



Symbiotic seed germination of *Grammatophyllum speciosum* Blume and *Dendrobium draconis* Rchb. f., native orchids of Thailand

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ABSTRACT

In vitro symbiotic seed germination is an important tool not only for the study of orchid-fungus specificity but also for the production of mycobiont-infected healthy seedlings that could be valuable for both horticultural and conservation purposes. The current study compared effectiveness of eight putative orchid mycorrhizal fungi obtained from mature orchids in the genera *Paphiopedilum*, *Cymbidium* and *Dendrobium*, in promoting *in vitro* seed germination and protocorm development of *Grammatophyllum speciosum* Blume and *Dendrobium draconis* Rchb. f., native Thai orchids. The developmental stages of seeds and protocorms cultured on Murashige and Skoog (MS) medium, oat meal agar (OMA), or OMA inoculated with one of the eight fungal isolates were evaluated weekly. Two isolates of *Epulorhiza repens* (Bernard) Moore (=anamorphic species of *Tulasnella calospora* (Boud.) Juel), Da-KP-0-1 and Pv-PC-1-1, were found to be the most effective fungi in promoting protocorm development of *G. speciosum*. At week 13, protocorms co-cultured with either one of these two fungal isolates, on the average, were significantly more advanced than those sown on OMA. Protocorms co-cultured with isolate Pv-PC-1-1 were also significantly more advanced than those cultured on MS medium. For *D. draconis* seed germination, three fungal isolates of different anamorphic species of *Tulasnella*, C1-DT-TC-1, Pv-PC-1-1, and C3-DT-TC-2, were found to be the most effective fungi in promoting protocorm development. However, none of these fungal isolates outperformed MS medium. Additionally, the compatibility between the fungal isolates tested and the two orchid species was discussed.

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1. Introduction

Grammatophyllum speciosum Blume and *Dendrobium draconis* Rchb. f. are native orchids of Thailand (Nanakorn and Indharamusika, 1998) that have attracted the attention of orchid growers globally. *G. speciosum*, known as the world's largest orchid, is considered to be endangered in its natural habitats. *D. draconis* is a heavily traded species. Wild-collected plants are often seen in local plant markets. Micropropagation of *G. speciosum* using shoot tips of an *in vitro* specimen as explants was reported by Sopalum et al. (2010) and that of *D. draconis* using thin cross-sections derived from young stems of *in vitro* plantlets was reported by Rangsayatorn (2009). Although the protocols established by these investigators successfully produced a large number of orchid plantlets that

will benefit the orchid propagation for commercial purposes, they might not be suitable for conservation purposes due to the lack of genetic diversity. The production of orchids from seed is the best way for conserving natural populations while still maintaining the genetic diversity and producing strong healthy plants (Butcher and Marlow, 1989).

Generally, seed germination of orchids can be done using either asymbiotic or symbiotic methods (for reviews, see Rasmussen, 1995; Yam and Arditti, 2009). However, several comparative studies between asymbiotic and symbiotic germination demonstrated that where successful symbiotic methods had been established they were more desirable methods because symbiotic protocorms could develop more rapidly than asymbiotic protocorms (Johnson et al., 2007; Muir, 1989; Rasmussen et al., 1990). Moreover, symbiotic seedlings could serve to inoculate soil with a germination promoting mycobiont (Johnson et al., 2007; Stewart and Zettler, 2002). To date, there have been relatively few studies dedicated to symbiotic seed germination of epiphytic orchids, especially Asian species.

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Members of the Orchidaceae show various degrees of fungal specificity during seed germination. Warcup (1973) reported that seed germination of two *Pterostylis* species and two *Diuris* species were stimulated only by *Ceratobasidium cornigerum* (Bourdot) D.P. Rogers and *Tulasnella calospora* (Boud.) Juel, respectively, whereas, seed germination of seven *Thelymitra* species was stimulated by more than one species of *Tulasnella* but not markedly by *C. cornigerum*. Bonnardeaux et al. (2007) tested the compatibility between six terrestrial orchids and 12 fungi (one *Ceratobasidium* isolate, two *Sebacina* isolates, eight *Epulorhiza* isolates and one endophytic ascomycete) and reported that *Disa bracteata* Sw. and the indigenous Australian *Microtis media* R. Br. were either fully or partially compatible with all the fungi studied, whereas *Pterostylis sanguinea* D.L. Jones & M.A. Clem. and *Caladenia falcata* (Nicholls) M.A. Clem. & Hopper germinated exclusively with the fungal isolates from the *Ceratobasidium* and *Sebacina* clades, respectively. Additionally, it was found that different isolates of the same fungal species could differ markedly in the efficiency of seed germination promotion (Stewart and Kane, 2006; Warcup, 1973) and the most efficient isolates were not necessarily those isolated from the same orchid species that they stimulated germination (e.g., Bonnardeaux et al., 2007; Muir, 1989; Warcup, 1973). Therefore, screening tests for fungal compatibility are a crucial step for successful symbiotic seed germination.

Previously, we isolated 27 isolates of *Rhizoctonia*-like fungi from root sections of mature orchids in the genera, *Paphiopedilum*, *Cymbidium* and *Dendrobium*. These fungi were identified and grouped into five subclades according to the sequences of internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) sequences (Nontachaiyapoom et al., 2010). In the current study, we evaluated eight fungal isolates in four of the five subclades for their effectiveness in promoting *in vitro* seed germination and protocorm development of *G. speciosum* and *D. draconis* orchids.

2. Materials and methods

2.1. Plant material

Initially, seeds of four orchid species were used in this study; however, the seeds of two orchid species (i.e., *Cymbidium sinense* (Jack.) Willd. and *Dendrobium crepidatum* Lindl. & Paxton) did not germinate in all treatments including Murashige and Skoog medium (MS; Murashige and Skoog, 1962), probably due to dormancy, therefore, the results presented in this article exclude them.

A mature (brown) undehiscent capsule of *G. speciosum* was collected in Kuraburi District, Pangnga Province on 20 January 2010 and a green capsule (with the zones between dehiscence zones turned to yellow) of *D. draconis* was collected from Plant Collection Center under the Royal Initiative of Her Majesty the Queen, Chatrakarn District, Phitsanulok Province on 9 December 2009. Both capsules were obtained from naturally pollinated plants. The capsule of *G. speciosum* and that of *D. draconis* were transported to Plant Tissue Culture Laboratory of Mae Fah Luang University in Chiang Rai Province in paper packets at room temperature on 25 January 2010 and 17 December 2009, respectively, where they were rewrapped in tissue paper put in silica gel-desiccated plastic bags. The capsules were kept at 4 °C in darkness until they were used for seed germination experiment on 31 January 2010.

2.2. Fungal material

Eight fungal isolates used in this study were: (1) two potentially new anamorph species in the genus *Tulasnella* from Clade I, Pv-QS-0-2 and C1-DT-TC-1; (2) three isolates identified as *Epulorhiza repens* (Bernard) Moore from Clade II, Cs-QS-0-1, Da-KP-0-1,

Table 1

Developmental stages of orchid seeds and protocorms.

Stage	Description
0	No germination, seed with intact seed coat
1	Enlarged embryo, seed coat ruptured by the enlargement of embryo (=germination)
2	Globular embryo, rhizoids present
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf and further development

Adapted from Johnson et al. (2007).

and Pv-PC-1-1; (3) one isolate identified as anamorph of *Tulasnella irregularis* Warcup & P.H.B. Talbot from Clade III, C3-DT-TC-2; (4) one isolate identified as *Epulorhiza calendulina*-like *Rhizoctonia* from Clade V, Pch-QS-0-3; and one isolate identified as potentially new anamorphic species in the family Tulasnellaceae, Pv-QS-0-1 (Nontachaiyapoom et al., 2010). The initial letters of fungal isolate codes (i.e., P, D, and C) indicate orchid genera (i.e., *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, respectively) from which the fungi were isolated. The fungal isolate in Clade IV, Cl-QS-0-1 was not used in this study because it grew very slowly compared to other fungal isolates (Nontachaiyapoom et al., 2010).

2.3. Seed germination

The seed germination experiment was carried out in a completely randomized design with 10 treatments [MS medium, oat meal agar (OMA; 10 g/l OMA, Himedia Laboratories, Mumbai, India, 8 g/l agar), and OMA inoculated with one of the eight fungal isolates] and three replicates. The orchid capsule was soaked in 95% ethyl alcohol and flamed. Seeds were transferred to a Petri dish and mixed to ensure the homogeneity. An approximately equal amount of seeds (approximately 600 seeds of *G. speciosum* and 800 seeds of *D. draconis*) was transferred to a piece of Whatman No. 1 filter paper (Whatman International, Kent, UK) placed on either MS medium or OMA. Inoculation was done by placing a 5 × 5 mm² block of potato dextrose agar (PDA) containing the fungal hyphae in the center of OMA medium. The plates were then wrapped in aluminum foil and incubated in the dark at 25 °C for one week in a plant growth chamber (Model 6150CP4 Contherm Scientific, Petone, New Zealand). After that, aluminum foil was removed and the plates were incubated at 25 °C in a 16-h-light/8-h-dark cycle for 15 weeks. Seed germination and protocorm developmental stages (adapted from Johnson et al., 2007; Table 1) were examined weekly under a stereomicroscope (Stemi DV4, Carl Zeiss, Jena, Germany). Photos of seeds and protocorms at different developmental stages were acquired at 13 weeks after sowing using the stereomicroscope attached with a digital camera (Moticam 2000, Motic, Xiamen, China), three photos at randomly chosen areas of the filter paper were taken for each replicate of each treatment. Percentage of seeds or protocorms at each developmental stage was calculated by dividing the number of seeds or protocorms at each stage by the total number of seeds and protocorms. Average developmental stage for each treatment was calculated by the formula $[(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d) + (4 \times e) + (5 \times f)]/100$ where *a*, *b*, *c*, *d*, *e*, and *f* are the percentages of seeds/protocorms at stages 0, 1, 2, 3, 4, and 5, respectively. Comparison of average developmental stages among different treatments was done using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test ($P < 0.05$). Randomly sampled protocorms were examined microscopically to confirm presence or absence of pelotons under a compound light microscope (Axiotech, Carl Zeiss) attached with a digital camera (PowerShot G9, Canon, Tokyo, Japan) at 14 weeks after seed sowing.

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