure called the protocorm, which in turn develops into plantlets with leaves and roots (seedlings) in green orchids [10]. A variety of fungi can stimulate seed germination, but few provide sufficient nutrients for protocorm development [11]. This imposes a growth bottleneck between seed germination and the protocorm stage. Orchid-seed germination is dependent on mycorrhizal fungi for carbon resources [2]. However, after leaves develop, plants obtain carbon by photosynthesis, but depend on mycorrhizal fungi for other nutrients such as minerals and moisture from soil [6]. Because plants' physiological needs differ from the protocorm to the leafy seedling stages, the fungi required for development may also change [6]. Thus, orchid mycorrhizal specificity during early developmental stages needs to be evaluated individually at the seed germination, protocorm growth, and leafy seedling development stages. In vitro seed germination tests, i.e., pure cultures of sterilized seeds and a fungal isolate, are used to identify fungal taxa linked to seed germination and protocorm growth [12]. Furthermore, inoculation of a fungal isolate on an asymbiotic leafy seedling enables identification of fungal taxa that promote seedling development. Although in vitro seed germination tests have been applied to a variety of orchid species [13,14], few studies have evaluated leafy seedling development [15].

The genus *Dendrobium*, one of the most species-rich genera of vascular plants [16], comprises 1826 species of epiphytic orchids distributed across Asia and Australia [17,18]. *Dendrobium* species have high medicinal and ornamental value [19,20], and some are extremely endangered [21]. Mycorrhizal specificity varies among adult individuals of *Dendrobium* species; populations of *D. fimbriatum* are associated with a single fungal group, and those of *D. officinale* with four to five fungal groups [22]. Eight coexisting *Dendrobium* species showed distinct mycorrhizal communities [23]. However, the extent of the differences in mycorrhizal specificity among *Dendrobium* species at early developmental stages is unclear. Although numerous studies have evaluated strains that promote *Dendrobium* seed germination [24,25], most examined a single *Dendrobium* species [21,26]; few evaluated the mycorrhizal specificity of multiple *Dendrobium* species [27]. Because *Dendrobium* is a highly diversified plant genus, comparison with closely related species will provide insight into the diversification of mycorrhizal specificity during plant speciation.

In this study, we evaluated mycorrhizal specificity during in vitro seed germination and advanced seedling development in three *Dendrobium* species (*D. officinale*, *D. okinawense*, and *D. moniliforme*) native to Japan. *D. officinale* is distributed from southern China to southern Japan, mostly in subtropical regions [18]. *D. moniliforme* is widely distributed from the Himalayas to Indochina, and from southern China to Japan; it is mostly found in warm temperate regions [18]. By contrast, *D. okinawense* is distributed only in Okinawa and Taiwan Islands, in subtropical regions [28]. Takamiya et al. (2014) demonstrated that these three species belong to the *Dendrobium* section *Dendrobium*, and that *D. okinawense* and *D. moniliforme* are sister taxa; *D. officinale* are closely related to these two taxa [29]. This allows investigation of mycorrhizal specificity among closely related species. Adult plants of *D. okinawense* have a highly specific association with a single Tulasnellaceae group [28], whereas those of *D. officinale* are associated with multiple Tulasnellaceae and Serendipitaceae groups [30]. Because mycorrhizal specificity at the adult stage differs between the two *Dendrobium* species, their specificity at early developmental stages may also differ.

In this study, to investigate whether mycorrhizal specificity at early developmental stages differs among *D. officinale*, *D. okinawense*, and *D. moniliforme*, seeds of the three species were cultured with nine fungal isolates obtained from these three species. Furthermore, to evaluate the effect of fungal isolates on seedling growth after leafing, seedlings from asymbiotic culture were cultured with nine fungal isolates. We examined compatible fungal isolates during three early developmental stages—seed germination, protocorm growth and seedling development—and compared in vitro mycorrhizal specificity among the three *Dendrobium* species.

2. Materials and Method

2.1. Sample Collection

Roots of *D. officinale*, *D. okinawense*, and *D. moniliforme* were collected from natural habitats or cultivated plants in Japan. We sampled three individuals from two wild populations of *D. officinale* in Kagoshima Prefecture, and one wild individual of *D. okinawense* in Okinawa Prefecture. Roots of *D. moniliforme* were collected from one individual from a wild population in Kagoshima Prefecture and one cultivated individual from Shizuoka Prefecture. Samples were stored in polyethylene bags mixed with humid moss/paper with labels and transferred to the laboratory for fungal isolation.

Seeds of *D. officinale* were obtained from three mature capsules from three individuals in Kagoshima Prefecture. Seeds of *D. okinawense* were collected from two mature capsules from one individual cultivated in a greenhouse at Okinawa Churashima Foundation. Seeds of *D. moniliforme* were obtained from three mature capsules from three individuals cultivated in a private garden in Shizuoka Prefecture. Capsules of the same species were mixed and used for symbiotic germination testing.

2.2. Fungal Isolation and Molecular Identification

Collected roots were washed with tap water, and mycorrhizal colonization was evaluated by microscopy using hand sections of roots. Colonized cortex layers of mycorrhizal roots were excised under a stereomicroscope, rinsed three times with sterile water, and cut open under sterile water to release the fungal pelotons. Sterile water mixed with pelotons was dropped onto water agar medium with 50 ppm each of streptomycin and tetracycline. Plates were incubated at 25 ± 1 °C in darkness for 3 days. Fungal colonies from actively growing isolates were subcultivated onto a potato dextrose agar (PDA) dish for purification.

DNA was extracted from the fungal isolates following the method of Izumitsu et al. (2012) [31]. Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA were amplified using the primer pairs ITS5/TW13. PCR amplification and sequencing were carried out as described by Rammitsu et al. (2021) [28]. PCR products were analyzed using the BigDye v. 3.1 terminator system [32] and 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. ITS sequences were analyzed by BLAST search against the GenBank sequence database to find the closest matching sequence. The ITS sequences of fungal isolates were submitted to the DNA Data Bank of Japan (DDBJ) (Table 1). All ITS sequences were assigned to operational taxonomic units (OTUs) defined by 97% sequence similarity. A total of nine isolates (including seven OTUs) were obtained (Table 1). To compare the effects of different isolates within the same OTU, two isolates were used for SE1 and CE18. SE1A and CE18A have 99.0% and 99.4% sequence similarity with SE1B and CE18B, respectively. FU1 showed 97% sequence similarity with *Fusarium oxysporum* (MT447552), which forms fungal coils in *Dendrobium candidum* root cells [33]. The isolates were deposited in the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC) (Table 1).

Table 1. Fungal isolates from *Dendrobium officinale*, *D. okinawense*, and *D. moniliforme* used for symbiotic culture.

Fungal Genus	Fungal Isolates	Isolate ID	Host Plants	NCBI Accession No.	NBRC Accession No.
Tulasnellaceae	TU11	F833	*D. okinawense*	LC683207	NBRC 115273
	TU22	F761	*D. moniliforme*	LC683208	NBRC 115545
	TU27	F763	*D. officinale*	LC683202	NBRC 115262
Serendipitaceae	SE1A	F358	*D. officinale*	LC683209	NBRC 114327
	SE1B	F809	*D. officinale*	LC683203	NBRC 115270
	SE2	F444	*D. moniliforme*	LC683210	NBRC 115521
Ceratobasidiaceae	CE18A	F356	*D. officinale*	LC597346	NBRC 114326
	CE18B	F828	*D. okinawense*	LC683211	NBRC 115272
Fusarium	FU1	F830	*D. okinawense*	LC683212	NBRC 115531

2.3. In Vitro Symbiotic Germination

Mature capsules were sterilized using 75% ethanol and dried for 1 week using silica gel desiccant until nearly ruptured. Only seeds from nearly dehisced capsules were collected, to ensure that they were mature, and stored at 5°C until use. Prior to use, the seeds were evaluated using the 2,3,5-triphenyl tetrazolium chloride (TTC) test to ensure high viability (>90%). A PDA medium in a 9-cm-diameter Petri dish was inoculated with a 5 × 5 mm agar cube of fungal inoculum in the middle of the Petri dish as a pre-culture and incubated in darkness at 25 ± 1 °C for 7 days. Seeds were sterilized for 3 min with 1% sodium hypochlorite solution, and about 100 seeds were sown onto the surface of an oatmeal agar medium (OMA; 2.5 g/L oatmeal and 15 g/L agar) in Petri dishes and incubated at 25 ± 1 °C for 1 week to check for contamination. Next, 1 × 1 cm discs were cut off (5–10 seeds per disc) and transplanted to a new OMA medium. A total of 20 seeds on 2–4 discs were placed on the OMA medium. Discs were placed at the same distance from the center, so that the seeds contacted the mycelium elongating from the center at the same time. Five replicates (100 seeds) were used for each fungal treatment. Petri dishes without a fungal inoculum were the control. A 6 mm plug of fungal culture was inoculated at the center of the Petri dish with the OMA medium and incubated under a 12/12 h light/dark photoperiod at 25 ± 1 °C.

After 3 months of incubation, the number of seeds was counted under a stereomicroscope. Seed development was classified as follows: stage, 0 no germination (Figure 1a); stage 1, an enlarged embryo with ruptured seed coat (Figure 1b); stage 2, a globular embryo (protocorm) with rhizoids (Figure 1c); stage 3, a protocorm with an apical meristem (Figure 1d); stage 4, emergence of the first leaf (seedling formation) (Figure 1e); and stage 5, a seedling with a second leaf and further development (Figure 1f). The seed germination (%) per stage was calculated using the following formula: Percentage seed germination = (number of seeds per germination stage ÷ total number of viable seeds) × 100. The germination stage corresponded to stage 2 and above. The standardized growth index (GI) modified from Spoerl (1948) [34] was calculated as follows: GI = (N1 + N2 × 2 + N3 × 3 + N4 × 4 + N5 × 5) ÷ (N0 + N1 +N2 + N3 + N4 + N5), where N0 is the number of seeds at stage 0, N1 is the number at stage 1, etc. [35]. The GI can range from 0 (no seeds germinated) to 5 (all seeds reached the seedling stage).

2.4. In Vitro Symbiotic Culture of Seedlings

Seedlings after leafing obtained from asymbiotic culture were cultured with the nine fungal isolates. Seeds were sterilized with 1% sodium hypochlorite solution for 3 min and transferred to a new Dogashima medium (NDM) [36]. Seedlings at stage 5 were used for symbiotic culture. Fungal isolates were pre-cultured on PDA in darkness for 10 days. Four seedlings and one colonized agar plug (diameter = 6 mm) from the edge of the fungal colony were transferred to a modified oatmeal agar medium (ONY; 0.38 g/L NH_4NO_3, 0.2 g/L KH_2PO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KCl, 0.1 g/L yeast extract, 2.5 g/L oatmeal, and 15 g/L agar). Each glass bottle (450 mL) contained 50 mL of ONY medium, and five

glass bottles were used per treatment. The control lacked a fungal isolate. The cultures were grown at 25 ± 1 °C under a 12 h/12 h light/dark photoperiod. After 3 months, the plant height, the longest root length, and the number of roots, leaves, and tillers were measured per plant. Seedlings were dried at 50°C for 48 h for fresh and dry weight measurements [37].

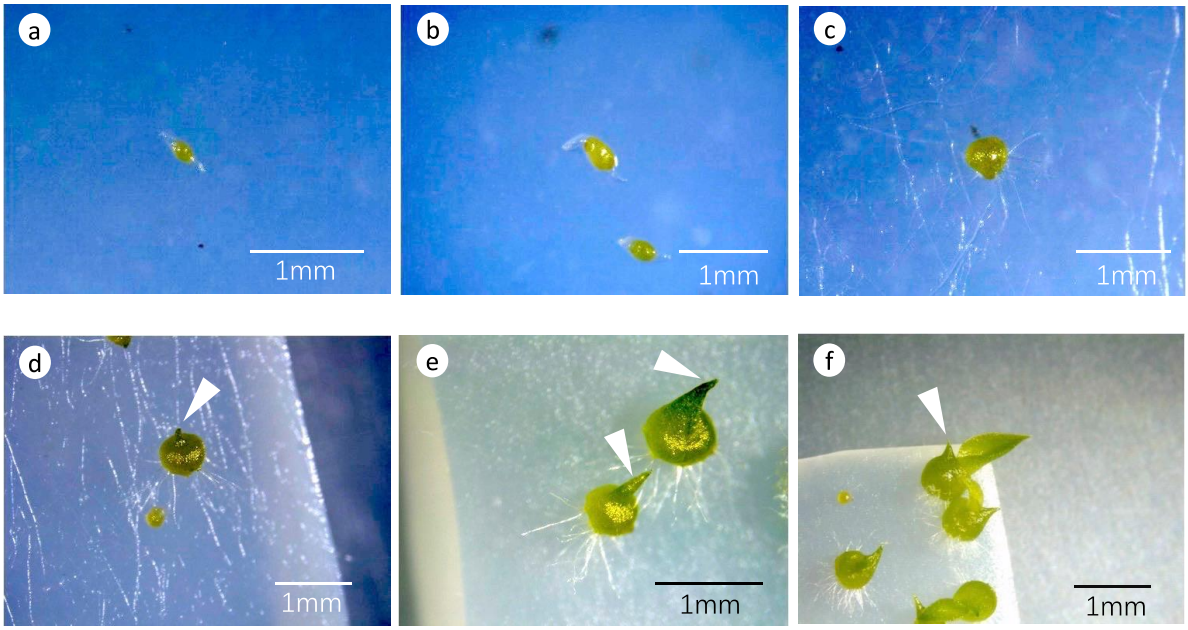

Figure 1. The six developmental stages of germinating *Dendrobium officinale* seeds. (**a**) Stage 0: no germination; (**b**) stage 1: an enlarged embryo with ruptured seed coat; (**c**) stage 2: a globular embryo (protocorm) with rhizoids; (**d**) stage 3: a protocorm with an apical meristem (arrow); (**e**) stage 4: emergence of the first leaf (arrow) (seedling formation); and (**f**) stage 5: a seedling with a second leaf (arrow) and further development.

2.5. Statistical Analysis

The effects of fungal isolates on seed germination and seedling development (GI, fresh and dry weight, plant height, root number, longest root, leaf number and tiller number) were statistically compared within each *Dendrobium* species using SPSS Statistics 27.0.1 (IBM Corp., Armonk, NY, USA) software. For seed germination testing, each treatment was replicated on five plates. Twenty plants per treatment were used for seedling growth testing. The data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test ($p < 0.05$).

3. Results

3.1. Effects of Fungal Strains on Symbiotic Germination

Seeds of *D. officinale*, *D. okinawense*, and *D. moniliforme* were cultured symbiotically with nine fungal isolates (three Tulasnellaceae, three Serendipitaceae, two Ceratobasidiaceae, and one *Fusarium*) (Table 1). Several OTU isolates promoted germination (Figure 2a), but fewer significantly increased the GI value in all three *Dendrobium* species (Figure 2b). For *D. officinale*, the TU22, TU27, SE1A, SE1B, and SE2 isolates promoted seed germination (Figure 2a), four of which significantly increased the GI value (Figure 2b). Seed germination of *D. okinawense* was significantly promoted by all isolates except for FU1 (Figure 2a), but only isolate SE2 significantly increased the GI value (Figure 2b). In *D. moniliforme*, the TU22, TU27, SE1B, SE2, and CE18A isolates promoted seed germination (Figure 2a), two of which (TU22 and SE2) had high GI values (Figure 2b).

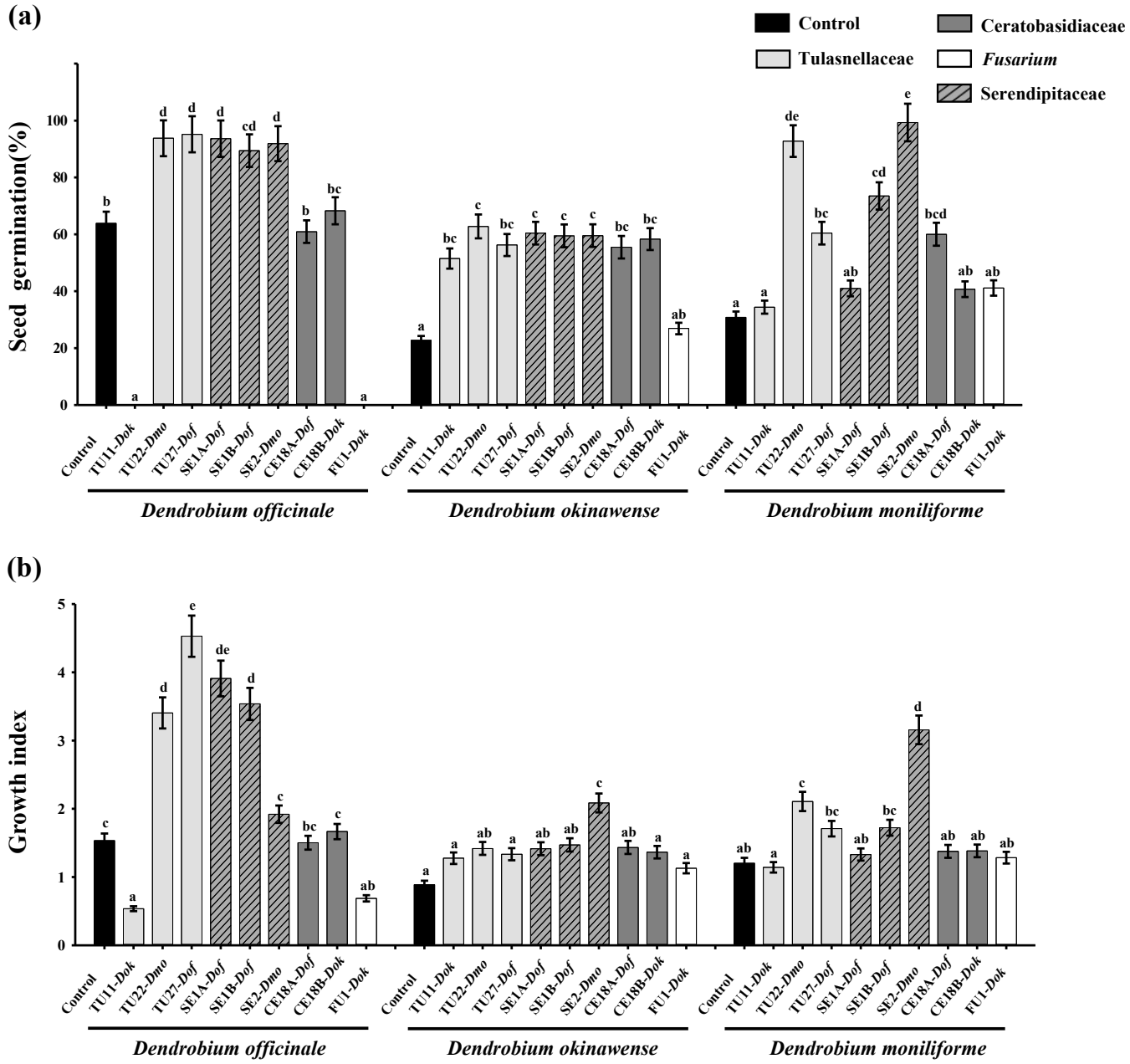

Figure 2. (**a**) Seed germination (%) and (**b**) seedling growth index (GI) of fungal isolates on *Dendrobium officinale* (*Dof*), *D. okinawense* (*Dok*) and *D. moniliforme* (*Dmo*) at 3 months after symbiotic culture. The host orchid is indicated after the fungal isolate name. Data are means of five replicates; bars indicate standard errors. Different letters denote significant differences according to the Tukey-Kramer test at the $p < 0.05$ level.

In *D. officinale* and *D. moniliforme*, the highest GI values resulted from the fungal isolates from their own roots (Figure 2b), whereas the isolate with the highest GI in *D. okinawense*, SE2, was isolated from *D. moniliforme*. In *D. officinale*, among four isolates with a high GI value (TU22, TU27, SE1A, and SE1B), three (TU27, SE1A, and SE1B) were from *D. officinale*; the isolate with the highest GI was TU27. *D. moniliforme* had two isolates with high GI values (TU22 and SE2), both of which were isolated from *D. moniliforme*. The effects of fungal isolates within the same OTU were compared between SE1 and CE18. Isolates within the same OTU showed similar effects, except that SE1B promoted seed germination of *D. moniliforme* more so than SE1A (Figure 2a). FU1 showed no positive effect in any of the *Dendrobium* species. In the control, no development beyond stage 2 was observed in any of the three *Dendrobium* species.

3.2. Effects of Fungal Strains on Symbiotic Cultures of Seedling

Seedling growth of *D. officinale*, *D. okinawense*, and *D. moniliforme* seedlings differed according to fungal strain (Figure 3 and Supplementary Figure S1). In *D. officinale*, seedlings inoculated with SE1B showed the highest fresh and dry weights with a significant difference in fresh weight seen compared with the other types of seedlings (Figure 3a,b). The number of roots was greatest after TU22 inoculation (Figure 3d). For *D. okinawense*, TU11 inoculation significantly increased the fresh weight and plant height and resulted in the highest dry weight (Figure 3a–c). Seedlings cultured with SE2 had the largest number of roots (Figure 3d). In *D. moniliforme*, TU22 significantly increased fresh weight, dry weight, plant height, and root number (Figure 3). TU27 and SE2 also increased seedling fresh weight, plant height, and leaf number (Figure 3a,c and Supplementary Figure S1).

Fungal isolates with an increased fresh/dry weight varied in whether they promoted above-or belowground growth. In *D. okinawense*, TU11, which showed the highest fresh/dry weight, markedly increased plant height but other indices were not promoted (Figure 3 and Supplementary Figure S1). In *D. moniliforme*, TU22 and TU27 significantly promoted all indices, including plant height, root number, longest roots, leaf number, and tiller number. On the other hand, SE1B, which induced the highest fresh/dry weight, showed no promotive effects on any of the other indices in *D. officinale*.

The most effective fungal isolates for *D. officinale*, *D. okinawense*, and *D. moniliforme* originated from their own roots. SE1B isolated from *D. officinale* increased the fresh weight of *D. officinale* (Figure 3a). TU11 from *D. okinawense* had the greatest effect on the fresh weight and plant height of *D. okinawense* (Figure 3a,c). Two fungal strains, TU22 and SE2, from *D. moniliforme* significantly increased the fresh weight of *D. moniliforme* (Figure 3a). Fungal isolates within the same OTU had similar growth-promoting effects, except that SE1B exerted a greater effect on *D. officinale* growth than SE1A (Figure 3). Also, CE18 and FU1 showed no positive effect, and sometimes a negative effect, on seedling development. The inoculation of CE18A caused seedling death in *D. okinawense* and *D. moniliforme*.

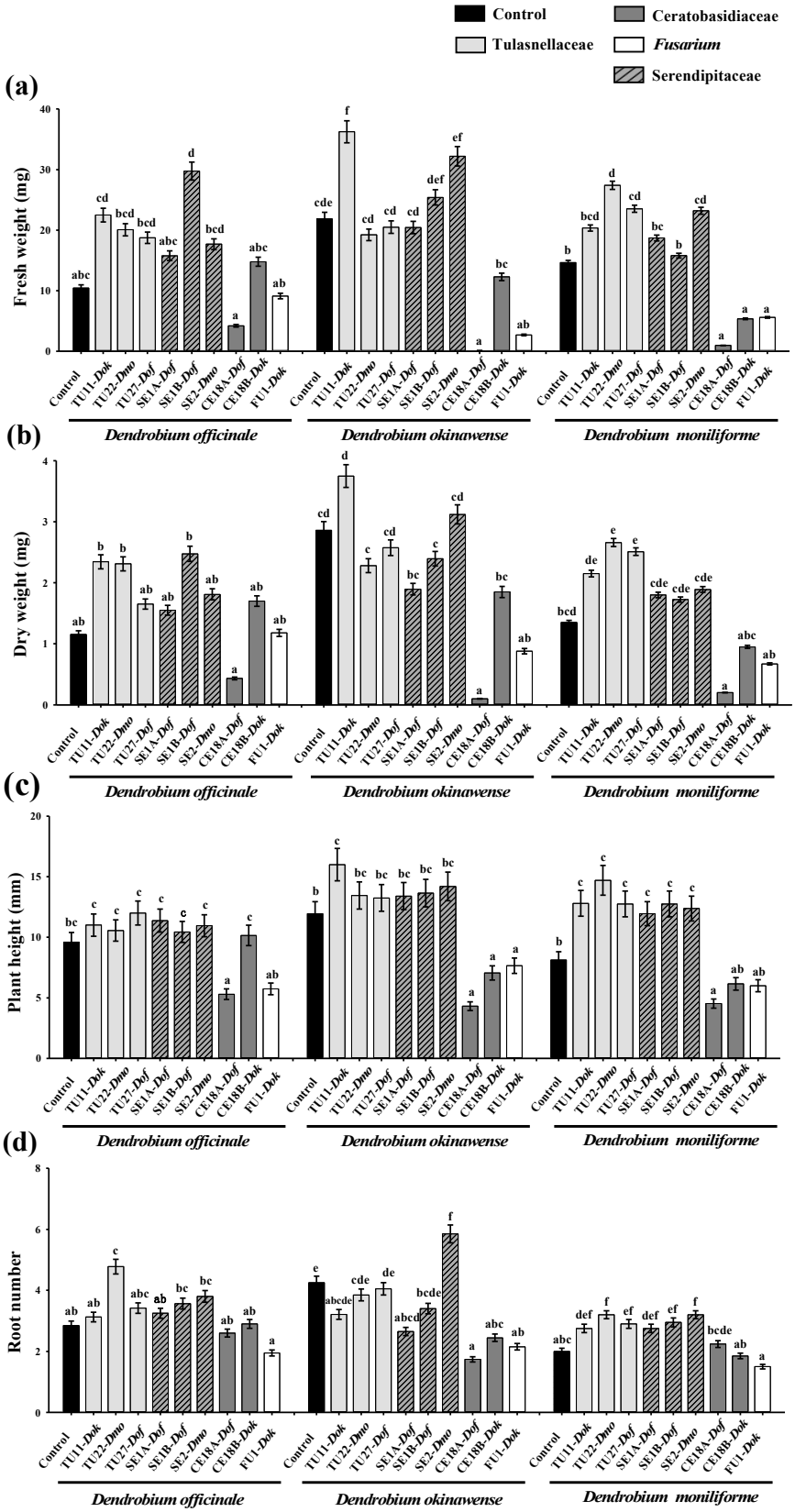

Figure 3. Effect of fungal isolates on the growth of asymbiotic cultured seedlings of *Dendrobium officinale* (*Dof*), *D. okinawense* (*Dok*), and *D. moniliforme* (*Dmo*) at three months after symbiotic culture. Fresh weight (**a**) and dry weight (**b**), plant height (**c**), and number of roots (**d**). Host orchid is indicated after the fungal isolate name. Bars show standard errors (n = 20 plants per treatment). Mean values with different letters are significantly different at $p < 0.05$ (Tukey-Kramer test).

4. Discussion

Three *Dendrobium* species showed a growth bottleneck during seed germination, and the fungal isolates that affected protocorm growth and seedling development varied among them (Figure 4). A variety of fungal isolates stimulated seed germination, but few promoted protocorm and seedling development (Figure 4). Five isolates (TU22, TU27, SE1A, SE1B, and SE2) contributed to *D. officinale* seed germination, four of which (TU22, TU27, SE1A, and SE1B) increased the GI value (Figure 2). However, only SE1B significantly increased seedling fresh weight (Figure 3a). Decreasing fungal diversity during development was found in *D. moniliforme*: five isolates (TU22, TU27, SE1B, SE2, and CE18A) promoted seed germination, whereas two (TU22, and SE2) and three (TU22, TU27, and SE2) isolates promoted protocorm growth and seedling development, respectively (Figures 2–4). The largest bottleneck was in *D. okinawense*: eight fungi (TU11, TU22, TU27, SE1A, SE1B, SE2, CE18A, CE18B) promoted seed germination, but only one (SE2 or TU11) accelerated protocorm and seedling development (Figures 2–4).

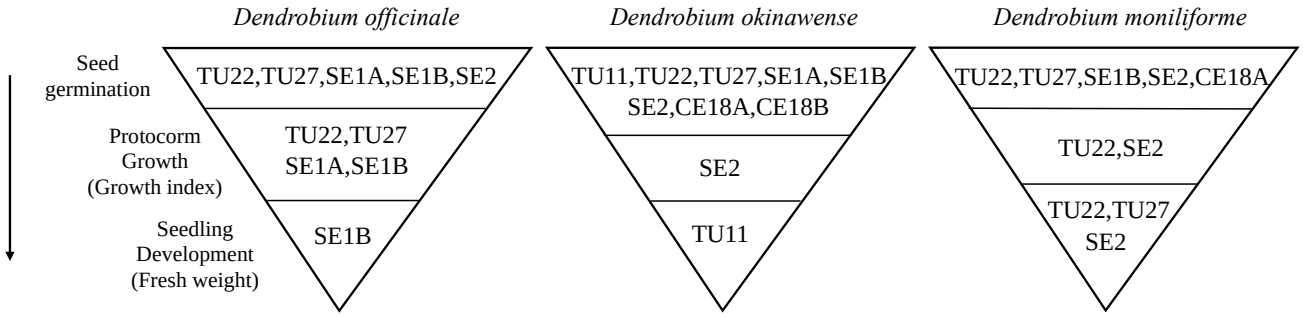

Figure 4. Fungal strains that affect early developmental stages (seed germination, protocorm growth, and seedling development) of *Dendrobium officinale*, *D. okinawense*, and *D. moniliforme*. Fungal isolates that significantly increased seed germination (%) and growth index (GI) values are classified as fungi promoting seed germination and protocorm growth, respectively. For the seedling developmental stage, fungal isolate(s) that significantly increased fresh weight are shown.

A similar bottleneck was found in previous studies on *Dendrobium* species. Only two of seven *Tulasnella* isolates promoted seedling development in *D. moniliforme* [11]. Two of six fungal strains, Serendipitaceae and *Tulasnella calospora*, promoted rapid seed germination up to the seedling stage in *D. officinale* [25]. Similar bottlenecks have been reported in *D. aphyllum* [21], *D. devonianum* [26], *D. exile* [24], and *D. huoshanense* [38]. Therefore, bottlenecking may be common among *Dendrobium* species. A taxonomic bottleneck during seed germination has been documented in other orchids, such as *Cephalanthera* [39], *Bletilla striata* [40], and *Cyrtopodium glutiniferum* [41]. Because orchid seeds have scant nutrient reserves, orchid-seed germination depends on mycorrhizal fungi for mineral and carbon resources [2]. A variety of fungi can stimulate seed germination, but few provide sufficient nutrients for subsequent protocorm growth, resulting in a major bottleneck. Stimulation of seed germination by diverse fungi may increase plant fitness. Swelling of an embryo and the development of rhizoids expand the surface area, resulting in a higher probability of encountering a growth-promoting fungus.

The same fungal OTUs contributed to *D. officinale* and *D. moniliforme* development at three developmental phases, whereas the fungal strain shifted from SE2 to TU11 during seedling development in *D. okinawense* (Figure 4). In *D. officinale*, SE1B supports plant growth from seed germination to seedling development. This fungal isolate shared 97% ITS sequence similarity to the fungal isolate *Thanatephorus* sp. SSCDO-8 (MH348617), which reportedly promotes seed germination, protocorm growth, and seedling development in *D. officinale* [42]. In *D. moniliforme*, TU22 and SE2 showed positive effects in all three developmental phases (Figure 4). The fungal OTU, SE1, is a dominant fungal partner for adult *D. officinale* individuals in wild populations [30], and associations with fungal strains

that contribute to growth in the early developmental stage may remain throughout the orchid life cycle. In *D. okinawense*, the effective fungal strain shifted from SE2 to TU11 during protocorm and seedling development (Figure 4). Eleven mature plants of *D. okinawense* from natural habitats were predominantly associated with a single Tulasnellaceae OTU, TU11 [28]. Therefore, the association of *D. okinawense* with TU11 continues to the adult stage. Switching of mycorrhizal fungi between juvenile to adult plants has been reported in other orchid species, such as *Oeceoclades maculata* [43]. Orchid-seed germination is dependent on mycorrhizal fungi for carbon resources, but plants obtain carbon by photosynthesis after leaf development [6]. Because plant physiological needs differ from the juvenile to adult stage, the fungi required for development may also change. In *D. okinawense*, SE2 likely contributes to carbon supply during early developmental stages, whereas TU11 provides the nutrients needed for post-leafing seedling development. Orchids with fungal switching may have a higher risk of mortality than non-switching orchids because they encounter other compatible fungi during growth. *D. okinawense* is distributed only on the islands of Okinawa and Taiwan. In addition, adult individuals of *D. okinawense* have a highly specific association with TU11 [28]. Fungal switching and narrow specificity might explain the rarity of *D. okinawense*.

Our findings imply that *D. okinawense* and *D. officinale*, adult plants which differ in mycorrhizal specificity, also differ in specificity at early developmental stages. At the adult stage, *D. okinawense* shows high mycorrhizal specificity toward TU11 [28], whereas *D. officinale* is associated with multiple Tulasnellaceae and Serendipitaceae fungi [30]. In this study, *D. okinawense* showed higher specificity in vitro than *D. officinale* at early developmental stages. Only one fungal isolate significantly promoted protocorm growth in *D. okinawense*, compared to four in *D. officinale* (Figure 4). *Dendrobium* species with high mycorrhizal specificity at the mature stage could also have high specificity at juvenile stages. The effective fungal isolate at the seedling development stage differed between *D. officinale* and *D. okinawense* (Figure 4). SE1 and TU11 were the dominant fungal partners for adult plants of *D. officinale* and *D. okinawense*, respectively [28,30]. Fungal strains that promote the development of leafy seedlings may also dominate during adult stages.

The results of our symbiotic seedling culture imply that an increase in above- or below-ground biomass is different among fungal species. In *D. okinawense*, TU11 promoted only plant height, while TU22 and TU27 facilitated all indices in *D. moniliforme* (Figure 3 and Supplementary Figure S1). The lack of increase in any indices except fresh/dry weight caused by the inoculation of *D. officinale* with SE1B may be due to an increase in other indices that were not examined in this study, such as root or stem thickness. An increase in aboveground biomass may increase photosynthesis while an increase in belowground biomass may expand nutrient acquisition from the roots and/or mycorrhizal associations. It is possible that fungal species mediate above- and belowground biomass ratios; further study is required to resolve this issue.

Under control conditions, seeds of the three *Dendrobium* species showed no seedling development, but they reached stage 2 on poor nutrient medium. The enlarged embryos quickly turned green, suggesting that nutrient acquisition through photosynthesis may have allowed them to develop to stage 2. Seed germination in *D. devonianum* was significantly delayed under dark compared to light conditions [26]. These results indicate that photosynthesis contributes to the initial germination of *Dendrobium*. Inoculation with Ceratobasidiaceae and *Fusarium* fungi often inhibited growth more than that with controls. Because these are also known plant pathogens [44,45], they may behave in a parasitic way during the juvenile stage in *Dendrobium* species.

Mycorrhizal specificity at early developmental stages in vitro differs among the three closely related *Dendrobium* species analyzed in this study. Divergence of mycorrhizal specificity at the adult stage has been observed in *Dendrobium* species, ranging from dominance by a single fungus to diverse mycobionts [22,23,28,30]. Our results imply diversity of mycorrhizal specificity during seed germination and seedling development in *Dendrobium* species. *D. okinawense* and *D. moniliforme* are sister taxa within the section

Dendrobium [29], but their mycorrhizal specificity differed markedly. At the seedling development stage, *D. okinawense* had high specificity for TU11, and *D. moniliforme* for TU22, TU27, and SE2 (Figure 4). Therefore, the mycorrhizal specificity of the genus *Dendrobium* became highly diversified during speciation. Distinct mycorrhizal communities might contribute to plant niche differentiation and reduce species competition among orchids [46]. The genus *Dendrobium* comprises 1826 species [17] and is one of the most species-rich genera of vascular plants [16]. Highly divergent mycorrhizal specificity could contribute to this extreme diversification of the genus *Dendrobium*. Our study elucidates the diversity of orchid mycorrhizal specificities and provides valuable information for conservation of endangered *Dendrobium* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121119/s1, Figure S1: Effects of fungal isolates on the seedling growth of *Dendrobium officinale*, *D. okinawense*, and *D. moniliforme* at three months after symbiotic culture. Longest root (a), leaf number (b), and tiller number (c).

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