CHAPTER 7

Investigation of selected AM fungal colonization in target plant roots

7.1 Introduction

The studies of AM fungal communities in environmental site samples are difficult because the fungi are obligate symbionts that can not culture in axenic condition and can only be identified morphologically in the spore stage (Vandenkoornhuyse *et al.*, 2003). AMF have been widely studied in the community by using polymerase chain reaction (PCR)-based method, including restriction fragment length polymorphism (RFLP) (Daniell *et al*., 2001), terminal-restriction fragment length polymorphism (T-RFLP) (Pandey *et al.*, 2007; Mummey and Rillig, 2008), nested PCR and single stranded conformation polymorphism (SCCP) (Kjoller *et al*., 2000) and denaturing gradient gel electrophoresis (DGGE) (Liang *et al*., 2008). AMF, which colonizes plant roots, can be detected by several methods. Zézé *et al*. (1997) investigated *Scutellospora castanea* in plant root by using highly repeated DNA sequence (SC1) specific probe. Complete sequence of SC1 was used to generate primers that were sufficiently specific for absolute detection of *S. castanea* in plant. Moreover, Lee *et al*. (2008) designed a set of PCR primers to facilitate rapid detection and identification directly from field-grown plant roots. Although morphological identification of AMF is the most widespread method and in many cases still unavoidable, it should be carried out along with other methods such as molecular for mutual confirmation (Jansa *et al*., 2002). Previously, DGGE were used to investigate AM fungal colonization and community in target plant roots or soils for over a decade. Muyzer *et al*. (1993) was first to use PCR-DGGE to profile microbial communities. The first use of this technique for fungal community analysis was by Kowalchuk *et al*. (1997). Since then, PCR-DGGE has proven to be a powerful technique for the culture-independent detection and characterization of fungal populations in plant material and

soil (Smit *et al*., 1999; Vainio and Hantula, 2000; Kowalchuk *et al*., 2002). PCR-DGGE was demonstrated by Smit *et al*. (1999) to be complimentary to cloning strategies for fungal community studies by tentatively identifying cloned 18S rDNA fragments by comparison to community DGGE banding patterns. Vainio and Hantula (2000) showed DGGE detected more fungal species from environmental samples than culturing techniques. Kowalchuk *et al*. (2002) noted discrepancies observed between the AMF-like groups detected in spore populations versus direct 18S rDNA analysis of root material by DGGE, corroborating previous suggestions that spore inspection alone may poorly represent actual AMF population structure. Moreover, PCR-DGGE was also used to detect AM fungal community in cultivated soils (Ma *et al*., 2005). In order to confirm the result of AM fungal colonization in host plant roots, our experiment was aimed to detect AM fungal colonization in target plant roots which were early inoculated with selected AM fungal spores by comparing DGGE pattern with reference spore inocula.

7.2 Materials and method

7.2.1 Source of AM fungal spores and colonized roots

AM fungal inoculated *T. grandis* roots from experiment 5.2.1.2 in Chapter 5, were sampled after maintain them in the green house for one year after AM fungal spores inoculation. Three replicates of root from each treatment (uninoculated plant (T0), *Claroideoglomus etunicatum* NNT10 (T1), *C. etunicatum* PBT03 (T2), *Entrophospora colombiana* CMU05 (T3), and *Funneliformis mosseae* RYA08 (T4)) were sampled to represent each treatment. Three reference spores of *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08 were used for DNA extraction as an inoculated spore species. They were selected because isolates of their species were found in rhizosphere of *T. grandis* and *A. crassna* in the field and had efficiency to enhance growth of *T. grandis* plantlets (Chapter 5). *Glomus intraradices* spore was also used as comparative spore species.

7.2.2 DNA extraction from roots and spores

Tectona grandis roots were sampled from pot experiment of one year-old after AM fungal inoculation with *T. grandis* plantlets (Chapter 5, preliminary experiment

5.2.1.1). Roots were washed with tap water and dried on tissue paper before keeping in -20°C until use. DNA was extracted from roots using the PowerPlant DNA isolation kit (MoBio Laboratories, CA) following the manufacturing construction. A single spore of *C. etunicatum* NNT10, *C. etunicatum* PBT03, *G. intraradices* and *F. mosseae* RYA08 were separated and used for DNA extraction. The spore was washed with sterile distilled water three times, then transferred into 0.5 ml microcentrifuge tubes and washed 3 times with 200 ml chloramine T (2% w/v), streptomycin sulfate (0.02% w/v) and gentamicin sulfate (0.01% w/v) (Bécard and Fortin 1988). Then, the spore was washed again with sterile distilled water and transferred to a 1.5 ml microcentrifuge tubes on ice containing 1.5 µl sterile distilled water. The cleaned spore was crushed within the microcentrifuge tubes with a micropestle under a dissection microscope. DNA was resuspended in 30 µl TE buffer and then used as template for PCR .

7.2.3 Nested PCR and conditions

PCR amplifications were individually performed on the DNA extracted from the root samples and spores in a first PCR using AML1-AML2 to amplify approximately 800 bp fragment of the partial 18S rDNA gene (Lee *et al*., 2008). PCR was done in 20 µl volume with 2.0 µl template DNA using 0.5 units of *i*-Taq™ plus DNA Polymerase (iNtRON Biotechnology, Inc., Kyungki-Do) with the manufacture's recommended buffer (10x *i*-Taq™ plus PCR buffer), 2.5 mM each dNTP and 10 pM of each the primers. Product was amplified on a MyCycler™ Thermal Cycler (Bio-Rad Laboratories Inc., CA) with an initial denaturating of 95˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 45 sec, annealing at 58˚C for 45 sec, extension at 72˚C for 45 sec, followed by a final extension of 72˚C for 10 min. PCR product was analyzed by agarose gel electrophoresis (1.0% (w/v) agarose; 100 V, 30 min) using 3 µl of the resulting products.

Second round PCR was conducted using 10 pmol of each the primers AM1 (Helgason *et al*. 1998) and NS31–GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGTCTGGTGCC, which corresponds to NS31 described by Simon *et al*. (1992) plus a GC clamp sequence described by Kowalchuk *et al*. (1997) to amplify a 550 bp 18S rDNA gene fragment. One microlitre of diluted DNA (1:100) was used as template for PCRs in a 20 µL volume containing: 1

U 10x *i*-Taq™ plus PCR buffer (iNtRON Biotechnology, Inc., Kyungki-Do), 1 U of *i*-Taq™ plus DNA Polymerase, 2.5 mM dNTP mixture and 10 pM AM1 and NS31-GC. The PCR cycling conditions were one cycle at 94ºC for 3 min, followed by 30 (94ºC, 45 sec; 58ºC, 45 sec; 72ºC, 45 sec) and a final extension at 72ºC for 10 min. PCR products were run on a 1% agarose gel electrophoresis, stained with a 10% ethidium bromide solution and then digitally captured using a gel imaging system, GeneFlash Syngene Bio Imaging (Syngene, Cambridge).

7.2.4 Denaturing gradient gel electrophoresis analysis

PCR products were individually run through DGGE, as described below, to assess their electrophoretic mobility to the analysis of the AM fungal inoculated and uninoculated samples. A 5 mL stacking gel containing no denaturants was added before polymerization was complete (1 h). Denaturing gradient gel electrophoresis was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., CA), with 10 µL of the second round (nested) PCR products in 1X TAE buffer at a constant temperature of 60ºC at 120 V for 3 h on a 6% (w⁄v) polyacrylamide gel (40% acrylamide⁄bis-acrylamide) with a 10–30% denaturant gradient [100% denaturant corresponding to 7 M urea and 40% (v/v) formamide].

7.2.5 Restriction fragment length polymorphism analysis

The DNA bands that had translocated into the same gradient with inoculated spore bands were existed from acrylamine gel and re-amplified using the same primers, AM1 and NS31. The PCR products were purified using NucleoSpin Gel and PCR Clean up (Macherey-Nagel GmbH & Co., Dueren). 8.4 µl of the purified PCR product were digested with 5 units of the restriction enzyme HinfI and Hsp92II separately for 3 h at 37˚C. The entire volume of the resulting PCR products was run on a 2% agarose gel at 80 V for 45 min and stained with a 10% ethidium bromide solution and then digitally captured using a gel imaging system, GeneFlash Syngene Bio Imaging (Syngene, Cambridge).

7.3 Results

7.3.1 Denaturing gradient gel electrophoresis analysis

This study aimed to investigate selected AM fungal spores colonization in inoculated *T. grandis* roots after one year-old inoculation. Amplification of DNA from reference species and colonized roots with primer pair NS31-GC/AM1 yielded products of the correct size (approximately 550 bp) visualized on an agarose gel. Analysis of AM fungal colonized root by PCR-DGGE was performed in duplicate (Figure 7.1: lane 5–14) to check specificity of primers used in this experiment. The reproducibility of DGGE was tested by loading two replicates of PCR products for each sample on DGGE gels. No distinguishable difference in DGGE pattern was observed between two replicates of PCR production after nested PCR with AML1-AML2 and NS31-GC/AM1.

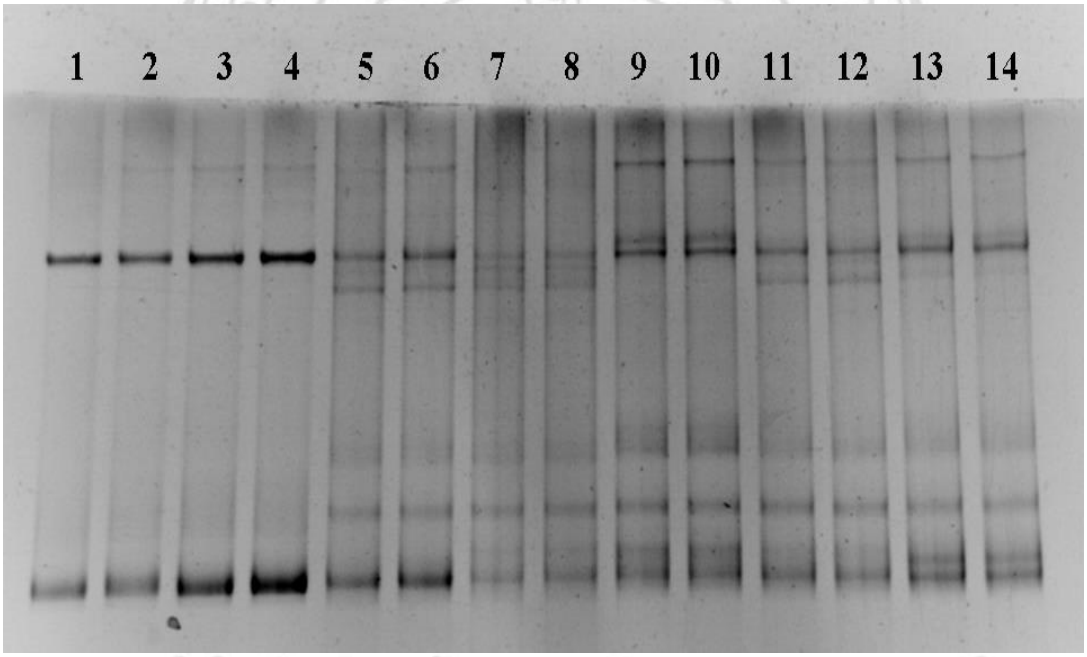

Figure 7.1 DGGE profiles of partial 18S rDNA fragments for reference AMF spores, Lane 1: *Glomus intraradices*; Lane 2: *Claroideoglomus etunicatum* NNT10; Lane 3: *C. etunicatum* PBT03; Lane 4: *Funneliformis mosseae* RYA08, and two replicates of each inoculated and uninoculated *T. grandis* roots, Lane 5–6: DNA amplified from *C. etunicatum* NNT10 inoculated plant root; Lane 7–8: uninoculated plant root; Lane 9–10: *C. etunicatum* PBT03 inoculated plant root; Lane 11–12: *F. mosseae* RYA08 inoculated plant root; Lane 13–14: *Entrophospora colombiana* CMU05.

The DGGE analysis of the NS31-GC/AM1 primer products yielded banding patterns within the range of 10–18% denaturant under our conditions (Figure 7.2). As shown in Figure 7.2, partial 18S rDNA gene of four AM fungal strains migrated into the same distance on acrylamide gel. Therefore, bands of the different species could not be combined into one lane as reference spores marker. DGGE profiles revealed the common AM fungal groups inside of the roots of *T. grandis*. Amplified DNA from reference fungal spores yielded distinguishable dominant band with sharp and intense DGGE bands within the range of 10–12% denaturant. The intensity of each band indicated its relative abundance in each root samples. Dominant DGGE of AM fungal inoculated *T. grandis* roots were also showed in those denaturing gradient ranging from 2–3 bands even in uninoculated roots. Other faint bands were also found in all *T. grandis* root samples, but only the bands that immigrated into the same denaturing gradient were used to determine certain species inside. The DGGE bands from reference spores and *T. grandis* roots were immigrated into the same denaturing gradient but those bands could not used to refer to species inside the roots. Therefore, the DGGE bands that had immigrated into the same denaturing gradient with inoculated spore bands were existed from acrylamine gel and re-amplified using the same primers, AM1 and NS31 in order to confirm AM fungal species in those bands using restriction fragment length polymorphism analysis (RFLP).

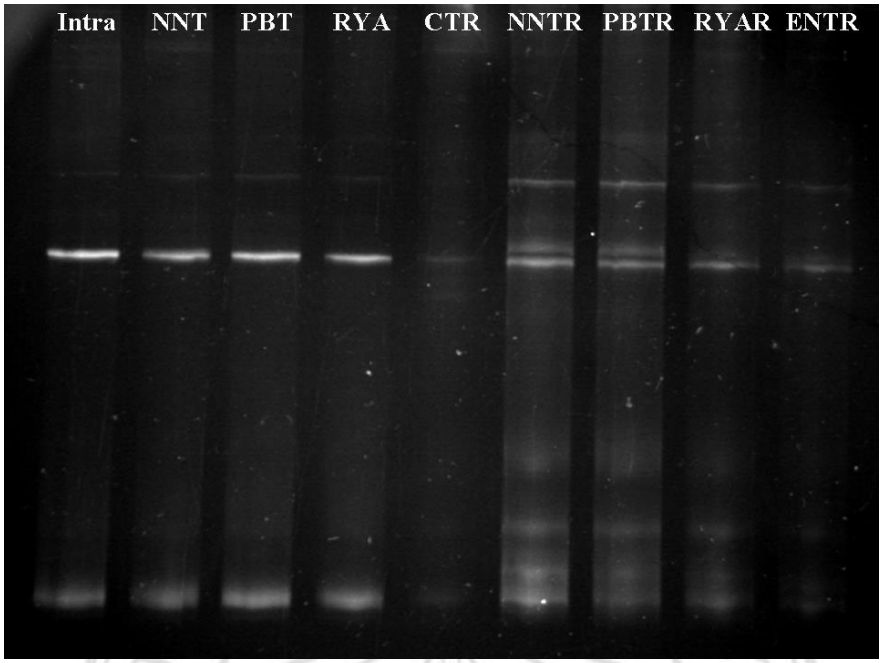

Figure 7.2 DGGE profiles of partial 18S rDNA fragments for reference AMF spores: Lane 1: *Glomus intraradices* (Intra) ; Lane 2: *Claroideoglomus etunicatum* NNT10 (NNT); Lane 3: *C. etunicatum* PBT03 (PBT); Lane 4: *Funneliformis mosseae* RYA08 (RYA), and *T. grandis* roots: Lane 5: DNA amplified from uninoculated plant root (CTR); Lane 6: *C. etunicatum* NNT10 inoculated plant root (NNTR); Lane 7: *C. etunicatum* PBT03 inoculated plant root (PBTR); Lane 8: *F. mosseae* RYA08 inoculated plant root (RYAR); Lane 9: *Entrophospora colombiana* CMU05 inoculated plant root (ENTR).

7.3.2 Restriction fragment length polymorphism analysis

The reamplified products from existing bands yield correct size of NS31-AM1 primer products. Restriction fragment length polymorphism profiles of partial 18S rDNA fragments restricted with HinfI and Hsp92II (Figure 7.3) showed the variation of RFLP profiles in both reference spores and colonized roots in each treatments. In Figure 7.3, *C. etunicatum* NNT10 (NNT) and *C. etunicatum* PBT03 (PBT) showed similar RFLP pattern while *G. intraradices* (Intra) and *F. mosseae* RYA08 (RYA) were quite differed from both *C. etunicatum* strains when using Hsp92II. Restriction fragments of all reference spores had similar dominant fragments size with approximately 600 and 550 base pairs when using HinfI and Hsp92II, respectively. Other faint bands of *C. etunicatum* NNT10 and *C. etunicatum* PBT03 were similar while in *G. intraradices*

and *F. mosseae* were quite similar but could not deferred when comparing fragment size with 100 base pairs plus ladder. AM fungal amplified products from inoculated and uninoculated *T. grandis* roots showed similar pattern both in HinfI and Hsp92II digested fragments. Therefore, RFLP pattern using those restriction enzymes could not identified the different of certain AM fungal species that have been inoculated into *T. grandis* roots but could estimated the possible AM fungal species inside the roots from each treatment by comparing RFLP patterns. All *T. grandis* roots tend to be colonized with *C. etunicatum* when comparing RFLP pattern from both HinfI and Hsp92II digestions.

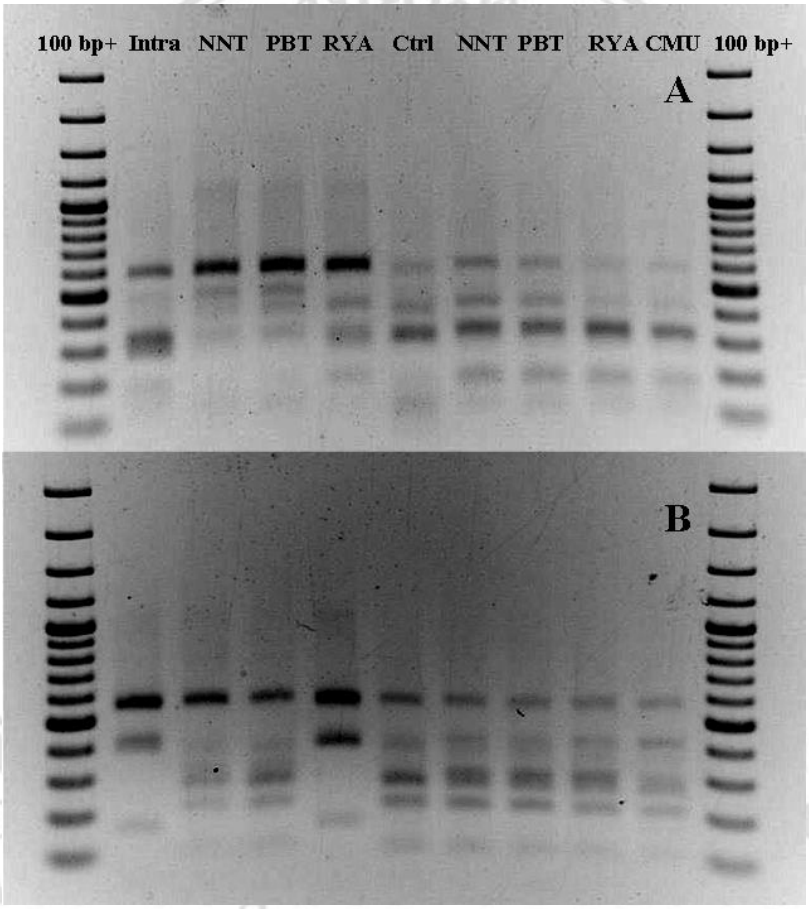

Figure 7.3 Restriction fragment length polymorphism profiles of partial 18S rDNA fragments restricted with A) HinfI and B) Hsp92II Intra: *Glomus intraradices* ; NNT: *Claroideoglomus etunicatum* NNT10; PBT: *C. etunicatum* PBT03; RYA: *Funneliformis mosseae* RYA08; Ctrl: uninoculated plant root; NNT: DNA amplified from *C. etunicatum* NNT10 inoculated plant root; PBT: *C. etunicatum* PBT03 inoculated plant root; RYA: *F. mosseae* RYA08 inoculated plant root; CMU: *Entrophospora colombiana* CMU05.

7.4 Discussion

Our study aimed to detect AM fungal colonization in target plant roots which were early inoculated with selected AM fungal spores using DGGE. In this study, there was no distinguishable difference in DGGE patterns between two replicates of PCR production after nested PCR with AML1-AML2 and NS31-GC/AM1. This result confirmed that the nested PCR with AM fungal specific primers, AML1-AML2, was increased the sensitivity of PCR-based technique and solved the problem of low resolution and yield using the primer pair AM1/NS31GC. According to Ma *et al*. (2005) who checked the specificity of AM1 in GenBank and found that AM1 primer sequence can amplify non-AMF templates. Therefore, specificity of primer for AM fungi in soil DNA extracts need to be restrictive to exclude non-AMF templates and increase the resolution and yield of DNA during PCR amplification (Anderson *et al*., 2003; Liang *et al*., 2008).

The similar DGGE mobility of reference spore *G. intraradices*, *C. etunicatum* NNT10, *C. etunicatum* PBT03, and *F. mosseae* RYA08 was unexpected under our DGGE condition. Because different species have differ in 18S rDNA sequence and GC-content but the high percentage of sequence similarity of spores may indicate insufficient nucleotide sequence and GC-content differences in the targeted 18S rDNA fragment to distinguish spore species by DGGE (Ma *et al*., 2005). Other faint bands that found in all *T. grandis* root samples may cause from the contamination of AM fungal spores during the pot maintenance after one year of inoculation. That problem may also subsequently affect to RFLP pattern which showed that all *T. grandis* roots tend to be colonized with *C. etunicatum*. The existing bands from DGGE gel before reamplification may obtain DNA fragments of closely band and also the co-migration of different sequences (Gonzalez *et al*., 2003; Liang *et al*., 2008). Though, DGGE-RFLP patterns could not specify the AM fungal species inside the colonized roots. However, the selected AM fungal colonization has been confirmed by the observation of AM fungal structure in *in vitro* inoculated *T. grandis* roots (Chapter 5, experiment 5.3.2). Therefore, those results confirmed that selected AM fungal spores inoculum (*Claroideoglomus etunicatum* NNT10, *C. etunicatum* PBT03, and *Funneliformis mosseae* RYA08) could colonized *T. grandis* roots.

Even though, the DGGE-RFLP patterns could not identify the different of certain AM fungal species in *T. grandis* roots in this study. But, those patterns could estimate the possible AM fungal species inside all roots from each treatment when comparing RFLP patterns of both restriction enzyme digestions. Therefore, it was suggested that the AM fungal colonization in target plant root could be easily investigate using AM fungal specific primers, if only one species has been inoculated to those plant. Previous studies revealed the molecular techniques used to detect specific AM fungi in mycorrhizal roots. For example, Zézé *et al*. (1996) first used a highly repeated DNA sequence SC1) screened from a partial genomic library of *S. castanea* as a probe with Southern analyses. In 2002, Yogoyama *et al*. (2002) designed an oligonucleotide probe based on the *Gigaspora margarita* DNA sequence and found that the combination of PCR and the probing successfully detected the diagnostic sequence from both single spores and colonized roots DNA. Therefore, the selection of detection technique depends on the application and value added in that work. Detection of multiple species inside the roots should be use high throughput molecular techniques such as DGGE-cloning technique, florescence probe that specific for selected species, DNA-DNA hybridization, or even next generation sequencing.