CHAPTER 8

GENERAL DISCUSSION

Based on the results of this study, the orchid mycorrhizal associations are found commonly in Thai terrestrial orchids due to the heavy colonization of mycorrhizal fungi in roots of 6 terrestrial orchid samples (*D. pulcherrima, E. spectabilis, P. bellatulum, P. susannae, P. tankervilleae,* and *S. affinis*). This colonization is in agreement with other reports to confirm that terrestrial orchids depend upon mycorrhizal associations in nature (McKendrick *et al.* 2000, 2002; Smith and Read, 2008; Swart and Dixon, 2009; Wright *et al.* 2009). Several studies tried to isolate the orchid mycorrhizal fungi from roots of orchids using single peloton isolation by crushing orchid roots to get single cell containing peloton and placed it on isolation agar media (Brundrett *et al.* 2001; Athipunyakom *et al.* 2004a; Yamato *et al.* 2005). In this study, the mycorrhizal fungi could be recovered by the classical methodology for isolation of endophytic fungi using surface sterilization and place a piece of root on isolation agar media, but many fungal endophytes were also recovered by this method. Therefore, the appropriated isolation methodology for avoiding contamination and decreasing other endophytes was presented by applied some other methods together with isolation method described by Zhu *et al.* (2008). In natural soil, there are a large number of microorganisms, running tap water before surface sterilization was

required to get rid of other free living soil microbes. Moreover, the poor nutrient media such as ¼ PDA and some antibiotics such as chloramphenicol or streptomycin were also required for decreasing the growth of other microbial contaminations (Brundrett *et al.* 2001; Zhu *et al.* 2008). The identification of endophytic fungi isolated from roots of all terrestrial orchid samples supports the view that the genus *Epulorhiza* is one of the most common and distinctive form-genera of *Basidiomycetes* forming mycorrhizal associations with orchids (Currah *et al.* 1997; Zettler and Hofer 1998; Ma *et al.* 2003; Stewart and Kane 2006; Taylor and McCormick 2007; Zhu *et al.* 2008; Shimura *et al.* 2009). However, the study on fungal diversity and distribution of orchid mycorrhiza are still required due to the diversity and distribution of orchid mycorrhizal fungi are poorly study and there are a few report especially in Thailand (Sangthong, 2002; Athipunyakom *et al.* 2004a; 2004b; Nontachaiyapoom *et al.* 2010).

Our results are also in agreement with many studies that reported the requirement of mycorrhizal associations of orchid seed germination and orchid seedlings growth (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, this is the first study that describes *in vitro* symbiotic seed germination of *P. susannae*. This study revealed that the mycorrhizal occurrence during seed germination *in vitro* of orchid depended on nutrient of agar media due to the enrich media such as Vacin and Went agar could support the growth of fungi caused orchid seed died. Moreover, some studies reported that orchid mycorrhizal fungi are able to be pathogenic or saprophytic fungi upon to quality of medium nutrition (Porras-

Alfaro and Bayman, 2007). Therefore, the suitable nutrient or agar media is required for symbiotic seed germination. the current work is the first experiment in which *in vitro* symbiotic seed germination has resulted in orchid protocorm development to stage 5 and presented the advantage of orchid survival rate when compared with asymbiotic seed sowing after sowing in the dark condition for 70 days followed by 12 h photoperiod, 1,000 Lux, (light: dark, 12: 12 h) for 63 days. The incubation conditions that were used are similar to the incubation conditions of *Habenaria repens*, terrestrial orchid from Florida, that developed protocorm to stage 5 (Stewart and Zettler, 2002). While, some studies used dark condition for symbiotic seed germination (Zettler and Hofer, 1998; Batty *et al.* 2001; Athipunyakom *et al.* 2004b; Stewart and Kane, 2006). The symbiotic orchid seed germination has several concerning factors and require different factors in each orchid species. It is quite complicate to understand and control all concerning factors. However, the study on these concerning factors for further development of symbiotic seed germination study of *P. susannae* is still required.

In this study, our findings supported that the *Epulorhiza* isolate was the most effective fungal endophytes to promote seed germination of several orchid species (Zelmer *et al.* 1996; Zettler and Hofer, 1998; Stewart and Zettler, 2002; Stewart and Kane, 2006; Johnson *et al.* 2007). Furthermore, one effective *Epulorhiza* isolate on symbiotic seed germination was isolated from two orchid species (*P. susannae* and *E. spectabilis*) that found in different habitat. This finding revealed that one orchid mycorrhizal fungi could form mycorrhiza with different orchid species. This suggests that some mycorrhizal fungi are not highly specific to one orchid species. In addition, this finding lead to use some

mycorrhizal fungi which non specific host range for propagation of general orchids. Nevertheless, the range of host plants for this isolate and others are unknown and will be evaluated in the future. The relationship between mycorrhizal fungi and orchid is quite complicate and the different specific mycorrhizal fungi in each stage of orchid's life cycle were reported from some studies (Kristiansen *et al.* 2004; Stewart and Kane, 2006; Dearnaley, 2007; Tao *et al.* 2008; Shimura *et al.* 2009). The symbiotic seed germination and protocorm developmental stage 5 of *P. susannae* was not high, it is possible that the isolated mycorrhizal fungi were not the most suitable for seed germination due to the fungi were isolated from roots of mature plant. Some reports suggested there are different specific mycorrhizal fungi in each stage of orchid's life cycle (Kristiansen *et al.* 2004; Stewart and Kane, 2006; Dearnaley, 2007; Tao *et al.* 2008; Shimura *et al.* 2009). Further research is required to study on fungal species in orchid life cycle, 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.

The growth optimization of mycorrhizal fungi and the effects of mycorrhizal fungi on growth of orchid seedlings of *D. pulcherrima* using coconut husk with PDB as a substrate for fungal inoculum was described in the present study. This study revealed that all isolated mycorrhizal fungi could grow well at 30 ˚C and pH 6-8 on agar media and the suitable potting media for growth of selected mycorrhizal fungi was coconut husk with PDB. This optimal fungal

growth condition can be applied easily for fungal inocula production and using in tropical orchid plantation or reforestation. Moreover, the coconut husk is widely used for orchid and plant potting media. In Thailand and other countries in tropical zone especially in Southeast Asia, the coconut husk is a low cost material and there is a large number of this product from coconut farm. Thus, this is the appropriated material for mycorrhizal inoculum production and easy to use as a potting media. Nonetheless, the mycorrhizal fungi could not grow well in pure substrate of coconut husk due to lack of nutrients for fungal growth. For production of mycorrhizal inoculum, nutrient media (PDB) was added into coconut husk to support the fungal growth and the unused nutrient media by mycorrhizal fungi was washed out by water before use as orchid potting media which similar to methodology of some mycorrhizal inoculum production from other studies (Trappe, 1977; Lee *et al.* 2008). Based on our results, the study of effects of orchid mycorrhizal inoculum using coconut husk with nutrient media on the growth of orchid seedlings can be used as a model for further application for orchid mycorrhizal inoculum production and use in a nursery or reforestation program and the results are in agreement with other reports to support that orchid seedlings require appropriate mycorrhizal fungi and the nursery management also require a good mycorrhizal fungi management (Trappe, 1977; Batty *et al.* 2006b; Lee *et al.* 2008). However, this report is the beginning of using orchid mycorrhizal inoculum for cultivation orchid seedlings. There are several factors such as environmental factors on fungal inoculum, the long term preservation of mycorrhizal inoculum and the suitable fungi for general using with many orchid species or host specific of orchid mycorrhizal fungi that still required for further

research to develop the fugal inoculum including finding the appropriate mycorrhizal fungi for production in commercial scales.

Not only the effects of mycorrhizal fungi on seed germination and growth of orchid seedlings was described in this study but some chemical productions such as IAA and siderphores productions by mycorrhizal fungi were also studied. For many plant growth factors, the plant growth hormone is one of the important factors. IAA is one of plant growth hormone that has ability to improve plant growth by stimulating cell elongation, root initiation and increasing of seed germination and seedlings growth (Ahmad *et al.* 2005; Tsavkelova *et al.* 2007; Khamna *et al.* 2010). IAA could be produced by plant and some microorganisms especially for plant associated microorganisms including endophytic and rhizospheric soil microorganisms (Robinson *et al.* 1998; Hasan, 2002; Niemi *et al.* 2002; Ahmad *et al.* 2005; Tsakelova *et al.* 2007; Tsavkelova *et al.* 2008; Khan *et al.* 2009; Khamna *et al.* 2010). In addition, the siderophores production by microorganisms is one of mechanisms for binding Fe^{3+} from the environment, transport it back to the microbial cells and make it available for growth (Neilands, 1995; Chaihan *et al.* 2009; Khamna *et al.* 2010). This advantage of siderophores producing microbes could stimulate plant growth directly by increasing the availability of iron in soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake system (Mahmoud and Abd-Alla, 2001; Chaihan *et al.* 2009; Khamna *et al.* 2010). This study found that all isolated mycorhizal fungi could produce IAA and some of isolated endophytic fungi could produce IAA and siderphores while, non isolated mycorrhizal fungi produced siderphores. However, only a few orchid mycorrhizal

isolates presented the high level of IAA production while, the highest of IAA production was produced by fungal endophyte (CMU-AU 006, *C. gloeosporioides*) isolated from root of *S. plicata*. These findings indicated that the orchid mycorrhizal fungi do not use the advantage of siderophores production to promote the growth of host plants and the growth of orchid also might be supported by the siderphores produced by some endophytic fungi. For further study, the effects of the siderophore producing endophytic fungi on growth of host plant are required to study. In addition, it is possible that the IAA produced by mycorrhizal fungi is one of the mechanisms of orchid mycorrhizal association to support plant growth. The evaluation of biological activities of fungal IAA by determination of root formation of kidney bean cuttings, the corn seed germination and elongation of corn roots and elongation of rice coleoptiles revealed that the function of fungal IAA are similar to standard IAA and in accordance with other reports (Tsavkelova *et al.* 2007; Khamna *et al.* 2010). Furthermore, the thin layer chromatography showed that the R_f values of the fungal IAA did not different with the standard IAA. This result of TLC technique is also in agreement with other reports to support that IAA was presented in all fungal supernatants (Ahmad *et al.* 2005; Khamna *et al.* 2010). Although, the present study on IAA supports the role of mycorrhizal fungi that promote seed germination and growth of plant. Nevertheless, the effects of the fungal IAA on orchid seed germination and growth of seedlings are therefore needed in order to study and understand the roles of orchid mycorrhizal fungi for further study.

In conclusion, this is the first study described the mycorrhizal fungi and some endophytic fungi isolated from roots of six Thai terrestrial orchids (*P.*

susannae, *P. bellatulum*, *P. tankervilleae*, *E. spectabilis*, *D. pulcherrima* and *S. affinis*) and effects of mycorrhizal fungi on symbiotic seed germination of *P. susannae* and seedlings growth of *D. pulcherrima* using mycorrhizal inoculum from coconut husk with PDB as a mixed potting media. In addition the IAA and siderophores production by orchid mycorrhizal fungi and some endophytic fungi were presented. This study can be leaded to use as model for orchid propagation and conservation including an application for using in industrial or commercial cultivation of orchids. However, there are several concerning factors that needed for further study to keep the research going on and improve the understandings about the orchid mycorrhizal fungi for application and using in the future.