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Effect of germination media on *in vitro* symbiotic seed germination of three *Dendrobium* orchids

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ABSTRACT

In vitro symbiotic seed germination has become increasingly popular for orchid propagation. With very few exceptions, *in vitro* symbiotic seed germination methods use oat meal agar (OMA) as the medium. However, effects of oat concentrations on symbiotic germination of orchid seeds have not been investigated. In our first experiment, effectiveness of three concentrations of ground oat (1, 5, or 10 g/l) in OMA inoculated with an orchid mycorrhizal fungus (*Tulasnella deliquescens* (Juel) Juel), uninoculated OMA (10 g/l of oat) and one fifth Murashige and Skoog medium containing 6 g/l of sucrose (1/5MS) on promoting seed germination of *Dendrobium lindleyi* Steud. were compared. Germination and protocorm development of *D. lindleyi* were found to be enhanced by higher oat concentrations. The OMA containing 10 g/l of oat with mycorrhizal inoculation also outperformed 1/5MS. In our second and third experiments, the use of common orchid cultivation substrates as alternative media for *in vitro* symbiotic orchid seed germination was investigated. For *Dendrobium fimbriatum* Hook., we demonstrated that sphagnum peat moss inoculated with *T. deliquescens* either isolate Da-KP-0-1 or Pv-PC-1-1 in plastic containers could be used to germinate the orchid to advanced seedling stage, although the germination percentages were low, probably due to the waterlogged characteristic of the peat moss and low ventilation of the plastic boxes. For *Dendrobium findlayanum* C.S.P. Parish & Rchb.f., we used the mixture of peat moss and coir dust packed in Petri dishes to solve the mentioned problems and compared the effectiveness of this system to OMA and 1/5MS. The three treatments resulted in similar germination percentages but the symbiotic methods were more effective than 1/5MS in promoting protocorm development of *D. findlayanum*. The findings of this study can be used to improve symbiotic germination media and they encourage the use of symbiotic germination method as mean for orchid seed propagation.

1. Introduction

Seed germination is an essential step in orchid conservation and commercial orchid production. In conservation programs, seed propagated orchids are used for the plant reintroduction (Stewart and Kane 2006; Paul et al. 2012). In commercial orchid production, germination methods are used to propagate tissue culture-recalcitrant species (Zeng et al. 2014, 2016) and to propagate hybrids after cross-pollination. As orchid seeds lack endosperms, they contain either small levels of food reserves or the forms of food reserves that can hardly be metabolized by orchid embryos (Yam and Arditti 2009). In nature orchid seeds depend on orchid mycorrhizal fungi to provide necessary nutrients for germination, a process called symbiotic seed germination (Yam et al. 2009). Asymbiotic seed germination method, invented by Lewis Knudson, requires the addition of soluble sugar which serves as the carbohydrate source for orchid embryos (Knudson 1922; Yam and Arditti 2009). Although the asymbiotic germination method has been used successfully for many orchid species, symbiotic germination especially *in vitro* methods are gaining popularity because they promoted higher germination rates and/or symbiotic protocorms could develop more rapidly than asymbiotic protocorms in many studies (*e.g.*, Rasmussen et al. 1990; Johnson et al. 2007; Øien et al. 2008; Nontachaiyapoom et al. 2011; Nikabadi et al. 2014). Moreover, the *in vitro*-grown symbiotic seedlings are likely to be more adaptable to *ex vitro* conditions than asymbiotic seedlings since orchid mycorrhizal fungi have been reported to facilitate the uptake of water and nutrients (Yoder et al. 2000; Smith and Read 2008), promote photosynthetic performance and increase radiation-use efficiency (Lee et al. 2014). *In vitro* symbiotic seed germination methods generally use oat meal agar (OMA) with various concentrations of oat as the culture media, *i.e.*, 2.5 g/l (Øien et al. 2008; Tan et al. 2014), 3.0 g/l (Zettler et al. 2007), 4.0 g/l (Zhou and Gao 2016), and 10 g/l (Nontachaiyapoom et al. 2011). Surprisingly, despite its popularity, no study has specifically investigated effects of oat concentrations on the performance of symbiotic seed germination of orchids. The first experiment of this study was aimed to investigate the effects of oat concentrations (*i.e.*, 1, 5, and 10 g/l) in OMA on seed germination and

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protocorm development of *Dendrobium lindleyi* Steud., a popular ornamental orchid.

Agar is known to affect growth and development of *in vitro* plants such as causing hyperhydricity (Aggarwal and Nirmala 2012), and plantlets from agar-based media require an extra step of washing to remove the agar from the roots. Moreover, the price of OMA is increasing dramatically because the global shortage of *Gelidium* seaweed has pushed up the price of agar (Callaway 2015). Therefore, alternative germination medium for *in vitro* symbiotic seed germination is desirable. Previously, Aewsakul et al. (2013) reported the use of common orchid cultivation media (*i.e.*, soil, coir dust and sphagnum peat moss) as the germination media for *ex vitro* symbiotic seed germination of *Spathoglottis plicata* Blume. In the second and third experiments of this study, common cultivation substrates reported to be suitable for *ex vitro* symbiotic seed germination (Aewsakul et al. 2013) were tested for *in vitro* symbiotic seed germination of two *Dendrobium* orchids. At generic level, *Dendrobium* is among the most heavily traded genera and the most important cut-flower orchids in the global trade (Swangmaneecharern et al. 2012). Moreover, many *Dendrobium* species have been used in traditional medicines and/or reported to contain phytochemicals that have pharmaceutical activities (Hossain 2011; Bhattacharyya et al. 2016a, 2016b).

2. Materials and methods

2.1. Orchid seeds

Flowers of *D. lindleyi* plants grown in a private orchid nursery at Muang District, Chiang Rai were hand-pollinated in March 2014. Four undehisced mature capsules were harvested on 12 October 2014 and seeds were removed from the capsules on 17 October 2014. All seeds were kept in silica-gel desiccated tubes at 4 °C until use. Seeds of *D. fimbriatum* were removed from twenty naturally pollinated mature capsules using aseptic technique in April 2013, and seeds of *D. findlayanum* were removed from two naturally pollinated mature dehisced capsules on 14 June 2013.

2.2. Orchid mycorrhiza fungi

Two isolates of *Tulasnella deliquescens* (Juel) Juel (previously known as *Epulorhiza repens* (N. Bernard) R.T. Moore) isolate Da-KP-0-1 and Pv-PC-1-1 (Nontachaiyapoom et al. 2010) were used in this study. Fungal isolate Da-KP-0-1 was obtained from a root of *Dendrobium anosmum* Lind. and fungal isolate Pv-PC-1-1 was obtained from a root of *Paphiopedilum villosum* (Lindl.) Stein. They were reported to be highly effective in seed germination promotion of many *Dendrobium* orchids (Nontachaiyapoom et al. 2011; Swangmaneecharern et al. 2012). Agar pieces containing hyphae of this fungus, stored in sterile water at room temperature at Mae Fah Luang University, Chiang Rai, were used for the initial fungal culture. The agar pieces were placed on potato dextrose agar (PDA) containing antibiotics (30 μg/ml oxytetracycline, 30 μg/ml streptomycin, and 30 μg/ml ampicillin) at 30 °C for one week.

2.3. Seed germination experiments

2.3.1. Effects of oat concentrations on symbiotic seed germination of *D. lindleyi*

The seed germination experiment of *D. lindleyi* was conducted in a completely randomized design with five treatments and seven replicates, *i.e.*, one fifth Murashige and Skoog medium (Murashige and Skoog 1962) containing 6 g/l of sucrose (1/5MS), OMA with 10 g/l of ground oat without mycorrhizal inoculation, OMA with one of the three concentrations of ground oat (1, 5, or 10 g/l) with mycorrhizal inoculation. All media were gelled with 0.8% agar (Criterion™ C5001, Hardy Diagnostics, Santa Maria, CA, U.S.A.). Seeds were surface-sterilized in 50 ml of 1% sodium hypochlorite solution containing two drops of Tween-20 for 10 min and subsequently rinsed in sterilized water three times. Approximately equal amount of seeds were then sprinkled onto the media using a spatula. Mycorrhizal inoculation was done by aseptically placing a 1.2×1.2 cm^2 block of PDA containing fungal hyphae of the mycorrhizal isolate Da-KP-0-1 at the center of OMA. For OMA without mycorrhizal inoculation, a 1.2×1.2 cm^2 block of PDA was placed at the center of the medium. All Petri dishes were then kept in the dark at 25 °C for 1 week, and then in a 16-h-light/8-h-dark cycle at 25 °C for 11 weeks. During the 16-h light, irradiation with an intensity of 25–32 μmol m^{-2} s^{-1} was provided by 36 W Cool white fluorescent lamps (Philips, Bangkok, Thailand). Seed germination and protocorm developmental stages (*i.e.*, Stage 0, no germination; Stage 1, seed coat ruptured by enlarged embryo; Stage 2, globular embryo and rhizoids present; Stage 3, appearance of protomeristem; Stage 4, emergence of first leaf; Stage 5, elongation of first leaf and further development) were examined weekly. Photos of seeds and protocorms were acquired at 12 weeks after sowing using a stereomicroscope (Zeiss-Stemi 2000-C, Zeiss, Oberkochen, Germany) attached with a digital camera (Canon Powershot G9, Tokyo, Japan), 10 photos at randomly chosen areas 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. Comparisons of percentages of germination and percentages of seeds/protocorms at each developmental stage among the treatments were done using analysis of variance (ANOVA) and Tukey's HSD Test with $P = 0.05$. Randomly sampled protocorms from all treatments were examined microscopically to verify the presence or absence of pelotons.

2.3.2. Symbiotic seed germination of *D. fimbriatum*

The seed germination experiment of *D. fimbriatum* was conducted in a completely randomized design with three treatments, *i.e.*, peat moss without fungal inoculation, peat moss inoculated with either one of the two isolates (*i.e.*, Da-KP-0-1 and Pv-PC-1-1) of *T. deliquescens*. Preparation of germination substrate was done by soaking the sphagnum peat moss (Pindstrup Mosebrug A. S., Ryomgaard, Denmark) in tap water overnight three times, packed into 473-ml clear round plastic containers with thickness of about 1.5 cm and subsequently sterilized by autoclaving for 2 h. Peat moss was selected as germination substrate in this experiment because Aewsakul et al. (2013) showed that peat moss resulted in better germination and protocorm development compared to soil and coir dust. Fungal inoculation on peat moss was done by aseptically placing a 1×1 cm^2 block of PDA containing fungal hyphae in the center of the germination medium. The containers were then kept at 30 °C in dark for one week and subsequently the agar blocks were removed. Seeds were mixed to ensure the homogeneity using scalpel knife, then approximately equal amount of seeds were sprinkled onto peat moss. After seed sowing, the edges of the germination containers were sealed with thin polyvinylchloride cling film (Aro, Bangkok, Thailand), kept in the dark at 25 °C for 2 weeks, and then in a 16-h-light/8-h-dark cycle at 25 °C for 15 weeks. During the 16-h light, irradiation with an intensity of 25–32 μmol m^{-2} s^{-1} was provided by 36 W Cool white fluorescent lamps (Philips, Bangkok, Thailand). Seed germination and protocorm developmental stages were examined weekly. Photos for assessment of seeds and protocorms in different stages were performed as described above at 17 weeks after seed sowing. Randomly sampled protocorms from all treatments were examined microscopically to verify the presence or absence of pelotons. Comparison of germination percentages among the treatments was done using ANOVA and Tukey's HSD Test with $P = 0.05$.

2.3.3. Symbiotic seed germination of *D. findlayanum*

The seed germination experiment of *D. findlayanum* was conducted in a completely randomized design with three treatments, *i.e.*, 1/5MS containing 1.5% sucrose and 0.8% agar, OMA (10 g/l of oat, 8 g/l of agar) pre-inoculated with *T. deliquescens* isolate Da-KP-0-1, and the

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