# CHAPTER 5

# Selection of arbuscular mycorrhizal fungi for growth enhancement of *Aquilaria crassna* and *Tectona grandis*

## 5.1 Introduction

AM fungal inocula are widely used in agriculture and forestry. They are used in seedling preparation for forestry (Brundrett *et al*., 1994). Mycorrhization of forest plants have recently been considered as the substitute of chemical fertilization to produce healthy seedlings and proved to be suitable for planting in the field (Brundrett *et al*., 1996). Many studies are being done to select the suitable indigenous AM fungal strains for the establishment and management of forests. These study make the foresters and people mindful about the role of mycorrhiza as a tools to maintain and manage the forests environment friendly (Dhar and Mridha, 2006). Habte *et al*. (2001) found that AM fungal inoculation of *Acacia koa* seedlings in the nursery has significant beneficial effect on early tree establishment in the field. According to Turjaman *et al*. (2006), AMF increase early growth of two non-timber forest product species *Dyera polyphylla* and *Aquilaria filaria* under greenhouse conditions. Rajan (2000) screened AM fungi for their symbiotic efficiency with *Tectona grandis* and observed that *Glomus leptotichum* was the best AM symbiont for teak compared to the others. Since, micropropagation techniques have been recognized as very successful applications for rapid production of several commercial important varieties of horticultural and forest tree species. However, low survival rates and poor growth rate occurred after transplant and exposure to field conditions. These are the most common problems which impedes its usage for commercial plant production. Nowadays, AM fungal inocula also applied for micropropagated plantlets. Kapoor *et al*. (2008) found that inoculation of AM fungi to the roots of micropropagated plantlets play a beneficial roles on their post-transplanting

efficiency. Therefore, AM fungi could be developed into effective inocula for field application in agriculture and reforestation. The achievement of AM fungal technology will depend on mycorrhizal host, well-adapted effective fungal strains and optimal soil condition. Therefore, in seedling production, the soil properties and level of fertilization should be evaluated to keep secondary effects caused by changed mycorrhizal association (Nandakwang *et al*., 2008).

## 5.2 Materials and methods

### 5.2.1 Plant growth experiment

Two experiments were performed to test the efficiency of selected AM fungal spore inocula for enhancement the growth of *T. grandis* and *A. crassna*; tissue culture plantlets. All micropropagate *T. grandis* and *A. crassna* used in this study was obtained from Thai Orchids Lab Co. Ltd. First experiment was done with hardening stage *T. grandis* and *A. crassna* plantlets and divided into two parts: (1) preliminary AM fungal inoculation with *T. grandis* and *A. crassna* plantlets, (2) confirmation experiment with *T. grandis* plantlets. Second experiment were performed *in vitro* with rooting stage *T. grandis* plantlets.

#### 5.2.1.1 AM fungal inoculation with hardening stage plantlets.

##### 5.2.1.1.1 Inoculum production

From Chapter 4, the most sequences found in rhizosphere soils and roots of *A. crassna* and *T. grandis* were belonged to the family *Glomeraceae* that includes *Claroideoglomus* spp., *Glomus* spp. and *Rhizophagus* spp. Therefore, spores of *C. etunicatum* NNT10, *C. etunicatum* PBT03 and *F. mosseae* RYA08 which produced from indigenous AM fungal communities associated with *A. crassna* and *T. grandis* in Thailand were chosen. Those spore isolates were propagated using trap culture and pot culture method with *Sorghum bicolor* L. *Entrophospora colombiana* CMU05 spores were obtained from Dr. Supattra Charoenpakdee, the Sustainable Development of Biological Resources Lab (SDBR), Department of Biology, Faculty of Science, Chiang Mai University. Spores of each AM fungal species were separated from cultivated soils by wet sieving and sucrose

centrifugation (Gerdemann and Nicolson, 1963). Each spore species was placed on a moisture filter paper and kept in 4ºC refrigerator until use.

#### 5.2.1.1.2 AM fungal inoculation

Preliminary AM fungal inoculation with *T. grandis* and *A. crassna* plantlets, were set up for four treatments as follows: control (uninoculated plant), *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *E. colombiana* CMU05 (except *A. crassna*). This experiment was performed in the greenhouse at Department of Biology, Faculty of Science, Chiang Mai University with natural light period during hot season (February–May 2011). Confirmation experiment were evaluated with *T. grandis* plantlets which were inoculated with *C. etunicatum* NNT10, *C. etunicatum* PBT03, *E. colombiana* CMU05, and *F. mosseae* RYA08 in the greenhouse at Department of Agronomy, Faculty of Agriculture, Chiang Mai University and grown with natural light period during cold season (November 2011–February 2012). Uniform 12–15 cm in height seedlings were chosen for both sub-experiments. The 20 plantlets (5 replicates × 4 treatments) for preliminary experiment and 95 plantlets (19 replicates × 5 treatments) were selected for confirmation experiment. All plantlets were washed in filtered tap water and each plantlet was transplanted into the 8 inch diameter black plastic pot containing 2 kg of the sterilized sandy soil substrate. The inside of the pot was lined with a bottom hole on a plastic bag to allow water drainage and the outside of the pot was covered with plastic bottom plate to prevent a contamination. To facilitate inoculation, 100 spores each were counted and placed on a moisture piece of 0.5 × 0.5 cm sterilized filter paper (Whatman No.1). AM fungal spores were inoculated close to the lateral roots of plantlet. Plantlets were grown for 120 days in the greenhouse and obtained modified Hoagland's nutrient solution (0.1 strength phosphorus (0.1 mM) pH 6.5) (Hoagland and Arnon, 1950) once per week. Plant height and stem circumference were measured. At harvest, roots were randomly collected from each plantlet and washed before staining with 0.05% trypan blue. Roots and soils of each pot were determined for root length colonization (detailed in Chapter 3).

#### 5.2.1.2 *In vitro* inoculation with rooting stage *T. grandis* plantlets.

##### 5.2.1.2.1 Inoculum production

Spores of *Claroideoglomus etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08 were obtained from the source as detailed in 5.2.1.1.1. Spores were surface sterilized using chloramine T (2% w/v), streptomycin sulfate (0.1% w/v) and gentamicin sulfate (0.05% w/v) (Bécard and Fortin, 1988), and stored at 4ºC in sterile distilled water. *Glomus intraradices* root organ culture was obtained from Dr. David D. Douds, USDA, Agricultural Research Service, Eastern Regional Research Center, Pennsylvania, USA.

##### 5.2.1.2.2 *In vitro* dual culture with *T. grandis* plantlets

Roots were subcultured on minimal (M) medium with 0.2% (w/v) gellan gum. Roots and surface sterilized AM fungal spores were grown together in 9 cm square Petri plates (*in vitro* culture methodology, detailed in Chapter 6) and observed for fungal hyphae growth for at least 3 months before using as inoculum. Sixty uniform *T. grandis* plantlets with 5 cm in height (12 replicates × 5 treatments; uninoculate, *C. etunicatum* NNT10, *C. etunicatum* PBT03, *G. intraradices* and *F. mosseae* RYA08) were used and each three plantlets were transferred into a culture bottle filled with MSR medium lacking sucrose and vitamins with 0.4% (w/v) gellan gum (Voets *et al*., 2005). Suspension of 20–25 surface sterilized spores were injected nearby roots of *T. grandis* plantlets in each culture bottle and incubated horizontally for 2 months at 27ºC. *Glomus intraradices* root organ culture was cut into 1 cm diameter disc using cock borer. Three fungal discs that contained both spores and colonized roots were inoculated nearby *T. grandis* plantlets roots in each culture bottle. The bottom of each culture bottle was half covered with aluminum foil to protect root from exceed florescence light and allow plant to receive light for their photosynthesis (Figure 5.1). Culture bottles were incubated for 2 months on shelf under florescence light (1,100 lux) under a 12-h-light (27ºC)/ 12-h-dark (25ºC) to allow spore germination and hyphae regrowth. All bottles were monitored for colonization (e.g. proliferation of hyphae) after 2 months of incubation. Then, the plantlets were transferred to 3 inch plastic pots filled with sterile vermiculite as culture substrate and covered whole pot with sun bags to control humidity. Ten milliliters of modified Hoagland's nutrient solution 0.1 strength

phosphorus (0.1 mM) was applied to each pot once. All plastic pots were incubated on the shelf in the same condition as above for a month. After 3 months of inoculation, *T. grandis* plantlets were measured for height, number of leave, wet weight and dry weight of shoot, wet weight of root, and percentage of root length colonization.

### 5.2.2 Statistical analysis

Univariate analysis was employed for plant height, number of leaves, stem circumference, wet weight and dry weight of shoot, wet weight of root, and percentage of root length colonization. Tukey's post hoc multiple mean comparison test was used to test significant differences between treatments at $P<0.05$. All statistical analyses were performed with Statistical Package for Social Sciences version 11.5 (SPSS Inc., IL).

**Figure 5.1** Culture bottles covering with aluminum foil incubated under florescence light.



## 5.3 Results

### 5.3.1 Plant growth experiment with hardening stage plantlets.

Preliminary experiment, *T. grandis* plantlets had the best growth responded to AM fungal spore inoculation than uninoculated plantlets ($P<0.05$). *Tectona grandis* plantlets that inoculated with *C. etunicatum* PBT03 had highest height followed by *C. etunicatum* NNT10, *E. colombiana* and uninoculated plantlets, respectively. There

were no significantly differed in number of leaf and wideness of leaf between treatments. In *A. crassna*, there was no significantly differed in height, number of leaf, and wideness of leaf between inoculated and uninoculated plantlets between treatments (Figure 5.2 and Table 5.1).

A confirmation experiment, *T. grandis* plantlets had highest height when inoculating with *C. etunicatum* PBT03 followed by *C. etunicatum* NNT10, *F. mosseae* RYA08**,** *E. colombiana* CMU05 and uninoculate control, respectively (Table 5.2). Inoculating *T. grandis* plantlets with *C. etunicatum* PBT03 gave higher stem diameter than *C. etunicatum* NNT10, *F. mosseae* RYA08**,** *E. colombiana* CMU05 and uninoculate control, respectively.

**Table 5.1** Growth responses of inoculated and uninoculated *T. grandis* and *A. crassna* plantlets after 4 months of inoculation (Preliminary experiment).

| Spore specie | *Tectona grandis* | | | *Aquilaria crassna* | | |
|---|---|---|---|---|---|---|
| | **Height (cm)** | **Number of leaves** | **Leaf Width** | **Height (cm)** | **Number of leaves** | **Leaf Width** |
| Control (uninoculate) | 32.67 b* | 8.67 a | 16.20 a | 52.70 a | 19.33 a | 4.80 a |
| *C. etunicatum* PBT03 | 43.90 a | 10.00 a | 15.83 a | 58.17 a | 22.67 a | 4.47 a |
| *C. etunicatum* NNT10 | 42.83 a | 10.67 a | 17.37 a | 69.13 a | 30.67 a | 4.80 a |
| *E. colombiana* CMU05 | 37.40 b | 10.67 a | 18.07 a | - | - | - |
| Significant | *P<0.05* | ns | ns | ns | ns | ns |

* The same letter in each column indicate no significant different (α=0.05, n=3) between treatment, ns = not significant.

**Table 5.2** Height and stem circumference of inoculated and uninoculated *T. grandis* plantlets after 4 months of inoculation (Confirmation experiment).

| Treatment | Plant height (cm) | Stem circumference (cm) |
|---|---|---|
| Control (uninoculate) | 18.63 b* | 3.86 d |
| *E. colombiana* CMU05 | 19.41 b | 4.28 c |
| *F. mosseae* RYA08 | 19.84 b | 4.65 b |
| *C. etunicatum* NNT10 | 20.69 b | 5.10 a |
| *C. etunicatum* PBT03 | 22.88 a | 5.12 a |
| Significant | *P<0.05* | *P<0.05* |

* The same letter in each column indicate no significant different (α=0.05, n=13) between treatments.

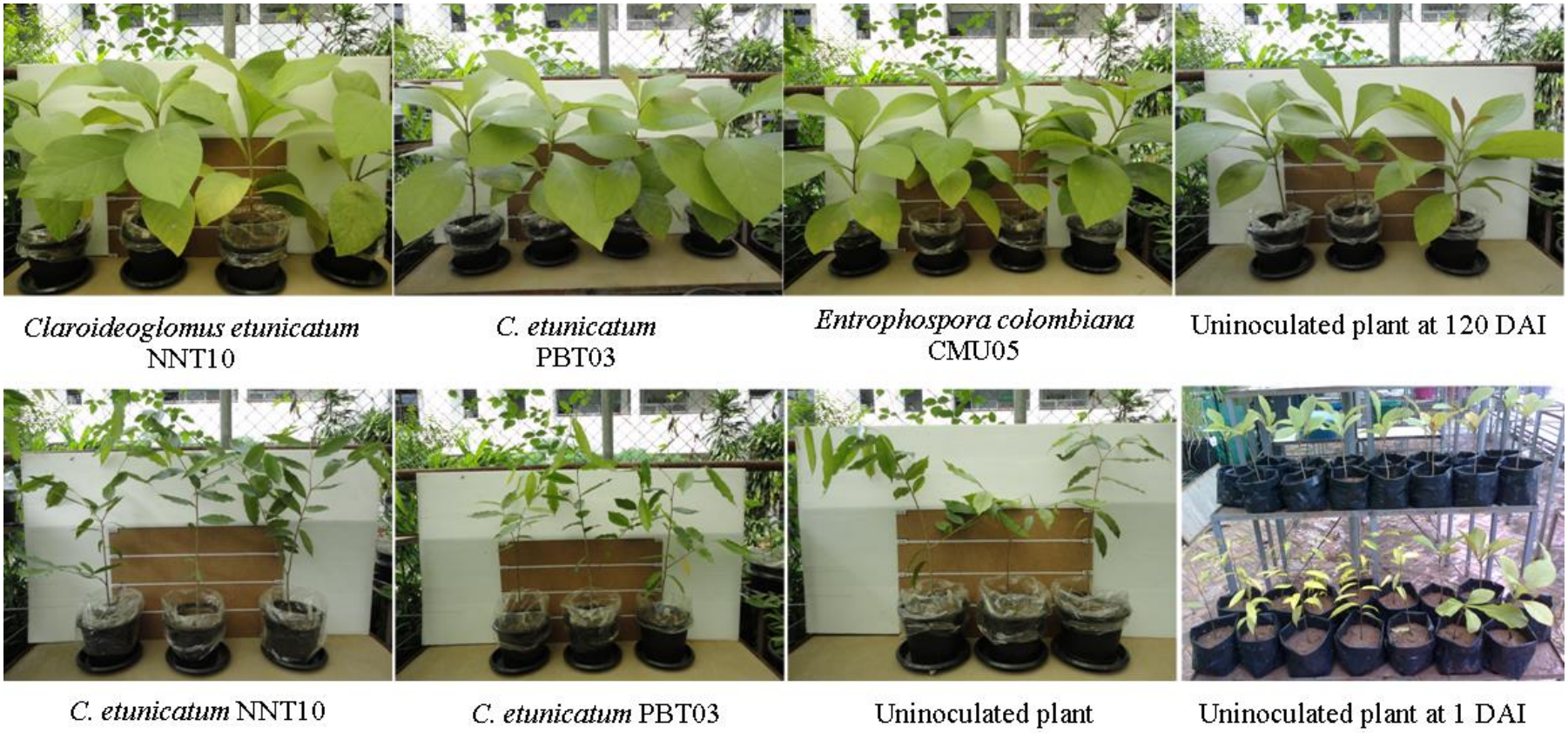


**Figure 5.2** Effect of inoculation with AM fungi (Part I experiment) on height of *T. grandis* (above row) and *A. crassna* (below row) plantlets compared with the uninoculated treatment. DAI = day after inoculation.

### 5.3.2 Plant growth experiment with rooting stage *T. grandis* plantlets.

The regrowth of AM fungal hyphae from initial inoculating root organ culture of *G. intraradices* and *F. mosseae* RYA08, and spore germination of *C. etunicatum* NNT10, *C. etunicatum* PBT03 were found (Figure 5.3). *Tectona grandis* plantlets inoculated with *F. mosseae* RYA08 had highest plant height follow by *G. intraradices*, *C. etunicatum* NNT10, *C. etunicatum* PBT03, and uninoculated plant, respectively (Figure 5.4 and Table 5.3). Inoculation with *G. intraradices* was affected on shoot wet weight but gave low shoot dry weight inferior to *F. mosseae* RYA08. There was no significant different of the number of leaves and root wet weight between inoculated and uninoculated plantlets.

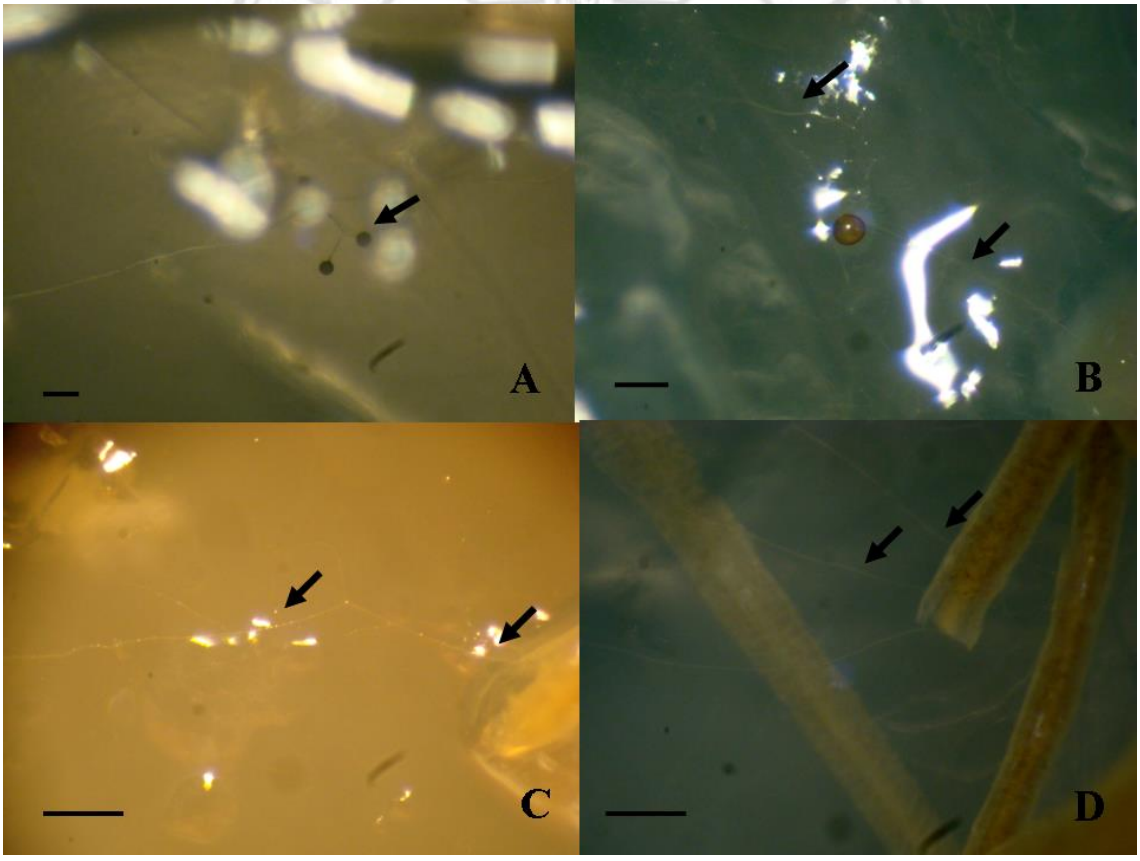


**Figure 5.3** Characterization of AM fungi *in vitro* inoculation (A) newly produced spores (arrow) of *G. intraradices* (B) spore germination and hyphae (arrowed) of *C. etunicatum* PBT03 (C) hyphae regrowth (arrowed) of *F. mosseae* RYA08 and (D) hyphae regrowth (arrowed) of *G. intraradices* from initial inoculating root organ culture.

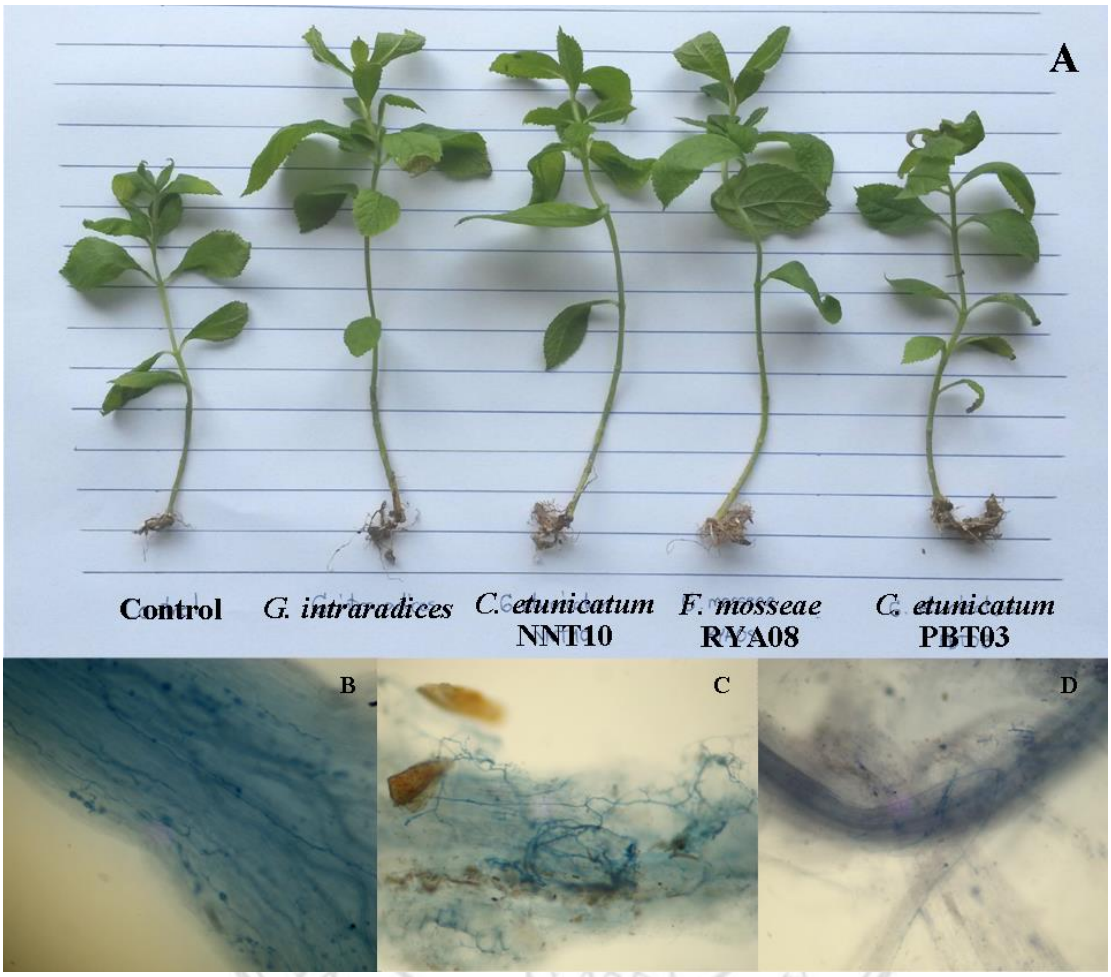


**Figure 5.4** Effect of *in vitro* inoculation with AM fungi on (A) height of *T. grandis* plantlets and extent of colonization with (B) *G. intraradices*, (C) *C. etunicatum* PBT03, (D) *C. etunicatum* NNT10.

**Table 5.3** Growth responses of inoculated and uninoculated *T. grandis* plantlets after 3 months of *in vitro* inoculation**.**

| **Treatment** | **Height (cm)** | **Number of leaves** | **Shoot WW (g)** | **Root WW (g)** | **Shoot DW (g)** | **Colonization (%)** |
|---|---|---|---|---|---|---|
| Control (uninoculated) | 8.06 b | 14.40 a | 0.290 ab | 0.0411 a | 0.0335 ab | 0.00 d |
| *G. intraradices* colonized root | 10.02 ab | 16.60 a | 0.454 a | 0.0919 a | 0.0451 ab | 38.50 a |
| *F. mosseae* RYA08 colonized root | 10.54 a | 15.20 a | 0.400 ab | 0.0628 a | 0.0497 a | 29.12 b |
| *C. etunicatum* NNT10 spores | 10.00 ab | 15.20 a | 0.286 ab | 0.0416 a | 0.0320 b | 19.38 c |
| *C. etunicatum* PBT03 spores | 8.68 ab | 14.80 a | 0.268 b | 0.0564 a | 0.0346 ab | 12.72 c |
| Significant | *P<0.05* | ns | *P<0.05* | ns | *P<0.05* | *P<0.05* |

* The same letter in each column indicate no significant different (α=0.05, n=5) between treatment, ns = not significant.

**5.4 Discussion**

Our study demonstrated that AM fungal inoculation could enhance growth of *T. grandis* both in rooting and hardening stage plantlets such as plant height, wet and dry weight of shoot. But there was no differed in growth response of *A. crassna* over those uninoculated plantlets. These results agreed with a number of previous researches on AM fungal inoculation in *T. grandis.* Verma and Jamaluddin (1995) performed that inoculation of *T. grandis* seedlings with *G. fasciculatum* and mix AM fungi separately showed better height growth, biomass and percent root infection in nursery compared to uninoculated (control) seedlings. The mixed AM inoculum was found more effective to boost the growth and biomass. Rajan *et al*. (2000) found that nine AM fungus increased in plant growth and plant nutritional of *T. grandis* stumps over those grown in uninoculated seedlings. Moreover, shoot N and P concentrations of AM colonized seedlings were increased by as much as 70–153% and 135–360%, respectively (Turjaman *et al*., 2006). In addition, Swaminathan and Srinivasan (2006) concluded that inoculation of phosphobacteria and AM fungi conjointly be done for better survival and growth of *T. grandis* seedlings. On the contrary, our study found that AM fungal inoculation had no effected on growth of *A. crassna*. Some replicate of uninoculated *A. crassna* had variation in height that may be caused by environmental condition during growing. To my acknowledge no published record of AM fungal inoculation of *A. crassna*, but previous studies were proposed the effect of AM fungi with other *Aquilaria* spp. Turjaman *et al*. (2006) studied the effect of *Glomus clarum* and *Gigaspora decipiens* on the early growth of two non-timber forest product species, *Dyera polyphylla* and *A. filaria* and found that they were increased plant height, diameter, survival rates and shoot and root dry weights after transplantation. Inoculation *A. agallocha* seedlings with *G. fasciculatum* was resulted in reduction the rotting incidence of damping-off disease caused by the pathogenic fungus (*Pythium aphanidermatum*) and also increased host plant height, total biomass and dry matter (Tabin *et al*., 2009).

The inoculation techniques performed here were aimed to find an appropriate method to inoculate micropropagate *T. grandis* and *A. crassna* both based on soil-base substrate or *in vitro* culture with AM fungal colonized roots. Many previous decade

studies have proposed success on mycorrhization with micropropagate plants as reported by Rai (2001) and other recent studies (Estrada-Luna *et al*., 2000; Declerck *et al*., 2002; Estrada-Luna and Davies, 2003; Joshee *et al*., 2007; Koffi *et al*., 2009). Most experiments were *ex vitro* inoculated AM fungi with acclimatization or hardening plantlets. This inoculation technique gave the good result for plantlets which strong enough to grow in open condition not in a close tunnel or growth chamber that can easily control humidity and temperature. In our study using soil-based substrate, micropropagate *T. grandis* and *A. crassna* in hardening stage were used to evaluate the efficiency of selected AM fungal strains on growth enhancement of both plants by *ex vitro* inoculation. We had no success on inoculating at acclimatization stage of *T. grandis* and *A. crassna* plantlets or the plantlets that transplant to culture substrate. Those plantlets started to die back and showed damping-off symptom at 1 month after AM fungal spores inoculation (data not show). The results may be caused from soil borne pathogen contamination on the surface of non-surface sterilized AM fungal spores. *In vitro* inoculation with rooting stage *T. grandis* plantlets experiment, showed the efficiency of both AM fungal colonized roots and surface sterilized spores to regrowth or germinate into new roots and enhance growth of plantlets. Covering the bottom of culture bottle with aluminum foil to protect root from exceed florescence light, was an easily way to handle the inoculation and not to complicate production system of tissue culture laboratory when producing AM fungal colonized plantlets. Previously, Voet *et al*. (2005) have developed an autotrophic culture system for the *in vitro* mycorrhization. Roots of plantlets were associated with AM fungus under *in vitro* conditions, while shoots developed under open air conditions. Recently, Voet *et al*. (2009) also found that extraradical mycelium network of AM fungi allows fast colonization of seedlings under *in vitro* conditions. Seedlings were readily colonized after 3 days of contact with the mycelium and newly colonized seedlings were able to reproduce the fungal life cycle, with the production of thousands of spores within 4 weeks. However, biomass and out planting survival during early phase are critical in any tree species. Therefore, AM fungi may serve as a powerful tool to enhance growth of any seedling not only micropropagate plants. It could be suggested that the AM fungal inoculation may be useable for commercial plantation of *T. grandis* and *A. crassna*; the economically important tree species in the future.