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Original article

Using *in situ* seed baiting technique to isolate and identify endophytic and mycorrhizal fungi from seeds of a threatened epiphytic orchid, *Dendrobium friedericksianum* Rchb.f. (Orchidaceae)

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ABSTRACT

All orchids require association with mycorrhizal fungi for seed germination and development under natural conditions but their identification and feasibility are not known. The *in situ* seed baiting germination was examined of *Dendrobium friedericksianum* Rchb.f., a native threatened epiphytic orchid species, to detect fungi that promote germination. It was found that seed germination percentages were lowered by 0.1%, with a total of seven protocorms formed. Six endophytic fungi were isolated from seven protocorms in seed packets. Three binucleate *Rhizoctonia*-like fungal isolates which formed a teleomorphic state were morphologically identified as *Tulasnella violea*, *Epulorhiza repen* (anamorph *Tulasnella*) and *Trichosporiella multisporum*. The species of *Beauvaria* and *Fusarium* which are endophyte fungi were also isolated and may play an important role for plant growth and survival of *D. friedericksianum*. The results in this study suggested that *in situ* seed baiting is beneficial for screening compatible mycorrhizal fungi to promote the growth and propagation of epiphytic orchids.

Introduction

Dendrobium is one of the largest genera of Orchidaceae, with over 1000 species worldwide (Leitch et al., 2009). Many *Dendrobium* orchids are important horticultural crops and have been used in commercial trade due to their popular flowering abundance, wide range of flower colors, sizes, shapes, year-round availability and lengthy post-harvest life (Hossain, 2011). Most *Dendrobium* species are suspected of becoming extinct. Of these, *Dendrobium friedericksianum* Rchb.f. is a native epiphytic orchid found in tropical rain forests in Eastern Thailand according to the checklist of international species, (Convention on International Trade in Endangered Species of Wild Fauna and Flora, 2014). Due to their beautiful, bright golden-yellow flowers with diameters around 4–5 cm each, the orchid has attracted collectors and so specimens are in high demand for illegal trading. Consequently, this species is on the verge of being seriously endangered in the wild.

Orchid seeds are usually small, dust-like and lacking an endosperm. Consequently, germination of orchid seeds and seedling development need compatible endophytic mycorrhizal fungi to provide the minute seeds with carbon, water and nutrients under natural conditions (Arditti and Ghani, 2000; Rasmussen and Rasmussen, 2009). Asymbiotic seed germination techniques and *in vitro* rapid propagation of seedlings have been applied to *D. friedericksianum* (Prasertsivivatna and Koolpluksee, 2011), but the seedlings have rarely survived after a transplant to nature despite being well grown in aseptic culture. It is assumed that they may be dependent on suitable mycorrhizal fungi for orchid seedling survival.

Most studies on orchid mycorrhizal fungi have been focused on *in vitro* symbiotic techniques using fungal isolates obtained from

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adult orchid roots, not from protocorms or seedling stages (Chutima et al., 2011; Nontachaiyapoom et al., 2011). It was not clear whether mycorrhizal fungi found in adult orchid roots are the same fungi necessary for seed germination until Rasmussen and Whigham (1993) studied and developed *in situ* seed germination methods which involved burying seed packets in the field during the growing season to assess the presence of fungi capable of supporting germination. This seed baiting has been successfully used by McKendrick et al. (2000) and Brundrett et al. (2003).

The present study hypothesized that the species-specific mycorrhizae isolated from the developed protocorms should have a greater impact on the success of germination and subsequent stages of seed development. The aim of this study was to isolate and identify mycorrhizal and non-mycorrhizal endophytic fungi from protocorms of *D. friedericksianum* using the *in situ* seed baiting method. This study is the first to report on culturable mycorrhizal fungi that may be valuable for orchid propagation and conservation.

Materials and methods

Study site and seed collection

In December 2012, 9–10 mth-old, hand-pollinated, yellow-green capsules of *D. friedericksianum* were collected from fruit orchards in Chanthaburi, a southeastern province of Thailand. The capsules were put in a paper bag and transported to Rambhai Barni Rajabhat University. The capsules were kept in silica gel-desiccated plastics boxes in the dark for approximately 1 wk at room temperature. The seeds were then transferred to sealed glass bottles stored at 4 °C in the dark until use, within 4–6 mth (Stewart and Kane, 2007).

In situ baiting method

In January 2013, approximately 300–400 *Dendrobium fridericksianum* seeds were placed in separate folding 5 cm × 10 cm nylon mesh pouches with a 45 μm pore size, which was large enough to allow fungal hyphae to enter but small enough to retain seeds inside the pouch. Each of these packets was placed inside a 35 mm plastic slide mount (Brundrett et al., 2003; Zi et al., 2014). All packets were secured onto the tree bark near the adult roots of *D. friderickssianum* and were then covered with a thin layer of sterile wet *Sphagnum* moss to prevent desiccation but suitable for light to penetrate. In July 2013, harvests were taken during the orchid growing season. Recovered seed packets were placed between moist sheets of sterilized wet cotton wool and stored at 4 °C.

Seed germination and development assessment

Seed germination and development were observed under a dissecting stereomicroscope and scored on a scale of 0–5 as described by Arditti and Ghani (2000). Seed germination and protocorm development percentages were calculated.

Fungal isolation from seed baiting packets

Mycorrhizal fungi were isolated from the collected germinated seeds which had approached stage 3 according to Brundrett et al. (2003) with slight modifications before dispersal on the agar surface as follows. The seed packet surfaces were washed with running tap water and sterilized by immersing in 70% ethanol for 15–30 s depending on the seed size. The seed packets were then dipped in sodium hypochlorite solution containing 0.5% available chlorine for 3–5 min and rinsed three times with sterile double-distilled water. They were opened under a dissecting microscope. The protocorms were then sown on modified Czapek Dox agar medium (Yamato et al., 2005) supplemented with 0.05 g/L streptomycin. The pH was adjusted to 7.3. After 4–7 d, the fungal hyphae growth that had emerged from the protocorms were excised and transferred onto fresh potato dextrose agar (PDA) and incubated at 25 ± 2.0 °C in the dark. Fungal colonies from actively growing isolates were subcultivated 2–3 times onto a fresh PDA dish to be purified and assigned a strain number (denoted with the code DFCT-B).

Cultural and morphological characterization

The growth rate and colony characteristics were recorded from the culture grown on PDA (Sneh et al., 1991; Currah et al. 1997; Roberts, 1999). Microscopic observations were made using a slide culture technique. Fungal hyphae stained with lactophenol cotton blue and monilioid cells were assessed visually and microscopically using light microscopy (Eclipse E400; Nikon; Tokyo, Japan). The number of nuclei per cell was determined after staining with safranin O–KOH using Bandoni's method (Bandoni, 1979).

Results

In situ symbiotic seed germination

The 30 seed packets placed *in situ* on the bark of trees in January 2013 (Fig. 1) were collected 7 mth later in July 2013. The numbers of seeds that reached or passed each germination stage out of the total seeds placed in each packet as successful events for the specific stage were counted. The results of seed germination are shown in Table 1. The total seed germination percentage of *D. friedicksianum* was 77.8 ± 9.3. Seed germination of orchid species up to the total achievement of stage-3 protocorms (appearance of protomeristems) were determined (Fig. 2A). The peloton and coiled mycelium (Fig. 2B) of the orchid's mycorrhizal fungi were observed in the protocorms. More than 50% of germinated seeds developed to stage-2.

Microbiont identification and morphological characteristics

In total, six endophyte mycorrhizal fungi were isolated from seven protocorms in the formation stage collected *in situ*. These isolates were divided into two groups based on cultural characteristics. Group I, containing isolates DFCT-B-05, DFCT-B-09 and DFCT-B-17 showed resemblance to *Rhizoctonia*-like orchid endophytes consisting of colorless colonies, right-angle branching, constrictions at branch point, septum in the branch hyphae near its point of origin, sclerotia formation, chains of monilioid cells and a number of nuclei in young cells. These three isolates are described below.

Isolate DFCT-B-05 grew slowly with a white-cream colony, submerged and reached 9 cm in diameter after 2 wk on PDA (Fig. 3A). Vegetative hyphae were 5–6 μm wide, binucleate, but no clamp connection was found. Basidia were swollen at the tips of subtending hyphae and bearing small, inverted-drop-shaped basidiospores, 4.5–5.5 μm (Fig. 3B) in length. This fungal isolate was identified to be *Tulasnella violea* (teleomorp).

Isolate DFCT-B-09 colony became white-to-cream in 1 wk and was submerged with glabrous surface (Fig. 3C). Hyphae were 3.5–4.0 μm wide, septate, binucleate and had a light brown, small sclerotia. Monilioid cells were ellipsoidal to spherical (Fig. 3D), hyaline were in unbranched short chains and it was identified as *Epulorhiza repens* (anamorph).

Isolate DFCT-B-17 colony was flat with white aerial hyphae (Fig. 3E). Fertile hyphae were 2.0–3.0 μm wide, septate and bearing a lateral conidia. The isolate, described as *Trichosporiella*