In vitro Symbiotic Seed Germination of *Dendrobium formosum* Roxb. ex Lindl.

Na-monrug Khamchatra[1/2/] Somsak Apisitwanich[4/] Kongkanda Chayamarit[3/] Sureeya Tantiwiwat[1/]

ABSTRACT

Most studies on orchid symbiotic seed germination involve with terrestrial and temperate orchids. There are few reports on association between epiphytic or tropical orchid species and symbiotic seed germination. This study was to determine the effect of mycorrhizal fungi on *Dendrobium formosum* symbiotic seed germination in vitro. Five mycorrhizal fungi isolated from *D. formosum* roots, were identified as genera *Ceratobasidium* (DFRN02), *Cylindrocarpon* (DFRN01 and DFRN03) and *Epacris* (DFRN04 and DFRN05). All fungal species were cultured into *D. formosum* seeds which were collected from provinces of Ranong and Chumphon in 2011-2012.,whilst on group of seeds were treated without fungal (control). After 3 months sowing, seeds of all treatments germinated over 60%. The germination (%) of DFRN02 treatment was significantly higher than other groups after 4 months sowing, followed by treatments of DFRN04, DFRN05 and DFRN01 respectively. The results indicates that *Ceratobasidium* sp. (DFRN02) can be a potential mycorrhizal fungus for *D. formosum* symbiotic seed germination and therefore for the conservation of this threatened orchid.

Key words: *Dendrobium formosum*, epiphytic orchid, mycorrhiza, symbiotic seed germination

[1/] Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

[2/] Department of Biology, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000, Thailand.

[3/] Department of National Parks, Wildlife and Plants, Phahon Yothin Rd., Chatuchak, Bangkok 10900, Thailand.

[4/] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

INTRODUCTION

The minute seeds of orchids are capable of dispersing across geographical and ecological barriers, but contain very little stored energy to support germination (Arditti and Ghani, 2000). Orchid seeds depend on the energy and nutrients supplied by a mycorrhizal fungus to germinate under natural conditions (Rasmussen, 1995). Although orchid seeds were cultured to maturity using asymbiotic techniques, asymbiotically grown seedlings rarely survive after being transferred to nature despite growing well *in vitro*, besides the survival rate of orchid seedlings from symbiotic sowing was higher than from asymbiotic sowing (Batty *et al*., 2006). The survival of orchids in managed or restored habitats may require the presence of appropriate mycobionts to support plant development and subsequent seedling recruitment (Zettler, 1997). This is supported by well documented cases where orchids have developed with fungi cultured on organic additives *in vitro*, or on organic debris (Batty *et al*., 2001).

Several cases have been documented for the transplant success from symbiotically germinated orchids. An efficient symbiotic seed germination protocol to germinate seeds of the rare subtropical terrestrial orchid, *Habenaria macroceratitis*, was described, and the seed germination percent was highest when seeds were sown with a fungal mycobiont, *Epulorhiza* sp. (Stewart and Kane, 2006). The seed of several Australian temperate terrestrial orchid taxa, *Caladenia arenicola* and *Pterostylis sanguinea*, germinated best when seeds were sown with mycorrhizal fungi, and the seedling survival of improved when actively growing symbiotic seedlings were transferred to natural habitats during the growing season (Batty *et al*., 2006). Athipunyakom *et al.* (2004) found that *Spathoglottis plicata* seeds, inoculated with *Epulorhiza repens* and *Rhizoctonia globularis* isolated from 11 terrestrial Thai orchid species, showed the initiation of leaves 60 days after sowing seed. Seed germination and protocorm development of the threatened terrestrial orchid, *Pecteilis susannae* (L.) Rafin were evaluated up to 70 days after sowing, and the percent of symbiotic seed germination was highest when seeds were cultured with *Epulorhiza* sp. (Chutima *et al*., 2011). *Dendrobium* is the second largest genus in the family Orchidaceae. *Dendrobium*

formosum Roxb. ex Lindl. is anendemic epiphytic wild orchid species in Southeast Asia, widely distributed in Himalayas, Thailand, Vietnam, and Myanmar (Nanakorn and Indharamusika, 1998). *D. formosum* is a species in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendix II listed plants (Convention on International Trade in Endangered Species of Wild Fauna and Flora, 2014), because they are large beautiful and fragrant flower and fragrant which make them worthy subjects for cultivation and hobbyists. Unfortunately, the species has become threatened with extinction due to the loss of its natural habitat as well as over-harvesting for sale in illegal trade. The knowledge about propagation is needed for the conservation of this species. However, little is known about their association with mycorrhiza fungi and their role in germination of *D. formosum.*

Thus the objective of this study was to investigate the association between orchid mycorrhizal fungal and *D. fomosum* symbiotic seed germination (invitro) using mycobionts isolated from *D. formosum* in Thailand.

MATERIALS AND METHODS

Plant materials

Whole plants of *D. formosum* (Figure 1a) were collected from anatural habitat near Ngao National Park located at Ranong (N.Khamchatra 11, *N. Khamchatra* 12 and *N. Khamchatra* 13) and ChumphonProvinces (*N. Khamchatra* 14 and *N. Khamchatra* 15), Thailand in 2011-2012. Nine to ten monthold *D. formosum* orchid capsules in yellow green color were collected and kept in desiccated plastics boxes and stored in the dark place for about one week at room temperature. Afterward, the orchid seeds were transferred to sealed glass bottles before being stored at cold (4 °C) in dark place until use within 6 months.

Standard methods for collection and preservation were used following Jain and Rao (1977). The voucher specimens were deposited at Plant Genetic Conservation Project Herbarium, Faculty of Science and technology, Rambhai Barni Rajabhat University, Chanthaburi Province, Thailand. Flowers were preserved in alcohol. Moreover, live specimens are being maintained at the first author's garden.

Fungal isolation

The roots of *D. formosum* (Figure 1b) were rinsed in tap water to remove debris and cut in 1 cm segments. The segments were sterilized in 0.5 % NaOCl for 5 min. and finally rinsed in sterile distilled water three times. The surface-sterilized segments were transferred to a sterile Petri dish and immersed in sterile water. The segments were cut longitudinally, and pelotons (Figure 1c) were removed from cortical cells using a dissecting needle under a stereomicroscope (Nikon SMZ-2B). The individual pelotons were that opaque, circular-oval and submersible were washed in five changes of sterile distilled water. Each peloton (Figure 1d) was then placed on a Petri plate containing 1/6 Nutrient Dextrose Yeast Extract agar (1/6NDY) supplemented with 100 mg/ml of streptomycin and 100 mg/ml tetracycline. Plates were incubated at 25C in the dark, and observed periodically for fungal colonies development. Fungal mycelia of each colony were sub-cultured 2-3 times onto another fresh Potato Dextrose Agar (PDA) plates to be purified and assigned the accession number, starting with DFRN01, DFRN02, DFRN03, DFRN04 and DFRN05.Pure cultures were kept on 1/5 PDA slants and also placed in sterile distilled water at 15 ˚C for further use and 20% glycerol at -20 ˚C for long-term preservation.

Morphological characterization of isolates

The 5 fungal isolates were first identified using morphological characteristics, according to the methods outlined by Robert (1999) and Athipunyakom etal. (2004). For determination of culture characteristics, all isolates were cultured on PDA and Oat Meal Agar (OMA) at 25 ± 2˚C in the dark. Radial growth of the isolates was measured 3 and 6 days after inoculation. Cultural morphology was recorded at first and fourth week. The characteristics of fungal isolates were recorded as color and growth of the colony, concentric rings formation and aerial hyphae, shape and size of the sclerotia. Hyphal and monilioid length and width were measured. Lactophenol cotton blue was used as a staining agent for observing hyphal characteristics and Safranin O-KOH was used for staining the fungal nuclei. The hyphal characteristics and the fungal nuclei were examined and photographed under a Nikon Eclipse E400 microscope.

Molecular analysis

Molecular identification was based on internal transcripbed spacer (ITS) sequences. All isolates were grown in 20 mL Potato Dextrose Broth (PDB) medium in the dark at room temperature for 7 to 10 days. A 50-100 mg mycelia aliquot was harvested from each culture and DNA was extracted with cetyltrimethy lammonium bromide (CTAB) method as described by Zhou *et al.* (1999).

The primers ITS1 (5-TCCGTAGG TGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTG ATATGC-3) (White *et al*., 1990) were used for ITS amplification. Polymerase chain reaction (PCR) technique was used to amplify the ITS sequences according to the Thermo Scientific PCR master mix kit (Fermentas, California, USA). The amplification was carried out in a thermocycler (Takara, Japan) with a PCR condition of 94 °C for 5 min, followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min, followed by 5 min. of final extension at 72 °C. PCR products were separated by 1.5% agarose gel electrophoresis, stained with SYBR Safe (Molecular Probes, Eugene, OR) DNA gel stain, i.e. 1:10,00 dilution of TBE buffer and visualized by ultraviolet light transillumination. PCR products were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA). Sequencing was performed on 23 ABI 3730 XLs by using Big Dye Terminator V3.1 Cycle Sequencing Kit (Macrogen, Inc., Korea). Each PCR template was sequenced in both direction using either forward (ITS1) or reverse (ITS4) primers. The sequenced were manually edited for ambigucus base with sequencer v. 4.0 (Gene Codes Corporations). In order to determine the identity of these ITS sequences, the "megablast" searches were performed on the U.S. National Center for Biotechnology Information (NCBI: blast.ncbi.nlm.nih.sov). Pairwise sequence comparisons were expressed as the percentage of the total number of nucleotide differences divided by total number of position sequence similarity of 95% or greater was considered.

Symbiotic seed germination

The effects of isolates on promoting *D. formosum in vitro* symbiotic seed germination were evaluated using a method of Stewart and Kane (2006) with modification. Seed were surface sterilized

as described by Batty *et al.* (2001). Seeds in a filtered paper pocketwere immersed in distilled water with tween-20 for 2 min twice, followed by 0.5% NaOCl for 10 min and finally rinsed 3 times in sterile distilled water. Approximately 150-200 surface sterilized seeds were sown onto the surface of a one-eighth of a sterile Whatman No.4 filtered paper and placed in Petri dish containing 20 mL of sterile Oat Meal Agar (OMA) at pH 6.0. A 5- mm diameter plug of each fungal inoculum, was taken from the actively growing hyphal edge 7 days after culturing on PDA, were placed at the middle of sown seeds plates. Uninoculated plates were used as control. Four replications were used for 6 treatments. All 24 culture plates were sealed with parafilm and then kept at 25 °C in the dark for 4 months. Seed germination and protocorm development were monitored monthly under stereomicroscope and returned to the dark conditions. Developmental stages were recorded and scored on a scale of 0-5 (adapted from Arditii and Ghani, 2000; Table 1). Protocorms were randomly sampled and microscopically examined to confirm the mycobiont structures under a compound light microscope. Percentages of seed germination were based on viable seeds using dissecting microscopy and protocorm development were calculated by dividing the number of seed in each germination and development stage by the total number of viable seeds in the sample (Arditii and Ghani, 2000). The data were analyzed using Microsoft Office Excel program for one-way analysis of variance (ANOVA), and means were compared by Duncan's Multiple Range test

RESULTS AND DISCUSSION

Fungal isolation and identification

Intense colonization of peletons was found in root of *D. formosum* (Figure 1). Intact or degraded pelotons were observed in approximately 30-40% of root cortical cells with brownish color.

Five fungal endophytic fungi isolates were extracted from *D. formosum* roots. Based on morphology and molecular characteristics (Table 2), the fungal isolates were divided into 2 groups. Group I was the isolate DFRN02 which showed resemblance to Rhizoctonia-like orchid endophyte. The colony color of isolate DFRN02 on PDA was creamy white. The ellipsoidal monilioid cells were

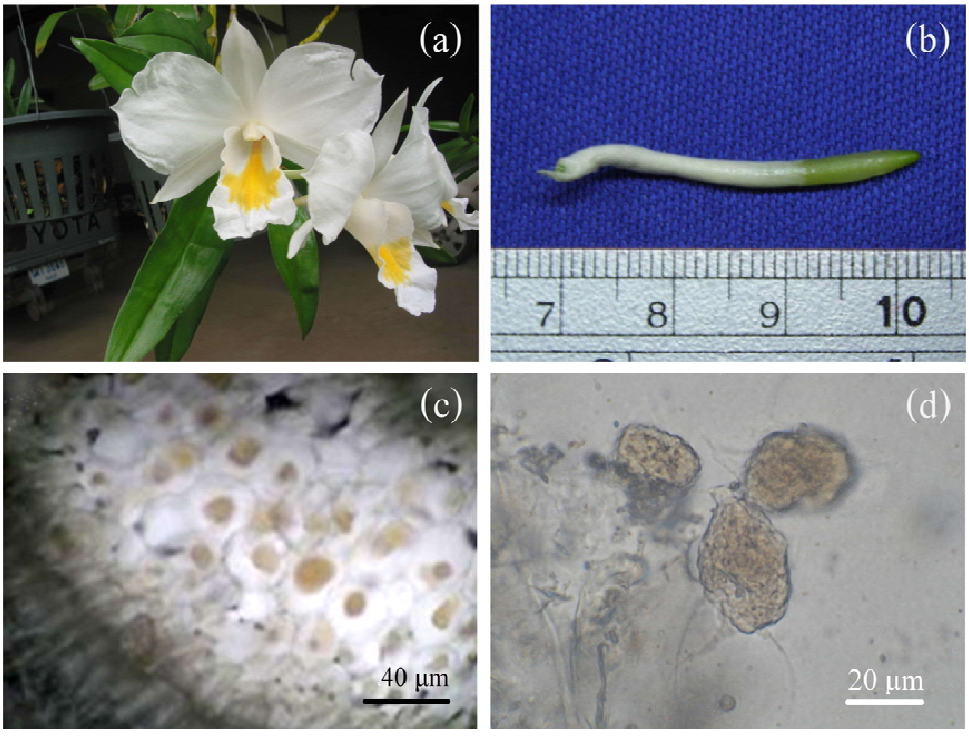

Figure 1 Dendrobiumformosum and peloton colonization: (a) Flower; (b) Root; (c) Pelotons in cortical cells; (d) Single peloton.

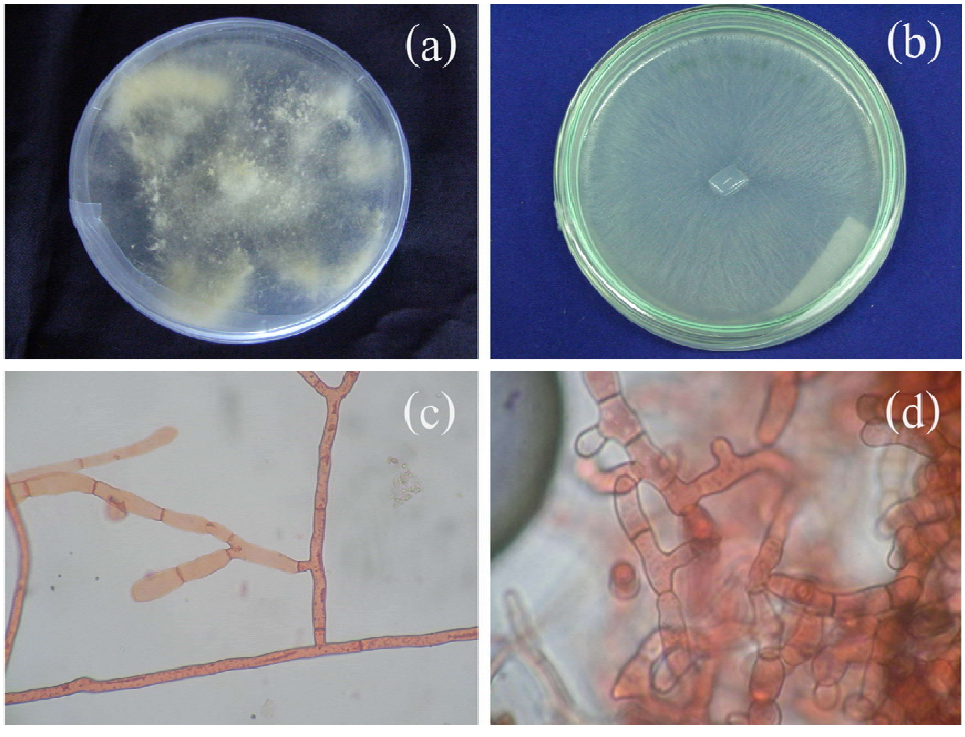

Figure 2 Fungal colonies on PDA and morphological characteristics: (a) Isolate DFRN02 colony on PDA at 14 days; (b) Isolate DFRN02 colony on PDA at 7 days; (c) Right-angle branching hyphae; (d) Cluster of monilioid cells.

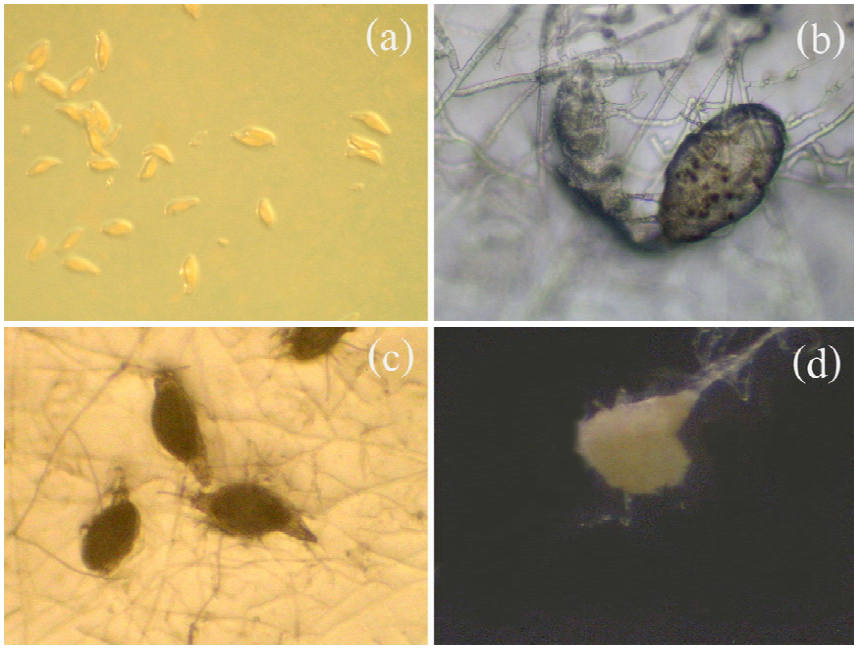

Figure 3 Symbiotic seed germination and protocorm development of *D. formosum* inoculated with Ceratobasidium isolated fungi (DFRN02) cultured on OMA: (a) control (uninoculated seeds); (b) Fungal colonization of *Ceratobasidium* sp. in protocorm; (c) Stage 2: continued embryo enlargement, testa ruptured; (d) Stage 3: appearance of protomeristem.

observed after 7 days (Figure 2). According to the BLAST search, isolate DFRN02 was identified as genus Ceratobasidium Gene Bank code: accession number GU937735 (Jiang *et al*., 2010). These fungal genera are well known as mycorrhizal fungi and associated with a diverse range of terrestrial and epiphytic orchids, including *Acianthus* R.Br., *Bipinnula* Comm. Ex Juss., *Caladenia* R.Br., *Cymbidium* Sw., *Dendrobium* Sw., *Diuris* Sm., *Goodyera* R.Br., and *Platanthera* Rich. The other isolates were Group II consisting of 4 isolates, identified as *Cylindrocarpon* Gene Bank code: accession number FJ605259 (Vaz *et al*., 2008), isolates DFRN01 and DFRN03 and *Epacris* Gene Bank code: accession number AY268191 (Williams *et al*., 2003), isolates DFRN04 and DFRN05 (Table 2). In this study, endophytic fungal were found as Cylindrocarpon (DFRN03) and Epacris microphylla (isolates DFRN04 and DFRN05), similar to Bayman and Otero (2006) which listed a large amounts of

fungal endophytes species isolated from epiphytic, terrestrial and lithophytic orchids roots.

Symbiotic seed germination

In vitro symbiotic seed germination is a powerful tool for both the production of mycobiont-infected seedlings for use in plant reintroduction and the study of fungal specificity within the Orchidaceae. However, there are a few reports that address in vitro symbiotic seed germination and development of Thai native orchid species (Athipunyakom *et al.*, 2004; Nonthachaiyapoom *et al.*, 2011). This is the first study that describes in vitro symbiotic seed germination of *D. formosum*. Over 60% of seed in this study germinated and developed to protocorm after sowing for 3 months (Table 3). After 4 months sowing, seed germination of DFRN02, DFRN04 and DFRN05 treatment increased to 100%, 89.13% and 83.89% respectively which were significantly higher than control. Moreover, percentage of seed germination in treatments inoculated with fungal isolates DFRN02 (100%), DFRN04 (89.13%) and DFRN05 (83.89%) were significantly different from control at 4 months. The most advanced protocorm development at stage 3 was promoted by isolate DFRN02 (*Ceratobasidium* sp.) for 4 months after seed sowing (Figure 3). The isolates DFRN04 (Cylindrocarpon) and DFRN05 (*Epacris microphylla*) also promoted seed germination at stage 1. Nevertheless, the difference between percentages of symbiotic and asymbiotic seed germination was not high (15%), and the final protocorm developmental stage is not sufficient for propagation and conservation of this epiphytic orchid species at present. There is a complicated relationship between orchid and endophytic fungi, and it is not clear whether the mycorrhizal fungi found in adult plants are the same fungi necessary for seed germination (Porras-Alfaro and Bayman 2007). Moreover, some reports suggest that there are specific endophytic fungi for different stages of growth in an orchid's life cycle (Dearnaley, 2007). Also it is possible that symbiosis may require particular environmental conditions that cannot be achieved in the laboratory to promote the symbiotic relationship between orchid and fungus. Finding an effective mycobiont for *D. formosum* is a promising step forward in continuing

Table 1 Different seed germination stages of *D. formosum.*

Stage	Characteristics
0	No germination, seed with intact seed coat
1	Enlarged embryo, seed coat ruptured by the enlargement of embryo (=germination)
2	Continued embryo enlargement, rupture of testa
3	Appearance of protomeristem
4	Emergence of rst leaf
5	Elongation of rst leaf and further leaf development

Table 2 Morphology and molecular characteristics identification of five endophytic fungal isolated from the wild *Dendrobium formosum* orchid.

Isolate	Morphology	Sequence match	Identity (%)	Fungal group
DFRN01	Colony color on PDA was white to cream, aerial hyphae in the center with white submerged margin	*Cylindrocarpon* sp. FJ605259	97	Hypocreales; Nectriaceae
DFRN02	Colony color on PDA was creamy white, monilioid cells ellipsoidal, aerial and submerged hyphae	*Ceratobasidium* sp. GU937735	93	Cantharellales; Ceratobasidiaceae
DFRN03	Colony color on PDA was white to cream, aerial and submerged hyphae	*Cylindrocarpon* sp. FJ605259	98	Hypocreales; Nectriaceae
DFRN04	Colony on PDA was white to pale yellow with aerial hyphae	*Epacris microphylla* AY268191	98	Hypocreales; Cordycipitaceae
DFRN05	Colony on PDA was white to cream with aerial hyphae, dense, forming granular aggregation in the center with submerged margin	*Epacris microphylla* AY268191	97	Hypocreales; Cordycipitaceae

Table 3 Percentages of seed germination and protocorm development of *D. formosum* seed after 1, 2, 3 and 4 months sowing

Fungus	Percentage of seed germination after			
	1 Months	2 Months	3 Months	4 Months
Control	0.00 c	11.81 d	60.88 c	68.44 d
DFRN01	2.56 c	3.63 d	70.44 c	71.63 cd
DFRN02	15.00 a	77.31 a	100.00 a	100.00 a
DFRN03	1.50 c	4.19 d	61.88 c	72.69 cd
DFRN04	12.06 ab	60.69 b	84.13 b	89.13 ab
DFRN05	6.94 a	42.44 c	70.31 c	83.89 bc

In a column, mean followed by a common letter are not significantly different at 5% level by DMRT

efforts to develop reintroduction and conservation procedures for this rare and threatened orchid species, as the techniques employed here can be relevant to other endangered and threatened wild orchid species. Further studies should focus on improving the efficiency of *D. formosum in situ* seedling development, such as mass propagation, transplant success to nursery and then to the natural habitats.

CONCLUSION

Association between symbiotic seed germination and mycorrhiza fungi of *D. formosum* was found. Only DFRN02 isolate, was a suitable mycobiont for *D. formosum* seed germination and seedling growth *in vitro*. Orchid-fungal symbiosis value in *D. formosum* propagation and their reintroduction into natural habitats. It can be applied to other endangered species. Moreover, we now need to understand the efficacy of *D. formosum* symbiotic seed germination insitu. If the orchid indeed requires different fungal strains within its lifetime, such knowledge about the fungi partners will be helpful for reintroduction and conservation of this species, as well as other commercial or endangered and threatened Thai orchid species. An *in situ* baiting technique is quite promising to be further used in orchid conservation program.

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