CHAPTER 6

Propagation of arbuscular mycorrhizal fungal spores for large scale production

6.1 Introduction

AM fungi are obligate symbionts, and as such can only be grown in the presence of roots. Therefore, AM fungal inoculum can propagate by growing the fungi with plants in greenhouse pot culture or other methods such as aeroponic systems (Hung and Sylvia, 1988; Jarstfer and Sylvia, 1995; Mohammad *et al*., 2000), hydroponic systems (Rai, 2001), and *in vitro* with root organ cultures (Brundrett *et al*., 1999; Jaizme-Vega *et al*., 2003; Fonseca *et al*., 2006). Commercially-available inocula of AM fungi are produced via many of these methods (Ijdo *et al*., 2011). An alternative to purchased inocula is the on-farm production of AM fungal inoculum.

Pioneering work on the on-farm production of AM fungal inoculum was first conducted in the tropics (Dodd *et al*., 1990; Sieverding, 1991; Gaur, 1997; Maiti *et al*., 2009). These methods utilize raised beds of soil, typically after fumigation, to produce introduced or indigenous isolates of AM fungi. Another method developed for temperate climates can be used to produce inoculum of introduced or native isolates of AM fungi in compost and vermiculite mixtures (Douds *et al*., 2005; Douds *et al*., 2006; Douds *et al*., 2010). This method can produce isolates of AM fungi introduced from off-site via colonization of the host plant prior to transplant into the mixture, while isolates of AM fungi indigenous to the site/farm can be propagated through the introduction of a small amount of field soil to the compost and vermiculite mixture (Douds *et al*., 2010). An alternative way to propagate axenic culture of AM fungi is *in vitro* production system with transformed root. Monoxenic cultures of AM fungi have been used for more than decade to comprehend the symbiosis and physiology of these fungi, but only a few AM fungal species have been successfully maintained in monoxenic culture with

Ri T-DNA transformed roots (Ijdo *et al*. 2011). Establishment of new AM species into this culture system is important for molecular, physiological, and taxonomical studies as well as the application with micropropagate plant *in vitro*. In Thailand, organic agriculture is gaining the attention of farmers because of the increasing cost of chemical fertilizer and growing concern about healthy food. Though these farmers have available to them many demonstrations that AM fungal inoculation can promote growth and nutrition of field grown crops (Charoenpakdee *et al*., 2010; Watanarojanaporn *et al*., 2011; Boonlue *et al*., 2012) and woody species under nursery conditions (Ramanwong, 1998; Youpensuk *et al*., 2005), methods for the production of an AM fungal inoculum using easily-available local materials are not available. The aim of this work was to use leaf litter compost, locally-available and produced on most farms, as a substrate component to produce an AM fungal inoculum using an on-farm methodology adapted for tropical climates from the method proposed by Douds *et al*. (2006). The first experiment in substrate-based production systems, examined the effect of different diluents and host plants to optimize sporulation in a sandy soil-based mixture. The second experiment used soil-based inocula from the first experiment and tested the efficiency of leaf litter compost and vermiculite mixture upon on-farm inoculum production. The *in vitro* axenic culture was also determined for the best suitable medium for selected AM fungal cultures (*Claroideoglomus etunicatum* NNT10, *C. etunicatum* PBT03 and *Funneliformis mosseae* RYA08) in order to use in the *in vitro* inoculation with micropropagate *Tectona grandis* and *Aquilaria crassna*.

6.2 Materials and method

6.2.1 Substrate-based production systems

Two experiments were conducted to develop a method for AM fungal inoculum production using greenhouse and on-farm methodology. Three AM fungi were utilized: *C. etunicatum* NNT10, *C. etunicatum* PBT03 and *F. mosseae* RYA08, all isolated from trap cultures of *T. grandis* and *A. crassna* rhizosphere soils. The first experiment examined the effect of different diluent amendments of a sandy soil and a variety of host plants to optimize sporulation. The second experiment utilized leaf litter compost and vermiculite mixed with starter inoculum in an on-farm setting to study the impact of a variety of host plants upon inoculum production.

6.2.1.1 Experiment I Selection of suitable diluents and host plants using pot culture (pot culture)

The first experiment was a complete factorial with three factors: host plant species (sweet corn (*Zea mays* var. *saccharata*), sorghum (*Sorghum bicolor* (L.) Moench) and thornless mimosa (*Mimosa invisa* Mart.), AM fungi (*C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08), and pot culture media (see below). Each treatment combination had three replicates (two seedlings/ replicate) laid out in a Completely Randomized Block design. Infertile sandy soil from Mae Hea Agricultural Research Station and Training Center (98º 56´ E, 18º 45´ N) was passed through a sieve with 4 mm mesh to remove large debris and standardize grain size. The soil was mixed with river sand (1:1, v/v) and later analyzed for its nutrient content (Table 6.1). The sandy soil then was mixed with either clay-brick granules, rice husk charcoal, or vermiculite (1:1 v/v) or left unamended and sterilized twice, with one day at room temperature in between, in an autoclave at 121°C, 15 psi for 30 min.

One hundred seeds of each species were disinfected with 0.05% sodium hypochloride (NaOCl) for 5 min and washed three times in sterile water. Seeds were germinated in sterile coarse river sand. Forty eight healthy and uniform seedlings of each host plant were selected after 7 days. Each host plant seedling was inoculated with 100 spores of one of three AM fungus isolates listed above which were placed on sterile filter paper (Whatmann No.1) and transplanted into greenhouse pots. Controls received blank sterilized paper. The pots were black polyethylene plastic pots, 25 cm in top diameter (14 cm height × 17.5 cm width) containing 2 L of one of the four potting mixtures described above. One hundred milliliters of modified Hoagland's nutrient solution [0.1 strength phosphorus (0.1 mM) pH 6.5] (Hoagland and Arnon, 1950) was applied to each pot once per month. The pots were watered every other day with filtered tap water from which chlorine was evaporated for 24 hours in a 100 L black tank before use. The pots were maintained in a greenhouse under natural photoperiods, temperature range of 25 to 38ºC, and relative humidity between 46 and 84% which are in the range for the rainy season of Thailand (May–July 2013).

Watering was stopped 7 days prior to harvesting at 90 days to allow the potting mixture and plants dried slowly. Spores were isolated from soil samples (100

cm^3) by wet sieving and sucrose density gradient centrifugation (Brundrett *et al*., 1996). Three replicates of culture material from each treatment combination were wet sieved separately. AM fungal spores were counted under a stereomicroscope to determine spore density per 100 cm^3 culture material. Three replicates of fine roots from each treatment combination were carefully removed and thoroughly washed in tap water. Percentage root length colonized by AM fungi was evaluated after treating a root sample with 10% (w/v) potassium hydroxide (KOH) solution and staining with 0.05% trypan blue in lactoglycerol (Phillips and Hayman, 1970) via the gridline intersect method under a dissecting microscope (Giovannetti and Mosse, 1980). The remaining culture materials were left to dry in pots for four months (August–November 2013) in the greenhouse and used as AM fungal inocula in Experiment II.

6.2.1.2 Experiment II On-farm inoculum production

Non-pasteurized leaf litter compost, produced in windrows from leaves of *Dimocarpus longan* Lour., *Mangifera* spp. L., and *T. grandis* at the Lumyong's Farm, Mueang, Chiang Mai, was sorted by hand to remove large debris. The remaining culture materials of each pot from Experiment I were mixed together and used as soil-based, mixed-species inoculum. Density of viable spores from the soil-based inocula of Experiment I was assessed microscopically. Viability was based upon appearance of the spores, particularly homogeneity and size of the internal vacuoles (large, coalesced vacuoles indicate loss of viability). Density of viable spores averaged 838.25±77.19 spores 100 cm^{-3} (mean ± SEM, n=3 samples). The soil-based inoculum was mixed with vermiculite and leaf litter compost (1:1:1, v/v/v) and fill to half of four well-water concrete tanks (50 cm height × 80 cm diameter) with drainage holes. The tanks were located 50 cm distance to each other and received the same amount of sunlight. The inoculum, compost, and vermiculite mix in four tanks was analyzed for nutrient content (Table 6.1).

Seeds of *Paspalum notatum* Flugge, *Tagetes patula* L., and *Z. mays* var. *saccharata*, and bulbs of *Allium cepa* L. were disinfected and sown in sterile coarse river sand. After 7 days, ten healthy and uniform seedlings of each host plant were randomly selected and transplanted into each tank. The plants were watered every other day with filtered tap water. The plants were maintained in the tanks with natural

photoperiods from January through March, 2014. After three months of growth, the shoots were removed and three replicate root samples were randomly collected from each tank. In addition, three samples of culture materials were randomly collected per tank. Spore density per 100 cm^3 soils and AM fungus colonization of roots were determined as above.

Table 6.1 Chemical analyses of substrates used in the pot culture (Experiment I) and on-farm production (Experiment II) of AM fungus inoculum.

Substrate	**pH**	**Organic matter** ($g\ 100g^{-1}$)	**N** ($g\ 100g^{-1}$)	**P** ($mg\ kg^{-1}$)	**K** ($mg\ kg^{-1}$)
Sandy soil (Experiment I)	6.70[a]	2.65	0.147	66.87	346.30
Compost: vermiculite: soil inoculum (Experiment II)	7.04	6.74	0.217	214.29	275.08

[a]Values are means of three (Experiment I) and four (Experimental II) replicatoins.

6.2.2 Experiment on *in vitro* axenic culture

This experiment was aimed to determine the best suitable medium for selected AM fungal cultures; *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08 and used *G. intraradices* as a positive control. The *in vitro* culture will used as inocula in *in vitro* inoculation with *Tectona grandis* plantlets.

6.2.2.1 Determination of the best medium suitable for AM fungi

Spores of *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08 were isolated from pot culture by wet sieving and 50% sucrose centrifugation (Gerdemann and Nicolson, 1963). 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 until use on Petri plates of Minimal (M) medium (Bécard and Fortin, 1988) with 0.4% (w/v) gellan gum (Phytagel, Sigma) as gelling agent and without sucrose. Spores were pre-germinated on M medium and modified Strullu Romand (MSR) medium (Declerck *et al*., 1998, modified from Strullu and Romand, 1986). Three replicate of 9 cm square plastic Petri plates with 20 spores individually inserted into the gel of each media were incubated vertically at 32ºC in 2%

CO_2 and dark for 25 days. Germination percentage and length of hyphae from individual germinated spores were observed after 25 days of incubation. Total hyphae length of individual germinated spores was measured microscopically using an ocular micrometer to determine the best medium suitable for AM fungi.

6.2.2.2 Dual culture propagation

Excised Ri T-DNA transformed carrot (*Daucus carota* L.) roots (DC3 clone), developed by Bécard and Fortin (1988), were used as host for *C. etunicatum* NNT10, *C. etunicatum* PBT03, *F. mosseae* RYA08, and *G. intraradices*. Roots were propagated on M medium with 0.2% (w/v) gellan gum (Phytagel, Sigma). A MSR medium solidified with 0.4% gellan gum on one-compartment Petri plate was used in the subsequent experiments. Three days-old root cultures were inoculated each with 25 decontaminated *C. etunicatum* NNT10, *C. etunicatum* PBT03, *F. mosseae* RYA08, and *G. intraradices* spores. Spores were dispensed on the surface of culture medium alongside the actively elongating root regions using sterile surgical blade No. 11. Each treatment was replicated four times. Plates were incubated horizontally for 3 months in the dark at 22±2°C. Spore germination and colonization (e.g. proliferation of hyphae) as well as development of contamination on spore surfaces were monitored routinely using a dissecting and an inverted microscope. After five months, gels were solubilized with 10 mM sodium citrate pH 6.0 (Doner and Bécard, 1991) following the incubation period and roots were cleared and stained with trypan blue (Phillips and Hayman, 1970) to detect colonization by *C. etunicatum* NNT10, *C. etunicatum* PBT03, *F. mosseae* RYA08, and *G. intraradices*. Root length was estimated using a line intersect method (Tennant, 1975).

6.2.3 Data collection and statistical analysis

At harvest, plant shoots were discarded and the substrates were assayed for spore numbers and root length colonization. Two-way analysis of variance (ANOVA) was used for spore numbers and root colonization in experiment I. Univariate analysis was employed for percent colonization and spore density in experiment II. Univariate analysis was used for spore numbers and root colonization in *in vitro* experiment. 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).

6.3 Results

6.3.1 Substrate-based production systems

6.3.1.1 Pot culture

Results of the two-way ANOVA indicated that the number of spores of each AMF isolate was significantly affected (*P<0.01*) by host plant (H), substrate (S), and interaction of host plant and substrate (H×S), except for H×S in *F. mosseae* (Table 6.2). Root colonization by each AM fungal species was significantly affected by host plant (H) and substrate (S), and their interaction (H×S).

Table 6.2 Summary of two-way analysis of variance for AM fungal spore numbers and root length colonization of *C. etunicatum* PBT03, *C. etunicatum* NNT10, and *F. mosseae* RYA08 using different diluents and host plants (Experiment I).

Factor		***C. etunicatum* PBT03**		***C. etunicatum* NNT10**		***F. mosseae* RYA08**	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Spore							
Host (H)	2	23.449	<0.001	52.000	<0.001	69.342	<0.001
Substrate (S)	3	13.465	<0.001	22.467	<0.001	11.987	<0.001
H × S	6	1.860	<0.001	11.438	<0.001	8.070	0.130
Root colonization							
Host (H)	2	49.442	<0.001	70.117	<0.001	170.408	<0.001
Substrate (S)	3	18.431	<0.001	26.238	<0.001	55.654	<0.001
H × S	6	3.623	0.011	4.722	<0.001	5.057	0.002

Table 6.3 Main effects of AM fungus isolates, host plants, and culture materials on resulting spore populations and percentage of root length colonized after three months in the greenhouse (Experiment I).

Main effects separation of means	Spores 100 cm^{-3} substrate	Colonization (% root length)
1. AM fungal isolate		
C. etunicatum PBT03	3,302.56 a[a]	51.95 a
C. etunicatum NNT10	2,406.00 ab	50.12 a
F. mosseae RYA08	1,282.38 b	50.12 a
2. Host plants		
Zea mays	3,690.00 a	64.98 a
Sorghum bicolor	3,068.89 a	59.87 a
Mimosa invisa	232.05 b	29.63 b
3. Culture materials		
Sandy soil	1,118.59 c	51.61 a
Sandy soil: clay-brick granules	2,963.78 ab	58.44 a
Sandy soil: rice husk charcoal	1,627.33 bc	33.09 b
Sandy soil: vermiculite	3,611.56 a	62.83 a

[a]Numbers in the same column, within a factor, followed by the same letter are not significantly different (α=0.05, Tukey's Method of Multiple Comparisons), means of 36 observations for AM fungus treatment, 36 for host plant, and 27 for culture material.

Spore production by *C. etunicatum* PBT03 was significantly greater than that of *F. mosseae* RYA08 (Table 6.3). The AM fungi produced more spores with *Z. mays* and *S. bicolo*r as host than with *M. invisa*. Cultures with *M. invisa* failed to produce spores in the presence of rice husk charcoal (data not shown). *Mimosa invisa* did not grow well in rice husk charcoal and exhibited browning of leaves. Indeed, no colonization *M. invisa* roots was observed when grown in the sandy soil mixed with rice husk charcoal (data not shown). Spore populations were higher in the presence of vermiculite as diluent compared to sandy soil and the sandy soil-rice husk charcoal mixture, and tended to be greater than those with clay-brick granules. Percentage of root

length colonized by AM fungi was higher with *Z. mays* and *S. bicolor* as host plant *vs. M. invisa*.

6.3.1.2 On-farm inoculum production

The number of spores produced in this experiment tended to be lower than that in Experiment I for all four host plants. For example, the number of AM fungal spores produced with *Z. mays* in Experiment I ranged from 1,081 to 9,050 spores 100 cm^{-3}, while in Experiment II the total number of spores of the three isolates ranged from 2,409 to 2,912 spores 100 cm^{-3}. Production of spores with *Z. mays* was significantly higher than those with other host plants, followed by *P. notatum, T. patula* and *A. cepa* (Figure 6.1A). Other AMF, not inoculated into the system, produced 24–83 spores 100 cm^{-3} (data not shown). These spores were identified as *Acaulospora rugosa* Morton, *A. foveata* Trappe and Janos, and *Scutellospora* sp. and were excluded from the data presented below and in Figure 1 because they represented only 1–3% of the total. Nevertheless, there was an approximate 10X increase in spores from the level present in the original inoculum used for this experiment. Given that the original inoculum had a spore density of 838 ± 77 per 100 cm^3 and this inoculum comprised one third of the media, the resulting initial population was 279 spores 100 cm^{-3}, approximately one tenth of the final population of 2,645 spores 100 cm^{-3} when cultured with *Z. mays*. Percentage root length colonization of *Z. mays* was greater than that of *A. cepa*, *P. notatum* and *T. patula* (Figure 6.1B).

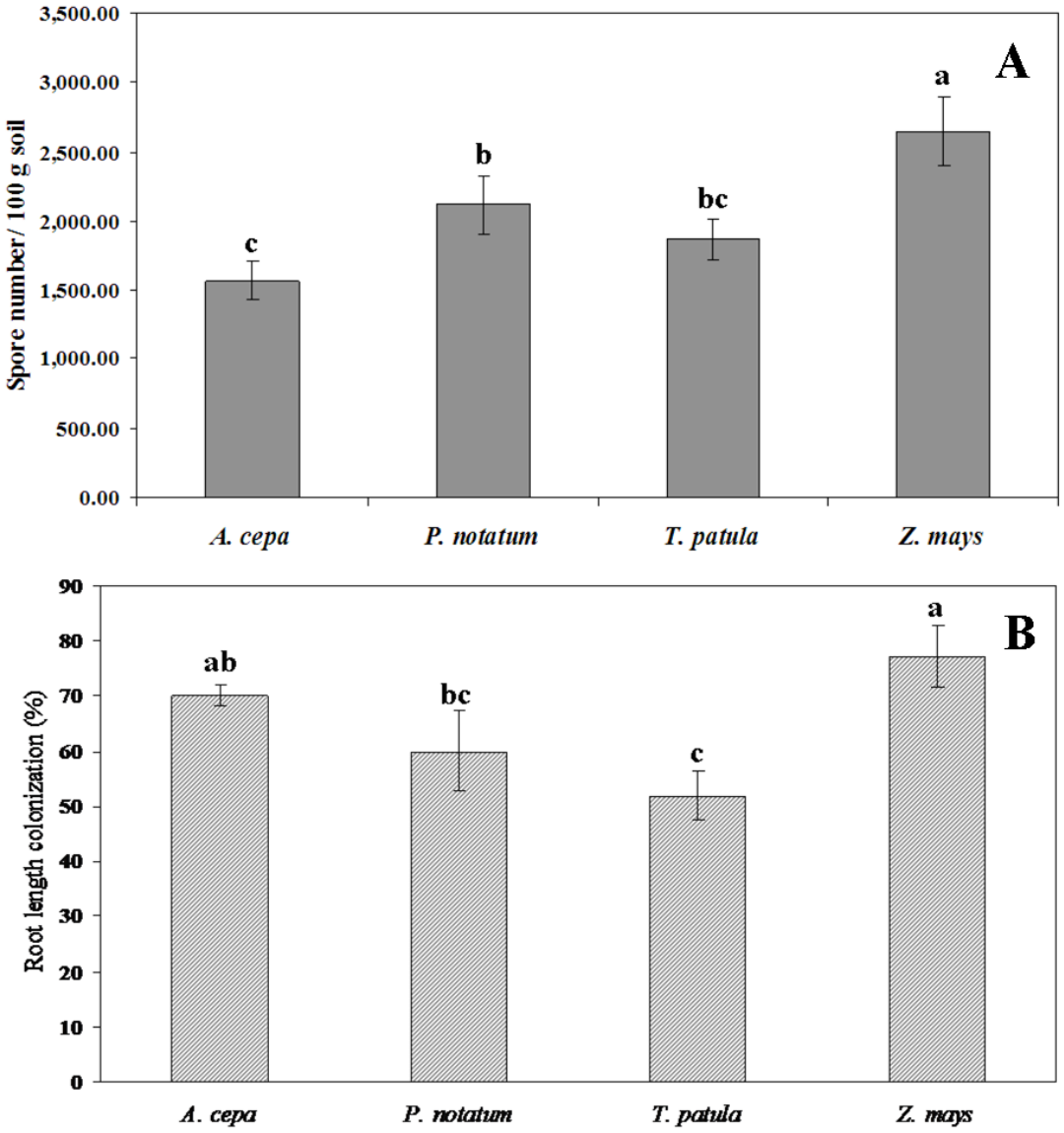

Figure 6.1 Mean number of spores from different host plants (A) and mean percentage root length colonization (B) in the on-farm inoculum production system using leaf litter compost mixed with vermiculite and soil inoculum (1:1:1), Experiment II. Means (n=3) ± SEM. Columns with the same letter are not significantly different (α=0.05).

6.3.2 Experiment on *in vitro* production system

Pre-germinated spore experiment showed germination of *C. etunicatum* NNT10, *C. etunicatum* PBT03, *F. mosseae* RYA08, and *G. intraradices* at 32ºC in 2% CO_2 in both M and MSR media (Figure 6.2). *Glomus intraradices* spores were started to germinate at two days after incubation on both medium, while other spore species

started to germinate at day 4 (data not show). Hypha of *G. intraradices* were germinated through subtending hypha and elongated straight hyphae with few branches into medium faster than other spore species (Figure 6.2A). *Claroideoglomus etunicatum* PBT03 spores were germinated with highly branched hyphae (Figure 6.2B) as well as in *F. mosseae* RYA08 spores (Figure 6.2D) while *C. etunicatum* NNT10 spores had few branched hyphae (Figure 6.2C). Germination percentage of *G. intraradices* was higher than that in *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08, respectively (Table 6.4). Germination percentages of most spore species tend to be higher in MSR medium. Hyphal length of individual germinated spore of *G. intraradices* was higher than that in *C. etunicatum* PBT03, *C. etunicatum* NNT10, and *F. mosseae* RYA08, respectively.

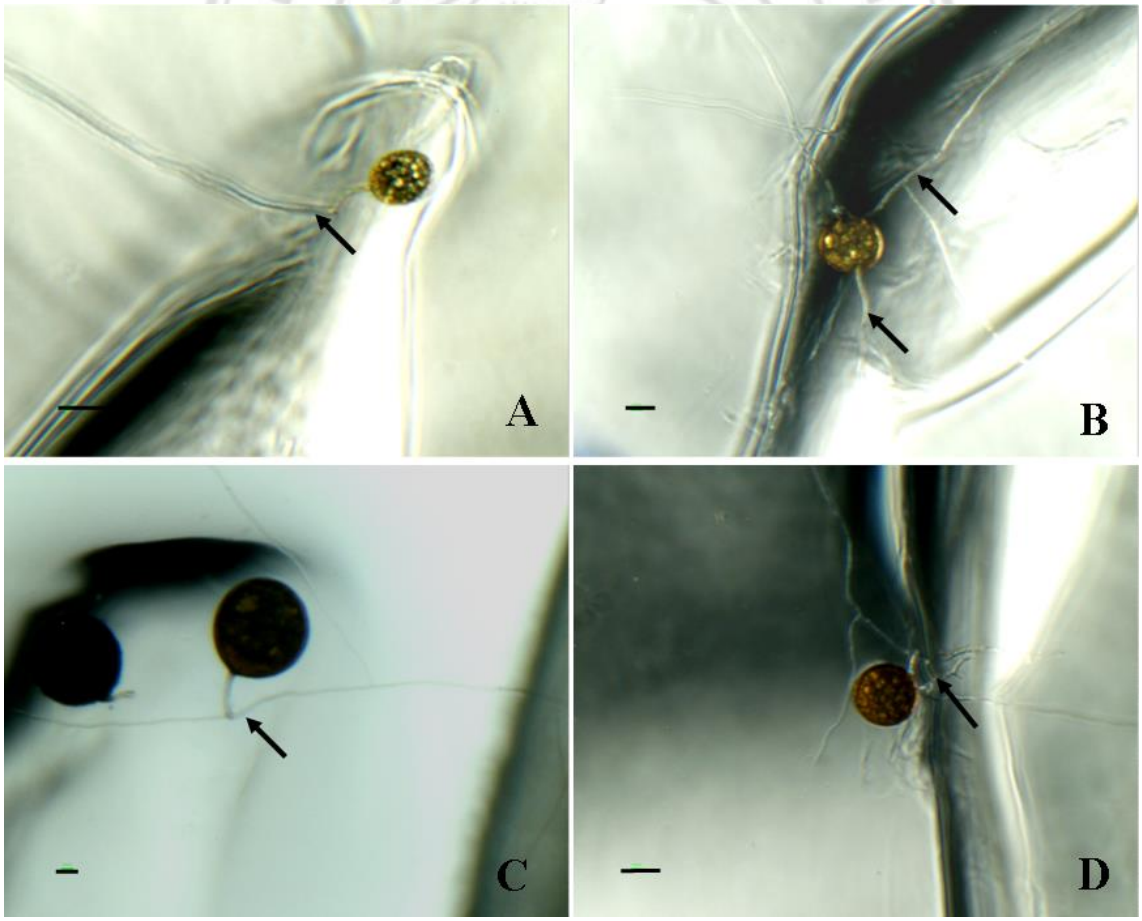

Figure 6.2 Germinated hyphae (arrow) from surface sterile spore on culture medium after 25 days of incubation: (A) *G. intraradices*, (B) *C. etunicatum* PBT03, (C) *C. etunicatum* NNT10, and (D) *F. mosseae* RYA08. Bar = 100 µm.

Table 6.4 Germination percentage and hyphal length of individual germinated spore on the minimal medium (M) and modified Strullu Roman (MSR) media after 25 days of incubation.

Spores specie	Germination percentage (%)		Hyphal length of individual germinated spore (mm)	
	M	MSR	M	MSR
G. intraradices	74.00a[a]	75.33a	7.08a	8.11a
C. etunicatum NNT10	29.50b	36.67b	1.45b	1.35c
C. etunicatum PBT03	20.67b	20.00c	5.40a	4.40b
F. mosseae RYA08	17.38b	28.00bc	1.13b	1.28c

[a]Numbers in the same column, within a factor, followed by the same letter are not significantly different (α=0.05, Tukey's Method of Multiple Comparisons).

Glomus intraradices and *F. mosseae* RYA08 hyphae were spreaded throughout the Petri plates and produced new spores successfully after 14 and 24 days of inoculation, respectively (Table 6.5 and Figure 6.3A–D). *Glomus intraradices* produced the longest hypha growing beyond a radius of Petri plate. *Claroideoglomus etunicatum* PBT03 (Figure 6.3E) and *C. etunicatum* NNT10 were produced only hyphae but not spread throughout the media and no new spores were produced at 5 months after inoculation. Number of new spores produced by *G. intraradices* and *F. mosseae* RYA08 were approximately 7,800 and 26 spores per Petri plate, respectively (Table 6.5). Percentage of root length colonization of *G. intraradices* was higher than those in *F. mosseae* RYA08, *C. etunicatum* PBT03 and *C. etunicatum* NNT10.

Table 6.5 Number of new spores and hyphal characterization of each spore species in *in vitro* propagation after 5 months of inoculation.

Spore species	Colonization (%)	Approximately production/plate	Hyphal growth characterization
G. intraradices	93.83 a*	7,800 a	hyphae spread throughout the Petri plates and start to produce new spores at 14 days after inoculation.
C. etunicatum NNT10	8.48 c	0.00 b	produced only hyphae but not spread
C. etunicatum PBT03	10.99 c	0.00 b	produced only hyphae but not spread
F. mosseae RYA08	69.00 b	26.33 b	hyphae spread throughout the Petri plates and new spores were produced at 24 days after inoculation.

* Numbers in the same column followed by the same letter are not significantly different (α=0.05, Tukey's Method of Multiple Comparisons).

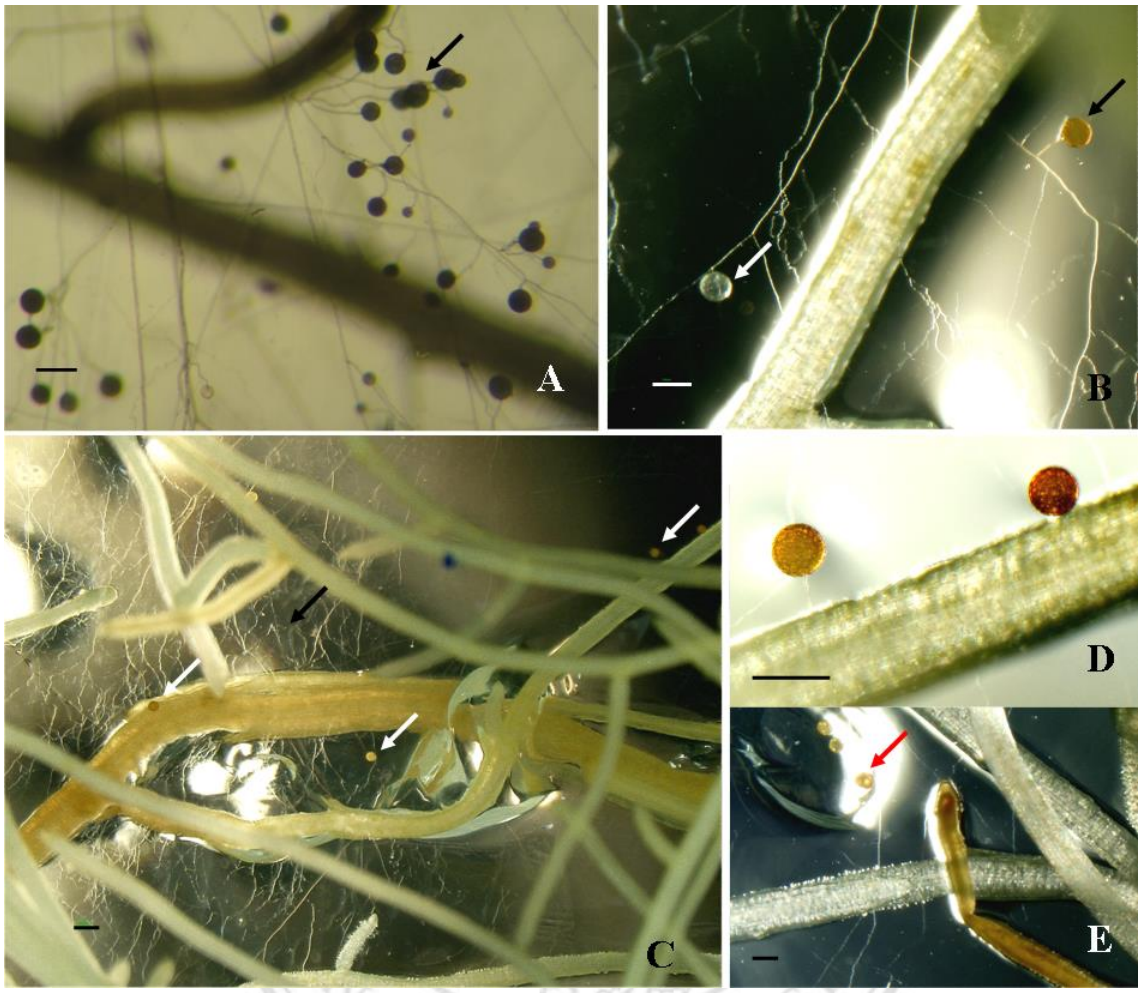

Figure 6.3 *In vitro* culture of AM fungal spores: (A) *G. intraradices*; showing a group of new produced spores (arrow), (B) *F. mosseae* RYA08; showing new young spore (white arrow) and mature spore (black arrow) adhered to hyphae, (C) *F. mosseae* RYA08 culture with dense hyphae (black arrow) and spores (white arrows) throughout the medium, (D) *F. mosseae* RYA08 spores produced outside the transform carrot root, and (E) *C. etunicatum* PBT03 spores (red arrow) germinated and colonized nearby roots. Bar = 500 µm.

6.4 Discussion

6.4.1 Substrate-based production systems

Our results demonstrate the use of leaf litter compost and other locally-available substrates to produce AM fungal inoculum on-farm for the tropical climate of Thailand. Spore production was significantly affected by host plant and substrate. Production of mycorrhizal fungus spores was greater with *Z. mays* in pot culture and on-farm production was considerably higher with it than with other host plants. These results agree with those of previous studies (Millner and Kitt, 1992; Talukdar and Germida, 1993; Gaur *et al*., 2000) that spore production may be related to an inherent ability of that AM fungal strain to sporulate or a favorable interaction (such as carbon availability) with a maize host crop. Gaur and Adholeya (2002) also inoculated five tropical fodder crops with a consortia of indigenous AM fungal species in non-sterile soil amended with organic matter and observed that the level of production of infective propagules was dependent on the host plant species.

In this study, spore number of *C. etunicatum* PBT03, *C. etunicatum* NNT10 and *F. mosseae* RYA08 tended to be higher when cultured with the C4 plants *P. notatum*, *S. bicolor* and *Z. mays*, but not with C3 plants *A. cepa*, *M. invisa* and *T. patula*. This result complements that of Egerton-Warburton *et al*. (2007) who demonstrated that certain *Glomus* species (rapid colonizers producing small spores) increased their spore production after N fertilization when associated with a C4 host. The INVAM website (http://invam.wvu.edu/) reports that spore numbers in some of the pot cultures in their collection decrease after successive propagation cycles and suggests alternation of hosts from C4 Sudan grass [*Sorghum sudanense* (Piper) Stapf], a host commonly used by INVAM, to C3 legume red clover (*Trifolium pretense* L.), a species that is unrelated to the former host (Ijdo *et al*., 2011).

Percentage of root length colonization in both pot and on-farm production tended to be higher for all three AM fungi using *Z. mays* as host plant followed by *A. cepa*, *P. notatum*, *S. bicolor*, *T patula*, and *M. invisa*, respectively. However, number of spores 100 cm^{-3} substrate was not directly correlated with root colonization levels, and was highest in *Z. mays* followed by *S. bicolor*, *P. notatum*, *T patula*, *A. cepa*, and

M. invisa. This result agrees with other reports that correlations between intraradical colonization and extraradical sporulation are often poor and are dependent on the plant/fungus association and particular culture conditions (Giovannetti *et al*., 1988; Douds, 1994; Hart and Reader, 2002; Ijdo *et al*., 2011).

Three diluents of the sandy soil used in this study included: clay-brick granules, rice husk charcoal, and vermiculite. Number of spores of all AM fungal isolates tended to be highest with vermiculite followed by clay-brick granules and rice husk charcoal and lowest in unamended sandy soil. Relatively inert substrates such as vermiculite, perlite, and horticultural potting mix have been used to dilute nutrient-rich compost for on-farm inoculum production in plastic bags (Douds *et al*., 2010). They serve an additional function because their larger particle size can enhance drainage, moisture retention, and aeration as well as having an influence on sporulation of AM fungi (Millner and Kitt, 1992; Gaur and Adholeya, 2000).

Mimosa invisa tended to grow slowly in rice husk charcoal and have lower root mass vs. when grown in other substrates, while other host plants exhibited no ill effects. Komaki *et al*. (2002) suggested that a small amount of rice husk charcoal could increase the growth of *Catharanthus roseus*, but that browning of leaves appeared with excessive application because of the higher concentrations of potassium and higher pH level in rice husk charcoal than wood charcoal. The media with the addition of charcoal is more alkaline and thus may be more favorable for growth of *S. bicolor* and *Z. mays*, but not for *M. invisa*, resulting in AM fungal growth in roots of those plants. Deenik *et al*. (2011) reported that there was a highly significant fertilizer effect of charcoal addition, which produced increases in maize growth. They also found that incompletely carbonized charcoals have high volatile matter content that stimulates microbial growth inducing N deficiency due to immobilization (Deenik *et al*., 2010). These results are in agreement with the findings of Gundale and DeLuca (2007) who showed that low temperature charcoals inhibited plant growth and high temperature charcoals promoted plant growth.

Spore number 100 cm^{-3} substrate in the pot culture experiment was higher than the on-farm experiment. This may have resulted from the concentration of phosphorus in the substrate (leaf compost) used for the on-farm system being three times higher

than those of the pot culture substrate (66.87 vs. 214.29 mg kg^{-1}). AM fungal colonization and spore production are favored under low-nutrient (mainly P) conditions (Smith and Read, 1997). Proper consideration of the compost nutrient concentration is necessary to determine the relative proportions of yard clippings compost and diluent in another on-farm production system (Douds *et al*., 2008) Therefore, farmers adopting this method should take concentration of phosphorus into account when using leaf litter compost as a component AM fungal spore production.

Leaf litter compost is a suitable farm waste for incorporation into the substrate for the on-farm production of AM fungal inoculum. The use of leaf litter compost for on-farm inoculum production also reduces agricultural waste and residue as well as reduces the cost of producing mycorrhizal fungus inoculum. On-farm production of AM fungus inoculum is especially appealing to organic farmers who seek to minimize off-farm inputs through the use of compost and locally-available diluents and appreciate the option to produce indigenous AM fungi. Further, the host plant can itself be a commercial crop, such as sweet corn.

6.4.2 *In vitro* production system

Our *in vitro* experiment revealed that germination percentage and hyphal length of individual germinated spore of *G. intraradices* was higher in both M and MSR medium than other spore species and spores were started to germinate at day 2 after incubation. Whereas the germination percentage and hyphal length of individual germinated spore of *C. etunicatum* PBT03, *C. etunicatum* NNT10, and *F. mosseae* RYA08 were lower than that in *G. intraradices* and started to germinate after. From this observation, the cold storage at 4ºC after surface sterilization may differently affected on spore germination of AM fungal species. This observation values were agreed with Juge *et al*. (2002) who studied the effect of cold storage period at 4ºC on spore germination of *G. intraradices* and found that more than 30% of the spores had germinated by day 3 in all cold treatments. They also found that there was no significant differences in germination at the beginning of the experiment (day 3) but cold stratification longer than 14 days significantly increased spore germination, clearly reduced spore mortality from 90% to 50% and considerably altered the hyphal growth pattern. This result may also caused from spore dormancy of each spore species which

controlled by basic internal biological clock and that cold storage prior to germination is not absolutely necessary to break dormancy of some AM fungal species (Juge *et al*. 2002; Eskandari and Danesh, 2010). However, Hepper and Smith (1976) increased germinability of *F. mosseae* by stratification at 6°C for several weeks. Safir *et al*. (1990) also obtained increased and synchronized spore germination of *F. mosseae* after 28 days at –10°C, whereas spore storage at 4°C did not improve germination rates over storage at room temperature. On the contrary, Douds and Schenck (1991) concluded that *F. mosseae* spores showed no dormancy, as identical germination rates were obtained with or without a storage period.

In dual culture experiment, *G. intraradices* and *F. mosseae* RYA08 hyphae were spreaded throughout the Petri plates and produced new spores but others was not produced. This result was in accordance with previous decade research on the *in vitro* of *G. intraradices* culture that these model fungi can grow well in response with transformed carrot roots factors and sporulate profusely after crossing a barrier into root-free medium (Chabot *et al*., 1992; St. Arnaud *et al*. 1996). In this experiment, we used one-compartment Petri plates to culture all fungal strains with transformed carrot roots as a preliminary *in vitro* experiment. The split plate were also used once to test the efficiency of *G. intraradices* and our selected spores on spore production but only *G. intraradices* hyphae could crossed the barrier (data not shown). Therefore, only the one-compartment Petri plates were used in this experiment. Germination and hyphal growth of *F. mosseae* were observed by previous published papers. The germination of *F. mosseae* spores was observed 80% germination within 14 days (Meier and Charvat, 1992) and maximal germination only after 35–40 days of incubation (Hardie, 1985). Douds (1997) manipulated buffer, pH, and phosphorus levels in M medium in order to increase the successful for culture *F. mosseae* with carrot roots but 14% success rate was achieved and no new spores were produced. Moreover, those cultures grew for 17–24 weeks before termination. In our dual culture, *F. mosseae* RYA08 hyphae had spread throughout the Petri plates and approximately 26 spores were produced after 5 months of incubation while other spores were produced trifling hyphae around the germinated spores. These results may cause from preferable growth of each AM fungal species on different culture media. For example, MSR medium that was used in this experiment gave more success in *F. mosseae* culture than M medium, when compared to the result

with Douds (1997). The differentiates between M and MSR media as small amount in milligram of macro elements ($MgSO_4$, KNO_3, KH_2PO_4, $Ca(NO_3)_2$), micro nutrients, and vitamins in those medium (Appendix B), may not be affected to the growth of AM fungal *in vitro* culture. Therefore, the future subculture of *F. mosseae* or other AM fungal species could be done from successful cultures, as with routine propagation of *G. intraradices in vitro* dual cultures (St. Arnaud *et al*. 1996) and should keep the different culture medium into account when selecting appropriate culture conditions for those fungi.