CHAPTER 4

EEFECTS OF ORCHID MYCORRHIZAL FUNGI ON SYMBIOTIC SEED GERMINATION

4.1 Introduction

Many studies reported many orchids have a symbiotic relationship with various fungi in their environment (Arditti *et al.* 1981; McKendrick *et al.* 2000, 2002; Athipunyakom *et al.* 2004b; Batty *et al.* 2006a; 2006b; Smith and Read, 2008; Swarts and Dixon, 2009). In nature, most orchid seeds cannot germinate or are able to start germinating but will not grow unless they are infected with mycorrhizal fungi which supplies young plants with a carbon source and inorganic nutrients (Brundrett *et al.* 1996, 2003; McKendrick *et al.* 2000; 2002; Swarts and Dixon 2009; Wright *et al.* 2009). The main genera of orchid mycorrhizal fungi associated with mature plants of six terrestrial orchid species were identified in chapter 3. Whether these isolated fungi can assist and enhance seed germination and early growth of seedlings remain to be tested.

Symbiotic seed germination is used to grow seeds with suitable fungi. The seeds become infected with the fungi and growth begins. Mycorrhizal plants are stronger and more resistant to pathogens, such as fungal diseases, than asymbiotic plants (Brundrett *et al.* 2001; 2003). For example, an efficient symbiotic seed germination protocol to germinate seeds of the rare sub-

tropical 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). Furthermore, Batty *et al.* (2006a) found that seed of several Australian temperate terrestrial orchid taxa, *C. arenicola* and *Pterostylis sanguinea*, germinated best when seeds were sown with mycorrhizal fungi but the fungi failed to stimulate tuber development. However, seedling survival improved when actively growing symbiotic seedlings were transferred to natural habitats during the growing season. This suggests that there may be a particular sequence of symbiotic events from seed germination to the adult plant with different fungi playing different roles.

In Thailand, there are only a few reports on orchid mycorrhizal fungi and their efficacy in facilitating seed germination and plant growth. Athipunyakom *et al.* (2004a) reported on seven genera and fourteen species of orchid mycorrhizal fungi isolated from eleven terrestrial orchid species. In another study, seed of one terrestrial orchid, *Spathoglottis plicata*, inoculated with *Epulorhiza repens* and *Rhizoctonia globularis* presented the initiation of leaves 60 days after the seeds were sown (Athipunyakom *et al.* 2004b).

Pecteilis susannae (L.) Rafin is a terrestrial orchid widely distributed in dry dipterocarp forest and rainforest throughout Thailand. The species has become threatened with extinction due to the loss of its natural habitat as well as over-harvesting for illegal trade. The orchid capsules appear to have few viable seeds. Micropropagation using tissue culture techniques are not yet available (Santisuk *et al.* 2006; Suyanee Vessabutr, personal communication). Therefore, this study aimed to investigate the orchid mycorrhizal fungi associated with *P. susannae* and

determine their benefit for seed germination. The data collected from this study will be used for further propagation and conservation of this orchid.

4.2 Materials and methods

4.2.1 Orchid seed collection

Seeds (Figure 4.1d) were obtained from mature capsules (Figure 4.1a, b and c) in natural habitat (Site 2; Sa-Lunag, Chiang Mai, Thailand) in October-November 2008. Immediately following collection, capsules were dried over silica gel for 2 weeks at 25 °C, followed by storage at 4 °C in the dark until used, within 4 months (Stewart and Kane, 2006).

4.2.2 Orchid seed viability test

Viability of seeds was determined using the tetrazolium test (Brundrett *et al.* 2001) before used in each symbiotic seed germination experiment. Orchid seeds were placed in 10 ml of 5% CaOCl (w/v) and 1% tween 80 (v/v) for 5 h. The bleach solution was pipetted off and replaced with sterile water 2 times, and the seeds left in water for 24 h. The water was pipetted off and replaced with 10 ml of 1% TTC solution (1 g of 2.3.5-triphenyltetrazolium chloride in 100 ml of sterile distilled water, adjust pH to 7 with 0.1 M NaOH) and the seeds were incubated in the dark at 30˚C for 24 h. The TTC solution was pipetted off and the seeds were washed 3 times for 5 min each in sterile distilled water. The seeds were placed in Petri dish and examined under a stereomicroscope to score viable

seed with red stained embryo. The percentage viability was calculated by dividing the number of seed with stained by the total number of seeds counted.

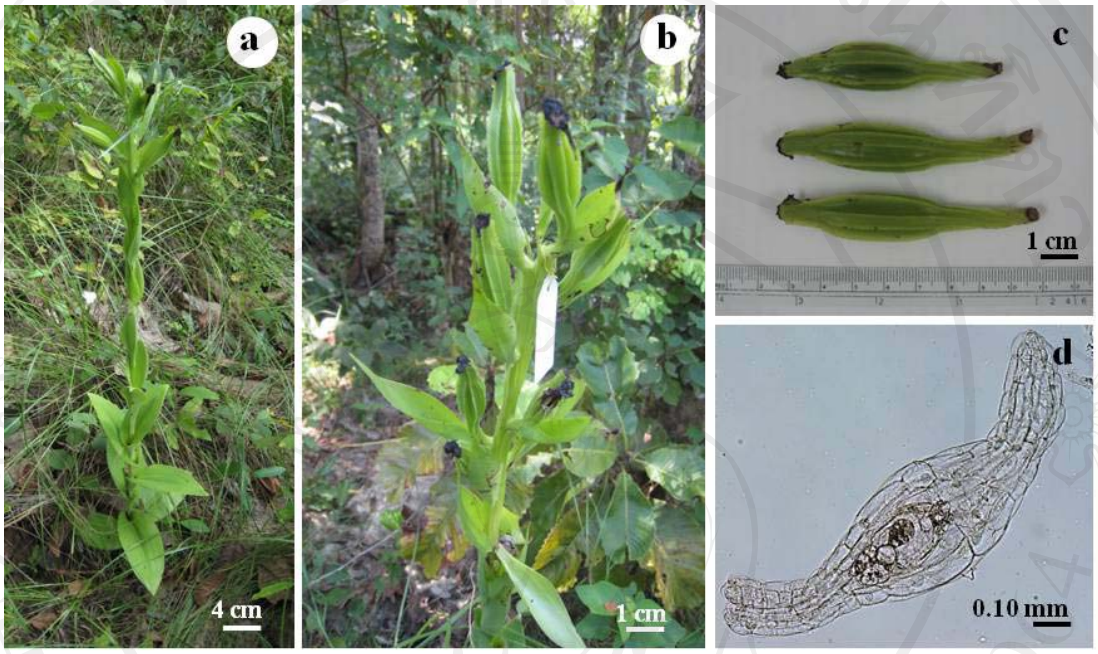

Figure 4.1 *Pecteilis susannae* in natural habitat (site 2: Sa-Lunag, Chiang Mai, Thailand): orchid plant with capsules (a) and (b), orchid capsules (c) and orchid seed (d)

4.2.3 Symbiotic seed germination study of *P.susannae*

4.2.3.1 Effects of agar media on symbiotic seed germination

The effects of 3 agar media (Oatmeal agar, Cellulose agar, and Vacin and Went agar, 1949) on symbiotic seed germination of *P. susannae* using 7 mycorrhizal fungi isolated from *P. susannae* (Table 4.1) were studied. The symbiotic effects of these fungal isolates on orchid seed germination were

evaluated using a modified procedure of Stewart and Kane, (2006). Seeds were sown from capsules after surface sterilization for 1 min in 95% ethanol: 5.25% NaOCl: sterile distilled water (1:1:1 v/v/v). Seeds (50-100) were placed over the surface of a 1 × 4 cm filter strip (Whatman No.4) within each 9 cm diameter Petri dish plate containing 20 ml of each medium. The pH of all agar media was adjusted to 6.0 with 0.1 M NaOH. Each plate was inoculated with a 1 × 1 cm plug of each fungal inoculum taken from the hyphal edge 5 days after culturing on PDA. Uninoculated plates served as a control. Each treatment was replicated on 4 plates. Plates were sealed with parafilm and wrapped in aluminum foil to exclude light and maintained in the dark at 25°C for 70 days. The plates were examined weekly using a stereomicroscope and returned to the dark conditions. Seed germination and protocorm development were scored on a scale of 0 - 5 (Table 4.2). Seed germination and protocorm development percentages were calculated by dividing the number of seeds in each germination and developmental stage by the total number of viable seeds in the sample. The data were statistically analyzed using SPSS V16.0 for one-way analysis of variance (ANOVA) and means were compared by Duncan's Multiple Range test ($P \leq 0.05$).

4.2.3.2 Effects of endophytic fungi recovered from *P. susannae* on symbiotic seed germination

The symbiotic effects of all endophytic fungi isolated from *P. susannae* (Table 4.3) on its seed germination using Oatmeal agar (pH 6.0) were evaluated using the modified procedure of Stewart and Kane, (2006) described in 4.3.2.1. Each treatment was also replicated on 4 plates. Plates were sealed with parafilm

and wrapped in aluminum foil to exclude light and maintained in the same condition mentioned in 4.3.2.1 for 70 days. The plates were examined weekly using a stereomicroscope and returned to the dark condition. The percentages of seed germination and protocorm development were scored, calculated and statistic analysis performed as described in 4.3.2.1.

4.2.3.3 Effects of mycorrhizal fungi on symbiotic seed germination

The symbiotic effects of 3 selected effective mycorrhizal fungi (CMU-AUG 007, CMU-AUG 013 and CMU-AUG 028) and mycorrhizal fungi isolated from *Eulophia spectabilis*, *Paphiopedilum bellatulum* and *Spathoglottis affinis* (Table 4.4) on seed germination of *P. susannae* were evaluated using a modified procedure of Stewart and Zettler, (2002) and Stewart and Kane, (2006). Symbiotic seed germination was undertaken using the method described in 4.2.3.2. Plates were sealed with parafilm and wrapped in aluminum foil to exclude light and maintained in the dark at 25°C for 70 days, followed by a 12 h photoperiod (light 1,000 Lux: dark, 12:12 h) at 25°C for 63 days. The plates were examined weekly using a stereomicroscope and returned to the incubation conditions. The percentages of seed germination and protocorm development were scored, calculated and statistically analyzed using the method described in 4.3.2.1.

Table 4.1 Mycorrhizal fungi isolated from roots of *Pecteilis susannae* and used to determined effects of agar media on symbiotic seed germination

Fungal isolate	Fungal taxa	Orchid location
CMU-AUG 002	*Epulorhiza* sp.	site 1 July 2005
CMU-AUG 028	*Epulorhiza* sp.	site 1 July 2005
CMU-AUG 007	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 013	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 040	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 031	*Epulorhiza* sp.	site 3 July 2007
CMU-AUG 025	*Epulorhiza* sp.	site 3 September 2007

Table 4.2 Seed germination and protocorm developmental stages use for determination of growth and development of *Pecteilis susannae in vitro*, adapted from Stewart and Kane, (2006)

Stage	Description
0	No germination, viable embryo
1	Swollen embryo, production of rhizoids
2	Continued embryo enlargement, rupture of testa, further production of rhizoids
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf

Table 4.3 Endophytic fungi isolated from roots of *Pecteilis susannae* and used in symbiotic seed germination

Fungal isolate	Fungal taxa	Orchid location
CMU-AUG 035	*Cladosporium* sp.	site 1 July 2005
CMU-AUG 038	*Cladosporium* sp.	site 1 July 2005
CMU-AUG 001	*Colletotrichum gloeosporioides*	site 1 July 2005
CMU-AUG 002	*Epulorhiza* sp.	site 1 July 2005
CMU-AUG 028	*Epulorhiza* sp.	site 1 July 2005
CMU-AUG 050	*Fusarium* sp.	site 1 July 2005
CMU-AUG 055	*Fusarium* sp.	site 1 July 2005
CMU-AUG 044	*Pestalotiopsis* sp.	site 1 July 2005
CMU-AUG 022	*Xylaria* sp.	site 1 July 2005
CMU-AUG 036	Mycelia sterilia	site 1 July 2005
CMU-AUG 078	*Cladosporium* sp.	site 2 August 2007
CMU-AUG 077	*Cladophialophora* sp.	site 2 August 2007
CMU-AUG 070	*Colletotrichum* sp.	site 2 August 2007
CMU-AUG 007	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 013	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 040	*Epulorhiza* sp.	site 2 August 2007
CMU-AUG 021	*Fusarium* sp.	site 2 August 2007
CMU-AUG 006	*Nodulis sporium*	site 2 August 2007
CMU-AUG 024	*Trichocladium pyriforme*	site 2 August 2007
CMU-AUG 019	*Xylaria* sp.	site 2 August 2007
CMU-AUG 003	*Dothiodeomycete* sp.	site 3 July 2007
CMU-AUG 031	*Epulorhiza* sp.	site 3 July 2007
CMU-AUG 005	*Fusarium* sp.	site 3 July 2007
CMU-AUG 020	Mycelia sterilia	site 3 July 2007

Table 4.3 (continued)

Fungal isolate	Fungal taxa	Orchid location
CMU-AUG 025	*Epulorhiza* sp.	site 3 September 2007
CMU-AUG 008	*Fusarium* sp.	site 3 September 2007
CMU-AUG 042	*Pestalotiopsis* sp.	site 3 September 2007
CMU-AUG 033	*Phoma* sp.	site 3 September 2007

Table 4.4 Mycorrhizal fungi isolated from roots of *Pecteilis susannae*, *Eulophia spectabilis*, *Paphiopedilum*, and *Spathoglottis affinis* and used in symbiotic seed germination

Fungal isolate	Fungal taxa	Orchid source	Orchid location
CMU-AUG 007	*Epulorhiza* sp.	*Pecteilis susannae*	site 2 August 2007
CMU-AUG 013	*Epulorhiza* sp.	*Pecteilis susannae*	site 2 August 2007
CMU-AUG 028	*Epulorhiza* sp.	*Pecteilis susannae*	site 1 July 2005
CMU-STE 003	*Gloeotulasnella* sp.	*Eulophia spectabilis*	site 4 Doi Suthep
CMU-STE 004	*Epulorhiza* sp.	*Eulophia spectabilis*	site 4 Doi Suthep
CMU-STE 011	*Tulasnella* sp.	*Eulophia spectabilis*	site 4 Doi Suthep
CMU-STE 014	*Epulorhiza* sp.	*Eulophia spectabilis*	site 4 Doi Suthep
CMU-SLP 007	*Epulorhiza* sp.	*Paphiopedilum bellatulum*	local market
CMU-SLP 008	*Epulorhiza* sp.	*Paphiopedilum bellatulum*	local market
CMU-AU 211	*Epulorhiza* sp.	*Spathoglottis affinis*	local market
CMU-AU 212	*Tulasnella* sp.	*Spathoglottis affinis*	local market

4.3 Results

4.3.1 Symbiotic seed germination of *P. susannae*

4.3.1.1 Effects of agar media on symbiotic seed germination

The tetrazolium test revealed that *P. susannae* seeds were 66.99% viable. The viable seed containing a red stained embryo, while dead seed was hyaline after being treated with tetrazolium (Figure 4.2). All symbiotic and asymbiotic seed (control) began to swell and germinate within 14 days after sowing. An effect of endophytic fungi on seed germination of *P. susannae* using 3 agar media (Oatmeal agar, Cellulose agar and Vacin and Went agar) was evaluated after sowing for 70 days (Table 4.5). The best protocorm developmental stage was found in stage 2 after sowing the seeds with fungal isolates, CMU-AUG 013 (2.4%) and CMU-AUG 028 (2.2%) on Oatmeal agar. No symbiotic seed germination treatments were reached to stage 2 of protocorm developmental stage using Cellulose agar and Vacin and Went agar. Moreover, the determination of seed germination and protocorm development were difficult on Vacin and Went agar due to fungal isolates being fast growing on this agar medium. Therefore, the Oatmeal agar was selected to use in symbiotic seed germination for next experiment.

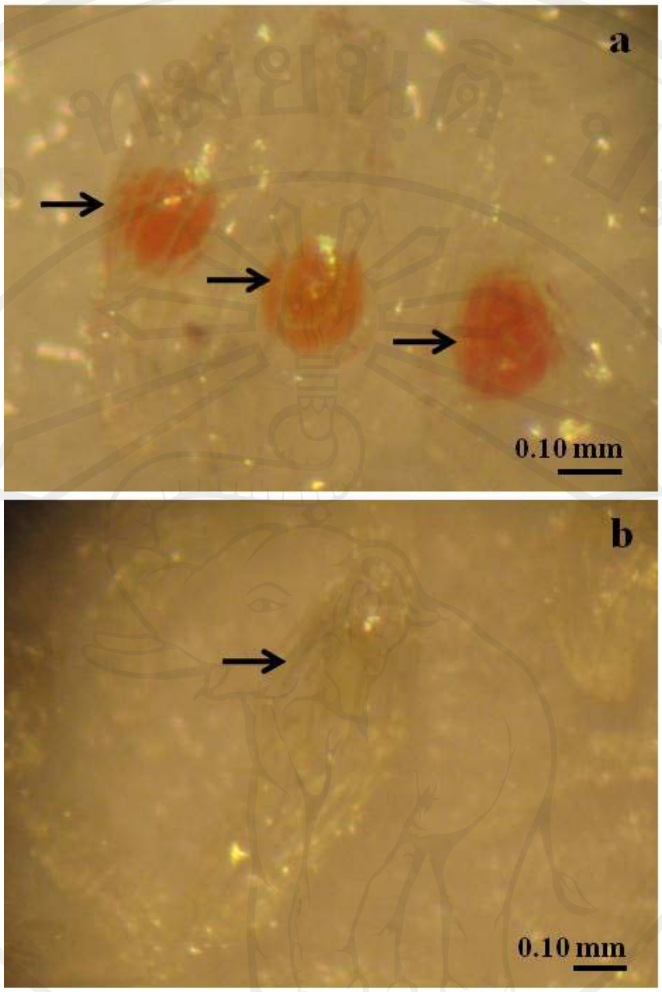

Figure 4.2 Viable seed with the embryo stained red (a) and hyaline embryo of dead seed (b) of *Pecteilis susannae* after being treated with tetrazolium

Table 4.5 Effects of 3 agar media (Oatmeal agar, Cellulose agar and Vacin and Went agar) on symbiotic seed germination and protocorm development of *Pecteilis susannae* 70 days after sowing

Agar medium	Oatmeal agar		Cellulose agar		Vacin and Went agar	
Protocorm development	Stage 1 (%)	Stage 2 (%)	Stage 1 (%)	Stage 2 (%)	Stage 1 (%)	Stage 2 (%)
Fungal isolate						
Control (uninoculated)	54.5±14.0^{a}	1.5±1.1^{ab}	41.2±9.7^{b}	0	65.2±13.1^{a}	1.2±0.9^{a}
CMU-AUG 002	42.3±4.3^{b}	0^{b}	0^{d}	0	0^{d}	0^{b}
CMU-AUG 007	62.3±6.8^{a}	0^{b}	53.0±4.7^{a}	0	48.9±12.1^{bc}	0^{b}
CMU-AUG 013	61.1±8.7^{a}	2.4±1.8^{a}	0^{d}	0	69.9±7.9^{a}	0^{b}
CMU-AUG 025	41.1±2.8^{bc}	0^{b}	44.6±3.4$_{b}$	0	37.2±8.0^{c}	0^{b}
CMU-AUG 028	57.6±8.7^{a}	2.2±1.8^{a}	0^{d}	0	47.7±6.7^{bc}	0^{b}
CMU-AUG 031	14.5±2.1^{d}	0^{b}	32.6±3.5^{c}	0	42.1±8.6^{c}	0^{b}
CMU-AUG 040	29.1±7.8^{cd}	0^{b}	40.4±6.4^{b}	0	59.0±3.5^{ab}	0^{b}

The results are means (n = 4) ± SE. Means with the same letter for each stage are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

4.3.1.2 Effects of endophytic fungi recovered from *P. susannae* on symbiotic seed germination

The tetrazolium test revealed *P. susannae* seeds to be 51.25% viable. All 27 fungal endophytes isolated from roots of *P. susannae* were tested for symbiotic seed germination of *P. susannae*. The seed in 9 treatments [Asymbiotic seed (control) and the symbiotic seed using 7 *Epulorhiza* isolates (CMU-AUG 002, CMU-AUG 007, CMU-AUG 013, CMU-AUG 025, CMU-AUG 028, CMU-AUG

031 and CMU-AUG 040), and 1 *Fusarium* isolate (CMU-AUG 021)] began to swell and germinate within 14 days after sowing. In the remaining 19 treatments, the orchid seeds were rapidly overgrown by the fungi. An effect of 8 endophytic fungi on seed germination of *P. susannae* was found and evaluated after sowing for 70 days, as shown in Figure 4.3. Seed germination and protocorm development to stage 2 was reached and the numbers were significantly higher than the uninoculated control (11.1%) when seeds were inoculated with fungal isolates CMU-AUG 007 (17.7%) and CMU-AUG 013 (17.6%). Moreover, seed germination and protocorm developmental stage 1 was significantly higher than the control (51.0%) for fungal isolates CMU-AUG 007 (62.2%), CMU-AUG 013 (68.5%) and CMU-AUG 028 (64.6%) (Figure 4.4). These 3 fungal isolates from this experiment will be selected for further symbiotic seed germination study of *P. susannae* and compared with fungal endophytes isolated from other orchids.

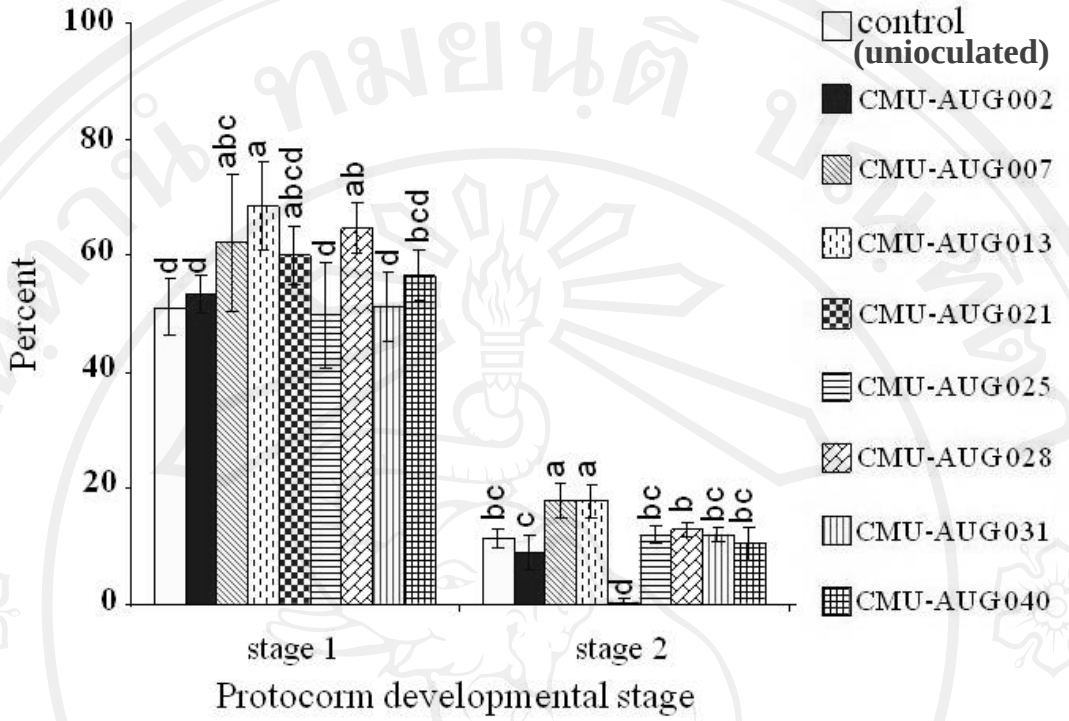

Figure 4.3 Effects of 8 endophytic fungi on seed germination and protocorm development of *Pecteilis susannae* 70 days after sowing. The results are means (n = 4) ± SE. Means with the same letter for each stage are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

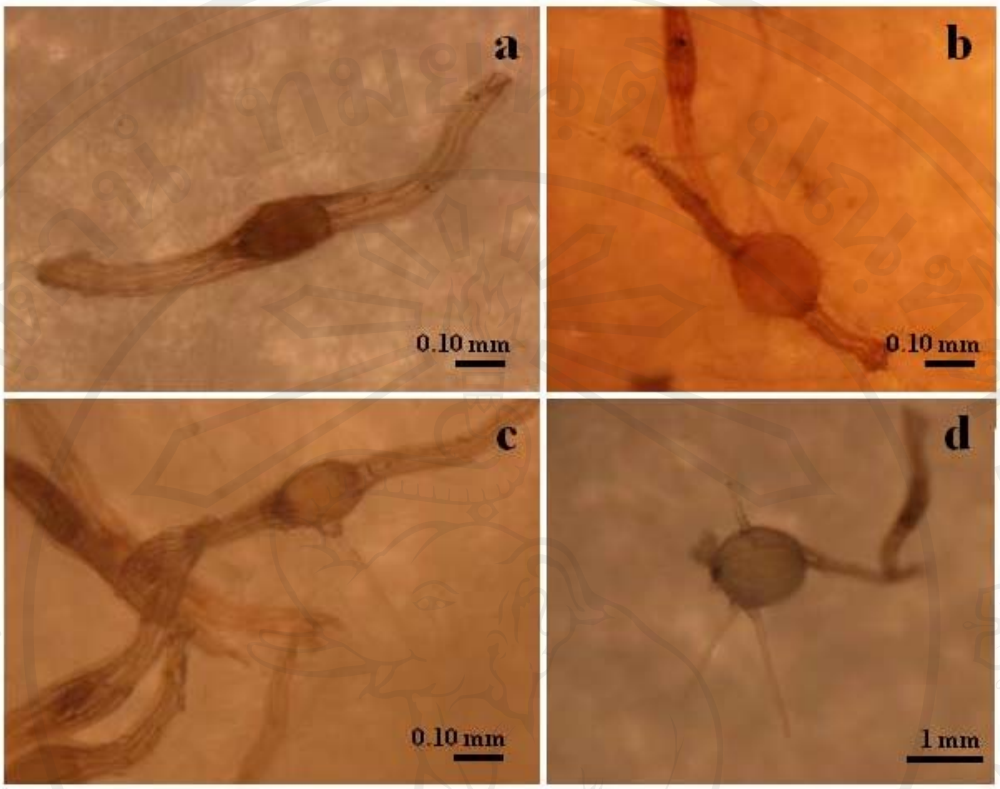

Figure 4.4 Symbiotic seed germination and protocorm developmental stages of *Pecteilis susannae* using fungal endophyte *Epulorhiza* sp., CMU-AUG 013, cultured on Oatmeal agar 70 days after sowing. (a) stage 0, (b and c) stage 1 and (d) stage 2 of protocrom development

4.3.1.3 Effects of mycorrhizal fungi on symbiotic seed germination

The tetrazolium test revealed *P. susannae* seeds to be 67.13% viable. As in previous experiment, all symbiotic and asymbiotic seed (control) treatments began to swell and germinate within 14 days after sowing. Effect of fungal isolate on seed germination of *P. susannae* was evaluated after sowing for 133 days as shown in Figure 4.5. Seed germination and protocorm developmental stages 2 and 3 were present 70 days after sowing in the dark. Furthermore, in this study

protocorm development was advanced up to stage 5 when seeds were inoculated with *Epulorhiza* isolates CMU-AUG 028 (4.3%), CMU-AUG 007 (4.2%), CMU-STE 014 (3.9%) and CMU-AUG 013 (2.2%) and incubated in 12 h of photo period (light: dark,12: 12 h) for 63 days after excluding light for 70 days. In addition, seed germination and protocorm developmental stage 4 were promoted with fungal isolates CMU-STE 011 (5.7%) and CMU-AU 212 (4.3%). Without fungi, protocorm development was arrested at stage 3. Lastly, fungal isolates CMU-SLP 007 (8.9%), CMU-SLP 008 (6.7%) and CMU-AU 211 (6.0%) increased seed germination at protocorm developmental stage 3 when compared with the control (3.5%), (Table 4.6 and Figure 4.6).

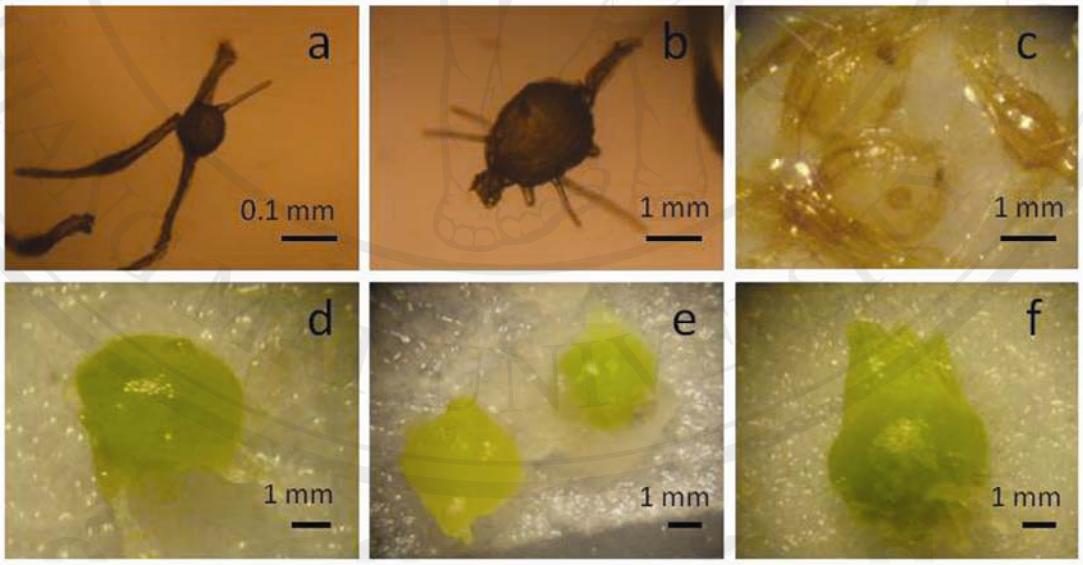

Figure 4.5 Symbiotic seed germination and protocorm developmental stages of *Pecteilis susannae* using mycorrhizal fungi *Epulorhiza* sp., CMU-AUG 007, cultured on Oatmeal agar 133 days after sowing. (a) stage 1, (b) stage 2, (c and d) stage 3, (e) stage 4 and (f) stage 5 of protocorm development

Table 4.6 Effects of 11 endophytic fungi on seed germination and protocorm development of *Pecteilis susannae* 133 days after sowing

Fungal isolate	Orchid source	Protocorm development (%)				
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Control (uninoculated)		20.5 ± 1.5^{c}	16.1 ± 0.7^{d}	3.5 ± 0.7^{h}	0^{d}	0^{c}
CMU-AUG 007	*Pecteilis susannae*	11.1 ± 0.7^{f}	21.2 ± 1.5^{c}	18.3 ± 1.4^{b}	15.1 ± 1.1^{a}	4.2 ± 1.1^{a}
CMU-AUG 013	*Pecteilis susannae*	17.7 ± 1.6^{de}	16.0 ± 0.8^{d}	24.5 ± 2.3^{a}	4.4 ± 0.8^{c}	2.2 ± 0.9^{b}
CMU-AUG 028	*Pecteilis susannae*	20.4 ± 1.6^{c}	15.7 ± 1.1^{d}	11.3 ± 0.7^{d}	6.0 ± 1.2^{b}	4.3 ± 0.2^{a}
CMU-STE 003	*Eulophia spectabilis*	30.7 ± 2.2^{a}	15.5 ± 1.7^{d}	0^{i}	0^{d}	0^{c}
CMU-STE 004	*Eulophia spectabilis*	18.7 ± 0.9^{cd}	29.1 ± 1.4^{a}	4.7 ± 0.8^{gh}	0^{d}	0^{c}
CMU-STE 011	*Eulophia spectabilis*	31.2 ± 1.7^{a}	12.4 ± 1.9^{e}	14.3 ± 0.7^{c}	5.7 ± 0.5^{b}	0^{c}
CMU-STE 014	*Eulophia spectabilis*	17.3 ± 1.5^{de}	15.7 ± 2.5^{d}	11.0 ± 1.0^{d}	4.1 ± 1.2^{c}	3.9 ± 0.7^{a}
CMU-SLP 007	*Paphiopedilum bellatulum*	18.1 ± 1.0^{de}	21.6 ± 0.7^{c}	8.9 ± 1.9^{e}	0^{d}	0^{c}
CMU-SLP 008	*Paphiopedilum bellatulum*	26.4 ± 0.4^{b}	28.3 ± 0.9^{a}	6.7 ± 1.5^{f}	0^{d}	0^{c}
CMU-AU 211	*Spathoglottis affinis*	20.8 ± 1.9^{c}	7.2 ± 0.5^{f}	6.0 ± 1.5^{fg}	0^{d}	0^{c}
CMU-AU 212	*Spathoglottis affinis*	15.8 ± 1.8^{e}	23.9 ± 0.5^{b}	14.2 ± 0.6^{c}	4.3 ± 1.0^{c}	0^{c}

The results are means (n = 4) ± SE. Means with the same letter for each stage are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

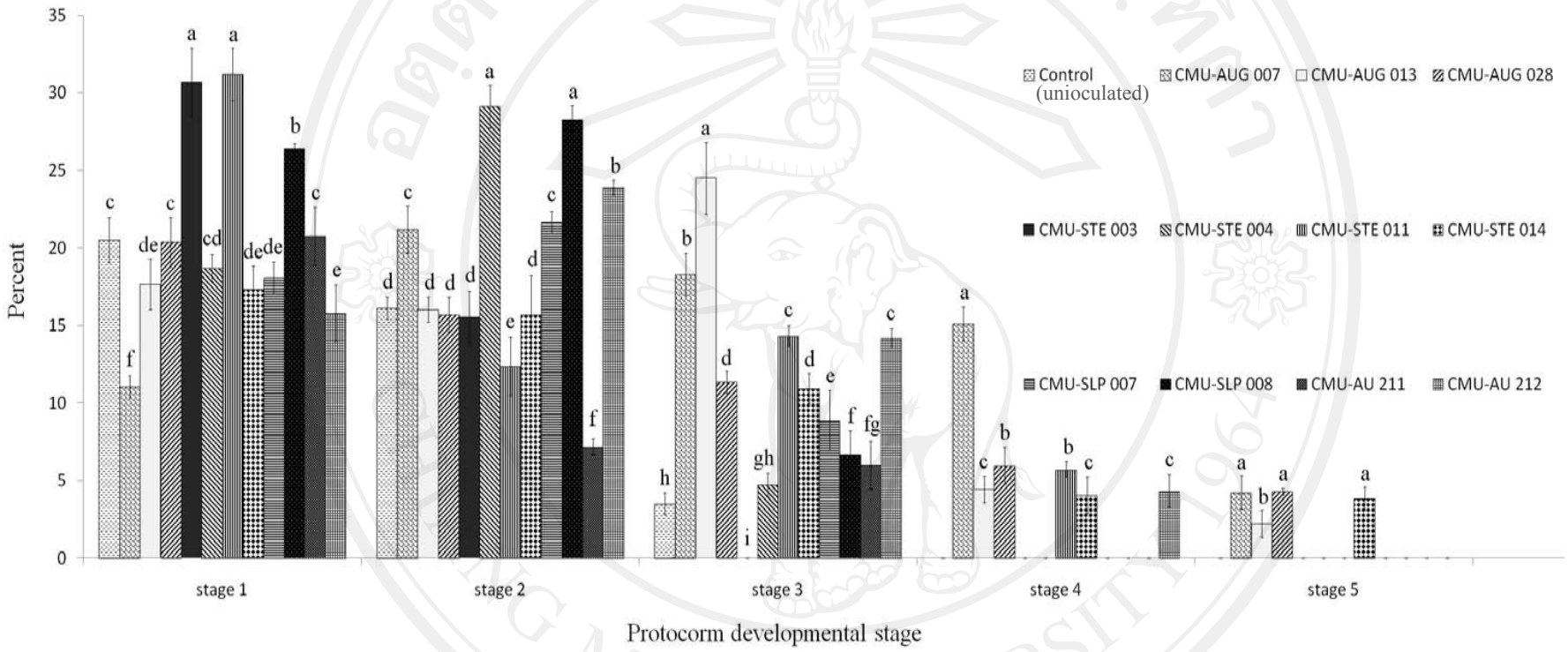

Figure 4.6 Effects of 11 endophytic fungi on seed germination and protocorm development of *Pecteilis susannae* 133 days after sowing. The results are means (n = 4) ± SE. Means with the same letter for each stage are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

4.4 Discussion

In vitro symbiotic seed germination has become a favoured and useful methodology for orchid seed propagation. Many studies have reported on orchid propagation and seed germination using symbiotic technique (Zettler and Hofer, 1998; Batty *et al.* 2001; Stewart and Zettler, 2002; Athipunyakom *et al.* 2004b; Kristiansen *et al.* 2004; Batty *et al.* 2006a; 2006b; Stewart and Kane, 2006; Johnson *et al.* 2007). However, there are few reports that address *in vitro* symbiotic seed germination of Thai native terrestrial orchid species (Athipunyakom *et al.* 2004b). This is the first study that describes *in vitro* symbiotic seed germination of *P. susannae*.

The most common medium used for *in vitro* symbiotic seed germination is Oatmeal agar (Zettler and Hofer, 1998; Stewart and Zettler, 2002; Stewart and Kane, 2006), while, Cellulose agar had limited use (Porras-Alfaro and Bayman, 2007). No asymbiotic culture media (plant tissue culture media) have been used for symbiotic seed germination. The Vacin and Went (1949) medium was chosen for the trial because it is of interested to know whether it would be possible to adapt routine plant propagation media for orchid culture with symbiotic fungi without the need to change the media between early and late stages of *in vitro* growth. The three agar media were used to sow orchid seeds with the isolated mycorrhizal fungi in order to select a suitable agar medium for later studies. The results support that Oatmeal agar was the most suitable for using in symbiotic seed germination and is in agreement with other reports (Zettler and Hofer, 1998; McKendrick *et al.* 2000; 2002; Stewart and Zettler, 2002; Stewart and Kane,

2006). Furthermore, the most advance protocorm developmental stage was presented on this agar medium. In addition, the stages of orchid protocorm development are easy to determine under the stereo microscope. In contrast, low seed germination and protocorm development was observed on Vacin and Went agar and, moreover, the symbiotic plates were difficult to determine because the fungal isolates grew rapidly and formed considerable aerial mycelium.

Some studies have reported that orchid mycorrhizal fungi are able to be pathogenic or saprophytic depending on the nutrient content of the media (Porras-Alfaro and Bayman, 2007). In nature, orchids have to control the balance between fungal invasion, symbiosis and digestion of peloton structures. This suggests that orchids control the infection process and the fungal symbionts are adapted to this control (Hadley, 1982; Brundrett *et al.* 2001; Sivasithamparam *et al.* 2002; Smith and Read, 2008). Therefore, the rich nutritive status of the Vacin and Went agar may cause the over growth of fungi making it difficult to control the balance in the orchid-mycorrhizal fungi relationship. Although the symbiotic plates using Cellulose agar were easy to observed, the protocorm developmental stages were low when compared with symbiotic seed germination using Oatmeal agar. Thus, the Oatmeal agar was selected to establish the symbiotic seed germination in this study.

Germination and protocorm development of *P. susannae* seed inoculated with fungal endophytes advanced to stages 2 and 3 occurred after incubation in the dark for 70 days. This is similar to the findings of Stewart and Kane, (2006). In their study, seed of *Habenaria macroceratitis*, a terrestrial orchid in Florida, were cultured with six fungi originating from the roots of *H. macroceratitis*. All

six fungal isolates were identified as belonging to the genus *Epulorhiza*, resulting in a maximum of 65.7% germination and maximum protocorm development of stage 2 after seed sowing for 58 days. Furthermore, the incubation condition in the current study was similar to other studies which have used dark conditions for symbiotic seed germination (Zettler and Hofer, 1998; Batty *et al.* 2001; Athipunyakom *et al.* 2004b; Stewart and Kane, 2006).

Of interest is the main finding that protocorm developmental stage 5 was reached after symbiotic sowing with *Epulorhiza* isolates obtained from roots of *P. susannae* and *E. spectabilis*. This occurred after incubation in the dark condition for 70 days followed by 12 h photoperiod, 1,000 Lux, (light: dark, 12: 12 h) for 63 days. Although the incidence of stage 5 was not high (< 10%), the current work is the first experiment in which *in vitro* symbiotic seed germination has resulted in orchid protocorm development to stage 5. The incubation conditions that were used to obtain these findings are similar to Stewart and Zettler, (2002) who obtained 10% of protocorm developmental stage 5 in *Habenaria repens*, a terrestrial orchid from Florida, when sown with *Epulorhiza* isolates and incubated in the dark for 69 days followed by a 12 h photoperiod (light: dark, 12: 12 h) for 13 days. This finding showed that there are likely to be more than one governing seed germination of orchid. Unraveling all of these factors is difficult and progress so far has been slow. Areas for further research are discussed in chapter 8.

In this study, the most advanced seed germination and protocorm development occurred with *Epulorhiza* isolates which suggests that this fungal genus contains the most effective fungal endophytes to promote seed germination of the tropical orchid *P. susannae* as well as some other orchids (Zelmer *et al.*

1996; Zettler and Hofer, 1998; Stewart and Zettler, 2002; Stewart and Kane, 2006; Johnson *et al.* 2007). The effective fungi that promoted symbiotic seed germination in this study were not only isolated from *P. susannae* but also from *E. spectabilis*. Stewart and Zettler (2002) reported that the most advanced protocorm development of *H. repens* occured after sowing with isolates of *Epulorhiza* obtained from other orchid species (*Spiranthes brevilabris* and *Epidendrum conopseum*). Athipunyakom *et al.* (2004b) showed that *R. globularis*, isolated from roots of *Goodyera procera*, could promote the most advanced protocorm development of *S. plicata*. In addition, some other studies have also found that orchid seeds could germinate with fungi isolated (e.g. *Tulasnella* spp. and *Ceratobasidium* spp.) from other species of orchids (Warcup, 1973; Otero *et al.* 2004; Porras-Alfaro and Bayman, 2007). This suggests that some mycorrhizal fungi are not highly specific to one orchid species. Generally, mycorrhizal fungi are host-specific, but the symbiotic relationship between orchid mycorrhizal fungi and orchid plants are mostly non-specific (Lee, 2002). The range of host plants for the fungal isolates are unknown and will be evaluated in the future to determine if the fungal isolates can be used for a wide range of terrestrial orchid species. However, it should be borne in mind that there is sometimes a complicated relationship between orchids and mycorrhizal fungi and the optimal conditions for orchid seed germination (Brundrett *et al.* 2001, 2003; Porras-Alfaro and Bayman, 2007). Some reports suggest there are different specific mycorrhizal fungi in each stage of an orchid's life cycle (Kristiansen *et al.* 2004; Stewart and Kane, 2006; Dearnaley, 2007; Tao *et al.* 2008; Shimura *et al.* 2009). The studies on symbiotic seed germination are still essential for orchid propagation and conservation (Batty

et al. 2006a; 2006b). Further research is required to optimize the later stages of *P. susannae* seedling development, followed by transplant success to the nursery and then to the field. The understanding of these symbiotic seed germination will assist further developmental method for orchid propagation of this orchid species and other terrestrial orchids in Thailand.