CHAPTER 7

MYCORRHIZAL COLONIZATION AND EEFECTS OF MYCORRHIZAL FUNGI ON GROWTH OF ORCHID SEEDLINGS

7.1 Introduction

Most plants depend on mycorrhizal fungi for absorption of nutrient from soils. Mycorrhizal formation increased root-soil interfaces, water and nutrient uptake of hosts, and host tolerance to environmental stresses such as extremes of temperature, moisture, pH, and toxicants (Harley and Smith, 1983; Hung and Trappe, 1987; Sánchez *et al.* 2001). Inoculation of plant seedlings with mycorrhizal inoculum may help the seedlings overcome the transplanting shock and increase the seedlings survival rate and growth thereafter. The need of inoculating nurseries with mycorrhizal fungi may begin with initial nursery development. Generally, the used potting media lack mychorrhizal fungi. Therefore, the mycorrhizal fungi must be added to produce mycorrhizal seedlings (Trappe, 1977; Hung and Trappe, 1987; Lee *et al.* 2008). The inoculation of media with mycorrhizal fungi also can increase significantly successful rooting of cuttings and growth of root plants that are difficult to root (Linderman and Call, 1977). There are several methods to do fungal inoculum. The method for successful fungal inoculation of nurseries has been increased by fumigating nursery soils to decrease populations of endemic soil organisms that are competitive with or antagonistic to mycorrhizal inoculations (Trappe, 1977). The

using fungal mycelium is the one of method for fungal inoculation, pure mycelia cultures are techniques for inoculation with pure cultures of selected mycorrhizal fungi. This method offers guaranteed success for mycorrhizal fungi that grow well in pure culture (Mikola, 1970; Trappe, 1977). In addition, non-mycorrhizal seedlings will not survive if the out-planting site lack of appropriate mycorrhizal fungi. To establish a good nursery management, a good mycorrhizal fungi management is required (Trappe, 1977; Lee *et al.* 2008).

Doritis pulcherrima Lindl (Figure 3.6) are common species and distributed in rainforest and dipterocarp forest of Thailand (Nanakorn and Indharamusika, 1998; Santisuk *et al.* 2006). At the present time, most of the forests are secondary or degraded and there is a high level of human impact due to slash and burn agriculture in the uplands which is having a negative impact on many orchids. In addition, this orchid species are one of favorite terrestrial orchid due to the beautiful flower and the mature plant of this orchid species are not complicate to cultivate. However, mycorrhizal associations are still necessary for most orchids to support the growth and survival rate of seedlings especially for transferred non-mycorrhizal seedlings from *in vitro* propagation to nursery or removing orchid to new habitat (Batty *et al.* 2006b; Roy *et al.* 2009).

A study on mycorrhizal fungi inoculation using mixed potting media of orchid seedlings of *D. pulcherrima* was established to determine the effectiveness of inoculum to form mycorrhizal association with orchid seedlings and to evaluate effects of mycorrhizal inoculum on the growth of orchid seedlings.

7.2 Materials and methods

7.2.1 Fungal inoculum

Two mycorrhizal fungi, CMU-DP 506, *Epulorhiza* sp., and CMU-DP 514, *Tulasnella* sp., recovered from roots of *D. pulcherrima*, were used in this experiment. Fungal inoculum was prepared by culturing each fungal isolate in 250 ml Erlenmeyer flasks containing a sterile potting medium comprised of coconut husk soaked in Potato dextrose broth (PDB) adjusted to pH 7 for 12 hours, and then incubated at 30˚C until there was extensive colonization of the medium by fungal mycelium (about 21 days; Figure 7.1).

7.2.2 Symbiotic cultivation of orchid seedlings

Non-mycorrhizal orchid seedlings (height, 1 cm) of *D. pulcherrima* were recovered from *in vitro* propagation from tissue culture Queen Sirikit Botanic garden and Department of Biology Faculty of Science, Chiang Mai University laboratories. The fungal inocula (Figure 7.1) were washed with sterile water 3 times to remove the unused nutrients from the PDB to minimize growth of other soil microorganisms before being used. The washed inoculum was mixed with sterile soil (1: 2 v/v of fungal inoculum: soil) for the orchid seedling potting media preparation. The sterile soil (autoclaved 2 times at 121˚C, 15 min) mixed with washed sterile coconut husk saturated with PDB was used as the control medium. The cultivated orchid seedlings with potting media without fungi were used as the control. There were 3 replicates per treatment with each replicate consisting of 10 orchid seedlings. All cultivated orchid seedlings treatments were placed in natural

conditions. Watering was established one time per day using sterile water. After cultivating the orchid seedlings for 90 days, mycoorhizal colonization and effects of mycorrhizal fungi inoculum on growth of the orchid seedlings were determined.

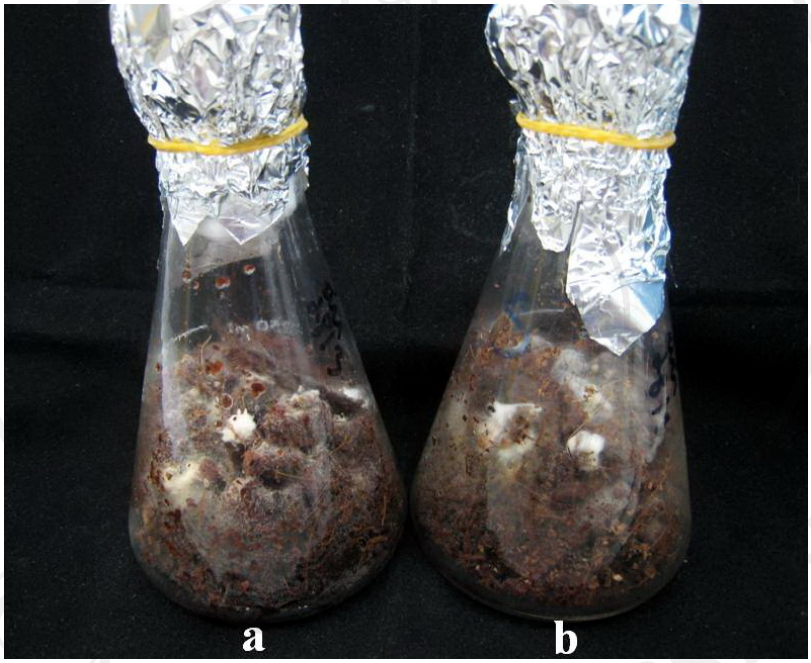

Figure 7.1 Orchid mycorrhizal inocula of fungal isolates CMU-DP 506, *Epulorhiza* sp., (a) and CMU-DP 514, *Tulasnella* sp., (b) using coconut husk supplemented with Potato dextrose broth after culturing at 30˚C for 21 days

7.2.3 Evaluation of symbiotic orchid seedlings

The effects of mycorrhizal fungi inoculum on the growth of orchid seedlings were evaluated by determination of survival rate, shoot dry weight and shoot height. The dry weights of orchid shoots were determined after cut off the

orchid roots and dried at 60˚C for 48 hours. The survival rate was calculated by dividing the number of survival seedlings by the total number of seedlings in each treatment. The cut orchid roots were used for determination of mycorrhizal colonization.

7.2.4 Determination of mycorrhizal colonization

Two to three roots of each orchid seedling were collected and cut into 1 cm segments. Then, the root segments were washed with tap water and cleared using 10% (w/v) KOH solution, stained in 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) and observed under a compound microscope. The stained root segments were randomly picked with fine tip forceps and mounted on glass slides. Peloton structures and colonization by mycorrhizal fungi were determined. Percentages of colonization in roots and percentages of crop colonization by mycorrhizal fungi were determined. The percentages of mycorrhizal colonization in roots were calculated by dividing the number of times of the presented peloton structure on the cross-hair position by the total number of determined times of the root samples under a compound microscope (McGonigle *et al.* 1990; Brundrett *et al.* 1996). The percentages of crop colonization by mycorrhizal fungi were calculated by dividing the number of seedlings that found peloton structure by the total number of determined seedlings.

7.2.5 Statistical analysis

All data were analyzed using SPSS V16.0 for one-way analysis of variance (ANOVA) and means were compared by Duncan's Multiple Range test ($P \leq 0.05$).

7.3 Results

7.3.1 Evaluation of symbiotic orchid seedlings

Both fungal inocula promoted the growth and survival of seedlings when compared with the control (non mycorhizal potting media) (Table 7.1). The fungal isolate CMU-DP 506 promoted the highest shoot height (2.72 cm), shoot dry weight (1.67 g) and survival rate (67 %) of orchid seedlings (Table 7.1 and Figure 7.2).

7.3.2 Determination of mycorrhizal colonization

Inoculation with fungal isolate CMU-DP 506, *Epulorhiza* sp., and CMU-DP 514, *Tulasnella* sp. resulted in extensive root colonization and the formation of peloton structures (Figure 7.3) of mychorrhizal fungi. Mycorrhizal colonization in roots of orchid seedlings by CMU-DP 506 and CMU-DP 514 were 83.25% and 76.83%, respectively while the control was 22.22%. Crop colonization by CMU-DP 506, CMU-DP 514 and control were 85.71%, 80.16% and 25.56%, respectively (Figure 7.4).

Table 7.1 Effects of mycorrhizal inoculum of fungal isolates CMU-DP 506, *Epulorhiza* sp., and CMU-DP 514, *Tulasnella* sp., used for potting media on growth (height and dry weight of shoots of seedlings) and survival rate of *Doritis pulcherrima* seedlings after cultivation for 90 days

Treatment	Height (cm)	Dry weight (g)	Survival rate (%)
Control	2.40 ± 1.33	1.26 ± 0.06^{c}	53 ± 5.77^{b}
Epulorhiza sp., CMU-DP 506	2.72 ± 0.12	1.67 ± 0.04^{a}	67 ± 5.77^{a}
Tulasnella sp., CMU-DP 514	2.63 ± 0.05	1.48 ± 0.04^{b}	63 ± 5.77^{ab}

The results are means (n = 3) ± SE. Means with the same letter are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

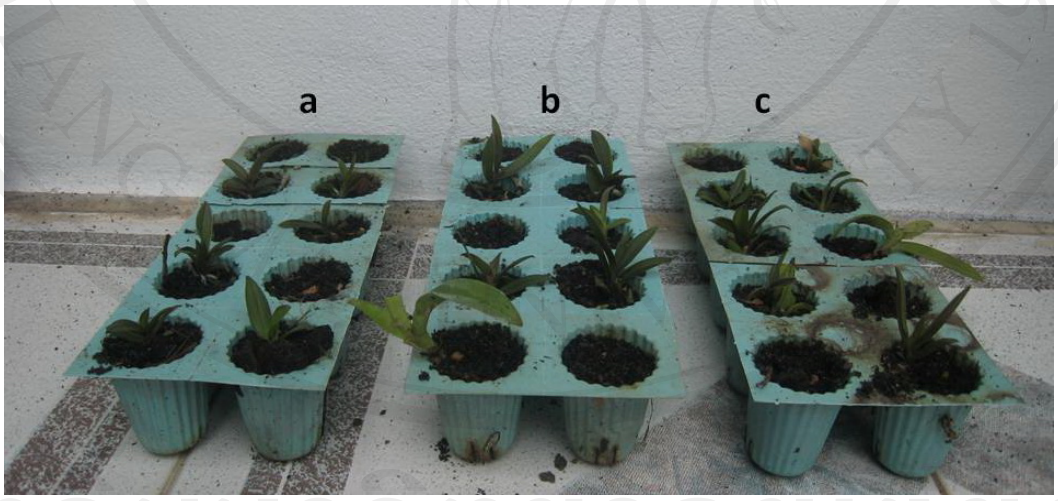

Figure 7.2 Seedlings of *Doritis pulcherrima* after cultivation using non mycorhizal potting medium (a), potting medium with fungal isolate CMU-DP 506, *Epulorhiza* sp., (b) and potting medium with fungal isolate CMU-DP 514, *Tulasnella* sp., (c) for 90 days

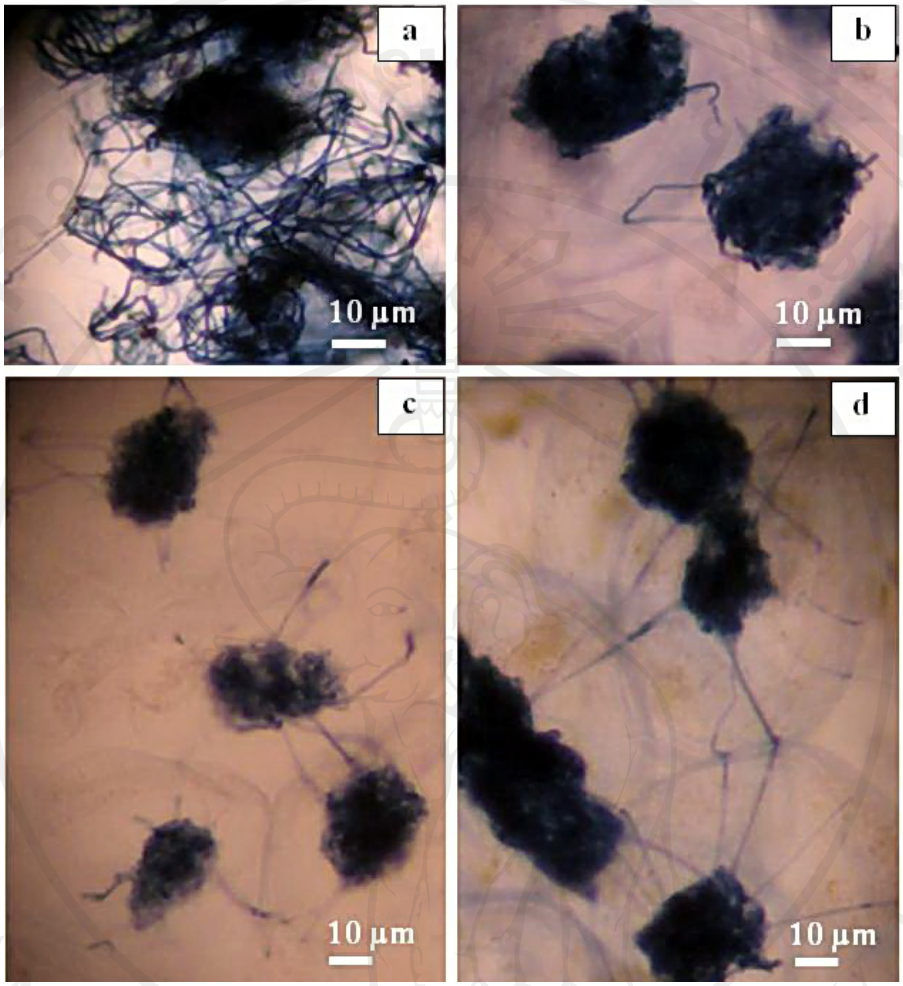

Figure 7.3 Peloton structures, coiled hyphae, in cortical cells of *Doritis pulcherrima* seedlings stained with 0.05% (w/v) trypan blue in lactoglycerol after cultivation using potting media containing CMU-DP 506, *Epulorhiza* sp., (a) and (b) and potting media containing CMU-DP 514, *Tulasnella* sp., (c) and (d) for 90 days

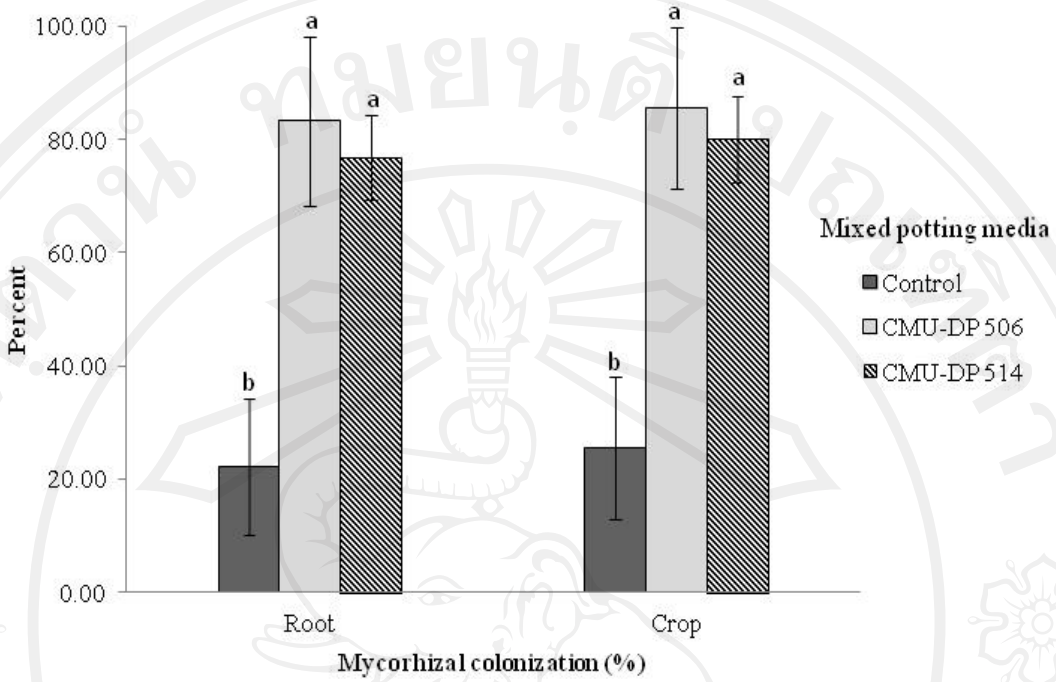

Figure 7.4 Root and crop colonization by mycorhizal fungi of *Doritis pulcherrima* seedlings after cultivation using potting media mixed with mycorrhizal inoculum of CMU-DP 506, *Epulorhiza* sp., and CMU-DP 514, *Tulasnella* sp., for 90 days. The results are means (n = 3) ± SE. Means with the same letter are not significantly different (Duncan's Multiple Range test; $P \leq 0.05$)

7.4 Discussion

In this study, mycorrhizal inocula of fungal isolate CMU-DP 506, and CMU-DP 514 were made using coconut husk, a general potting medium for orchids, supplemented with PDB nutrient media for the fungal substrate. This procedure was effective in delivering live inoculum to the roots and the fungi were able to persist long enough to effect colonization. Most orchids especially, terrestrial orchids, that are cultivated in nurseries require potting media for

planting and appropriate mycorrhizal fungi for supporting the growth of seedlings (Trappe, 1977; Batty *et al.* 2006b; Lee *et al.* 2008; Roy *et al.* 2009). The mycorrhizal inoculum especially for ectomycorrhizal and abuscular mycorrhizal (AM) fungi are generally studied and used for plant cultivation or reforestation (Trappe, 1977; Hung and Trappe, 1987; Lee *et al.* 2008). The vegetative fungal mycelium of ectomycorhizal fungi were used as the inoculum by cultured pure culture of fungi in potting media (etc. peatmoss, pine bark, vermiculite). Lee *et al.* (2008) presented the good potential of fungal inoculum using a peatmoss-vermiculite as a substrate for inoculum of ectomycorrhizal fungi identified as a member of *Theleporaceae* for improving dipterocarp seedlings establishment. Hung and Trappe, (1987) produced vegetative inoculum in a vermiculite-peatmoss of *Laccaria laccatar* and *Hebeloma crustuliniforme* and reported the successful mycoorhizal formation with Douglas-fir transplanted seedlings. However, the orchid mycorrhizal fungi inoculum is poorly studied when compared with ectomycorrhiza and AM. There are some studies described the soil or nursery transfer of orchid seedlings after symbiotic seed germination (Batty *et al.* 2001: 2006b). This is the first report to describe the use of orchid mycorrhizal inoculum for non-mycorrhizal seedlings of *D. pulcherrima*.

In this study, the fungal inocula were washed using sterile water before mixed with the potting medium to remove unused nutrients and avoid the growth of other soil microorganisms. After the non-mycorrhizal orchid seedlings were cultivated with the fungal inocula for 90 days, the peloton structures found in cortical root cells of seedlings indicated effective colonization by the mycorrhizal fungi. The orchid seedlings cultured with both fungal inocula had high levels of

colonization in roots and crops when compared with the control. In addition, the growth and survival rate of orchid seedlings increased significantly after used the both fungal inocula mixed with potting media. These findings were similar to symbiotic seedlings of three native Western Australian terrestrial orchids (*Caladenia arenicola*, *Diuris magnifica,* and *Thelymitra crinita*) that showed the higher percentage of seedlings survival rate than asymbiotic seedlings in greenhouse (Batty *et al.* 2006b). For the control was slightly found mycorrhizal colonization, it might be contaminated by mycorrhizal fungi in the nature due to the experiment was established in the open system, natural conditions.

Based on our results, it is 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). Furthermore, it is possible that the orchid mycorrhizal inoculum from coconut husk will be applied to add into orchid potting media and used in nursery program. Nevertheless, the effects of environmental factors on fungal inoculum, the long-term preservation of fungal inoculum and the host specific of orchid mycorrhizal fungi are still required for further study to understand and select the suitable candidate fungi for general orchid species in commercial scales.