CHAPTER 2

Literature review

2.1 Arbuscular mycorrhiza fungi

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil fungi in the phylum *Glomeromycota* (Schüßler *et al*., 2001) which are mutually associated with plant roots of most vascular plants. A new, broader definition of mycorrhizas that embraces the full diversity of mycorrhizas while excluding all other plant-fungus associations is a symbiotic association essential for one or both partners, between a fungus (specialised for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer (Brundrett, 2004). The term symbiosis is often used to describe these highly interdependent mutualistic relationships where the host plant receives mineral nutrients and the fungus obtains photosynthetically derived carbon compounds (Harley and Smith, 1983).

Mycorrhizas occur in a plant root where intimate contact results from synchronized plant-fungus development. AM hyphae form highly branched tree-like haustoria called "arbuscules" within the cortical cells of plant root during periods of active plant growth (Figure 2.1A, 2.1B). Many AMF form vesicles, which are lipid-filled storage structures produced in intercellular spaces (Figure 2.1C, 2.1D). Some produce auxiliary vesicles and large asexual spores (20–1000+ µm diameter) are formed in the soil (Figure 2.1E) (Sylvia *et al*., 2005). AM fungi produce a network of hyphae in the soil with relatively thin, highly branched hyphae which are thought to absorb nutrients, and thicker hyphae which interconnect roots, spores and absorptive hyphae (Brundrett *et al*., 1996).

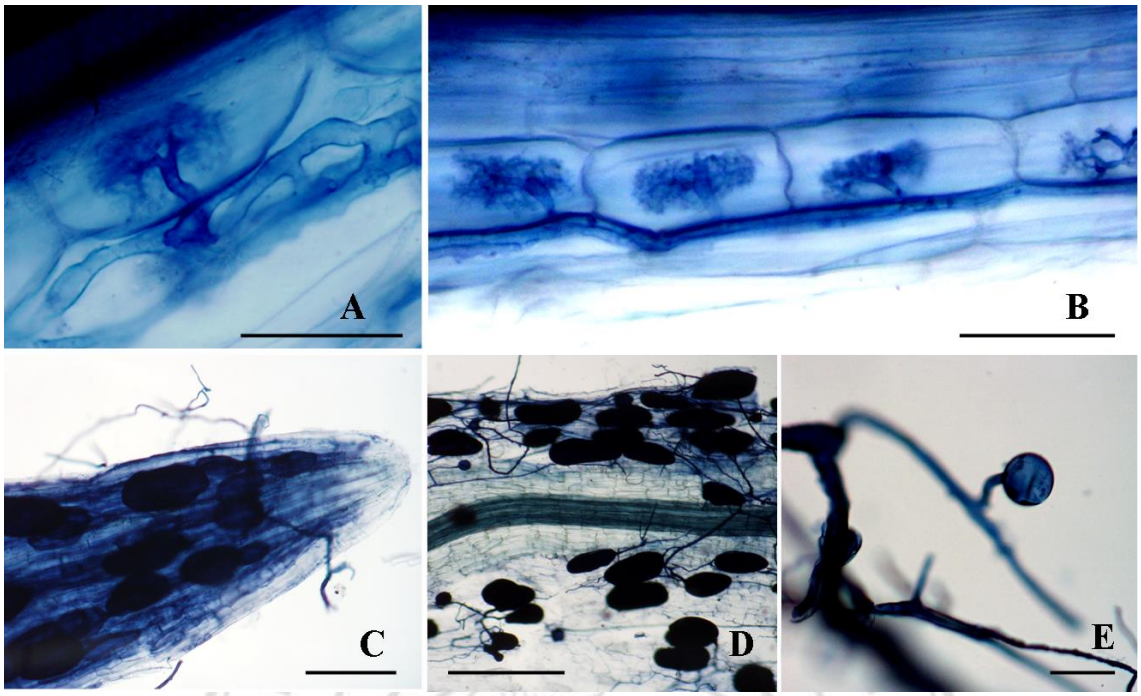

Figure 2.1 AM fungal structures produced in marigold's root (A–B) and teak's root (C–E): (A) tree-like arbuscule, (B) arbuscules within the cortical cells of plant root, (C) AM fungal colonized root tip showing vesicles, (D) vesicles in root cortex, and (E) spore attached with hyphae. Bar = 100 µm.

2.1.1 Mycorrhizal symbiosis

AM fungi are obligate symbiotic fungi that propagate by growing with a living host plant that and therefore currently cannot be culture without host plant (Harley and Smith, 1983). AM fungi are known to form symbiosis with embryophytes (land plants) as well as vascular land plants such as hornworts, *Anthoceros punctatus* (Schüßler, 2000) and liverworts (Fonseca and Berbera, 2008). Major morphological classes of AM symbioses appear to be plant-related, so they were named after the plants in which they were originally observed (Dickson, 2004). The two different types of arbuscular mycorrhizas were named *Arum*- and *Paris*- mycorrhizas. *Arum*-type mycorrhizal colonization produces intercellular hyphae and arbuscules, whereas *Paris*-type forms intracellular hyphae, coils and arbusculate coils. Most cultivated plants produce *Arum*-type mycorrhiza, while many trees and forest herbs form *Paris*-type (Dickson, 2004). Diagrams of colonization types observed in the root material are shown in Figure 2.2. Colonization type is dependent upon both the host plant and the

fungus, and the structures do not change over time (Cavagnaro *et al*., 2001). The rate of spread of colonization is slower in the *Paris*-type than in the *Arum*-type perhaps because the *Paris*-type colonization keeps reduces the energy supply to the fungi which might be desirable for plants of slow growth in a woodland environment (Becerra *et al*., 2007).

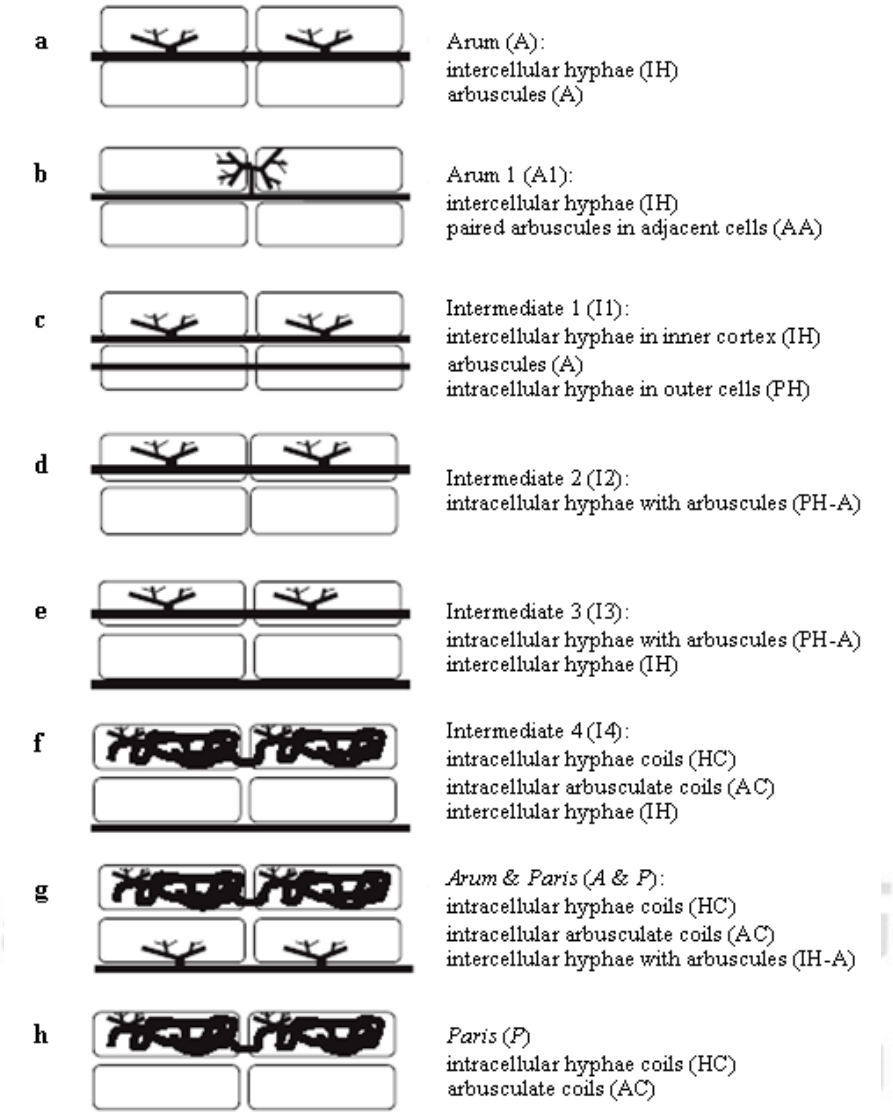

Figure 2.2 Diagrams of colonization types observed in the root material. (a) *Arum*, (b) *Arum* 1, (c) Intermediate 1, (d) Intermediate 2, (e) Intermediate 3, (f) Intermediate 4, (g) *Arum* and *Paris*, and (h) *Paris*. IH, intercellular hyphae; PH, intracellular hyphae; A, arbuscules, AA, paired arbuscules; HC, hyphal coils; AC, arbusculate coils; IH–A, arbuscules associated with IH; PH–A, arbuscules associated with PH (Dickson, 2004).

2.1.2 Classification of *Glomeromycota*

The classical AM fungal identification was based on spore morphology, spore formation, spore wall structure and staining reaction with Melzer's reagent under a microscope (Gerdemann and Trappe, 1974; Walker and Sanders, 1986; Morton and Benny, 1990; Schenck and Pérez, 1990). In 1990, AM fungi were organized in three families (*Acaulosporaceae*, *Gigasporaceae*, and *Glomeraceae*) and six genera (*Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*) within one order, *Glomerales* of the fungal phylum *Zygomycota* (Morton and Benny, 1990). Later, Morton and Redecker (2001) classified two other families, the *Archaeosporaceae* and *Paraglomaceae* with two new genera, *Archaeospora* and *Paraglomus*, respectively. These families were characterized based on the combination of DNA sequence, immunological reactions against specific monoclonal antibodies, fatty acid distance, and mycorrhizal morphology which provide the basis for recognizing *Archaeospora* and *Paraglomus*. In 2001, Schüßler *et al*. used all phylogenetic computations along with evidence published in the past, and demonstrated that the AM fungi (and *Geosiphon pyriformis*) belong to a monophyletic clade that probably represents a sister group to the clade comprising the *Basidiomycota* and *Ascomycota* (Figure 2.3). They elevated AM fungal group to the level of phylum *Glomeromycota* with three new orders (*Archaeosporales*, *Paraglomerales* and *Diversisporales*). In Figure 2.3, the *Zygomycota* and the *Chytridiomycota* do not form monophyletic clades and shown as the respective taxa representing the clade. The *Glomeromycota* divide into four statistically highly supported main clades with many of the classical AM fungi remaining in the order *Glomerales* (Figure 2.4). Then, Schüßler and Walker (2010) officially published a species list with new families and new genera of *Glomeromycota* by depositing paper copies in several scholarly libraries in Germany, the United Kingdom, and the United States.

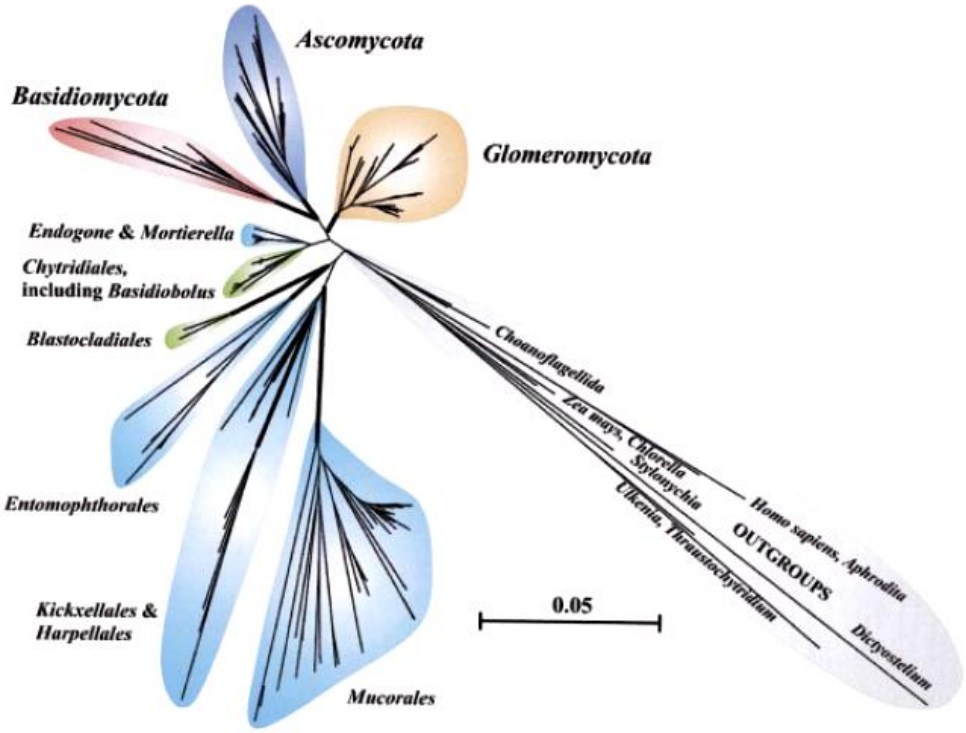

Figure 2.3 Phylogeny of fungi based on SSU rRNA sequences (Schüßler *et al*., 2001).

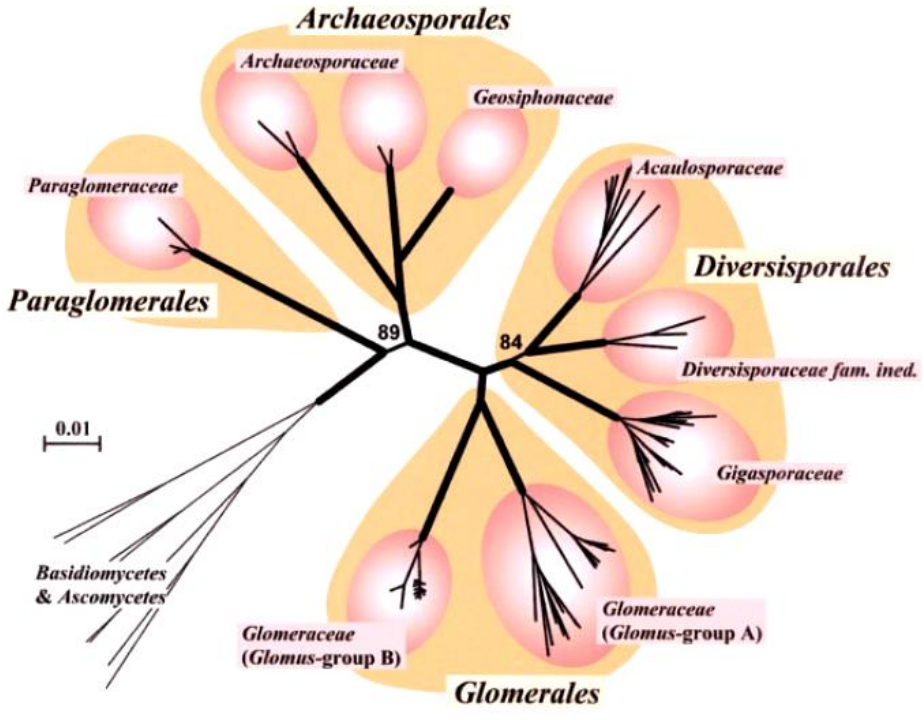

Figure 2.4 Taxonomic structure of the AM and related fungi (*Glomeromycota*), based on SSU rRNA gene sequences (Schüßler *et al*., 2001).

In 2011, the phylogeny of *Glomeromycota* based on small subunit rRNA sequences (SSU), ITS region, partial large subunit rRNA sequences (LSU) of the rRNA gene, and partial β-tubuline gene were modified by Oehl *et al*. (2011a) classifies the relationships among orders and genera, and accepted three classes (*Archaeosporomycetes*, *Glomeromycetes* and *Paraglomeromycetes*) of AM fingi (Figure 2.5, right panel). Then, Redecker *et al*. (2013) concerned that the lack of morphological evidence such as spore dimorphism which served as the basis for erection of the *Archaeosporomycetes* is not emphasized when considering that the range of analogous characters used to rank classes in other groups of fungi are homoplasies in *Glomeromycota*. Therefore, they consider that current data do not support splitting the phylum *Glomeromycota* into three classes as published by Oehl *et al*. (2011a) and thus concluded that all glomeromycotan orders group into only one class, the *Glomeromycetes* (Redecker *et al*., 2013) (Figure 2.5, left panel). Recent AM fungal classification could be update online through a web site (http://schuessler.userweb .mwn.de/amphylo/amphylo_species.html) that established by Schüßler and Walker (2010) (Table 2.1)

Table 2.1 Classification of *Glomeromycota* by Schüßler and Walker (2010)

Order	**Family**	**Genera**
Archaeosporales	*Ambisporaceae*	*Ambispora*
	Archaeosporaceae	*Archaeospora*
	Geosiphonaceae	*Geosiphon*
Diversisporales	*Acaulosporaceae*	*Acaulospora*
	Diversisporaceae	*Diversispora, Entrophospora, Otospora, Redeckera*
	Gigasporaceae	*Dentisculata, Gigaspora, Racocetra, Scutellospora*
	Pacisporaceae	*Pacispora*
Glomales	*Claroideoglomeraceae*	*Claroideoglomus*
	Glomeraceae	*Funneliformis, Glomus, Rhizophagus, Septoglomus, Sclerocystis*
Paraglomerales	*Paraglomeraceae*	*Paraglomus*

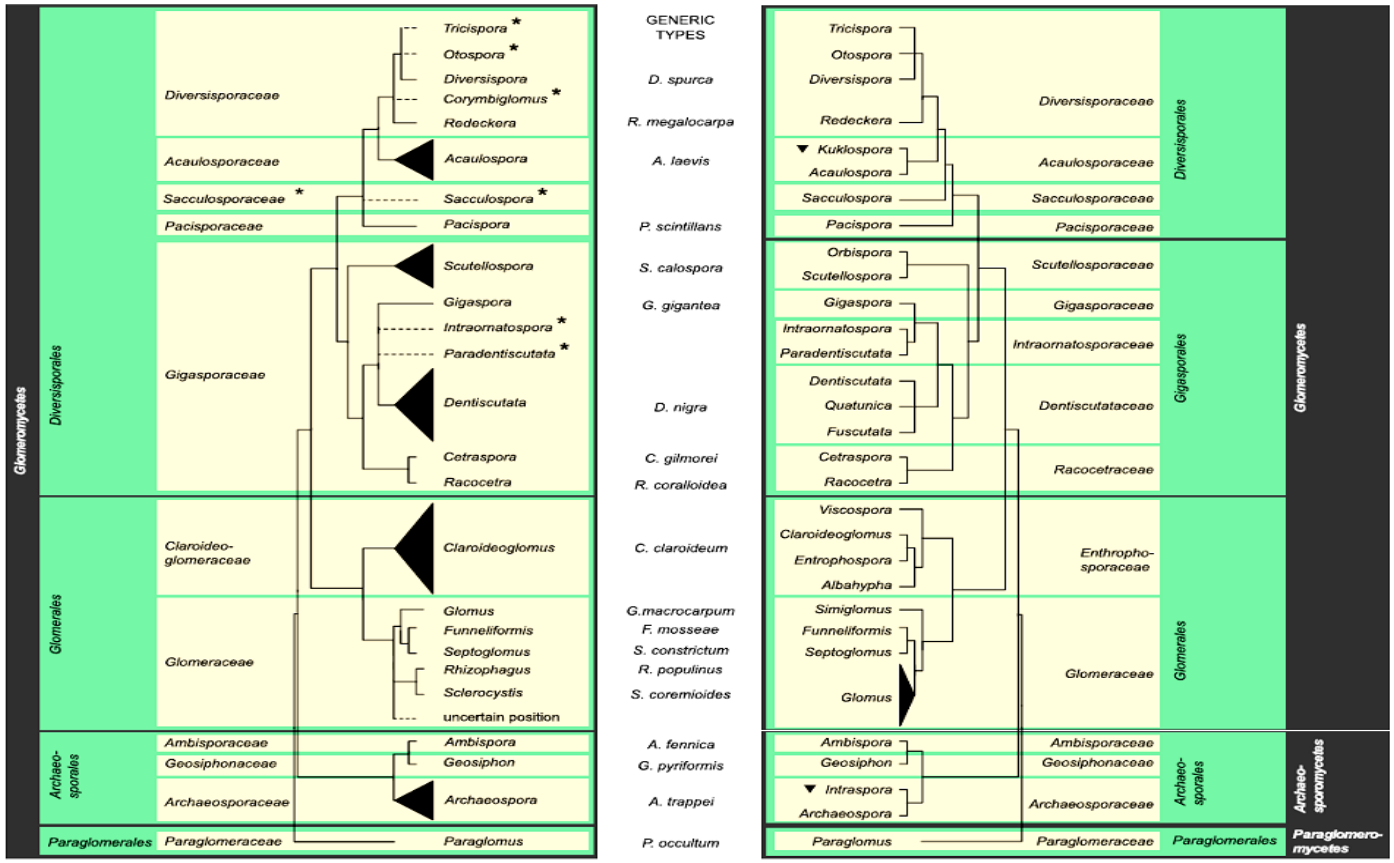

Figure 2.5 Consensus classification of the *Glomeromycota* (left panel) in comparison to the system summarized by Oehl *et al*. (2011b) (right panel), including additional taxa proposed by Goto *et al*. (2012). Dashed lines indicate genera of uncertain position, asterisks indicate insufficient evidence, but no formal action taken, inverted triangles indicate taxa already rejected in previous publications (Redecker *et al*., 2013).

2.2 Arbuscular mycorrhiza identification

2.2.1 Morphological identification

The identification of the *Glomeromycota* is based classically on the structure of their spores including development, arrangement, shape, size, colour, ornamentation, spore wall layers and staining reaction with Melzer's reagent under a microscope (Walker and Trappe, 1993; Brundrett *et al.*, 1996), but limited morphological differentiation may caused incorrect identification. Moreover, a single AMF culture can sometimes produce spores that differ in morphology and color, so they may not match fresh reference material produced under controlled conditions. Reliable identification requires a good deal of patience and experience, and only a handful of experts worldwide can confidently identify the whole range of AMF by examining spores (Young, 2012). Information on species descriptions is available in the web site (http://www.lrz.de/~schuessler/amphylo/). Accurate identification of mycorrhizal fungi from field samples is required to allow information about their biology to be exchanged, but it is difficult or even not possible if spores are old or damaged by microbial activity (Walker, 1992; Morton, 1993). It may be necessary to first establish pot cultures of AM fungi to allow their accurate identification and confirm their mycorrhizal status (Brundrett *et al*., 1996). Additionally, environmental factors such as temperature, light intensity and soil moisture content may influence AM morphology, as these factors affect the growth and morphology of roots. Some AMF species are prolific spore producers while others are not, this fact may explain some of spore density differences (Clapp *et al*., 1995). Thus it is essential that good quality specimens of any fungus used in experiments are lodged in a herbarium to allow the identity of fungi to be reassessed in the future.

2.2.2 Molecular identification and community structure

The studies of AM fungal communities in environmental samples are difficult because the fungi are obligate symbionts that cannot cultured in axenically and can only be identified morphologically in the spore stage (Vandenkoornhuyse *et al*., 2003). It is even possible that the inoculum values do not reveal the intensity of root colonization in a community. The 'classical' characterization based on spore structures

may be flawed because some species form more than one spore morphology and several cryptic taxa were only uncovered as a result of molecular biological analyses (Krüger *et al*., 2012). The nuclear rDNA region sequences are becoming taxonomically sufficiently broad to permit molecular ecological studies of AMF communities. The main reasons for the success of the ribosomal DNA (rDNA) as an evolutional marker is that its sequence encode for multiple-copy loci, whose repeated copies in tandem are happen together by concerted evolution, and it is present in all organisms with a common evolutionary origin (Gherbawy and Voigt, 2010). Schematic drawing of the organization of the fungal ribosomal rRNA gene cluster is shown in Figure 2.6.

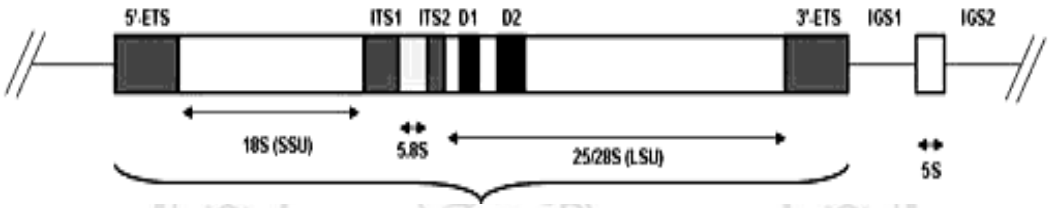

Figure 2.6 Schematic drawing of the organization of the fungal ribosomal rRNA gene cluster. All cluster components are including: SSU, small subunit (18S rRNA gene); LSU, large subunit (25-28S rRNA gene); D1 and D2, highly divergent regions of the LSU rRNA gene; ITS1, ITS2 and ET1, ET2: intergenic and extragenic spacers and intergenic spacers (IGS1 and IGS2), respectively (Gherbawy and Voigt, 2010).

The internal transcribe spacer (ITS) region is often used to determine fungal species and will be formally proposed for adoption as a universal DNA barcode marker for fungi to the Consortium for the Barcode of Life, with the possibility that supplementary barcodes may be developed for particular narrowly circumscribed taxonomic groups (Schoch *et al*., 2012). But AMF most environmental ITS region phylotypes cannot be affiliated to species, or species-level identities are not determinable using only this short and highly variable region (Stockinger *et al*., 2009). The most comprehensive taxon sampling for the *Glomeromycota* covers the SSU rDNA region (Schüßler *et al*., 2001) but it is inadequate for species resolution of AMF. Various studies have designed sets of specific primers for AM fungi (Helgason *et al*., 1998; Lee *et al*., 2008; Krüger *et al*., 2009) to facilitate rapid detection and identification directly from field-grown plant roots (Table 2.2). Recently, Krüger *et al*.

(2012) have been present the phylogenetic evidence to support the taxonomy of AM fungi based on the three rDNA markers: individual small subunit, internal transcribed spacer, and large subunit (SSU-ITS-LSU) rDNA sequences that provide reliable and robust resolution from phylum to species level.

AMF have been widely studied in the community using polymerase chain reaction (PCR)-based methods, 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 (SSCP) (Kjoller *et al*., 2000) and denaturing gradient gel electrophoresis (DGGE) (Liang *et al*., 2008). For fungi, it has been suggested that T-RFLP is more sensitive than DGGE (Brodie *et al*., 2003; Singh *et al*., 2006). DGGE is relatively insensitive with less abundant ribotypes tending not to form distinct bands but contributing to a diffuse background, or structurally different DNA molecules coincidentally co-migrating to the same place on a gel (Singh *et al*., 2006). T-RFLP has been shown to typically distinguish a greater proportion of ribotypes from environmental samples. It is efficient to use T-RFLP to process large numbers of samples and then to make clone libraries from selected samples for sequencing to obtain identities of key species (Figure 2.7) (Dickie and FitzJohn, 2007).

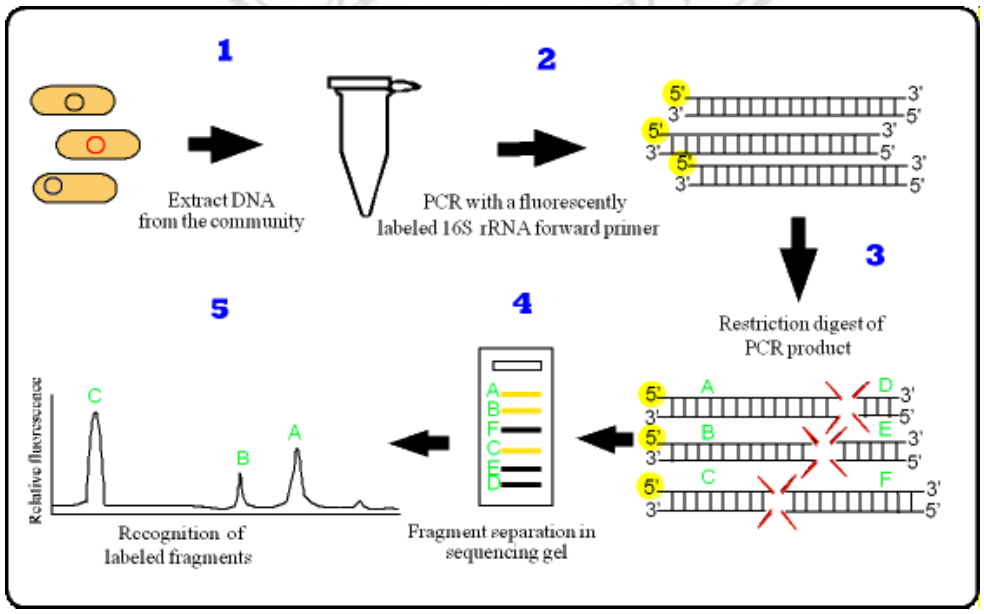

Figure 2.7 Diagram show outline of terminal-restriction fragment length polymorphism method.

Table 2.2 Description of the group-specific primers used in PCR targeting the AM fungal rDNA gene

Phylum or domain	Targeted group(s)	Primer sequence (5'–3')	Primer name	Reference
Glomeromycota: small subunit (SSU) rDNA	*Glomus* group A, *Acaulosporaceae*, and *Gigasporaceae*	GTT TCC CGT AAG GCG CCG AA	AM1	Helgason *et al*., 1998
Eucarya: SSU	All groups	TTG GAG GGC AAG TCT GGT GCC	NS31	Simon *et al*., 1992
Glomeromycota: LSU	All groups	TTG AAA GGG AAA CGA TTG AAG T TAC GTC AAC ATC CTT AAC GAA	FLR3 FLR4	Gollotte *et al*., 2004
Glomeromycota: small subunit (SSU) rDNA	*Glomus* group B *Diversisporaceae* (*Glomus* group C)	GTT TCC CGT AAG GTG CCA AA GTT TCC CGT AAG GTG CCG AA	AM2 AM3	Santos-González *et al*., 2007
Glomeromycota: SSU	All groups	ATC AAC TTT CGA TGG TAG GAT AGA GAA CCC AAA CAC TTT GGT TTC C	AML1 AML2	Lee *et al*., 2008

Table 2.2 (continued)

Phylum or domain	Targeted group(s)	Primer sequence (5'–3')	Primer name	Reference
Glomeromycota: partial SSU, whole internal transcribed spacer (ITS) region and partial LSU rDNA	All groups	TGG GTA ATC TTT TGA AAC TTY A	SSUmAf1	Krüger *et al*., 2009
		TGG GTA ATC TTR TGA AAC TTC A	SSUmAf2	
		TCG CTC TTC AAC GAG GAA TC	SSUmCf1	
		TAT TGT TCT TCA ACG AGG AAT C	SSUmCf2	
		TAT TGC TCT TNA ACG AGG AAT C	SSUmCf3	
		GCT CAC ACT CAA ATC TAT CAA A	LSUmAr1	
		GCT CTA ACT CAA TTC TAT CGA T	LSUmAr2	
		TGC TCT TAC TCA AAT CTA TCA AA	LSUmAr3	
		GCT CTT ACT CAA ACC TAT CGA	LSUmAr4	
		DAA CAC TCG CAT ATA TGT TAG A	LSUmBr1	
		AAC ACT CGC ACA CAT GTT AGA	LSUmBr2	
		AAC ACT CGC ATA CAT GTT AGA	LSUmBr3	
		AAA CAC TCG CAC ATA TGT TAG A	LSUmBr4	
		AAC ACT CGC ATA TAT GCT AGA	LSUmBr5	

2.3 Benefits of arbuscular mycorrhiza

2.3.1 Plant nutrient uptake

AM fungi can enhance the nutrient uptake by plant roots (especially phosphorus), particularly in low fertility soils (Brundrett *et al*., 1996). AMF produce prodigious hyphae which extend from roots into soil providing much of the absorption surface area beyond nutrient depletion zones in mycorrhizal plants, most notably in the case of phosphorus. The diagram in Figure 2.8 demonstrates why a plant with fine, extensive roots and long root hairs (A) would normally receive much less benefit from the presence of mycorrhizas than a plant with coarse roots (B) (Brudrett *et al*., 1996). Phosphate is present in the soil in 3 forms: soluble inorganic phosphorus in the soil solution, insoluble inorganic phosphorus in crystal lattices, and organic compounds such as phytate (Cooper, 1984). Phosphate is relatively immobile in soil and diffuses slowly to the plant root. The importance of adequate tissue phosphorus concentrations during early-season growth has been reported in many different crop species (Grant *et al*., 2001). Where early-season phosphorus supply is low, phosphorus fertilization may improve phosphorus nutrition and crop yield potential but alternately, encouragement of AM associations under low-phosphorus conditions may enhance phosphorus uptake improving crop yield potential and replacing starter fertilizer phosphorus applications (Grant *et al*., 2004). Karandashov *et al*. (2004) found that mycorrhiza-specific phosphate transporter genes and their regulation are conserved in phylogenetically distant plant species, and they are activated selectively by fungal species from the phylum *Glomeromycota*. This research also found that expression of the phosphate transporter gene StPT3 is induced in root cells hosting diverse AM structures, not only in cortical cells colonized by heavily branched hyphae (i.e. arbuscules and arbusculate hyphae) but also in cells containing only thick coiled hyphae (*Paris*-type). Moreover, Govindarajulu *et al*. (2005) reported that inorganic nitrogen is taken up by the extraradical hyphae and that the nitrogen is incorporated into amino acids, translocated from the extraradical to the intraradical mycelium as arginine but transferred to the plant without carbon.

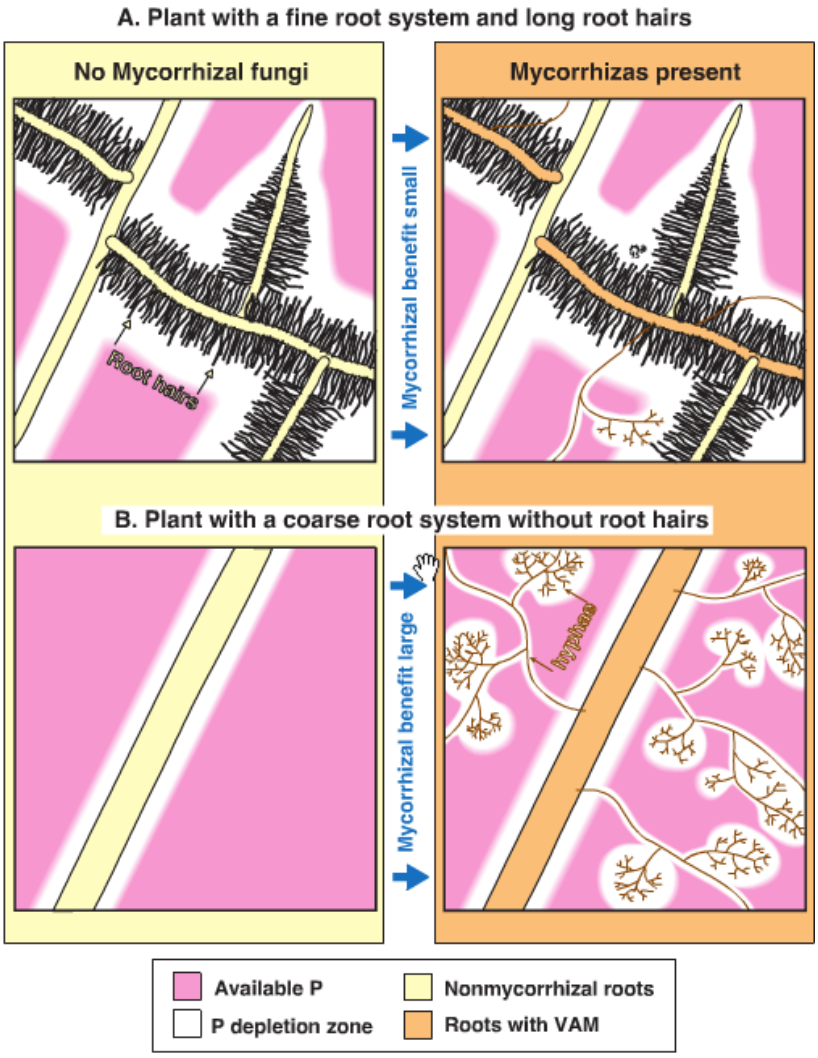

Figure 2.8 The role of root hairs and mycorrhizal fungus hyphae in acquiring poorly mobile nutrients such as phosphorus from soils in plant with fine, extensive roots and long root hairs (A) and plant with coarse roots without root hairs (B) (Brudrett *et al.*, 1996).

2.3.2 Enhance root water uptake and tolerance of water stress

AM fungi play important roles in water uptake ability and water use efficiency in host plants growth and stability of seedlings that grow in water stress area. The relevant hypotheses of the mechanism by which the fungus modifies host-plant water relations are (1) an indirect effect of improved phosphorus nutrition in mycorrhizal plants with high stomata conductance, (2) improvement of water uptake in mycorrhizal root systems either by the extraradical hyphal contribution,

increasing effective root hydraulic conductivity or modifying root physiology, (3) modifying biochemical of water regulation in the host plant through changes in hormonal signaling, (4) induction of osmoregulatory responses in mycorrhizal plants or changes in soil water-retention properties (Morte *et al*., 2000; Ruiz-Lozano, 2003; Al-Karaki *et al*., 2004). A number of studies have demonstrated the effect of AM fungal inoculation on water stress in host plants. For example, Porcel and Ruiz-Lozano (2004) showed that AM plants were protected plant against drought, as shown by their significantly higher shoot-biomass production. Moreover, the leaf water potential was also higher in stressed AM plants (-1.9 MPa (midday leaf water potential)) than in non-AM plants (-2.5 MPa). Doubková *et al*. (2013) provide strong evidence that the role of AM symbiosis in the performance of *Knautia arvensis* plants in stressful environment of serpentine soils consists not only in the improved phosphorus nutrition, but also in the alleviation of drought stress.

2.3.3 Improve structure and stability of soil by glomalin production

Aggregate stability is a good predictor of the ability of a soil to maintain good water infiltration rates, good cultivation and adequate aeration for plant growth. AM fungi can improve soil aggregate stability through the activities of mycelia and glomalin (Wright and Upadhyaya, 1998; Rillig, 2004). Glomalin is a glycoprotein produced by hyphae of AM fungi (Wright *et al*., 1996) and this protein is abundant in soils (Wright and Upadhyaya, 1996) and acts as a soil particle-binding agent. Glomalin in soils is quantified as glomalin-related soil protein (GRSP) that is an alkaline-soluble protein material with a low turnover (Rillig, 2004). It contributes to the soil carbon pool in native grasslands (Purin *et al*., 2006). Rillig and Steinberg (2002) reported that with small beads (< 106 μm; simulating a non-aggregated soil) fungal hyphal length was reduced over 80% compared to large beads (710–1180 μm; simulating an aggregated soil). Conversely, glomalin yield (immunoreactive protein) was more than seven times higher in the small bead growing space. Figure 2.9 showed various mechanisms (including hypothesized processes) that are hyphal mediated and influence the formation or stabilization of soil at macroaggregate and microaggregate scales. Mechanisms are divided into (1) physical process: hyphal enmeshment of particles can altered water regime and alignment of particles for exerting pressure, (2) biochemical

process: release of mycelium products (including glomalin) from decomposing or living hyphae, and (3) biological process: Fungal interactions with the soil food web and influence microbial communities (Rillig and Mummey, 2006).

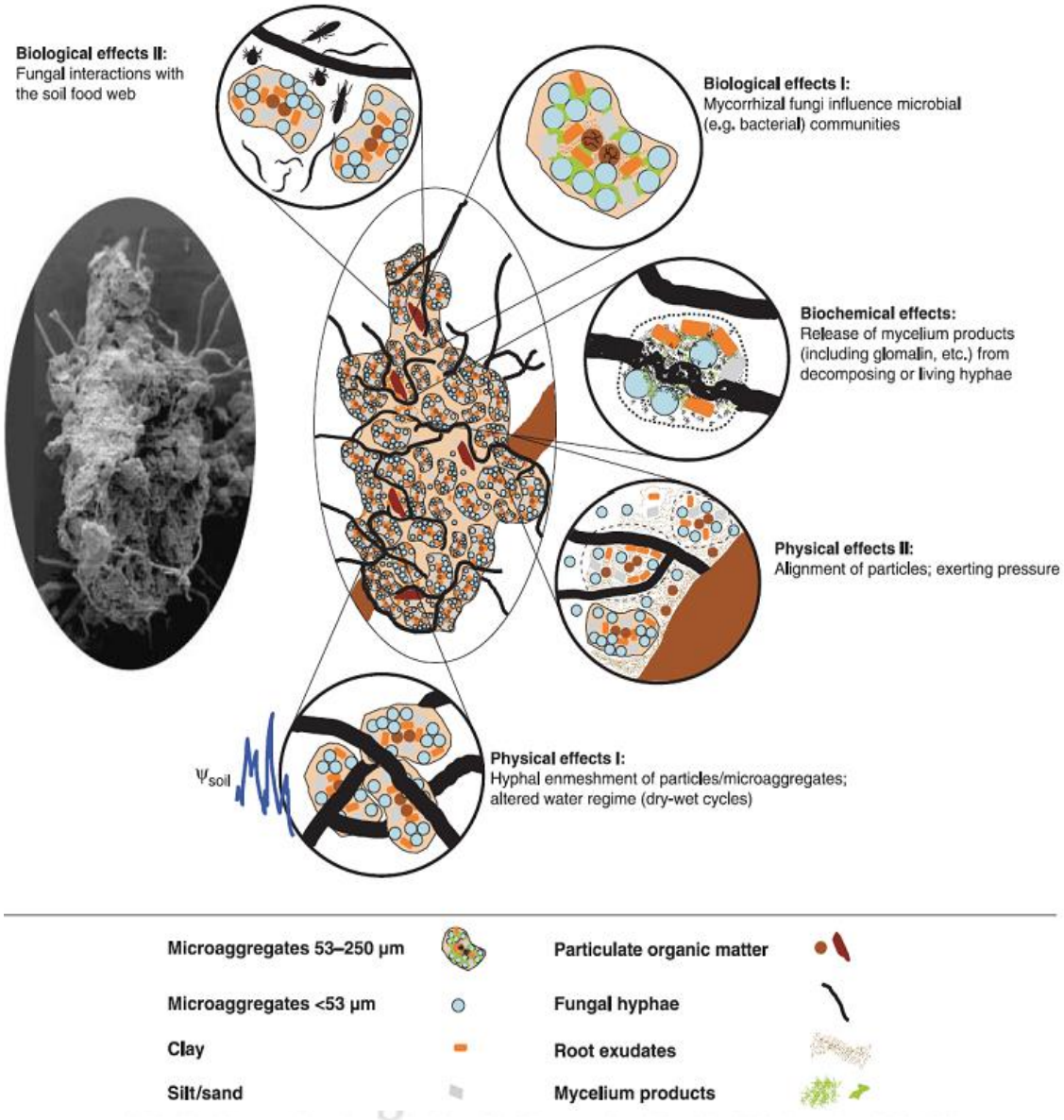

Figure 2.9 Overview of various mechanisms that are hyphal mediated and influence the formation or stabilization of soil at macroaggregate and microaggregate scales. Mechanisms are divided into physical, biochemical and biological processes (Rillig and Mummey, 2006).

2.3.4 Protect plant pathogen infection

Previous studies have showed that AM associations appear to reduce damage caused by soil-borne plant pathogens such as fungal pathogens causing root rots and vascular damage (*Aphanomyces*, *Fusarium*, *Phytophthora*, *Sclerotium* and *Verticillium*) and parasitic nematodes causing root galls and root lesions (*Meloidogyne*, *Pratylenchus* and *Radophulus*) (Gianinazzi *et al*., 2002). They also provide protection from soil borne plant pathogen such as *Pythium ultimum* by suppress and compete phytopathogen infection (Calvet *et al*., 1993; Cruz *et al*., 2008). In 2012, Youpensuk et *al*. also found that inoculation with AM fungi could reduce disease severity of six varieties of tangerine or citrus species from *P. parasitica*. The effective protection against root pathogens conferred by AM fungi is probably a consequence of several, and likely interacting, mechanisms that have been described by Azcón-Aguilar *et al*., (2002) as follows:

1. Improvement of plant nutrient status by the AM association may help the host plant to overcome a pathogen's attack and compensate for root tissue damage.

2. Growth of AM fungi and pathogens depends on host photosynthates, so competition for carbon compounds at colonization sites may result in reduced pathogen growth.

3. AM fungi induce changes in anatomy and architecture of root system which may reduce susceptibility to pathogenic infection.

4. AM fungi induce some soil microorganisms to exhibit antagonistic activities against pathogens in the rhizosphere of mycorrhizal plants.

5. AM fungi activate host plant against pathogen attack by producing elicitors able to induce plant defense responses.

2.4 Inoculum production of arbuscular mycorrhiza

The difficulties of obtaining large quantities of pure inoculum from obligate symbionts have limited the broad application of AM fungi. In the last decade, there are many different cultivation techniques that have been developed and proved to have specific advantages and limitations considering to their design and application. Ijdo *et al*. (2011) classified the production systems for AM fungi in three main categories

(Figure 2.10).

1. The classical sand/soil and more advanced substrate based production systems. These systems are widely used and represent a cost-effective way to large-scale produce AM fungal inoculum.

2. The substrate-free cultivation systems (hydroponics and aeroponics) that have been developed to produce relatively clean (sheared) AM fungal inoculum. However, the limitation of these production systems are higher costs associated with their use to smaller scale applications and research purposes.

3. The *in vitro* cultivation systems are based either on excised roots or root organ cultures (ROC) or on whole autotrophic plants under contaminant-free condition. *In vitro* cultivation of AM fungi is particularly adapted to the production of high-added-value crops such as crops generated via micropropagation techniques.

2.4.1 Substrate–based production systems

Classical AM fungal production is generally performed by the cultivation of host plants and a single identified specie or consortium of selected AM fungi in a soil- or sand-based substrate or a range of substrate substitutes and diluents. This procedure have been used to identify AM fungi from soils collected in the field, when these do not contain spores of sufficient quality or quantity to allow fungi to be accurately identified (Brundrett *et al*., 1996). The production process is often conducted in greenhouses under controlled or semicontrolled conditions or in growth chambers for the easy control of parameters such as humidity, light intensity and temperature. The control of phosphorus level in cultivation substrates is most concerned for inoculum production that can be varies between host plants, fungal isolates and soils. Large-scale production may be performed in pots of various materials (e.g. earthenware or plastic) and different sizes (e.g., Sylvia and Schenck, 1983; Millner and Kitt, 1992) or scaled up to medium-size bags and containers and to large raised or grounded beds (Gaur and Adholeya, 2002; Douds *et al*., 2005, 2006). However, the most considerations for selecting host plants concern their tolerant to growing conditions such as temperature, light intensity and drought stress. Large-scale production is sometimes conducted in open air, e.g. on-farm production (Gaur and Adholeya, 2002; Douds *et al*., 2005, 2006),

and infrequently on field plots (e.g. Dodd *et al*., 1990).

2.4.2 Substrate-free cultivation systems

The substrate-free cultivation techniques also termed as solution culture techniques. These methods provide an alternative to soil-based production system for mass production of clean and soilless AM fungal inoculum. These techniques mainly differ in the mode of aeration and application of the nutrient solution. In which the solution is not flowing or static systems, the nutrient solution needs to be aerated via an aeration pump to prevent roots to suffer from oxygen deprivation, while in substrate-free production systems (i.e. hydroponics and aeroponics) precolonized plants are produced prior to their introduction into the systems (Ijdo *et al*., 2011). Plant seedlings are preinoculate with AM fungal propagules in pots containing a substrate (e.g., mixture of sand and perlite) for several weeks to allow AM fungal colonization. Several plant species have been used with substrate-free production systems such as wheat (*Triticum aestivum* L.), linseed (*Linum usitatissimum* L.), maize (*Zea mays* L.), Bahia grass (*Paspalum notatum* Flügge), Sudan grass (*Sorghum sudanese* Staph.) and sweet potato (*Ipomoea batatas* L.) (Elmes and Mosse, 1984; Hung and Sylvia, 1988; Dugassa *et al*., 1995; Jarstfer and Sylvia, 1995; Wu *et al*., 1995; Hawkins and George, 1997; Mohammad *et al*., 2000). The host plant may influence on some AM fungal colonization levels and possibly also impact their sporulation. In addition, it should be taken into account that nutrient solution requirements might differ among host species and use diluted and modify (e.g., solution with low P content) from the existing nutrient solutions e.g. Knop's, Hoagland's or Long Ashton (Ijdo *et al*., 2011).

2.4.3 *In vitro* production systems

Pioneering work attempts to culture AM fungi *in vitro* date back the late 1950s (Mosse, 1959), who then reported the first association of an *Endogone* species with a plant. After that, several studies have prepared the way to mass-produce AM fungi. In 1975, Mosse and Hepper successfully cultured AM fungus with excised roots of tomato (*Lycopersicum esculentum* Mill.) and red clover (*Trifolium pratense* L.) on a gelled medium. Ten years later, Mugnier and Mosse (1987) and Bécard and Fortin (1988) used Ri T-DNA in *Agrobacterium rhizogenes* transformed carrot roots as host in

the root organ culture system. In order to increase AM fungal propagation, St-Arnaud *et al*. (1996) used a split-plate method, separating the root compartment from AM fungus compartment which only the AM fungus developed. Douds (2002) demonstrated that replacement of the gelled media in the distal compartment and resupply of 200 mg glucose to the proximal (root) compartment coincident with harvest of spores resulted in an approximate threefold increase in spore production over the standard split-plate culture technique. In parallel to the excised roots systems, Voets *et al*. (2005) system enabled the shoot to develop outside the Petri plate while the roots and AM fungus were associated inside the Petri plates. In Dupré de Boulois *et al*. (2006) system, the shoot developed in a sterile tube vertically connected to the top of a Petri plate in which the AM fungus and roots developed.

At present, commercial AM fungal inocula are produced in nursery plots, containers with different substrates and host plants, aeroponic systems, hydroponics system or *in vitro*. Biotechnology science linked to industrial activity needs to be considered to (1) the development of molecular probes for monitoring AM fungal inocula in the field, (2) increasing knowledge on the ecophysiology of AM fungi in human being disturbed ecosystems and the interactions of AM fungi with other rhizosphere microbes, and (3) selection of new plant varieties with enhanced mycorrhizal traits and of AM fungi with new symbiotic traits (Gianinazzi and Vosátka, 2004). The continued development of high quality and low-cost inoculum methods can be expected, which could lead to more new and advanced methods for AM fungal large-scale inoculum production to serve organic agriculture and global warning about food consumption in the future.

Figure 2.10 Production of AM fungal inoculum by A) substrate-based production system: large raised bed cultures, B) *In vitro* production system: *in vitro* culture systems based on autotrophic plants, and C) substrate-free cultivation system: hydroponics (Ijdo *et al*., 2011).

2.5 Botanical description of studied host plants and interaction with AM fungi

2.5.1 *Aquilaria crassna* Pierre ex H. Lecomte

Aquilaria crassna is a threatened forest species that occur typically in understory growing to 25–30 m in evergreen and semi-evergreen rainforests of south-east Asia. Barden *et al*. (2000) reported that the use of agarwood was mainly classified into 4 types: incense, perfume, medicine and other uses such as carving, aromatic ingredient for food and beverage and authentic agarwood bead or necklaces. The incense has been used for religious purposes by Muslims, Buddhists, and Hindus since the ancient times. *Aquilaria crassna* is heavily exploited for its aromatic resin-infused wood (agarwood) and of economic importance as it is one of the most highly valuable forest products currently traded internationally as one of natural fragrant sources

(Osoguchi, 2002). Agarwood has been extracted from natural stands of *Aquilaria* species including *A. crassna* since the many decades because the general people believed that high-graded agarwoods were derived from the natural stands and resin formation was caused by the natural phenomena. *Aquilaria crassna* has been listed on the Appendix II of The Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) for the purpose of preventing the over-exploitation of the natural Aquilaria trees for commercial purposes (CITES, 2004). The differences of botanical characteristics between *A. crassna* and *A. malaccensis* are the length of pedicels and the size of calyx-lobes. The length of pedicels of *A. crassna* is 5–10 mm as compared to 2–5 mm in *A. malaccensis*, while the size of calyx- lobes of *A. crassna* is 3–4 by 2–3.5 mm large as compared to 2–3 by 1.5–2 mm in *A. malaccensis*. The calyx-lobes of *A. crassna* during the fructification are much enlarged up to 15 mm (Peterson, 1997). Characterization of *A. crassna* bush, fruit, leave and seeds are show in Figure 2.11.

Figure 2.11 Characterization of *Aquilaria crassna*, (A) *A. crassna* planted in plantation, (B) *A. crassna* leave and fruits, and (C) fruit showing a pair of seeds.

2.5.2 *Tectona grandis* Linn.f.

Tectona grandis is a large deciduous tree reaching over 30 m in height in favorable conditions and grow in tropical and sub-tropical countries. It is well known worldwide for its high quality timber used for making furniture, panel work, railway carriages and traditional medicine in Southeast Asia. Crown open with many small branches; the bole is often buttressed and may be fluted, up to 15 m long below the 1st branches, up to 1 m diameter at breast height. Bark is brown, distinctly fibrous with shallow, longitudinal fissures. The root system is superficial, often on deeper than 50 cm, but roots may extend laterally up to 15 m from the stem. The very large, 4-side leaves are shed for 3–4 months during the later half of the dry season, leaving the branchlets bare. Leaves are opposite, elliptic or obovate, shiny above and hairy below, vein network clear, about 30–50 × 15–20 cm but young leaves up to 1 m long (Figure 2.12). Flowers small, about 8 m across, mauve to white and arranged in large, flowering heads, about 45 cm long; found on the top most branches in the unshaded part of the crown. Fruit is a drupe with 4 chambers; round, hard and woody, enclosed in an inflated, bladder–like covering; pale green at first, then brown at maturity. Each fruit may contain 0 to 4 seeds. There are 1000–3500 fruits/kg (Orwa *et al*., 2009).

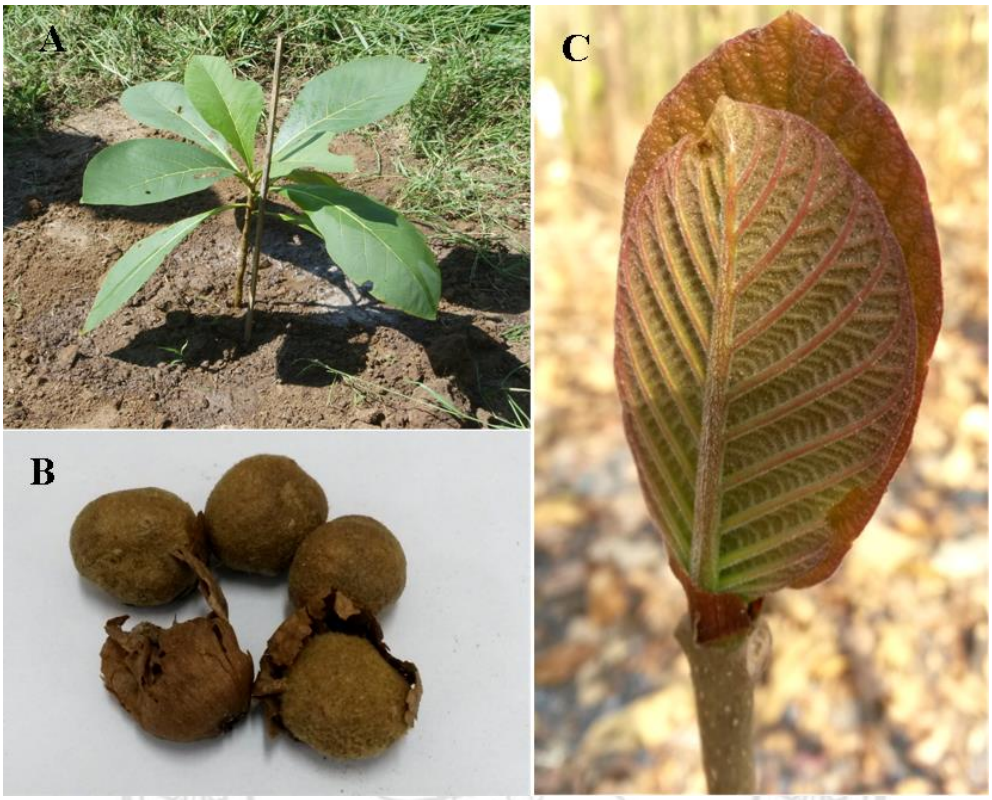

Figure 2.12 Characterization of *Tectona grandis*, (A) *T. grandis* seedling planted in plantation, (B) round, hard and woody fruit enclosed in an inflated, bladder-like covering, and (C) young leave showing hairy below leave and clear vein network.

2.5.3 Interaction between AM fungi and studied host plants

AM fungal diversity in *A. crassna* and *T. grandis* rhizosphere soils has been reported mostly in India. For example, Venkataramanan *et al.* (1985) studies AM fungal occurrence in *A. agallocha* and found the abundant of AM fungal structure in the fine lateral roots. Tamuli and Boruah (2002) collected roots and rhizosphere soils of *A. malaccensis* from various plantations in Assam, India and found the variation of AM fungal colonization and number of spores in those soils. In 1988, Thapar and Klan reported that spore numbers of AM fungi in *T. grandis* increased after rains during summer. Several AM fungal species were also found in rhizosphere soils of *T. grandis* both from natural forest (Kanakdurga *et al*., 1990; Rani *et al*., 1995; Dhar and Mridha, 2012) and plantations (Talukdar and Thakuria, 2001). Previously, inoculation of AM fungal inocula has been reported to enhance the growth of *Aquilaria* sp. (Turjaman *et al*., 2006; Tabin *et al*., 2009) and *T. grandis* (Rajan *et al*., 2000; Swaminathan

and Srinivasan, 2006). Colonization of AM fungus increased plant height, shoot diameter, shoot and root dry weights, shoot N and P concentrations of the seedlings and survival rates after transplantation. Arbuscular mycorrhizal fungi have been applied to promote the growth of various plantlets and have benefited early tree establishment in the field (Habte *et al*., 2001; Urgiles *et al*., 2009).

Since, micropropagation techniques have been recognized as very successful applications for large scale 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.