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Mycorrhization in Sustainable Agriculture

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Abstract

Arbuscular mycorrhizal (AM) fungi are ubiquitous and widespread in soils all around the world. They form a link between the plant roots and soil. The root systems of most terrestrial plants (80%) and majority of crops harbour diverse communities of mycorrhizal fungi. The AM species form an intimate relationship with plant root systems that enables the flow of soil minerals to the plant in exchange for photosynthates. The fungus penetrates into the root cortical cells forming specialized structures such as arbuscules and vesicles. AM fungi are generally known to benefit plant health and play an essential role in plant nutrient uptake, soil aggregation, water relations, ecosystem establishment, plant diversity, and plant productivity. They also improve plant resistance against root pathogens and enhance foliar resistance. The improvement of phosphorus (P) nutrition by AM fungi has received most attention. The importance of AM fungi in restoration and revegetation of disturbed lands is also well known. The significance of mycorrhizal symbiosis in association with biodiversity and ecosystem functioning is now being revealed, particularly with respect to their potential to influence plant diversity and productivity. In this study, the diversity and structure of AM fungal communities, AM fungal inoculum production and multiplication for utilization as biofertilizer will be discussed, along with the importance of the AM role in growth of agro-economically important plants in sustainable agriculture and the functioning of natural ecosystems.

Key words: Arbuscular mycorrhizal fungi, symbionts, soil-based systems, sustainable agriculture

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Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are multikaryotic, asexual and obligate symbionts that colonize around 80 per cent of vascular plants. The origin of these fungi, based on molecular clock dating, is estimated to have occurred around 600 million years ago (Redecker et al 2000). Fossil evidence clearly demonstrated the presence of AM-like fungi in the primitive Bryophyte-like land plants in the early Devonian, approximately 400 million years ago (Remy et al 1994; Taylor et al 1995). Being obligate biotrophs, AM fungi are unable to complete their life cycle in the absence of a host plant (Azcon-Aguilar et al 1998). AM fungal propagules exist as spores, living hyphae, vesicles, mycorrhizal root segments or colonized soil (Diop et al 1994). After contact with a suitable host root, inter- and/or intra-cellular colonization of the root cortex occurs (Smith and Smith 1997) that allows the completion of the AM fungal life cycle.

Diversity and Structure of AM Fungal Communities. Fossil and molecular studies suggest that AM association is evolutionarily an ancient form of symbiosis and its occurrence with the first terrestrial plants has been reported (Lee et al 2013). These fungi have low species diversity with approximately 240 identified species (Schubler and Walker 2010, Kruger et al 2012). However, molecular reports suggest the existence of a considerably larger number of species (Redecker 2002). The taxonomy of AM fungi is based on the traditional methods of identification *i.e.* morphological characteristics of spores such as size, colour, and wall layers (Morton 1988). Earlier AM fungi were placed within six genera of order Glomales (Zygomycota) using morphological characteristics and phylogenetic analysis (Morton and Benny 1990). However, sequence analyses of ribosomal RNA showed that all AM species are included in a monophyletic clade and are separate from all other major fungal groups, yet are

extremely diverse within the group and were therefore placed in a new phylum Glomeromycota (Schubler et al 2001).

Arbuscular mycorrhizal associations are initiated by spore germination. Following successful signaling recognition events, appressoria formation takes place on root epidermal cells. The localized production of wall-degrading hydrolytic enzymes by the fungus and the exertion of hydrostatic pressure by the hyphal tip help in successful hyphal penetration through the epidermis (Bonfante and Perotto 1995). The hyphae extend apically and distally, inter- and intra-cellularly in the cortical cells (Plate 1a). Inter-cellular hyphal lateral branches penetrate the cortical cells and form branched, tree-like structures called 'arbuscules' and are major sites of nutrient exchange between the fungus and host (Plate 1b). The arbuscules are short-lived and in most host-fungus interactions, degenerate within 7 to 12 days period. Vesicles are balloon-like structures formed within the cortical cells that store lipids (Plate 1c). They may be intercalary or terminal and also function as propagules or develop into intra-radical spores. AM species belonging to genera *Gigaspora, Scutellospora* and *Dentiscutata* form auxiliary cells (external vesicles), which are thin walled clustered swellings with spines or knobs. They help in temporary storage of carbon compounds (Plate 1d).

Both intra- and extra-radical spores are formed with thick walls that contain lipids, cytoplasm and many nuclei, and function as propagules (Plate 1e, f). Spore colour, shape and size may vary considerably depending upon developmental stage and environmental conditions. Spore colour varies from hyaline to white to yellow, red, brown and black with all intermediate shades (Plate 1g-j). The shape of spores is governed by the genotype of the fungus and the substrate in which the spores are formed. Further variation seen in the spore shape may be due to environmental stress. Colour difference may be due to pigmentation in spore wall or in the spore content (Morton, 1988). *Glomus tenue* is the smallest AM spore with an average diameter of 10-12 µm, while in contrast, *Gigaspora gigantea* is the largest spore with dimensions ranging from 183-500 x 291-812µm.

Spores may also be aggregated into groups called 'sporocarps'. In sporocarpic species, the spores are either in a loose or highly ordered arrangement around a hyphal plexus. The sporocarps may be formed in soil, root, empty seed coats, insect carapaces or rhizomes. The external colour of the sporocarp ranges from white to brown, while internal colour ranges from white to black and brown.

The region of small subunit (SSU) rDNA has been most widely used for phylogenetic analysis of AM fungi, because the intra-specific variation of the internal transcribed spacer region rDNA, which is generally used for identification and bar-coding of fungal species, is too high to be used in distinguishing AM fungal species (Sanders et al 1995, Lloyd-Macgilp et al 1996). However, the use of SSU rDNA may also underestimate the diversity of the phylum Glomeromycota (Husband et al 2002). In a recent study, Kruger et al (2012) analyzed sequences of 136 AM species, including 27 undescribed species, and suggested that the number of described species was absolutely underestimated. It has been suggested that information from sequences such as large subunit (LSU) and β-tubulin, would be required for a closer estimation of actual AM fungal diversity (Schubler and Walker 2010, Msiska and Morton 2009, Morton and Msiska 2010).

Ecological studies of AM fungi until recently relied upon spores collected from rhizosphere soil. However, spore count data collected from soil does not necessarily reflect on the actual diversity and function of active AM colonizing plant roots. Therefore, identification of AM species within plant roots is critical. However, identification of AM hyphae to species level within roots using morphological features is not possible. In addition, due to the complexity of root systems, it is not possible to distinguish active symbionts of AM fungi in an individual plant from spore communities in soil.

Development of suitable primers and molecular analysis protocols has enabled identification of AM fungi within plant roots (Lee et al 2013). Specific primers have been developed for all AM fungal

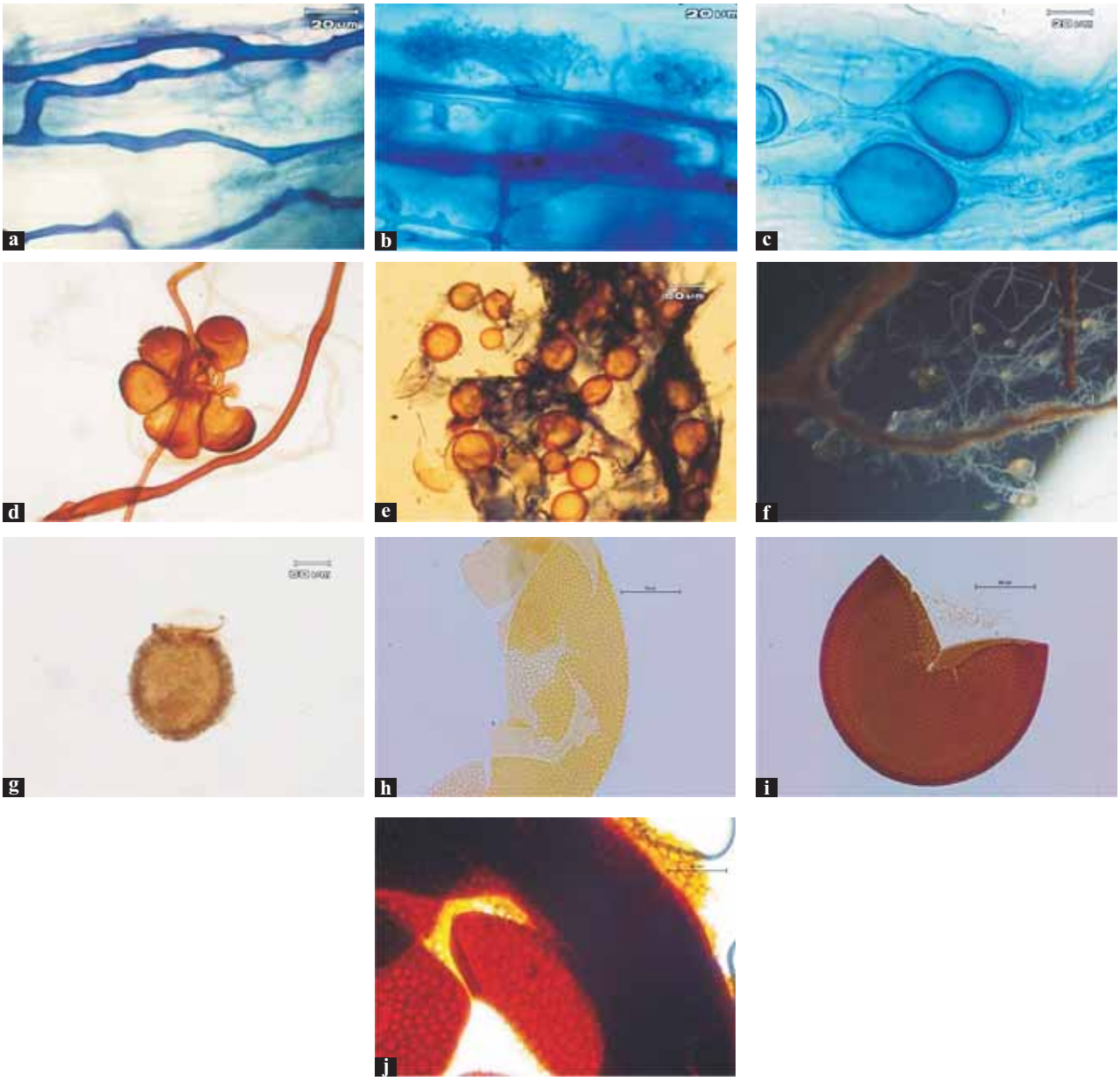

Plate 1. a. Hyphal colonization; b. Arbuscules; c. vesicles; d. Soil borne auxilary cells; e. intra-radical spores; f. Extra-radical spores; g. Spore of *Sacculospora felinovii*; h. Ornamentation in *Acaulospora bireticulata*; i. Spore of *Acaulospora scrobiculata*; j. Ornamentation in *Scutellospora reticulata*

lineages for use in PCR (Helgason et al 1999, Lee et al 2008,Wubet et al 2006), and the LSU sequences were used for identification of AM fungi and for construction of a more accurate system (Gollotte et al 2004, Pivato et al 2007; Rosendahl et al 2009). The classification system of AM fungi is being revised constantly as the number of new species being identified steadily increases. The most recent classification of AM fungi by Redecker et al (2013) is based on a consensus of regions spanning ribosomal RNA genes: 18S (SSU), ITS1-5.8S-ITS2 (ITS), and/or 28S (LSU). Kruger et al (2009) designed four primer mixtures, covering the partial SSU, whole internal transcribed spacer (ITS) rDNA region and partial LSU. These primers will be helpful for the molecular characterization of AM fungi, including species descriptions (Gamper et al 2009), resulting in a sequence database that will allow the design of further primers for the detection of AM fungi from field samples.

AM fungi as symbionts with plants are widely distributed among a variety of ecosystems ranging from sub-polar to tropical latitudes and from swamps in low lands to high elevation mountainous areas (Read 1991). D'Souza and Rodrigues (2013) recovered 28 AM fungal species of five genera, *viz., Glomus, Acaulospora, Scutellospora, Gigaspora* and *Entrophospora*; with *Glomus* as the dominant genus from mangroves of Goa. Khade and Rodrigues (2009) recorded 13 AM fungal species belonging to four genera *viz., Acaulospora, Glomus, Gigaspora* and *Dentiscutata* associated with six varieties of *Carica papaya* L. in a tropical agro-based ecosystem of Goa, India. Radhika and Rodrigues (2010) carried out AM fungal diversity studies in 36 medicinal plants from different localities in the Goa region of the Western Ghats. They recorded 42 AM fungal species belonging to five genera *viz., Glomus, Acaulospora, Scutellospora, Gigaspora* and *Ambispora,* with *Glomus* being the most dominant genus and *G. fasciculatum* the dominant AM species. Dessai and Rodrigues (2012) assessed AM fungal diversity associated with vegetable crop plants cultivated in Goa. A total of 51 AM species were recovered from rhizosphere soil samples of 27 vegetable plants with *Glomus* as the dominant genus followed by *Acaulospora*, *Gigaspora* and *Scutellospora. Acaulospora scrobiculata* was the dominant AM species. Rodrigues and Jaiswal (2001) recorded 15 AM species from coastal sand dune vegetation of Goa with *Glomus* as the dominant genus. Khade and Rodrigues (2003) surveyed the prevalence of AM fungi in Mollem forest area of Western Ghats of Goa. They screened 25 tree species belonging to 18 families. The highest mean root colonization (100%) was reported in *Macaranga peltata, Xylia xylocarpa, Zanthoxylum rhetsa* and *Randia ruglosa*. Maximum mean spore density of 745 spores $100g^{-1}$ rhizosphere soil was recorded in *Leea indica*. A total of 18 AM fungi belonging to five genera, *viz., Acaulospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* were found to be associated with the studied tree species.

Importance of AM Symbiosis. AM fungi are known to be a major microbial component of plant and soil ecology. The co-existence of different AM fungal isolates in soils results in healthy plant growth and seedling establishment, nutrient cycling, and resistance to drought, temperature and salinity. Further they control plant pathogens and facilitate plant access to soil nutrients. Additionally, AM fungi have a role in structuring plant communities (Van der Heijden et al 1998), ameliorating soil quality by improved aggregation of soil particles and organic carbon content in soils (Rillig and Mummey 2006, Bedini et al 2009) and are useful indicators of ecosystem change (McGonigle and Miller 1996). Beside yield increase, AM fungi enhance crop quality by enrichment of macro- and micro-nutrients (Veresoglou et al 2012, White and Broadley 2009, Antunes et al 2012).

The interconnected network of external hyphae acts as additional catchment and absorption surface in the soil beyond the depletion zone enhancing uptake of P (Bolan, 1991), nitrogen (N) (Hodge et al 2001) and micro-nutrients (Clark and Zeto 2000). The retrieved nutrients are transported directly to roots and exchanged by mechanisms controlled by both partners (Kiers et al 2011), in exchange for photosynthetically derived carbon compounds (Smith and Read 2008). Furthermore the fungi have a direct influence on plant growth resulting from effects on soil structure stabilization and accumulation of humic substances (Bethlenfalvay and Linderman 1992, Bethlenfalvay and Schuepp 1994).

Inoculum Production and Multiplication of AM Fungi. Over the last four decades, AM fungal inoculum has been utilized in agriculture, horticulture, forestry programs, and for environmental reclamation, to increase crop yield and health and to limit the application of agrochemicals (Johansson et al 2004). According to Maeder et al (2000) in order to enhance AM symbiosis, common practices should be chosen that favour optimal soil properties for the development of AM symbiosis. Various cultivation techniques for inoculum production of AM fungi have been attempted in the last few decades. Besides sand/soil- and substrate-based production techniques, substrate-free culture techniques (hydroponics and aeroponics) and *in vitro*

cultivation methods have been attempted in large-scale production of AM fungi.

Sand/soil-based systems are not sterile and are prone to contamination even with good phytosanitary care (Ames and Linderman 1978). Here, the inoculum is composed of dried root fragments or colonized root fragments, AM spores, sporocarps, and fragments of hyphae. Spores can be extracted from the soil and used as inoculum but such spores tend to have very low viability or may be dead or parasitized. In such a case, initially the rhizosphere soil is taken to prepare a 'trap culture' using a suitable host plant. This enables increase in the number of viable spore propagules for further isolation, multiplication and production of pure or monospecific cultures. These cultures are obtained after a known isolate of AM and a suitable host are grown together in a medium (sterilized soil/sand) optimized for development of AM association and spore formation. The pure culture inoculum thus produced consists of spores, colonized root fragments, and AM hyphae.

The plant used as host in inoculum production should be carefully selected. It should grow fast, adapt to the prevailing growing conditions, be readily colonized by AM, and produce a large quantity of roots within a relatively short period (45–60 days). It should be resistant to pests and diseases common to the inocula production environment.

Gilmore (1968) recommended *Fragaria* sp. (strawberry) for open pot culture propagation of AM fungi. Since then, a range of plant species have been used as hosts. Some common temperate host plants included *Zea mays* (corn), *Allium cepa* (onion), and *Arachis hypogaea* (peanut). Widely used tropical hosts included *Stylosanthes* spp., *Paspalum notatum* (bahia grass) and *Pueraria phaseoloides* (kudzu) (http://invam.wvu.edu/methods/cultures/host-plant-choices).

At the Goa University Arbuscular Mycorrhizal Culture Collection (GUAMCC), *Plectranthus scutellarioides* (L.) R Br is used as host plant for mass multiplication (for both trap cultures as well as pure or monospecific cultures) of AM fungal inoculum (Plate 2a). Commonly known as coleus, it has advantages as it is easily propagated through cuttings, requires less maintenance and it serves as a good host for AM fungal colonization and multiplication (Plate 2b). The host plant should be fertilized by periodic additions of a nutrient solution such as Hoagland's solution (minus P). To ensure that most of the spores in the inoculum are mature, it is essential to grow the host plant for 12-14 weeks. The medium is then allowed to dry gradually by reducing the frequency of watering over a week and then withdrawing water completely. The inoculum can then be further multiplied.

Soil-based systems for cultivation of AM fungi are the most widely adopted technique for AM fungal inoculum production. They are considered a convenient system for large-scale production that is able to reach inoculum densities set for mass production of 80-100 propagules^{-3} (Feldmann and Grotkass 2002). In soil-based production systems, the nutrient requirements for AM fungus and plant can be monitored and regulated (Lee and George 2005). Major disadvantages of soil-based cultivations systems are that, in most cases, the absence of unwanted contaminants cannot be guaranteed and the systems consume more space.

Production of AM propagules under aseptic conditions is one of the most promising methods of obtaining high quality pathogen-free inoculum required for research. Non-soil based approaches include *in vitro* systems to grow on media under sterile conditions. Here, the host plant is replaced by Ri t-DNA transformed roots (genetically modified with *Agrobacterium rhizogens*). The fungus is able to colonize and produce spores that are morphologically and structurally similar to those produced in pot cultures. The ability of the *in vitro* produced propagules to retain their viability to colonize and initiate new mycorrhizal symbiosis indicates that the fungus is able to complete its life cycle. The success thus achieved by *in vitro* culture of AM species using Ri t-DNA transformed roots indicates that this technique can be exploited for large scale inoculum production. The absence of undesirable contaminants or microorganisms makes *in vitro* cultivation systems suitable for large-scale production of high-quality inoculum.

Ri-plasmid transformed root cultures were pioneered by Mugnier and Mosse (1987). A natural genetic transformation of plants by the ubiquitous soil bacterium *Agrobacterium rhizogenes* Conn. (Riker et al 1930) produces a condition known as hairy roots. This stable transformation produces Ri T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. Their modified hormonal balance makes them particularly vigorous and allows profuse growth on artificial media (Tepfer 1989). *Daucus carota* L. (carrot) and *Convolvulus sepium* L. (bindweed) were among the earliest species transformations using *A. rhizogenes* (Tepfer and Tempé 1981). For *in vitro* culture of AM fungi using Ri T-DNA roots, the disinfected AM fungal propagules (spores and vesicles) are plated on to Modified Strullu Romand (MSR) media for germination after which the germinated propagules are associated with actively growing Ri T-DNA transformed roots for establishment of AM symbiosis (Plate 2c-h).

Due to their obligate biotrophic nature, the *in vitro* culture and large-scale production of these fungi has been limited there by reducing their potential for use as inoculum in agricultural and horticultural practices (Plenchette et al 1996). With the advancement in research on AM fungi, novel tools have been developed for growing these fungi under *in vitro* conditions one of them being root organ culture (ROC). The establishment of *in vitro* root-organ cultures has greatly increased our understanding of the AM symbiosis. Although the *in vitro* system is artificial, it allows non destructive, morphological and physiological investigations of the AM symbiosis. Moreover, it is possible to increase similarity to a natural system by allowing the development of the AM symbiosis in a nutrient environment which is closer to that of the mineral soil. This technique permits production of pure, viable, contamination free and abundant propagules in a smaller space. Therefore *in vitro* dual culture of Ri T-DNA transformed roots and AM fungi has proved to be a powerful tool for the study of these symbionts.

The culture plates may be placed in a growth chamber requiring minimal space for incubation with no requirement of light. The sporulation dynamics can be controlled and optimal harvesting time can be determined. Factors that influence optimal production *viz.,* nutrient availability, culture conditions, presence of contaminants, etc. are more easily manipulated in *in vitro*. However, the number of AM species that have been grown in *in vitro* is fewer than those obtained under pot cultures. Also, the costs involved using *in vitro* production are higher as it requires skilled technicians and sophisticated laboratory equipment such as sterile work flows, controlled incubators for ROC, and growth chambers for plant systems. Another advantage of the *in vitro* system is the low requirements in the follow-up of the cultures. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention (Ijdo et al 2011). To harvest of the *in vitro* produced AM fungal propagules, citrate buffer is used to solubilize the culture medium.

D'Souza et al (2013) reported an inhibitory effect of sucrose on spore germination and germ-tube growth in the AM species *Rhizophagus irregularis.* They reported greater and earlier spore germination in MSR medium without sucrose compared to sucrose addition. The study also revealed that the germ tube length was significantly greater in MSR medium without sucrose. Rodrigues and Rodrigues (2012) reported successful establishment of dual culture in *G. clarum* and transformed chicory roots on the MSR medium that produced abundant sporulation. Following infection, considerable internal root colonization and extensive proliferation of extra-radical mycelium was observed. Rodrigues and Rodrigues (2015) successfully demonstrated the capability of viable monoxenically produced spores of *Funneliformis mosseae* to induce AM colonization *in vivo*, indicating suitability of the method for large-scale inoculum production.

Role in Sustainable Agriculture. Sustainable agriculture is farming using natural sustainable ways and plays an important role in agro-ecosystems. It is ecologically sound, economically feasible and socially responsible (Siddiqui and Pichtel 2008). Beneficial soil microorganisms such as AM fungi are widely distributed in natural and agricultural lands. They form symbiotic associations with majority of agricultural and

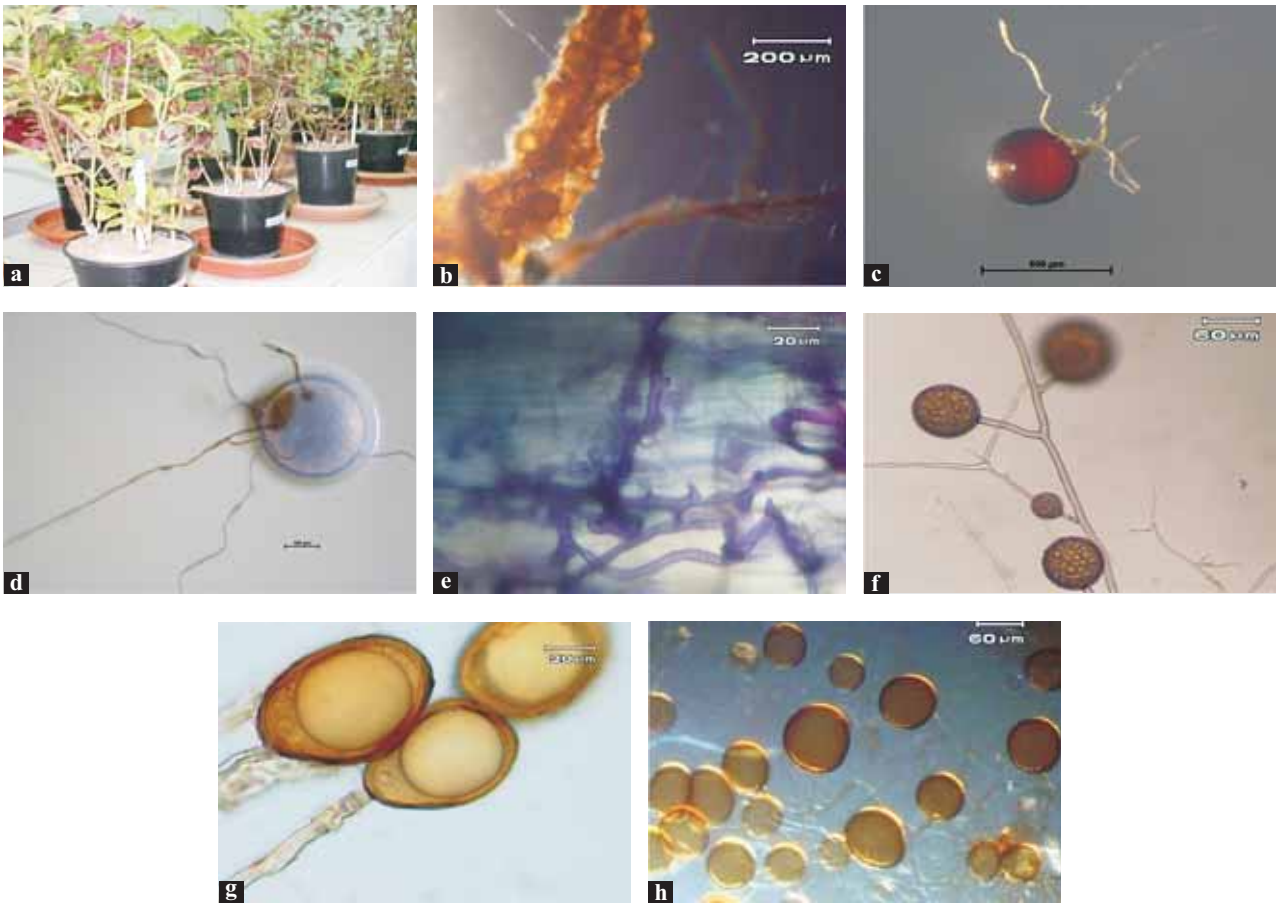

Plate 2. a. Pure cultures of AM fungi; b. Intra-radical spores; c. *In vitro* spore germination in *Scutellospora gregaria*; d. *In vitro* spore germination in *Scutellospora scutata*; e. *In vitro* hyphal coils; f. *In vitro* sporulation in *Rhizophagus intraradices*; g. Vesicles of *Rhizophagus clarus*; h. Sporulation in *Rhizophagus clarus* using vesicles as propagules

horticultural crops. They make nutrient resources especially P (Abo-Rekab et al 2010) available to the crops. Besides they increase the soil fertility (Rillig and Mummey 2006) and provide numerous other ecosystem services. Being responsible for overall plant health and development, they can be applied in agro-ecosystems. Plant health and productivity are rooted in the soil, and the quality of soil depends on the diversity and viability of the microbiota (Doran and Linn 1994; Visser, 1985) that shapes the structures which support a stable and healthy agro-ecosystem (Bethlenfalvay and Schuepp 1994). The role of AM fungi has been described as a fundamental link between plant and soil (Bethlenfalvay and Linderman 1992) and thus AM fungi have attracted interest as ecosystem engineers and biofertilizers (Fitter et al 2011). AM inoculum has been produced and used in agriculture, horticulture, landscape restoration, and site remediation for almost two decades (Hamel 1996). Although AM fungi are ubiquitous in agricultural soils, field experiments showed that a further addition of AM by inoculation can positively affect plant root colonization and thereby increase crop productivity (Lekberg and Koide 2005; Lehmann et al 2012).

The application of sterile inoculum (*in vitro* produced) can be of great value for *in vitro* propagation of high-value crops and ornamental plants. In addition, *in vitro* propagation in association with AM fungi could reduce mortality rates and the transplantation shock of reintroduced endangered plant species. It could also be used to enhance the production of secondary metabolites used in the pharmaceutical industry (Kapoor et al 2008).

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