CHAPTER 3

Identification and efficiency of arbuscular mycorrhizal fungi for growth promotion of *C. alismatifolia* Gagnep.

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are beneficial fungi associated with roots of the host plant, including the important agricultural and horticultural crops. The beneficial role of AMF is to improve plant growth via increased mineral nutrient uptake and water absorption. Identification of AMF is usually based on the morphology of spores, structural character and related structures formed in the roots. Morphological characters of spore can be determined in some genus and species. Since molecular biology technique has been used to identify characters of AMF, it is important to determine each AMF species. Sometimes, both morphological and molecular techniques are combined for identification of AMF. AMF colonization has also been found in scale leaves and leaf bases of *Curcuma decipiens* Dalz. and *Curcuma longa* L. The total infection percentages are 26.6% and 19.4%, respectively (Khade and Rodrigues, 2007).

Therefore, this research was aimed to select and identify AMF species which was beneficial to promote growth of *C. alismatifolia*, an important flower crop in Thailand by using morphological characterization and molecular techniques.

3.2 Materials and methods

3.2.1 Soil collection and morphological study

The preliminary investigation of AMF were carried out by sampling soil from rhizosphere of *C. alismatifolia* at five locations, i.e., Saithong National Park (ST), Paa Hinngarm National Park (PH), Chiayapoom province, San Sai district farmers (BL and DD) and Yangkram subdistrict (YK), Chaing Mai province. Morphological study of AMF was conducted by isolating spores from soil samples. Soil samples were determined by wet-sieving and decanting technique with a sucrose solution centrifugation method (Brundrett *et al*., 1996; Asif, 1997). Spores of AMF isolates from the field soil were separated by spore size and spore shape by stereo microscope (Olympus SZ-40, Japan). Then, the spores were stained with PVLG and Melzer's reagent to observe spore color and layers of spore wall by microscrope (Olympus CX31, Japan.). The root samples were long-sectioned to determine infection. The roots staining with 0.05% trypan blue were assessed for mycorrhizal infection by modified grid line intersect method (Brundrett *et al*., 1996).

3.2.2 Spore propagation

About 400 spores of each isolate were propagated on host plants using *Zea mays*. The inoculated plants were grown in plastic bag using sterilized sand as

growing medium and weekly supplemented with Hoagland's nutrient solution (Millner and Kitt, 1992) for three months.

3.2.3 Plant materials and cultivation

C. alismatifolia Gagnep. cv. 'Chiang Mai Pink' plantlets derived from *in vitro* propagation were grown for 2 months before fungi inoculation. The plantlets were inoculated with 3 characters of 200 spores AMF grouped by morphological study of 5 locations. The inoculated plants were grown in sterilized sand, three inch pot and weekly supplemented with Hoagland's nutrient solution (Millner and Kitt, 1992) for three months. One group of plantlets were grown in sterilized sand without AMF inoculation as control treatment. The plantlets were grown in controlling room with fluorescent lamp and temperature at 25 °C.

The plant height, number of leaves, total leaf area, diameter of rhizome, fresh and dry weight, infection percentages and phosphorus concentration in roots and leaves were determined for three months after planting (MAP). Phosphorus concentration was analyzed by the modified method of Ohyama *et al.* (1991). The experimental design was in Completely Randomized Design (CRD) with 16 treatments, four replications per treatment. Means of each pair of data combinations within the same stage were analyzed by using a statistical analysis program the Statistic 8 (SXW Tallahassee, FL, USA). The least significant difference (LSD) was used to interpret significant difference among the means (<0.05).

3.2.4 Identification of AMF by 18S rDNA sequence

DNA extraction

About 20 spores of each sample were added with 1% Tween20 and 500 µl dH_2O, then sonicated for 5 sec. Spores were taken to new microcentrifuge tube with 500 µl dH_2O and sonicated 3 times for 5 sec. After sonication, these spores were then taken to new microcentrifuge tube. After that, these spores were broken by pessel and added 50 µl of InstaGene^{TM} Matrix, then incubated at 56 ˚C for 30 min and at 95 ˚C for 10 min. Next, centrifuged at 10,000 rpm for 1 min and took 30 µl of supernatant to new microcentrifuge tube as a DNA extraction. Volumes of 5 µl of each DNA extract were used as PCR template.

PCR conditions

The 2x Mighty Amp buffer (TaKaRa) and Mighty Amp DNA polymerase (TaKaRa) were used for PCR with the SSUmAf1-LSUmAr3 or SSUmCf3-LSUmBr1 primer pairs. SSUmCf3 and LSUmBr1 were also applied as nested primers. Thermal cycling (Applied Biosystems 9700) was followed, conditions for the first PCR: 5 min initial denaturation at 99 ˚C; 40 cycles of 10 sec denaturation at 99 ˚C, 30 sec annealing at 60 ˚C and 1 min elongation at 72 ˚C; and a 10 min final elongation. The same conditions were used for the nested PCR primers except that the annealing temperature was 63 ˚C and only 30 cycles were carried out. The 5 µl of nested PCR products were loaded on 1.5% agarose gels with 1xTAE buffer at 100 V, and visualized after ethidium bromide staining (1 µg/ml) (Krüger *et al*., 2009).

Cloning PCR products and sequencing

1.5 µl of PCR product was cloned into pGEM-T easy vector (Promega) as in manufactures' method. 1 µl of ligation reaction was added to 50 µl of competent cells (DH5α, Invitrogen). Heat-shock the cells for 45-50 sec at 42 ˚C and on ice for 2 min. Next, 500 µl of SOC medium was added and incubated for 1 hr at 37 ˚C with shaking (160 rpm). After that 100 µl of each transformation culture was spread onto duplicate LB/IPTG/X-Gal plates and incubated it overnight at 37 ˚C. White colonies were selected into YT medium and incubated for 16 hr at 37 ˚C with shaking. Plasmids were isolated by Pure Yield^{TM} Plasmid Miniprep System (Promega). Plasmid DNA was used for sequencing. The clones were sequenced, using M13R primer with the BigDye v3.1 (Applied Biosystems) by 3130xl Genetic Analyzer, Applied Biosystems ABI. The BLAST search system available in the GenBank databases was used to compare the resulting sequence with those identified. Phylogenetic analysis was carried out using CLUSTAL W software, and the evolutionary tree for the datasets was inferred, employing the neighbor-joining method with MEGA version 4.0.2.

3.3 Results

3.3.1 Morphological study of AMF

Three characters of AMF spores from rhizosphere soil were determined. Isolate No. 1 had single and globose, spores with diameter of approximately 125-250 µm and the color was dark red-brown (Figure 3.1 A). The spore wall did not react with Melzer's reagent (Figure 3.1 C). Spore wall had three layers (Figure 3.1 D). The

investigation of infection showed that this isolate could infect cortex and formed both arbuscule and vesicle (Figure 3.1 E). The type of AMF colonization was the *Arum*-type. These characteristics of isolate No. 1 were similar to those in the genus of *Acaulospora* which forms spores laterally from the neck of a swollen hyphal terminus.

Isolate No. 2 had also single and globose- spores with diameter of approximately 125-250 μm, the same as isolate No. 1. However, the color was light white (Figure 3.1 F). Melzer's staining reaction occurred in layers of this spore. This layer was stained dark red-brown to very dark red-purple (Figure 3.1 H). Spore wall had three layers (Figure 3.1 I). Spores were formed terminally on a bulbous sporogenous cell of subtending hyphae. The investigation of infection indicated that it could infect into cortex and formed arbuscule only (Figure 3.1 J). The type of AMF colonization was the *Arum*-type. These AMF spore characteristics were distinctively determined to be the family of Gigasporaceae.

Isolate No. 3 was singly and globosely, the same as in isolate No.1 and No. 2 but the spore size varied between 45-125 μm and spore color was light white (Figure 3.1 K). This isolate had four layers of spore wall (Figure 3.1 N). The forth layer was laminate layer which often appear to be quite thin. Melzer's staining reaction was not present in layers of isolate No.3 (Figure 3.1 M). The infected roots showed that the isolate No.3 could infect cortex and formed both arbuscule and vesicle (Figure 3.1 O). The type of AMF colonization was the *Arum*-type. The hyphal network consisted of numerous H branches and occasional coils. These AMF spore characteristics were distinctively determined to be the genus of *Glomus*.

3.3.2 Effect of AMF on growth of *Curcuma* plantlets

The results showed that plant height of all inoculated plants was significantly greater than uninoculated plant (control) at 1st, 2nd and 3rd month (Table 3.1). The number of leaves of inoculated plants were not significantly different when compared to control at the 1st and 2nd month but it was higher than the control at the 3rd month. After growing for 3 months, the leaf area, diameter of rhizome, fresh and dry weight of plantlets, % infection and P concentration were measured. The results showed that inoculated plants produced better results than control. Plant inoculated with ST isolate No. 3 gave the best of leaf area, fresh and dry weight, % infection and P concentration in roots and leaves (Table 3.2). P concentrations in roots of inoculated plants were approximately 7.08-9.48 mg/gDW, compared with 1.98 mg/gDW in control roots. The concentrations in leaves of inoculated plants were also the highest at 9.2 mg/gDW after being inoculated with ST isolated No. 3 (Table 3.2).

3.3.3 18S rDNA sequence of AMF

Spore sample of ST isolate No. 3 which had the highest potential for plant growth was identified. The nested-PCR-amplified products were 1.5-1.8 kb (Figure 3.2). On the basis of a BLAST analysis of 18S rDNA gene sequences, the *C. alismatifolia* AMF isolate No. 3 was almost identical to *Glomus claroideum* (AJ567744) showing 97% similarity (Figure 3.3).

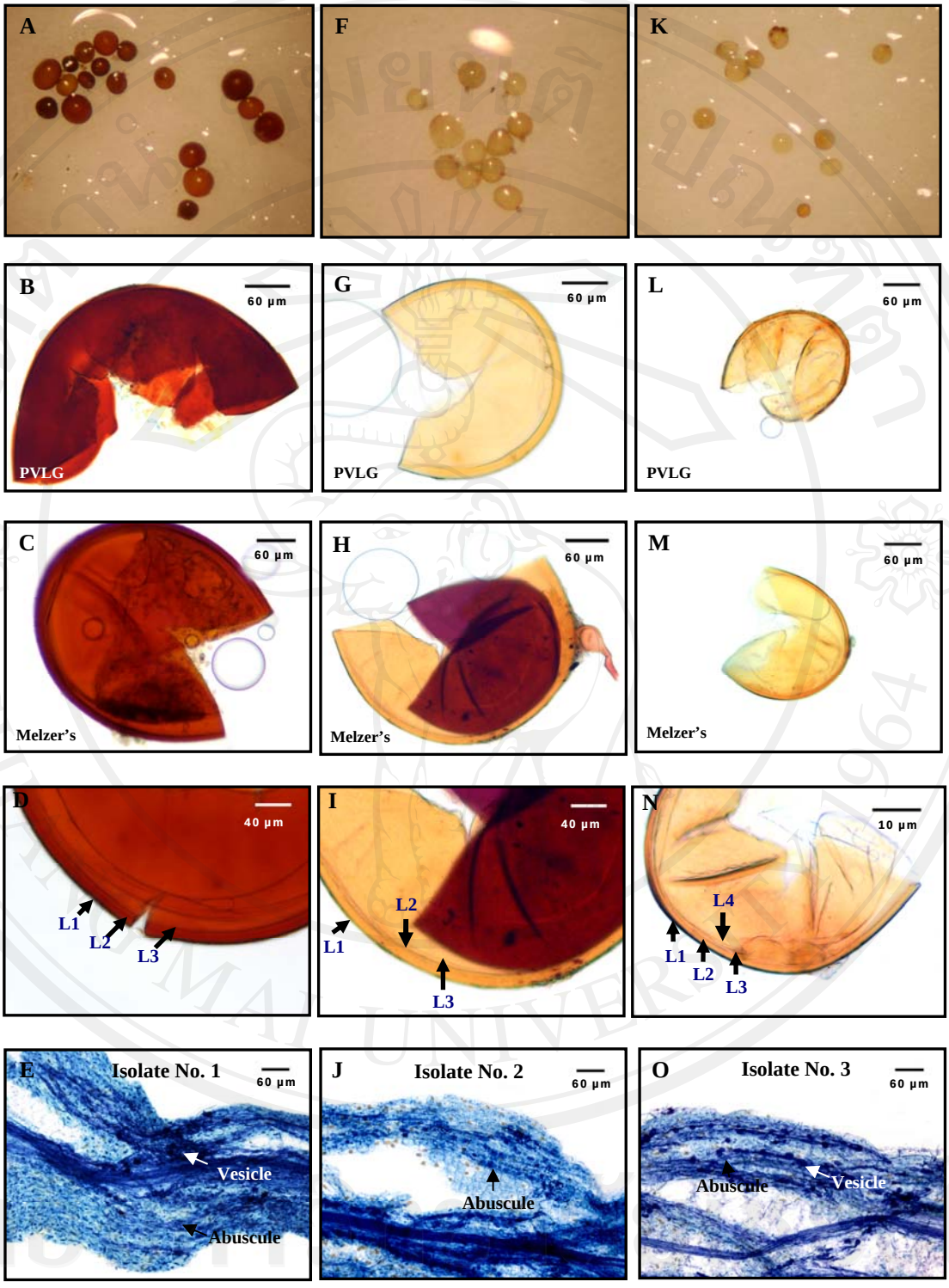

Figure 3.1 The morphology of AMF isolated from rhizosphere soil of *C. alismatifolia*

Table 3.1 Effect of inoculated plants with AMF on plant height and number of leaves of *Curcuma* plantlets

Treatment	1st month		2nd month		3rd month	
	Plant height[1/] (cm)	Number of leaves	Plant height[1/] (cm)	Number of leaves	Plant height[1/] (cm)	Number of leaves[1/]
Control	12.4 f	3.8	19.22 e	5.5	22.29 f	6.2 d
PH isolate No. 1	14.76 abc	3.8	23.40 b	5.7	33.09 bc	7.1 a
ST isolate No. 1	15.25 ab	3.7	23.83 b	5.6	31.71 bcd	6.9 abc
BL isolate No. 1	13.16 ef	3.6	18.47 e	5.6	26.02 e	6.4 cd
DD isolate No. 1	13.41 de	3.7	18.05 e	5.6	25.98 e	6.5 bcd
YK isolate No. 1	14.33 bcd	3.8	21.05 d	5.6	30.3 d	6.5 bcd
PH isolate No. 2	15.03 ab	3.6	23.79 b	5.6	32.04 bcd	7.0 ab
ST isolate No. 2	15.07 ab	3.6	22.19 bcd	5.4	31.37 bcd	7.0 ab
BL isolate No. 2	15.22 ab	3.6	22.14 bcd	5.6	31.88 bcd	7.0 ab
DD isolate No. 2	14.87 ab	3.6	21.22 d	5.7	30.55 cd	7.0 ab
YK isolate No. 2	14.89 ab	3.7	23.22 bc	5.7	31.02 bcd	7.1 a
PH isolate No. 3	14.84 ab	3.6	22.14 bcd	5.7	32.68 bcd	7.3 a
ST isolate No. 3	15.56 a	3.5	30.21 a	5.9	38.63 a	7.3 a
BL isolate No. 3	15.47 a	3.6	23.57 b	5.8	33.61 b	7.2 a
DD isolate No. 3	14.81 ab	3.5	21.51 cd	5.6	32.17 bcd	7.0 ab
YK isolate No. 3	13.83 cde	3.6	21.31 d	5.7	32.24 bcd	7.0 ab
f-test	*	ns	*	ns	*	*
$LSD_{0.05}$	0.96	0.44	1.8	0.45	2.75	0.6

[1/] Mean with the same columns followed by different characters showed significant difference between treatment *f*-test at $P<0.05$

The symbol "ns" is : not significantly different and "*" is significantly different at the $P<0.05$

Table 3.2 Effects of AMF inoculation on leaf area, diameter of rhizome, fresh and dry weight, % infection of *C. alismatifolia* planlets.

Treatment	Total leaf area[1/] (cm^2)	Diameter of Rhizome[1/] (cm)	Plant Weight[1/] (g)		%infection[1/]	P concentration (mg/gDW)	
			fresh	dry	(%)	roots	leaves
Control	17.18 c	0.58 e	3.98 e	0.4 d		1.98 i	3.65 f
PH isolate No. 1	49.02 ab	0.76 ab	5.25 abc	0.57 abc	36.41 gh	8.32 de	7.34 cd
ST isolate No. 1	42.75 ab	0.76 ab	5.09 abcd	0.58 abc	35.91 gh	7.93 ef	7.02 cd
BL isolate No. 1	36.07 b	0.71 cd	4.56 cde	0.49 bcd	33.49 ij	7.41 fg	4.61 ef
DD isolate No. 1	36.15 b	0.70 d	4.51 de	0.48 cd	35.39 ghi	8.08 fgh	4.88 e
YK isolate No. 1	37.39 b	0.74 bc	4.8 bcd	0.5 bcd	34.65 hi	7.17 gh	7.07 cd
PH isolate No. 2	43.96 ab	0.72 cd	5.19 abcd	0.56 abc	32.56 j	7.08 h	6.79 d
ST isolate No. 2	38.07 b	0.70 d	4.85 bcd	0.51 abc	35.73 gh	7.83 efg	6.80 cd
BL isolate No. 2	41.15 ab	0.77 a	5.07 abcd	0.55 abc	38.86 ef	8.39 cde	7.37 cd
DD isolate No. 2	39.3 ab	0.74 bc	4.96 bcd	0.5 bcd	37.19 fg	8.24 de	7.12 cd
YK isolate No. 2	37.92 b	0.76 ab	4.88 bcd	0.5 bcd	40.89 e	8.73 bcd	7.12 cd
PH isolate No. 3	40.26 ab	0.72 cd	5.25 abc	0.57 abc	64.07 c	9.13 abc	8.35 ab
ST isolate No. 3	52.64 a	0.76 ab	5.75 a	0.61 a	70.65 a	9.48 a	9.20 a
BL isolate No. 3	52.57 a	0.78 a	5.47 ab	0.59 ab	40.77 e	8.57 cde	7.42 bcd
DD isolate No. 3	41.09 ab	0.72 cd	5.30 ab	0.56 abc	67.96 b	9.43 ab	7.78 bc
YK isolate No. 3	42.56 ab	0.70 d	5.09 abcd	0.57 abc	61.27 d	8.78 abcd	7.50 bcd
f-test	*	*	*	*	*	*	*
$LSD_{0.05}$	14.29	0.02	0.71	0.10	0.02	0.75	0.98

[1/] Mean with the same columns followed by different characters showed significant difference between treatment *f*-test at $P<0.05$

The symbol "ns" is : not significantly different and "*" is significantly different at the $P<0.05$

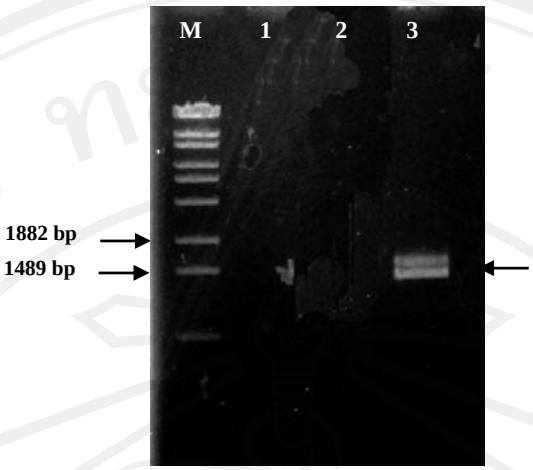

Figure 3.2 Nested PCR amplification of AMF isolate No. 1 (lane 1), isolate No. 2 (lane 2), isolate No. 3 (lane 3) and λEcoT marker (M)

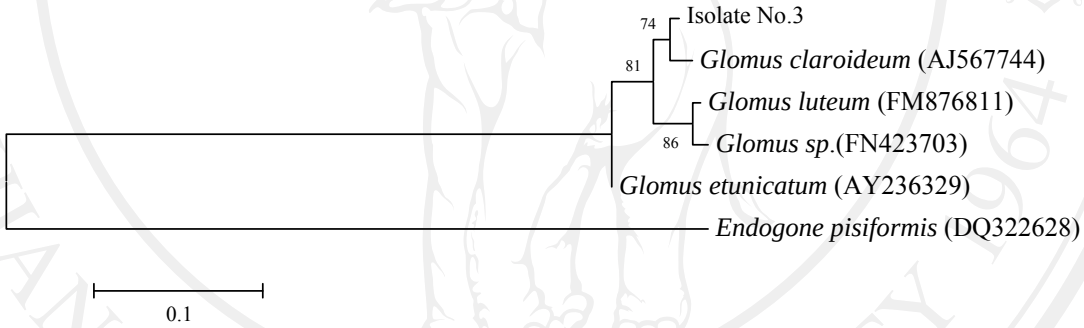

Figure 3.3 Phylogenetic relationships of AMF isolate No.3.

The Neighbor-Joining method utilized a 18S rDNA sequence. *Endogone pisiformis* (DQ322628) was used as an outgroup. Accession numbers were shown in parentheses. Bar, 1 substitutions per 100 nucleotide positions.

3.4 Discussion

Morphological characterization could separate AMF spores into 3 groups with the different genera. The spore propagation result showed that isolate No. 1 and isolate No. 2 could not be propagated, using *Zea may*s as a host plant. Only isolate No. 3 could be propagated by this host. This indicated that AMF were specific to type of host plant. The level of specificity of AMF could give important consequences on plant ecology. The specificity of plant response to the AMF, the diversity and composition of the AMF community had been shown to exert large effects on plant diversity and composition (Hijden *et al*., 1998). However, when *C. alismatifolia* was used as host plant, the result showed that the isolate No.1 and No.3 could infect into cortex and formed both arbuscule and vesicle whereas isolated No.2 could form arbuscule only. Saouy (2008) found that two isolates of AMF spores in *Lactuca sativar* var crispa L were also specific to host plant. In the present study, the morphological identification revealed that two isolates were different, i.e., isolate No.1 was determined to be the genus of *Glomus* by morphological identification. Spore was globosely, spore size varied between 125-250 µm and spore color was yellow-brown. The spore wall had four layers and no reaction was produced in Melzer's reagent. The type AMF of association was *Arum*-type. Isolate No.2 was determined to be of the genus of *Gigaspora* with spore size varying from 250-500 µm and spore color was white. The spore wall had three layers and occurred with Melzer's staining reaction. Spore formed bulbous sporogenous cell of subtending hyphae. The AMF identification using morphology was also reported in many researches. Namanusart (2003) found that the morphology of AMF spore isolated from *Acacia mangium* rhizosphere were identified into 4 genera; *Acaulosopora,*

Gigaspora, Glomus, Scutellospora and one unknown *Glomus*-like species. Sizes of spore varied from 106 to 330 μm, having many forms such as globose, subglobose, ovoid shape. Charoenpakdee *et al*. (2007) reported that AMF spores of physic nut were identified by wet sieving and sucrose centrifugation. Thirty two species have been identified, comprising 4 genera: *Acaulospora* (19 species), *Gigasopora* (2), *Glomus* (8) and *Scutellospora* (3). The most dominant species were *Acaulospora* sp. 1NK12. *Acaulospora* sp. 1NK15, *Acaulospora* sp. 1NK16, *Acaulospora* sp. 3CM17 and *Gigaspora rosea* 3CM 02, and *Gigaspora* sp. 1LO01.

Three isolates (No. 1, No. 2 and No. 3) of AMF from 5 different locations were inoculated into *C. alismatifolia* plantlets. ST isolate No.3 gave the best results in terms of plant height, leaf area, fresh and dry weight, infection percentage and P concentration. Paraskevopoulou-Paroussi *et al*. (1997) reported that shoot dry weight, P concentration in leaves, total P uptake, leaf area, number of leaves were significantly higher in AMF plants than in non-AMF plants when *Glomus clarum* or *Gigaspora decipiens* were inoculated in *Aloe vera*. This result suggested that AMF colonization could increase the nutrient uptake and growth (Tawaraya *et al*., 2007). The infection percentage of *C. alismatifolia* AMF varied from 32.56-70.65% (Table 3.2). It was similar to the report in physic nut (*Jatropha curcas* L.) by Charoenpakdee *et al*. (2007) which showed that infection percentage varied between 37.7 - 94.3%. The infection of *Glomus caledonium* in green leaf and red leaf lettuce were 90% and 97%, respectively, while the infection of *Gigasapora margarita* to green leaf and red leaf lettuce were 19.5% and 55.8% (Saouy, 2008). This indicated that AMF was specific to the type of host plant. The level of specificity of AMF could give important consequences on plant ecology (Hijden *et al*., 1998). However, the

responsiveness of plant species to AMF infection was highly variable. Benefit from AMF included improved access to limiting soil resources, especially immobile nutrients, such as P, Cu, Zn, and ammonium, and these benefits might be significant (Wilson *et al*., 2001). Marschner and Dell (1994) estimated that AMF hyphae could accumulate up to 80% of a plant's phosphorus requirements and 25% of a plant's nitrogen requirements. AMF significantly increased P- concentration, P-uptake, % P derived from fertilizer and P-availability in vetiver (Techapinyawat *et al*., 2002). Moreover, AMF could improve the soil and crop productivity by allowing farmers to reduce their inputs of chemical fertilizers and/or by enhancing plant survival, thus AMF had beneficial effect on soil aggregation and soil fertility (Mridha, 2003). The increase of plant height, number of leaves and leaf area by ST isolate No. 3 might be due to the increase of P concentrations in roots and leaves, it brought about the increase of fresh and dry weight and new rhizome diameter, indicating that AMF inoculation could accelerate growth of new rhizome which was beneficial for rhizome production by tissue-cultured plantlets.

The molecular fingerprinting techniques were independent of culturing, and bases of small-subunit (SSU) rDNA genes could be amplified by the SSUmAf1-LSUmAr3 or SSUmCf3-LSUmBr1 primer pairs. SSUmCf3 and LSUmBr1 were also applied as nested primers. The 18S rDNA gene sequence could identify only isolated No. 3. It was *Glomus claroideum* showing 97% homology. *G. claroideum* occurred in many species, i.e., *Plantago lanceolata*, *Zea mays* (Blaszkowski *et al*., 2003), *Allium porrum* (Vandenkoornhuyse *et al*., 2001) *and Glycine max* (Schenck and Smith, 1982). Vandenkoornhuyse *et al*. (2001) identified *G. claroideum* by using SSU rDNA PCR-RFLPs. The primers used were MH2 and MH4. Namanusart (2003)

reported that the 18S and 5.8S rDNA were used for sequencing of AMF from *Acacia mangium* rhizosphere. Five species, i.e., *Gigaspora albida* (99% homology), *Gigasora gigantean* (97% homology), *Glomus mosseae* (94% homology), *Glomus claroideum* (90% homology) and *Scutellospora persica* (96% homology) were identified. Saouy (2008) found that two AMF isolates from lettuce (*Lactuca sativa*) rhizophere soil were identified as *Glomus caledonium* (65% homology) and *Gigaspora margarita* (67% homology) by AM1/NS31 primer. AMF in *Taxus baccata* was identified using Glomeromycota-specific primer and the cloned and 5.8 S rDNA sequences. The results indicated four sequence type of *Glomus* and one sequence type of *Archaeospora* (Wubet *et al*., 2003).

3.5 Conclusion

AMF were collected from rhizosphere soil of *Curcuma* production at five different locations. They could be separated into three characters with the difference of morphological characteristics, i.e., spore size, spore color, Melzer's staining, spore wall and infection. The ST isolate No. 3-inoculated plantlets could produce the best results of growth and rhizome size of *C. alismatifolia*. The result of 18S rDNA gene sequences revealed that the isolate No. 3 was almost identical to *Glomus claroideum* (AJ567744), showing 97% similarity.