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The Diversity and Growth-Promoting Potential of the Endophytic Fungi of *Neuwiedia singapureana* (Orchidaceae) in China

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Abstract: *Neuwiedia singapureana* is a rare and endangered plant of the Apostasioideae subfamily. The Apostasioideae subfamily has a unique evolutionary status, as it is considered to be the most primitive group forming the base of the Orchidaceae evolutionary tree. Therefore, *N. singapureana* has high scientific research and conservation value. The endophytic fungal communities associated with orchids are rich and diverse, but few studies have investigated the endophytic fungi of *Neuwiedia* orchid plants. In the present study, the aim was to examine the endophytic fungal community structures associated with wild *N. singapureana* rhizomes and normal roots in the ground and with bare prop roots in the air at two sampling sites in China. High-throughput sequencing of nuclear ribosomal DNA fragments of the internal transcribed spacer regions was conducted, and cultivable methods were adopted. A total of 2161 endophytic fungal operational taxonomic units (OTUs) were obtained at a 97% sequence similarity threshold. The endophytic fungal diversity differed among the samples but not significantly. There were many more non-mycorrhizal endophytic fungal than orchid mycorrhizal (OM) fungal species detected in the *N. singapureana* orchid, about 98.33% OTUs of non-mycorrhizal fungi contrasting with 1.67% OTUs of potential orchid mycorrhizal fungi, among which Ceratobasidiaceae, Russulaceae, and Thelephoraceae were the dominant orchid mycorrhizal fungi. One culturable OM fungal *Epulorhiza* sp. isolated from the rhizome was capable of significantly promoting the seed germination and seedling growth of *Dendrobium officinale* and *Epidendrum secundum* orchids, respectively, with different efficiencies. These endophytic fungal strains with growth-promoting functions will provide materials for orchid conservation and for the study of the mechanisms underlying orchid symbiotic associations.

Keywords: orchid; mycorrhizal; rhizome; *Epulorhiza*; *Dendrobium*; germination

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1. Introduction

In nature, most of Orchidaceae plants depend on mycorrhizal fungi from the seed germination and development stages to the adult stage, which is accompanied by flowering and fruit bearing. Furthermore, as one of the most important ecological factors, orchid mycorrhizal fungi (OMF) have been increasingly recognized to affect orchid distribution. The OMF mainly belong to eight fungal clades: Ceratobasidiaceae, Tulasnellaceae, Sebacinales, Russulaceae, Thelephoraceae, Cortinariaceae, Serendipitaceae, and Tricholomataceae [1–4]. The availability of appropriate fungi may be correlated with host selection. Consequently, screening symbiotic fungi that effectively promote the germination and growth of Orchidaceae plants is vital for understanding the orchid–mycorrhizal fungi symbiotic mechanism. Many non-standard mycorrhizal fungi can also be detected and isolated from adult roots, and these are termed root-associated endophytic fungi [5]. Endophytic fungi have been found in almost all plant groups and are described as fungi that live in living plants at

certain stages of the life cycle without causing obvious disease symptoms [6–8]. Endophytic fungi comprise an important part of plant microecosystems. The endophytic fungi that have been reported have no obvious phylogenetic relationship with known OMF [5,9–11]. Compared with mycorrhizal fungi, the diversity of non-mycorrhizal endophytic fungi in orchids roots has been found to be more abundant [6,11–13]. Most of these endophytic fungi belong to Ascomycota and Basidiomycota [11,14].

Endophytic fungi have been reported in some Orchidaceae genera such as *Dendrobium* [15], *Doritis* [16], *Anoectochilus*, *Bletilla*, *Cleisostoma*, *Coelogyne*, *Eria*, *Gastrochilus*, *Gymnadenia,* and *Vanda* [17,18]. Among the reported endophytic fungi isolated from Orchidaceae, *Fusarium*, *Xylaria*, *Alternaria*, *Phoma,* and *Colletotrichum,* amongst others, were prevalent and dominant genera, and some were potentially promising beneficial fungi [15]. Some common Orchidaceae endophytic fungi can also infect the roots of orchid seedlings and form hyphal groups resembling the typical structures of OMF in the root cortex cells; these infected seedlings have been shown to grow well. Examples of this can be seen when *Cladosporium* is incubated with *Dendrobium officinale* and *Bletilla* [19], or when *Fusarium oxysporum* is incubated with *Bletilla* [20]. Some of these endophytic fungi may have positive effects on germination or seedling growth, as has been described for *Trichoderma* [15,21,22], *Clonostachys* [15], *Fusarium* [20,23], and *Mycena* [24] species. Given that, the use of non-OMF for their mycorrhizal-associated potential should be advocated [25].

Neuwiedia singapureana is a rare and endangered plant of the Apostasioideae subfamily. The Apostasioideae subfamily has a unique evolutionary status, as it is considered to be the most primitive group forming the base of the Orchidaceae evolutionary tree [26,27]. *N. singapureana* is mainly distributed in southeastern China and is also rarely distributed in Vietnam, Thailand, Malaysia, Singapore, and Indonesia in forests at approximately 500 m above sea level [28]. The *Neuwiedia* genus has attracted wide attention due to its special evolutionary position. However, the distribution area of *Neuwiedia* plants is narrow, and the individuals are rare, and thus there is a lack of related research. The limited number of previous studies has focused on flower structure, development, and pollination biology [29,30], systematic anatomy [31], classification and evolution [27,32,33], genome size [34,35], seedling morphological structure [36], and mycorrhizal fungi [37]. During the study of mycorrhizal fungi in *Neuwiedia*, Kristiansen et al. [36] found peloton-forming fungi in *Neuwiedia* orchid protocorms. The researchers then isolated *Ceratobasidium* and *Tulasnella* strains from *Neuwiedia veratrifolia* roots and cultured them [37]. Since this study, there have been few reports on the endophytic and mycorrhizal fungi associated with *Neuwiedia* plants.

Revealing the endophytic fungal communities associated with endangered orchid plants is very important for understanding how to maintain biodiversity and promote community stability. Endophytic fungal diversity, including the diversity associated with changes among different rhizome and root samples from *N. singapureana* plants from different locations, has not been adequately studied. In the present study, amplicon sequencing and culturable methods were used to study the fungal community structure and diversity in *N. singapureana* rhizomes and roots. The aim was to determine the fungal community characteristics related to the different organs and locations of the endangered *N. singapureana* plants.

2. Materials and Methods

2.1. The Habitat and Sampling of N. singapureana

Healthy populations of the terrestrial orchid, *N. singapureana,* located in the Diaoluoshan and Jianfengling National Nature Reserves of Hainan, China, were used in this study (Figures 1 and S1). There were four sample groups in total—wild *N. singapureana* rhizomes in the ground sampled from the Diaoluoshan Reserve (Neu-DF), wild *N. singapureana* rhizomes and normal roots in the ground (Neu-JF and Neu-JR), and bare prop roots in the air (Neu-JA)—sampled from the Jianfengling Reserve. All samples were collected in July 2019. Bare prop roots are those that are exposed to the air but are not deeply rooted in the

soil. In this study, while some *N. singapureana* roots were partially exposed to the air, other parts of the same roots were buried deep in the soil. Thus, the bare parts exposed to the air of prop root were samples in this study to investigate endophytic fungal diversity.

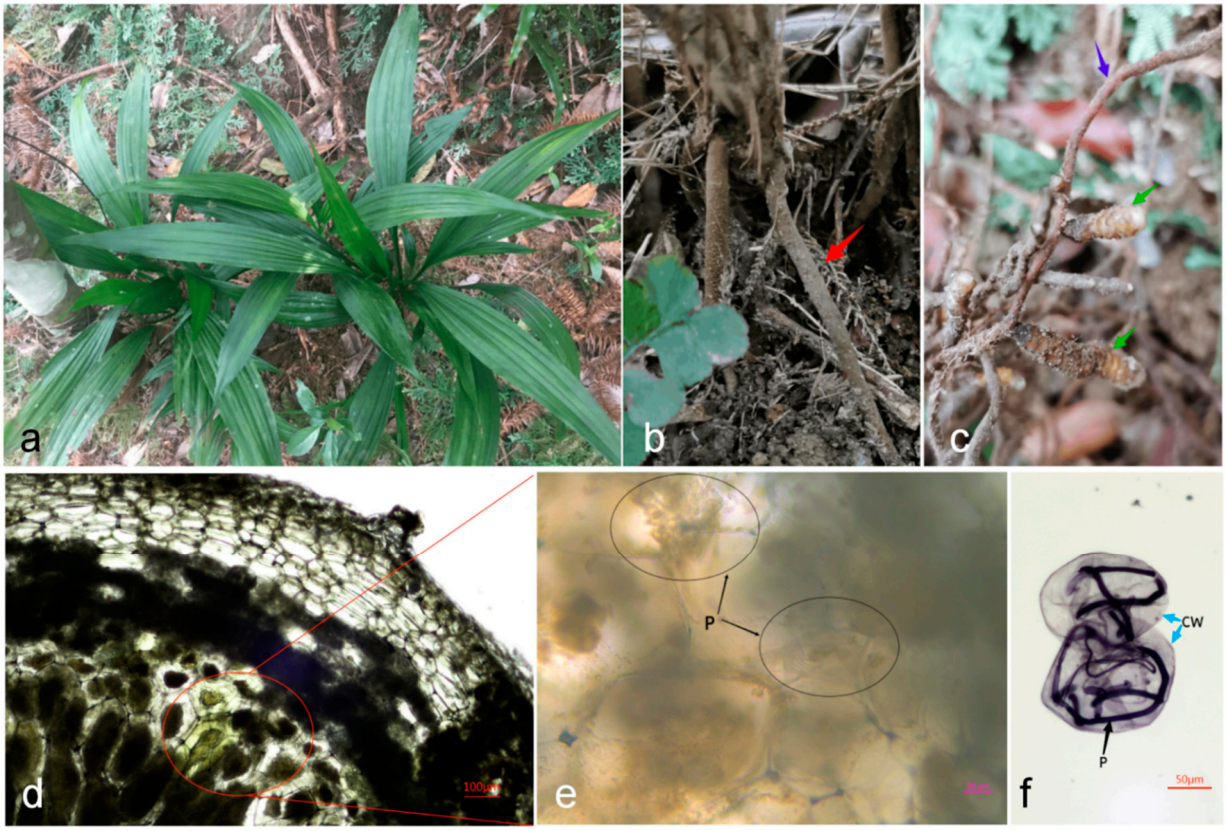

Figure 1. Tissues and microscopic observation of the colonization of endophytic fungi in the rhizome and root of the wild orchid *Neuwiedia singapureana*. The wild *N. singapureana* plants were sampled from National Nature Reserves in Hainan, China (**a**). The red arrow indicates the bare prop roots in the air (**b**), the green arrows indicate the rhizomes in the ground, and the purple arrow indicates the normal roots in the ground (**c**). The red circle indicates the location of hypha in the cortex of the root cross-section (**d**). The black arrows indicate the pelotons (P) of the intracellular fungi (**e**), the blue arrows indicate the cell wall (CW), and the black arrow indicates the intracellular fungal hypha stained with trypan blue (**f**).

Five rhizomes or roots were collected from each individual (five individuals in total each 10 m apart) and were transported to the laboratory with ice. The surface soil was then flushed from the roots by running them under water for 1 h. The roots were then surface sterilized by submersion for 30 s in 0.5% NaClO. The samples were then rinsed for 30 s three times in sterile distilled water, and mycorrhizal colonization was checked using a microscope (Leica DFC450 C, Leica Microsystems Ltd., Weztlar, Germany). Subsequently, these samples were sectioned into 1 cm fragments, frozen in liquid nitrogen, and stored at −80 °C prior to DNA extraction.

2.2. Internal Transcribed Spacer (ITS) Sequencing and Operational Taxonomic Unit (OTU) Analysis

Amplicon preparation for Illumina NovaSeq 6000 sequencing: Fungal DNA was extracted using HiPure DNA Kits according to the manufacturer's protocols (Magen, Guangzhou, China). Optical density (OD) readings were taken for the DNA samples using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). DNA samples with an OD 260/280 ratio of 1.8–2.0 and an OD 260/230 ratio of less than 2.0

and that did not produce smears on 1.5% agarose gel were selected for use for subsequent experiments. The ITS2 amplicons of the ribosomal DNA gene were amplified using the ITS3_KYO2/ITS4 primers. Purified amplicons were pooled in equimolar ratios and were paired-end sequenced (PE250) on an Illumina platform according to the standard protocols.

Quality control and OTU analysis: The raw data were filtered using FASTP v0.18.0 [38]. Paired-end clean reads were merged using FLASH v1.2.11 [39]. Noisy sequences were filtered using QIIME v1.9.1 [40] under specific filtering conditions [41] to obtain high-quality clean tags. The effective tags were clustered into OTUs using a ≥97% similarity threshold via the UPARSE v9.2.64 pipeline [42]. The tag sequence with the highest abundance was selected as the representative sequence within each cluster. Shannon and Chao diversity indexes were calculated in QIIME v1.9.1 [40]. UpSet plot analysis was performed using the UpSetR package v1.3.3 [43] to identify unique and common OTUs among the sampling groups. The representative sequences were assigned to organisms by a naive Bayesian model using RDP classifier v2.2 [44] based on the Greengene database vgg_13_5 [45]. The species abundance was plotted in R project using the pheatmap package v1.0.12 [46].

2.3. Isolation and Identification of Culturable Endophytic Fungi

The normal belowground rhizomes of 20 individual *N. singapureana* plants that were spaced 10 m apart from each other were chosen for endophytic fungal isolation in Diaoluoshan National Nature Reserve. The rhizomes were flushed with running water for 2 h. The rhizomes were then surface sterilized for 3 min in 1% NaClO and five times in sterile water in a Clean Bench. The rhizomes were cut into 1–2 mm long segments. The segments were placed in a 90 mm Petri dish containing 2% potato dextrose agar (PDA) with 50 mg/L benzylpenicillin sodium. The Petri dishes were incubated at 25 °C. When fungal colonies developed, they were transferred to new PDA medium for purification.

The purified fungal isolates were selected for further identification. DNA extraction was performed using the same procedure as described above. The amplification, sequencing, and identification were conducted according to Toju et al. [47]. The ITS region of ribosomal DNA was amplified using the primer pair ITS4 [48] and ITS1F [49]. The representative strains identified based on their morphological and molecular characteristics were transferred to PDA slants for further study.

2.4. Symbiotic Germination of Orchid Seeds and Fungal Isolates

The symbiotic germination of orchid seeds and the fungal isolates obtained from the above procedure was investigated. Mature *Dendrobium officinale* and *Epidendrum secundum* capsules were harvested from the scientific research greenhouse of Beijing Botanical Garden, China. The full sterile seed packets of *E. secundum* and *D. officinale* were surface sterilized before opening. The seeds were then cultured with endophytic fungal isolates on 10 g/L oatmeal agar (OMA) medium according to the procedures described by Xu et al. [50]. The fungal treatments and blank controls were placed in germination chambers (Beijing Botanical Garden, Beijing, China). The germinated protocorms or seedlings were transferred to new OMA medium every 2 months. The asymbiotic and symbiotic protocorms and seedling roots were surface sterilized for 3 min in 0.5% NaClO according to the above methods used for surface-sterilized rhizomes. Then, the sterilized protocorms and seedling roots were sampled for DNA extraction, ITS amplification, and sequencing; then, the ITS sequences were blast to identify whether they were the fungal isolates used in the above symbiotic germination experiment. Moreover, the mycorrhizal colonization was checked in the asymbiotic and symbiotic protocorms and seedling roots using a microscope (Leica DFC450 C, Leica Microsystems Ltd., Weztlar, Germany) and field-emission scanning electron microscope (SEM) (Hitachi SU8020, Hitachi Ltd., Tokyo, Japan).

3. Results

3.1. Endophytic Fungal Diversity

After quality filtering and chimera removal, 815,975 high-quality sequences were obtained and could be assigned to the different samples. The number of high-quality sequences per individual sample varied from 56,934 to 72,665. A total of 2161 OTUs were identified at 97% sequence similarity (Table 1), including 890 OTUs in Neu-DF, 724 OTUs in Neu-JF, 1141 OTUs in Neu-JA, and 789 OTUs in Neu-JR. The endophytic fungal OTU abundance was higher in Neu-JA than in the Neu-JR and Neu-JF groups, which were all from the same habitat at Jianfengling National Nature Reserve. Moreover, the endophytic fungal OTU abundance was higher in the rhizomes sampled from Diaoluoshan (Neu-DF) than in those from Jianfengling.

Table 1. Internal transcribed spacer sequencing data from wild *Neuwiedia singapureana* roots and rhizomes.

Sample ID	Total Clean Tags	Unique Tags	Taxon Tags	OTUs	Total OTUs
Neu-DF-1	65,930	15,807	52,159	584	
Neu-DF-2	62,061	13,741	47,490	365	890
Neu-DF-3	69,822	15,161	51,898	365	
Neu-JF-1	70,228	10,823	64,926	380	
Neu-JF-2	71,261	14,196	53,733	401	724
Neu-JF-3	71,661	15,041	45,758	465	
Neu-JA-1	72,147	22,521	54,120	790	
Neu-JA-2	62,355	21,768	47,549	795	1141
Neu-JA-3	70,931	14,580	54,053	424	
Neu-JR-1	69,980	15,857	47,969	444	
Neu-JR-2	56,934	11,866	45,044	298	789
Neu-JR-3	72,665	15,147	66,269	601	

Note: OTU, operational taxonomic unit; Neu-DF, *N. singapureana* rhizome samples from Diaoluoshan Reserve; Neu-JF, *N. singapureana* rhizome, Neu-JR, *N. singapureana* root, and Neu-JA, *N. singapureana* air-exposed root samples from Jianfengling Reserve.

The endophytic fungal community Shannon diversity (Figure 2a) and Chao richness (Figure 2b) indexes of all samples were calculated to illustrate the complexity of each sample. The endophytic fungal diversity differed among the four sample groups but not significantly ($p > 0.05$). The alpha diversity was higher in Neu-JA than in Neu-DF, Neu-JF, and Neu-JR. This result suggests that the aerial roots above the ground had greater fungal community diversity and richness than the rhizomes and normal roots belowground. Moreover, the fungal community diversity and richness were slightly higher in Neu-DF than in Neu-JF, but this difference was not significant ($p > 0.05$).

The similarities and differences in fungal communities among the different sampling groups were analyzed using an UpSet diagram (Figure 3). The diagram showed that there were large numbers of specific and common fungal OTUs among different samples. The number of specific fungal OTUs was the highest in the bare roots (Neu-JA; 703 OTUs), followed by Neu-DF (530 OTUs), Neu-JR (253 OTUs), and finally Neu-JF (196 OTUs). In total, there were 87 OTUs that were common to all four sampling groups, most of which belonged to *Penicillium* (Trichocomaceae), Nectriaceae, and Herpotrichiellaceae. The rhizomes from the two different collection sites (Neu-DF and Neu-JF) had 23 OTUs in common, with most belonging to Trichocomaceae. The Neu-JA, Neu-JR, and Neu-JF samples, which were from the same collection site, had 55 OTUs in common, with most belonging to Trichocomaceae and Herpotrichiellaceae (Table S1 and Figure 3).

The relative abundance of fungal OTUs found in the different sample groups significantly differed at various taxa levels, as shown by a heatmap (Figure 4). Eurotiomycetes was the dominant class in the Neu-DF and Neu-JA group, Tremellomycetes was the dominant class in Neu-JF, and Leotiomycetes was the dominant class in Neu-JR. Of the endophytic fungi, five families were dominant in the Neu-DF rhizomes, including Trichocomaceae,

Ophiostomataceae, and Trichosporonaceae. In the Neu-JF rhizomes, five families were dominant, including Russulaceae, Trimorphomycetaceae, and Didymellaceae. The dominant families in the Neu-JR roots were Dermateaceae, Hypocreaceae, and Didymellaceae. Five families were dominant in the Neu-JA roots, including Telephoraceae, Cucurbitariaceae, and Phaeosphaeriaceae.

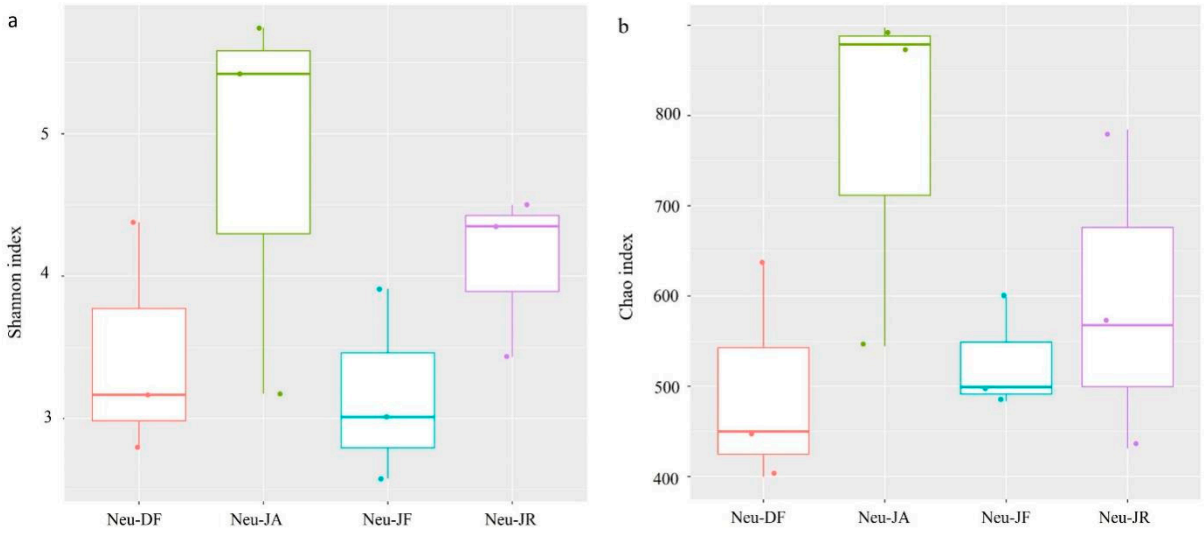

Figure 2. The diversity and richness of endophytic fungal communities associated with different *Neuwiedia singapureana* sampling groups. (**a**) Shannon and (**b**) Chao indexes of the operational taxonomic units (OTUs) from different wild *N. singapureana* root and rhizome samples. Neu-DF, the rhizomes of *N. singapureana* from the Diaoluoshan Reserve; Neu-JA, the air-exposed bare roots of *N. singapureana* from the Jianfengling Reserve; Neu-JF, the rhizomes of *N. singapureana* from the Jianfengling Reserve; Neu-JR, the normal roots of *N. singapureana* from the Jianfengling Reserve.

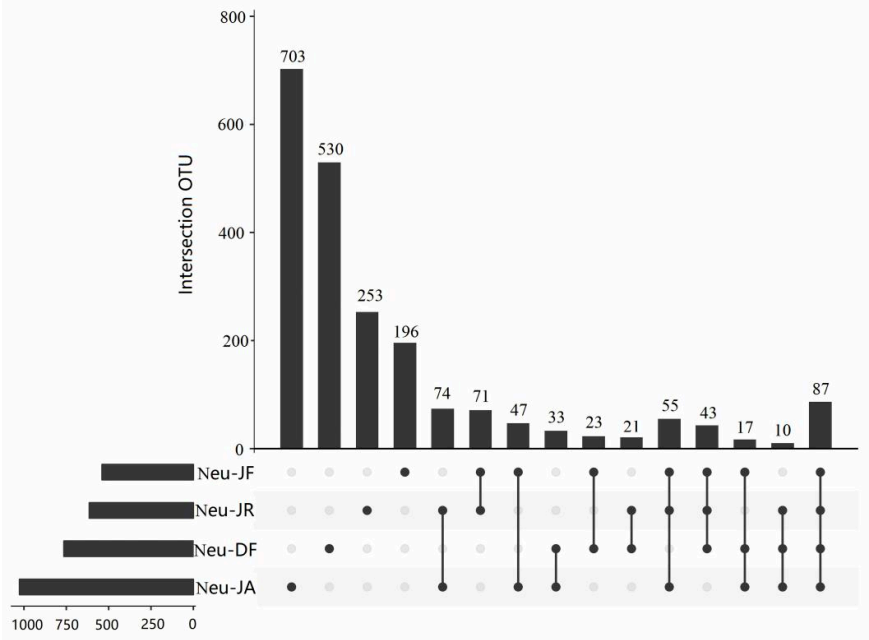

Figure 3. Common and specific operational taxonomic units (OTUs) from different *Neuwiedia singapureana* root and rhizome samples. The dots indicate specific OTUs, and the lines indicate common OTUs. Neu-JF, the rhizomes of *N. singapureana* from the Jianfengling Reserve; Neu-JR, the normal roots of *N. singapureana* from the Jianfengling Reserve; Neu-DF, the rhizomes of *N. singapureana* from the Diaoluoshan Reserve; Neu-JA, the air-exposed bare roots of *N. singapureana* from the Jianfengling Reserve.

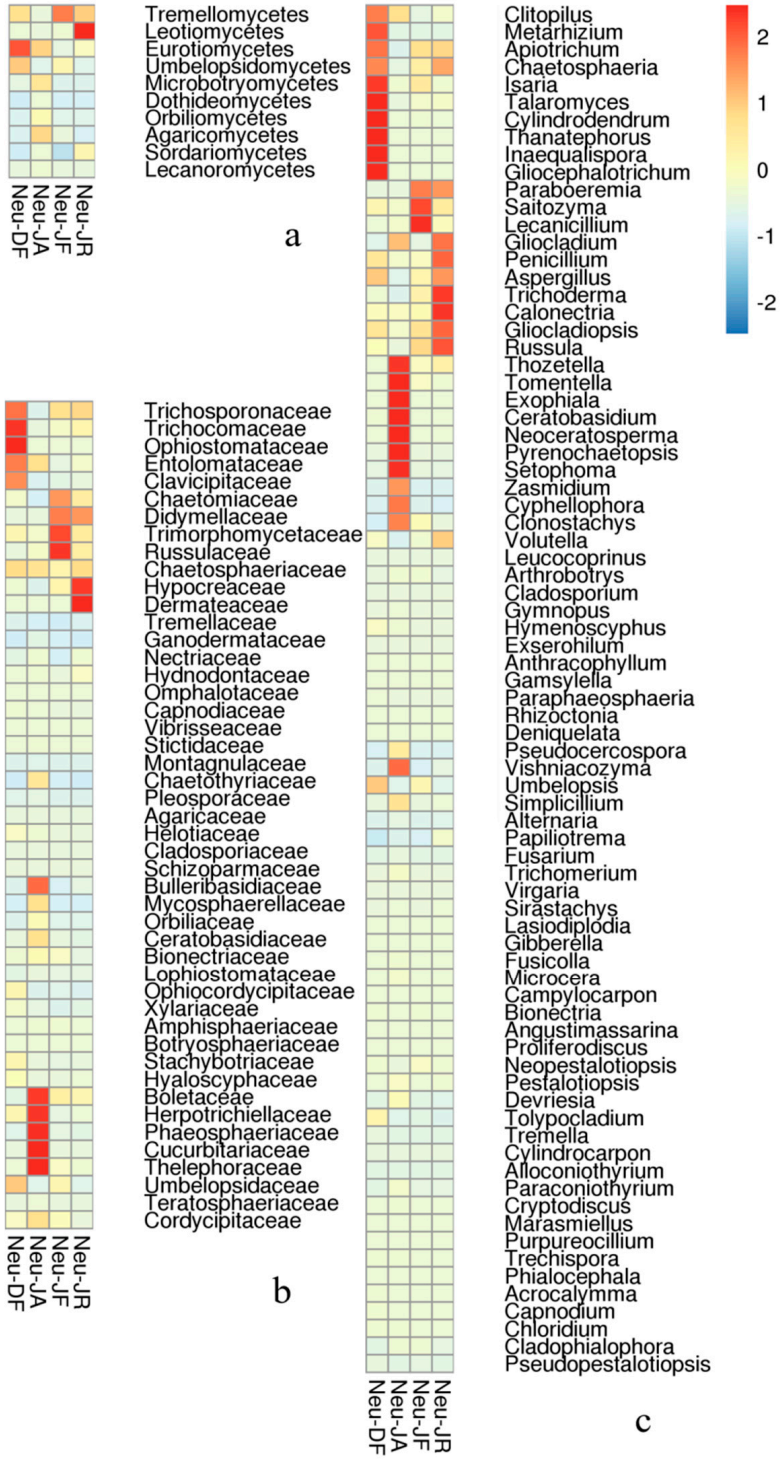

Figure 4. Heatmap showing relatively abundant fungal operational taxonomic units (OTUs) among the *Neuwiedia singapureana* sampling groups. The diversity of endophytic fungi is shown at the (**a**) class, (**b**) family, and (**c**) genus levels. OTUs with >1000 reads are shown. Neu-DF, the rhizomes of *N. singapureana* from the Diaoluoshan Reserve; Neu-JA, the air-exposed bare roots of *N. singapureana* from the Jianfengling Reserve; Neu-JF, the rhizomes of *N. singapureana* from the Jianfengling Reserve; Neu-JR, the normal roots of *N. singapureana* from the Jianfengling Reserve.

There were also clear differences in endophytic fungal diversity among sample groups at the genus level. Seven genera were dominant in the Neu-DF rhizomes, including *Gliocephalotrichum*, *Inaequalispora*, and *Thanatephorus*. *Lecanicillium*, *Saitozyma*, and *Paraboeremia*

were dominant in the Neu-JF rhizomes. Nine genera were dominant in the normal roots of Neu-JR, including *Trichoderma*, *Calonectria*, and *Gliocladiopsis*. In the bare roots of Neu-JA, eight genera were dominant, including *Thozetella*, *Tomentella*, and *Exophiala*.

3.2. OM Fungal Communities in Different Sample Groups

In total, 36 (about 1.67% of 2161) OTUs corresponded to OMF according to [1]. The majority of the OMF detected (17 OTUs) belonged to the Ceratobasidiaceae family (13,408 sequences). In addition, a number of other OM fungal taxa that have previously been reported were detected in this study. These included Russulaceae (6 OTUs, 7071 sequences), Thelephoraceae (10 OTUs, 3972 sequences), Cortinariaceae (1 OTU, 28 sequences), Serendipitaceae (1 OTU, 17 sequences), and Tricholomataceae (1 OTU, 3 sequences).

However, the abundances of these OM fungal families differed between the *N. singapureana* sampling groups. Ceratobasidiaceae fungi were mainly found in the rhizomes (Neu-JF and Neu-DF) and bare roots (Neu-JA). Meanwhile, Thelephoraceae members were abundant in the OM fungal community associated with the bare roots (Neu-JA) and normal roots (Neu-JR). The OTU members of Russulaceae were similar in all sample groups. Serendipitaceae fungi were detected only in Neu-DF. Cortinariaceae and Tricholomataceae were detected in Neu-DF, Neu-JR, and Neu-JA, but not in Neu-JF (Table 2).

Table 2. The operational taxonomic units (OTUs) corresponding to the orchid-associated mycorrhizal families identified in different *Neuwiedia singapureana* sampling groups.

OTU	Family	Closest Match in GenBank	Description	Identity (%)	Sampling Group
OTU000006	Ceratobasidiaceae	AJ318438.1	*Rhizoctonia* sp. Bi8	99.63	DF, JF, JR
OTU000034	Russulaceae	MF433036.1	*Russula pseudobubalina* strain K15060707	99.65	DF, JF, JR, JA
OTU000036	Ceratobasidiaceae	GU937737.1	*Ceratobasidium* sp. F7	98.70	DF, JF, JR, JA
OTU000072	Ceratobasidiaceae	GU937740.1	*Thanatephorus* sp. G5	98.63	DF, JF, JR, JA
OTU000075	Thelephoraceae	MK770303.1	Uncultured Thelephoraceae clone 3RDZ	98.43	DF, JF, JR, JA
OTU000096	Ceratobasidiaceae	GU937737.1	*Ceratobasidium* sp. F7	98.69	DF, JF, JA
OTU000157	Ceratobasidiaceae	GU937737.1	*Ceratobasidium* sp. F7	98.37	JA
OTU000236	Thelephoraceae	MK770303.1	Uncultured Thelephoraceae clone 3RDZ	96.86	JA
OTU000313	Ceratobasidiaceae	MG707439.1	Uncultured Ceratobasidiaceae clone OTU-C1	92.60	DF
OTU000347	Ceratobasidiaceae	KF823616.1	Uncultured *Ceratobasidium* clone AEW1_04	91.43	JF
OTU000362	Ceratobasidiaceae	MG707444.1	Uncultured Ceratobasidiaceae clone OTU-C6	92.56	DF
OTU000513	Russulaceae	EU819421.1	*Russula aeruginea* voucher JMP0057	97.39	DF, JF, JR, JA
OTU000586	Ceratobasidiaceae	KF267010.1	Ceratobasidiaceae sp. CBS 570.83	94.25	JF
OTU000700	Thelephoraceae	MK770317.1	Uncultured Trechisporales clone 4RDN	96.56	JF, JR, JA
OTU001323	Thelephoraceae	KF359624.1	*Tomentella* sp. 2 CC 14-01	97.81	JA, JR
OTU001489	Russulaceae	KU886599.1	*Russula rubra* voucher SAV:F-4216	96.83	JF, JR, JA
OTU001522	Thelephoraceae	MK770310.1	Uncultured Thelephoraceae clone 3RDD	97.19	JA
OTU001568	Thelephoraceae	EU668202.1	Uncultured *Tomentella* isolate 7754.1.R	96.24	JA, JR
OTU001629	Cortinariaceae	KT875178.1	*Cortinarius cramesinus* voucher PDD:107699	92.90	DF, JR, JA
OTU001729	Ceratobasidiaceae	LC511146.1	Uncultured Ceratobasidiaceae Cer5-M3402-CE7	97.03	JA, JR
OTU001891	Ceratobasidiaceae	MK336472.1	*Ceratobasidium* sp. strain Y. H. Yeh I0717	94.87	DF
OTU002091	Serendipitaceae	DQ520096.1	*Sebacina vermifera* AFTOL-ID 1877	96.95	DF
OTU002490	Thelephoraceae	GU452529.1	Uncultured *Thelephora* clone UBCOFE635Ar	97.19	JF, JR, JA
OTU002572	Russulaceae	MF433036.1	*Russula pseudobubalina* strain K15060707	98.95	JA, JR
OTU002807	Thelephoraceae	MK770275.1	Uncultured *Russula* clone 1RNN	96.56	JA, JR

Table 2. *Cont.*

OTU	Family	Closest Match in GenBank	Description	Identity (%)	Sampling Group
OTU003491	Ceratobasidiaceae	GU937740.1	*Thanatephorus* sp. G5	97.95	JA
OTU003881	Ceratobasidiaceae	GU937740.1	*Thanatephorus* sp. G5	97.59	JA
OTU004335	Thelephoraceae	MT678910.1	*Tomentella* sp. isolate LL_50	90.55	JF, JR
OTU004340	Ceratobasidiaceae	JF691537.1	Uncultured Ceratobasidiaceae clone TP362.1	99.67	JA
OTU005158	Tricholomataceae	MN906230.1	*Delicatula integrella* voucher S.D.	97.43	DF, JR, JA
OTU005219	Ceratobasidiaceae	MH862733.1	*Rhizoctonia solani* strain CBS 101382	91.62	JF
OTU005319	Ceratobasidiaceae	LT988374.1	Uncultured fungus genomic DNA sequence	99.63	JA
OTU005780	Ceratobasidiaceae	JQ926741.1	*Ceratobasidium* sp. HBESXF	90.35	JF
OTU006075	Thelephoraceae	MK770310.1	Uncultured Thelephoraceae clone 3RDD	95.94	JA
OTU006421	Russulaceae	KU886599.1	*Russula rubra* voucher SAV:F-4216	96.82	JR
OTU006489	Russulaceae	KU141303.1	Uncultured *Russula* clone HS3-15	100.00	DF

Note: DF, *N. singapureana* rhizome samples from Diaoluoshan Reserve; JF, *N. singapureana* rhizome samples from Jianfengling Reserve; JR, *N. singapureana* root samples from Jianfengling Reserve; JA, *N. singapureana* air-exposed root samples from Jianfengling Reserve.

3.3. Culturable Endophytic Fungal from Rhizomes

One culturable OM fungus, *Epulorhiza* sp. (Tulasnellaceae), was isolated, and this had high similarity (97.56%) with *Epulorhiza* sp. Pca-QS-0-1, which was isolated from *Paphiopedilum callosum* (Orchidaceae) roots sampled in Thailand. The hyphae were regular with binucleate cells (Figure 5b) and separated with branching at right angles (Figure 5c).

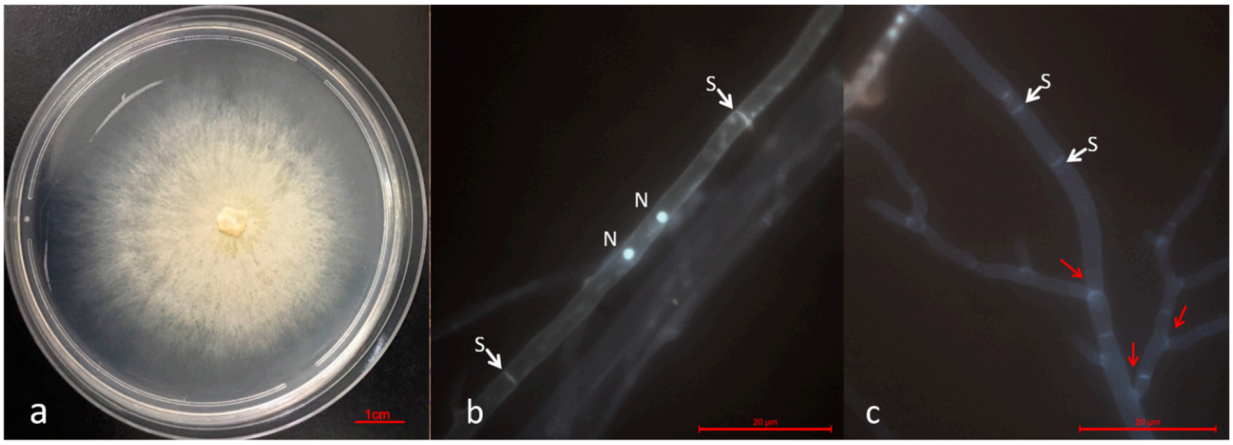

Figure 5. Colony morphology (**a**) and hyphae stained with SYBR Green I showing binucleate cells (**b**,**c**) of *Epulorhiza* sp. obtained from *Neuwiedia singapureana* rhizomes. N = nuclei; S = septa; the red arrows indicate hyphae with branching at right angles.

3.4. Promotion of Symbiotic Seed Germination by Fungal Isolates In Vitro

The ability of the fungal strain, *Epulorhiza* sp. (Epu) (Figure 5 and Table S2), to promote the seed germination of *D. officinale* and *E. secundum* orchids was tested. The symbiotic germination tests showed that the Epu strain promoted orchid seed germination and seedling growth with different efficiencies.

With the use of the standards for classifying seed germination stage set by Zettler et al. [51], the orchid seed germination and protocorm development were regularly observed and recorded. The *D. officinale* and *E. secundum* seeds developed faster when inoculated with the Epu isolate than when cultured individually under the same incubation time. When the protocorms of seeds inoculated individually ceased development at stage 2 (protocorm formation with the continued enlargement of the embryo to burst the

testa; Figure 6a) after 130 days, the *E. secundum* protocorm inoculated with Epu developed to stage 7, with root emergence and seedling growth (Figure 6b).

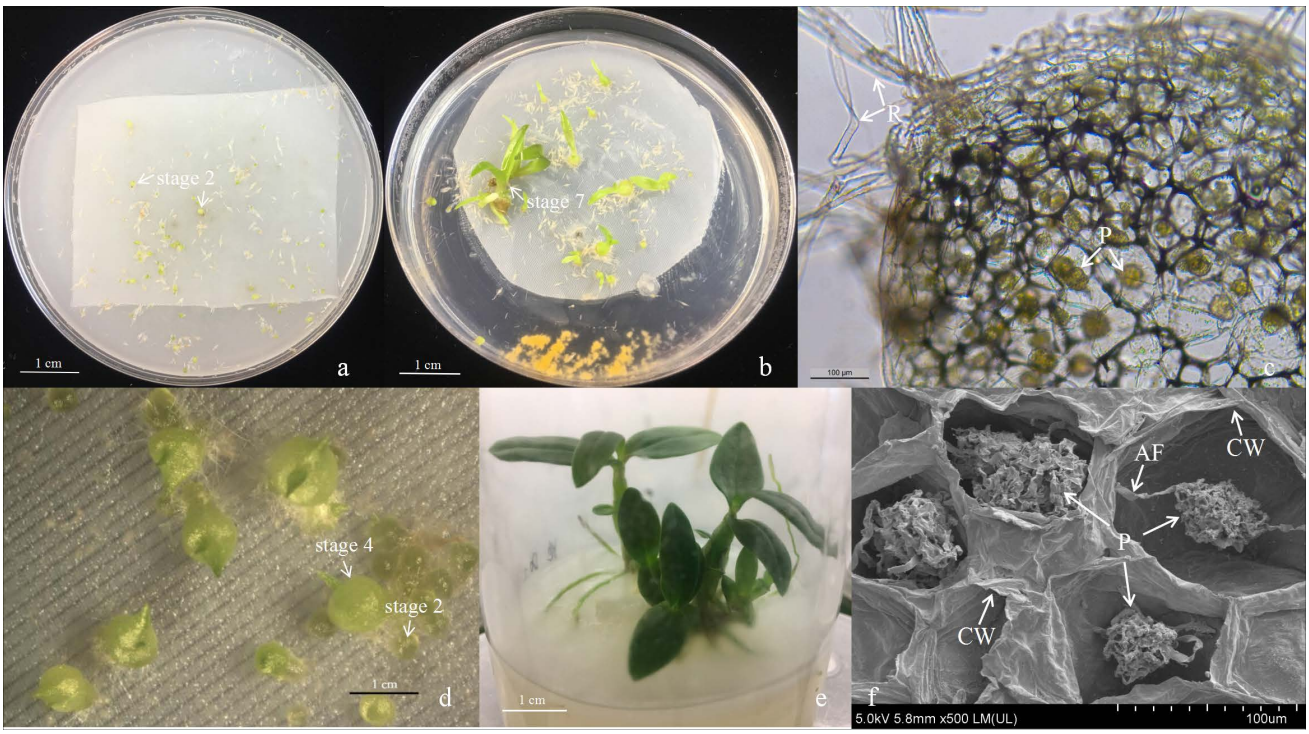

Figure 6. Symbiotic germination effects of endophytic fungal isolates on the seeds of the two orchids. The asymbiotic germination of the protocorms of mature *Epidendrum secundum* seeds (**a**). The symbiotic germination of the protocorms or seedlings of *Epidendrum*–Epu (**b**) and *Dendrobium*–Epu (**d**,**e**) combinations. The intracellular colonization of fungal pelotons in the symbiotic roots of the (**c**) *Epidendrum*–Epu and (**f**) *Dendrobium*–Epu combinations. a and b show the results after 130 days, d after 30 days, and e after 180 days. AF, active fungus; CW, cell wall; P, peloton; R, Rhizoid.

When inoculated with Epu, the *D. officinale* protocorm developed to stage 4, with cotyledon formation and rhizoid emergence after 30 days (Figure 6d). After 180 days of incubation, the *D. officinale* seedling could grow up to 5 cm in height on the 10 g/L OMA medium (Figure 6e) while the protocorms of seeds inoculated individually still ceased development at stage 2.

In the symbiotic roots of both the *Epidendrum*–Epu and *Dendrobium*–Epu seedlings, the fungal ITS sequences were amplified successfully, but not in the asymbiotic protocorms. By sequences alignment analysis, it was found that the amplified sequences were identical with the *Epulorhiza* sp. strain used in the symbiotic germination experiments. Moreover, the intracellular colonization of fungal pelotons in the symbiotic roots of the *Epidendrum*–Epu and *Dendrobium*–Epu seedlings was detected by both microscopic (c) and SEM observation (f) but was not detected in the asymbiotic protocorms. The results showed that the *Epulorhiza* sp. strain promoted the symbiotic seed germination and seedling growth and successfully colonized the *D. officinale* and *E. secundum* roots.

4. Discussion

4.1. Endophytic Fungal Diversity

An array of endophytic fungi is ubiquitous in healthy plant tissues and organs. Some of these fungi have been reported as root-associated endophytic fungi that can help orchids to absorb rhizosphere soil nutrients [52,53] and also affect plant yield and change secondary metabolite contents [54]. Thus far, different types of endophytic fungi have been detected and isolated from many orchid species [11,14].

In this study, the endophytic fungal diversity in orchid tissues was analyzed by high-throughput sequencing. Among the four sampling groups, a wide variety of endophytic fungi were detected with different richness values, belonging mainly to Basidiomycota, Mortierellomycota, and Mucoromycota. Many of these fungi have already previously been identified as orchid root colonizers. For example, some potentially beneficial fungi, such as *Trichoderma,* have been found in the roots of *Dendrobium nobile* [15], *Bulbophyllum neilgherrense,* and *Pholidota pallida* [14,55]. The endophytic fungi *Trichoderma koningiopsis* and *Trichoderma rogersonii* were isolated from *Dendrobium* and were found to have an inhibitory effect on *Pythium ultimum,* which is a pathogenic fungus of *D. nobile* [22]. Furthermore, some other *Trichoderma* spp. have been shown to promote the growth of several other plants [21]. In the present study, *Trichoderma* fungi were detected as the dominant fungal group in *N. singapureana* roots and rhizomes.

Earlier studies have found that there is no, or very low, presence of mycorrhizal fungi on the aerial roots of orchids [56]. An increasing number of recent studies has shown that almost all orchids are associated with mycorrhizal fungi in nature. For example, a large community of fungal associates was found in *Acampe praemorsa* orchid roots exposed in the air [57]. In addition to an abundance of common OMF, non-mycorrhizal endophytic fungi were also abundant in the air-exposed roots of *N. singapureana* in the present study. In these air-exposed roots, the dominant endophytic fungal genera included *Ceratobasidium*, *Neoceratosperma*, and *Exophiala*. These dominant genera were considerably different from those found associated with the belowground roots. This result indicated that the different intracellular microecologies in the roots of different niches (air-exposed and belowground) might have specific biological and ecological functions.

With respect to endophytic fungi in other vegetative tissues, stem-associated endophytic fungi have been previously detected. It has also been reported that geographical factors affect the distribution and diversity of endophytic microbial populations in plants [57,58]. The rhizomes of *N. singapureana* orchids grow belowground and are surrounded by soil microbes, and thus a complex microecological environment forms inside the rhizomes.

In the present study, Trichocomaceae and Ophiostomataceae were dominant fungal families in the rhizomes from plants from Diaoluoshan. Meanwhile, Russulaceae and Trimorphomycetaceae were dominant in the rhizomes from plants from Jianfengling. The fungal community composition and abundance displayed clear differences between samples from the two different geographical regions, which are approximately 200 km apart. This result is consistent with the idea of geographical regional differences [59,60]; the endophytic fungal composition in a plant is dependent on environment variations and host plant adaptation [57].

Common OMF have mainly been reported to belong to the families Ceratobasidiaceae, Tulasnellaceae, and Sebacinaceae. With the rapid development of modem molecular biology, newer and more efficient isolation techniques may be more successful [2]. Common endophytic fungi of other plants, such as Thelephoraceae and Russulaceae, have also been classified in the OM fungal group [1,3]. Although most types of common OMF were detected in the samples tested in the present study, the abundances of these fungal families differed in the roots and rhizomes. For example, Ceratobasidiaceae and Thelephoraceae mainly occurred in the rhizomes from both Diaoluoshan and Jianfengling. This indicated that similar to the roots, the rhizomes might also participate in mycorrhizal regulation. These fungi were also detected in the bare roots from Jianfengling. Moreover, Russulaceae was a dominant fungal family in the rhizomes from Jianfengling. Although the Tulasnellaceae family was not detected in the *N. singapureana* samples by high-throughput amplicons, one culturable *Epulorhiza* sp. strain was isolated from the rhizomes. In addition, the Sebacinaceae family was absent in the present study. This result was similar to the results of previous studies in which the most common culturable OM fungal strains were also not successfully obtained [15]. This could be attributed to the primer bias against this

fungal group [61]. Multigene primer pairs should be considered to reduce the data error caused by primer bias in future studies.

4.2. Growth-Promoting Function of OMF

Mycorrhizal fungi are found in many Orchidaceae plants and perform functions that promote the germination and growth of orchids. Under in vitro culture conditions, *Epulorhiza* strains and inoculated protocorms or seedlings of *Cymbidium hybridum* [62], *Coelogyne nervosa* [63], *Dendrobium* spp. [64,65], *Paphiopedilum* spp. [66,67], and *Anoectochilus roxburghii* [68] have been shown to effectively form symbioses; the growth of these seedlings was promoted by the mycorrhizal fungus. In orchid–*Epulorhiza* symbionts, the contents of Abscisic Acid (ABA) or partial mineral elements were shown to increase [64,65]. In addition, the growth of the lateral roots of the inoculated seedlings was induced, and root vitality was significantly improved by the symbiotic interaction [65]. In the present study, the *D. officinale* and *E. secundum* seeds inoculated with a *Epulorhiza* sp. isolate germinated faster; the protocorm developed into seedlings successfully in a shorter time than the symbiotic protocorms, which ceased development at the initial stage of protocorm formation. These results may thus highlight the universality of the orchid growth-promoting effects of *Epulorhiza*.

5. Conclusions

In this study, high-throughput ITS sequencing was carried out, and fungi were isolated via cultivable methods. The aim was to examine the endophytic fungal community structures associated with the rhizomes and roots of wild *N. singapureana* plants from two sampling sites in China. The results indicated that the endophytic fungal diversity differed among the samples but not significantly. Moreover, *Trichoderma* strains were isolated primarily, followed by *Fusarium* and *Apiotrichum* strains. There were many more non-mycorrhizal endophytic fungal than OM fungal species detected in the *N. singapureana* orchid. A *Epulorhiza* sp. isolate was found to be capable of significantly promoting the seed germination and seedling growth of *D. officinale* and *E. secundum* orchids, respectively, with different efficiencies. These endophytic fungal strains with growth-promoting functions will provide potential materials for orchid conservation and for further study of the mechanisms underlying orchid symbiotic associations.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d16010034/s1. Table S1: All of the OTU sequences, profiling, and annotations; Table S2: Possible identities of endophytic fungi isolated from *Neuwiedia singapureana* based on internal transcribed spacer (ITS) sequences; Figure S1: Map of China showing the two sampling locations of *Neuwiedia singapureana* marked with green and blue dots. (Color figure online).

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