การเพาะเมล็ดกล้วยไม้แบบสมชีพอย่างง่าย

Simplified *Ex Vitro* Symbiotic Seed Germination of Orchids

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บทคัดย่อ

การเพาะเมล็ดกล้วยไม้ เป็นวิธีขยายพันธุ์ที่ใช้ในการคัดเลือกกล้วยไม้ลูกผสม และการขยายพันธุ์กล้วยไม้ในโครงการอนุรักษ์ แม้ว่าการเพาะเมล็ดกล้วยไม้บนอาหารสังเคราะห์จะเป็นวิธีที่ใช้กันอย่างแพร่หลาย การเพาะเมล็ดกล้วยไม้โดยการเลี้ยงร่วมกับราไมคอร์ไรซากล้วยไม้ หรือการเพาะเมล็ดกล้วยไม้แบบสมชีพกำลังได้รับความนิยมเพิ่มขึ้น การเพาะเมล็ดกล้วยไม้แบบสมชีพนอกหลอดทดลอง เป็นวิธีเพาะเมล็ดแบบง่ายที่ไม่ต้องใช้เทคนิคปลอดเชื้อตั้งแต่การหว่านเมล็ดเป็นต้นไป การศึกษานี้ได้ดัดแปลงวิธีเพาะเมล็ดกล้วยไม้แบบสมชีพที่เคยมีรายงานก่อนหน้านี้ โดยการปรับปรุงวัสดุเพาะเมล็ดให้เหมาะสมยิ่งขึ้น และใช้กล่องพลาสติกบรรจุอาหารที่ฝามีรูติดฟิล์มที่สามารถระบายอากาศได้ เพื่อลดการปนเปื้อนจากไรและรา การศึกษานี้ยังได้สาธิตการเพาะเมล็ดกล้วยไม้ จำนวน 4 ชนิด ได้แก่ เอื้องหัวข้าวเหนียว (*Spathoglottis affinis* de Vriese) รองเท้านารีเหลืองเลย [*Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein] รองเท้านารีอินทนนท์ลาว (*Paphiopedilum gratrixianum* Rolfe) และเอื้องครั่งสายสั้น (*Dendrobium parishii* Rchb.f.) โดยใช้กล่องเพาะเมล็ดที่พัฒนาขึ้น และการใช้ไอโซเลทของราไมคอร์ไรซากล้วยไม้ *Tulasnella deliquescens* (Juel) Juel ที่มีประสิทธิภาพ แม้ว่าวิธีที่พัฒนาขึ้นจะให้เปอร์เซ็นต์การงอกและเปอร์เซ็นต์ของโพรโทคอร์มระยะที่ 5 ต่ำกว่าอาหารสังเคราะห์ (ได้แก่ อาหาร Murashige and Skoog ที่มีความเข้มข้นครึ่งสูตร) ในกล้วยไม้ 3 ชนิดที่ทดสอบ แต่วิธีที่พัฒนาขึ้นส่งเสริมการเจริญเติบโตของโพรโทคอร์ม/กล้ากล้วยไม้รองเท้านารีได้ดีกว่า บทความนี้ยังได้อภิปรายถึงศักยภาพและข้อจำกัดอื่น ๆ ของวิธีเพาะเมล็ดกล้วยไม้แบบสมชีพดังกล่าว

ABSTRACT

Orchid seed germination is a propagation method used in the selection of hybrids and conservation programs. Even though asymbiotic germination is the most widely used approach, symbiotic germination is gaining popularity. *Ex vitro* symbiotic germination is a simple seed germination method that does not require axenic condition from seed sowing step onwards. The present study modified the previously reported *ex vitro* symbiotic germination method by optimizing components of germination substrate and using plastic food containers with lids vented with air-permeable film to reduce the problem of mite and fungal contamination. We also demonstrated successful seed germination of four orchid species [i.e., *Spathoglottis affinis* de Vriese, *Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein, *Paphiopedilum gratrixianum* Rolfe and *Dendrobium parishii*

Rchb.f.] using the established method and effective isolates of an orchid mycorrhizal fungus, *Tulasnella deliquescens* (Juel) Juel. Although the established method resulted in less germination percentages and percentages of Stage 5 protocorms compared to asymbiotic medium (i.e., half strength of Murashige and Skoog medium) in three studied orchids, it supported more rapid growth of *Paphiopedilum* protocorms/seedlings. Other potentials and limitations of the established method are discussed in this article.

คำสำคัญ: การเพาะเมล็ดแบบสมชีพ ไมคอร์ไรซากล้วยไม้ รา *Tulasnella*
Keywords: Symbiotic seed germination, Orchid mycorrhiza, *Tulasnella*

INTRODUCTION

Orchidaceae is one of the largest flowering plant families with approximately 27,800 species worldwide (The Plant List, 2013). Orchids are economically important because they are sold as both cut flowers and potted plants. The high demand for orchids especially for those that cannot be successfully propagated in laboratory/ nursery prompts wild collection which leads to declining number of orchid populations in nature (Chugh et al., 2009; Nontachaiyapoom, 2013). The situation has been worsened by deforestation and climate change. Therefore, an easy and highly efficient propagation method of orchid is necessary to reduce the pressure of demand.

Seed germination of orchids is the best propagation method for orchid conservation because it conserves genetic diversity. Seed germination is also a routine step to propagate hybrids after cross-pollination. In nature, orchid seeds cannot germinate by themselves because they contain small levels of food reserves and must depend on orchid mycorrhizal fungi to provide them necessary nutrients for germination, a process called symbiotic seed germination (Yam and Arditti, 2009). Asymbiotic seed method, invented by Lewis Knudson, requires the addition of soluble sugar which is then used as carbohydrate source for orchid seeds (Knudson, 1992; Yam and Arditti, 2009). Although asymbiotic method has been used for propagation of many orchids, asymbiotic germination of some orchid species was not very successful (Tsutsui and Tomita, 1986; Chugh et al., 2009). Moreover, several comparative studies between asymbiotic and symbiotic seed germination demonstrated that symbiotic methods were more effective since symbiotic protocorms developed more rapidly than asymbiotic protocorms (e.g., Johnson et al, 2007; Nontachaiyapoom et al, 2011). Symbiotic seed germination techniques can be classified into four categories: *in situ*, *ex situ*, *ex vitro* and *in vitro* seed germination. *In vitro* and *ex vitro* methods have advantages over the other two methods in that the quality and quantity of orchid mycorrhizal fungus can be controlled. *Ex vitro* method is particularly advantageous because axenic condition is not required (Aewsakul et al, 2013). The first report of *ex vitro* method involved in cumbersome preparation of potting mix and yielded low percentages of seed germination (Quay et al, 1995). Later, Aewsakul et al. (2013) reported a simpler and more efficient *ex vitro* symbiotic seed germination method. However, the method of Aewsakul et al. (2013) still cannot be directly used by general orchid growers because a growth chamber and Petri dishes were required. The objectives of this study were to further simplify the *ex vitro* symbiotic seed germination method of Aewsakul et al. (2013) and to test the new method on several orchid species. *Tulasnella* fungi (previously known as

Epulorhiza spp.) are mycorrhizal fungi in roots of many orchid genera and species (Rasmussen, 2002; Dearnaley, 2007), and have been observed to promote seed germination of orchids which were not those from which the fungi were isolated (Swangmaneecharern et al, 2012). Therefore, *Tulasnella* fungi are potential germination promoting mycobionts for many genera and species of orchids (Swangmaneecharern et al., 2012). In this study, two effective isolates of *Tulasnella deliquescens* (Juel) Juel were used to germinate the tested orchids.

RESEARCH METHODOLOGY

1. Orchid seeds

Four undehisced mature capsules of *Spathoglottis affinis* de Vriese were collected from naturally pollinated plants in the nursery of Queen Sirikit Botanic Garden (QSBG), Chiang Mai, on 7 December 2014. Flowers of *Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein and *Paphiopedilum gratrixianum* Rolfe orchids grown in a private nursery in Chiang Mai were hand-pollinated on 4 April 2014 and on 14 November 2015, respectively. Twelve undehisced mature capsules of *P. hirsutissimum* were harvested on 7 December 2014 (247 days after pollination). Six undehisced mature capsules of *P. gratrixianum* were harvested on 11 August 2016 (241 days after pollination). Flowers of *Dendrobium parishii* Rchb.f. plants grown in the nursery of QSBG were hand-pollinated in May 2016. One dehisced mature capsules of *D. parishii* was harvested on 25 January 2017. All capsules were transported to Mae Fah Luang University, Chiang Rai, in silica-gel desiccated plastic bags and kept at 4ºC until the removal of the seeds.

Seeds of *S. affinis* and *P. hirsutissimum* were removed from the capsules on 10 December 2014 and subsequently stored in silica gel desiccated tubes at 4ºC until use on 18 and 16 September 2016, respectively. Seeds of *P. gratrixianum* were removed from the capsules on 1 September 2016 and subsequently stored in a silica-gel desiccated tube at 4ºC until use on 21 November 2016. Seeds of *D. parishii* were removed from the capsules on 7 February 2017 and subsequently stored in a silica gel desiccated tube at 4ºC until use on 3 March 2017.

2. Orchid mycorrhiza fungi

Tulasnella deliquescens (previously known as *Epulorhiza repens* (N. Bernard) R.T. Moore) isolates 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* Lindl. 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 (Nontachaiya-poom 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 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) (Otero et al., 2002) at 30°C for one week.

3. Seed germination experiments

The mixture of coir dust, peat moss and sand at a ratio of 12:7:1 by volume was used for the seed germination of *S. affinis*, *P. hirsutissimum* and *P. gratrixianum*, and the mixture of coir dust and peat moss at a ratio of 2:1 by volume was used for the seed germination of *D. parishii.* Sand was washed several times with plenty of water and autoclaved at 121°C for 30 minutes. Coir dust and peat moss were soaked in tap water overnight three times. The

germination substrates were mixed and dried in a hot air oven at 80°C for 45 min to reduce the moisture content of the substrate. Two hundred ml of the substrate mixture were packed with 1.5 cm of thickness to each germination pot and force was applied to flatten the surface. This pot was then put into a 473-ml clear round plastic box (Figure 1a). Then, three pieces of 1.5 cm^2 100-lb drawing papers were placed on the substrate and the germination containers were sterilized by autoclaving (45 minutes for the substrate mixture containing sand and 30 minutes for the substrate mixture containing only peat moss and coir dust). After autoclaving, the germination containers were transferred into a laminar air flow and dried with lids open for 30 minutes. Fungal inoculation was done by placing three pieces of 1.2 cm^2 blocks of PDA containing mycorrhizal mycelium on the papers. For uninoculated control, three pieces of 1.2 cm^2 blocks of PDA were used. Lids were closed and then the edge between the lids and the germination boxes were sealed with thin polyvinylchloride cling film (Aro, Bangkok, Thailand). The germination boxes were incubated at 30°C in dark for one week. Subsequently, the agar blocks and the papers were removed. The original lids were then replaced by lids vented with 4-cm^2 air-permeable cellophane.

Control treatments included water agar (WA), half strength of Murashige and Skoog medium (Murashige and Skoog, 1962) containing 20 g/l of sucrose (1/2MS), and oat meal agar with 10 g/l of ground oat (OMA; Mala et al., 2017). All media were gelled with 0.8% agar (Criterion™ C5001, Hardy Diagnostics, Santa Maria, CA, U.S.A.). They were sterilized at 121°C for 15 minutes. Subsequently, approximately 30 ml of the medium were poured into Petri dishes (100 mm x 20 mm). The seed germination experiments of *S. affinis*, *P. hirsutissimum* and *P. gratrixianum* were conducted in a completely randomized design (CRD) with four treatments, i.e., WA, 1/2MS, *ex vitro* symbiotic seed germination boxes with fungal isolate Da-KP-0-1, and *ex vitro* symbiotic seed germination boxes with fungal isolate Pv-PC-1-1. The seed germination experiment of *D. parishii* was conducted in the CRD with six treatments, i.e., WA, 1/2MS, OMA pre-inoculated with fungal isolate Da-KP-0-1, OMA pre-inoculated with fungal isolate Pv-PC-1-1, *ex vitro* symbiotic seed germination with fungal isolate Da-KP-0-1, *ex vitro* symbiotic seed germination with fungal isolate Pv-PC-1-1. After seed sowing, the germination plates/boxes were kept in dark for either four weeks (for seeds of terrestrial orchids, i.e., *S. affinis*, *P. hirsutissimum* and *P. gratrixianum)* or one week (for seeds of *D. parishii).* Then, Petri dishes of the *in vitro* treatments (i.e., WA, 1/2MS and OMA) were transferred to a 16-h-light/8-h-dark cycle at 25°C, while the *ex vitro* symbiotic seed germination boxes were kept on a shelf at room temperature under natural light. 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; Johnson et al., 2007) were examined weekly. Photos of seeds and protocorms of all treatments and replicates were acquired when protocorms in the best performing treatment reached stage 5 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. 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.

RESULTS AND DISCUSSION

Our previous attempts to use the plastic food containers as seed germination boxes and to keep the boxes after seed sowing in the non-controlled natural light, temperature and humidity were not successful because of serious fungal and mite contamination. In those experiments, the number of mites found in the *ex vitro* germination boxes was as high as 100-200 mites per box (unpublished data). Fungi and mites are common contaminants in orchid potting media. Aewsakul et al. (2013) reported fungal contamination on the *ex vitro* symbiotic germination media in all treatments in their study but the mycorrhizal inoculation treatments showed a lower degree of contamination. Similarly, we observed that the germination substrate of the uninoculated control in our previous experiments was heavily covered with contaminating fungi. The optimization of the germination substrate components and the use of container lids vented with the cellophane could dramatically reduce the mite contamination (the number of mites observed was reduced to 0-12 mites/box) in both uninoculated and mycorrhizal inoculated treatments and effectively reduced fungal contamination in mycorrhizal inoculated treatments but inadequately reduced the fungal contamination in *ex vitro* uninoculated treatments. Therefore, in the seed germination experiments in this study we used *in vitro* WA, and 1/2MS as the negative and positive controls, respectively.

Seeds of *S. affinis* sown on 1/2MS and on *ex vitro* symbiotic germination medium inoculated with either fungal isolate Da-KP-0-1 or Pv-PC-1-1 started to germinate at 2 weeks after sowing. Only protocorms in these treatments could develop to the most advanced stage (Stage 5, Table 1 and Figure 1c-1e). Seeds of *S. affinis* on WA germinated much later *(*6 weeks after sowing) and protocorms in this treatment did not develop beyond Stage 3 (Table 1 and Figure 1b). For this experiment, *ex vitro* symbiotic germination methods were found to be inferior to asymbiotic seed germination (1/2MS) since they resulted in significantly lower germination percentage and percentage of Stage 5-protocorms (Table 1). For *ex vitro* symbiotic seed germination of *S. affinis*, the two *T. deliquescens* isolates, Da-KP-0-1 and Pv-PC-1-1, performed equally well at promoting seed germination and protocorm development. Microscopic observation confirmed the presence of pelotons (i.e., hyphal coils of orchid mycorrhizal fungus) in protocorms in the two *ex vitro* symbiotic germination treatments (partly shown in Figure 1f) and the absence of peloton in protocorms on 1/2MS and WA (data not shown).

Table 1 Effects of seed germination methods and orchid mycorrhizal isolates on the seed germination and protocorms development of *S. affinis* at 9 weeks after seed sowing

Treatments[a]	Percentage of seeds and protocorms development[b]						Germination (%)
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
WA	83.04±1.83a	5.27±3.45ab	11.67±3.53a	0.01±0.04c	0.00±0.00c	0.00±0.00c	16.96±1.83b
1/2MS	69.82±2.39b	0.72±0.44c	0.49±0.25b	1.47±0.49bc	1.68±0.51bc	25.81±2.85a	30.16±2.39a
Da-KP-0-1	83.00±6.04a	2.22±2.28bc	1.62±1.24b	3.55±1.01a	3.73±2.07a	5.89±3.61b	17.00±6.04b
Pv-PC-1-1	78.28±1.74a	6.50±2.49a	2.62±1.34b	2.50±1.09ab	2.59±0.90ab	7.49±2.11b	21.72±1.74b

[a]WA and 1/2MS were done *in vitro*. Da-KP-0-1 and Pv-PC-1-1 represent *ex vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively.

[b]Values are means ± standard deviations of 8 replicates. Means in the same column marked with different letters are significantly different ($P<0.05$).

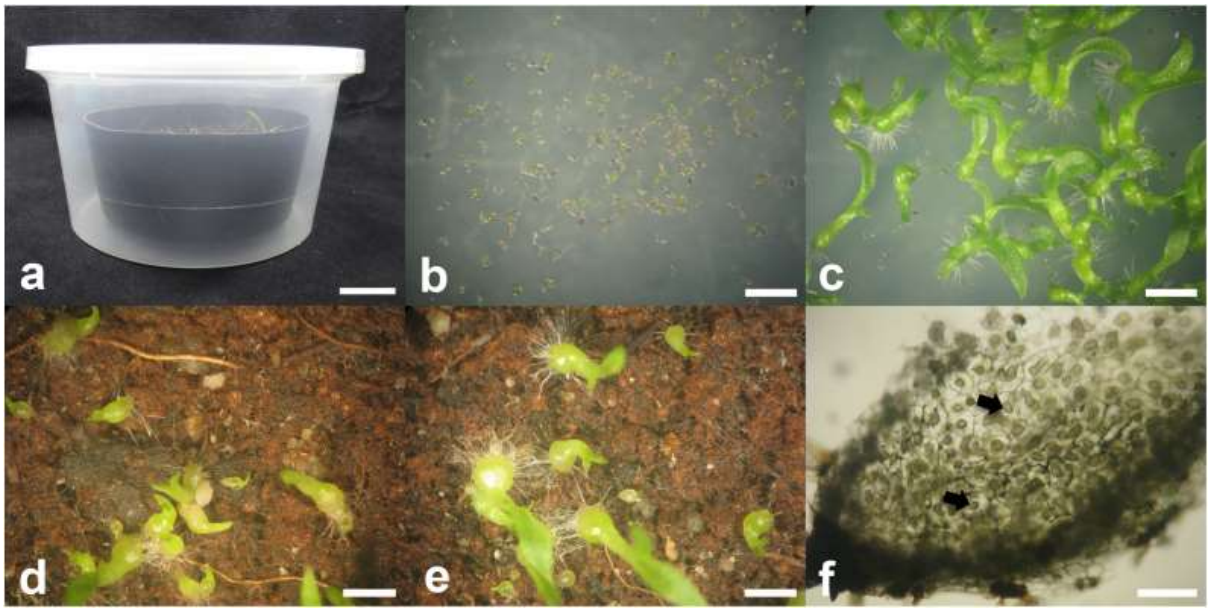

Figure 1 The *ex vitro* symbiotic seed germination box (a). Seeds and protocorms of *S. affinis* at 9 weeks after seed sowing on WA (b), 1/2MS (c), and *ex vitro* symbiotic germination medium inoculated with *T. deliquescens* isolate Da-KP-0-1 (d) or Pv-PC-1-1 (e). Pelotons (arrows) in a protocorm cultured on *ex vitro* symbiotic germination medium with the isolate Pv-PC-1-1 (f). Scale bars: (a) 2 cm, (b-e) 2 mm, (f) 0.2 mm.

Seeds of *P. hirsutissimum* on 1/2MS and on *ex vitro* germination medium inoculated with either fungal isolate Da-KP-0-1 or Pv-PC-1-1 started to germinate at 7 weeks after sowing, while seeds on WA did not germinate at all. Similar to the result of *S. affinis* seed germination, only protocorms on either 1/2MS or *ex vitro* germination medium with mycorrhizal inoculation could develop to Stage 5, and 1/2MS was the best performing treatment in term of germination percentage and percentage of Stage 5-protocorms (Table 2 and Figure 2). However, for this experiment, the sizes of symbiotic protocorms and seedlings of *P. hirsutissimum* were found to be much larger than those cultured on 1/2MS (Figure 2b-2d). For *ex vitro* symbiotic seed germination of *P. hirsutissimum*, the isolate of *T. deliquescens* highly

affected the germination percentage and protocorm development. The fungal isolate Pv-PC-1-1 resulted in significantly higher germination percentage (3.85%) and percentage of Stage 5 protocorms (1.14%) compared to those inoculated with the fungal isolate Da-KP-0-1 (0.88% and 0.18%, respectively; Table 2). Microscopic observation confirmed the presence of pelotons in protocorms in both symbiotic treatments and the absence of pelotons in protocorms on 1/2MS and WA (data not shown).

Table 2 Effects of orchid mycorrhizal isolates on the seed germination and protocorms development of *P. hirsutissimum* at 31 weeks after seed sowing

Treatments[a]	Percentage of seeds and protocorms development[b]						Germination (%)
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
WA	100.00±0.00a	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00c
1/2MS	92.37±3.90c	0.93±1.10b	0.13±0.23ab	1.38±1.14a	0.91±0.93a	4.27±3.64a	7.63±3.90a
Da-KP-0-1	99.12±0.58a	0.70±0.46b	0.00±0.00b	0.00±0.00b b	0.00±0.00b	0.18±0.41b	0.88±0.58c
Pv-PC-1-1	96.15±1.94b	2.34±1.42a	0.20±0.19a	0.07±0.14b	0.09±0.15b	1.14±0.98b	3.85±1.94b

[a]WA and 1/2MS were done *in vitro*. Da-KP-0-1 and Pv-PC-1-1 represent *ex vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively.

[b]Values are means ± standard deviations of 10 replicates. Means in the same column marked with different letters are significantly different ($P<0.05$).

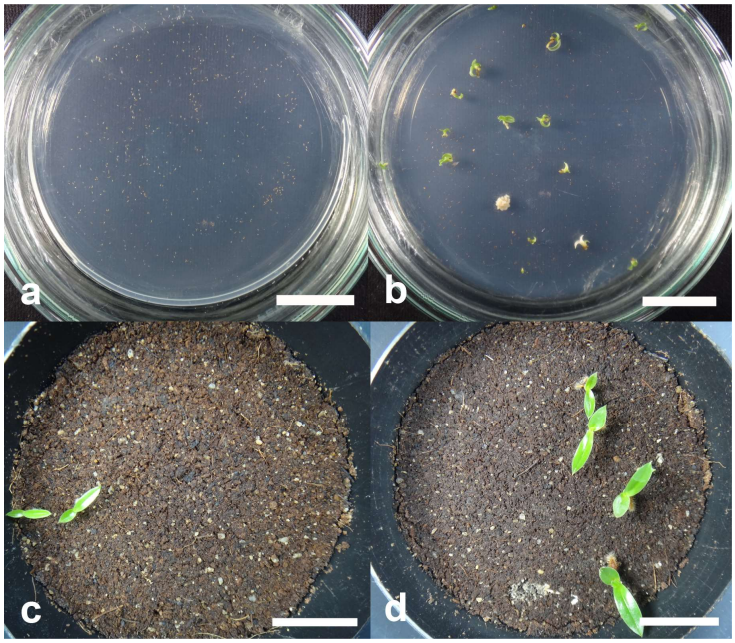

Figure 2 Seeds and protocorms of *P. hirsutissimum* at 31 weeks after seed sowing on WA (a), 1/2MS (b), and *ex vitro* symbiotic germination medium inoculated with fungal isolate Da-KP-0-1 (c) or Pv-PC-1-1 (d). Scale bars: 2 cm.

Seeds of *P. gratrixianum* germinated only on 1/2MS or on *ex vitro* symbiotic germination medium inoculated with the fungal isolate Pv-PC-1-1 after 7 weeks of sowing. Seeds of *P. gratrixianum* cultured on either WA or *ex vitro* symbiotic germination medium inoculated with the fungal isolate Da-KP-0-1 did not germinate at all. Microscopic observation confirmed the presence of pelotons in protocorms co-cultured

with the fungal isolate Pv-PC-1-1 and the absence of pelotons in the protocorms co-cultured with the fungal isolate Da-KP-0-1 and protocorms on WA and 1/ 2MS (data not shown) . Therefore, unlike *P. hirsutissimum* which was fully compatible with both fungal isolates Da-KP-0-1 and Pv-PC-1-1, *P. gratrixianum* was fully compatible with only the isolate Pv-PC-1-1. Interestingly, for *P. gratrixianum* seed germination, *ex vitro* symbiotic germination with the fungal isolate Pv-PC-1-1 and 1/2MS performed equally well in terms of germination percentage (3.16% and 3.18% , respectively) and the percentage of Stage 5-protocorms (2.43% and 0.74%, respectively; Table 3). Moreover, the sizes of symbiotic protocorms (and later developed into seedlings) inoculated with the isolate Pv-PC-1-1 were must larger than those on 1/ 2MS (Figure 3b, 3d). It is also important to note that low seed germination percentages of *Paphiopedilum* orchids in this study were observed. We hypothesized that the long period and the method of seed storage might be responsible to this problem. In this study, seeds of *P. hirsutissimum* and *P. gratrixianum* were stored in silica-gel desiccated tubes. Silica gel is known for its high water adsorbing capacity. Pritchard (2008) suggested that excessive drying could reduce the viability of orchid seeds in the long-term storage. For future studies of orchid seed germination, seed drying and storage can be performed as suggested by Seaton and Ramsay (2005).

Because of the availability of the seeds, we were able to include two more treatments (i.e., *in vitro* symbiotic seed germination using *T. deliquescens* either isolate D a -K P -0 -1 or Pv-PC-1-1) into the germination experiment of *D. parishii*. The germination of *D. parishii* seeds in all treatments was observed within two weeks after sowing. At 11 weeks after seed sowing, the 1/2MS and *in vitro* symbiotic germination with fungal isolate Pv-PC-1-1 resulted in significantly highest and lowest germination percentages, respectively (Table 4). Even though seeds on WA and *ex vitro* symbiotic germination medium with fungal isolate Pv-PC-1-1 could germinate, they did not develop beyond Stage 1 (Table 4 and Figure 4a, 4f). Only protocorms on 1/ 2MS, those co-cultured with fungal isolate Da-KP-0-1 either *in vitro* or *ex vitro*, and those co-cultured with fungal isolate Pv-PC-1-1 *in vitro* could develop to the most advanced stage (Table 4 and Figure 4b-4e) . The percentage of Stage 5-protocorms on 1/ 2MS was the highest (30. 85%), followed by those in the treatment of *in vitro* symbiotic germination with fungal isolate Da-KP-0-1 (5.72%) and *ex vitro* symbiotic germination with fungal isolate Da-KP-0-1 (1.84%), respectively. These results clearly showed that *D. parishii* seeds preferred fungal isolate Da-KP-0-1 and for the first time demonstrated variation in the orchid-mycobiont compatibility in the *in vitro* and *ex vitro* symbiotic seed germination systems. Orchid-mycobiont specificity was likely stricter in *ex vitro* condition compared to *in vitro* condition. Microscopic observation confirmed the presence of pelotons in protocorms in the treatments of *in vitro* and *ex vitro* symbiotic germination with fungal isolate Da-KP-0-1 and *in vitro* symbiotic germination with fungal isolate Pv-PC-1-1, and the absence of pelotons in protocorms in the remaining treatments. Furthermore, a significant number of protocorms in the *in vitro* symbiotic seed germination treatments appeared to be degraded and did not develop to advanced protocorm stages, while protocorm degradation was not observed in the *ex vitro* symbiotic seed germination treatments *(*Table 5 and Figure 4c, 4d*).* The source of nutrition *(*i.e., oat*)* of the *in vitro* symbiotic germination treatments might be responsible for the virulence of the orchid mycorrhizal

fungus. Beyrle et al. (1995) reported that levels of nitrogen combined with carbohydrate supply affected mycorrhizal interactions between *Orchis morio* L. and a mycorrhizal *Rhizoctonia*. Mycorrhizal interaction was established when a relatively low nitrogen supply to the fungus was combined with a high carbohydrate supply. Rejection of the fungus was associated with high-carbon and high-nitrogen medium. Mala et al. (2017), using *in vitro* symbiotic seed germination system, reported that germination and protocorm development of *Dendrobium lindleyi* Steud. were found to be enhanced by higher oat concentrations. Interestingly, in this study both mycorrhizal and saprotrophic interactions were observed simultaneously on the same germination medium. Further investigation will shade light on how nutrition affects mycorrhizal interaction.

Table 3 Effects of seed germination methods and orchid mycorrhizal isolates on the seed germination and protocorms development of *P. gratrixianum* at 22 weeks after seed sowing

Treatments[a]	Percentage of seeds and protocorms development[b]						Germination (%)
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
WA	100.00±0.00a	0.00±0.00a	0.00±0.00b	0.00±0.00b	0.00±0.00a	0.00±0.00b	0.00±0.00b
1/2MS	96.82±1.89b	0.06±0.22a	0.62±0.97a	1.24±0.98a	0.52±0.94a	0.74±0.79ab	3.18±1.89a
Da-KP-0-1	100.00±0.00a	0.00±0.00a	0.00±0.00b	0.00±0.00b	0.00±0.00a	0.00±0.00b	0.00±0.00b
Pv-PC-1-1	96.84±1.57b	0.29±0.49a	0.09±0.30ab	0.15±0.22ab	0.20±0.24a	2.43±1.23a	3.16±1.57a

[a]WA and 1/2MS were done *in vitro*. Da-KP-0-1 and Pv-PC-1-1 represent *ex vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively.

[b]Values are means ± standard deviations of 11 replicates. Means in the same column marked with different letters are significantly different ($P<0.05$).

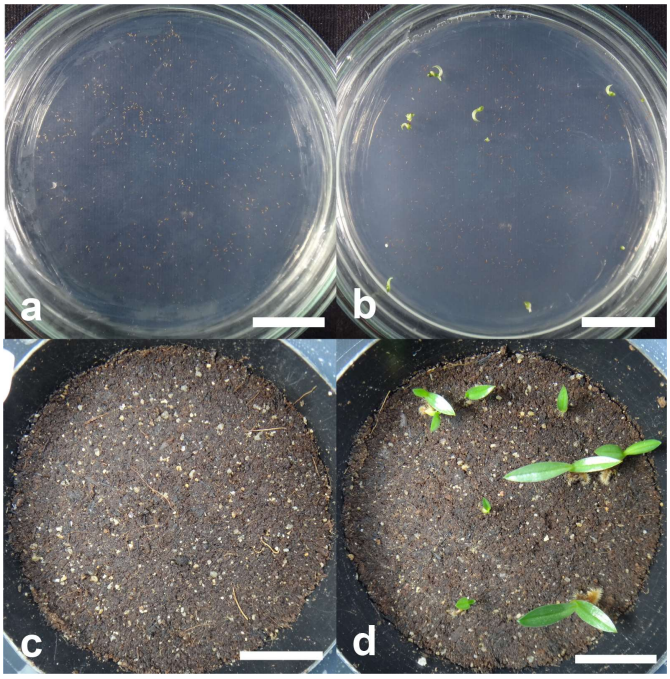

Figure 3 Seeds and protocorms of *P. gratrixianum* at 22 weeks after seed sowing on WA (a), 1/2MS (b), and *ex vitro* symbiotic germination medium inoculated with fungal isolate Da-KP-0-1 (c) or Pv-PC-1-1 (d). Scale bars: (a-d) 2 cm.

Table 4 Effects of seed germination methods and orchid mycorrhizal isolates on the developmental stages of seeds and protocorms of *D. parishii* at 11 weeks after seed sowing

Treatments[a]	Percentage of seeds and protocorms development[b]						Germination (%)
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
WA	75.66±4.13b	24.34±4.13a	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	24.34±4.13b
1/2MS	43.34±6.77c	1.14±0.63c	7.23±1.14a	13.16±2.02a	4.28±3.40a	30.85±9.06a	56.66±6.77a
Da-KP-0-1 (*in vitro*)	78.27±4.01b	9.18±7.14ab	1.58±1.59b	2.60±2.75b	2.65±2.78ab	5.72±5.91b	21.73±4.01b
Pv-PC-1-1 (*in vitro*)	92.53±5.42a	3.11±4.43ab	1.16±0.86b	2.77±2.83b	0.13±0.26b	0.30±0.27b	7.47±5.42c
Da-KP-0-1 (*ex vitro*)	82.28±2.90b	11.52±5.83b	1.46±0.21b	1.77±0.72b	1.13±1.02ab	1.84±1.70b	17.72±2.90b
Pv-PC-1-1 (*ex vitro*)	77.93±3.90b	22.07±3.90a	0.00±0.00b	0.00±0.00b	0.00±0.00b	0.00±0.00b	22.07±3.90b

[a]WA and 1/2MS were done *in vitro*. Da-KP-0-1 (*in vitro*) and Pv-PC-1-1 (*in vitro*) represent *in vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively. Da-KP-0-1 (*ex vitro*) and Pv-PC-1-1 (*ex vitro*) represent *ex vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively.

[b]Values are means ± standard deviations of five replicates with the exception that the treatment of Pv-PC-1-1 (*in vitro*) had four replicates because of contamination. Degraded protocorms in the *in vitro* treatments were excluded from the data analysis. Means in the same column marked with different letters are significantly different ($P<0.05$).

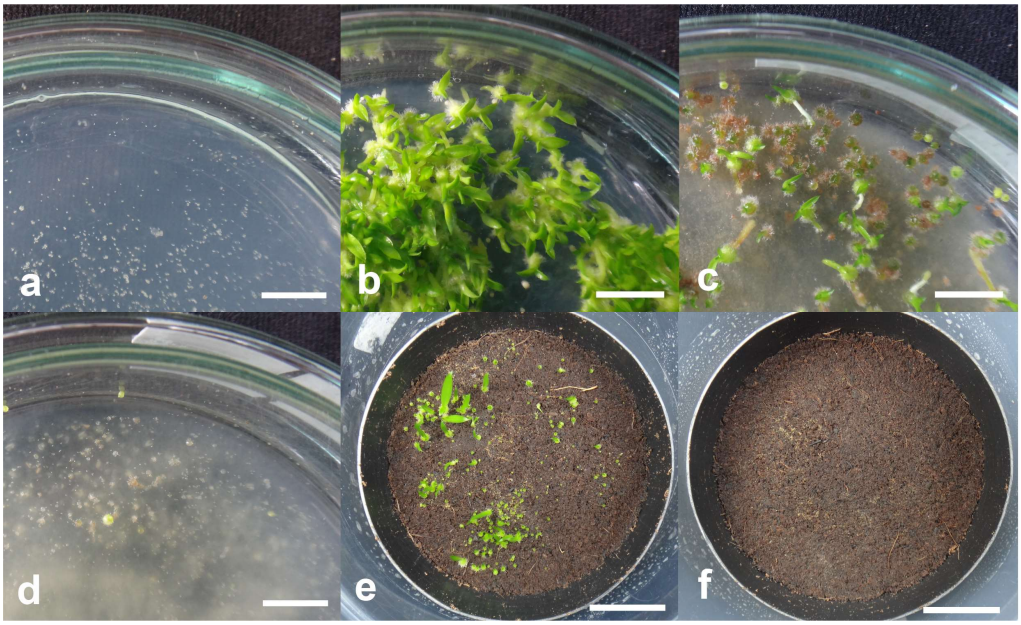

Figure 4 Seeds and protocorms of *D. parishii* at 11 weeks after seed sowing on WA (a), 1/2MS (b), OMA with fungal isolate Da-KP-0-1 (c) or Pv-PC-1-1 (d), and *ex vitro* symbiotic germination medium inoculated with fungal isolate Da-KP-0-1 (e) or Pv-PC-1-1 (f). Scale bars: (a-d) 1 cm, (e and f) 2 cm.

Table 5 Percentage of normal and degraded seeds and protocorms of *D. parishii* at 11 weeks after seed sowing

Treatments[a]	Percentage of[b]	
	Normal seeds/protocorms	Degraded seeds/protocorms
WA	100.00±0.00a	0.00±0.00b
1/2MS	99.84±0.35a	0.16±0.35b
Da-KP-0-1 (*in vitro*)	86.39±8.25b	13.61±8.25a
Pv-PC-1-1 (*in vitro*)	85.08±4.07b	14.92±4.07a
Da-KP-0-1 (*ex vitro*)	99.99±0.02a	0.01±0.02b
Pv-PC-1-1 (*ex vitro*)	100.00±0.00a	0.00±0.00b

[a]WA and 1/2MS were done *in vitro*. Da-KP-0-1 (*in vitro*) and Pv-PC-1-1 (*in vitro*) represent *in vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively. Da-KP-0-1 (*ex vitro*) and Pv-PC-1-1 (*ex vitro*) represent *ex vitro* symbiotic germination treatments using *T. deliquescens* isolate Da-KP-0-1 and Pv-PC-1-1, respectively.

[b]Values are means ± standard deviations of five replicates with the exception that the treatment of Pv-PC-1-1 (*in vitro*) had four replicates. Means in the same column marked with different letters are significantly different ($P<0.05$).

Lastly, although the established *ex vitro* symbiotic germination method resulted in less germination percentages and percentages of Stage 5 protocorms in three out of four experiments in this study, we would like to argue that the established *ex vitro* symbiotic germination method has several advantages over the conventional *in vitro* asymbiotic seed germination. Firstly, the established method does not require axenic condition during the seed germination step. Therefore, it is feasible for general orchid growers that have no access to expensive laboratory and scientific instruments. Secondly, orchid seedlings obtained from *ex vitro* symbiotic seed germination method does not require root washing and *ex vitro* acclimatization step. Many *in vitro*-propagated orchid plantlets died during the *ex vitro* transplanting (Arditti, 1994). Last but not least, several comparative studies between asymbiotic and symbiotic seed germination demonstrated that mycorrhizal protocorms developed more rapidly than asymbiotic protocorms (e.g., Johnson et al., 2007; Nontachaiyapoom et al., 2011). In this study, symbiotic protocorms/seedlings of *Paphiopedilum* orchids were larger than asymbiotic protocorms/seedlings.

CONCLUSIONS

The present study modified the *ex vitro* symbiotic seed germination method of Aewsakul et al. (2013) by optimizing components of germination substrate and using plastic food containers with lids vented with air-permeable film to reduce the problem of mite and fungal contamination. We also demonstrated successful seed germination of four orchid species (i.e., *S. affinis*, *P. hirsutissimum*, *P. gratrixianum* and *D. parishii*) using the established method and *T. deliquescens* either isolate Da-KP-0-1 or Pv-PC-1-1. Although *in vitro* asymbiotic seed germination (1/2MS) was found to be superior to *ex vitro* symbiotic germination method in term of germination percentages and the percentages of Stage 5-protocorms. However, the sizes of symbiotic protocorms/seedlings of *P. hirsutissimum* and *P. gratrixianum* in the *ex vitro* symbiotic seed germination treatments were larger than those on 1/2MS. Our experiments also clearly showed that different orchid

species preferred different orchid mycorrhizal isolates for symbiotic seed germination. The seeds of *S. affinis*, *P. hirsutissimum*, and *D. parishii* were fully compatible with either fungal isolate Da-KP-0-1 or Pv-PC-1-1 but the seeds of *P. hirsutissimum* showed preference towards fungal isolate Pv-PC-1-1 and the seeds of *D. parishii* showed preference towards fungal isolate Da-KP-0-1. The seeds of *P. gratrixianum* were fully compatible only to fungal isolate Pv-PC-1-1. Furthermore, we demonstrated for the first time that the compatibility between orchid and mycobiont in the *in vitro* and *ex vitro* symbiotic seed germination systems could differ greatly and that orchid-mycobiont specificity was likely stricter in the *ex vitro* condition compared to the *in vitro* condition. Interestingly, in this study both mycorrhizal and saprotrophic interactions between *D. parishii* protocorms and *T. deliquescens* either isolate Da-KP-0-1 or Pv-PC-1-1 were observed simultaneously on the same *in vitro* germination medium but not on *ex vitro* germination medium. It is interesting to further investigate how nutrition affects mycorrhizal interaction.

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