ARBUSCULAR MYCORRHIZAL (AM) FUNGAL INOCULUM PRODUCTION USING *IN VITRO* TECHNIQUE FOR REVEGETATION OF DEGRADED SAND DUNES

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This thesis is examined by me and no corrections have been suggested.

25/1/2018

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HOD Botany

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Guide

DECLARATION

I hereby declare that the matter embodied in this thesis entitled, **"Arbuscular mycorrhizal (AM) fungal inoculum production using *in vitro* technique for revegetation of degraded sand dunes"** is the result of investigations carried out by me, under the supervision of Prof. B. F Rodrigues and it has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or the other such similar title.

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CERTIFICATE

This is to certify that the work incorporated in this thesis entitled, **"Arbuscular mycorrhizal (AM) fungal inoculum production using *in vitro* technique for revegetation of degraded sand dunes"** submitted by Ms. Kim Maria Rodrigues, constitutes her independent work and the same has not been previously submitted for the award of any other degree, diploma, associate ship, fellowship or the other such title.

Goa University
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The root does anchor a plant, but it's the mycorrhizae that become the main system to absorb water and nutrients from the soil.

-Larry Simpson

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CHAPTER 1

Introduction

1.1: Coastal sand dunes

Sandy shores are highly organized natural dynamic systems that undergo continuous transformation due to changing weather conditions and geomorphological processes. They comprise of a near shore zone where water currents and waves are involved in sand movement, the foreshore zone where the transport is by water currents or waves and rarely by wind action, the backshore zone where the transport is primarily due to wind with breaking waves having least influence, and the dunes where the sand movement is largely due to wind action (Krumbein and Slack, 1956).

Coastal sand dunes are natural structures which shield the coastal zone by absorbing energy from wind, tide and wave action (McHarg, 1972). Coastal sand dunes are built up by the deposition of dry beach sand blown through wind action on the inland side of the beaches. The dune systems are the most capable and provide least expensive protection against shoreline erosion. Generally sand dunes consist of vegetation cover that traps the sand (Clowes and Comfort, 1987). The variability in the structure of the dune systems is influenced by factors such as its sediment property, climate and ecological conditions. The basic pre-requisite for the formation of sand dunes is a plentiful source of sediment transported by wind (Labuz, 2005) followed by stabilization of the deposited sand by vegetation. According to the degree of exposure to coastal stress conditions the vegetation on the dunes tends to occur in zones. Nearest to the sea is the pioneer zone, extending landward from the debris line at the top of the beach in the area of the fore dune or frontal dune. Only specialized pioneer plants that can withstand the stress conditions colonize areas exposed to salt spray, sand blast, strong winds and flooding by the sea. These plants have specialized structures such as a waxy coating on stems and leaves, are prostrate, and have well developed and rapidly spreading root systems. The creeping stems or stolons can interconnect, so if one part is

buried in shifting sand or is uprooted, another part continues to grow; and so serve to stabilize the sand, forming and building the dunes (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html).

The State of Goa, situated on the West coast of India has a beautiful coastline and beaches with a characteristic sand dune ecosystem of economic significance. The sandy beaches of Goa are backed by several rows of 1-10 meters high sand dunes that extend almost half a kilometer or more before merging with the hinterland coastal plain (http://www.goaenvis.nic.in/sanddunes.htm). According to Dessai (1995), the coastal sand dunes of Goa are classified as: **a) embryonic dune**, the zone nearest the sea just above the high tide level with its steeper face inland and is often not vegetated. The zone is formed by sand delivered to the beach by wave action and is the most vulnerable. The pioneer plants found growing on the embryonic dunes are *Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae), *Spinifex littoreus* L. (Poaceae) and other herbaceous species; **b) mid shore dune**, where the vegetation is characterized by shrubs and is more or less stable, species commonly found are *Spermacoce stricta* L. f. (Rubiaceae), *Leucas aspera* (Willd.) L. (Lamiaceae), *Vitex negundo* L. (Lamiaceae) and *Clerodendrum inerme* (L.) Gaertn. (Lamiaceae), and **c) hind shore dune**, the zone that has trees with well developed root systems. Dominant plant species growing on hind shore dune include *Vitex negundo* L. (Lamiaceae), *Clerodendrum inerme* (L.) Gaertn. (Lamiaceae), *Anacardium occidentale* L. (Anacardiaceae), *Pandanus tectorius* Park. (Pandanaceae), *Casuarina equisetifolia* L. (Casuarinaceae), *Cocos nucifera* L. (Arecaceae).

Sand dune vegetation plays a crucial role in the dune formation process by acting as a wind break and trapping the deposited sand particles, thereby influencing dune

morphology. The pioneer plants have the ability to grow up through the sand frequently producing new stems and roots which help to stabilize the ground as more sand is accumulated and the dune grows (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html). Fixed dunes play a key role in the protection of the coastline as they act as a buffer against wave damage during storms and protect the landward side from salt water intrusion, thus helping in the development of more complex plant communities. They also function as a sand reservoir to replenish and maintain the coastal ecosystem during times of weathering and erosional processes (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html).

Plants established on sand dunes are subjected to various environmental fluctuations which affect their growth, survival and community structure. The most important factors include temperature, desiccation, low moisture retention, soil erosion, sand accretion and burial, soil salinity, salt spray, changes in organic matter and pH (Maun, 1994). Loss of vegetation that traps and holds sand leaves the dunes and beach more susceptible to wind and water erosion (Gomez-Pina et al., 2002), resulting in degradation. Sand particles shift to another place through long-shore drift or littoral drift (Healy and de Lange, 2014). If the vegetation cover is damaged then strong winds may cause 'blowouts' or gaps in the dune ridge. Unless repaired, these can increase in size and lead to the migration of whole dune system on the inland side by covering everything in its path. With a diminished reservoir of sand, erosion of the beach may lead to coastal recession. The vegetation cover can also be adversely affected and destroyed by natural disturbances such as storms, cyclones, droughts, fire or by human intervention such as clearing, grazing, vehicles or excessive foot traffic (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html). Recreational and tourism activities, land reclamation, and excavation activities also

result in sand dune degradation (Gomez-Pina et al., 2002). Protection of the vegetation is thus vital (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html).

1.2: Arbuscular Mycorrhizal (AM) fungi

Ecosystems are occupied by large numbers of diversified microorganisms that interact in intricate networks (Moënne-Loccoz et al., 2015). Soil formation is the result of such complex network processes, biological, physical and chemical. Soil microbes are of great significance, as they are responsible for most biological transformations including nutrient recycling thereby facilitating the subsequent establishment of plant communities (Schulz et al., 2013).

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that form symbiotic association with plant roots (Smith and Read, 2008), belonging to phylum Glomeromycota. These fungi are a monophyletic lineage of obligate mycobionts (Schüßler et al., 2001). As the phylum is evolutionarily an ancient form of symbiosis in plants, about 90% of extant plant species are mycorrhizal (Moënne-Loccoz et al., 2015). The fungus penetrates plant root cell walls and develops intra-radical structures (hyphae, arbuscules, vesicles) in the cortical cells of the host root and extra-radical structures (hyphae, spores) in soil. This mutualistic association is characterized by a bidirectional flux wherein the mycobiont helps the phytobiont in acquisition of soil nutrients (mainly P) while the phytobiont provides photo-assimilates (carbon sources) to the mycobiont (Buscot et al., 2000; Brundrett, 2009).

Plant-microbe interactions have a major impact on plant functioning and plant community ecology (Moënne-Loccoz et al., 2015). It is assumed that fungi are the most

effective soil microorganisms involved in soil structure stabilization (McCalla 1946; Swaby 1949; Foster 1994), and AM fungi often comprise the major portion of the soil microbiome (Hayman, 1978). Mycorrhizae being vital components at the soil-root interface, through their extra-radical hyphae together with plant root hairs increase soil-root contact area (Geelhoed et al., 1997b). Hence primarily improving plant nutrient uptake of immobile nutrient mainly phosphorus (P) (Bell et al., 1989; Jakobsen et al., 2005; Bucher, 2007), also contributing in uptake of calcium (Ca) (Azcón and Barea, 1992), iron (Fe) (Treeby, 1992), manganese (Mn) (Kothari et al., 1991), zinc (Zn) (Bell et al., 1989) and nitrogen (N) (Nasholm et al., 2009). AM fungi are most beneficial in improving plant nutrient acquisition in low-fertility soils (Brundrett, 2009). It is assumed that they can serve as a substitute for reduced fertilizer input (Galvez et al., 2001), thereby leading to sustainable agriculture. Plant benefit other than nutritional attributed by AM fungi includes: 1. Enhanced plant tolerance to biotic stress (pathogenic infection, herbivory) and abiotic stress (drought, metal pollution, salinity) (Augé, 2004; Al-Karaki, 2006; Bennett and Bever, 2007); 2. Improved rooting of micro-propagated plantlets (Strullu, 1985) resulting in overall increase in plant growth and development; 3. Improved nutrient cycling, energy flow and plant establishment in disturbed ecosystems (Tiwari and Sati, 2008); 4. Enhanced diversity of plant community. AM fungi through their extensive mycelial network interconnect a number of unrelated individual plant species consequently impacting the function and biodiversity of entire ecosystem (Smith et al., 1997; Bonfante and Genre, 2010); 5. Besides the ramifying extra-radical mycelial network, secretion of hydrophobic 'sticky' proteinaceous substance known as 'glomalin' by the AM fungal hyphae in the soil also results in improved soil stability, binding, and water retention thereby reducing soil erosion (Rillig et al., 2002; Rillig and Mummey, 2006; Bedini et al., 2009); 6.

Influencing microbial and chemical environment of the mycorrhizosphere (plant root-associated microbial communities especially mycorrhizae present in the rhizosphere) to contribute in plant nutrient acquisition (Azcón-Aguilar and Barea, 2015); more precisely the hyphosphere, the zone surrounding individual hyphae (Johansson et al., 2004); 7. Bioremediation of soil. AM fungi are mainly involved in phytoremediation, use of plants for uptake of pollutants. The fungi help in alleviating metal toxicity to plants by reducing metal translocation from root to shoot (Leyval et al., 1997). Therefore they contribute in revegetation and restoration of disturbed or contaminated lands; and 8. AM fungi also provide protective nutrient components or antioxidants to human beings through agricultural products. AM symbiosis can stimulate synthesis of plant secondary metabolites, which are important for increased plant tolerance to environmental stresses or beneficial to human health through their antioxidant activity (Seeram, 2008). Thus AM fungi also contribute in the earth's ecosystem services (Gianinazzi et al., 2010).

1.3: Significance of AM fungi in coastal sand dune systems

Survival of plant communities under harsh conditions depends on interaction with different soil microbes *viz.*, mycorrhizae, plant growth-promoting bacteria (PGPB) and endophytes. AM fungi are widespread in sand dunes throughout the world and are known to extensively contribute to the stabilization and development of plant community structure (Nicolson 1960; Koske and Polson, 1984; Puppi and Riess, 1987; Koske and Gemma, 1996). AM fungal interaction in the rhizosphere is known to facilitate establishment and sustenance of dune vegetation. The vegetation cover and soil microbes play an elemental role in sand binding and stabilization in dune ecosystems. Profuse AM fungal colonization has been reported in coastal dune plants

(Giovannetti and Nicolson, 1983; Puppi et al., 1986). The AM fungal hyphae bind sand particles forming sand aggregates that resist strong winds and storms and stay intact even after death of roots and hyphae (Sutton and Sheppard, 1976; Koske and Polson, 1984).

Sand dune systems encourage the occurrence of abundant and diverse AM fungal communities (Nicolson and Johnston, 1979; Giovannetti and Nicolson, 1983; Koske, 1987; Mohankumar et al., 1988; Dalpé, 1989; Blaszkowski 1993; Stürmer and Bellei, 1994; Tadych and Blaszkowski, 2000a; Blaszkowski et al., 2002), mainly because of their low content of soil minerals especially P (Nicolson and Johnston, 1979; Koske, 1988). The adversity imposed on plants by low P levels is compounded by the low mobility of P in the soil. The absorption of readily available P by plant roots creates P depletion zones (where the total available P has been scavenged) around the roots (Marschner, 1995). Hence, the low availability of P affects its uptake by roots. The AM fungal extra-radical hyphae, which are thinner and more extensive rather than the root hairs themselves (Novero et al., 2008), are able to cross this zone and provide the plant with P (Koske and Polson, 1984).

Mycorrhizal plants are effective colonizers of disturbed habitats and absence of AM fungi exerts intense pressure on plant community species composition (Tommerup and Abbot, 1981). Close mutualistic plant microbe interactions facilitate existence of disturbed ecosystems. Physical destruction of hyphae in the web which forms close association between plants and AM has contributed to degradation of many ecosystems and restoring these relations is important to attain revegetation (Trappe, 1981). The increasing knowledge on importance of efficacy of AM association in ecosystem functioning and AM fungal diversity has encouraged attempts to identify native AM

species that colonize plants in their natural habitats. It is well known that native AM species have adapted to natural soil conditions during the course of evolution (Brundrett, 1991; Jaiswal and Rodrigues, 2001; Johnson, 2010), and that indigenous species can enhance plant growth and development more than exotic species (Schultz et al., 2001; Klironomos, 2003; Pánková et al., 2011; Pellegrino et al., 2011; Johnson et al., 2012; Estrada et al., 2013).

The understanding of AM symbiosis with dune plants and their distribution in soil is necessary for the sensible management of this fragile habitat (Jaiswal and Rodrigues, 2001). The occurrence of AM fungal propagules in sand dunes results in mycorrhizal plants as effective pioneer dune colonizers contributing to stabilization (Koske, 1987). Stabilization of disturbed habitats like coastal dunes is dependent upon successful establishment of an effective plant community. As mycorrhizal plants serve this purpose, there seems to be a great potential for restoration of degraded dune systems by encouraging AM symbiosis. It is evident however that the mycorrhizal status of early successional plants is governed by the AM fungal species available, by community composition and by inoculum potential (Koske and Gemma, 1997; Jaiswal and Rodrigues, 2001).

1.4: Significance of AM fungal diversity and dominance

Ecosystems and plant life are widely influenced by the functionally diverse nature of AM fungi, the symbiosis is considered as a tripartite relationship between plant, fungus and soil (Kernaghan, 2005), where the fungus creates an intimate link between the soil and the plant (Harrison, 1998). AM fungi co-exist as assemblages of mixed species in terrestrial ecosystems with certain species being dominant and a plant may be colonized by several AM species at one time. Most of the AM fungal species are widespread

occurring in different terrestrial habitats and are considered 'generalists' (Öpik et al., 2006) however some species appear to be restricted to particular ecosystem types and are considered 'specialists' (Oehl and Sieverding, 2004; Castillo et al., 2006; Oehl et al., 2007). Different soil types showing variation in AM species can be characterized by AM fungal community structure (Oehl et al., 2010). AM taxa differ significantly in their life histories and are believed to be nonspecific with regard to their capability to infect and colonize different plant species, although there may be exceptions (Helgason et al., 2002; Sanders, 2002; Vandenkoornhuyse et al., 2002a). There is also variation in response to soil structure, mineral acquisition, plant health, growth rate, biomass allocation and symbiotic effects (Abbott and Robson, 1985; Miller et al., 1995; Klironomos et al., 2000; Bever et al., 2001). AM species diversity is more distinct in undisturbed ecosystems compared to disturbed ecosystems as in undisturbed ecosystems there is greater degree of variability in terms of critical determinants. AM diversity and abundance can be affected by various factors/determinants such as habitat type, edaphic conditions, climatic or seasonal variations, host genotype and vegetation cover. AM dominance can also be affected by the severity and extent of disturbance in a habitat (Bhatia et al., 2013). Plant diversity and productivity are enhanced by AM symbiosis and AM species richness (van der Heijden et al., 1998; Moora et al., 2004). Increase in AM fungal diversity results in an increase in species richness and hence higher plant productivity. This suggests that changes in below ground AM fungal diversity can affect changes in above ground plant diversity and productivity (Finlay, 2008). Assessment of AM fungal diversity is essential if the benefits associated with the symbiosis are to be exploited. Knowledge of AM species diversity in functioning ecosystems is crucial for the development of inocula for agricultural and horticultural crops and for revegetation of degraded ecosystems.

1.5: AM fungal propagules and inoculum cultivation

AM fungi are present as chlamydospores or vegetative infective propagules colonizing plant roots in the rhizosphere. Upon germination, AM fungal hyphae penetrate the root cortical cells, bifurcating intra- and inter-cellularly from the point of entry. Within the cortical cells, the fungus forms tree-like branched structures called arbuscules that serve as the sites of metabolite exchange between fungus and host plant. Vesicles are lipid storage organs that also function as reproductive structures (chlamydospores).

Being obligate symbionts, the AM fungi can grow only in association with a living host plant root to complete its life cycle. Rhizosphere soil is used as a source of AM propagules that comprise of spores, sporocarps, hyphal fragments and dried colonized root fragments. For isolation of utmost quantity of AM fungal propagules from soil it is necessary to have knowledge about the diversity, abundance, viability, and colonization activity by the indigenous AM species in the selected soil sample. Since AM fungal spore propagules from natural soils can be non-viable, empty or parasitized, trap cultures can be set using a suitable host plant to increase the density of viable propagules. This enables identification of viable inoculum used in the generation of monospecific (single species) or pure cultures. The catch plant to be used as host should be adaptable to existing growing conditions, fast growing, readily colonized, producing a large quantity of roots in a relatively short period, and tolerant to pests and diseases. Some commonly used host plants include *Zea mays* L. (corn), *Allium cepa* L. (onion), *Arachis hypogaea* L. (peanut), *Stylosanthes* Sw. spp., *Paspalum notatum* Flüggé (bahia grass) and *Pueraria phaseoloides* (Roxb.) Benth. (Kudzu) (http://invam.wvu.edu/methods/cultures/host-plant-choices). The host plant should also be fertilized bi-weekly with nutrient solution such as Hoagland's solution (minus P) to maintain the nutrient status of the substrate. To ensure that the inoculum contains

mature spores, it is necessary to grow the catch plant for 12-14 weeks after which it is dried slowly by reducing water. The propagules from the inoculum can then be extracted and multiplied.

Presently, AM inoculum production techniques depend on soil/substrate-based cultures, which may not be sterile and can be mixed cultures involving other AM species or contaminants such as endophytes (Gianninazzi and Bosatka, 2004). Non-soil based cultures include *in vitro* or monoxenic or Root Organ Culture (ROC) systems involving the use of Ri T-DNA transformed plant root organs (genetically modified with *Agrobacterium rhizogenes*) able to grow on media under sterile conditions. Utilization of Ri-plasmid transformed root organ cultures for growth of AM fungi was pioneered by Mugnier and Mosse (1987). The ubiquitous soil bacterium *A. rhizogenes* Conn. (Riker et al., 1930) produces hairy roots in plants by natural genetic transformation. This stable transformation (Tepfer, 1989) produces Ri T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. The modified hormonal balance of the transformed roots allows profuse and vigorous growth on synthetic medium (Tepfer, 1989). *Daucus carota* L. (carrot) and *Convolvulus sepium* L. (bindweed) were among the first species to be transformed using *A. rhizogenes* Conn. (Tepfer and Tempé, 1981). For *in vitro* culture of AM fungi, the AM fungal propagules (spores, vesicles and colonized root fragments) after disinfection with a suitable sterilizing agent are plated on to Modified Strullu Romand (MSR) media for germination. The germinated propagules are then associated with actively growing Ri T-DNA transformed roots for establishment of AM symbiosis (Bécard and Fortin, 1988). This technique produces contamination free inoculum (Declerck et al., 2005).

1.6: Carrier based inoculum

AM fungal inoculum is available commercially in the form of carrier materials and potting media, with the former containing high concentrations of AM fungal propagules and the latter containing low concentrations of inoculum (Douds et al., 2010). Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms (Accinelli et al., 2009). A carrier is a delivery vehicle which is used to transfer live microorganism from laboratory conditions to a rhizosphere (Brahmaprakash and Sahu, 2012). The carrier is the major portion (by volume or weight) of the inoculant that helps to deliver a suitable amount of plant growth promoting microorganisms (PGPM) in good physiological condition (Smith, 1992). The carrier formulation should also provide a suitable microenvironment for the PGPM, assure a sufficient shelf life of the inoculant (at least 2-3 months for commercial purposes, possibly at room temperature) and allow easy dispersion or dissolution in the volume of soil near the rhizosphere (Nehra and Choudhary, 2015). A suitable biofertilizer carrier should posses as much as the following features *viz.*, it should be in powder or granular form; should support the growth and survival of the microorganism, and should be able to release the functional microorganism easily into the soil; should have high moisture absorption and retention capacity, good aeration characteristics and pH buffering capacity; should be non-toxic and environmentally friendly; should be easily sterilized (autoclaving and gamma-irradiation) and handled in the field; have good long term storage qualities; and should be inexpensive (Stephens and Rask, 2000; Rebah et al., 2002; Rivera-Cruz et al., 2008). Considering the above mentioned properties it is evident that not a single universal carrier is available which fulfills all the desirable characteristics, but good quality ones should have as many as possible (Brahmaprakash and Sahu, 2012).

Organic, inorganic or synthetic substances can be used as carrier materials. Commonly used carriers include soils like peat, coal, pumice or clay, sand, and lignite; inert materials like perlite, vermiculite, soilrite, alginate beads, polyacrylamide gels and bentonite (Mallesha et al., 1992; Redecker et al., 1995; Bashan, 1998; Gaur and Adholeya, 2000; Herridge et al., 2008; Malusá et al., 2012). Organic wastes from animal production and agriculture, and byproducts of agricultural and food processing industries such as charcoal, composts, farmyard manure, cellulose, soybean meal, soybean and peanut oil, wheat bran, press mud, corn cobs also meet the requirements of a carrier and thus could be good carrier materials (Herrmann and Lesueur, 2013; Wang et al., 2015). It is also possible to find carrier combinations comprising of a mixture of soil and compost; soil, peat, bark, and husks among others (Herridge et al., 2008). Peat is the most commonly used carrier material. However, it is a limited natural resource which is not readily available worldwide and its use has a negative impact on the environment from which it is extracted. This highlights the need for development of new carrier formulations using alternative resources to compete with the existing inoculants (John et al., 2011).

1.7: Shelf life of carrier based inoculum

Maintaining and maximizing the viability or shelf life of carrier based inocula is the foremost pre-requisite to enable and utilize the benefits provided by the microbial inoculant. A sufficiently long shelf life of the microbial inoculant (up to at least one season), maintaining its biological traits at an adequate level, is key for assuring the effectiveness of the biofertilizer, though being a major challenge for any kind of formulated product (Bashan et al., 2014). Therefore, the formulation of the inocula is a multi-step process which results in mixing one or more strains of microorganisms

(inoculum) with a particular carrier, with or without additives such as sticking agents or other additives like strigolactones synthetic analogs, vitamins (Ruyter-Spira et al., 2011; Palacios et al., 2014) to support the growth, plays a significant role in assuring the efficiency of the biofertilizer. It allows the protection of the microbial cells during storage and transport, possibly enhancing the persistence of the inocula in soil, in order to obtain the maximal benefits after inoculation (Manikandan et al., 2010; Schoebitz et al., 2012).

Different carrier materials can be used in the carrier formulation process, and each of them can comprise of specific positive traits and drawbacks, thus affecting the overall quality and efficacy of the biofertilizers (Herridge, 2008; Malusà et al., 2012; Herrmann and Lesueur, 2013; Bashan et al., 2014). Non-availability of good and suitable carrier materials can result in contamination problems and shorter shelf life of microbial inoculants. Drying process (Larena et al., 2003), moisture content (Roughley, 1968; Date and Roughley, 1977; Kannaiyan, 2000), storage conditions (Connick et al., 1996; Elzein et al., 2004b; Hong et al., 2005; Friesen et al., 2006) as well as storage temperature (Connick et al., 1996; Hong et al., 2005) are also important determinants of the shelf life of microbial inoculants or formulations and can affect their activity pre- or post-application. The effect of storage conditions on growth and survival of microorganisms is subjective to both the purity of the culture and the amount of moisture loss during storage (Roughley, 1968). Temperature optima and limits also vary with different microorganisms (Pindi and Satyanarayana, 2012).

The pH of a microbial formulation or product also plays an important role in determining its activity and stability. Field studies of AM fungal communities in a wide range of soil pH suggest that it is also the major driving factor for structuring these

communities (Wang et al., 1993; Dumbrell et al., 2010), thus affecting the colonization potential and efficacy of all kinds of PGPM included in biofertilizers. Adaptations of AM fungi to abiotic factors such as soil temperature and nutrient availability can also strongly influence the effect of the AM symbiosis on plant growth (Treseder and Allen, 2002 ; Antunes et al., 2011). Nevertheless, in terms of expected efficacy of AM fungi based biofertilizers, it is important to consider that the overall fertility of soil is supposed to regulate the kind of relation between the AM and the plant (Malusá et al., 2016).

1.8: Potential of AM fungi from coastal sand dunes and *in vitro* culture technique

AM fungi play a very important role in growth of pioneer vegetation that brings about stability in the coastal habitats mainly with regard to primary and secondary succession of plant life. Coastal sand dune systems are prone to natural as well as human disturbances that affect the structure and stability of dune plant communities. AM fungi with their widespread underground mycelial web, link a number of different plant species thereby impacting the ecology of a habitat. Because of their numerous positive effects on terrestrial ecosystems, examining the AM associations in dune plant species and their distribution in the sandy soils is needed for the sustainable management of these habitats. AM fungal inoculum production *via in vitro* culture technique is preferred over the traditional soil based pot culture technique. It results in the production of a large number of pure, viable and contamination free spores in a single Petri plate. The sterile conditions exclude undesired microbes and regular monitoring of the cultures make the technique more suitable for the mass production of high quality inoculum. Since biofertilizers are normally developed as carrier based inoculants containing the efficient microbes, there is a need for the development of a suitable

carrier formulation for mass multiplication of *in vitro* produced AM fungal propagules for its utilization as carrier based inoculum.

Recognizing the potential of AM spore production by *in vitro* culture technique, an effort was made to produce carrier based AM fungal bio-inocula. The work bridges the gap of developing a suitable carrier formulation to facilitate the transfer, multiplication and increase the efficacy of *in vitro* produced AM fungal propagules in the rhizosphere, and encourages the use of carrier based *in vitro* produced AM fungal bio-inocula for revegetation strategies of degraded sand dune ecosystems. The present work is undertaken with the following objectives:

1. To identify the dominant AM fungal species from the sand dune ecosystem.
2. To prepare pure culture inoculum using trap and pot cultures.
3. To prepare and standardize the protocol for *in vitro* culture technique for dominant AM fungal species.
4. To develop viable inoculum using suitable carrier for re-inoculation.
5. To maximize the shelf life of the *in vitro* prepared inoculum.
6. To study the effect of *in vitro* produced carrier based bio-inocula on selected plant species suitable for revegetation of the sand dunes.

CHAPTER 2

Review of Literature

2.1: History of discovery of the Arbuscular Mycorrhizal (AM) fungi

AM fungi are a group of ever-present obligate biotrophs having a pre-requisite to develop close mutualistic association with plant roots in order to grow and complete their life cycle (Parniske, 2008). They are found in almost all ecosystems (Read, 1991; Brundrett, 2009). Frank (1885) coined the term "mycorhiza", a peculiar relationship between tree roots and ecto-mycorrhizal fungi and was probably the first to identify the association between plant roots and mycorrhizal fungi (Frank and Trappe, 2005). The term "mycorrhiza" literally derives from the Greek words 'mycos' and 'rhiza', meaning fungus and root, respectively (Wang and Qiu, 2006). A detailed discussion of the derivation of the word "mycorrhiza", including the incorporation of the second "r" is given by Kelley (1931, 1950). As early as 1842, Nägeli described AM fungi. Trappe and Berch (1985) and Rayner (1926-1927) cite early observations of the AM symbiosis. Schlicht (1889), Dangeard (1896), Janse (1897), Petri (1903), Gallaud (1905), Peyronel (1924), Jones (1924) and Lohman (1927) conducted extensive surveys of host plants and gave anatomical descriptions of AM fungi. Phillips and Hayman (1970), Harley and Smith (1983) and Gardes and Bruns (1993) studied the partners and processes involved in this symbiosis.

The name for the arbuscular mycorrhizal fungal symbiosis has changed through the years. The symbiosis was once frequently called "phycomycetous endo-mycorrhiza" to distinguish it from the endo-mycorrhizal symbioses formed between members of the Ericaceae or Orchidaceae and higher fungi. The name "Phycomycete", however, no longer carries any systematic significance (Koide and Mosse, 2004). Frank (1887) recognized the difference between ecto- and endo-mycorrhizas. The name "vesicular-arbuscular mycorrhiza" was established, as fungal structures *i.e.* vesicles and arbuscules were observed within the roots (Janse, 1897; Gallaud, 1905). But the

detection of that 'not all fungi formed vesicles' led to the renaming of this symbiosis as "arbuscular mycorrhiza" which is now widely accepted (Koide and Mosse, 2004).

2.2: Diversification of AM fungal symbiosis

As AM fungi have widespread distribution, they have been reported to occur in the roots of most angiosperms and pteridophytes, along with some gymnosperms and the gametophytes of some lower plants like mosses and lycopods (Smith and Read, 1997). The earliest evidence for AM symbiosis in seed plants occurs in silicified roots of the Triassic cycad *Antarcticycas schopfii* (Stubblefield et al., 1987; Phipps and Taylor, 1996). The earliest known fossil evidence for AM symbiosis is seen in stems of an early vascular land plant *Aglaophyton major* dating 400 million-years-ago from the Rhynie chert (Remy et al., 1994). It is reported that *Aglaophyton major* also contained well preserved *Scutellospora*- and *Acaulospora*-like spores (Dotzler et al., 2006, 2009). Wide phylogenetic distribution and the presence of 450 million-year-old fossils of mycorrhizal fungal-like structures in early land plants from the Rhynie chert in Scotland (Remy et al., 1994; Redecker et al., 2000a; Dotzler et al., 2006), suggest that the AM symbiosis is ancestral among land plants and it probably allowed their transition from water to land (Selosse and Le Tacon, 1998). Simon et al. (1993) using molecular clock analysis (estimation of approximate dates of evolution of particular lineages based on rates of DNA sequence changes in different organisms) of ribosomal DNA sequence data from present day Glomales, stated that AM symbiosis has a single phylogenetic origin and that AM fungi evolved 353-462 million years ago. It is possible that the AM symbiosis had developed with early freshwater-aquatic phototrophic gametophytes previously before the Ordovician colonization of dry land and the development of mycorrhizal rhizoidal bryophytes (Willis et al., 2013). This is proved

from Wang et al. (2010) wherein the authors confirmed that the genes isolated from nearly all major plant lineages, required for formation of plant-AM symbiosis were present in the common ancestor of land plants and their functions were largely conserved during invasion of land by plants. Additionally only one living member of the ancient *Geosiphonaceae*, *Geosiphon pyriformis* forms a different type of AM symbiosis. It produces specialized bladders that harbor symbiotic cyanobacteria *Nostoc punctiforme* (Schüßler et al., 1994, 1996) giving a further indication of primitive ancestry. Nonetheless, molecular phylogenetic analysis has revealed that *Geosiphon* is a representative of the Glomeromycota (Schüßler et al., 2001). Molecular data also suggest that considerable phylogenetic radiation of Glomales taxa occurred parallel with the colonization of land (Redecker et al., 2000b). Molecular and fossil evidences suggest that the ancestors of all current land plants probably formed AM association and some plant taxa that do not form AM have lost the genetic ability to do so (Fitter and Moyersoen, 1996). The non-mycorrhizal plants and plant taxa forming other types of mycorrhiza must have evolved from an ancestral mycorrhizal condition (Pirozynski, 1981; Fitter and Moyersoen, 1996) and has probably evolved numerous times (Smith and Read, 1997).

2.3: Phylogenetic relationships

AM fungi form a monophyletic group in the phylum Glomeromycota (Schüßler et al. 2001). About 288 taxonomically described species are currently included in this group (Öpik and Davison, 2016). The nuclear-encoded rDNA phylogenies have revealed a considerable polyphyly of some genera, which has been used to reassess taxonomic concepts (Redecker and Raab, 2006). rDNA phylogenies have revealed that the genus *Glomus* is several times polyphyletic (Redecker et al., 2000b; Schwarzott et al., 2001).

AM species which form *Glomus*-like spores can be found in six different lineages within the Glomeromycota. Genus *Paraglomus* emerges to be the most primitive diverging glomeromycotan lineage as revealed in rDNA phylogenies. The separation of *Pacispora* and *Diversispora* clades from other '*Glomus* lineages' is well-supported by rDNA phylogenies (http://tolweb.org/Glomeromycota). *Glomus* groups A and B represented by the species *Glomus mosseae* and *Glomus claroideum* respectively, are genetically rather distant but still form a monophyletic group in rDNA phylogenies (Schwarzott et al., 2001). The formation of 'sporiferous saccule' was thought to be a characteristic feature solely of the Acaulosporaceae (*Acaulospora* and *Entrophospora*), but now it is known to occur in the *Archaeospora*. The Gigasporaceae (*Scutellospora* and *Gigaspora)* members are well distinguished by the formation of 'bulbous suspensor' which is exemplified by molecular data (http://tolweb.org/Glomeromycota). Gigasporaceae and Acaulosporaceae representatives form a clade in most rDNA phylogenies, which is in conflict with previous investigations based on cladistic analysis of morphological features that placed *Glomus* and Acaulosporaceae together (Morton and Benny, 1990).

2.4: Phylogenetic relationships of Glomeromycota to other fungi

The Glomeromycota is a monophyletic group which is supported by rDNA phylogenies (Schüßler et al., 2001; Helgason et al., 2003; James et al., 2006). The 'Glomales' were previously placed in the Zygomycota. But their symbiotic nature, the absence of zygospores and the rDNA phylogenies indicated that they form a monophyletic group distinct from other Zygomycotan lineages (http://tolweb.org/Glomeromycota). Based on this data, Schüßler et al. (2001) erected the phylum Glomeromycota. The authors also corrected the formerly used name 'Glomales' to 'Glomerales'. Phylogenetic trees

based on rDNA analyses place the Glomeromycota as the sister group of Asco- and Basidiomycota, although not strongly supported (http://tolweb.org/Glomeromycota).

2.5: Classification of AM fungi

The isolation of spores from soil is necessary for classification of AM fungi. Routine extraction from soil is made possible by Wet Sieving and Decanting technique, a method commonly used to extract nematodes from soil and adapted to AM fungi by Gerdemann (Gerdemann, 1955a; Gerdemann and Nicolson, 1963). Earlier many attempts of developing a classification system or method of recognition of all AM spore types have been carried out. Nicolson and Gerdemann, both plant pathologists, decided on the classical system with Latin names. Mosse (a plant anatomist) and Bowen (an ecologist) attempted a more descriptive system of classification based mainly on spore wall structure, colour and cytoplasmic characteristics (Mosse and Bowen, 1968). Nicolson and Gerdemann (1968) divided the fungi into two groups of genus *Endogone*, one forming extra-radical azygospores/zygospores arising from the tip of a swollen hyphal suspensor but producing no intra-radical vesicles, and the other forming extra-radical chlamydospores and intra-radical vesicles. Schüßler et al. (2001) used molecular data to establish relationships among AM fungi and between AM fungi and other fungi. The group of AM fungi was elevated to the level of phylum Glomeromycota, which was shown to be distinct from other fungal groups.

The identification techniques employed by taxonomists have become increasingly sophisticated. Primarily, taxonomies were based upon morphological and anatomical characteristics of the fungi. Later on, methods based on serology (Aldwell and Hall, 1987), isozyme variation through gel electrophoresis (Hepper, 1987) and fatty acid variation (Bentivenga and Morton, 1994) were introduced. Presently, systematists have

come to rely increasingly on DNA-based methods (Cummings, 1990; Davidson and Geringer, 1990; Simon et al., 1990, 1992, 1993; Redecker, 2000) which are considered to be the best measure of genealogical relationships among organisms (Koide and Mosse, 2004). DNA target regions mostly used for AM fungal identification are located on the ribosomal genes (Small and Large ribosomal Subunits – SSU and LSU and the Internal Transcribed Spacers – ITS1 and ITS2) as they show variation that is sufficient to distinguish between AM species or isolates (Krüger et al., 2012). All this has led to the modern era of molecular identification of AM species (Redecker et al., 2013). Next-Generation Sequencing (NGS) tools represent a further step forward for biodiversity surveys of all organisms (Shokralla et al., 2012), including AM fungi. Over the last few years, the number of NGS based AM fungal biodiversity studies has increased, while the spectrum of the target environments has broadened (Öpik et al., 2013). Furthermore, new sets of primer pair for the specific amplification of AM fungal DNA sequences, capable of providing higher accuracy and a broad coverage of the whole phylum Glomeromycota have been developed (Krüger et al., 2009). Nowadays, AM fungal assemblages are no longer studied only in plant roots, but also in the bulk rhizosphere soil (Lumini et al., 2010; Borriello et al., 2012; Davison et al., 2012). The main result obtained from the application of NGS to the study of AM biodiversity has been the discovery of an unpredictable diversity within the phylum Glomeromycota (Öpik et al., 2013). However, this series of novel molecular tools has introduced a new issue *i.e.* the continuously increasing number of unidentified AM fungal DNA sequences from environmental samples with no correspondence whatsoever to sequences of known species (Öpik et al., 2010). This has naturally made scientists aware of the fact that the number of AM species could be larger than expected. However, it is not reliable to have new species described on just the basis of short DNA

sequences obtained by means of NGS tools. Instead, for each new suggested taxon, a series of steps needs to be followed to characterize the morphotype, the functional traits, and the ecological role offered when present in combination with other organisms in a given environment. Therefore, NGS tools cannot be considered as complete replacements of the traditional methods of identification and description of new species (Berruti et al., 2014). Routine identification of arbuscular mycorrhizal fungi will probably continue to be based primarily on morphological characters and thus an increased acceptance of the combined approach between anatomy and DNA will be important. The ability to properly name the fungi, avoid duplication of names and relate the species to one another also depends heavily on international culture collection centre's such as the International Culture Collection of Arbuscular and Vesicular-arbuscular Mycorrhizal Fungi (INVAM), and the International Bank for the Glomeromycota (BEG/IBG) (Koide and Mosse, 2004).

The most recent classification of Glomeromycota **(Table 2.1)** is based on a consensus of regions spanning rRNA genes: 18S (SSU), ITS1-5.8S-ITS2 (ITS), and/or 28S (LSU). The phylogenetic reconstruction underlying this classification is discussed in Redecker et al. (2013).

Table 2.1: Consensus classification of the *Glomeromycota* by Redecker et al. (2013).

Class	Order	Family	Genus
Glomeromycetes	Diversisporales	Diversisporaceae	*Tricispora**
			*Otospora**
			Diversispora
			*Corymbiglomus**
			Redeckera
		Acaulosporaceae	*Acaulospora*
		Sacculosporaceae*	*Sacculospora**
		Pacisporaceae	*Pacispora*
		Gigasporaceae	*Scutellospora*
			Gigaspora
			*Intraomatospora**
			*Paradentiscutata**
			Dentiscutata
			Cetraspora
			Racocetra
	Glomerales	Claroideoglomeraceae	*Claroideoglomus*
		Glomeraceae	*Glomus*
			Funneliformis
			Septoglomus
			Rhizophagus
			Sclerocystis
		Ambisporaceae	*Ambispora*
	Archaeosporales	Geosiphonaceae	*Geosiphon*
		Archaeosporaceae	*Archaeospora*
	Paraglomerales	Paraglomeraceae	*Paraglomus*

(* indicate genera of uncertain position, insufficient evidence, but no formal action taken).

2.6: Morphological characters used for identification of AM fungi

The AM fungal spores have unique morphological and biochemical characters. Regardless of the species, each spore forms one spore wall (Morton, 2002).

2.6.1: Spore wall

Its formation occurs through a sporogenous hypha. It either forms at the tip of the sporogenous hypha as seen in species belonging to *Diversispora*, *Glomus*, *Gigaspora*, *Pacispora*, *Paraglomus*, *Racocetra*, *Scutellospora*. The spore wall can also develop from inside the sporogenous hypha as seen in species belonging to *Acaulospora*, *Entrophospora*, *Intraspora*, some *Glomus* spp. The spore wall can also form from the side of the sporogenous hypha as seen in species belonging to *Acaulospora*, *Ambispora*, *Archaeospora*, *Otospora*. Changes in the spore wall occur as the spore size increases *i.e.* it grows, thickens and differentiates. Once the spore ceases to expand small changes in colour, thickness and rigidity appear in the spore wall. The spore wall layers can be either permanent or impermanent structures (Błaszkowski, 2012).

2.6.2: Inner walls

These are colourless, permanent structures present in AM fungi of the genera *Acaulospora*, *Entrophospora*, *Intraspora*, *Ambispora*, *Archaeospora*, *Otospora*, *Pacispora*, *Racocetra*, *Scutellospora*. The number of inner walls can range from 1-3, with the innermost wall being frequently called as a germinal wall. Inner wall 1 usually forms after spore wall formation is complete. The subsequent inner wall layers arise only after the surrounding inner wall has completed its differentiation (Błaszkowski, 2012). Spore germination can occur upon full differentiation of the innermost wall (Morton, 2002). The germ tubes arise from the pre-germination structures associated with the innermost wall. The pre-germination structures are called germination orb

(seen in *Acaulospora* spp.), germination shield (seen in *Scutellospora*, *Racocetra*, *Pacispora* spp.) or germination structure (seen in *Ambispora appendicula*) (Błaszkowski, 2012).

2.6.3: Pre-germination structures

2.6.3.1: Germination orb

It is formed by a centrifugally rolled hypha which is hyaline in colour. It is an impermanent structure that decomposes with time (Błaszkowski, 2012).

2.6.3.2: Germination shield

These are formed by a coiled hypha and are generally elliptical, irregular plate-like, more or less flexible. They may be divided into 1-30 compartments which contain germ tube initial (Błaszkowski, 2012).

2.6.3.3: Germinal layer

It is a semi-flexible layer from which the germ tube emerges (Błaszkowski, 2012).

2.6.4: Sporocarp

Spores are formed in a highly ordered or loose arrangement around a hyphal plexus (Gerdemann and Trappe, 1974). The sporocarps may be surrounded by a loose or compact interwoven hyphal network called peridium.

2.6.5: Subtending hpha

It is the point of attachment from which the spore arose. It can be simple, recurved, constricted or swollen. The shape and the width of the hypha can vary within different genera and AM species.

2.7: Development of AM fungi

There are three growth phases in the AM fungal life cycle: asymbiotic, pre-symbiotic and symbiotic.

2.7.1: The asymbiotic phase

Multiple, successive rounds of spore germination and retraction of nuclei and cytoplasm occur in this phase of AM development (Parniske, 2008). Spores germinate and germ tube growth occurs for about 2-3 weeks without any association with roots or root exudates under appropriate water and temperature conditions. During this phase, the fungal growth is solely dependent on its triacylglyceride spore reserves (Garg and Chandel, 2010). The hyphal growth ceases after about 2-4 weeks and formation of septa takes place in the absence of a host root (Mosse, 1988).

2.7.2: The pre-symbiotic phase

The exploratory hyphal growth changes dramatically in the presence of signals derived from plant root exudates (strigolactones), inducing profuse hyphal branching, increased physiological activity and continued hyphal growth (Parniske, 2008). It takes one to several weeks for the establishment of host root contact by the fungal hyphae (Declerck et al., 1998). Once the fungus-plant root contact is established, changes in the fungal morphology and metabolism occur radically, marking the initiation of symbiotic phase (Besserer et al., 2006).

2.7.3: The symbiotic phase

On penetrating the root surface, AM fungi produce mycorrhiza (Myc) factors that have the ability to induce calcium oscillations in root epidermal cells and activate plant AM symbiosis-related genes (Kosuta et al., 2003, 2008). AM fungi form unique type of

appressoria called hyphopodia which develop from mature AM fungal hyphae (Bastmeyer et al., 2002). As a consequence of root contact, plant cells produce a pre-penetration apparatus (PPA). The PPA is a sub-cellular membrane structure across the vacuole of the host cell that determines the course of fungal growth through the plant cell. It is formed 4-5 hr after the formation of hyphopodia (Parniske, 2008). Within the PPA, a 'trans-cellular tunnel' is formed by the cytoskeletal microtubules and microfilaments, and dense endoplasmic reticulum cisternae that connect the plant cell nucleus with the site of appressorial contact allowing the fungal hypha to penetrate the host cell (Genre et al., 2005; Siciliano et al., 2007). Endoplasmic reticulum membranes present within the tunnel are responsible for the synthesis of the peri-fungal membrane (Parniske, 2008). However, the signals that stimulate the development of the PPA are still unknown (Genre et al., 2005). The fungal hypha that extends from the hyphopodium enters the PPA and guides the fungal growth towards the root cortex. The fungal hypha leaves the plant cell and enters the apoplast, wherein branching and lateral growth of the hypha occurs along the root axis (Parniske, 2008). Subsequently, these hyphae induce the development of PPA-like structures in inner root cortical cells (Genre et al., 2005), and on entering these cells they branch to form arbuscules. Arbuscule formation precedes vesicle formation. Vesicles, which act as storage organs of the fungus by accumulating lipids, are also formed within the apoplast. Sporulation occurs at the leading tip of individual fungal hyphae outside of the plant root (Parniske, 2008).

2.8: Arbuscules

A synchronized sub-cellular development of the host plant cell and the AM fungus results in the formation of arbuscules. Repeated branching of the fungal hyphae forms

the tree-shaped structure of arbuscules (Parniske, 2008). However, the arbuscule structure can vary depending on the fungal and host genotype (Smith and Read, 2008). The plant-derived peri-arbuscular membrane (PAM) excludes the fungus from the host cytoplasm. Signals and nutrients are exchanged across the symbiotic interface between the fungus and the plant comprising of the PAM, the fungal plasma membrane and the peri-arbuscular space that exists between these two membranes (Harrison, 2005). The transporter PT4 mediating the metabolic exchange at the plant and fungus interface is specifically localized to the PAM (Harrison et al., 2002). Arbuscules have a shorter lifespan *i.e.* as short as 8.5 days (Alexander et al., 1989). They undergo a growth phase until a certain maximum size is reached, after which they degrade or senescence is stimulated and the arbuscular hyphae get separated from the cytoplasm by septa formation. Subsequently they collapse over time and eventually disappear. The lifespan of arbuscules is mainly influenced by their ability to deliver nutrients especially phosphate (Javot et al., 2007).

There are two morphologically distinct types of AM fungi, characterized by intra-radical hyphal modifications within the root. The *Paris*-type, where the hyphal development is extensively intra-cellular in the form of hyphal coils formed within host root cortical cells, and the *Arum*-type, where the intra-radical hyphae spread inter-cellularly between the root cortical cells, penetrating cells only to form tree-like structures (Smith and Smith, 1997). The *Paris*-type AM colonization is a characteristic of plants growing in low-nutrient and high-stress environments, while the *Arum*-type colonization is associated with fast growing plant species (Brundrett and Kendrick, 1990). However, the presence of both the types of colonization has also been noted in some plants (Kubota et al., 2005).

2.9: Vesicles

They are terminal, globose to sub-globose structures which function as lipid storage bodies and fungal propagules/intra-radical spores (Dodd et al., 2000). They are formed by intercalary or terminal swellings of AM fungal hyphae in the inter- or intra-cellular areas of the root cortex (Javaid, 2009). The importance of vesicles as potential carbon sinks has been noted in many studies (Graham et al., 1997; Rouhier and Read, 1998; Staddon, 1998). Representatives of Glomeraceae and Acaulosporaceae form vesicles where as those belonging to Gigasporaceae do not form vesicles but instead form auxiliary cells on the extra-radical mycelium (Oehl et al., 2011).

2.10: Auxiliary cells

They are clusters of thin-walled cells formed on the extra-radical hyphae. Auxiliary cells of *Gigaspora* species have spiny surface where as those formed by *Scutellospora* species usually have knobby or smooth surface (Bentivenga and Morton, 1995; Morton, 1995). The auxiliary cells allow partitioning of nutrients and nuclei prior to spore formation (Boddington and Dodd, 1999).

2.11: Spores

They are multinucleate single cells produced as terminal swellings on the tip of sporogenous hyphae continuous with extra-radical hyphae (Koske, 1985). Sometimes, they also arise inside sporogenous hyphae (intercalary spores), inside roots and rhizosphere soil (Sieverding, 1987; Berch and Fortin, 1983). The number of spores produced depends on the AM species, plant species, host phenology, soil fertility and competitiveness among AM fungal species (Hayman, 1970; Giovannetti, 1985; Hetrick and Bloom, 1986; Gemma et al., 1989; Błaszkowski, 1993a).

2.12: AM fungal mycelium

The AM fungal mycelium is multinucleate and coenocytic (Dodd et al., 2000). The intra-radical mycelium undergoes constant development and re-organization within the root cells by forming arbuscules and vesicles (Dickson and Smith, 2001). The extra-radical mycelium spreads through the soil creating a network that subsequently associates with different plants (Puschel et al., 2007). The extra-radical mycelium connects the root systems of same or different plant species (Heap and Newman, 1980; Newman, 1988; Jasper et al., 1991). The extra-radical mycelium has a dynamic structure comprising of i) the runner hyphae which grow along the roots and are generally thick-walled and aseptate. Their primary function is nutrient acquisition and translocation (Harrison, 1999); ii) the thin-walled absorptive hyphae which branch dichotomously and colonize the rhizosphere; iii) the fertile hyphae which form new spores (Friese and Allen, 1991). The extra-radical mycelial network acts as an extension of the plant root system by improving nutrient uptake (Khan et al., 2000). It also improves soil structure and stability by secretion of glomalin (Miller and Jastrow, 1992; Wright and Upadhyaya, 1996; van der Heijden et al., 2006). The development of the intra-radical- and the extra-radical-mycelium is most likely related to the unique life strategies of the AM species from the different genera (Gazey et al., 1993; Brundrett et al., 1996; Boddington and Dodd, 1999; INVAM, 1999) and the production of their propagules (Boddington and Dodd, 1998, 1999; INVAM, 1999).

2.13: Coastal sand dune systems

Coastlands are highly organized natural dynamic systems undergoing continuous change due to geomorphological processes and varying climatic conditions. Coastal land is characterized by a stressful environment with low fertility, high salinity,

intermittent drought, variable temperatures, and an unstable sandy substrate (Yamato et al., 2012; Cui et al., 2016). Sand dunes are generally of two types *viz.*, an extremely dry interior desert of continental land masses such as Sahara in Africa or Victoria desert in Australia, and the coastal sand dunes that occur along Atlantic and Pacific coast of North America, along the Australian coast. In Asia, the coastal sand dunes occur in Japan, India and several other countries. In coastal sand dunes, the sand is the byproduct of weathered rocks from inland region eroded by rain and wind. The wave action and sea currents are responsible for shifting sand between the sea floor, beach and the dunes. In the sand dune habitats, the sand is coarse with low levels of inorganic nutrients (Desai and Untawale, 2002).

2.14: Coastal sand dune vegetation

Plant species found in coastlands are specifically adapted to the persisting extreme environmental conditions. Characteristic vegetation has adapted to temperate and tropical sand dunes. Members of Poaceae are dominant plant species in temperate dunes, while plant members of Asteraceae, Convolvulaceae, Fabaceae and Poaceae are dominant in tropical dunes (Sridhar, 2009). Usually, a transition in the occurrence of coastal plant species is found in dune ecosystem corresponding to the environmental gradient with distance from the sea, the vegetation closest to the seaside experiencing the most stressful conditions (Yamato et al., 2012). Sand dune vegetation is usually arranged into three main zones that are roughly parallel to the coastline. 1. The pioneer zone which is closest to the sea and extends landward in the area of the fore dune. Only specific pioneer plants such as *Spinifex littoreus* L. (Poaceae), *Ipomoea pes-caprae* (L.) R. Br. (Convolvulaceae) and few other herbaceous species that can withstand the harsh conditions colonize this zone that is exposed to salt spray, sand blast, strong winds and

flooding by the sea. These plants have specialized structures such as a waxy coating on stems and leaves, are prostrate, and have well developed and rapidly spreading root systems. The creeping stems or stolons can interconnect, so if one part is buried in shifting sand or is uprooted, another part continues to grow; and so serve to stabilize the sand, forming and building the dunes (https://www.ehp.qld.gov.au/coastal/ecology/beaches-dunes/coastal_dunes.html). 2. Plant species on the fore dunes or frontal sand dunes are more complex than those in the pioneer zone. Scrub or woodland plants occupy the fore dunes as there are more nutrients that support the growth of such plants. Plant species in this zone are generally semi-permanent windswept and include *Spermacoce stricta* L. f. (Rubiaceae), *Leucas aspera* (Willd.) Link (Lamiaceae), *Vitex negundo* L. (Lamiaceae), *Clerodendrum inerme* (L.) Gaertn. (Lamiaceae), *Casuarina equisetifolia* L. (Casuarinaceae), besides vines and few herbs. 3. The hind dune is characterized by the presence of more complex and well developed vegetation such as stunted trees, low shrubs and forest plants. Protected by the strong winds and salt spray experienced closer to the beach, this area is more protected making it easier for less hardy and specialized trees to grow and survive. Plants in this zone include *V. negundo* L. (Lamiaceae), *C. inerme* (L.) Gaertn. (Lamiaceae), *Anacardium occidentale* L. (Anacardiaceae), *Pandanus tectorius* Parkinson (Pandanaceae), *C. equisetifolia* L. (Casuarinaceae), *Cocos nucifera* L. (Arecaceae). Occurrence of these plants in the hind dunes results in the production of more humus and organic matter thus providing sufficient nutrients for the growth of more plants species. Eventually plant communities are established in this region, further contributing to the nutrients of the area (http://www.beachapedia.org/Vegetation;http://www.ozcoasts.gov.au/indicators/beach_dune.jsp; Desai, 1995).

2.15: Importance of coastal dune systems and its vegetation

Sand dunes serve as natural buffers, protecting the landward side from storm tides, waves and wind action. Stabilization of large, mobile dunes by the vegetation cover has been recognized as an effective means to decelerate the inland movement of sand (Woodhouse, 1982). The dune vegetation traps and holds windblown sand grains on the fore dunes. It contains many native plant species and is valued as a habitat of its own natural biodiversity. Loss of dune vegetation can trigger dune erosion wherein the exposed, dry sand particles are flown by high-velocity winds resulting in shifting of large volumes of sand, sometimes resulting in formation of large depressions in the dunes. These shifting sand particles can smother the surrounding vegetation and areas. Erosion of beaches and fore dunes may be a natural process and is often balanced by the supply of sand from the near shore continental shelf to the beaches by currents and waves. In some cases, sand from adjacent dunes may replenish beach systems during erosion periods. However anthropogenic activities can also induce dune erosion. Some of the human activities which can lead to erosion include grazing, fires, tracks and foot traffic resulting in loss of dune vegetation; urban development on fore dunes; clearance of dunes for agriculture, *etc.* ultimately result in sand dune degradation (http://www.ozcoasts.gov.au/indicators/beach_dune.jsp).

2.16: AM fungi and its benefits to sand dune ecosystems

AM fungi are widespread in coastal sand dune systems (Sturmer and Bellei, 1994). Coastal sand dunes favour the occurrence of AM fungi mainly because of low P content (Ranwell, 1972). Mycorrhizal diversity in sand dunes results in an increase in longevity of feeder roots and improvement in soil texture through increased aggregation of soil particles (Nasim, 2005). The AM fungal colonization of plant roots greatly increases

the uptake of phosphorous, nitrogen, calcium, potassium and zinc (Gupta et al., 2000). AM fungi provide plants with P that enables AM plants to grow better than non-mycorrhizal plants when P is limiting. An increase in yield or biomass of AM plants is often observed as compared to non-mycorrhizal plants (Mosse, 1972). Dune vegetation benefits greatly by AM association through improved establishment, greater biomass accumulation, faster colonization of bare areas, improved water relations, large increase in relative growth rate, leaf area, total biomass and increased seed output (Corkidi and Rincón, 1997b). Increased nutrient supply, salinity tolerance, reduced abiotic stresses and formation of wind-resistant soil aggregates are also some of the major benefits derived by the dune vegetation through AM fungal association (Gemma and Koske, 1989). Read (1989) showed that plant communities in successional sand dune chronosequences are governed by an interaction between biotic and physico-chemical properties of the sand. Not only does the composition of plant species change with seasons and age of the dune systems but also the association with soil microorganisms changes with succession because of an increase in organic matter, improved substrate stability and nutrient enrichment (Koske and Gemma, 1997). Most important function of mycorrhizal fungi at the ecosystem scale is their contribution to soil structure. Soil aggregation is also important in non-agricultural ecosystems, such as in the context with restoration of disturbed lands, erosion control, global change, or soil carbon storage (Niklaus et al., 2003). Many physical, chemical and biological factors (and their interactions) contribute to soil aggregation, yet among the biological aspects, AM fungi are of special significance. They create conditions contributing to formation of micro aggregates, and they chemically enmesh and stabilize micro-aggregates and smaller macro-aggregates into macro aggregate structures. Localized drying of soil, in close proximity to roots, promotes binding between root exudates and clay particles, directly

facilitating micro aggregate formation (Augé et al., 2004). Besides this, other functions of AM in sand dune ecosystems include increased resistance of plants to root pathogens and increased plant tolerance to salt and drought stress (Koske et al., 1975; Nelson, 1987; Newsham et al., 1995; Koske et al., 2004).

2.17: AM fungi in sand dunes and their association with dune vegetation

Coastal plant communities are faced by poorly formed soils with shifting sands and nutrient deficit environment. Sand dune vegetation is essential for the formation and preservation of sand dunes and protection of coastline. Dune vegetation is highly adapted to salt laden winds of the coast, and maintains the fore dunes by holding the sand in the dunes, trapping sand particles blown up from the beach, and aid in repairing the degraded dunes (Desai and Untawale, 2002). Coastal sand dunes face harsh environments, where AM fungi play an important ecological role in promoting growth, establishment and survival of plant species that colonize dunes (Dalpe, 1989; Tadych and Błaszkowski, 2000a).

Extra-radical hyphal network of AM fungi is involved in the transfer of nutrients from the soil nutrient deficiency zones formed around the plant roots. They play a vital role in building and maintaining the structure of sand dunes and in stabilization of dune vegetation. Jehne and Thompson (1981) reported considerable amount of hyphal connections of fungal mycelium in the top 20 cm of mobile sand in Cooloola (Queensland), Australia. These fungi bind loose sand grains into larger aggregates through secretion of hydrophobic 'sticky' glycoproteinaceous substance known as 'glomalin' which results in improved soil stability, binding, and water retention and hence limits sand dune loss or erosion (Bedini et al., 2009). Forster and Nicolson

(1981) reported that 1.5 % of the aggregate sand grains reach a diameter of 2 mm in Scotland dunes.

AM fungal association is common in sand dune plants. Stahl (1900) and Asai (1934) initially reported the occurrence of AM associations with roots of sand dune plants. Since then, several surveys have been carried out in temperate and sub-tropical regions (Lee and Koske, 1994); a few from tropical coast of Hawaiian Islands (Koske, 1988; Koske and Gemma, 1996), India (Mohankumar et al., 1988; Kulkarni et al., 1997; Visalakshi, 1997; Rodrigues and Jaiswal, 2001) and Singapore (Louis, 1990). AM fungi have also been reported from beaches in Australia (Koske, 1975; Jehne and Thompson, 1981; Brockhoff, 1985), as well as from maritime sand dunes of other countries (Nicolson and Johnston, 1979; Koske and Halvorson, 1981; Giovannetti and Nicolson, 1983; Bergen and Koske, 1984; Sylvia, 1986). About 65 AM fungal species have been reported from sand dune habitats around the globe, representing 28 % of the total AM species (Stürmer et al., 2010), including 32 new species (Błaszkowski and Czerniawska, 2011). Studies on AM fungal associations in sand dune plants in Australia, USA, India, and Europe indicate that dominant dune plants and pioneer grasses are normally associated with AM fungi. These fungi help in dune stabilization through successful establishment of plant communities by improving their nutrient status. Koske (1988) reported that among all other mycorrhizal types, the AM fungi lead in their benefit to plant species in sand dune ecosystems.

The most common AM fungal genera in coastal sand dune systems worldwide are *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora*. Variations in AM fungal spore densities per unit volume of soil have been reported which depend upon different factors such as season, host genotype and phenology, and environment, ranging from 1

to >300 100 g^{-1} soil (Maun, 2009). The greater the number of viable propagules, the more the chances of formation of symbiosis and utilization of its benefits by plants. *Scutellospora erythropa* in Bahamas, *Acaulospora scrobiculata* and *Gigaspora albida* in North America (Koske and Walker, 1984), *Glomus* spp. in Japanese dunes (Abe et al., 1994) formed the most dominant AM fungal species in dune systems. Koske (1987) reported 14 AM fungal species from the sandy soils of Wisconsin, with *Claroideoglomus etunicatum* being the most frequently isolated species. Lee and Koske (1994a) reported *Gigaspora* as the dominant AM genus in sand dunes of Atlantic coast of U.S. Kulkarni et al. (1997) recorded a total of 16 AM species in Mangalore coast of Karnataka with *Gigaspora ramisporophora*, *Glomus albidum*, *Rhizoglomus clarum* and *Racocetra gregaria* as dominant species. Rodrigues and Jaiswal (2001) recorded AM association in six plant species growing on sand dune vegetation of Goa and reported the presence of three AM fungal genera *viz.*, *Acaulospora*, *Glomus* and *Sclerocystis*. Stutz et al. (2000) observed that the taxonomic range of AM fungi was mostly limited to Glomeraceae and Acaulosporaceae at El Socorro, near Ensenada, Baja California. Sadhana (2015) reported the presence of 36 AM species belonging to the genera *Acaulospora*, *Diversispora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Rhizoglomus*, *Sclerocyctis* and *Septaglomus* from sand dune vegetation of Tamil Nadu. The author recorded *Acaulospora* and *Glomus* as the predominant genera in the study area. Jobim and Goto (2016) recorded AM association with 48 plants growing in maritime dunes from Brazil and reported that AM species belonging to the genera *Acaulospora* and *Gigaspora* as most frequently occurring species. Moradi et al. (2017) studied AM diversity from afforested dunes of Iran. They identified 16 AM species belonging to six genera, with *Funneliformis* and *Glomus* as the most frequently occurring genera.

The roots of dune plants are intimately associated with AM fungi (Koske and Gemma, 1997). Most plant species that colonize sand dunes are known to have AM symbioses with variable degrees of root colonization (Druva-Lusite and Ievinsh, 2010). However, on heterogeneous and unpredictable coastal ecosystems, AM colonization might be expected to be greater in the more stable habitats (Ievinsh, 2006). Giovannetti and Nicolson (1983) reported presence of *Funneliformis mosseae* and *Rhizoglomus fasiculatum* in Italian sand dunes. They observed that plant species of cosmopolitan families were found to be heavily colonized by AM fungi. Mobile to stable sand dunes of the Gulf of Mexico revealed AM colonization in 97 % of plant species (Corkidi and Rincon, 1997). El-Giahmi et al. (1976) studied AM fungi from coastal sandy soils of Libya, wherein most of the host plant species recorded colonization levels between 40-60 %. Giovannetti (1985) reported higher AM fungal root colonization in plant species belonging to families Asteraceae, Papilionaceae and Poaceae on the Italian dunes.

Koske (1975) reported higher spore density in older, more stabilized dunes than in younger dunes in Australia. Koske and Halvorson (1981) reported greatest AM fungal spore density in the rhizosphere of *Ammophila breviligulata* dominating the dune vegetation in Rhode Island. Bergen and Koske (1984) investigated occurrence of AM fungi from sand dunes of Capecod-Massachusetts. They recorded five AM species belonging to the genus *Gigaspora* in association with roots of *Ammophila breviligulata*, and *Gi. gigantea* was reported to be the dominant species. Sylvia (1986) reported spatial and temporal distribution of AM fungi associated with *Uniola paniculata* in fore dunes of Florida. The study reported that the spore densities in non-vegetated areas adjacent to vegetated dunes averaged less than 6 % of the spore densities found in the rhizosphere of sea oats. Beena et al. (2001b) reported occurrence of 30 AM species from 28 sand dune plant species belonging to 14 families from the

West Coast of India. *Ipomoea pes-caprae* and *Launaea sarmentosa* growing on sand dunes of West coast of Karnataka harboured 41 and 28 AM species respectively (Beena et al., 1997, 2000b). Ragupaty et al. (1998) reported occurrence of 14 AM species in rhizosphere of 31 plant species from sand dunes in Tamil Nadu. Khan (1971) studied and reported illustrations of 6 types of AM fungal spores from West Pakistan soils. Mohankumar et al. (1988) studied the distribution of AM fungi in the sandy beaches of Madras coast and reported the presence of *Entrophospora* and *Glomus* species. According to them, soil temperature and moisture status of the soil influenced the colonization of AM fungi in coastal soils.

Several edapho-climatic factors have been shown to affect spore germination, root colonization and efficiency of AM fungi. In the sand dunes, nutrient input is intermittent by salt spray or precipitation (Kellman and Roulet, 1990) and organic matter serves as a major energy resource (John et al., 1983). The main growth constraints faced by the dune vegetation are low availability of N, P, K, water and organic matter (Maun, 1994). According to van der Valk (1974b) calcium (Ca) and magnesium (Mg) are usually adequate for plant growth, while N, P and K are limiting in dune systems. AM fungal activity in dune systems mainly depends upon accessibility of organic matter. Preferential association of AM fungi is observed with decaying organic matter (John et al., 1983). High organic matter resulted in increased growth of AM fungi in soil (Joner and Jakobson, 1995; Sridhar, 2006). Significant difference was seen in the edaphic factors or determinants such as moisture, pH, P, Na, K and N between naturally vegetated and non-vegetated dunes of the West Coast of India (Beena et al., 1997; Kulkarni et al., 1997; Beena et al., 2000b, 2000c). Organic matter supplies P for acid and alkaline phosphatases of AM fungi. It is observed that alkaline phosphatase activity of AM fungi decreases in soil devoid of organic matter

(Sridhar, 2009). Uptake of Ca by AM fungi plays an important role in P and water uptake by plants (Pai et al., 1994). Enhancing P nutrition is believed to be the major benefit to the host plant in an AM fungal association. In coastal sand dunes, the level of available P to the plants is typically very low. The hardship imposed on plants by such low P levels is compounded by the low mobility of P in the soil. Available P is removed from near the absorbing surface of roots creating a narrow depletion zone. The AM fungal extra-radical hyphae are able to cross this zone and provide the plant with P (Koske, 1984). AM fungi also play a significant role in N acquisition by plants (Hodge et al., 2010; Smith et al., 2011). However, plant species differ in their mycorrhizal response in complex ways across gradients of N and P availability (Hoeksema et al., 2010). It is reported that AM abundance decreases with higher P (Richardson et al., 2011) and N concentration (Treseder, 2004) in soil, while soil pH affects mostly fungal community composition (Dumbrell et al., 2010). According to Sieverding (1991) one of the most critical soil physico-chemical factor appears to be pH, and many fungi show a wide tolerance to distinct pH ranges, which is reflected in the occurrence of species rather than genera (Koske, 1987). Whilst species from different genera can be found in soils covering a broad pH range, others like *F. mosseae* have only been reported from soils with pH value greater than 5.5 (Sieverding, 1991). Temperature also appeared to be the main factor determining the structure and distribution of AM fungal communities along the latitudinal temperature gradient (Koske, 1987). Although variations in the behaviour of AM fungal species are known to exist with respect to other soil factors (heavy metals, texture, moisture, nutrient levels, salinity, *etc.*), the significance of these for AM diversity in native or natural habitats is still poorly understood. The distribution of AM fungal species appears to be more closely related to host plant, soil structure, and environmental conditions than to

competition by other AM species (http://mycorrhizae.ifas.ufl.edu/Files/THESIS.pdf). However, during primary dune succession, changes in AM community composition are more related to soil factors rather than plant species (Sikes et al., 2012, 2014).

Sturmer and Bellei (1994) studied composition and seasonal variation of AM spore populations in dune soils on the Island of Santa Catarina, Brazil. They observed that spore numbers of *F. constrictum*, *C. etunicatum* and *Acaulospora* species were highest in winter, whereas that of *Gi. albida* peaked in spring season. Beena et al. (1997) reported that AM root colonization peaked during post monsoon, while AM fungal species richness and spore diversity were highest during monsoon. Ramos-Zapata et al. (2011) reported higher spore density during the rainy season when compared with dry season. A two year seasonal study by Beena et al. (2000b) on the coastal sand dunes of West coast of India revealed that the percent AM root colonization was least during monsoon and highest during post-monsoon, but the mean spore density was least during post-monsoon and highest during summer season. *Glomus* was the most common genera with mean species richness being highest in *I. pes-caprae* (Beena et al., 2001).

Burrows and Pfleger (2002) reported that plant cover can be predictive of spore volume or number. Nicolson (1960) examined the level of AM colonization in dune grass in more complex dune system and found a dramatic increase in the AM activity from the fore dunes to the recently fixed dunes. According to Mayr (1965) disturbed habitats result in reduced number of AM fungal propagules because of the reduction in host plants. Disturbance of soil leads to elimination or reduction in number of viable propagules of AM fungi (Reeves et al., 1979). Sylvia and Will (1988) reported changes in AM populations and other soil microorganisms in replenished sand planted with

Uniola paniculata and *Panicum* species. They observed a shift in dominant AM fungi found in the planted zone with respect to those in established dunes. Beena et al. (2000b) reported that the vegetation cover, AM fungal colonization, species richness and diversity were greater in moderately disturbed dunes than in severely disturbed dunes of West Coast of India. Cordoba et al. (2001) reported that the AM fungal community was dominated by distinct families along a gradient of dune stabilization. They observed that Gigasporaceae dominated in the embryonic dunes, while Acaulosporaceae and Glomeraceae dominated in the foredunes and fixed dunes. da Silva et al. (2015) studied the community composition of AM fungi on a vegetation gradient in coastal dune areas of northeastern Brazil. They reported that AM spore density was higher in the arboreal dunes as compared to the shrubby and herbaceous dunes, whereas the highest numbers of infective propagules were observed in the herbaceous dunes, followed by the shrubby and arboreal dunes. Their results indicated that the areas closest to the sea had greater AM diversity compared to the later successional dunes in farer distance from the sea. Generally, the AM fungal diversity appears to be greater in more stabilized dunes than in younger or disturbed dunes (Giovannetti and Nicolson, 1983). Currently high throughput sequencing technologies have enabled the survey and examination of AM fungal communities at different spatial scales (Lekberg et al., 2012; Öpik et al., 2013; Wehner et al., 2014; Davison et al., 2015). These surveys also enable the examination of AM community existing in host plant roots (Hazard et al., 2013) which was previously problematic due to the difficulties in characterization of AM species through intra-radical structures produced in the roots only by microscopic examinations (Alkan et al., 2006).

AM associations can be potential determinants of plant diversity in ecosystems. Since plant species differ in their response to AM fungi in the soil, the presence or absence of

AM has been linked to the composition of plant communities that grow in the dunes (Francis and Read, 1995). AM fungi can probably modify the structure and functioning of a plant community in a complex and unpredictable way (Read, 1990). Any shift in the AM fungal population can result in survival, competition and floristic diversity of plant community composition, causing changes in the ecology of natural habitats (Miller and Allen, 1992). Therefore, knowledge of the different factors influencing the population biology of AM fungi is essential for their utilization in conservation of the environment (Allen, 1991), biotechnology (Mulongoy et al., 1992) or in sustainable agriculture (Bethlenfalvay and Linderman, 1992).

2.18: *In vitro* culture of AM fungi

The conventional method used to study the life cycle of AM fungi *in situ* is to associate them with root organ culture (ROC) (Fortin et al., 2002). This technique has greatly influenced our understanding on various aspects of AM symbiosis by allowing non-destructive observations throughout the fungal life cycle. Although the host plant is replaced by Ri T-DNA transformed roots, the fungus is able to colonize and sporulate. The development of spores, morphologically and structurally similar to those produced in pot cultures, and 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. ROC technique has proved to be successful for cultivation and mass inoculum production of AM fungi (Rodrigues and Rodrigues, 2013).

2.19: System description

Since Mosse and Hepper (1975) first established cultures of AM fungi using excised roots, tremendous improvements have been made in the use of Ri T-DNA transformed roots (Mugnier and Mosse, 1987), in the manipulation of the culture media to induce sporulation (Bécard and Piché, 1992), and in the development of a bi-compartment system that allowed the production of root-free AM fungal mycelium and spores (St-Arnaud et al., 1996). This improvement has enabled studies in sporulation dynamics (Declerck et al., 2001), spore ontogeny (Pawlowska et al., 1999), stimulation of germination and hyphal growth by CO_2 (Bécard and Piché, 1989a), regulation of hyphal growth and branching by root exudates (Nagahashi et al., 1996b), reactions to compounds from the host and non-host roots (Schreiner and Koide, 1993), uptake, transfer and metabolic fate of ^{13}C-labeled metabolites (Pfeffer and Shachar-Hill, 1996), response of AM fungi to cell wall-associated phenolics (Douds et al., 1996) and flavonoids (Morandi et al., 1992), lipid metabolism (Bago et al., 2002), transport of mineral nutrients to roots (Dupré de Boulois et al., 2005) and isolation of microbe free AM fungal mycelium and spores for molecular analysis (Pawlowska and Taylor, 2004).

Using the split-plate method, Douds (2002) demonstrated that AM fungi continue to sporulate after medium from the distal compartment has been partially replaced, and glucose provided to the proximal compartment, resulting in repeated harvests from the same Petri plate culture. Different production systems have been derived from the basic ROC in Petri plates. Tiwari and Adholeya (2003) and Adholeya et al. (2005) cultured root organs and AM fungi in small containers, by which large-scale production was obtained. Large-scale cultivation of AM fungi has also been performed in an airlift bioreactor (Jolicoeur et al., 1999), in a mist bioreactor with perlite as the substrate (Jolicoeur, 1998), and in a bioreactor containing solid medium (Fortin et al., 1996). In

the patented container-based hydroponic culture system of Wang (2003), the root organs and AM fungus were periodically exposed to a liquid culture medium. Gadkar et al. (2006) further developed a container in which a Petri plate containing ROC was used to initiate fungal proliferation in a separate compartment filled with sterile expanded clay balls. In parallel to the systems based on excised roots, Voets et al. (2005) and Dupré de Boulois et al. (2006) developed two *in vitro* culture systems based on autotrophic plants. In the system developed by Voets et al. (2005), the shoot developed outside the Petri plate while the roots and AM fungus were associated inside the Petri plates that were filled with a suitable gelled medium, resulting in more than 12,000 spores per Petri plate after 22 weeks of culturing. In another system (Dupré de Boulois et al., 2006), the shoot developed in a sterile tube vertically connected to the top of a Petri plate in which the AM fungus and roots developed. The cultures were then placed in growth chambers to provide controlled environmental conditions adequate for plant growth and ~1,600 spores were obtained in a period of 12 weeks in the root compartment of a bi-compartmental Petri plate. A derived plant production system has recently been detailed in a patent proposal (Declerck et al., 2009) where each pre-inoculated produced plant (Voets et al., 2009) is individually introduced into a sterile growth tube. A nutrient solution circulates in this closed system flowing onto the mycorrhizal roots. These studies have thus greatly improved our earlier understanding of AM fungi propagation processes and life cycles (Strullu et al., 1997). Other potential uses for this system are the production of pure, concentrated inoculum and sterile fungal tissue for genetic and physiological studies.

Advances in the development of *in vitro* systems have opened new prospects in the study of the AM symbiosis. Research areas such as fungal colony architecture, physiology, biochemistry, cytology and molecular biology, traditionally affected by the

intrinsic problems presented by culturing AM in soil, have especially benefited from this revolution (Declerck et al., 2005).

2.20: AM fungal species cultivated on ROC

Up till now, several Glomeraceae and a few Gigasporaceae genera have been successfully cultivated *in vitro* on ROC **(Table 2.2)** and are maintained in international culture collections (Declerck and Dalpé, 2001).

2.21: Culture media

Two media frequently used to culture AM fungi on ROC are the minimal (M) medium (Bécard and Fortin, 1988) and the modified Strullu Romand (MSR) medium (Strullu and Romand, 1986, modified by Declerck et al., 1998). Both these media contain micro- and macro-nutrients as well as vitamins and sucrose (Cranenbrouck et al., 2005). Both media are solidified with a gelling agent such as PhytaGel and GelGro. The successful development of fungal isolates into sustainable culture has been achieved using minimal M medium (Bécard and Fortin, 1988). While this medium has been widely used for the study of AM fungi *in vitro*, it appears unsuitable for the culture of other AM fungal species (Douds, 1997). Manipulation of medium composition and pH to suit new fungal isolates could lead to a better understanding of factors affecting the complex biology underlying the symbiosis. MSR medium lacking sucrose promoted higher germination rates in *Rhizoglomus irregulare* (D'Souza et al., 2013). ROC systems in bioreactors (Jolicoeur et al., 1999) and containers (Gadkar et al., 2006) were performed with liquid M medium. In the compartmented culture system (Gadkar et al., 2006), glucose-soaked cotton rolls were supplied to the ROC and AM fungus, while the compartment containing expanded clay was filled with a layer of

Table 2.2: Arbuscular mycorrhizal (AM) species cultivated on Root Organ Culture (ROC).

AM species	Reference
Acaulospora rehmii Sieverd. and Toro	Dalpé and Declerck, 2002
Gigaspora rosea Nicolson & Schenck	Bago et al., 1998b
Gi. margarita Becker & Hall	Miller-Wideman and Watrud, 1984; Diop et al., 1992; Gadkar and Adholeya, 2000
Gi. gigantea (Nicolson & Gerd.) Gerd. & Trappe	Gadkar et al., 1997
Gi. decipiens Hall & Abbott	Fernández Bidondo et al., 2012
Claroideoglomus etunicatum (Becker & Gerd.) Walker & Schüßler	Schreiner and Koide, 1993
Glomus versiforme (Karst.) Berch	Diop et al., 1994a; Declerck et al., 1996a
G. deserticola Trappe, Bloss & Menge	Mathur and Vyas, 1995
G. fistulosum Skou & Jakobsen	Nuutila et al., 1995; Gryndler et al., 1998
Rhizoglomus clarum (Nicolson & Schenck) Sieverd., Silva & Oehl	de Souza and Berbara, 1999; Rodrigues and Rodrigues, 2012
R. intraradices (Schenck & Smith) Sieverd., Silva & Oehl	Chabot et al., 1992a; St-Arnaud et al., 1996
R. fasciculatum (Thaxter) Sieverd., Silva & Oehl	Declerck et al., 1998
R. proliferum (Dalpé & Declerck) Sieverd., Silva & Oehl	Declerck et al., 2000
Funneliformis caledonium (Nicolson & Gerd.) Walker & Schüßler	Hepper, 1981; Karandashov et al., 2000
F. geosporum (Nicolson & Gerd.) Walker & Schüßler	Declerck et al., 1998
F. mosseae (Nicolson & Gerd.) Walker & Schüßler	Douds, 1997
Sclerocystis sinuosa Gerd. & Bakshi	Bi et al., 2004

liquid M-medium without sugars and vitamins. Similar to the *in vivo* hydroponic culture systems, sufficient aeration of the liquid medium is needed in the *in vitro* solution culture techniques (Jolicoeur et al., 1999). Whole-plant *in vitro* culture systems were conducted on the MSR medium lacking sucrose and vitamins (Dupré de Boulois et al., 2006) that were similarly solidified with either Phytagel or GelGro. The addition of vitamins and sucrose is not required in whole-plant culture systems as the autotrophic plant provides sugars obtained by photosynthesis and metabolizes the vitamins required for plant growth.

2.22: AM host root

Ri T-DNA transformed roots have been used effectively in studying the interaction between various plant hosts and AM fungi. ROC was first developed by White and coworkers (White, 1943; Butcher, 1980) who used excised roots on synthetic media supplemented with vitamins and sucrose. However, extensive root growth on medium, characterized by the formation of numerous lower order branches, has been obtained in relatively few plant species. The formation of lower order roots is essential for increase in root biomass and the establishment of continuous cultures. ROC was first performed successfully by Mosse and Hepper (1975) using an *in vitro* system based on a dual culture of spores and excised roots of *Trifolium* (clover) species. Mugnier and Mosse (1987) obtained similar results using *Daucus carota* L. (carrot) roots genetically transformed by *Agrobacterium rhizogenes* Conn. Later, Strullu and Romand (1986, 1987) showed that it was also possible to re-establish mycorrhiza on excised roots of *Fragaria* × *Ananassa* Duchesne (strawberry), *Allium cepa* L. (onion), and *Solanum lycopersicum* L. (tomato), using the intra-radical phase (vesicles or entire mycorrhizal root pieces) of several *Glomus* species as inoculum. The *in vitro* large scale production

of *R. intraradices* spores was initially attempted on ROC (Declerck et al., 2001) and later extended to plant systems (Voets et al., 2009). This system of dual culture allowed abundant production of spores of *Gi. margarita* (Diop et al., 1992). A natural genetic transformation of plants by the ubiquitous soil bacterium *A. rhizogenes* (Riker et al., 1930) induces a condition known as 'hairy roots'. This stable transformation (Tepfer, 1989) produces Ri T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. A modified hormonal balance encourages vigour and allows profuse growth on artificial media (Tepfer, 1989). Carrot and *Convolvulus sepium* L. (bindweed) were among the earliest species to be transformed using *A. rhizogenes* (Tepfer and Tempé, 1981). These Ri T-DNA transformed roots have since served in a wide range of fundamental and applied studies on AM symbiosis. ROC is commonly initiated on carrot root tissue and its use has permitted an increase in spore production of *F. mosseae* (Mugnier and Mosse, 1987). In recent years however, different excised roots, notably of *Cichorium intybus* L. (chicory) and *Medicago truncatula* Gaertn. (barrel medic), have been successfully used to culture AM fungi (Boisson-Dernier et al., 2001; Fontaine et al., 2004). It has been revealed that a change of root clone impacts AM fungal spore production (Tiwari and Adholeya, 2003). Voets et al. (2005) used *Solanum tuberosum* L. (potato) and obtained production of ~12,000 spores in 12 weeks of cultivation. Fernández et al. (2009) carried out *in vitro* monoxenic symbiosis between *R. intraradices* and transformed *Glycine max* (L.) Merr. (soybean) roots (TSRs) and showed that TSR cultures were able to support the growth and characteristic development of the fungus. Pratap Chandran et al. (2011) co-cultivated transformed roots *Canavalia* species with *G. microcarpum* in Petri plate and observed 60 % AM colonization on the 20th day. Other hosts, such as tissue cultured

banana (*Musa acuminata* Colla) were found suitable for association (Koffi et al., 2009) but were less effective for large scale production of spores.

2.23: AM Fungal Inocula

Many species and strains of AM fungi have been cultured in the ROC system. However, only a few species are fast growers and colonizers, able to produce thousands of *in vitro* propagules in a few months and thus have potential in large scale production. In most cases, two types of fungal inocula *viz.*, extra-radical spores or propagules from the intra-radical phase (mycorrhizal root fragments and isolated vesicles) of the fungal ontogeny can be used to initiate monoxenic cultures. Cultures of AM fungal species that do not produce vesicles (*Scutellospora* and *Gigaspora* species) are systematically produced using spores, which are usually large and germinate vigorously. Recently, sporocarps of *F. mosseae* have also been used in an attempt to establish *in vitro* cultures (Budi et al., 1999). The intra-radical forms of AM fungi have been less commonly used as starter material despite being a potentially good source of inoculum. Strullu and Romand (1987) demonstrated that intra-radical vesicles and hyphae within root pieces or extracted from roots by enzymatic maceration were able to regenerate vegetative mycelium in *Glomus* species. When associated with tomato roots, the mycelium formed new and typical mycorrhizae. This system was successfully used by Diop et al. (1994a) for dual axenic culture of mycorrhizal root-segments containing *G. versiforme* or *R. intraradices* associated with non-transformed tomato roots. The cultivation system was further improved by using transformed carrot roots as host with several *Glomus* species (Diop, 1995). Diop et al. (1994a) obtained approximately 2000 spores per Petri dish over a period of three months using a dual culture of leek (*Allium* sp.) root-segments colonized by *G. versiforme* associated with tomato roots. They further demonstrated

that the fungus both in tomato root segments and as spores, produced in sterile conditions, germinated well and was able to complete its life cycle in association with isolated tomato roots. Inoculation of *Faidherbia albida* (Delile) A. Chev. plantlets with the newly produced spores and mycorrhizal root-segments were also successfully demonstrated (Diop et al., 1994a). Species from *R. intraradices* clade/species complex are found among the most productive so far. This species *sensu lato* is the most frequently cultured AM fungus *in vitro*.

Strullu and Plenchette (1990a, b) demonstrated the ability of entrapped, disinfected mycorrhizal root fragments to form new mycorrhizae, even after storage for 1 month at 4°C. Intra-radical vesicles separated from roots and encapsulated were also shown to retain their inoculum potential (Plenchette and Strullu, 2003) and hence represent another practical source of inoculum. In a study by Declerck et al. (1996a), *in vitro* produced spores of *G. versiforme* were entrapped in alginate beads and their inoculum potentials were evaluated by a biological assay. The results showed that the encapsulated spores were able to germinate and the regenerated mycelium retained its ability to colonize roots under controlled conditions. Declerck and Angelo-van Coppenolle (2000) developed a cryopreservation technique based on the entrapment of monoxenically produced spores of *R. intraradices* in alginate beads. These studies indicate the feasibility of encapsulation of *in vitro* produced spores and therefore represent a new kind of high quality inoculum, free of pathogens.

For all AM propagules, appropriate selection and efficiency of sterilization process are keys to the success of axenic or monoxenic AM fungal cultures. Isolated spores are often surface sterilized using the two-step procedure of Mertz et al. (1979) as modified by Bécard and Fortin (1988). AM sheared inocula are surface sterilized according to

Diop et al. (1994a, b) method. Vesicles are then easily isolated by enzymatic digestion of the heavily colonized roots. Surface sterilization involves baths in chloramine T (2 %) solution with traces of a surfactant (Tween 20/80) and antibiotics, such as streptomycin or gentamycin. To maintain spore dormancy, all steps from spore isolation to rinsing should be done on ice. If spores are not to be used immediately, they should be stored at 4°C, either in distilled water, on water agar, or on 0.1 % $MgSO_4 \cdot 7H_2O$ solidified with 0.4 % gellan gum. To limit the risk of contamination by bacteria or fungi that were not eliminated during the sterilization process, spore number should be limited in each Petri plate.

Generally AM fungal spores do not need specific conditions or the presence of a host root to germinate. However, root exudates and 2 % CO_2 can stimulate germination and/or post germination hyphal growth (Buée, 2000). Recalcitrant spores can be placed alongside a growing root. If spores fail to germinate within 20 days, either the sterilization treatment is possibly strong or the spores are immature, dormant, or dead. It is well known that spores of some AM fungal species require cold stratification (4°C) prior to germination (Smith and Read, 2008). This requirement can vary within a genus. *Gi. gigantea* (Koske, 1981) and *Gi. margarita* require cold treatment, whereas *Gi. rosea* did not (Bécard and Fortin, 1988). The cold treatment (14-21 days) is best applied prior to spore isolation, when the spores are still attached to the extra-radical mycelium (Fortin et al., 2002).

2.24: Continuous Cultures

The first continuous culture was achieved by Strullu and Romand (1986) and is now commonly used for a wide range of *Glomus* species (Declerck et al., 1998). Continuous culture is obtained by transferring mycorrhizal roots to fresh medium either with or

without spores (St-Arnaud et al., 1996; Declerck et al., 1996a). Following this transfer, the pre-existing root-fungus association continues to proliferate. While using older mycorrhizal roots, it is preferable to transfer them to a Petri plate containing an actively growing root (Declerck et al., 1998). In the method by St-Arnaud et al. (1996), apical segments of actively growing mycorrhizal roots with or without extra-radical mycelium that are supporting the spores are transferred to a fresh medium. The root and associated fungus continues to grow across successive transfers onto fresh medium. This procedure requires the use of young, actively growing cultures, to allow continuous growth of the host root. The method is effective for *Glomus* species having a well-developed intra-radical phase, such as *R. intraradices*. For AM fungal species that do not produce intra-radical vesicles (*Gigaspora* and *Scutellospora* species), direct sub-culturing is possible but it is more difficult to achieve (Fortin et al., 2002). Alternatively, with older cultures, *in vitro* produced spores can be used to inoculate new roots (Bécard and Fortin, 1988).

Diop (1995) established a bank of germplasm of AM fungi monoxenically cultivated in association with isolated tomato or transformed carrot roots. The propagules produced (spores, hyphae, colonized roots) germinated and re-colonized new plants efficiently. Encapsulation stabilizes biological properties of mycorrhizal roots and the isolated vesicles or spores (Declerck et al., 1996b). This also preserves infectivity of AM propagules under *in vitro* or *in vivo* assays.

2.25: Fungal Morphological Features in Root Organ Culture System

The use of ROC of AM enables the aseptic production of spores of various AM fungal species. Germination of the AM fungal propagules usually proceeds from the pushing of the inner spore wall through the lumen of the subtending hyphae (de Souza and

Berbara, 1999), directly through the spore wall (Tommerup and Kidby, 1980), or the subtending hyphal wall (Giovannetti et al., 1991). Spore germination gives rise either to straight, thick-walled hyphae (de Souza and Berbara, 1999) or to stunted hyphae, depending on the physiological status of spore (Juge et al., 2002). The germination of isolated intra-radical vesicles was clearly demonstrated by Diop et al. (1994a, b). Germination occurred through the lumen of the subtending hyphal attachment (Declerck et al. 1998), the germ tubes generating runner and ramified hyphae similar to those of AM fungi spores. To date, no systematic investigation has been conducted on factors influencing vesicle germination. Among other fungal structures capable of re-growth are hyphae from the peridium of *F. mosseae* sporocarps which have the capability to elongate and differentiate into vesicle-like structures (Budi et al., 1999). Furthermore, the "germination" of colonized root segments is currently used to replicate AM fungi monoxenic cultures (Strullu et al., 1991). The root vesicles and eventually intra-radical spores are certainly the fungal propagules involved in root segment "germination" as colonized root segments, where vesicles and spores were absent, remained unsuccessful in propagation. Germ tube growth is dependent on the availability of spore reserves (Sancholle et al., 2001), and the protoplasm contains all the organelles required to ensure development (Meier and Charvat, 1992). This consists of a straight-growing hypha (runner hyphae, RH) exploring the media by successive branching into thinner-diameter filaments (Declerck et al., 2000). In the case of no hyphal root contact or host signal detection, germ tube growth stops within a few days (Bécard and Piché, 1989b). The protoplasm shrinks back from the hyphal apex, and is sequestered from the empty hyphae by repeated septation (Logi et al., 1998). These germination attempts resemble a well-orchestrated survival scenario, providing repeated chances for the fungus to establish symbiosis. Spore germination does not

generally require the presence of a host root (the non-symbiotic stage). However, for further growth and development, the AM fungus becomes dependent upon the presence of, but not necessarily physical contact with an adequate host (Giovannetti et al., 1996). Using *Gi. rosea*, it was shown that this activated physiological stage (the pre-symbiotic stage) requires the simultaneous presence of root exudates and CO_2 (Poulin et al., 1993). Bécard and Piché (1989a) suggested that *Gi. rosea* was capable of fixing CO_2 as a mineral source of carbon. *In vitro* labeling with $^{13}CO_2$ and NMR spectroscopic analysis has confirmed that substantial dark fixation of CO_2 occurs in *R. intraradices* during spore germination (Bago et al., 1999b).

In monoxenic cultures, root colonization levels vary according to the host plant species and fungal isolates (Elsen et al., 2003). Acidification of the media directly influences AM fungal development. The pH 5.5 value of standard monoxenic culture systems might limit the growth of some isolates, but an increase in pH of the nutritive media may alter the solubility and balance of the media components. Buffered media may counteract such imbalances. Most monoxenic culture plants support the *Arum*-type colonization (Glorian, 2002). A contrary situation occurs with carrot root culture, an Apiaceae (Umbelliferae) recognized as supporting both *Paris*- and *Arum*-type colonization (Smith and Smith, 1997), whereby *Arum*-type colonization is differentiated more. Only one *F. caledonium* isolate has differentiated *Paris*-type colonization with a carrot root culture (Karandashov et al., 2000), and one *C. etunicatum* isolate had mixed types, differentiating hyphal coils in the first layer of cortical cells (Pawlowska et al., 1999). *Paris* and *Arum* morphotypes were long considered to be determined by the plant genome (Smith and Smith, 1997), but the typical *Paris* anatomical type observed in carrot root culture colonized by *F. caledonium* emphasizes the impact of the fungal genome on the regulation of fungal

morphology (Cavagnaro et al., 2001). Budi et al. (1999) reported that the hyphae from the peridium of *F. mosseae* sporocarps have the capability to elongate and differentiate into vesicle-like structures (VLS). When differentiated, VLS occurred within 2-8 days after root contact and their size ranged between 20 and 100 μm, depending on the species (Declerck et al., 2005). VLS are small, hyaline thin-walled swellings resembling miniature spores (Strullu and Romand, 1987). Hypotheses about their role range from a survival process during the pre-symbiotic stage to an aborted sporulation tentative (Declerck et al., 2005).

The basic structure of the mycelium is composed of large, straight growing relatively un-branched thick walled runner hyphae (RH) (Friese and Allen, 1991), small-diameter thin walled branched hyphae called arbuscule-like structures (ALS) (Bago et al., 1998a) or fine branching (FB) (Juge et al., 2009), and spores. Runner hyphae are similar to pre-symbiotic hyphae in their capacity to extend rapidly, to colonize the substrates, and to establish root contact. Microscopical cellular and sub-cellular observations allow detection of protoplasmic streaming, nuclei migration and organelle morphology (Bago et al., 2001). Hyphae are either single-walled, as with *G. versiforme* (Garriock et al., 1989), or double-walled as found through ultra-structure work on *R. fasciculatum* (Bonfante-Fasolo and Grippiolo, 1982). The abundance of runner and branched hyphae determines the mycelium architecture. Once a successful symbiosis is established, numerous ALS are differentiated along hyphae (Bago et al., 1998a). Ultra-structural investigations revealed that ALS (renamed as branched absorbing structures or BAS; Bago et al., 1998d) are very similar to intra-radical arbuscules and, like arbuscules, they are sites of intense metabolic activity. Arbuscules and BAS are also similar in terms of their gross morphology (thinner diameter with increased dichotomous branching). The extent to which these structures are functionally comparable remains to be elucidated.

However, prolific branching of the fungus to form BAS results into an important increase in surface area producing a structure better adapted for nutrient uptake. It has also been shown that increased acidification of the medium coincides with a higher production of spore-associated BAS. This change in pH could be a direct consequence of a greater phosphate uptake, to provide storage products for the spores (Bago et al., 1998b, 1998c). It also appears that inorganic nitrogen and phosphate absorption by extra-radical mycelium is closely correlated with BAS development (Bago et al., 1998d). BAS may adopt variable morphologies, the most striking being the large and stunted ramified structures of *F. caledonium* (Karandashov et al., 1999).

Spore differentiation occurs either apically or intercalary along lateral branches of RH, often in association with BAS (Bago et al., 1998d). The outer evanescent spore wall then originates from the hyphal wall. The spore apical hyphae, even though collapsed, remain attached to the spore during most of the maturation process. Intra-radical spores have sometimes been observed in monoxenic cultures (de Souza and Barbara, 1999). Spore production differs considerably between species and between isolates of a single species, and seems to be related to spore size. With the small to medium size spore species *R. proliferum* and *R. intraradices*, an average of 7,800 and 8,200 spores were differentiated in mono-compartment (Declerck et al., 2001) and bi-compartment growth systems (St-Arnaud et al., 1996) respectively. Most *Glomus* species exhibit an asynchronous mode of sporulation, *i.e.* with a lag, log and plateau phase (Declerck et al., 1996a, 2001).

Research into major differences between AM fungal cultures has dealt primarily with mycelium architecture, hyphal network density, pattern of ramification, spore abundance, and positioning and clustering of spores. Large-spore species usually

exhibit a less dense mycelium and fewer anastomoses. Stunted BAS, together with typical *Paris*-type root colonization, characterize *F. caledonium* isolates (Karandashov et al., 2000). Spore maturation of monoxenic cultured AM fungi follow similar ontogeny steps as those in pot-culture. Differences reside essentially in the clean, contaminant-free quality of monoxenic cultured spores, with abundant fungal material available at precise age and physiological stages. The comparison between monoxenically cultured species shows ready segregation between large- and small-spore species in terms of apical mode of development, single spore differentiation, and low sporulation levels. By contrast, smaller spore species present a variable growth pattern, mainly with intercalary sympodial spore growth, clustered spores, and high sporulation levels. Spore wall morphology of monoxenically differentiated spores does not differ fundamentally from field collected ones, apart from the lower mean spore diameter measured for some AM fungal isolates (Pawlowska et al., 1999). With monoxenic cultures, all elements of spore wall architecture remain observable throughout maturation, including the evanescent outer wall, often absent in soil propagated AM fungal spores, due to abrasion and/or digestion by soil microorganisms.

2.26: Fundamental and Practical Studies

Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspects of AM symbiosis, complementing experimental approaches. The compartmentalized Petri dish system (St-Arnaud et al., 1996) is particularly suitable for the study of nutrient uptake and translocation in AM fungi under strictly controlled conditions. It also allows the differentiation between intra-radical and extra-radical fungal metabolism (Bago et al., 2000). The compartmentalized system has been used for example, by Joner et al. (2000) to study P transport by the

extra-radical hyphae of *R. intraradices*, and in N nutrition, the compartmentalized *in vitro* system was used to show that the extra-radical hyphae of *R. intraradices* facilitate nitrate (Bago et al., 1996) and ammonium absorption (Villegas, 2001).

The first report of interactions between soil microbes and AM fungi under aseptic *in vitro* conditions was by Mosse (1962), who observed that root colonization could not be established without adding either a suspension of *Pseudomonas* species or various types of bacterial filtrates. Following this pioneering work, a wide range of soil bacteria and fungi has been shown to enhance *in vitro* germination of spores and hyphal growth of *F. mosseae* without direct contact between the organisms. These results suggest involvement of volatile (*e.g.* CO_2) or highly diffusible substances (Azcón, 1989). Simultaneously, spore-associated bacteria have been identified from the genera *Pseudomonas* and *Corynebacterium* (Mayo et al., 1986), and that cell-free fractions from rhizosphere bacteria cultures have the same stimulatory effect as complete bacterial cultures (Azcón, 1987). AM fungi can contribute to root disease suppression through mechanisms not well understood (Linderman, 1994) but the most obvious effect of AM fungi has been attributed to amelioration of nutrient uptake (P and others), resulting in more vigour in growing plants that are better able to ward off or tolerate root disease. St-Arnaud et al. (1995a) proposed a compartmentalized *in vitro* system to elucidate interactions between *R. intraradices* and the root pathogen *Fusarium oxysporum* f. sp *chrysanthemi*. Significant negative correlations were found between conidia production and *R. intraradices* hyphae or spore concentrations. McAllister et al. (1994) found no *in vitro* interactions between spores of *F. mosseae* and *Trichoderma koningii* Oudem or *Fusarium solani* (Mart.) Sacc. Life cycles of *R. intraradices* and the burrowing nematode, *Radopholus similis* (Cobb) Thorne, were achieved in monoxenic cultures (Elsen et al., 2001). The AM fungus reduced the nematode population by 50

%. Also, AM root-organ cultures showed a synergistic interaction between the extra-radical mycelium of *R. intraradices* and soil bacteria in a study of rhizosphere nutritional dynamics (Villegas, 2001). In this study, species-specific interactions were obtained between *R. intraradices* and *Pseudomonas aeruginosa* (Schröter) Migula, *P. putida* Trevisan and *Serratia plymutica* Lehman and Neumann. Although the inherent ability of the fungus and the bacteria to solubilize a recalcitrant form of calcium phosphate was low, *P. aeruginosa* and *P. putida* interacting with the extra-radical mycelium markedly increased P availability in the growth medium. This increase was dependent on the N source, which allowed a reduction of the pH (Villegas and Fortin, 2001). Associations found between some bacterial strains and AM fungal propagules may have a promotional effect on short-term pre-symbiotic mycelium development but little impact on AM propagule germination (Fernández Bidondo et al., 2011).

2.27: Advantages and Disadvantages of the Root Organ Culture System

Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspects of AM symbiosis, complementing the experimental approaches. The most evident advantage shared by all *in vitro* cultivation systems is the absence of undesirable microorganisms due to controlled conditions, rendering greater suitability for large-scale production of high-quality inoculum. Contamination by other microorganisms may occur either at the establishment of the cultivation process or at later stages of culture. Therefore, it may be useful to control the cultures visually, by standard plate-counting techniques and by molecular techniques. The cultures may be placed in a growth chamber requiring minimal space for incubation with no light required in the case of ROCs. Following sporulation dynamics during cultivation also provides a means to control the level of spore production and to determine the optimal

harvesting time. Factors that influence optimal production (*e.g.* nutrient availability, presence of contaminants) can be more easily detected and controlled in liquid cultures. As a drawback, the diversity in terms of genera of AM fungi that have been grown *in vitro* is lower than under pot cultivation systems. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention. The harvesting method of solid *in vitro* cultures involves solubilization of the medium by citrate buffer *i.e.* the gelling agent may be removed from the culture medium so as to stimulate re-growth of the fungus (Doner and Bécard, 1991). Monoxenic cultures provide access to abundant and high-quality fungal material suitable for taxonomic and evolutionary studies (Fortin et al., 2002). In terms of biodiversity, monoxenic cultures provide a tool for basic comparative analyses of root populations and strain potential, long-term propagation capabilities, and fungal adaptation to environment.

CHAPTER 3

To identify the dominant AM fungal species from the sand dune ecosystem. (Objective 1)

3.1: INTRODUCTION

Sand dunes are mounds of drifted sand formed by the accumulation of immense amount of shifting sand grains on the beach. The structure of the dunes and sandy beaches is subjected to constant change due to their dynamic nature (http://www.goaenvis.nic.in). The major pre-requisites for the formation and building of sand dunes are wind, sand and vegetation, three essential elements that have multi-faceted interactions (Carter, 1988).

Sand dune ecosystems perform numerous functions: the dunes are characteristic features of coastal stability; they act as nature's line of protection, sources of beach sustenance, provide protection from coastal erosion, they replenish the loss of sand due to wave and current energies, act as sand banks to sustain the sedimentary and dynamic balance of the coastal ecosystem, they support a rich diversity of flora and fauna, serve as a perfect location for recreation, they protect the hinterlands from winds, and hence are of great ecological significance to coastal population. However, coastal sand dune ecosystems have been under constant threat due to population pressure, developmental activities, tourism and construction. Some of the factors responsible for dune degradation are construction activities, sand extraction, recreation activities, litter on beaches and salt water ingress. Natural disturbances like continuing abrasion, salt spray, erosion and accretion, and wave attack are some of the natural factors that affect the coastline frequently and have always been a usual part of beach and dune building processes. Sand dune systems are an indication of the ecological equilibrium between the prevailing physical forces of the ocean and thus act as nature's shield to prevent monsoon storms, waves and cyclonic surges (http://www.goaenvis.nic.in).

Dune vegetation which constitutes the inland flora that grows immediately behind the beaches also plays an essential part in dune formation since these plants act as wind breakers due to which wind is forced to transport sand along its course. Dune vegetation thus traps and stabilizes moving sand particles. These plants are adapted to a harsh environment characterized by a mobile substrate, saline atmosphere and a frequent bombardment of moving sand grains (Paskoff, 1989). Poaceae members are the main coastal sand dune stabilizing plants in temperate dunes (Read, 1989), while in tropics plant species belonging to Asteraceae, Convolvulaceae, Fabaceae and Poaceae contribute towards the stabilization of coastal sand dunes (Moreno-Casasola and Espejel, 1986; Koske and Gemma, 1990; Devall, 1992; Kulkarni et al., 1997). West coast of India supports a wide variety of coastal sand dune vegetation (Rao and Meher-Homji, 1985).

Arbuscular mycorrhizal (AM) fungi are widespread in coastal sand dunes throughout the world (Sturmer and Bellei, 1994). The sand dune ecosystems exhibit favourable environment for the association and development of AM fungi with dune plants, because dunes are deficient in major nutrients especially phosphorus and the symbiosis also improves stress tolerance (Ranwell, 1972; Koske and Halvorson, 1981; Koske and Gemma 1995). AM fungi play an important role in growth and succession of pioneer vegetation in coastal sand dunes (Nicolson, 1959). Colonization of dune plants for better nutrient uptake and binding of sand grains into stable aggregates by AM fungi significantly stabilizes the sand dunes (Sutton and Sheppard, 1976; Koske and Polson, 1984). Most of the plants on fore dunes and fixed dunes are associated with AM. The most common AM fungal genera in sand dunes are *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora*. AM fungi produce asexual spores singly or aggregated (sporocarps). Species richness and spore density of AM fungi in sand dunes varies according to the

host plants and geographical region (Maun, 2009). AM fungal root colonization is also highly influenced by the density of propagules (spores, extra-radical hyphae and colonized root fragments) in the rhizosphere, soil type, climatic conditions, host type and the fungal species (Smith and Read, 1997).

AM fungi are a taxonomically, physiologically and morphologically diverse group and can colonize a varied group of herbaceous and woody plants (Maun, 2009). Below ground diversity of AM fungi is one of the chief variable governing the plant diversity and functioning of dune habitat. Loss of AM fungi decreases plant equilibrium as well as productivity therefore resulting in dune instability (Sridhar, 2009).

The knowledge and understanding of AM association with dune vegetation and their distribution in the sandy soils is very important for achieving revegetation objectives by effectively using them in sand dune ecosystem conservation and management. Therefore, the present study is aimed at evaluating the AM fungal association and identifying the diversity of indigenous AM species associated with some dominant sand dune plants.

3.2: MATERIALS AND METHODS

3.2.1: Study sites

Goa, the smallest state of India, covering an area of 3702 sq km is located on the west coast of the Indian Peninsula between the longitudes 74°20'13" to 73°40'33"E and latitudes 15°48'00" to 14°53'54"N. It is bounded by the Arabian Sea on the west and by the Western Ghats on the east. It is bordered by the state of Maharashtra to the north and by Karnataka to the south and east. The coastline of Goa consists of a combination of beaches, rocky shores and headlands protruding into the sea. Of the 105 km long

shore, more than 70 km comprise of sandy beaches, all backed by a number of sand dunes (Mascarenhas, 1998).

Coastal sand dunes from 12 different locations of Goa *viz.,* Betalbatim (S1) (Grid Ref. 15°17'33.37"N, 73°54'28.34"E), Colva (S2) (Grid Ref. 15°16'34.62"N, 73°54'47.49"E), Benaulim (S3) (Grid Ref. 15°14'59.01"N, 73°55'15.97"E), Varca (S4) (Grid Ref. 15°13'58.68"N, 73°55'31.95"E), Sinquerim (S5) (Grid Ref. 15°29'57.06"N, 73°46'1.74"E), Candolim (S6) (Grid Ref. 15°30'30.08"N, 73°45'55.50"E), Vagator (S7) (Grid Ref. 15°36'7.58"N, 73°44'0.45"E), Morjim (S8) (Grid Ref. 15°37'54.60"N, 73°43'20.62"E), Mandrem (S9) (Grid Ref. 15°39'29.22"N, 73°42'46.98"E), Arambol (S10) (Grid Ref. 15°41'11.72"N, 73°42'10.19"E), Kerim (S11) (Grid Ref. 15°42'47.52"N, 73°41'29.16"E) and Siridao (S12) (Grid Ref. 15°26'39.92"N, 73°51'20.35"E) were selected for the study **(Fig. 3.1)**. From the study sites, the dominating plant species were selected correspondingly and rhizosphere soils and root samples were collected.

3.2.2: Sample collection

Continuous one time sampling of dominant plant species from chosen sand dune sites was carried out to assess their AM status. In all, roots and rhizosphere soil samples of 18 plant species were collected from a depth of 0-25 cm, placed in zip-loc bags, labeled and then brought to the laboratory. Soil samples were extracted from all quarters of the plants so as to cover the entire rhizosphere, 3 samples from each of the dominant plant species. The sub-samples were mixed thoroughly to obtain a composite sample from each site. The roots were processed to determine root colonization whereas the soil samples were stored at 4°C until processed.

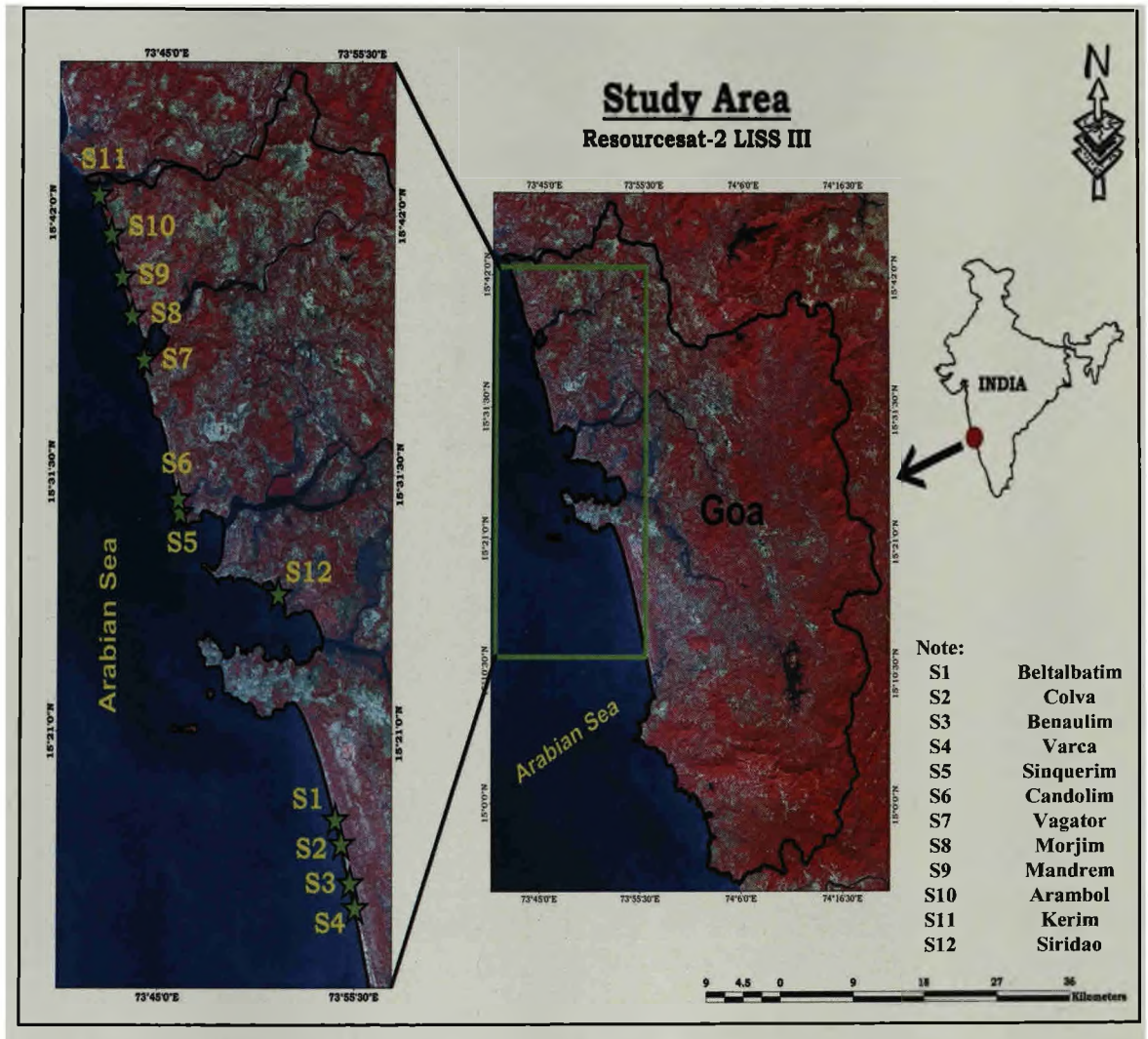


Fig. 3.1: Map of Goa showing the study sites.

3.2.3: Soil chemical analysis

Composite soil samples from each of the study sites were air-dried and sieved to 2 mm to remove larger soil particles. The samples were then submitted to Government of Goa Agricultural Department, Soil Analysis Laboratory, Margao, Goa and Italab House, Margao, Goa for chemical analysis. Analyses followed procedures laid out by Singh, Chhonkar and Dwivedi (2005), soil pH by pH meter (Elico L1 120) in a 1:2 ratio soil water solution, Electrical Conductivity (E. C.) by conductivity meter (Elico CM 180) in a 1:5 ratio soil suspension, Organic Carbon (OC) by Walkley and Black (1934) titration method, available Phosphorus (P) by Bray and Kurtz (1945) method, and available potassium (K) by ammonium acetate method from Hanway and Heidal (1952) using flame photometer (Systronic 3292). Available micro-nutrients zinc (Zn), iron (Fe), manganese (Mn), copper (Cu) were assessed by DTPA-$CaCl_2$-TEA method from Lindsay and Norvell (1978) using atomic absorption spectrophotometer (AAS-EC Element AS AAS 4139), and boron (B) by hot water soluble method from Berger and Truog (1939).

3.2.4: Processing of root segments for AM fungal colonization

Assessment of mycorrhizal colonization in roots was carried out by using modified Trypan blue staining technique (Phillips and Hayman, 1970) wherein roots were gently washed with tap water to remove the attached soil or organic particles followed by cutting into 1 cm segments. The cleaned root bits were then cleared in 2 % KOH by heating at 90°C for 30-45 min in oven, thoroughly rinsed in water, acidified with 5N HCl for 5 min and kept overnight in 0.05 % Trypan blue stain.

The stained root segments were examined using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x) for AM fungal structures.

Micrographs were imaged by Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras, and were not digitally edited.

Presence or absence of AM intra-radical and extra-radical non-septate hyphae, arbuscules, vesicles, hyphal coils and auxiliary cells in the root segments was recorded.

3.2.5: Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using Root Slide method (Read et al., 1976). The Trypan blue stained root fragments were mounted in polyvinyl-lacto-glycerol (PVLG) and the presence or absence of AM colonization was scored using the following formula:

Percent colonization = No. of root segments colonized ÷ Total no. of root segments scored × 100

3.2.6: Extraction of AM fungal spores

Extraction of AM fungal spores was carried out by Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963) wherein 100 g of rhizosphere soil sample (fresh or fridge-stored 4°C) was placed in a container and tap water added. The soil suspension was stirred using a glass rod and then allowing the sediment to settle for 10-15 seconds. The aliquot was decanted through sieves arranged in descending order (250-37 µm). The procedure was repeated a minimum of four times for each sample. The residues from each sieve were then washed into separate beakers. The aliquot was filtered separately through Whatman No. 1 filter paper. The filter paper was then placed in Petri plate and care was taken to ensure that it remained moist. The filter paper was examined for presence of spores and sporocarps under Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom).

3.2.7: Quantification of spore density of AM fungi

Quantification of AM spore numbers was carried out by a modified method of Gaur and Adholeya (1994) wherein Whatman No. 1 filter paper was folded into two equal halves followed by a second fold resulting in four equal halves. The filter paper was opened and two lines were drawn denoting four equal quadrats. Vertical lines were drawn on one half of the filter paper, dividing it into ten columns with each column about 0.5 cm apart. Columns were numbered and the direction of counting was marked with arrows. The filter paper was then folded in such a way that the marked portion received the aliquot during filtration and the other portion remained free of spores. The filter paper was then placed in a Petri plate and observed under the stereo microscope. The spores occupying the space between the numbered columns were counted. Intact spores were picked up using a needle and mounted in PVLG for taxonomic identification. Spore density was expressed as total number of spores recorded per 100 g of sample.

3.2.8: Taxonomic identification of spores

Clean, intact, un-parasitized spores mounted in PVLG were used for taxonomic identification. Initially spore identification was carried out as per traditional taxonomically general method of classification at 'genus' level, comprising of genera *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora*, which are most prevalent in the sand dunes (Maun, 2009). Several morphological characteristics play an informative role in spore identification at species level *viz.,* colour, dimension, ornamentation pattern, number of wall layers, pore occlusion, pattern and shape of germination shield, and reaction to Melzer's reagent. However with the development of molecular methods for elucidating the phylogenetic relationships among the AM genera and species, their

classification has been in transition and is re-assessed constantly. For taxonomical identification of AM fungal spores by using morphological criteria, reference was made to original species protologues described by Morton and Benny (1990), Schenck and Pérez (1990), Rodrigues and Muthukumar (2009), Schüßler and Walker (2010), Redecker et al. (2013), and online species descriptions provided by INVAM (International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi at the West Virginia University, USA (https://invam.wvu.edu/), arbuscular mycorrhizal fungi (Glomeromycota), *Endogone* and *Complexipes* species deposited in the Department of Plant Pathology, University of Agriculture in Szczecin, Poland (http://www.zor.zut.edu.pl/Glomeromycota/index.html), and amf-phylogeny.com.

3.2.9: AM species richness, Spore abundance and Relative abundance

Species richness, spore abundance and relative abundance (RA %) of AM fungi were calculated using the following formulae (Beena et al., 2000c, 2001).

3.2.9.1: Species richness: Species number per 100 g soil sample or species number per study site.

3.2.9.2: Spore abundance: Number of spores of a particular species per 100 g of soil sample.

3.2.9.3: Relative abundance (RA %) = Number of spores of a particular species ÷ total number of spores × 100

3.3: RESULTS

3.3.1: Soil chemical analysis

Results of physico-chemical properties of sand from 12 study sites revealed that pH was acidic to alkaline ranging from 6.3-8.6. Electrical Conductivity (E. C.) was in normal range <1 m.mhos/cm. Organic carbon percentage was low and it ranged from 0.04-0.43 %. Available P content was low to high ranging from 16.4-60.1 Kg/Ha. Available K content was low to medium ranging from 50.4-347 Kg/Ha. Micro-nutrients *i.e.* Zn (0.00-61.66 ppm), Fe (0.78-5.67 ppm), Mn (0.26-40.18 ppm), Cu (0.03-364.3 ppm), and B (traces-4.40 ppm) were present in traces-high levels **(Table 3.1)**.

3.3.2: Percent root colonization and spore density of AM fungi

Arbuscular mycorrhizal fungal colonization was observed in roots of dune plant species sampled from all the 12 sites; however the extent of colonization varied in the sand dune vegetation sampled. Spore density in the rhizosphere soil samples also exhibited great variation.

At Betalbatim (S1), highest root colonization was observed in *Spinifex littoreus* (90.00 %) and least root colonization was observed in *Leucas aspera* (15.66 %). Spore density was highest in *Spinifex littoreus* rhizosphere soils (388.00 spores 100 g^{-1}) and least in *Vitex trifolia* (34.66 spores 100 g^{-1}) **(Table 3.2)**.

At Colva (S2), highest root colonization was observed in *Ipomoea pes-caprae* (72.00 %) and least root colonization was observed in *Cocos nucifera* (14.66 %). Spore density was highest in *Ipomoea pes-caprae* rhizosphere soils (184.00 spores 100 g^{-1}) and least in *Lantana camara* (32.66 spores 100 g^{-1}) **(Table 3.3)**.

Table 3.1: Physico-chemical properties of sand from selected study sites.

Sites	*pH	*E. C. m.mhos/cm	*Macro-nutrients			*Micro-nutrients (ppm)				
			O C %	P Kg/Ha	K Kg/Ha	Zn	Fe	Mn	Cu	B
S1	8.60	<1	0.09	16.40	70.00	0.01	1.55	0.26	0.06	0.70
S2	7.90	<1	0.13	54.60	206.00	61.66	3.00	40.18	364.30	4.40
S3	7.70	<1	0.13	49.20	50.40	0.59	2.66	0.66	0.03	2.00
S4	8.50	<1	0.13	21.90	80.00	0.00	1.18	0.26	0.03	1.30
S5	8.50	<1	0.13	32.80	101.00	0.36	2.00	2.29	0.03	Traces
S6	8.10	<1	0.04	60.10	216.00	0.06	4.22	4.68	0.03	0.70
S7	7.50	<1	0.22	21.90	347.00	0.03	0.87	2.83	0.03	Traces
S8	8.00	<1	0.17	27.30	236.00	0.01	3.81	4.88	0.03	0.70
S9	7.80	<1	0.26	43.70	156.00	0.35	2.82	1.48	0.03	0.70
S10	7.00	<1	0.35	49.20	101.00	0.04	4.86	5.13	0.10	Traces
S11	6.30	<1	0.43	21.90	196.00	0.19	5.67	17.52	0.03	Traces
S12	8.60	<1	0.22	27.30	101.00	0.02	0.78	1.73	0.03	Traces

Legend: *Values are derived from a composite sample; S = site; S1 = Betalbatim, S2 = Colva, S3 = Benaulim, S4 = Varca, S5 = Sinquerim, S6 = Candolim, S7 = Vagator, S8 = Morjim, S9 = Mandrem, S10 = Arambol, S11 = Kerim, S12 = Siridao.

Table 3.2: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Beltalbatim (S1).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/700 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	+	+	70.00 ± 2.00	197.33 ± 4.50 *A. sc, F. geo, Gi. al*	1400.65
2.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	+	+	90.00 ± 3.00	388.00 ± 5.00 *A. sc, A. re, F. geo, Gi. al*	
3.	*Vitex trifolia* L. (Lamiaceae)	+	+	+	52.33 ± 2.51	34.66 ± 8.02 *A. re, A. sc, A. ele, F. geo, Gi. al*	
4.	*Ageratum conyzoides* L. (Compositae)	+	-	+	77.00 ± 4.00	214.00 ± 4.00 *A. re, A. sc, A. ele, R. man, Gi. al*	
5.	*Lantana camara* L. (Verbenaceae)	+	-	+	86.66 ± 1.15	218.33 ± 9.50 *Gi. al, A. sc, A. re, A. de*	
6.	*Leucas aspera* (Willd.) Link (Lamiaceae)	+	-	+	15.66 ± 2.08	40.00 ± 13.00 *A. sc, F. geo, Gi. al*	
7.	*Tridax procumbens* L. (Compositae)	+	-	+	28.00 ± 6.24	308.33 ± 34.50 *A. re, A. sc, F. geo, F. mos, Gi. al*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. de = A. delicata, A. re = A. rehmii, A. ele = A. elegans, R. man = Rhizoglomus manihotis, F. geo = Funneliformis geosporum, F. mos = F. mosseae, Gi. al = Gigaspora albida.*

Table 3.3: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Colva (S2).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/400 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	+	+	72.00 ± 3.00	184.00 ± 6.00 *A. sc, A. re, R. fas, Gi. al*	312.66
3.	*Vitex trifolia* L. (Lamiaceae)	+	+	+	52.66 ± 6.02	61.00 ± 6.00 *A. sc, A. re, A. ele, F. geo, Gi. al*	
5.	*Lantana camara* L. (Verbenaceae)	+	-	+	68.00 ± 3.00	32.66 ± 2.08 *A. sc, A. re, A. de, A. ele, F. geo, Gi. al*	
4.	*Cocos nucifera* L. (Arecaceae)	+	-	+	14.66 ± 2.08	35.00 ± 3.00 *A. sc, A. re*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. ele = A. elegans, A. de = A. delicata, R. fas = Rhizoglomus fasciculatum, F. geo = Funneliformis geosporum, Gi. al = Gigaspora albida.*

At Benaulim (S3), highest root colonization was observed in *Ipomoea pes-caprae* (83.00 %) and least root colonization was observed in *Tridax procumbens* (18.00 %). Spore density was highest in *Spinifex littoreus* rhizosphere soils (323.00 spores 100 g^{-1}) and least in *Ipomoea pes-caprae* (38.66 spores 100 g^{-1}) **(Table 3.4)**.

At Varca (S4), highest root colonization was observed in *Anacardium occidentale* (88.33 %) and least root colonization was observed in *Tridax procumbens* (24.66 %). Spore density was highest in *Spinifex littoreus* rhizosphere soils (208.00 spores 100 g^{-1}) and least in *Leucas aspera* (53.66 spores 100 g^{-1}) **(Table 3.5)**.

At Sinquerim (S5), highest root colonization was observed in *Ipomoea pes-caprae* (92.22 %) and least root colonization was observed in *Portulaca oleracea* (27.77 %). Spore density was highest in *Dactyloctenium aegyptium* rhizosphere soils (97.33 spores 100 g^{-1}) and least in *Portulaca oleracea* (14.66 spores 100 g^{-1}) **(Table 3.6)**.

At Candolim (S6), highest root colonization was observed in *Alternanthera ficoidea* (82.35 %) and least root colonization was observed in *Launaea nudicaulis* (22.22 %). Spore density was highest in *Alternanthera ficoidea* rhizosphere soils (60.66 spores 100 g^{-1}) and least in *Launaea nudicaulis* (16.00 spores 100 g^{-1}) **(Table 3.7)**.

At Vagator (S7), highest root colonization was observed in *Zoysia matrella* (55.55 %) and least root colonization was observed in *Vitex trifolia* (20.00 %). However, no colonization was observed in *Launaea nudicaulis*. Spore density was highest in *Launaea nudicaulis* rhizosphere soils (53.33 spores 100 g^{-1}) and least in *Zoysia matrella* (16.00 spores 100 g^{-1}) **(Table 3.8)**.

At Morjim (S8), highest root colonization was observed in *Anacardium occidentale* (84.00 %) and least root colonization was observed in *Ipomoea pes-caprae* (21.62 %).

Table 3.4: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Benaulim (S3).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/600 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	+	+	83.00 ± 4.00	38.66 ± 7.02 *A. sc, A. re, R. fas, C. cl, Gi. al*	666.98
2.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	-	+	77.00 ± 4.00	323.00 ± 32.00 *A. sc, A. re, A. fov, A. ni, A. bi, F. geo, R. cl, Gi. gi, Ra. gr, S. pe, Gi. al*	
3.	*Vitex trifolia* L. (Lamiaceae)	+	-	+	36.33 ± 3.51	44.33 ± 5.50 *A. sc, A. re, A. ele, F. geo, Gi. al*	
4.	*Lantana camara* L. (Verbenaceae)	+	-	+	60.33 ± 12.50	108.33 ± 8.50 *A. sc, A. re, A. de, A. ele, Gi. al*	
5.	*Leucas aspera* (Willd.) Link (Lamiaceae)	+	-	+	23.66 ± 7.02	40.66 ± 3.05 *A. sc, A. re, F. geo, Gi. al*	
6.	*Tridax procumbens* L. (Compositae)	+	-	+	18.00 ± 7.00	112.00 ± 8.00 *A. sc, A. re, F. geo, Gi. al*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. fov = A. foveata, A. ni = A. nicolsonii, A. bi = A. bireticulata, A. ele = A. elegans, A. de = A. delicata, R. fas = Rhizoglomus fasciculatum, R. cl = R. clarum, C. cl = Claroideoglomus claroideum, F. geo = Funneliformis geosporum, Gi. gi = Gigaspora gigantea, Gi. al = Gi. albida, Ra. gr = Racocetra gregaria, S. pe = Scutellospora pellucida.*

Table 3.5: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Varca (S4).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/700 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	+	87.33 ± 4.50	111.66 ± 15.01 *A. sc, A. re, R. fas, C. cl, Gi. al*	778.98
2.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	-	+	80.00 ± 7.00	208.00 ± 8.00 *A. sc, A. re, A. fov, A. ni, A. bi, F. geo, Gi. gi, Ra. gr, S. pe, Gi. al*	
3.	*Vitex trifolia* L. (Lamiaceae)	+	-	+	45.00 ± 5.00	66.33 ± 3.51 *A. sc, A. re, A. ele, F. geo, Gi. al*	
4.	*Lantana camara* L. (Verbenaceae)	+	-	+	63.66 ± 6.02	97.00 ± 9.00 *A. sc, A. re, A. de, A. ele, Gi. al*	
5.	*Leucas aspera* (Willd.) Link (Lamiaceae)	+	-	+	32.00 ± 6.00	53.66 ± 6.02 *A. sc, A. re, Gi. al*	
6.	*Tridax procumbens* L. (Compositae)	+	-	+	24.66 ± 3.05	80.33 ± 3.51 *A. sc, A. re, Gi. al*	
7.	*Anacardium occidentale* L. (Anacardiaceae)	+	+	+	88.33 ± 11.50	162.00 ± 35.00 *A. di, A. re, F. geo, R. fas, R. in, Gi. al, Gi. gi*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. fov = A. foveata, A. ni = A. nicolsonii, A. bi = A. bireticulata, A. ele = A. elegans, A. de = A. delicata, A. di = A. dilatata, R. fas = Rhizoglomus fasciculatum, R. in = R. intraradices, C. cl = Claroideoglomus claroideum, F. geo = Funneliformis geosporum, Gi. al = Gigaspora albida, Gi. gi = Gi. gigantea, Ra. gr = Racocetra gregaria, S. pe = Scutellospora pellucida.*

Table 3.6: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Sinquerim (S5).

Sr. No.	Plant species	Type of colonization				*Colonization (%)	*Spore density/100 g soil	Total spore density/500 g soil
		H	HC	A	V			
1.	*Digitaria ciliaris* (Retz.) Koeler (Poaceae)	+	-	+	+	68.25 ± 12.2	65.00 ± 7.00 *A. sc*	325.32
2.	*Dactyloctenium aegyptium* (L.) Willd. (Poaceae)	+	+	+	+	79.62 ± 9.81	97.33 ± 9.50 *A. de, A. sc*	
3.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	-	+	92.22 ± 11.12	63.00 ± 5.00 *A. sc, A. de*	
4.	*Boerhavia diffusa* L. (Nyctaginaceae)	+	-	-	+	33.33 ± 6.73	85.33 ± 10.50 *A. de*	
5.	*Portulaca oleracea* L. (Portulacaceae)	+	-	-	-	27.77 ± 6.43	14.66 ± 5.03 *A. de, A. sc*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, HC = hyphal coils, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc* = *Acaulospora scrobiculata*, *A. de* = *A. delicata.*

Table 3.7: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Candolim (S6).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/600 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	+	25.00 ± 7.63	30.66 ± 6.02 *R. fas, F. geo, Gi. al*	218.65
2.	*Alternanthera ficoidea* (L.) Sm. (Amaranthaceae)	+	-	+	82.35 ± 8.93	60.66 ± 5.03 *A. sc, A. de, A. re*	
3.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	+	78.78 ± 9.72	43.00 ± 8.00 *A. de, Gi. al*	
4.	*Acanthospermum hispidum* DC. (Compositae)	+	-	-	33.33 ± 6.73	19.00 ± 8.00 *A. de, Gi. al*	
5.	*Cyperus arenarius* Retz. (Cyperaceae)	-	-	+	40.00 ± 10.14	49.33 ± 2.51 *A. de, A. re, Gi. al*	
6.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	-	-	+	22.22 ± 6.14	16.00 ± 4.00 *A. de, A. sc*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. de = A. delicata, R. fas = Rhizoglomus fasciculatum, F. geo = Funneliformis geosporum, Gi. al = Gigaspora albida.*

Table 3.8: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Vagator (S7).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/500 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	+	25.00 ± 7.63	20.00 ± 5.00 *A. sc*	148.33
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	+	55.55 ± 7.88	16.00 ± 4.00 *A. sc, Gi. al*	
3.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	-	+	52.94 ± 6.78	28.00 ± 2.00 *A. sc, Gi. al*	
4.	*Vitex trifolia* L. (Lamiaceae)	+	-	+	20.00 ± 5.77	31.00 ± 6.00 *A. sc, Gi. al*	
5.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	-	-	-	-	53.33 ± 8.50 *Gi. al*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, Gi al = Gigaspora albida.*

Spore density was highest in *Anacardium occidentale* rhizosphere soils (143.00 spores 100 g^{-1}) and least in *Ageratum conyzoides* (18.00 spores 100 g^{-1}) **(Table 3.9).**

At Mandrem (S9), highest root colonization was observed in *Launaea nudicaulis* (77.77 %) and least root colonization was observed in *Vitex trifolia* (28.57 %). Spore density was highest in *Launaea nudicaulis* rhizosphere soils (90.66 spores 100 g^{-1}) and least in *Ipomoea pes-caprae* (21.66 spores 100 g^{-1}) **(Table 3.10).**

At Arambol (S10), highest root colonization was observed in *Launaea nudicaulis* (60.00 %) and least root colonization was observed in *Ipomoea pes-caprae* (27.77 %). However, no colonization was observed in *Ageratum conyzoides*. Spore density was highest in *Ipomoea pes-caprae* rhizosphere soils (20.66 spores 100 g^{-1}) and least in *Launaea nudicaulis* (14.66 spores 100 g^{-1}) **(Table 3.11).**

At Kerim (S11), highest root colonization was observed in *Leucas aspera* (60.00 %) and least root colonization was observed in *Ipomoea pes-caprae* and *Zoysia matrella* (20.00 %). Spore density was highest in *Launaea nudicaulis* rhizosphere soils (160.66 spores 100 g^{-1}) and least in *Zoysia matrella* (16.00 spores 100 g^{-1}) **(Table 3.12).**

At Siridao (S12), highest root colonization was observed in *Zoysia matrella* (74.07 %) and least root colonization was observed in *Vitex trifolia* (20.00 %). Spore density was highest in *Launaea nudicaulis* rhizosphere soils (26.00 spores 100 g^{-1}) and least in *Zoysia matrella* (14.66 spores 100 g^{-1}) **(Table 3.13).**

Table 3.9: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Morjim (S8).

Sr. No.	Plant species	Type of colonization					*Colonization (%)	*Spore density/100 g soil	Total spore density/600 g soil
		H	HC	A	V	Aux			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	-	+	-	21.62 ± 6.29	56.00 ± 11.00 *A. sc, Gi. al*	411
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	+	+	+	61.11 ± 8.24	59.00 ± 3.00 *A. sc, Gi. al*	
3.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	+	+	-	+	42.85 ± 5.06	65.00 ± 7.00 *A. de, A. sc*	
4.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	+	-	-	+	-	58.33 ± 9.17	70.00 ± 8.00 *A. sc, Gi. al*	
5.	*Ageratum conyzoides* L. (Compositae)	+	-	-	+	-	70.00 ± 7.63	18.00 ± 4.00 *A. de, Gi. al*	
6.	*Anacardium occidentale* L. (Anacardiaceae)	+	-	+	+	+	84.00 ± 7.93	143.00 ± 46.00 *G. ma, Ra. gr, S. sc, Gi. al, Gi. gi, Gi. ra*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, HC = hyphal coils, A = arbuscules, V = vesicles, Aux = auxiliary cells; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. de = A. delicata, G. ma = Glomus macrocarpum, Ra. gr = Racocetra gregaria, S. sc = Scutellospora scutata, Gi. al = Gigaspora albida, Gi. gi = Gi. gigantea, Gi. ra = Gi. ramisporophora.*

Table 3.10: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Mandrem (S9).

Sr. No.	Plant species	Type of colonization			*Colonization (%)	*Spore density/100 g soil	Total spore density/600 g soil
		H	A	V			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	+	44.44 ± 7.39	21.66 ± 7.02 *A. de, A. sc, A. re, A. bi, Gi. al*	343.31
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	+	33.33 ± 6.93	85.00 ± 8.00 *A. sc, A. re, Gi. al*	
3.	*Vitex trifolia* L. (Lamiaceae)	+	-	+	28.57 ± 9.29	37.00 ± 4.00 *A. bi, A. re, Gi. al*	
4.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	+	-	+	77.77 ± 6.43	90.66 ± 9.01 *A. bi, A. sc, Gi. al*	
5.	*Tridax procumbens* L. (Compositae)	+	-	+	30.00 ± 4.89	56.66 ± 9.01 *A. sc, A. bi, Gi. al*	
6.	*Leucas aspera* (Willd.) Link (Lamiaceae)	+	-	+	33.33 ± 6.93	52.33 ± 7.50 *A. de, A. sc, A. bi, Gi. al*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. de = A. delicata, A. bi = A. bireticulata, Gi. al = Gigaspora albida.*

Table 3.11: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Arambol (S10).

Sr. No.	Plant species	Type of colonization					*Colonization (%)	*Spore density/100 g soil	Total spore density/400 g soil
		H	HC	A	V	Aux			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	-	+	-	27.77 ± 6.71	20.66 ± 9.01 *A. sc*	70.32
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	+	-	+	-	40.00 ± 7.50	19.00 ± 8.00 *A. sc*	
3.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	+	-	-	+	+	60.00 ± 7.63	14.66 ± 4.04 *A. de, A. sc*	
4.	*Ageratum conyzoides* L. (Compositae)	-	-	-	-	-	-	16.00 ± 4.00 *A. de, A. sc*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, HC = hyphal coils, A = arbuscules, V = vesicles, Aux = auxiliary cells; + = observed, - = not observed; AM species: *A. sc* = *Acaulospora scrobiculata*, *A. de* = *A. delicata.*

Table 3.12: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Kerim (S11).

Sr. No.	Plant species	Type of colonization				*Colonization (%)	*Spore density/100 g soil	Total spore density/400 g soil
		H	A	V	Aux			
1.	*Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae)	+	-	+	-	20.00 ± 5.77	56.00 ± 4.00 *A. sc*	319.66
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	+	-	20.00 ± 5.77	16.00 ± 5.00 *A. sc*	
3.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	+	-	+	+	50.00 ± 8.39	160.66 ± 6.02 *A. sc, Gi. al*	
4.	*Leucas aspera* (Willd.) Link (Lamiaceae)	+	-	+	+	60.00 ± 7.63	87.00 ± 10.00 *A. sc*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, A = arbuscules, V = vesicles, Aux = auxiliary cells; + = observed, - = not observed; AM species: *A. sc* = *Acaulospora scrobiculata*, *Gi. al* = *Gigaspora albida.*

Table 3.13: Arbuscular mycorrhizal (AM) fungal association in sand dune vegetation at Siridao (S12).

Sr. No.	Plant species	Type of colonization				*Colonization (%)	*Spore density/100 g soil	Total spore density/500 g soil
		H	HC	A	V			
1.	*Ipomoea pes-caprae* (L.) R. Br. (Convolvulaceae)	+	-	-	+	25.00 ± 7.63	20.00 ± 8.71 *A. sc*	103.99
2.	*Zoysia matrella* (L.) Merr. (Poaceae)	+	-	-	+	74.07 ± 5.21	14.66 ± 3.05 *A. re, Gi. al*	
3.	*Spinifex littoreus* (Burm. f.) Merr. (Poaceae)	+	-	-	+	33.33 ± 6.93	25.00 ± 7.00 *A. de, A. sc*	
4.	*Vitex trifolia* L. (Lamiaceae)	+	-	-	+	20.00 ± 5.77	18.33 ± 6.50 *A. de, A. sc*	
5.	*Launaea nudicaulis* (L.) Hook.f. (Compositae)	+	+	-	+	37.50 ± 6.29	26.00 ± 14.00 *A. de, A. sc, F. geo*	

Legend: *Values are means of three replicates ± standard deviation; H = hyphae, HC = hyphal coils, A = arbuscules, V = vesicles; + = observed, - = not observed; AM species: *A. sc = Acaulospora scrobiculata, A. re = A. rehmii, A. de = A. delicata, F. geo = Funneliformis geosporum, Gi. al = Gigaspora albida.*

In general, AM colonization was characterized by the presence of intra-radical and extra-radical non-septate hyphae, hyphal coils, arbuscules (*Arum*- and *Paris*- type), vesicles, intra-radical spores, extra-radical spores and auxiliary cells (knobby and spiny/papillate) **(Plate 3.1 a-h, Plate 3.2 a-h)**. Overall maximum percent root colonization was observed in *Ipomoea pes-caprae* (92.22 %) at Sinquerim (S5) and minimum in *Cocos nucifera* (14.66 %) at Colva (S2). Maximum spore density was observed in *Spinifex littoreus* (388.00 spores 100 g^{-1} soil) at Betalbatim (S1) and minimum in *Portulaca oleracea*, *Launaea nudicaulis*, and *Zoysia matrella* (14.66 spores 100 g^{-1} soil) at Sinquerim (S5), Arambol (S10), and Siridao (S12) respectively. Site-wise, total spore density was highest at Betalbatim (S1) (1400.65 spores 100 g^{-1} soil) and least at Arambol (S10) (70.32 spores 100 g^{-1} soil).

3.3.3: AM species communities

A total of 22 AM fungal species belonging to 8 genera were recorded from the selected twelve sites. *Acaulospora* (8) was the dominant genus followed by *Rhizoglomus* (4), *Gigaspora* (3), *Scutellospora* (2), *Funneliformis* (2), *Claroideoglomus* (1), *Glomus* (1), and *Racocetra* (1) (species numbers given in parenthesis) **(Plate 3.3 a-h, Plate 3.4 a-f, Plate 3.5 a-f, Plate 3.6 a-f)**. *Acaulospora scrobiculata* was the most abundant AM species recorded from all the study sites.

Plate 3.1: Micrographs of intra- and extra-radical components of AM fungi in roots of sand dune plant species.

a) Intra-radical hyphal colonization in roots of *Ipomoea pes-caprae* (L.) R.Br.

b) Extra-radical hyphae in *Anacardium occidentale* L.

c) Hyphal coils in roots of *Dactyloctenium aegyptium* (L.) Willd.

d) Hyphal coils in roots of *Ageratum conyzoides* L.

e) *Arum*-type of arbuscular colonization in roots of *Dactyloctenium aegyptium* (L.) Willd. Arrows indicating arbuscules, inter-cellular longitudinal hyphae.

f) Enlarged view of *Arum*-type of arbuscular colonization in roots of *Dactyloctenium aegyptium* (L.) Willd. Arrows indicating presence of arbuscules intra-cellularly.

g) *Paris*-type of arbuscular colonization in roots of *Dactyloctenium aegyptium* (L.) Willd. Arrows indicating arbusculate coils extended from cell to cell intra-cellularly.

h) Degenerating *Arum*-type arbuscules in roots of *Zoysia matrella* (L.) Merr. Arrows indicating degenerating arbuscules, intra-radical hypha.

Plate 3.1

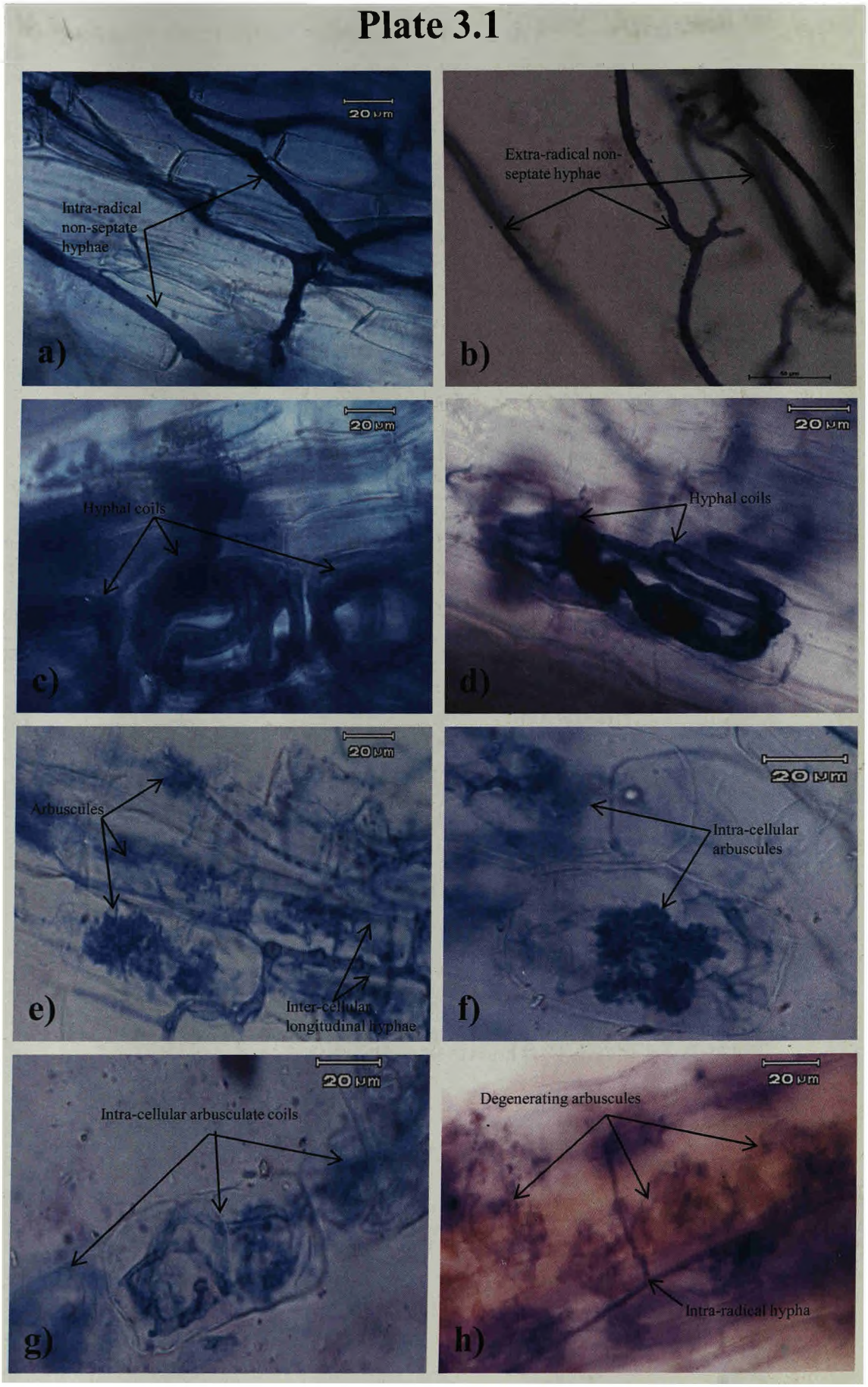

20 µm
Intra-radical non-septate hyphae
a)
Extra-radical non-septate hyphae
b)
20 µm
Hyphal coils
c)
20 µm
Hyphal coils
d)
20 µm
Arbuscules
Inter-cellular longitudinal hyphae
e)
20 µm
Intra-cellular arbuscules
f)
20 µm
Intra-cellular arbusculate coils
g)
20 µm
Degenerating arbuscules
Intra-radical hypha
h)

Plate 3.2: Micrographs of intra- and extra-radical components of AM fungi in roots of sand dune plant species.

a) Globose vesicles in roots of *Alternanthera ficoidea* (L.) Sm.

b) Sub-globose vesicle in roots of *Zoysia matrella* (L.) Merr.

c) Cluster of globose vesicles in roots of *Ipomoea pes-caprae* (L.) R.Br.

d) Cluster of elongated vesicles in roots of *Ipomoea pes-caprae* (L.) R.Br.

e) Formation of intra-radical spores in roots of *Anacardium occidentale* L. Arrows indicating intra-radical spores, intra-radical hyphae.

f) Extra-radical spores in roots of *Anacardium occidentale* L.

g) Knobby auxiliary cells in roots of *Anacardium occidentale* L. Arrows indicating extra-radical auxiliary cells, extra-radical hypha.

h) Spiny/papillate auxiliary cells in roots of *Zoysia matrella* (L.) Merr. Arrows indicating extra-radical auxiliary cells, extra-radical hypha, spiny structure.

Plate 3.2

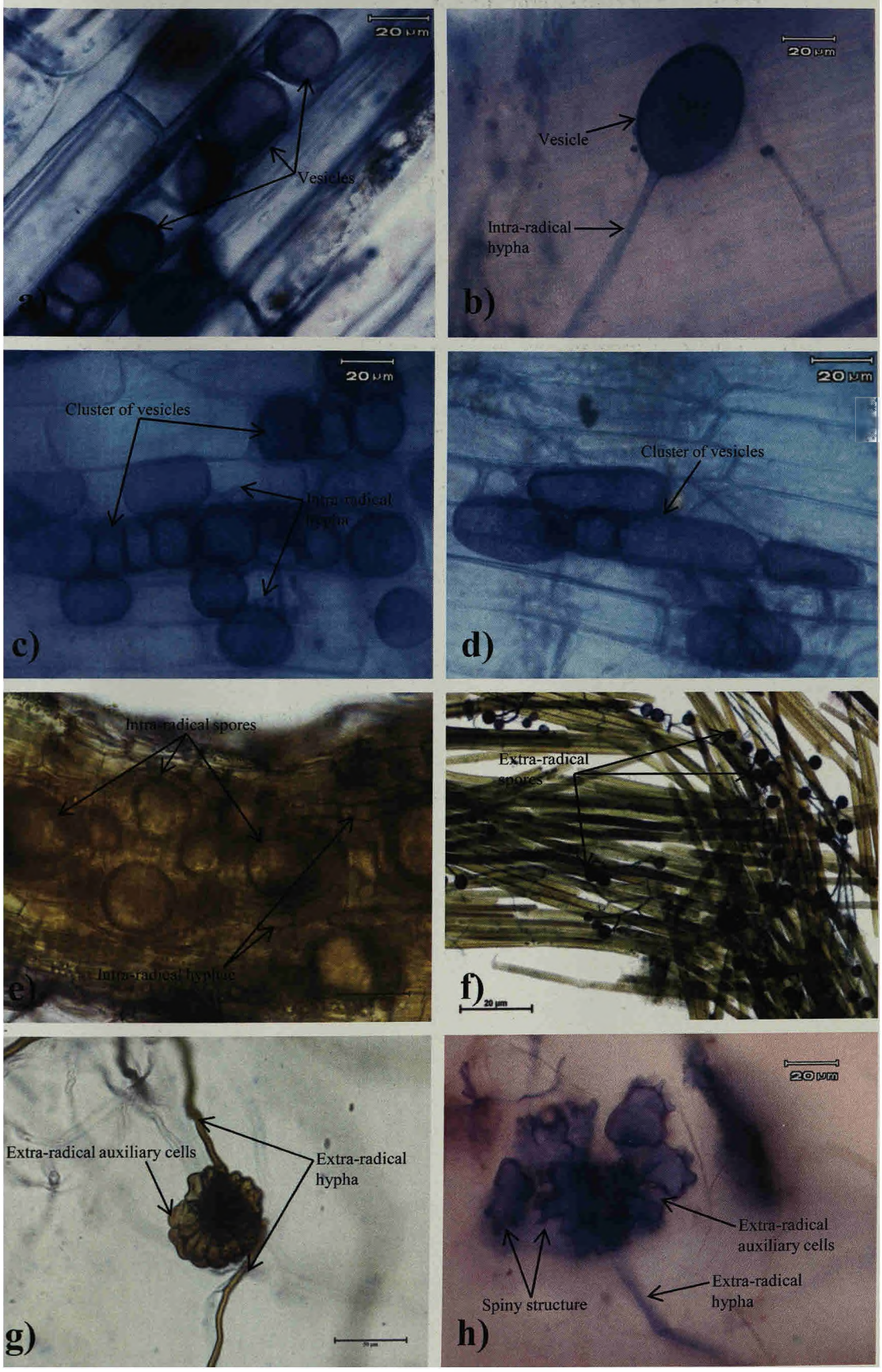

20 µm
Vesicles
a)
20 µm
Vesicle
Intra-radical hypha
b)
20 µm
Cluster of vesicles
Intra-radical hypha
c)
20 µm
Cluster of vesicles
d)
Intra-radical spores
Intra-radical hyphae
e)
Extra-radical spores
f)
20 µm
Extra-radical auxiliary cells
Extra-radical hypha
g)
20 µm
Extra-radical auxiliary cells
Extra-radical hypha
Spiny structure
h)

Plate 3.3: Micrographs of AM fungal species extracted from selected study sites describing morphological features.

a) Broken spore of *Acaulospora delicata* Walker, Pfeiff & Bloss. Arrows indicating wall structure, spore content.

b) Broken spore of *Acaulospora scrobiculata* Trappe. Arrows indicating wall structure, ornamentation pattern, spore content.

c) Enlarged view of ornamentation pattern (pits of different shapes) of *Acaulospora scrobiculata* Trappe.

d) Broken spore of *Acaulospora bireticulata* Rothwell & Trappe. Arrows indicating wall structure, ornamentation pattern (polygonal reticulum), spore content.

e) Broken spore of *Acaulospora rehmii* Sieverding & Toro. Arrows indicating wall structure, spore content.

f) Enlarged view of ornamentation pattern (cerebriform folds) of *Acaulospora rehmii* Sieverding & Toro.

g) Broken spore of *Acaulospora elegans* Trappe & Gerdemann. Arrows indicating wall structure, ornamentation pattern (light brown spines developing an alveolate reticulum), spore content.

h) Broken spore of *Acaulospora nicolsonii* Walker, Reed & Sanders. Arrows indicating wall structure.

Plate 3.3

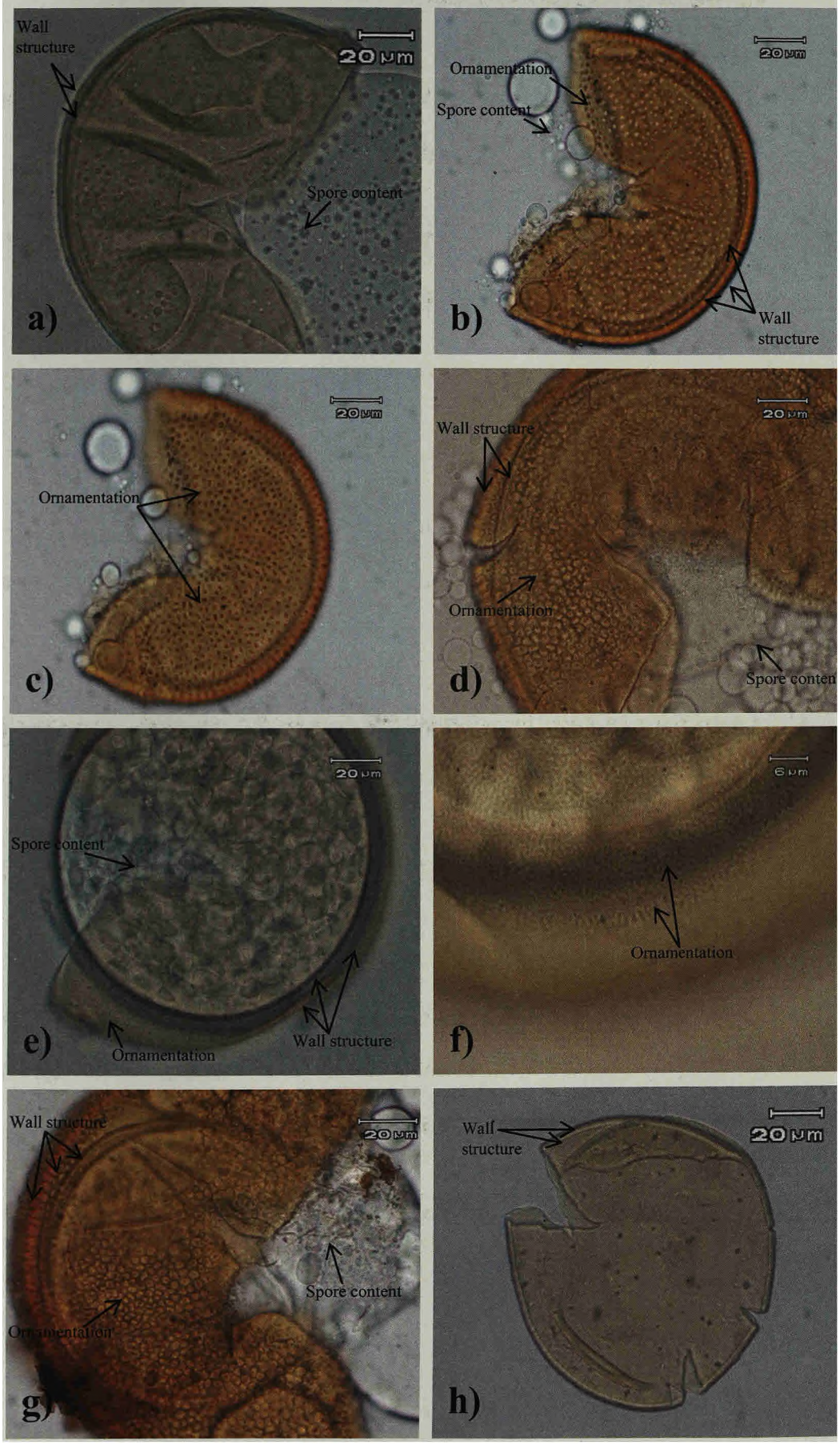

Wall structure
20 µm
Spore content
a)
Ornamentation
Spore content
20 µm
b)
Wall structure
20 µm
Ornamentation
c)
Wall structure
20 µm
Ornamentation
d)
Spore content
20 µm
Spore content
e)
Ornamentation
Wall structure
6 µm
Ornamentation
f)
Wall structure
20 µm
Spore content
Ornamentation
g)
Wall structure
20 µm
h)

Plate 3.4: Micrographs of AM fungal species extracted from selected study sites describing morphological features.

a) Broken spore of *Acaulospora foveata* Trappe & Janos. Arrows indicating wall structure, ornamentation pattern.

b) Enlarged view of ornamentation pattern (circular to ovoid concave depressions/pits) of *Acaulospora foveata* Trappe & Janos.

c) Broken spore of *Acaulospora dilatata* Morton. Arrows indicating wall structure.

d) Broken spore of *Rhizoglomus fasciculatum* (Thaxter) Sieverding, Silva & Oehl. Arrows indicating wall structure, subtending hypha.

e) Spore of *Rhizoglomus intraradices* (Schenck & Smith) Sieverding, Silva & Oehl. Arrows indicating wall structure, subtending hypha.

f) Broken spore of *Rhizoglomus manihotis* (Howeler, Sieverding & Schenck) Sieverding, Silva & Oehl. Arrows indicating wall structure, spore content.

Plate 3.4

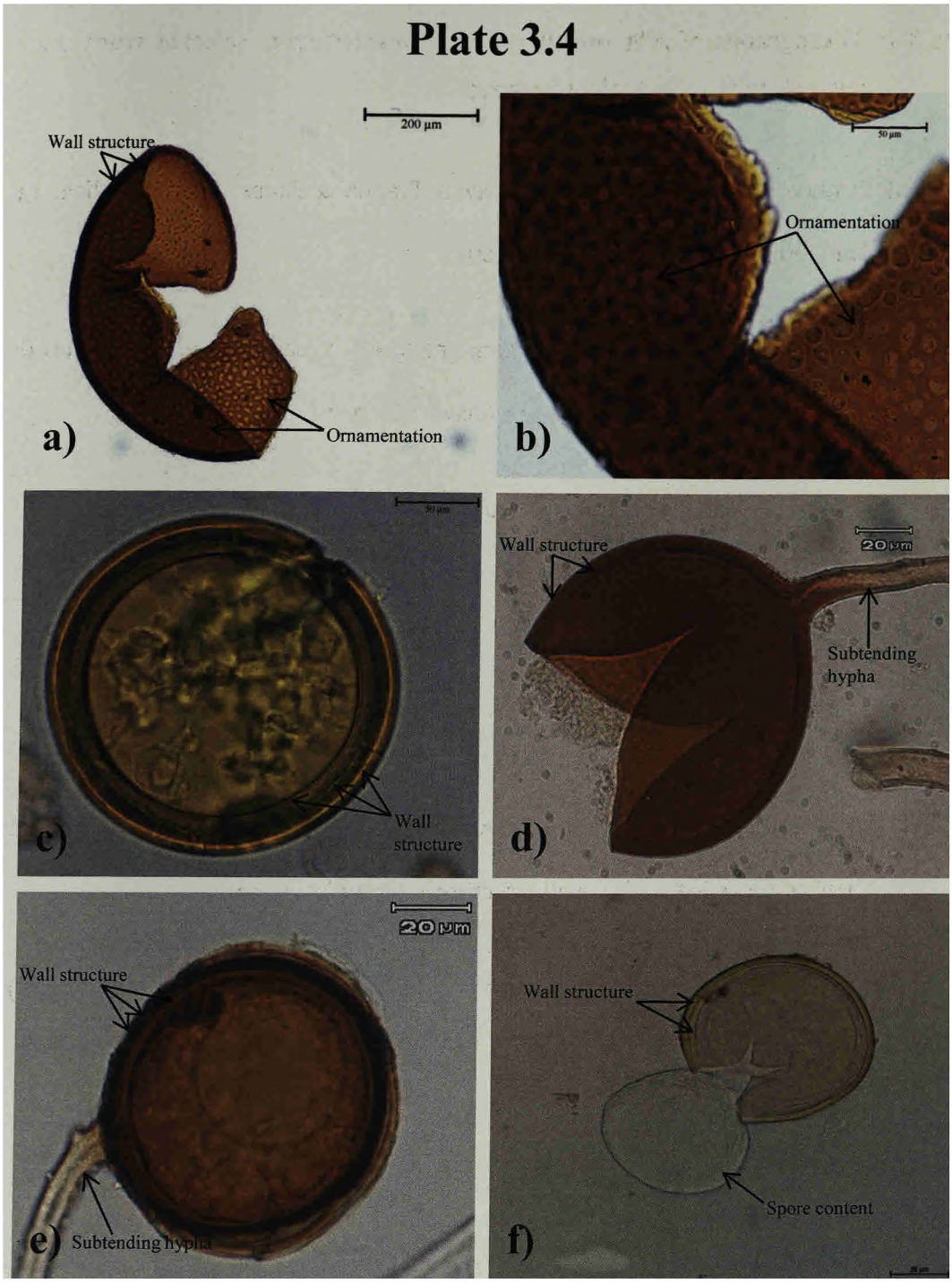

200 µm
Wall structure
Ornamentation
a)
50 µm
Ornamentation
b)
Wall structure
c)
20 µm
Wall structure
Subtending hypha
d)
20 µm
Wall structure
Subtending hypha
e)
Wall structure
Spore content
f)

Plate 3.5: Micrographs of AM fungal species extracted from selected study sites describing morphological features.

a) Broken spore of *Rhizoglomus clarum* (Nicolson & Schenck) Sieverding, Silva & Oehl. Arrows indicating wall structure, spore content.

b) Spore of *Funneliformis geosporum* (Nicolson & Gerdemann) Walker & Schüßler. Arrows indicating wall structure, subtending hypha.

c) Enlarged view of subtending hypha of *Funneliformis geosporum* (Nicolson & Gerdemann) Walker & Schüßler. Arrows indicating wall structure, septum, inner wall of subtending hypha, subtending hypha.

d) Spore of *Funneliformis mosseae* (Nicolson & Gerdemann) Walker & Schüßler. Arrows indicating wall structure, funnel-shaped subtending hypha.

e) Broken spore of *Claroideoglomus claroideum* (Schenck & Smith) Walker & Schüßler. Arrows indicating wall structure, subtending hypha.

f) Sporocarp of *Glomus macrocarpum* Tulasne & Tulasne. Arrows indicating wall structure, sporocarpic hyphae, subtending hyphal attachment.

Plate 3.5

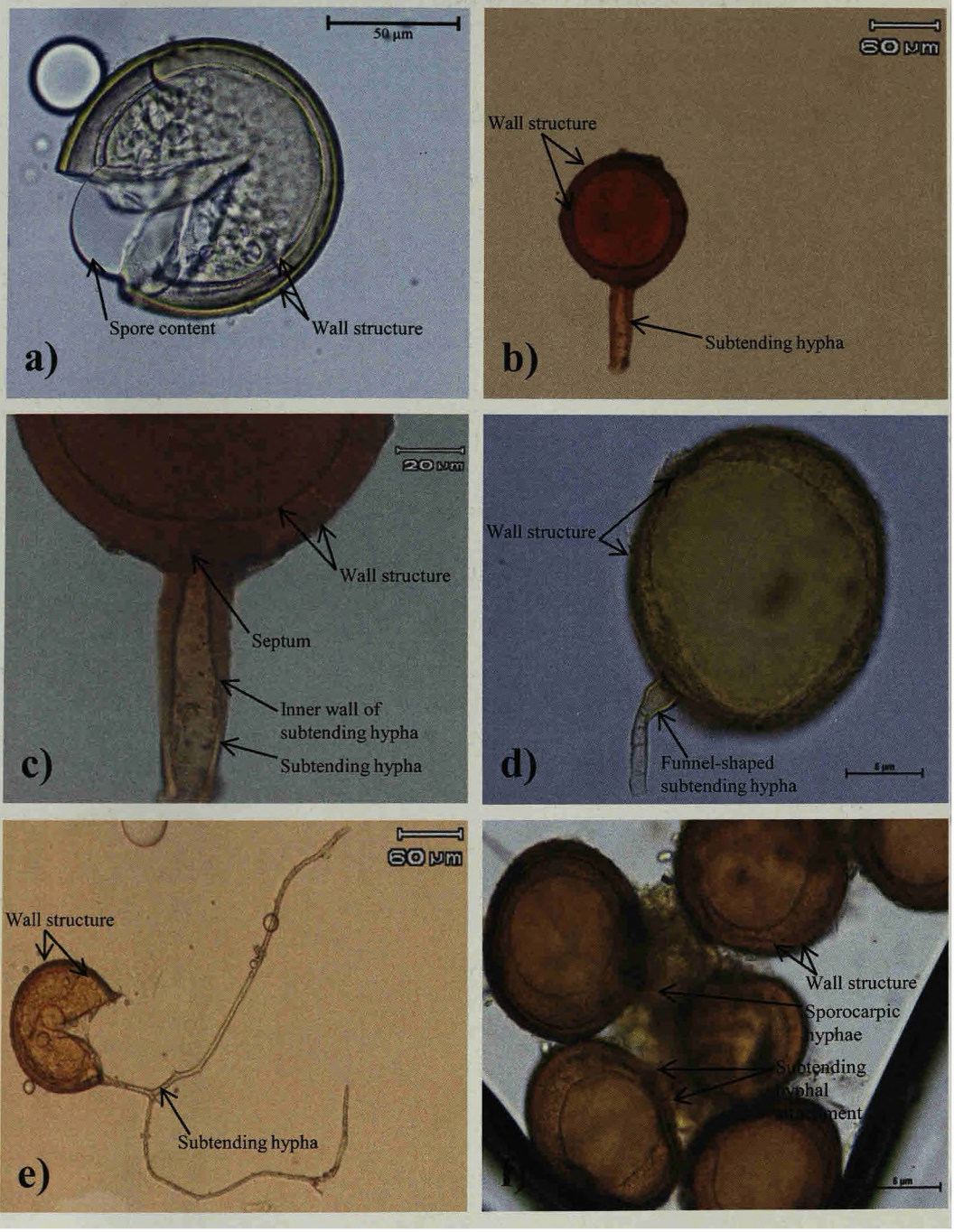

50 µm
Spore content
Wall structure
a)
60 µm
Wall structure
Subtending hypha
b)
20 µm
Wall structure
Septum
Inner wall of subtending hypha
Subtending hypha
c)
Wall structure
Funnel-shaped subtending hypha
d)
60 µm
Wall structure
Subtending hypha
e)
Wall structure
Sporocarpic hyphae
f)

Plate 3.6: Micrographs of AM fungal species extracted from selected study sites describing morphological features.

a) Broken spore of *Gigaspora albida* Schenck & Smith. Arrows indicating wall structure, sporogenous cell.

b) Broken spore of *Gigaspora gigantea* (Nicolson & Gerdemann) Gerdemann & Trappe. Arrows indicating wall structure, sporogenous cell.

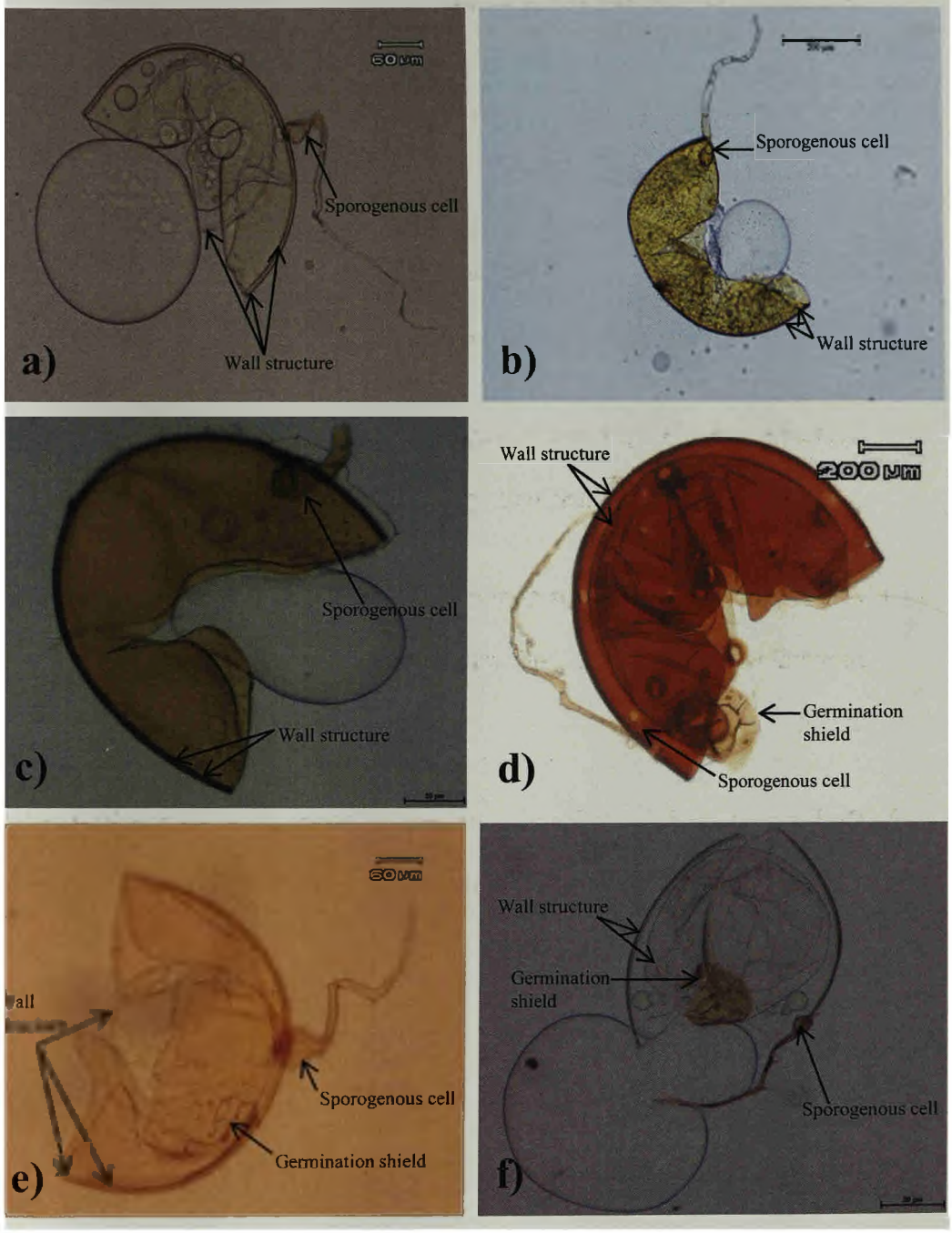
c) Broken spore of *Gigaspora ramisporophora* Spain, Sieverding & Schenck. Arrows indicating wall structure, sporogenous cell.

d) Broken spore of *Racocetra gregaria* (Schenck & Nicolson) Oehl, Souza & Sieverding. Arrows indicating wall structure, sporogenous cell, germination shield.

e) Broken spore of *Scutellospora pellucida* (Nicolson & Schenck) Walker, Sanders. Arrows indicating wall structure, germination shield, sporogenous cell.

f) Broken spore of *Scutellospora scutata* Walker & Diederichs. Arrows indicating wall structure, germination shield, sporogenous cell.

Plate 3.6


50 µm
Sporogenous cell
Wall structure
a)
Sporogenous cell
Wall structure
b)
Sporogenous cell
Wall structure
c)
Wall structure
200 µm
Germination shield
Sporogenous cell
d)
50 µm
Sporogenous cell
Germination shield
e)
Wall structure
Germination shield
Sporogenous cell
f)

3.3.4: AM species richness

AM species richness was highest at Varca (S4) (16 AM species site^{-1}) and least at Sinquerim (S5), Vagator (S7), Arambol (S10) and Kerim (S11) (2 AM species site^{-1}) each respectively **(Fig. 3.2)**.

3.3.5: AM Spore abundance and relative abundance

Highest AM spore abundance was recorded for *Acaulospora scrobiculata* (314 spores 100 g^{-1} soil) at Kerim (S11) and least for *Funneliformis geosporum* (2 spores 100 g^{-1} soil) at Colva (S2) and *Racocetra gregaria* (2 spores 100 g^{-1} soil) at Morjim (S8) **(Table 3.14)**.

Highest relative abundance of AM species was recorded for *Acaulospora scrobiculata* (98.22 %) at Kerim (S11) and least for *Claroideoglomus claroideum* (0.38 %) at Varca (S4) **(Table 3.15)**.

3.4: DISCUSSION

Edaphic factors play an essential role in establishment of AM symbiosis. The relationship between mycorrhizal colonization and physico-chemical parameters of soil varies distinctly (Smith and Read, 2008). AM root colonization levels can vary over a wide range of soil pH, phosphate, and salinity concentrations (Muthukumar and Udaiyan, 2002; Abdel Latef and Chaoxing, 2011; Chmura and Gucwa-Przepióra, 2012; Owens et al., 2012). In the present study, it was observed that the pH was acidic to alkaline ranging from 6.3-8.6. Soil pH is one of the important factors regulating spore germination and AM development (Maun, 2009). AM species have a variable pH optimum for spore germination, root colonization and growth (Hayman and Tavares,

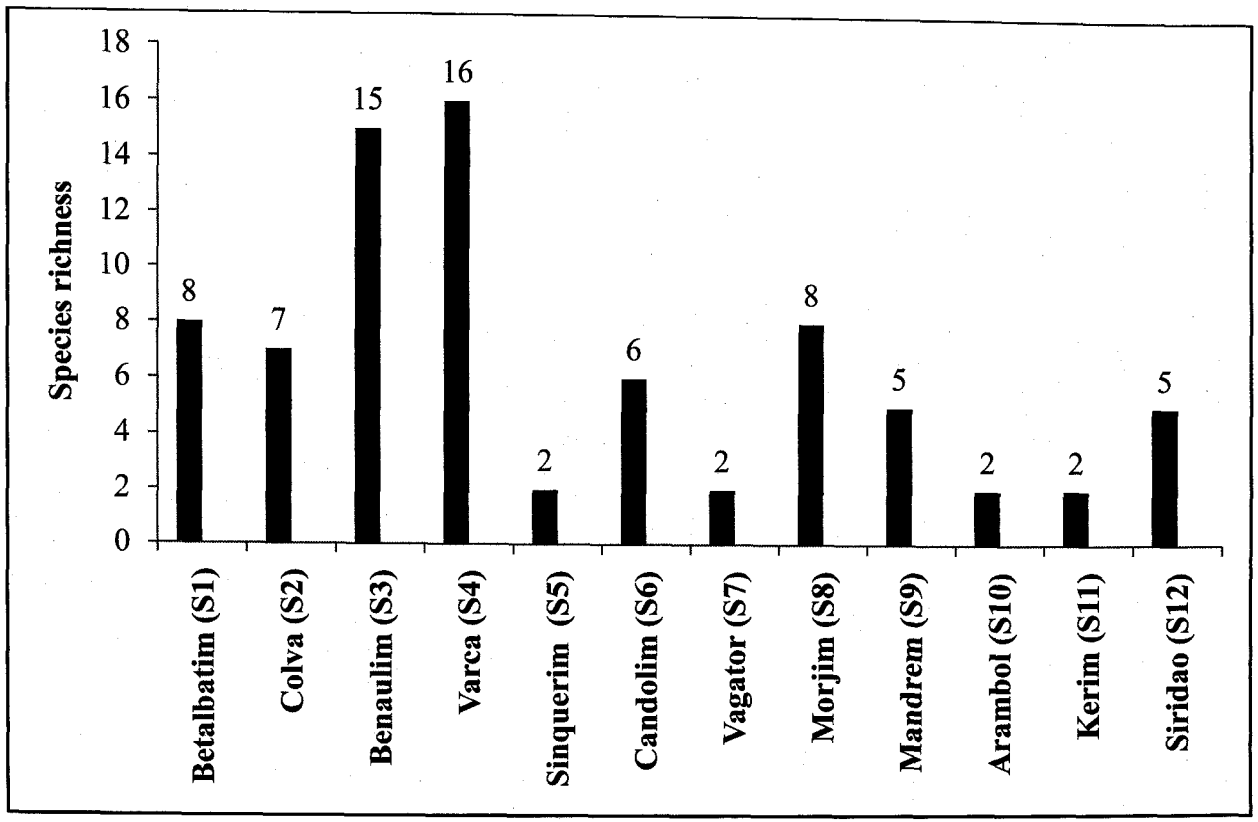


Fig. 3.2: AM fungal species richness in the selected sites.

Table 3.14: Spore abundance of AM fungal species in the selected sites.

Sr. No.	AM species	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
1.	*Acaulospora delicata*	257	14	101	30	179	84	nd	44	13	10	nd	42
2.	*Acaulospora scrobiculata*	299	108	144	130	146	26	70	143	147	60	314	44
3.	*Acaulospora bireticulata*	nd	nd	4	5	nd	nd	nd	nd	40	nd	nd	nd
4.	*Acaulospora rehmii*	285	96	111	112	nd	28	nd	nd	38	nd	nd	10
5.	*Acaulospora elegans*	111	10	17	63	nd	nd	nd	nd	nd	nd	nd	nd
6.	*Acaulospora nicolsonii*	nd	nd	10	33	nd	nd	nd	nd	nd	nd	nd	nd
7.	*Acaulospora foveata*	nd	nd	3	6	nd	nd	nd	nd	nd	nd	nd	nd
8.	*Acaulospora dilatata*	nd	nd	nd	21	nd	nd	nd	nd	nd	nd	nd	nd
9.	*Rhizoglomus fasciculatum*	nd	5	7	6	nd	16	nd	nd	nd	nd	nd	nd
10.	*Rhizoglomus intraradices*	nd	nd	nd	56	nd	nd	nd	nd	nd	nd	nd	nd
11.	*Rhizoglomus manihotis*	30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12.	*Rhizoglomus clarum*	nd	nd	60	nd	nd	nd	nd	nd	nd	nd	nd	nd
13.	*Funneliformis geosporum*	134	2	5	16	nd	8	nd	nd	nd	nd	nd	3
14.	*Funneliformis mosseae*	32	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15.	*Claroideoglomus claroideum*	nd	nd	4	3	nd	nd	nd	nd	nd	nd	nd	nd
16.	*Glomus macrocarpum*	nd	nd	nd	nd	nd	nd	nd	3	nd	nd	nd	nd
17.	*Gigaspora albida*	252	77	62	149	nd	56	78	144	105	nd	5	4
18.	*Gigaspora gigantea*	nd	nd	57	41	nd	nd	nd	64	nd	nd	nd	nd
19.	*Gigaspora ramisporophora*	nd	nd	nd	nd	nd	nd	nd	7	nd	nd	nd	nd
20.	*Racocetra gregaria*	nd	nd	54	77	nd	nd	nd	2	nd	nd	nd	nd
21.	*Scutellospora pellucida*	nd	nd	27	30	nd	nd	nd	nd	nd	nd	nd	nd
22.	*Scutellospora scutata*	nd	nd	nd	nd	nd	nd	nd	4	nd	nd	nd	nd

Legend: nd = AM species not detected in the sites; S = site; S1 = Betalbatim, S2 = Colva, S3 = Benaulim, S4 = Varca, S5 = Sinquerim, S6 = Candolim, S7 = Vagator, S8 = Morjim, S9 = Mandrem, S10 = Arambol, S11 = Kerim, S12 = Siridao.

Table 3.15: Relative abundance (%) of AM fungal species in the selected sites.

Sr. No.	AM species	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
1.	*Acaulospora delicata*	18.34	4.47	15.14	3.85	55.02	38.41	nd	10.70	3.78	14.22	nd	40.38
2.	*Acaulospora scrobiculata*	21.34	34.54	21.58	16.68	44.87	11.89	47.19	34.79	42.81	85.32	98.22	42.31
3.	*Acaulospora bireticulata*	nd	nd	0.59	0.64	nd	nd	nd	nd	11.65	nd	nd	nd
4.	*Acaulospora rehmii*	20.34	30.70	16.64	14.37	nd	12.80	nd	nd	11.06	nd	nd	9.61
5.	*Acaulospora elegans*	7.92	3.19	2.54	8.08	nd	nd	nd	nd	nd	nd	nd	nd
6.	*Acaulospora nicolsonii*	nd	nd	1.49	4.23	nd	nd	nd	nd	nd	nd	nd	nd
7.	*Acaulospora foveata*	nd	nd	0.44	0.77	nd	nd	nd	nd	nd	nd	nd	nd
8.	*Acaulospora dilatata*	nd	nd	nd	2.69	nd	nd	nd	nd	nd	nd	nd	nd
9.	*Rhizoglomus fasciculatum*	nd	1.59	1.04	0.77	nd	7.31	nd	nd	nd	nd	nd	nd
10.	*Rhizoglomus intraradices*	nd	nd	nd	7.18	nd	nd	nd	nd	nd	nd	nd	nd
11.	*Rhizoglomus manihotis*	2.14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
12.	*Rhizoglomus clarum*	nd	nd	8.99	nd	nd	nd	nd	nd	nd	nd	nd	nd
13.	*Funneliformis geosporum*	9.56	0.63	0.74	2.05	nd	3.65	nd	nd	nd	nd	nd	2.88
14.	*Funneliformis mosseae*	2.28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15.	*Claroideoglomus claroideum*	nd	nd	0.59	0.38	nd	nd	nd	nd	nd	nd	nd	nd
16.	*Glomus macrocarpum*	nd	nd	nd	nd	nd	nd	nd	0.72	nd	nd	nd	nd
17.	*Gigaspora albida*	17.99	24.62	9.29	19.12	nd	25.61	52.58	35.03	30.58	nd	1.56	3.84
18.	*Gigaspora gigantea*	nd	nd	8.54	5.26	nd	nd	nd	15.57	nd	nd	nd	nd
19.	*Gigaspora ramisporophora*	nd	nd	nd	nd	nd	nd	nd	1.70	nd	nd	nd	nd
20.	*Racocetra gregaria*	nd	nd	8.09	9.88	nd	nd	nd	0.48	nd	nd	nd	nd
21.	*Scutellospora pellucida*	nd	nd	4.04	3.85	nd	nd	nd	nd	nd	nd	nd	nd
22.	*Scutellospora scutata*	nd	nd	nd	nd	nd	nd	nd	0.97	nd	nd	nd	nd

Legend: nd = AM species not detected in the sites; S = site; S1 = Betalbatim, S2 = Colva, S3 = Benaulim, S4 = Varca, S5 = Sinquerim, S6 = Candolim, S7 = Vagator, S8 = Morjim, S9 = Mandrem, S10 = Arambol, S11 = Kerim, S12 = Siridao.

1985) and for most AM species it ranges between pH 5.0 and 8.0 (Maun, 2009). Most Gigasporaceae members occur in soils with pH 5.3 or lower, whilst most Glomeraceae and Acaulosporaceae members occur at pH 6.1 or higher (Tiwari et al., 2008). However, changes in soil pH can also result in changes in the soil chemical composition. Mycorrhizal association also depends upon soil type, available forms of nutrients especially P, plant species as well as AM species involved (Gaur and Kaushik, 2011). In the present study, organic carbon percentage was low ranging from 0.04-0.43 %. Organic matter content also affects AM spore number (Gaur and Kaushik, 2011). In the present study, available P content was low-high ranging from 16.4-60.1 Kg/Ha. Phosphorus is one of the chief plant macro-nutrients. The total spore density was highest in soil with P content around 16.4 Kg/Ha. Benefits of AM fungi increase especially in P deficient soils (Ruiz et al., 2011). In the present study, available K content was low-medium ranging from 50.4-347 Kg/Ha. Potassium is reported to have a stimulatory effect on AM fungi and soil K minimal level is often a pre-requisite for root colonization in some plant species (Ouimet et al., 1996; Gamage et al., 2004). In the present study, micro-nutrients *i.e.* Zn, Fe, Mn, Cu, and B were present in traces-high levels. AM fungi also have a positive influence on uptake of micro-nutrients that are vital for metabolic processes in plants (Marschner, 1995; Lehmann and Rillig, 2015). Concentrations of micro-nutrients play significant roles in shaping AM fungal communities either by triggering or suppressing spore germination, root colonization and mycelial growth (Gildon and Tinker, 1981; Moreira and Siqueira, 2002; Luis et al., 2006; Ortas and Akpinar, 2006; Motha et al., 2014; Alguacil et al., 2016).

In the present study, majority of the coastal plant species sampled showed characteristic AM fungal structures in the roots, signifying functionally active AM symbiosis. However, a variation was observed in the degree of root colonization of the plant

species sampled from the different sites. Environmental conditions, habitat differences, soil disturbance, and form of interaction between host plant, symbiont and the environment can lead to variations in the root colonization levels (Brundrett, 1991; Boddington and Dodd, 2000; Hindumathi and Reddy, 2011). Mycorrhizal colonization was also observed in root fragments of *Alternanthera ficoidea* (Amaranthaceae). Amaranthaceae and other non-mycorrhizal families such as Cactaceae, Chenopodiaceae, Cyperaceae and Juncaceae have been reported to be mycorrhizal under natural stress conditions (Neeraj et al., 1991). Low levels of soil nutrients in coastal ecosystems can also lead to the dependency of dune vegetation on mutualistic associations such as by AM fungi. AM fungal colonization is also observed in these non-mycorrhizal families when they co-occur with mycorrhizal families/plants (Hirrel et al., 1978; Miller et al., 1983).

In the present study, variations were observed in the AM spore density of rhizosphere soils of all the plant species sampled. The levels of spore density also varied between the same plant species sampled at different sites. Total spore density also varied at different sites. AM fungal spore density in coastal sand dunes is generally low and varies in different geographic locations depending upon environmental stresses, climate, sand movement and plant communities (Maun, 2009). However, sub-tropical regions usually contain higher spore numbers g^{-1} of soil than temperate regions (Sridhar and Beena, 2001). Physico-chemical characteristics of the respective dunes also contribute to the differences in spore density (Rose, 1988) and brisk changes in these characteristics can greatly affect AM spore numbers (Abbott and Robson, 1991). In general, the key factors involved in shaping natural AM fungal communities worldwide are macroclimatic changes followed by overall environmental variables such as vast differences in geology, soil type and soil pH, and the plant communities that arise under

such edapho-climatic conditions (Kivlin et al., 2011; Öpik et al., 2013). At local or regional levels, AM communities are more influenced by microclimatic changes followed by a sequence of soil properties such as pH, organic matter, texture and hydrology as well as shifts in natural plant communities (Ji et al., 2012; Silva et al., 2014b; Njeru et al., 2015). Intricate and competitive interactions between plant species and AM fungi, even within AM species, can also affect AM fungal community structure both on macro- and micro-scales (Horn et al., 2014).

The present study recorded a rich diversity of AM species associated with dune vegetation. The study recorded *Acaulospora* as the dominant genus followed by *Rhizoglomus*, *Gigaspora*, *Scutellospora*, *Funneliformis*, *Claroideoglomus*, *Glomus*, and *Racocetra*. *Acaulospora scrobiculata* was the most abundant AM species recorded from all the study sites. Willis (2013) recovered four AM genera *viz.*, *Acaulospora*, *Gigaspora*, *Scutellospora* and *Glomus* in dune vegetation from Morjim in north Goa, India. Members of AM fungal genera such as *Acaulospora*, *Glomus*, *Gigaspora* and *Scutellospora* are most prevalent in coastal areas worldwide (Maun, 2009). A study of the AM fungal status of plants from semi-arid and arid regions of India revealed association of the genera *Glomus*, *Gigaspora* and *Acaulospora* with most of the plants (Mukerji and Kapoor, 1986). Acaulosporaceae and Glomeraceae members usually produce more spore numbers as compared to Gigasporaceae members within the same environment (Bever et al., 1996; Suresh and Nagarajan, 2010). This may be due to the difference in their development, Acaulosporaceae and Glomeraceae members are thought to require less time for sporulation as compared to Gigasporaceae members which typically establish an extensive extra-radical mycelial network in the rhizosphere and hence produce less spore numbers (Hart and Reader, 2002; Piotrowski et al., 2004).

Acaulospora scrobiculata has been reported as the most common AM species in coastal sand dunes around the world (Jobim and Goto, 2016).

In this study, AM species richness was highest at Varca (S4) (16 AM species site^{-1}). AM species richness is a measure of the number of species whose spores are extracted from rhizosphere soil samples (McGee, 1989; Abbott and Gazey, 1994). It has been reported that AM species richness is dependent on the sample size *i.e.* the greater the number of rhizosphere samples collected, the greater the number of AM species is likely to be extracted (Radhika and Rodrigues, 2010). The sampling depth can also influence the composition and richness of AM species (Tiwari et al., 2008). AM fungal species richness and composition also varies with habitat type, landscape, plant community, and climatic conditions (Helgason et al., 1998; Wubet et al., 2004; Maun, 2009). It can also vary among the samples from a particular site and among different sites (Mueller, 2011) which can overall have an impact on the sporulation potential. However in general, relatively low AM species richness has been recorded in semiarid and arid regions (Tiwari et al., 2008).

In the present study, the dominant AM species was determined according to spore abundance and relative abundance. *Acaulospora* was recorded as the dominant genus with eight species *viz., A. bireticulata, A. delicata, A. dilatata, A. elegans, A. foveata, A. nicolsonii, A. rehmii* and *A. scrobiculata.* Spore abundance and relative abundance was highest in *A. scrobiculata* indicating its wide distribution at all study sites. Jaiswal (2002) reported *Acaulospora spinosa, A. scrobiculata, Glomus macrocarpum, Gigaspora margarita* and *Scutellospora weresubiae* as dominating AM species in terms of occurrence and abundance in coastal sand dune vegetation of Goa. Willis (2013) reported *Acaulospora spinosa, A. scrobiculata, Gigaspora margarita* and *Scutellospora*

gregaria as dominating species in terms of spore abundance in dune vegetation from Morjim in north Goa, India. Spore population results from complex interactions between fungus, plant and habitat, and is governed by a number of factors *viz.,* initial spore counts, physico-chemical properties of soil, host plant genotype and vegetation cover which greatly affect AM spore abundance (Tiwari et al., 2008; Wang et al., 2013). The incidence of an AM species and its abundance are determined chiefly by the presence and abundance of compatible host plant species that allow the fungus to grow and sporulate (Mueller, 2011). AM fungal communities in arid sites are made up of small-spore species mostly belonging to the genera *Glomus*, *Acaulospora* and *Entrophospora* (Stutz and Morton, 1996; Stutz et al., 2000). Fast growing and reproducing AM species with small spore size spread easily and have sporulation patterns adapted to fluctuating environmental conditions, thus having better chance of survival than large spore species (Dandan and Zhiwei, 2007; Yang et al., 2011). AM fungal species have a specific multi-dimensional niche that is modulated by the plant community present at a site and also by the physical and chemical composition of soil at that particular site, thus resulting in large variations in the composition and volume of AM fungal taxa between and within the site (Burrows and Pfleger, 2002; Ahulu et al., 2006).

3.5: CONCLUSION

Results obtained in the present study depicted good AM fungal association with sand dune vegetation. This indicates a dependency of dune vegetation on AM fungi for beneficial effects of the symbiosis, supporting adaptation to the dune ecosystem. The results of the present study also depicted that AM fungal root colonization, spore density, spore abundance and species richness varied among the selected study sites.

The data suggest a variable influence of several ecological factors, each of which may play an influential role in shaping AM communities in sand dune systems.

Diversity of AM fungi is one of the key factors contributing to the sustainable maintenance of plant biodiversity and ecosystem functioning (Van der Heijden et al., 1998; Bender et al., 2016). Thus, signifying the need for inclusion of these beneficial soil fungi in environmental preservation policies implemented for conservation of dune habitats.

CHAPTER 4

To prepare pure culture inoculum using trap and pot cultures. (Objective 2)

4.1: INTRODUCTION

In addition to the numerous beneficial effects of AM fungal symbiosis on overall growth and development of plant communities, the importance of AM fungi for agriculture, horticulture, reforestation programs and for reclamation of degraded areas is well supported (Jarstfer and Sylvia, 1992; Caravaca et al., 2002; Johansson et al., 2004; Douds Jr et al., 2007; Souza et al., 2010; Ijdo et al., 2011). As AM fungi are ubiquitous in nature their taxonomical, functional and genetic diversity is directly connected to plants and soils (Bever et al., 1996; Oehl et al., 2003), emphasizing the importance of assessment of diversity of AM communities (Lovelock and Ewel, 2005).

Diversity of AM communities is mainly described by extracting, counting and identifying the field collected propagules (spores). However, field collected spores can be problematic as they may be parasitized, not viable or present in low numbers under certain circumstances, they may lose or changes may occur in their appearance as the spore wall structure is prone to deterioration caused by various factors (root pigments, soil chemistry, temperature, moisture and microbial activity) in the rhizosphere (https://invam.wvu.edu/methods/cultures/trap-culture) thereby hindering a more accurate species identification. Sometimes the AM diversity recovered in the rhizosphere may represent only those AM species which are sporulating at the time of sampling (Brundrett et al., 1999). Therefore preparation and establishment of trap cultures of field collected rhizosphere soil samples represents an approach to increase spore number and to recover a large number of intact, viable, healthy spores which can be readily identified and used for initiation of monospecific or single species cultures as well as giving an idea of indigenous species diversity present in a habitat. Trap culture method, however, may not necessarily allow the identification of all the AM species

present as sporulation might be affected by host plant chosen for trapping or in some cases it may promote sporulation of cryptic AM species which weren't sporulating at the time of field sampling (Bever et al., 1996; Stürmer, 2004). Regardless, the trap culture method is still widely used to isolate local/native AM fungi, to establish monospecific cultures and develop inoculum for various research objectives, and to obtain knowledge of indigenous species diversity of an ecosystem all of which may provide an insight about AM fungal biology. Therefore, the present study is aimed at establishing and mass multiplying pure cultures of indigenous AM fungi isolated from rhizosphere soils of dune plants.

4.2: MATERIALS AND METHODS

4.2.1: Preparation of trap cultures

Propagation of AM fungi was carried out by a trap culture method modified from Morton et al. (1993) **(Plate 4.1 a-b)** wherein rhizosphere soil (along with roots) was mixed with sterilized sand in 1:1 ratio. The mixture was then transferred to 15 cm plastic pots which were pre-wiped with absolute alcohol. *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) cuttings were used as the host plant. The pots were maintained for a period of 90 days in the greenhouse for establishment of AM symbiosis and continuance of AM life cycle (colonization and sporulation). After 90 days, the pots were left to dry undisturbed (1-2 weeks), following which the shoot portion was cut off at the soil interface. The pot contents were then transferred in zip-loc plastic bags, labelled and stored at 4°C to eliminate or reduce dormancy period for at least 30 days. The spores were then extracted for taxonomic identification and to set up monospecific cultures.

Plate 4.1: Micrographs of preparation of pure culture inoculum using trap and monospecific cultures.

a) Rhizosphere soil sample collected from study site.

b) Trap cultures established by using rhizosphere soil sample.

c) Mixed AM fungal spores extracted and isolated from trap cultures.

d) Monospecific (single species) cultures established by isolating and identifying spores extracted from trap cultures.

e) *Funneliformis mosseae* spores extracted and isolated from monospecific cultures.

f) *Rhizoglomus intraradices* spores extracted and isolated from monospecific cultures.

g) Root fragments colonized by *Rhizoglomus intraradices* extracted and isolated from monospecific cultures.

h) Mass multiplication of AM fungal inocula using cuttings of *Plectranthus scutellarioides* (L.) R.Br. as the host plant.

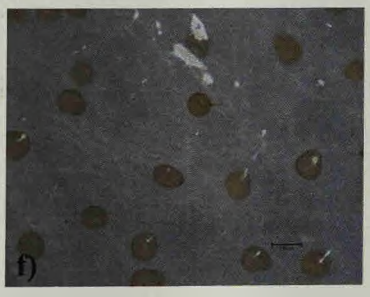
Plate 4.1

Rhizosphere soil sample

Trap cultures

Mixed spores isolated from trap cultures

Monospecific cultures



Spores and colonized root fragments isolated from monospecific cultures

Mass multiplication

4.2.2: Preparation of monospecific (single species) cultures

Monospecific cultures of AM species isolated from trap cultures were prepared (Gilmore, 1968) **(Plate 4.1 c-d)** wherein spores were extracted from trap cultures by Wet Sieving and Decanting method (Gerdemann and Nicolson, 1963) as described in Chapter 3. After repeated washing, the spores of each single species were isolated and stored in a Petri-plate at 4°C. The isolated spores were examined daily by viewing under Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom) for any changes in morphology *i.e.* loss of spore contents, colour change, damage or parasitism and such spores were excluded. Before inoculation, any soil particles or hyphal fragments or atypical spores were removed and the spores were washed with autoclaved distilled water. Plastic pots (15 cm) were filled with sterilized 1:1 sand:soil mix, in which the isolated spores (single species) were placed along with the Whatman filter paper at a depth of 2-3 cm, covered with additional medium and planted with *P. scutellarioides* cuttings in the pots. The pots were maintained in the glasshouse (27°C, 63 % relative humidity) and watered twice a week. Hoagland's solution (Hoagland and Arnon, 1950) minus P was applied after every 20 days. After 90 days of growth period, watering was ceased so as to allow the pot contents to dry. The pot contents were then harvested for analysis of spores and to develop mass inoculum **(Plate 4.1 e-h)**.

Microscopic observations were carried out using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x). Micrographs were imaged by Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras, and were not digitally edited.

4.3: RESULTS

4.3.1: Monospecific (single species) cultures

Eight out of 22 AM fungal species recovered from selected study sites were successfully mass multiplied as monospecific cultures using *P. scutellarioides* as the host plant. These included *Acaulospora scrobiculata*, *A. delicata*, *A. rehmii*, *Funneliformis geosporum*, *F. mosseae*, *Gigaspora albida*, *Racocetra gregaria* and *Rhizoglomus intraradices*. These AM species were mass multiplied and maintained as a live culture in the glasshouse of Arbuscular Mycorrhizal Culture Collection unit at Goa University. The cultures were subsequently used for the further studies described in later chapters.

4.4: DISCUSSION

In the present study, 8 out of 22 AM species were mass multiplied as monospecific cultures prepared from trap cultures. It has been reported that frequent collection of rhizosphere soil samples or preparation of successive trap cultures can greatly assist in the assessment of AM species composition in natural habitats (Stutz et al., 2000). Freshly collected rhizosphere soil samples can be maintained in trap culture so as to minimize the loss or viability of spores (Brundrett et al., 1999). However many variables such as variations in host root type and morphology, carbon biomass, and nutrient levels can affect composition/richness of AM fungi isolated from trap cultures (Cuenca and Meneses, 1996; Stutz and Morton, 1996; Brundrett et al., 1999).

Preparation of trap cultures and monospecific cultures are the most widely adopted methods used to maintain AM spores or inocula because of relatively low technical support needed and inexpensive consumables. Although the culture obtained from these

methods may not be highly pure *i.e.* no guarantee can be made of the absence of unwanted contaminants even if strict quality control measures are applied (Ijdo et al., 2011). However these methods are the least artificial (live host plants are used) and help in understanding the biology of AM fungal lifecycle as well as in supporting production and storage of the inocula for a longer duration.

CHAPTER 5

To prepare and standardize the protocol for *in vitro* culture technique for dominant AM fungal species. (Objective 3)

5.1: INTRODUCTION

The most common method used to propagate AM fungal propagules has been the pot culture technique. Though this technique is the least artificial, the culture obtained may not be high in purity *i.e.* there is no guarantee of contaminant-free inoculum even if strict quality control measures are applied (Ijdo et al., 2011). Further the technique is space and time consuming, and requires regular assessment of viability and monospecificity of inocula produced, all complicating the attainment of quality cultures (http://invam.wvu.edu).

The monoxenic culture or root organ culture (ROC) technique based on *Agrobacterium rhizogenes* Conn. transformed roots and untransformed roots allows the establishment of AM fungi under *in vitro* conditions (Mosse and Hepper, 1975; Mugnier and Mosse, 1987; Bécard and Fortin, 1988; Diop et al., 1992; Bago et al., 1996; Adholeya et al., 2005; Ijdo et al., 2011). When grown on a synthetic growth medium in association with transformed roots, AM fungal cultures can be maintained pure and viable for long duration. Advantages of the ROC technique include reliability of cultures *i.e.* production of contaminant free cultures, monospecificity of inoculum, morphological observations of the fungal life cycle can be conducted without disturbing the system, lower space and time requirements as the cultures are maintained under controlled growth chamber conditions, easy fungal growth quantification and inoculum quality control. Despite being artificial in nature, the *in vitro* technique is efficient for physiological, molecular and biochemical studies and mass inoculum production of AM fungi. There are however downsides to monoxenic culture system, such as the necessity of technical expertise, laboratory equipment, working under sterile conditions of laminar airflow, low sporulation levels for some species, number of strains cultivated

in vitro, difficulty in continuous cultivation of some strains, and physiological and genetic changes overtime due to successive culturing (http://invam.wvu.edu).

Nonetheless, due to the production of high quality and contamination free inocula that is both effective and efficient for large scale inoculum production the monoxenic culture system is an appropriate practice in the study of AM fungi. To scale up AM fungal inoculum production, it is essential to identify and select the species with the maximum potential for spore production using this system (Declerck et al., 2001; Ijdo et al., 2011). Therefore, the present study is aimed at preparing and standardizing the protocol of *in vitro* culture technique for dominant AM fungal species.

5.2: MATERIALS AND METHODS

5.2.1: Extraction of AM fungal propagules

Extraction of AM propagules (spores and colonized root fragments) from rhizosphere soil samples and monospecific cultures was carried out by Wet Sieving and Decanting technique (Gerdemann and Nicolson, 1963) as described in Chapter 3.

5.2.2: Monoxenic culture establishment:

5.2.2.1: AM fungal propagule disinfection process

For monoxenic culture establishment, isolated propagules were first rinsed twice with sterilized distilled water after which they were disinfected. The disinfection process was modified from Mosse (1959), Mertz et al. (1979), Daniels and Menge (1981), and Bécard and Fortin (1988) wherein five different combinations of sterilizing agents were tested *i.e.* i) 0.05 % Tween 20 + 2% chloramine T (10 min), ii) 0.5 % NaClO (sodium hypochlorite) + 2 % chloramine T (3 min), iii) 2 % chloramine T + ethanol (95-98 %) +

0.5 % NaClO (5-10 min), iv) 0.1-0.5 % $MgSO_4$+ 0.5 % NaClO (3-5 min), v) 250-400 µl NaClO (3-5 min). The concentrations and sterilization times varied from species to species (based on size of spores) and also depended upon the type of propagules used (spores or colonized root fragments). 250-400 µl NaClO (3-5 min) was found to be effective for all. Following disinfection, the propagules were rinsed three times with sterilized distilled water and treated with antibiotic solution (Streptomycin 0.02 % + Gentamycin 0.01 %) for 10 min.

5.2.2.2: Germination of disinfected propagules

For germination of disinfected AM propagules of eight AM species, 50 disinfected propagules (5 spores or colonized roots/Petri plate × 10 Petri plates) of each species were plated in triplicate on a Modified Strullu-Romand (MSR) minus sucrose medium and incubated in the dark at 27°C; the plates were kept in an inverted position. The pH, sucrose, nutrient compositions were varied so as to obtain maximum germination percentage. Although the pH varied from 4.8-5.5, pH 5.5 was found to be suitable for all AM species tested. The sucrose content was reduced to half the strength of as described by Declerck et al. (1998) and was found to be suitable for all AM species tested.

5.2.2.3: Culture media for cultivation of AM propagules and transformed roots

MSR medium (Declerck et al., 1998) was used as culture media for both cultivation of AM propagules and transformed roots.

5.2.2.4: Measurement of hyphal length of the germinated spores

Upon germination, hyphal length of the germinated spores was measured using ocular micrometer after every alternate day until the establishment of dual cultures.

5.2.2.5: Establishment of dual culture

For establishment of dual culture, clarigel plugs with germinated AM propagules were associated with actively growing Ri T-DNA transformed roots *i.e.* Chicory (*Cichorium intybus* L.) and Linum (*Linum usitatissimum* L.) roots **(Plate 5.1 a-b)**. The Petri plates were incubated in the dark in an inverted position at 27°C. The transformed roots were procured from Prof. B. F. Rodrigues, Department of Botany, Goa University.

5.2.2.6: Establishment of continuous culture

Continuous cultures were established by propagule re-association onto fresh MSR media (Chabot et al., 1992a), for which initially the monoxenically produced propagules were extracted from the gel (MSR media) using method by Cranenbrouck et al. (2005). The extracted monoxenic spores along with attached extra-radical mycelium were then associated with new actively growing transformed roots on fresh MSR media.

5.2.2.7: Extraction of monoxenically produced AM propagules

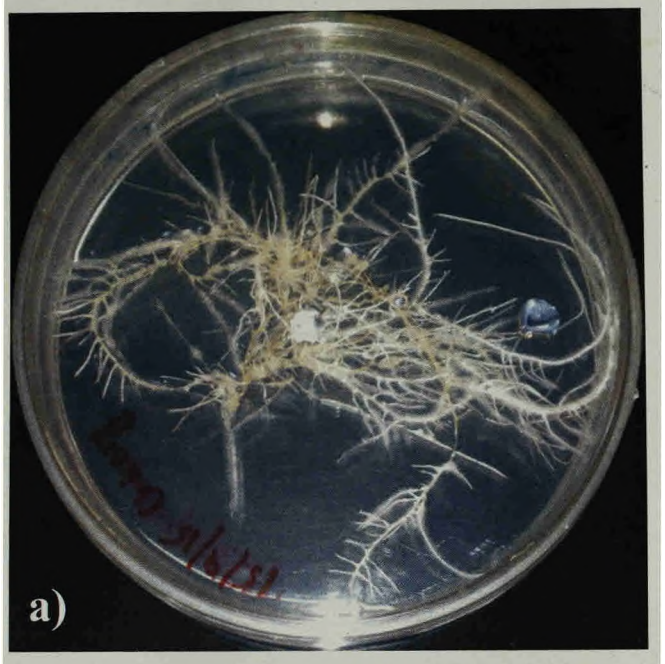
The monoxenically produced propagules were extracted from the gel (MSR media) by using method of Cranenbrouck et al. (2005), wherein a small piece of gel containing sufficient monoxenically produced spores with extra-radical mycelia was extracted from the mother culture and transferred into an empty sterile Petri plate. 25 ml citrate buffer (0.01 M) was filtered through sterile 0.22 µm syringe-driven MF Millipore Membrane Filter Unit (Millex ®- GS) and added to the Petri plate. The Petri plate was then agitated slowly so as to enable the gelling agent to dissolve. The spores attached to extra-radical mycelium were then transferred to a new sterile Petri plate containing sterile distilled water using micropipette by viewing under Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom), the entire procedure carried out under laminar flow.

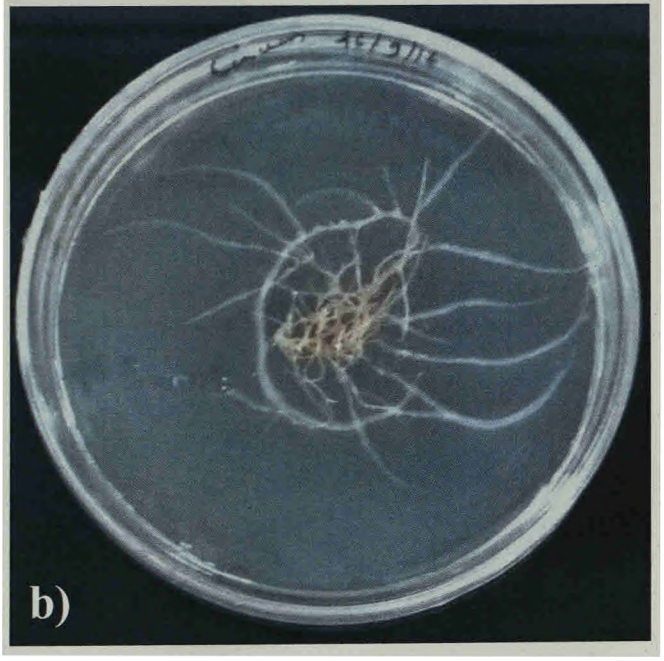
Plate 5.1: Micrographs of Ri T-DNA transformed roots growing on MSR media.

a) Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots.

b) Ri T-DNA transformed Linum (*Linum usitatissimum* L.) roots.

Plate 5.1





The extracted monoxenic spores along with attached extra-radical mycelium were then associated with new actively growing transformed roots on fresh MSR media.

5.2.3: Processing of transformed root segments for AM fungal colonization

Assessment of AM fungal colonization in transformed roots was carried out by using Trypan blue staining technique (Phillips and Hayman, 1970) as described in Chapter 3.

Microscopic examinations were carried out using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x). Micrographs were imaged by Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras, and were not digitally edited.

5.3: RESULTS

5.3.1: *In vitro* germination

In vitro germination was observed in 8 AM species *viz., Claroideoglomus claroideum, Rhizoglomus manihotis, R. clarum, R. intraradices, Funneliformis mosseae, Gigaspora albida, Racocetra gregaria* and *Acaulospora scrobiculata* **(Plate 5.2 a-g, Plate 5.3 a-f)**. The spore germination time was observed to vary from species to species **(Table 5.1)**. The percent spore germination *in vitro* was also found to vary from species to species, with maximum observed in *R. intraradices* and least in *Gi. albida* **(Fig. 5.1)**.

5.3.2: Hyphal length of the germinated spores

The hyphal length of *in vitro* germinated spores varied from species to species and it ranged from 72.90 µm to 656.10 µm. Average hyphal length was observed to be maximum in *R. intraradices* (573.07 ± 67.17 µm) and minimum in *A. scrobiculata* (153.90 ± 52.49 µm) (n = 6) **(Table 5.2)**.

Plate 5.2: Micrographs of *in vitro* germination of AM fungal propagules (spores and colonized root fragments) on MSR (-sucrose) media.

a) *In vitro* spore germination of *Claroideoglomus claroideum*. Arrows indicating germ tube emerging from spore wall, branching of hypha from germ tube.

b) *In vitro* spore germination of *Rhizoglomus manihotis*. Arrows indicating germ tube emerging from spore wall, branching of germ tube.

c) *In vitro* spore germination of *Rhizoglomus manihotis*. Arrows indicating two germ tubes emerging from spore wall.

d) *In vitro* germination of colonized root fragment of *Rhizoglomus clarum*. Arrows indicating multiple germination in the colonized root fragment.

e) *In vitro* spore germination of *Rhizoglomus clarum*. Arrows indicating germ tube emerging from spore wall, branching of germ tube.

f) *In vitro* germination of colonized root fragment of *Rhizoglomus intraradices*. Arrows indicating multiple germination in the colonized root fragment.

g) *In vitro* spore germination of *Rhizoglomus intraradices*. Arrows indicating two germ tubes emerging from spore wall.

Plate 5.2

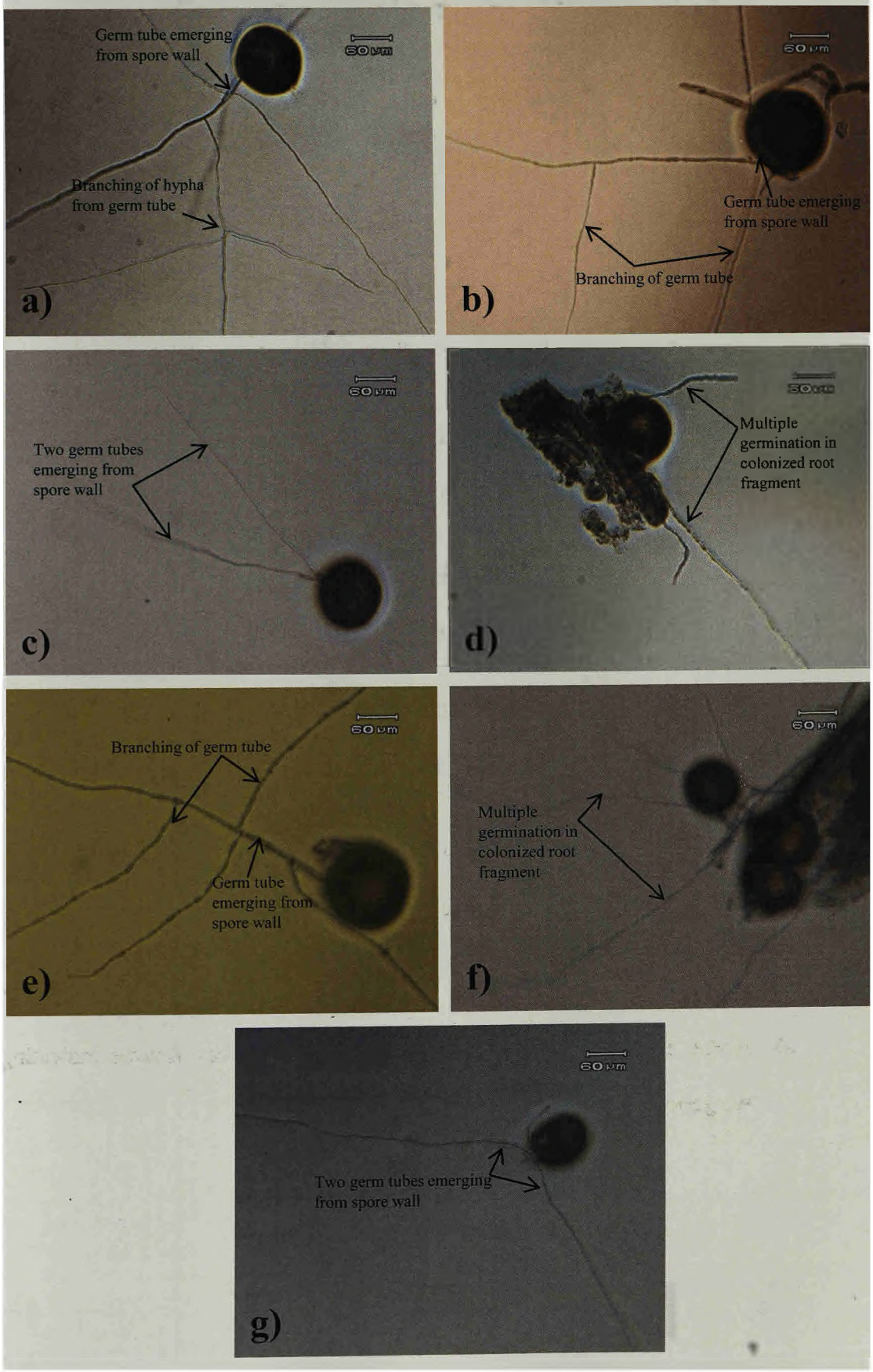

Germ tube emerging from spore wall
Branching of hypha from germ tube
a)
60 µm
Germ tube emerging from spore wall
Branching of germ tube
b)
Two germ tubes emerging from spore wall
c)
Multiple germination in colonized root fragment
d)
Branching of germ tube
Germ tube emerging from spore wall
e)
Multiple germination in colonized root fragment
f)
Two germ tubes emerging from spore wall
g)

Plate 5.3: Micrographs of *in vitro* germination of AM fungal propagules (spores and colonized root fragments) on MSR (-sucrose) media.

a) *In vitro* spore germination of *Funneliformis mosseae*. Arrows indicating re-growth of germ tube from the end of subtending hypha, branching of germ tube.

b) *In vitro* spore germination of *Funneliformis mosseae*. Arrows indicating re-growth of germ tube from the end of subtending hypha, two germ tubes emerging from spore wall.

c) *In vitro* spore germination of *Gigaspora albida*. Arrows indicating germ tube emerging from spore wall.

d) *In vitro* spore germination of *Racocetra gregaria*. Arrows indicating germ tube emerging from spore wall and branching.

e) *In vitro* spore germination of *Racocetra gregaria*. Arrows indicating multiple germ tubes emerging from spore wall.

f) *In vitro* spore germination of *Acaulospora scrobiculata*. Arrows indicating multiple germ tubes emerging from spore wall.

Plate 5.3

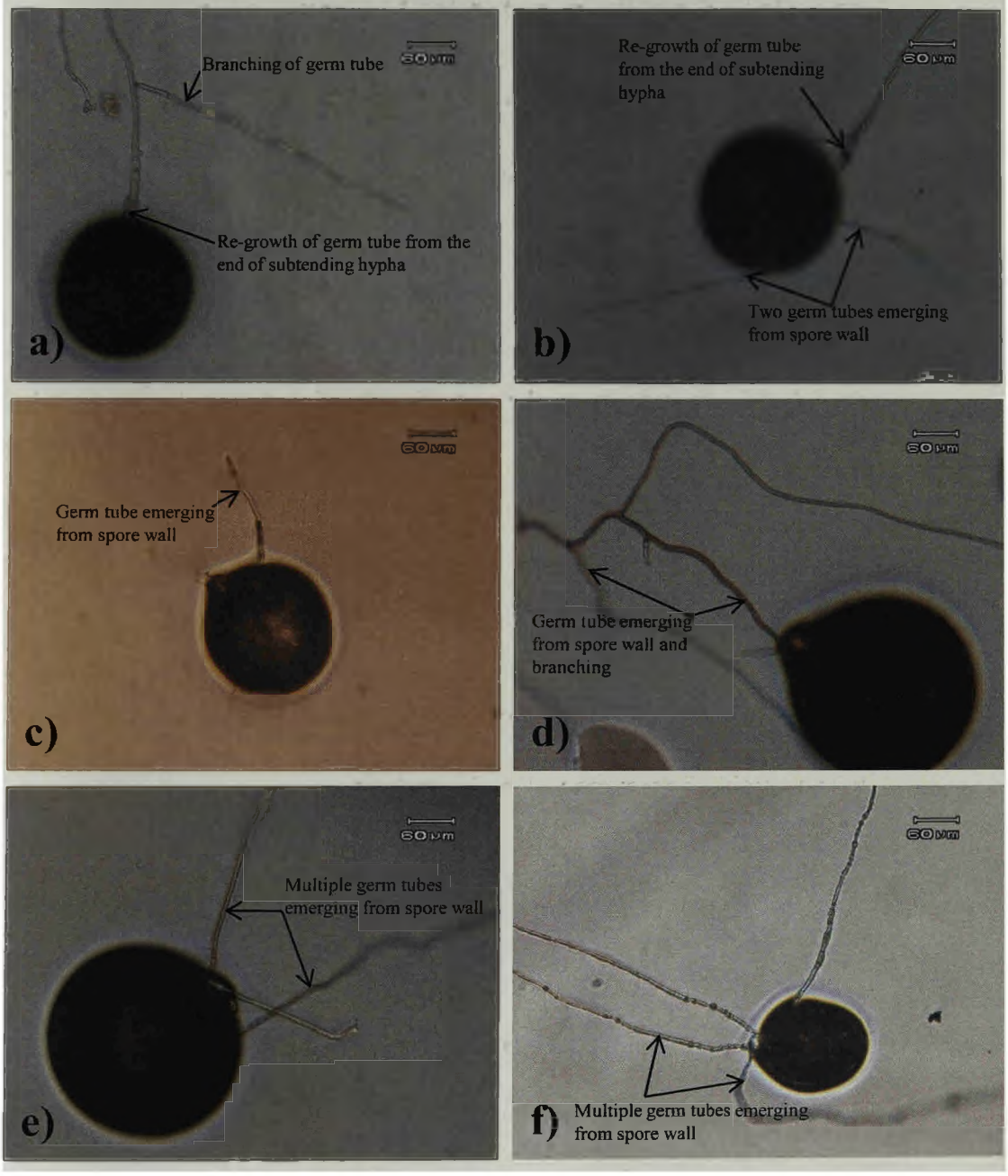

Branching of germ tube
Re-growth of germ tube from the end of subtending hypha
a)
Re-growth of germ tube from the end of subtending hypha
Two germ tubes emerging from spore wall
b)
Germ tube emerging from spore wall
c)
Germ tube emerging from spore wall and branching
d)
Multiple germ tubes emerging from spore wall
e)
Multiple germ tubes emerging from spore wall
f)

Table 5.1: Maximum spore germination time.

Sr. No.	AM species	Germination time (days after incubation)
1.	*Claroideoglomus claroideum*	19
2.	*Rhizoglomus manihotis*	18
3.	*Rhizoglomus clarum*	6-10
4.	*Rhizoglomus intraradices*	5-8
5.	*Funneliformis mosseae*	10-14
6.	*Gigaspora albida*	20
7.	*Racocetra gregaria*	6
8.	*Acaulospora scrobiculata*	23

Legend: n = 50.

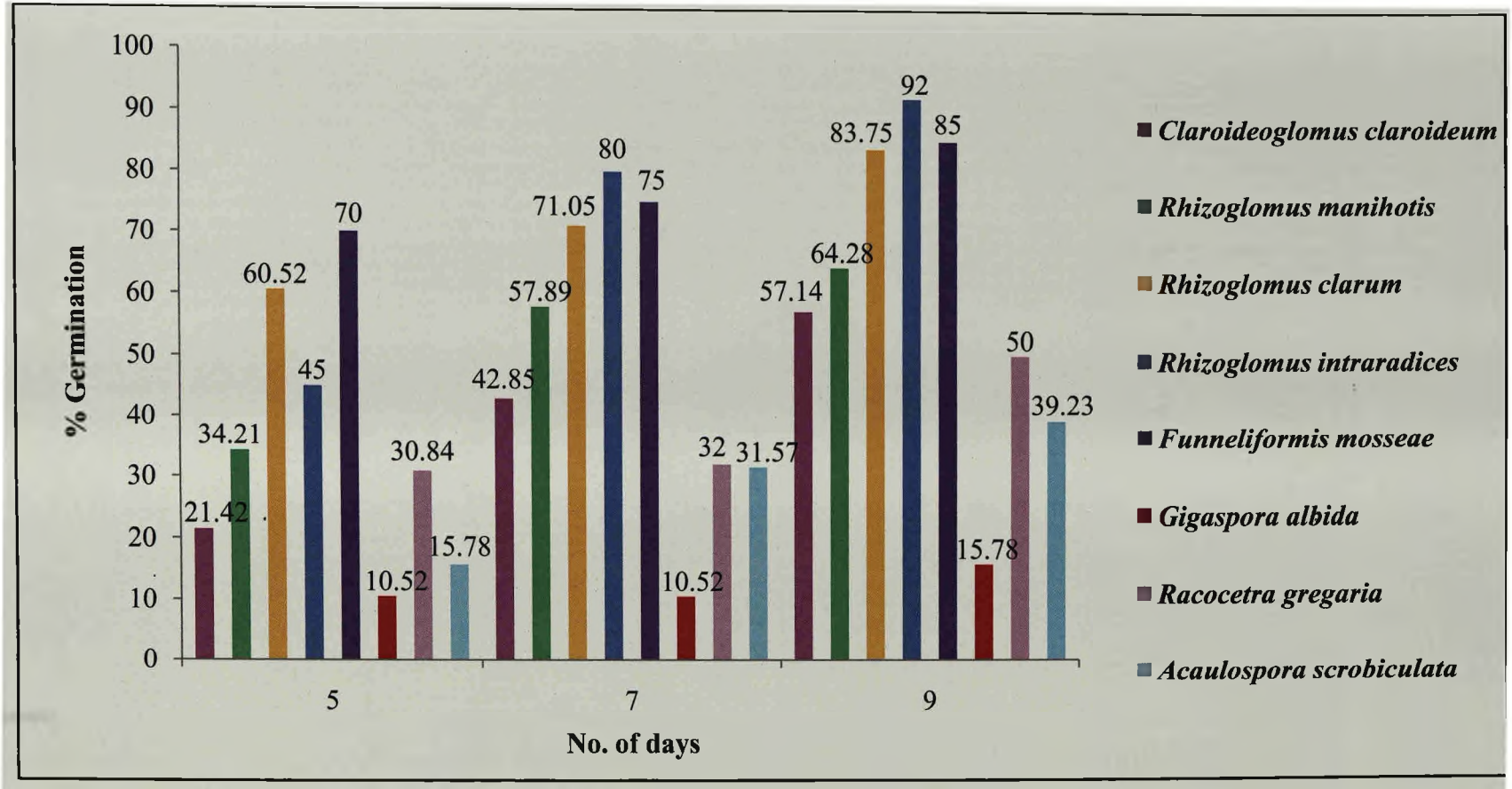


Fig. 5.1: Percent spore germination *in vitro*.

Table 5.2: Hyphal length in spores of AM species grown in *in vitro*.

AM fungi	Range of hyphal length (µm)	Average hyphal length (µm)
Claroideoglomus claroideum	291.60 – 413.10	352.35 ± 45.46
Rhizoglomus manihotis	340.20 – 486.00	413.10 ± 57.50
Rhizoglomus clarum	388.80 – 607.50	497.91 ± 59.75
Rhizoglomus intraradices	486.00 – 656.10	573.07 ± 67.17
Funneliformis mosseae	194.40 – 388.80	291.6 ± 70.42
Gigaspora albida	145.80 – 243.00	200.47 ± 37.45
Racocetra gregaria	170.10 – 340.20	281.46 ± 59.72
Acaulospora scrobiculata	72.90 – 218.70	153.90 ± 52.49

Legend: n = 6.

5.3.3: Dual cultures

Upon establishment of dual cultures, sporulation was observed in 4 AM species *viz., R. clarum* **(Plate 5.4 a-h, Plate 5.5 a-g),** *R. intraradices* **(Plate 5.6 a-h, Plate 5.7 a-h),** *F. mosseae* **(Plate 5.8 a-h, Plate 5.9 a-f)** and *A. scrobiculata* **(Plate 5.10 a-h).** In case of *A. scrobiculata*, adjacent attachment of the spore at the neck of a sporiferous saccule could not be observed. **Table 5.3** depicts comparison of sporulation dynamics of the monoxenically cultured species. In case of *Gi. albida* and *Ra. gregaria in vitro* growth was observed up to auxiliary cell formation in monoxenic culture **(Plate 5.11 a-b, Plate 5.12 a-h)** and sporulation did not occur even after repeated establishments of dual cultures.

5.3.4: Continuous cultures

Continuous cultures of *R. clarum*, *R. intraradices* and *F. mosseae* showing active re-growth of hyphae were obtained upon sub-culturing the colonized transformed root fragments and monoxenically produced spores along with extra-radical hyphae from the starter culture. In *R. clarum* re-growth was observed in 20 days, in *R. intraradices* re-growth was observed in 3 days, and in *F. mosseae* re-growth was observed in 12 days. In *A. scrobiculata* no re-growth was observed.

It was found that in *R. intraradices* and *F. mosseae,* the percent spore germination, frequency of culturing (sporulation) and sub-culturing (re-growth) were consistently high. Hence, these two AM species were screened for further studies.

Plate 5.4: Micrographs of monoxenic culture of *Rhizoglomus clarum* with Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots on MSR media.

a) Association of germinated colonized root fragment of *Rhizoglomus clarum* with transformed chicory root for establishment of dual culture. Arrows indicating colonized root fragment, transformed root, multiple germination of colonized root fragment.

b) Enlarged view of multiple germination of colonized root fragment of *Rhizoglomus clarum* in dual culture. Arrow indicating germ tube growth into runner hypha.

c) Initial contact between the hypha and the transformed root observed on tenth day after initiation of dual culture. Arrow indicating hyphal penetration into the root.

d) Germ tube growth as a straight growing runner hypha in the medium. Arrow indicating branching of runner hypha at right angles.

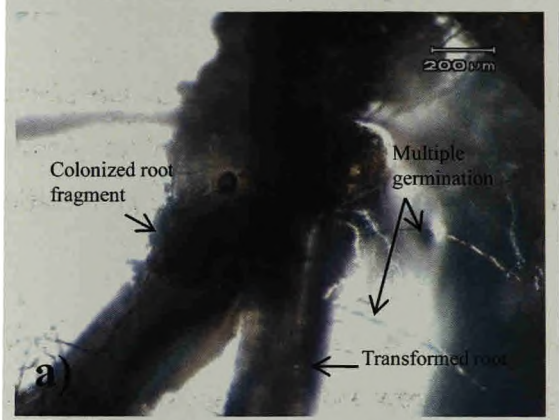
e) Well established extra-radical mycelium exploring the media. Arrow indicating extra-radical mycelium.

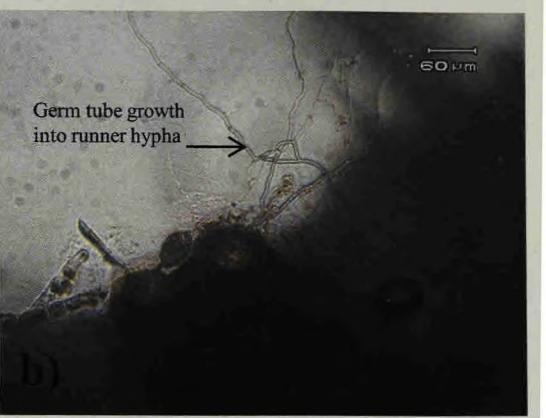
f) Branched absorbing structures (BAS) formed by extra-radical hypha. Arrows indicating BAS with dichotomous branching pattern.

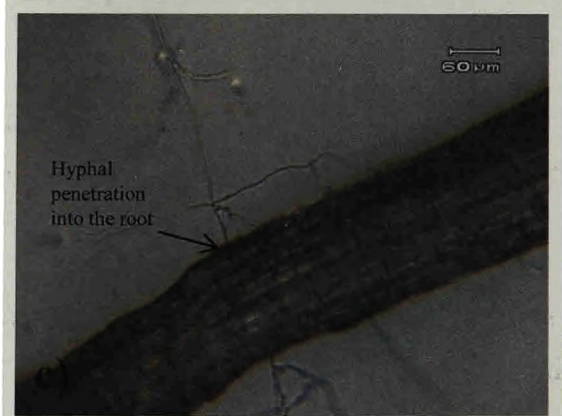
g) Intercalary position of spore along lateral branches of runner hypha. Arrows indicating intercalary spore, apical hypha.

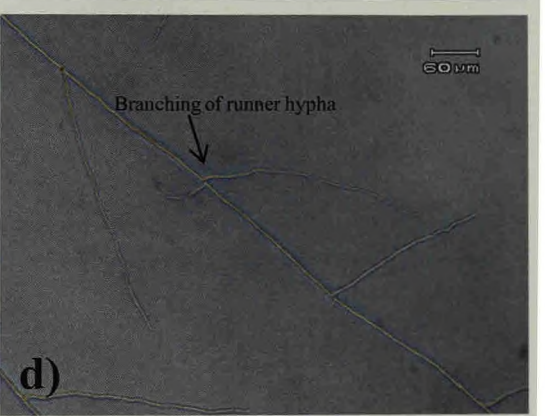
h) Terminal position of spore along lateral branches of runner hypha. Arrow indicating terminal spore.

Plate 5.4

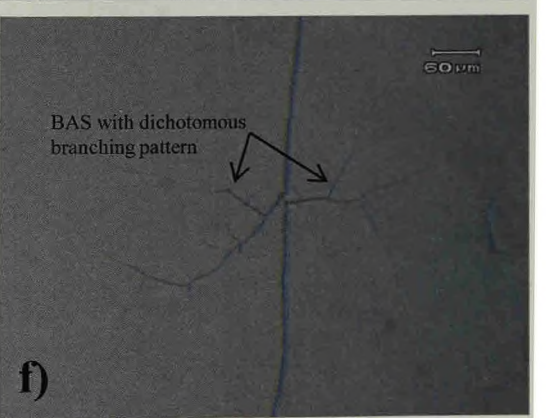

200 µm
Colonized root fragment
Multiple germination
Transformed root
a)

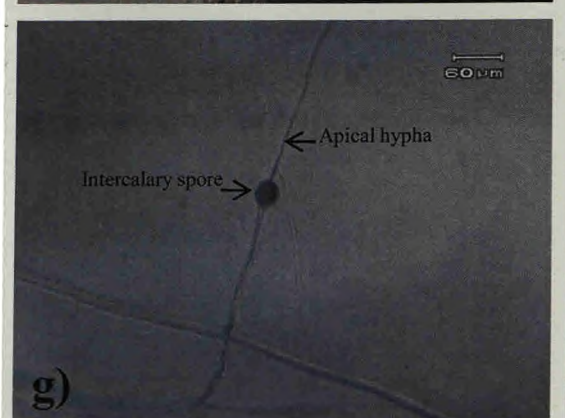

60 µm
Germ tube growth into runner hypha
b)


60 µm
Hyphal penetration into the root
c)


60 µm
Branching of runner hypha
d)


60 µm
Extra-radical mycelium
e)


60 µm
BAS with dichotomous branching pattern
f)


60 µm
Apical hypha
Intercalary spore
g)

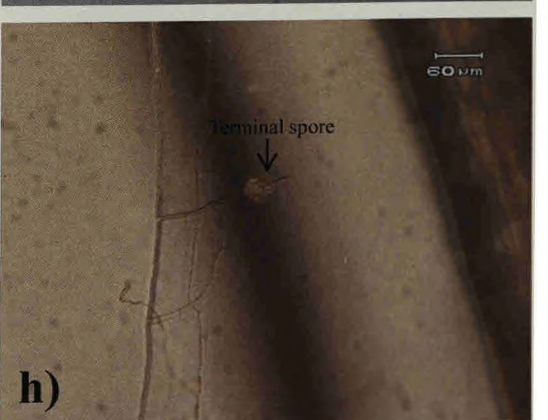

60 µm
Terminal spore
h)

Plate 5.5: Micrographs of monoxenic culture of *Rhizoglomus clarum* with Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots on MSR media.

a) Cluster of extra-radical spores on ramifications of runner hypha. Arrow indicating terminal spores.

b) Mass production of spores.

c) *In vitro* produced terminal spores. Arrow indicating thick spore wall.

d) Trypan blue stained transformed root showing intra-radical hyphal colonization. Arrow indicating intra-radical hyphae.

e) Trypan blue stained transformed root showing intra-radical hyphal and vesicular colonization. Arrows indicating mature vesicles, intra-radical hypha.

f) Trypan blue stained transformed root showing extra-radical hyphal colonization. Arrow indicating extra-radical hypha.

g) Trypan blue stained cluster of extra-radical spores. Arrows indicating terminal spores.

Plate 5.5

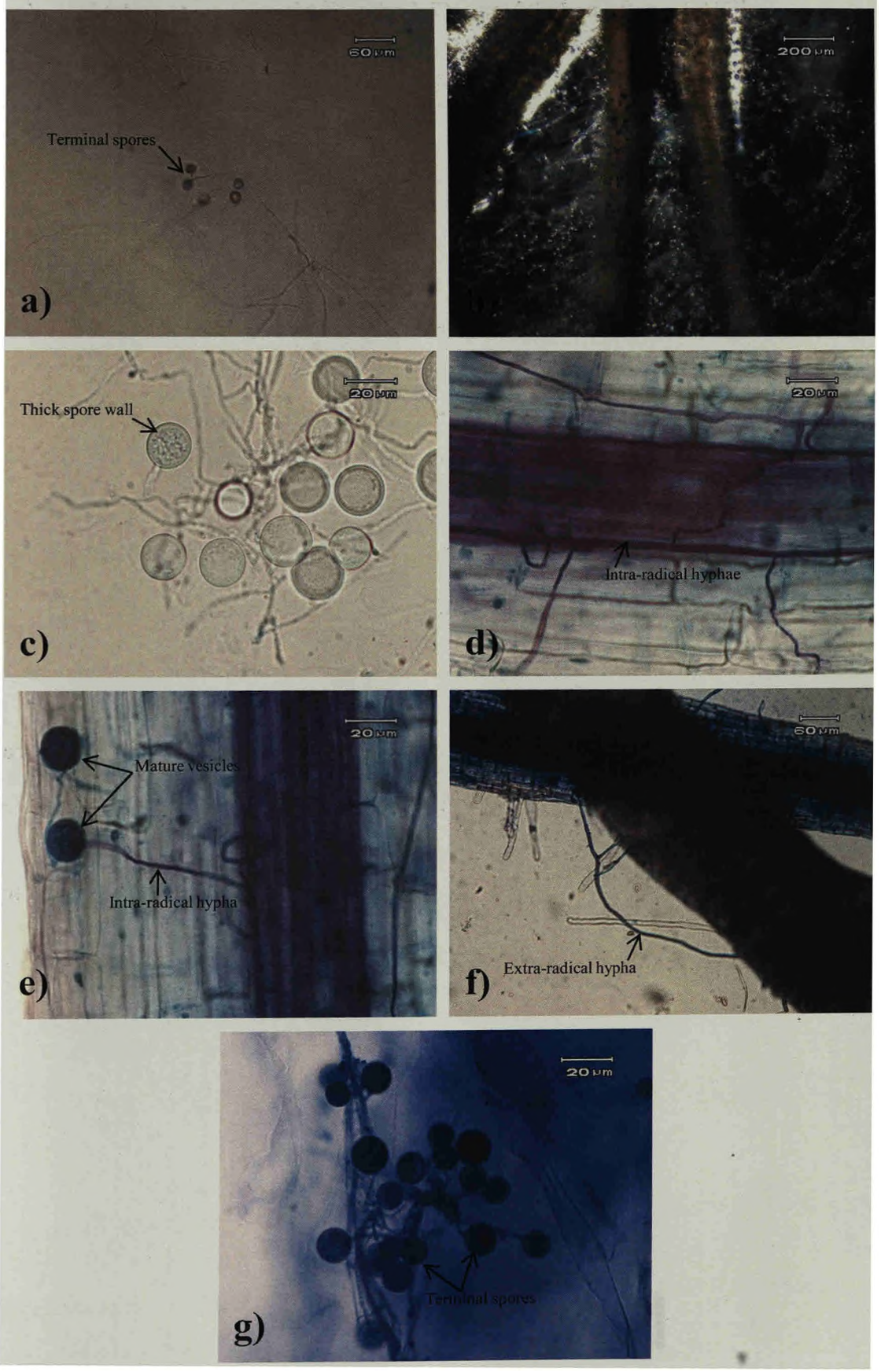

60 µm
Terminal spores
a)
200 µm
b)
20 µm
Thick spore wall
c)
20 µm
Intra-radical hyphae
d)
20 µm
Mature vesicles
Intra-radical hypha
e)
60 µm
Extra-radical hypha
f)
20 µm
Terminal spores
g)

Plate 5.6: Micrographs of monoxenic culture of *Rhizoglomus intraradices* with Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots on MSR media.

a) Association of germinated colonized root fragment of *Rhizoglomus intraradices* with transformed chicory root for establishment of dual culture. Arrows indicating colonized root fragment, transformed root, multiple germination of colonized root fragment.

b) Initial contact between the hypha and the transformed root observed on third day after initiation of dual culture. Arrow indicating hyphal penetration into the root

c) Germ tube growth as a straight growing runner hypha in the medium. Arrow indicating branching of runner hypha at right angles.

d) Well established extra-radical mycelium exploring the media. Arrow indicating extra-radical mycelium.

e) Branched absorbing structures (BAS) formed by extra-radical hypha. Arrows indicating BAS with dichotomous branching pattern.

f) Juvenile spore formation. Arrow indicating juvenile spore.

g) Terminal position of spores on sporogenic hypha. Arrows indicating terminal spores.

h) Disrupted senescing spore with differentiation of thin walled hyphae from inside. Arrows indicating senescing spore, thin walled hyphae.

Plate 5.6

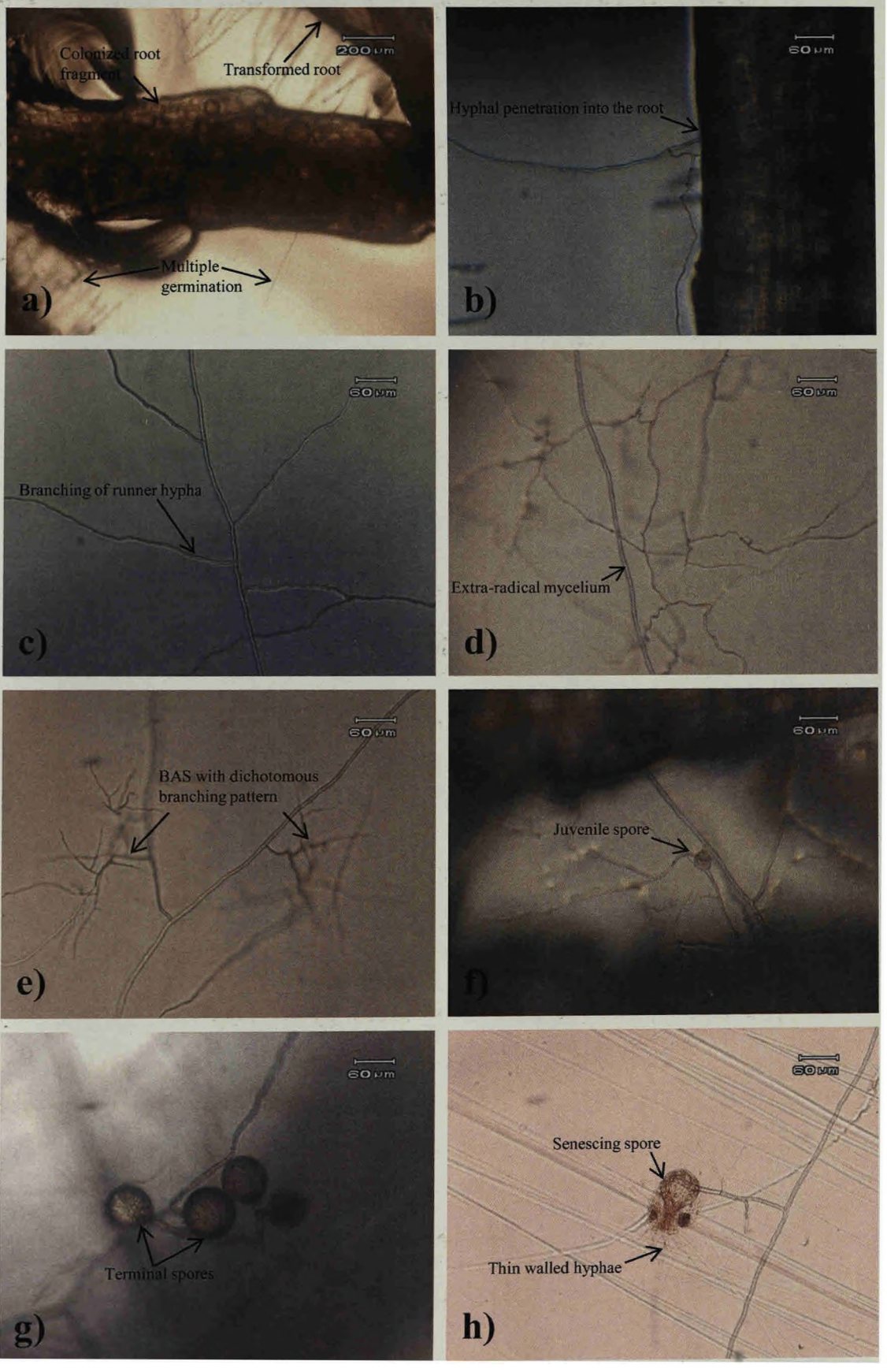

Colonized root fragment
Transformed root
200 µm
Multiple germination
a)
60 µm
Hyphal penetration into the root
b)
60 µm
Branching of runner hypha
c)
60 µm
Extra-radical mycelium
d)
60 µm
BAS with dichotomous branching pattern
e)
60 µm
Juvenile spore
f)
60 µm
Terminal spores
g)
60 µm
Senescing spore
Thin walled hyphae
h)

Plate 5.7: Micrographs of monoxenic culture of *Rhizoglomus intraradices* with Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots on MSR media.

a) Intra-radical vesicular colonization observed within the medium. Arrows indicating vesicles.

b) Intra-radical sporulation observed within the medium. Arrows indicating intra-radical spores.

c) Extra-radical sporulation observed within the medium. Arrows indicating extra-radical spores.

d) *In vitro* produced terminal spores. Arrow indicating thin laminated spore wall.

e) Trypan blue stained transformed root showing intra-radical hyphal and vesicular colonization (scale 50 µm). Arrows indicating intra-radical hyphae, vesicles.

f) Trypan blue stained transformed root showing intra-radical sporulation (scale 50 µm). Arrows indicating intra-radical spores.

g) Trypan blue stained cluster of extra-radical spores. Arrows indicating terminal spores.

h) Trypan blue stained intercalary spore (scale 50 µm). Arrows indicating intercalary spore, apical hypha.

Plate 5.7

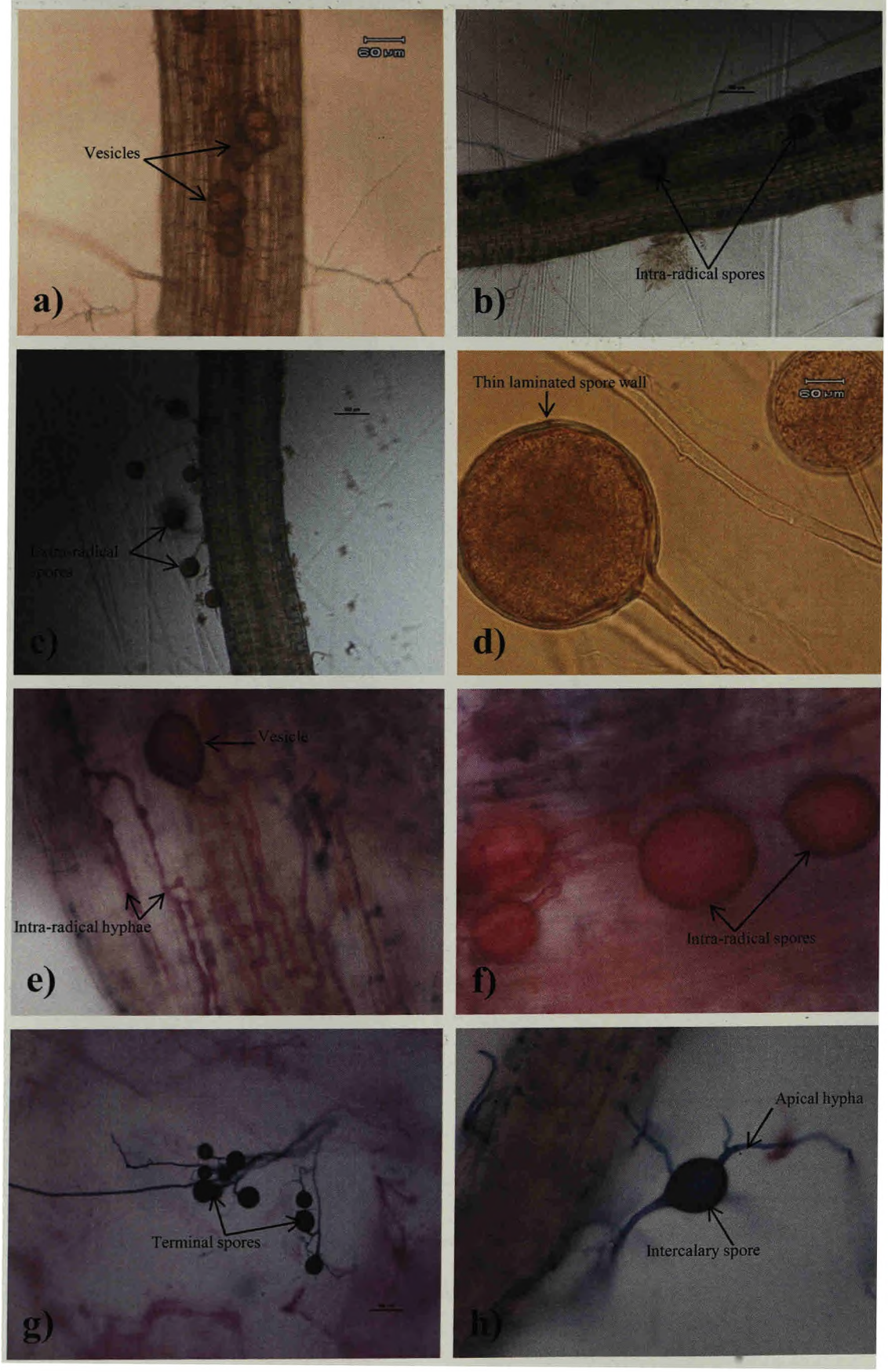

Vesicles
a)
Intra-radical spores
b)
c)
Thin laminated spore wall
d)
Vesicle
Intra-radical hyphae
e)
Intra-radical spores
f)
Terminal spores
g)
Apical hypha
Intercalary spore
h)

Plate 5.8: Micrographs of monoxenic culture of *Funneliformis mosseae* with Ri T-DNA transformed Linum (*Linum usitatissimum* L.) roots on MSR media.

a) Association of germinated spore of *Funneliformis mosseae* with transformed linum root for establishment of dual culture. Arrows indicating spore, germ tube, transformed root hair.

b) Initial contact between the hypha and the transformed root observed on third day after initiation of dual culture. Arrow indicating hyphal penetration into the root.

c) Germ tube growth as a straight growing runner hypha in the medium. Arrow indicating branching of runner hypha at right angles.

d) Well established extra-radical mycelium exploring the media. Arrow indicating extra-radical mycelium.

e) Branched absorbing structures (BAS) formed by extra-radical hypha entering degeneration process by cytoplasm retraction and septa formation. Arrows indicating BAS with dichotomous branching pattern, septa.

f) Formation of spore primordia on sporogenic hypha. Arrow indicating spore primordia.

g) Terminal juvenile spores on sporogenic hypha. Arrows indicating juvenile spores

h) Enlarged view of terminal juvenile spore on sporogenic hypha. Arrow indicating juvenile spore.

Plate 5.8

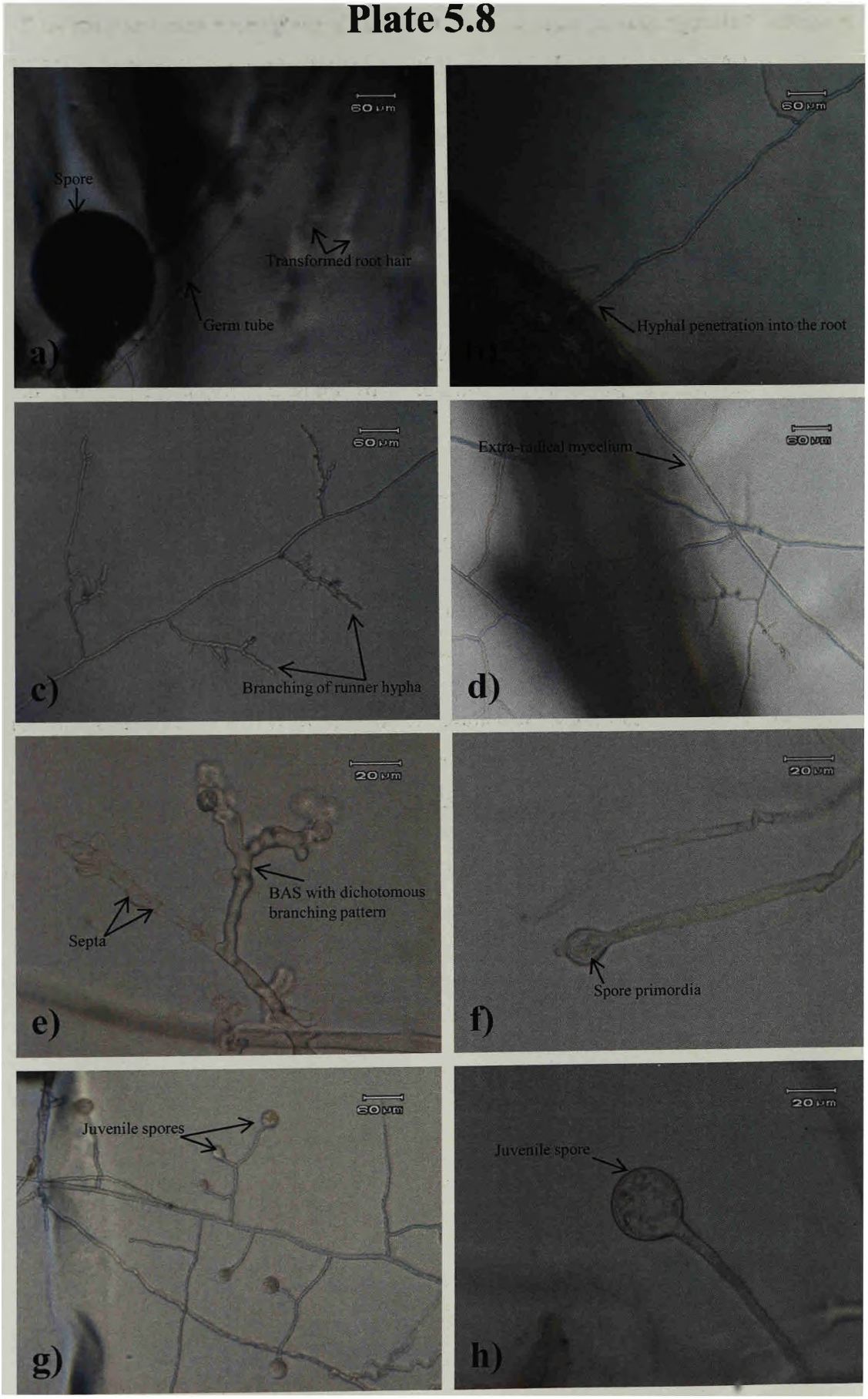

60 µm
Spore
Transformed root hair
Germ tube
a)
60 µm
Hyphal penetration into the root
b)
60 µm
Branching of runner hypha
c)
60 µm
Extra-radical mycelium
d)
20 µm
BAS with dichotomous branching pattern
Septa
e)
20 µm
Spore primordia
f)
60 µm
Juvenile spores
g)
20 µm
Juvenile spore
h)

Plate 5.9: Micrographs of monoxenic culture of *Funneliformis mosseae* with Ri T-DNA transformed Linum (*Linum usitatissimum* L.) roots on MSR media.

a) Mature terminal spores on sporogenic hypha. Arrows indicating terminal spores.

b) *In vitro* produced terminal spore. Arrows indicating thin laminated spore wall, funnel shaped hyphal attachment.

c) Intercalary spore. Arrows indicating intercalary spore, apical hypha.

d) Both patterns of sporulation on the same sporogenic hypha. Arrows indicating intercalary spore, terminal spore.

e) Trypan blue stained transformed root showing intra-radical hyphal colonization. Arrow indicating intra-radical hyphae.

f) Trypan blue stained transformed root showing hyphal coil. Arrow indicating hyphal coil.

Plate 5.9

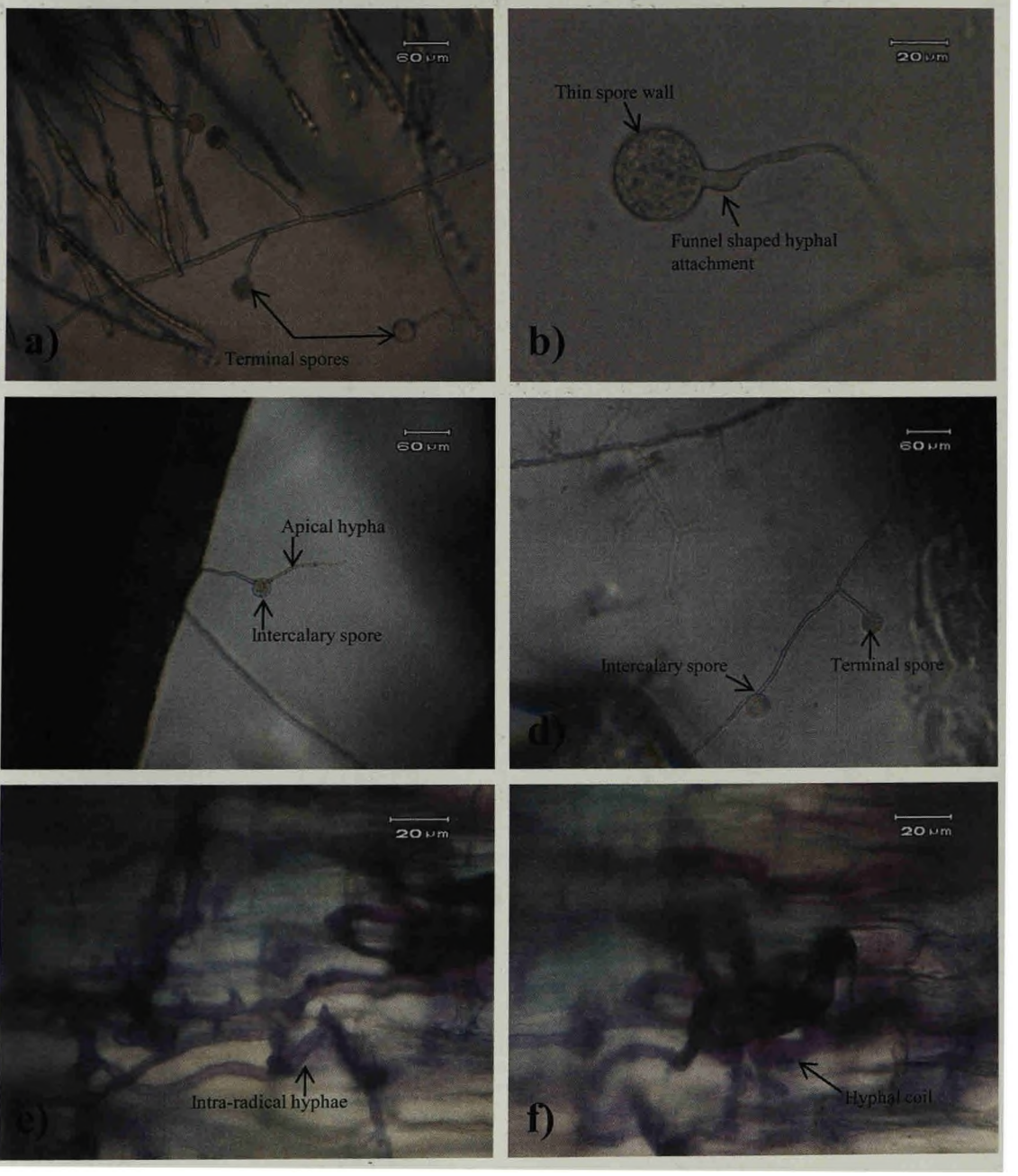

60 µm
a)
Terminal spores
20 µm
Thin spore wall
Funnel shaped hyphal attachment
b)
60 µm
Apical hypha
Intercalary spore
60 µm
Intercalary spore
Terminal spore
d)
20 µm
Intra-radical hyphae
e)
20 µm
Hyphal coil
f)

Plate 5.10: Micrographs of monoxenic culture of *Acaulospora scrobiculata* with Ri T-DNA transformed Linum (*Linum usitatissimum* L.) roots on MSR media.

a) Association of germinated spore of *Acaulospora scrobiculata* with transformed linum root for establishment of dual culture. Arrows indicating spore, germ tube, transformed root hair.

b) Initial contact between the hypha and the transformed root observed on tenth day after initiation of dual culture (scale 60 µm). Arrow indicating hyphal penetration into the root.

c) Germ tube growth as a straight growing runner hypha in the medium. Arrow indicating branching of runner hypha.

d) Well established extra-radical mycelium exploring the media (scale 60 µm) Arrow indicating extra-radical mycelium.

e) Branched absorbing structures (BAS) formed by extra-radical hypha (scale 60 µm) Arrow indicating BAS with dichotomous branching pattern.

f) Inflation of sporogenous hyphal apex into a claviform shape to form the globose sporogenous structure or sporiferous saccule (scale 60 µm). Arrow indicating inflation of sporogenous hyphal apex.

g) Formation of sporiferous saccule (scale 60 µm). Arrow indicating sporiferous saccule.

h) Mature terminal spore on sporogenic hypha. Arrow indicating spore.

Plate 5.10

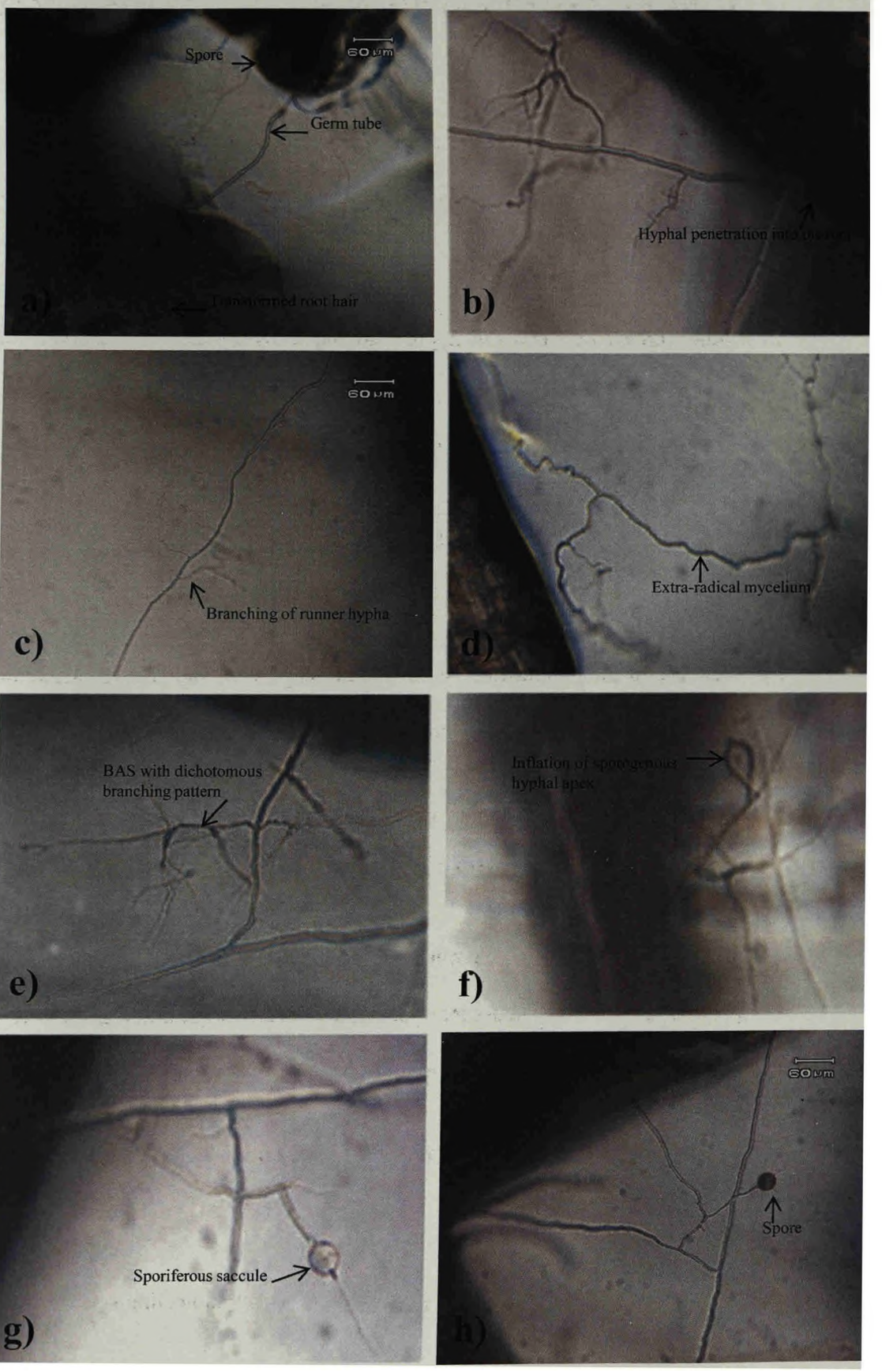

Spore
60 µm
Germ tube
a)
b)
Hyphal penetration into
60 µm
Branching of runner hypha
c)
Extra-radical mycelium
d)
BAS with dichotomous branching pattern
e)
f)
Sporiferous saccule
g)
60 µm
Spore
h)

Table 5.3: Sporulation dynamics in the four AM species grown under *in vitro* conditions.

Sr. No.	Monoxenic culture	Extra-radical mycelium			Spore position		Spore size (µm)	Spore colour	Number of spores/ Petri-plate	Sporulation time (days)	Percent colonization
		RH	BH	BAS	Intercalary	Terminal /Apical					
1.	*Rhizoglomus clarum* + transformed Chicory roots	+	+	+	+	+	72.90	Hyaline	>1000	120	95.23
2.	*Rhizoglomus intraradices* + transformed Chicory roots	+	+	+	+	+	121.50	Hyaline-reddish brown	>525	16	92.30
3.	*Funneliformis mosseae* + transformed Linum roots	+	+	+	+	+	102.50	Hyaline-reddish brown	>300	15	85.00
4.	*Acaulospora scrobiculata* + transformed Linum roots	+	+	+	-	+	80.00	Sub-hyaline-yellow	12	60	nd

Legend: RH = runner hyphae, BH = branched hyphae, BAS = branched absorbing structures; + = observed, - = not observed; nd = not detected.

Plate 5.11: Micrographs of monoxenic culture of *Gigaspora albida* with Ri T-DNA transformed Chicory (*Cichorium intybus* L.) roots on MSR media.

a) Association of germinated spore of *Gigaspora albida* with transformed chicory root for establishment of dual culture. Arrows indicating spore, germ tube, hyphal penetration into the root, transformed root.

b) Auxiliary cell formation by extra-radical hyphae. Arrow indicating papillate auxiliary cell.

Plate 5.11

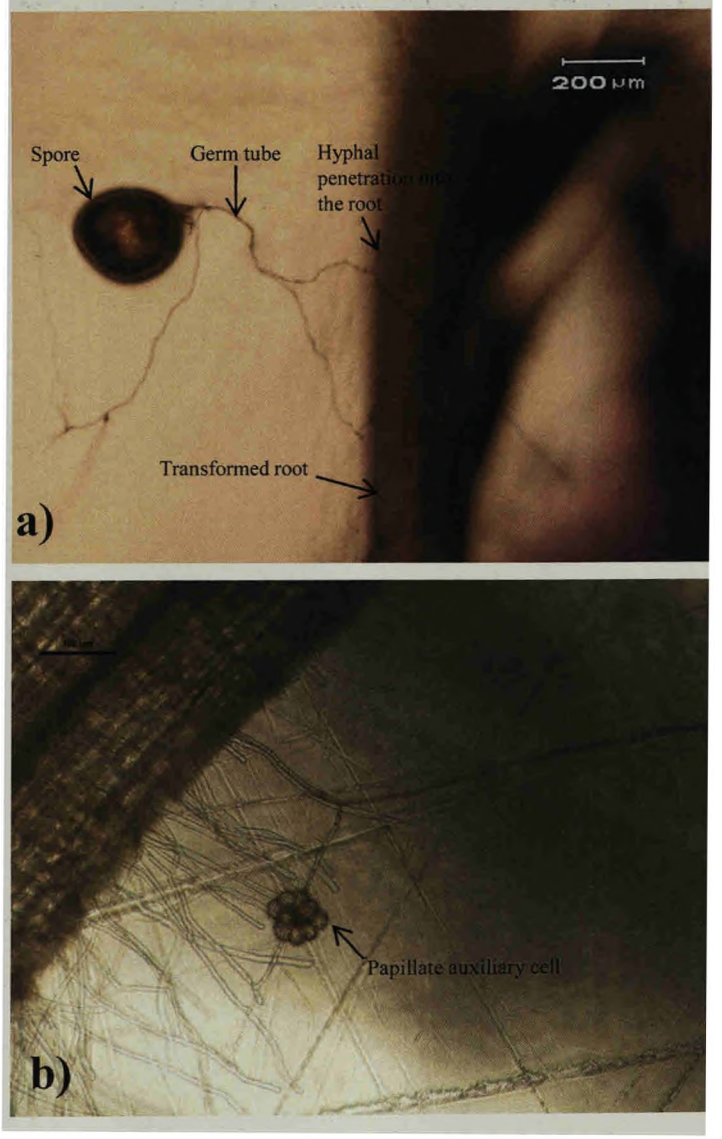

200 µm
Spore
Germ tube
Hyphal
the root
Transformed root
a)
Papillate auxiliary cell
b)

Plate 5.12: Micrographs of monoxenic culture of *Racocetra gregaria* with Ri T-DNA transformed Linum (*Linum usitatissimum* L.) roots on MSR media.

a) Association of germinated spore of *Racocetra gregaria* with transformed linum root for establishment of dual culture. Arrows indicating spore, germ tube, transformed root.

b) Initial contact between the hypha and the transformed root observed on tenth day after initiation of dual culture. Arrow indicating hyphal penetration into the root.

c) Infection units showing appressorium formation on the root. Arrows indicating infection units, hypha.

d) Germ tube growth as a straight growing runner hypha in the medium. Arrow indicating branching of runner hypha.

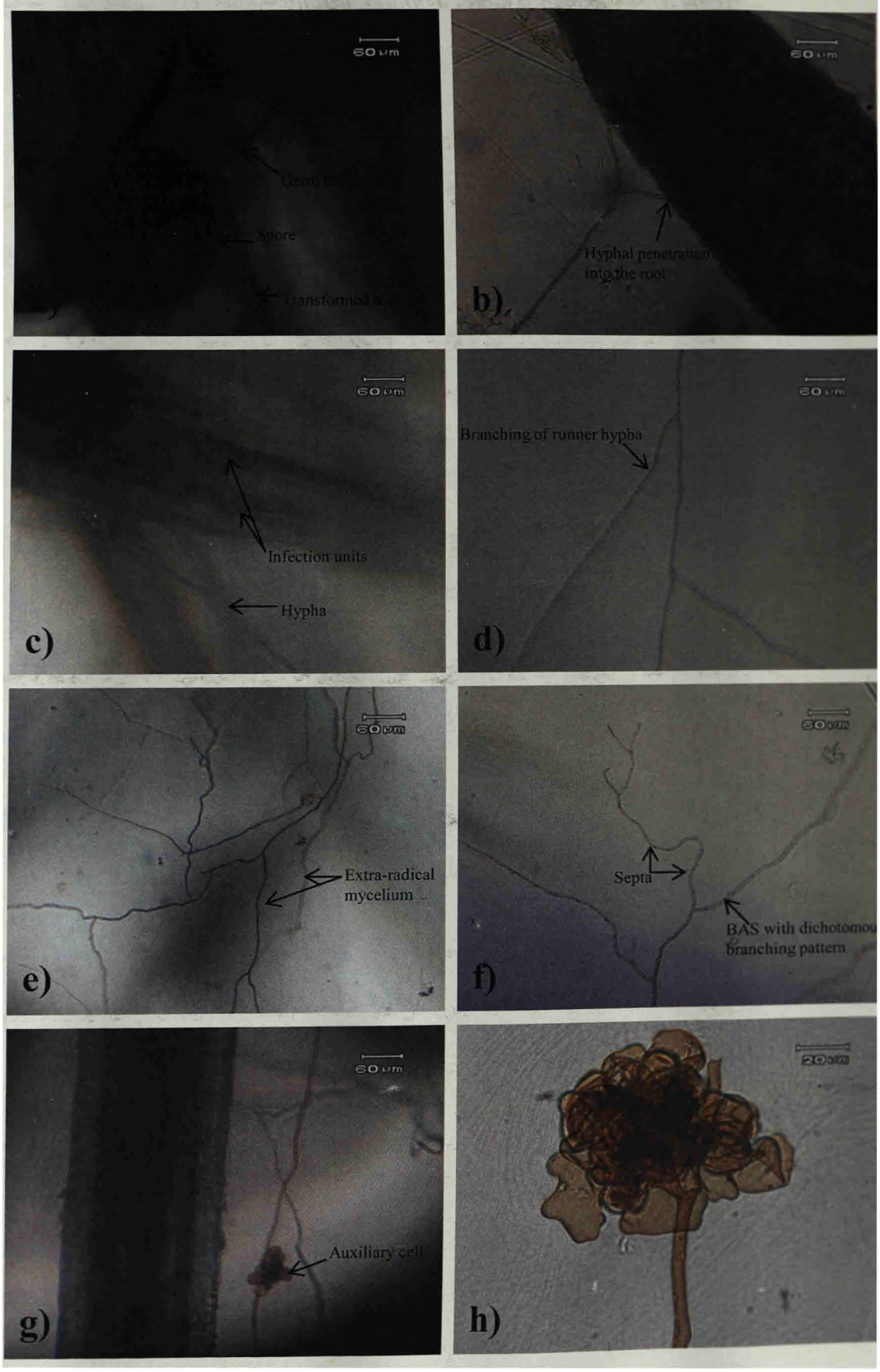
e) Well established extra-radical mycelium exploring the media. Arrows indicating extra-radical mycelium.

f) Branched absorbing structures (BAS) formed by extra-radical hypha entering degeneration process by cytoplasm retraction and septa formation. Arrows indicating BAS with dichotomous branching pattern, septa.

g) Auxiliary cell formation by extra-radical hyphae. Arrow indicating auxiliary cell.

h) Enlarged view of auxiliary cell depicting knobby structure.

Plate 5.12


60 µm
Spore
b)
Hyphal penetration into the root
c)
Infection units
Hypha
d)
Branching of runner hypha
e)
Extra-radical mycelium
f)
Septa
BAS with dichotomous branching pattern
g)
Auxiliary cell
h)
20 µm

5.4: DISCUSSION

In vitro cultivation system is a very powerful tool for the experimental establishment and study of AM association and its life cycle. Small but growing research on monoxenic culture has reported the successful establishment of AM associations using a number of AM fungal species (Fortin et al., 2002). In our study we report for the first time *in vitro* germination of indigenous species of *Claroideoglomus claroideum, Rhizoglomus manihotis, Gigaspora albida, Racocetra gregaria* and *Acaulospora scrobiculata.* However sporulation was observed only in indigenous species of *R. clarum, R. intraradices, F. mosseae* and *A. scrobiculata* in ROC. The characteristics of *R. clarum, R. intraradices* and *F. mosseae* in relation to the emergence of the germ tube, hyphal growth and the process of spore formation in monoxenic culture were similar to those described in the previous studies (Mosse and Hepper, 1975; Strullu and Romand, 1987; Friese and Allen, 1991; Chabot et al., 1992a; Douds Jr., 1997; Bago et al., 1998a; de Souza and Berbara, 1999; Declerck et al., 2001; Raman et al., 2001; Eskandari and Danesh, 2010; Maia et al., 2010).

In the present study, AM propagules *viz.,* isolated spores and colonized root fragments were used to initiate and establish monoxenic cultures. It has been reported that spores are the most effective propagules for Gigasporaceae members, while Acaulosporaceae and Glomeraceae members have been demonstrated to induce new colonization using spores and colonized root fragments (Biermann and Linderman, 1983; Brundrett et al., 1999; Klironomos and Hart, 2002).

In the present study, *in vitro* spore germination occurred by two modes *i.e.* normal mode by re-growth of germ tube through the subtending hypha and emergence of germ tube directly through spore wall. Some of the spores produced more than one germ tube

from spore wall, followed by growth and branching of the hyphae from the germ tubes. In *F. mosseae*, two percent of the spores showed both modes of germination. Different modes of germination have been reported in Glomeromycotan fungi depending on the type of genus. Spores of most *Glomus* species germinate by re-growth from the end of subtending hypha (Godfrey, 1957; Mosse, 1959) and although direct germination through spore wall has been reported in only a few Glomeraceae species *viz., Glomus albidum, G. caledonium, G. monosporum* and *G. albidum* (Tommerup and Kidby, 1980; Walker and Rhodes, 1981; Meier and Charvat, 1992). *Paraglomus* species also form glomoid spores that germinate directly through the spore wall (Oehl et al., 2011). Contrastingly, the germ tubes of *Gigaspora, Scutellospora* and *Acaulospora* species emerge directly through the spore wall (Giovannetti et al., 2010). Many germ tubes can also emerge from the subtending hypha as in *R. clarum* or a single germ tube as in *F. mosseae* and *G. caledonium* (Giovannetti et al., 2010). In the case of colonized root fragments, multiple germination arises from the root extremities (Diop et al., 1994a; Declerck et al., 1996a) thereby increasing the infectivity potential. Multiple germination is the ability of fungal spores to germinate several times by producing successive germ tubes (Koske, 1981b). This capacity was described in spores of a *Glomus* species (Mosse, 1959) and later studied in *Gi. gigantea* (Koske, 1981b). Multiple germination is considered an additional survival strategy of the germinating spores to increase the probability of successful contact and colonization of a host root (Giovannetti et al., 2010; Maia et al., 2010; Costa et al., 2013).

In the present study, the spore germination time and percent spore germination *in vitro* were observed to vary from species to species. The minimum time required for spore germination was observed to be 5 days after incubation and maximum 23 days after incubation. Maximum percent spore germination occurred within 9 days for most of the

indigenous AM species. Meier and Charvat (1992) reported 80 % germination in *F. mosseae* within 14 days wherein 1 % Hoagland's agar without P was used for spore germination.

In our study, the hyphal length of *in vitro* germinated spores was observed to vary from species to species and it ranged from 72.90 µm to 656.10 µm. Hyphal lengths of over 10 mm per germinated spore have been reported under the best experimental conditions (Azcon, 1987; Douds and Schenck, 1991).

In the present study, minus-sucrose media was used for spore germination, as it enables more rapid spore germination under *in vitro* conditions (D'Souza et al., 2013). Germ tube growth is usually dependent on the spore reserves (Bécard and Fortin, 1988; Sancholle et al., 2001). The protoplasm contains all the organelles essential for ensuring the development of germ tube *i.e.* pre-symbiotic phase (Meier and Charvat, 1992). The germ tube growth occurs as a straight growing hypha (runner hypha) exploring the media by successively branching into thinner-diameter filaments (Diop et al., 1994a; de Souza and Berbara, 1999; Declerck et al., 2000). Although AM spores are able to germinate *in vitro* in response to the different culture conditions, they are not capable of extensive independent hyphal growth and in the absence of a host root, no host signal detection occurs and the germinated spores or germlings cease growth within 8-20 days (Mosse, 1959; Daniels and Graham, 1976; Beilby and Kidby, 1980; Koske, 1981a; Hepper, 1984b; Bécard and Piché, 1989a; Giovannetti et al., 1993b; Schreiner and Koide, 1993b; Logi et al., 1998).

In the present study, monoxenic cultures of *R. clarum, R. intraradices, F. mosseae* and *A. scrobiculata* shared common colony morphology. AM fungal isolates under monoxenic culture conditions share a common colony architecture *i.e.* general

mycelium with runner hyphae (RH), branched hyphae (BH) and branched absorbing structures (BAS) or arbuscule like structures (ALS). All these elements are differentiated under a growth continuum with no ordered pattern. However, major differences between AM fungal cultures arise with density of hyphal network, pattern of ramification, spore abundance and positioning of spores (Declerck et al., 2005). In the present study, in monoxenic cultures of *R. clarum, R. intraradices, F. mosseae* and *A. scrobiculata*, spores were formed within several days and spores of various ages were observed simultaneously within a single colony. Both patterns of sporulation *i.e.* terminal and intercalary were observed separately on sporogenic hypha in *R. clarum, R. intraradices* and *F. mosseae*. In the case of *F. mosseae* both terminal and intercalary sporulation were also observed on a single sporogenic hypha. Terminal and intercalary spore formation have been reported for *G. versiforme* and *R. irregularis* in ROC (Chabot et al., 1992a; Bonfante and Bianciotto, 1995; Declerck et al., 1996a; Bago et al., 1998a, 1998b). Sporulation differs considerably between AM species and between isolates of a single species and is related to spore size (Declerck et al., 2005). However, in our study sporulation did not follow the classic three-phase (lag, exponential and plateau) spore development which has been previously reported in AM fungi (Declerck et al., 1996a; Bago et al., 1998a). This may be due to the observation time scales or it may be an innate character of the indigenous fungal isolates studied or due to the culture conditions. Our results are also in accordance to Chabot et al. (1992a) and Pawloska et al. (1999) who reported that spores formed under *in vitro* conditions displayed general morphological and anatomical likeness to soil-borne spores, but they were smaller and had either thick or thin laminated spore walls depending on the stage of development. In the present study, in case of *A. scrobiculata*, adjacent attachment of the spore at the neck of sporiferous saccule could not be observed. This may be due to

the time scales of observation. In case of *Gi. albida* and *Ra. gregaria in vitro* growth was observed up to auxiliary cell formation in monoxenic culture system and sporulation did not occur upon repetitive establishment of dual cultures. This may be due to their adapting time to the *in vitro* culture conditions and long vegetative phase. Kandula et al. (2006) reported that *Scutellospora* species have a long vegetative phase of 2-3 months before sporulation and a lengthy process of spore development. Bidondo et al. (2012) reported that as AM strains are adapted to the host plant and the natural environmental conditions of the isolation site, it is difficult to obtain first generation sporulation under *in vitro* conditions, a phenomenon termed as 'maternal effect'. It is also reported that Gigasporaceae members are more difficult to propagate and sub-culture under monoxenic conditions (Dalpé et al., 2005). Ijdo et al. (2011) suggested that Gigasporaceae members have different life strategies *i.e.* they invest their energy and resources to grow and survive (somatic growth) contrasting to Glomeraceae members which allocate their resources in reproduction which makes them more adapted to *in vitro* conditions. Gigasporaceae members do not form vesicles but form auxiliary cells on extra-radical hyphae (Dodd et al., 2000). The number of auxiliary cells observed in the present study was nine on average per plate for both *Gi. albida* and *Ra. gregaria*. It has been reported that over 600-700 auxiliary cells formation is necessary for substantial spore production, and they act as carbon storage structures spending their energy in sporulation (de Souza and Declerck, 2003). Declerck et al. (2004) estimated that to produce one spore, resources are needed from at least 19 auxiliary cells. In our study, the stability of the morphological characters of *R. clarum, R. intraradices, F. mosseae* and *A. scrobiculata* spores displayed under monoxenic culture conditions remained constant throughout. These properties *i.e.* the identity and the stability of monoxenic cultures have been reported as desirable culture characters

(Declerck et al., 2005). In the present study, disrupted senescing spores of *R. intraradices* with differentiation of thin-walled hyphae from within were also observed infrequently. The presence of aborted or senescent spores has been intermittently observed in healthy monoxenic cultures of *Glomus* species (Pawlowska et al., 1999; Karandashov et al., 2000; Dalpé, 2004). Hypothetical explanations of this phenomenon suggest the effects of inadequate growth under *in vitro* conditions, and self-strain protection against mutation (Marbach and Stahl, 1994; Pawloswka et al., 1999).

In the present study during the development of extra-radical hyphae, formation of so-called arbuscule-like structures (ALS) or branched absorbing structures (BAS) was observed. The structures were comprised of finely branching/branched hyphae resembling intracellular arbuscules. It has been reported that under sterile culture conditions, the growth of extra-radical hyphae is usually accompanied by the production of arbuscule-like structures or branched absorbing structures (Mosse and Hepper, 1975; Bécard and Fortin, 1988; Bago et al., 1998a, 1998b; Chabot et al., 1992a). Further, the appearance of these structures is in response to a more- or less-close interaction with the root (Bécard and Fortin, 1988) or a localized response to an unknown stimulus within the growth medium (Mosse and Hepper, 1975). It has been also been reported that ALS/BAS play an important role as special sites for nutrient acquisition by the extra-radical mycelium and help in increasing nutrient supply to the developing spores (Bago et al., 1998a, 1998b). In the present study, degeneration process of BAS by cytoplasm retraction and septa formation was also observed. BAS are ephemeral structures of the extra-radical mycelium which, like arbuscules, also undergo degeneration (Bago et al., 1998b).

In the present study, continuous cultures of *R. clarum, R. intraradices* and *F. mosseae* showing active re-growth of hyphae were obtained upon sub-culturing the colonized transformed root fragments and monoxenically produced spores along with extra-radical hyphae from the starter culture. Re-growth of hyphae from colonized mycorrhizal root segments (intra-radical structures) has been described in several studies (Strullu and Romand, 1986; Strullu et al., 1991; Diop et al., 1994a). However, in case of *A. scrobiculata*, no re-growth was observed in our study. Similar unsuccessful continuous cultures over several generations have been reported with *A. rehmii* (Dalpé and Declerck, 2002) and mentioned only as "difficult to achieve" (Fortin et al., 2002). Production of first spore daughter generation does not mean that the fungus can be maintained continuously under monoxenic culture conditions, and numerous monoxenically cultured species have certainly failed to be sub-cultured under the same growth conditions (Declerck et al., 2005).

In the present study, it was found that in *R. intraradices* and *F. mosseae* the percent spore germination, frequency of culturing (sporulation) and sub-culturing (re-growth) was high. Hence, these two AM species were screened for further studies. Ijdo et al. (2011) suggested that Glomeraceae members invest their energy in reproduction which makes them more adapted to the *in vitro* culture systems. Declerck et al. (2005) has described two pre-requisites for including AM fungi in monoxenic culture collections: (i) the ability of the fungus to complete its life cycle with adequate sporulation and (ii) the capability of the fungus to be continuously cultured under the same *in vitro* conditions. Considering these two pre-requisites, the monoxenic cultures of the indigenous species of *R. intraradices* and *F. mosseae* in our study met both the criteria.

5.5: CONCLUSION

Monoxenic cultivation of AM fungi on root culture allows detailed observations and long-term experimentations on the life cycle of AM fungi as well as offers production of high quality and microbiologically clean inocula that is both effective and efficient for large scale inoculum production. Our results depict the successful establishment of dual cultures with maximum potential for spore production using monoxenic culture system. The stable and homogeneous monoxenic material obtained, especially with regard to indigenous species of *R. intraradices* and *F. mosseae* could facilitate the mass production of pure and viable inocula.

CHAPTER 6

To develop viable inoculum using suitable carrier for re-inoculation. (Objective 4)

6.1: INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate biotrophic symbionts that after root colonization exert widely accepted beneficial effects to a wide range of host plant species (Adholeya et al., 2005). They represent a key link between soil and plants, and have gained a growing attention as ecosystem engineers and bio-inoculants (Gianinazzi et al., 1990; Gianinazzi and Vosatka, 2004; Fitter et al., 2011). Currently, AM fungal bio-inoculants are being increasingly considered in agriculture, horticulture and forestry, as well as for environmental reclamation and remediation, to increase overall crop health and yield, and to limit the usage of agrochemicals (Gianinazzi et al., 2002; Johansson et al., 2004). Many field and greenhouse experiments have been limited to the single inoculation of one of the following three species *viz., Rhizoglomus intraradices*, *R. irregulare* and *Funneliformis mosseae* (Krüger et al., 2012; Pellegrino and Bedini, 2014; Berruti et al., 2015). These AM species are very generalist symbionts that colonize a diversity of host plants. They can be easily and massively propagated, survive long-term storage and have a wide geographical distribution all over the world (Öpik et al., 2010). The abovementioned characteristics have made these AM fungal species suitable as premium inoculum components (Berruti et al., 2015). Several reports however, have highlighted that different isolates within the same species, rather than different species, can cause larger variations in host plant response (Munkvold et al., 2004; Gai et al., 2006; Angelard et al., 2010). This suggests that the widespread use of single AM fungal species, such as *R. intraradices*, *R. irregulare*, and *F. mosseae*, in inoculation experiments should not be considered as a flaw as these AM species can contain considerable functional heterogeneity (Berruti et al., 2015).

Recently, interest in AM fungi has focused on finding a viable method to optimize the production of high quality AM fungal inoculum to use as a bio-inoculant in cropping systems (Gianinazzi and Vosátka, 2004; Ijdo et al., 2011). Monoxenic cultivation is now a reality offering several-fold increase in mass production of effective propagules over conventional bulking techniques. This system of AM inoculum production provides potentially high and economically attractive options to chemical fertilizations thus emphasizing its potential importance in sustainable agriculture. AM monoxenic cultivation systems are particularly relevant to the tropical conditions wherein AM fungal counts are poor (Adholeya et al., 2005).

The assessment of viability, germination potential and colonization potential of monoxenically produced spores is highly essential and desirable for its use as bio-inoculum. In addition to spore germination and colonization tests, the use of vital stains offers a substitute technique for assessing spore viability. Tetrazolium salts such as 2,3,5-triphenyl tetrazolium chloride and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Nelson and Olsen, 1967; Sutherland and Cohen, 1983; Jiang and Erwin, 1990) have been used to assess the viability of fungal spores. MTT has also been used to evaluate viability of AM fungal spores (An and Hendrix, 1988; Meier and Charvat, 1993). Tetrazolium salts act as electron acceptors for coenzyme-linked cellular dehydrogenases and upon reduction, form highly coloured insoluble compounds called formazans (Altman, 1976). The formation of coloured formazan products by the reduction of tetrazolium salts has been recognized as an indicator of biological reducing systems (Kuhn and Jerchel, 1941).

Besides estimation of viability of monoxenically produced spores, the importance of selection of efficient organic carrier formulation is also of primary concern for

development of a viable AM fungal bio-inoculant. AM fungal inoculum is commercially available in a variety of forms ranging from high concentrations of AM fungal propagules in carrier materials to potting media containing inoculum at low concentrations (Douds et al., 2010). Bio-inoculants are usually prepared as carrier-based inoculants containing effective microorganisms (Accinelli et al., 2009). A carrier is a delivery vehicle which is used to transfer live microorganism from an agar slant to the rhizosphere (Brahmaprakash and Sahu, 2012). A suitable biofertilizer carrier should comprise of certain characteristic features *viz.*, it should be in powder or granular form; should support the growth and survival of the microorganism, and should be able to release the functional microorganism easily into the soil; should have high moisture absorption and retention capacity, good aeration characteristics and pH buffering capacity; should be non-toxic and environmentally friendly; should be easily sterilized and handled in the field; have good long term storage qualities; and should be inexpensive (Stephens and Rask, 2000; Rebah et al., 2002; Rivera-Cruz et al., 2008). Considering the above mentioned features it is apparent that no singular universal carrier is available which fulfills all the desirable characteristics, but good quality ones should retain as many as possible.

AM fungal inoculum comprises of spores, colonized root fragments and mycelium/hyphae. Isolated AM fungal spores and hyphae can then be mixed with the carrier material. The carrier materials can be organic, inorganic or synthetic. Commonly used carriers include soils like peat, coal, pumice or clay, sand, and lignite; inert materials like perlite, vermiculite, soilrite, alginate beads, polyacrylamide gels and bentonite (Mallesha et al., 1992; Redecker et al., 1995; Bashan, 1998; Gaur and Adholeya, 2000; Herridge et al., 2008; Malusá et al., 2012). Organic wastes from animal production and agriculture, and byproducts of agricultural and food processing

industries such as charcoal, composts, farmyard manure, cellulose, soybean meal, soybean and peanut oil, wheat bran, press mud, corn cobs also meet the requirements of a biofertilizer carrier and thus could be good carrier materials (Herrmann and Lesueur, 2013; Wang et al., 2015). It is also possible to find carrier combinations comprising of a mixture of soil and compost; soil, peat, bark, and husks among others (Herridge et al., 2008). Peat is the most commonly used carrier material. However, it is a limited natural resource which is not readily available worldwide and its use has a detrimental effect on the environment from which it is extracted. This highlights the need for development of new carrier formulations using alternative resources to compete with the existing inoculants (John et al., 2011).

This chapter is aimed at evaluating viability, germination and colonization potential of monoxenically produced spores, and selecting an efficient organic carrier formulation to sustain *in vitro* produced AM fungal propagules for its utilization as viable carrier based bio-inocula.

The chapter is divided into four parts *viz.,* I) estimation of viability of *in vitro* produced spores, II) assessment of the germination potential of monoxenically (*in vitro*) produced spores, III) assessment of the colonization potential of monoxenically (*in vitro*) produced spores and IV) selection of efficient carrier formulation.

6.2: MATERIALS AND METHODS

6.2.1: I) Estimating the viability of *in vitro* produced spores:

Tetrazolium salt 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (TTC) was evaluated as a vital stain for estimating the viability of *in vitro* produced spores of two AM species *viz., Funneliformis mosseae* and *Rhizoglomus intraradices.*

Extra-radical hyphae and spores of both AM species were extracted from an *in vitro* propagating dual culture and subjected to TTC test (An and Hendrix, 1988) to determine the viability.

6.2.1.1: Extraction of monoxenically produced AM propagules

Extraction of monoxenically produced AM fungal propagules from MSR media was carried out using method by Cranenbrouck et al. (2005) as described in Chapter 5. The extracted spores along with attached extra-radical mycelium were then subjected to TTC test.

6.2.1.2: TTC test

0.5 mg stock solution of TTC/ml was prepared with deionized water and stored at 4°C in dark. 1 ml of TTC stock + 1 ml aqueous suspension of spores along with attached extra-radical mycelia was mixed in a porcelain spot plate using micropipette and incubated at room temperature for a minimum of 40 hours. The spores along with attached extra-radical mycelia were then removed by viewing under Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom) and placed on microscope glass slides with cover glass for observation. Viable spores stain bright red whereas non-viable spores remain unstained.

6.2.2: II) Assessment of germination potential of monoxenically produced spores:

Assessment of germination potential of monoxenically produced spores of *R. intraradices* and *F. mosseae* was carried out for its utilization as carrier based inocula.

The monoxenically produced spores of *R. intraradices* and *F. mosseae* in different stages of development were inoculated on fresh Modified Strullu-Romand (MSR) minus sucrose media to study their germination potential.

6.2.2.1: Extraction of monoxenically produced AM propagules

Extraction of monoxenically produced AM fungal propagules from MSR media was carried out using method by Cranenbrouck et al. (2005) as described in Chapter 5. The extracted spores along with attached extra-radical mycelium were then inoculated on fresh MSR media to study their germination potential.

6.2.3: III) Assessment of colonization potential of monoxenically produced spores:

In vitro establishment of *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) seedlings was carried out to test the colonization potential of the *in vitro* produced spores of *R. intraradices* and *F. mosseae.*

Seeds of *P. scutellarioides* were surface sterilized with sodium hypochlorite and inoculated on MSR media. Upon germination (7-14 days) the seedlings were transferred to conical flasks containing sterilized vermiculite and were associated with *in vitro* produced spores of both AM species separately **(Plate 6.1 a-b)**. Hoagland's solution (Hoagland and Arnon, 1950) minus P was added after 20-day intervals. After 90 days of growth, roots of *P. scutellarioides* were examined for AM colonization.

6.2.3.1: Extraction of monoxenically produced AM propagules

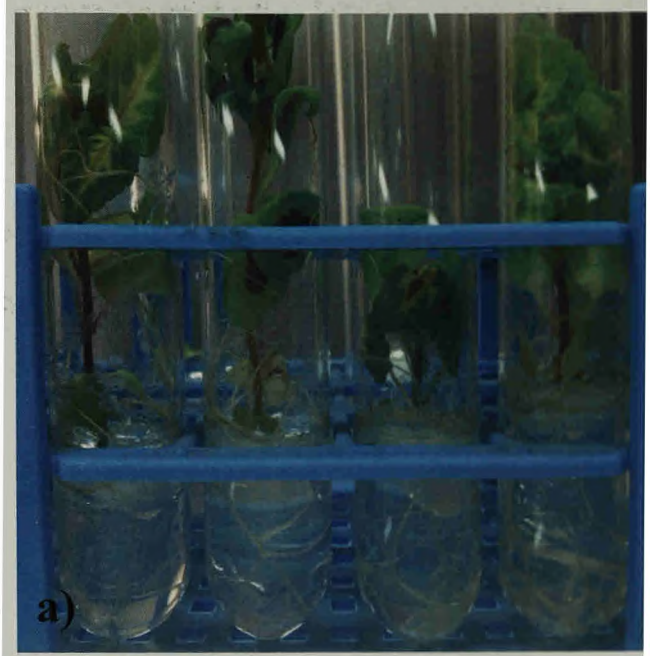
Extraction of monoxenically produced AM fungal propagules from MSR media was carried out using method by Cranenbrouck et al. (2005) as described in Chapter 5. The extracted spores along with attached extra-radical mycelium were then associated with seedlings of *P. scutellarioides.*

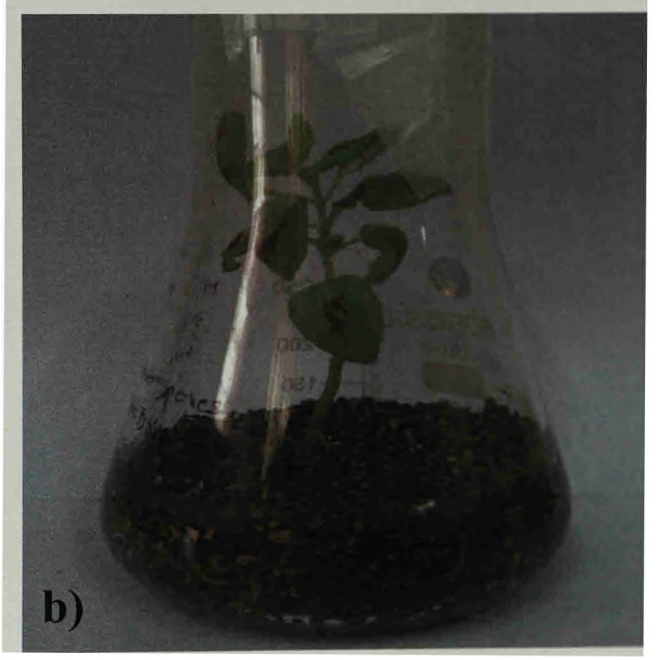
Plate 6.1: *In vitro* establishment of *Plectranthus scutellarioides* (L.) R.Br. seedlings to test the colonization potential of the *in vitro* produced spores.

a) *Plectranthus scutellarioides* seedlings growing on MSR media.

b) *Plectranthus scutellarioides* seedling transferred to conical flask containing sterilized vermiculite for association with *in vitro* produced AM spores.

Plate 6.1





6.2.3.2: Processing of root segments for AM fungal colonization

Assessment of AM colonization in roots of *P. scutellarioides* was carried out by using Trypan blue staining technique after 90 days of growth (Phillips and Hayman, 1970) as described in Chapter 3.

6.2.3.3: Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using Root Slide method (Read et al., 1976) as described in Chapter 3.

6.2.4: IV) Selection of efficient carrier formulation:

Selection of efficient carrier formulation was carried out for the development of carrier based bio-inocula.

6.2.4.1: Carrier preparation

For formulation of the carrier, initially sterilized sand and vermiculite were used separately as base components to formulate the carrier supplemented with sterilized cow dung powder, wood powder and wood ash in different proportions **(Table 6.1)**. It was observed that the treatments containing vermiculite as the base component showed maximum AM colonization as compared to sand **(Fig. 6.1)**, thus vermiculite was selected and used as the base component for the carrier supplemented with sterilized cow dung powder, wood powder and wood ash in different proportions resulting in 19 formulations/treatments in all, in order to review favourable or unfavourable effects of each material in the combination **(Table 6.2)**. The plant source of wood powder and wood ash was *Mangifera indica* L. (Anacardiaceae). The carrier materials were sterilized by autoclaving for two consecutive days at 121°C for 2 h. The wood powder was washed 3-4 times with tap water followed by drying in the oven and then

Table 6.1: Concentrations of carrier formulated in parts (ratios) and percentages with soil and vermiculite separately as base components.

Components	Treatment 1[a]	Treatment 2[b]	Treatment 3[a]	Treatment 4[b]	Treatment 5[a]	Treatment 6[b]
Soil	20 (60.60 %)	--	20 (62.50 %)	--	20 (64.51 %)	--
Vermiculite	--	20 (60.60 %)	--	20 (62.50 %)	--	20 (64.51 %)
Cow dung powder	8 (24.24 %)	8 (24.24 %)	8 (25.00 %)	8 (25.00 %)	8 (25.80 %)	8 (25.80 %)
Wood powder	4 (12.12 %)	4 (12.12 %)	3 (9.37 %)	3 (9.37 %)	2 (6.45 %)	2 (6.45 %)
Ash	1 (3.03 %)	1 (3.03 %)	1 (3.12 %)	1 (3.12 %)	1 (3.22 %)	1 (3.22 %)

Legend: -- = absent.

[a] Treatments 1, 3, 5 contain soil as the base component.

[b] Treatments 2, 4, 6 contain vermiculite as the base component.

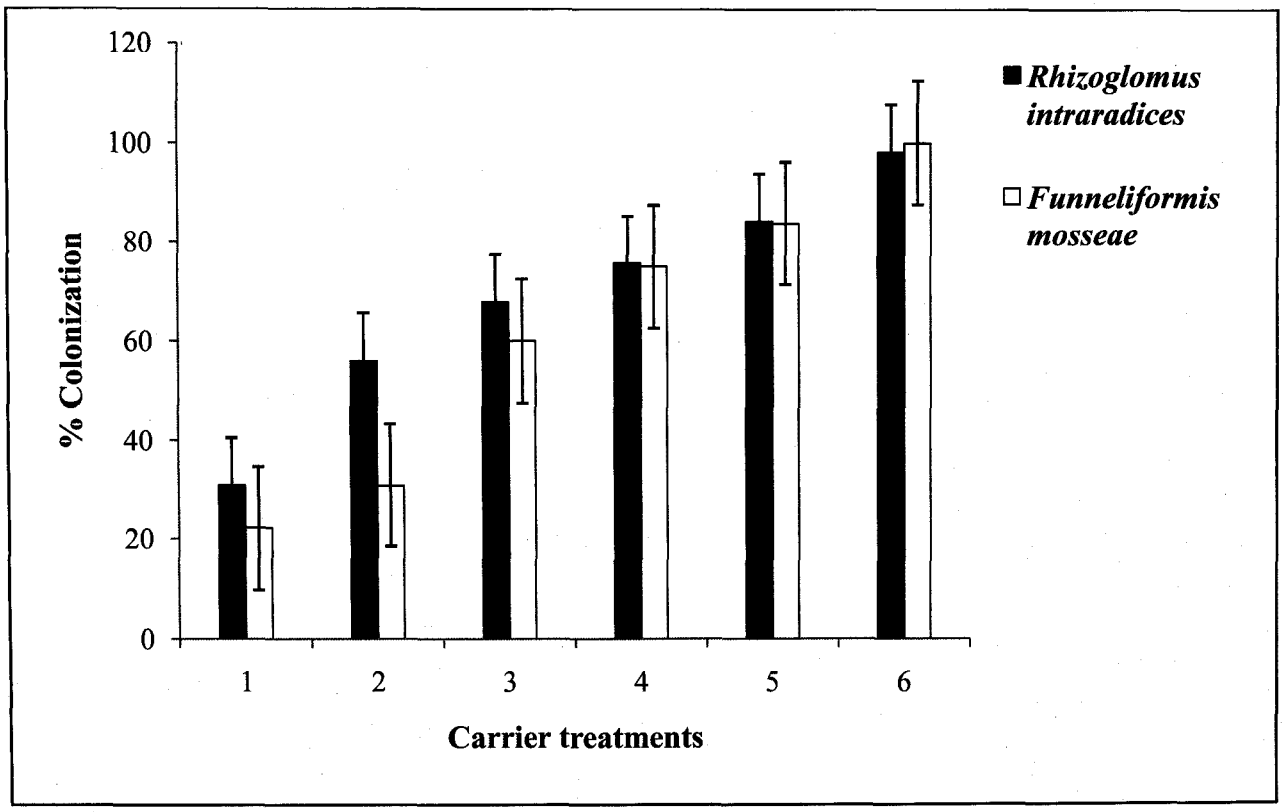


Fig. 6.1: Percent colonization in roots of *Eleusine coracana* Gaertn. plants inoculated with *Rhizoglomus intraradices* and *Funneliformis mosseae* with soil and vermiculite separately as base components.

Legend: Treatments 1, 3, 5 = contain soil as the base component; treatments 2, 4, 6 = contain vermiculite as the base component.

Table 6.2: Carrier formulations in various ratios and percentages.

Treatments	Vermiculite	Cow dung powder	Wood powder	Wood ash
1	20 (60.60 %)	8 (24.24 %)	3 (9.09 %)	2 (6.06 %)
2	20 (74.07 %)	4 (14.81 %)	2 (7.40 %)	1 (3.70 %)
3	20 (100.00 %)	--	--	--
4	20 (62.50 %)	8 (25.00 %)	2 (6.25 %)	2 (6.25 %)
5	20 (64.51 %)	8 (25.80 %)	2 (6.45 %)	1 (3.22 %)
6	20 (62.50 %)	8 (25.00 %)	3 (9.37 %)	1 (3.12 %)
7	20 (68.96 %)	4 (13.79 %)	3 (10.34 %)	2 (6.89 %)
8	20 (71.42 %)	4 (14.28 %)	3 (10.71 %)	1 (3.57 %)
9	20 (71.42 %)	4 (14.28 %)	2 (7.14 %)	2 (7.14 %)
10	20 (80.00 %)	--	3 (12.00 %)	2 (8.00 %)
11	20 (83.33 %)	--	3 (12.50 %)	1 (4.16 %)
12	20 (83.33 %)	--	2 (8.33 %)	2 (8.33 %)
13	20 (86.95 %)	--	2 (8.69 %)	1 (4.34 %)
14	20 (90.90 %)	--	--	2 (9.09 %)
15	20 (95.23 %)	--	--	1 (4.76 %)

16	20 (86.95 %)	--	3 (13.04 %)	--
17	20 (90.90 %)	--	2 (9.09 %)	--
18	20 (71.42 %)	8 (28.57 %)	--	--
19	20 (83.33 %)	4 (16.66 %)	--	--

Legend: -- = absent.

autoclaving. For physico-chemical characterization of the carrier materials, samples were submitted to Government of Goa Agricultural Department, Soil Analysis Laboratory, Margao, Goa and Italab House, Margao, Goa. Analyses followed procedures laid out by Singh, Chhonkar and Dwivedi (2005), wherein pH and EC were measured in a 1:1 (v/v) water solution using pH meter (LI 120 Elico, India) and conductivity meter (CM-180 Elico, India). Organic carbon (OC) was analyzed by rapid titration method (Walkley and Black, 1934). Available phosphorus (P) was estimated using Bray and Kurtz (1945) method. Available potassium (K) was estimated by ammonium acetate method (Hanway and Heidel, 1952) using flame photometer (Systronic 3292). Available micronutrients *viz.,* zinc (Zn), iron (Fe), manganese (Mn) and copper (Cu) were quantified by DTPA-$CaCl_2$-TEA method (Lindsay and Norvell, 1978) using atomic absorption spectrophotometer (AAS-EC Element AS AAS 4139). Boron (B) was quantified by the hot water soluble method (Berger and Truog, 1939).

6.2.4.2: Experimental Setup

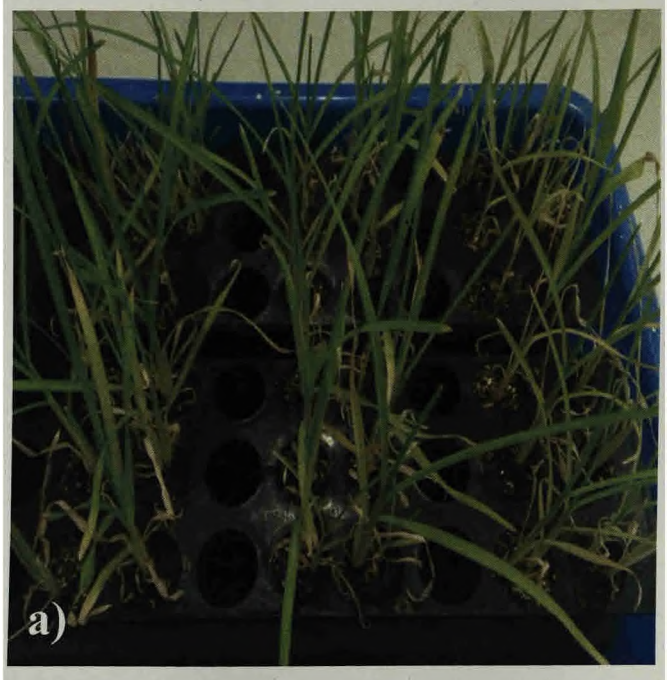
The experiment was set up using deep cell plug trays for a period of 3 months. Twenty *in vitro* produced spores of *R. intraradices* along with colonized transformed chicory (*Cichorium intybus* L.) roots and *F. mosseae* spores along with colonized transformed linum (*Linum usitatissimum* L.) roots were used as inocula in each deep cell plugs containing the carrier formulations and planted with pre-germinated seeds of *Eleusine coracana* Gaertn. (Poaceae) used as host plant **(Plate 6.2 a-b)**. The plants were maintained in the phytotron (Daihan Labtech, LGC-6201G) at 260 lux (16 h photoperiod), 26°C, 41.1 % humidity and 100 ppm CO_2 and Hoagland's solution (Hoagland and Arnon, 1950) minus phosphorus (P) was applied every 20 days. There were six replicates for each treatment. As the replicates were in a single tray, the trays were repositioned at the end of every week.

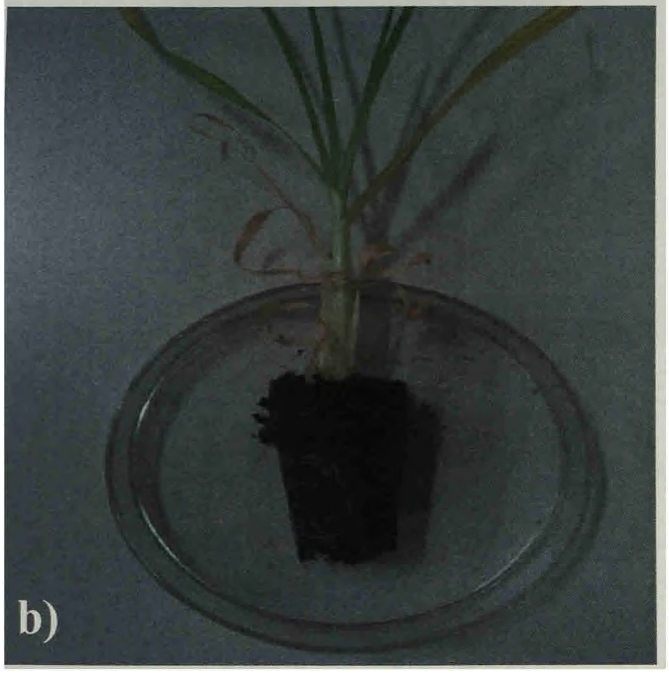
Plate 6.2: Selection of efficient carrier formulation.

a) Experimental set up using deep cell plug trays, with *in vitro* produced spores of *R. intraradices* and *F. mosseae* used separately as inocula along with carrier formulations and *Eleusine coracana* Gaertn. as host plant.

b) Deep cell plug content with attached intact plant.

Plate 6.2





6.2.4.3: Processing of root segments for AM fungal colonization

Assessment of AM colonization in roots of *E. coracana* was carried out by using Trypan blue staining technique after 3 months of growth (Phillips and Hayman, 1970) as described in Chapter 3.

6.2.4.5: Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using Root Slide method (Read et al., 1976) as described in Chapter 3.

Average number of entry points in 1cm root segment, root length, total number of infective propagules were also calculated as per Fertilizer (Control) Order, 1985 (Ministry of Agriculture, Government of India 2009).

Total number of infection points or infective propagules (IP) = average number of entry points formed in 1 cm root segment × total root length. Extrapolate the IP present as numbers per gram of substrate or inoculum.

6.2.4.6: Statistical Analysis

The experimental data was subjected to one-way analysis of variance (ANOVA) followed by Tukey post-Hoc pairwise comparison test. Parameters were correlated using Pearson's correlation coefficient. Statistical Package for Social Sciences (SPSS) (ver. 22.0 Armonk, NY: IBM Corp.) was used for all statistical analyses.

All microscope observations were made using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x), and Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom). Micrographs were imaged by Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras, and were not digitally edited.

6.3: RESULTS

6.3.1: I) Spore colour reaction in TTC

At 12-28 weeks, majority of spore primordia for both the AM species tested positive for viability with TTC by staining distinctly red. Maximum red colour reaction in *R. intraradices* spores was achieved within 48 h whereas in spores of *F. mosseae* maximum colouration was seen after 72 h **(Plate 6.3 a-d)**. Having achieved maximum colour reaction, colours remained relatively stable for spores of both AM species. Approximately 82 % of the *F. mosseae* spores and 92 % of the *R. intraradices* spores from 90 days old and 197 days old cultures were viable as assessed by vital dye staining. The level of viability remained unchanged throughout the spore maturation period (90-197 days old).

6.3.2: II) Germination potential of monoxenically produced spores

The study revealed that *in vitro* produced spores of both the AM species retained maximum germination potential up to 28 weeks *i.e.* fully matured spores at 197 days old **(Fig. 6.2)**, and therefore were selected for preparation of carrier based inocula.

6.3.3: III) Colonization potential of monoxenically produced spores

Presence of hyphae, arbuscules, vesicles and extra-radical spores indicated successful colonization of *P. scutellarioides* by the monoxenically produced spores. The study recorded 77.05 % colonization by *R. intraradices* and 63.66 % colonization by *F. mosseae* in the roots of *P. scutellarioides* **(Table 6.3, Plate 6.4 a-f)**.

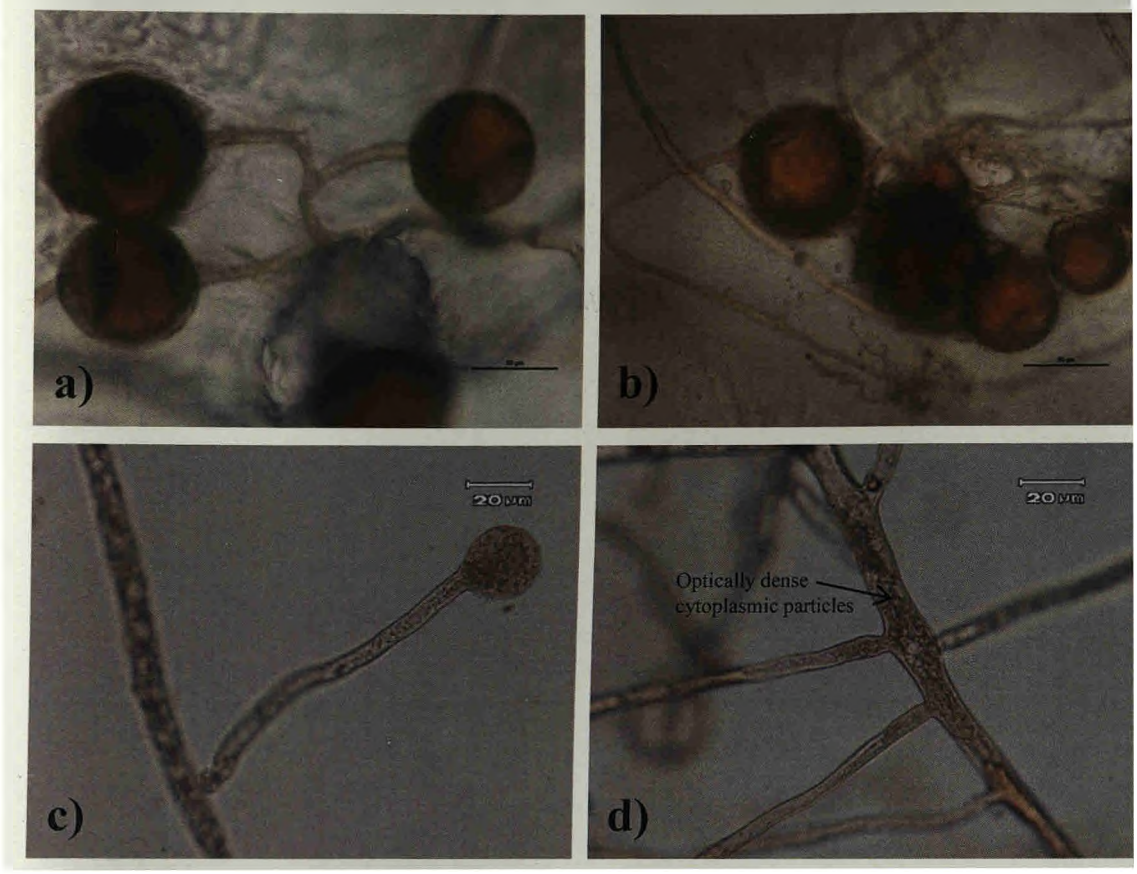
Plate 6.3: Micrographs of spore colour reaction in TTC.

a), b) Terminal spores of *Rhizoglomus intraradices* stained red by vital dye.

c) Terminal spore of *Funneliformis mosseae* stained red by vital dye.

d) Extra-radical hypha of *Funneliformis mosseae* stained red by vital dye- Arrow indicating optically dense cytoplasmic particles.

Plate 6.3



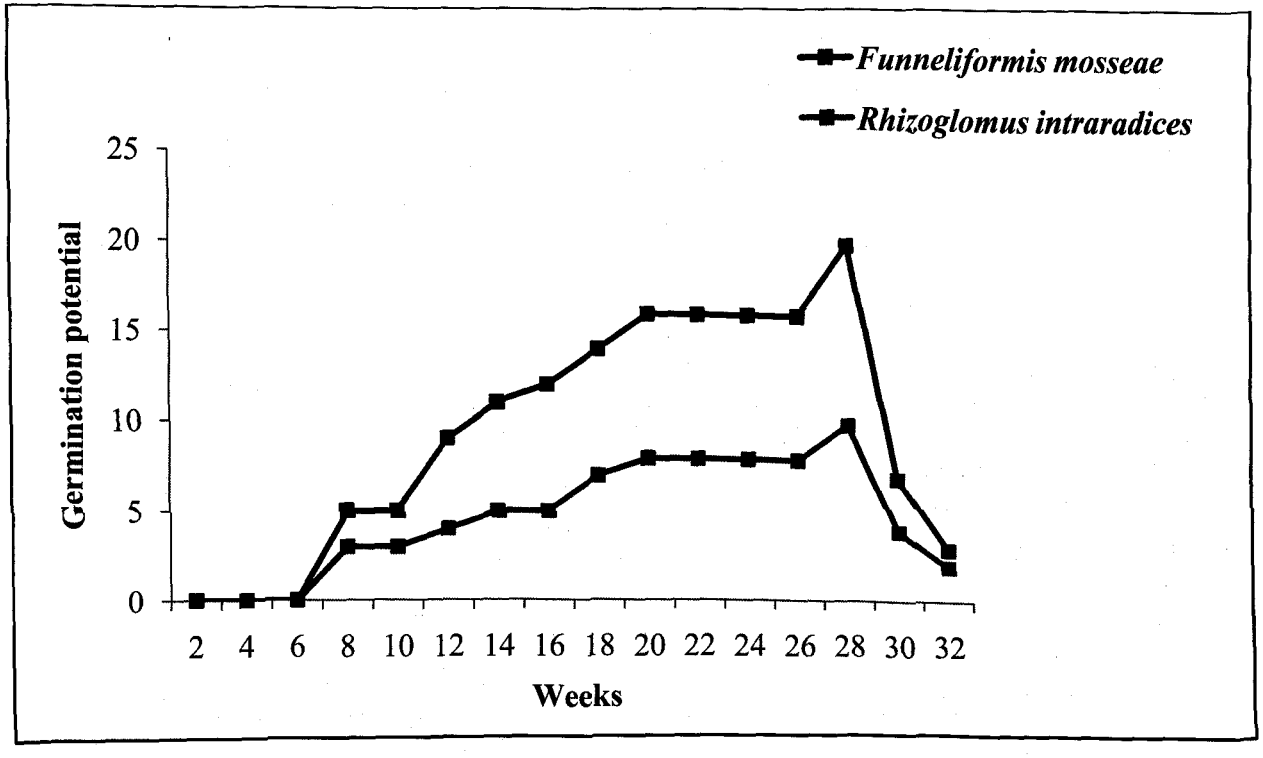


Fig. 6.2: Germination potential of monoxenically produced spores of *Rhizoglomus intraradices* and *Funneliformis mosseae.*

Table 6.3: Root colonization in *Plectranthus scutellarioides* (L.) R.Br. inoculated with monoxenically produced spores of *Rhizoglomus intraradices* and *Funneliformis mosseae.*

Sr. No.	AM species	Type of colonization			Colonization (%)
		H	A	V	
1.	*R. intraradices*	+	+	+	77.05 ± 4.59
2.	*F. mosseae*	+	+	+	63.66 ± 4.04

Legend: Values are means of 3 replicates ± standard deviation; H = hyphal colonization, A = arbuscules, V = vesicles, + present.

Plate 6.4: Micrographs of root colonization in *Plectranthus scutellarioides* (L.) R.Br. by monoxenically produced spores of *Rhizoglomus intraradices* and *Funneliformis mosseae* (assessment of the colonization potential).

a) Arbuscular colonization by *Rhizoglomus intraradices* (scale 50 µm). Arrows indicating arbuscules.

b) Vesicular colonization by *Rhizoglomus intraradices* (scale 50 µm).

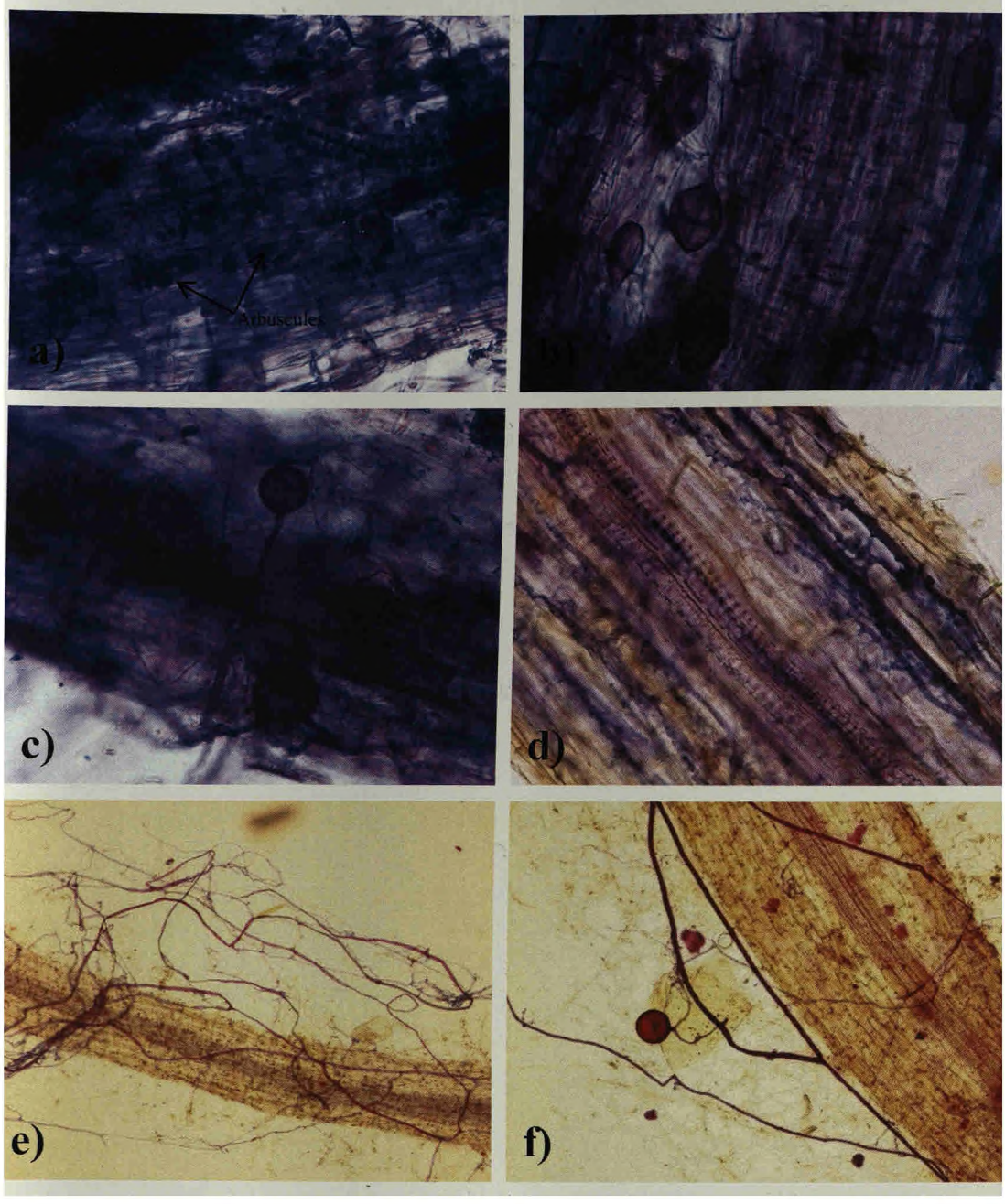
c) Extra-radical spores of *Rhizoglomus intraradices* (scale 50 µm).

d) Hyphal (intra-radical) colonization by *Funneliformis mosseae* (scale 50 µm).

e) Extra-radical hyphal colonization by *Funneliformis mosseae* (scale 100 µm).

f) Extra-radical spore of *Funneliformis mosseae* (scale 100 µm).

Plate 6.4



6.3.4: IV) Selection of efficient carrier formulation:

6.3.4.1: Physico-chemical characterization of the materials used for carrier formulation

Physico-chemical properties of the carrier materials are depicted in **Table 6.4**. It was observed that the materials used for formulation of carrier had different characteristics *viz.,* cow dung powder had higher amount of organic carbon (OC) and P, higher potassium (K) was found in wood ash, and the micro-nutrient contents were higher in cow dung powder except for copper (Cu) which was higher in wood ash.

6.3.4.2: Effect of different carrier treatments on re-inoculation/colonization potential of *in vitro* produced AM fungal inoculum

The *in vitro* produced spores of *R. intraradices* and *F. mosseae* along with the attached extra-radical mycelia were used separately as inocula to colonize *E. coracana* plants. The average root length of *E. coracana* plants in all the 19 carrier treatments for both the AM species were in the range of 1.70-3.06 cm. For *R. intraradices*, maximum number of entry points (13) per root segment in *E. coracana* was recorded in treatment 5 (vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1) while for *F. mosseae*, the maximum number of entry points (7.4) per root segment in the same host plant was also recorded in the same treatment **(Fig. 6.3)**. Similarly, the total number of infective propagules was highest (148.2 infection points g^{-1} of inoculum used) for *R. intraradices* in treatment 5 while for *F. mosseae*, it was highest (126.6 infection points g^{-1} of inoculum used) in the same treatment **(Fig. 6.4)**. Percent colonization was highest (100 %) in treatment 5 for both the AM species **(Fig. 6.5, Plate 6.5 a-h)**. Treatment 5 was optimal for both species.

As treatment 5 was observed to be the optimum for both AM species, the total propagules of treatment 5 were extracted by Wet Sieving and Decanting technique

Table 6.4: Physico-chemical parameters of carrier materials.

Carrier material	pH	E.C. m.mhos/ cm	Macro-nutrients			Micro-nutrients (ppm)				
			Organic Carbon %	Phosphorus Kg/Ha	Potassium Kg/Ha	Zinc	Iron	Manganese	Copper	Boron
Vermiculite	7.50	<1	0.78	10.90	170.80	0.54	1.13	17.27	0.26	1.30
Cow dung powder	6.60	2.80	4.07	1038.00	2952.00	4.41	14.44	25.54	1.25	50.60
Wood powder	5.80	<1	1.91	92.90	185.90	3.27	2.84	1.91	0.22	13.40
Wood ash	10.30	12.20	0.65	65.60	4435.20	4.10	10.85	7.23	25.00	25.30

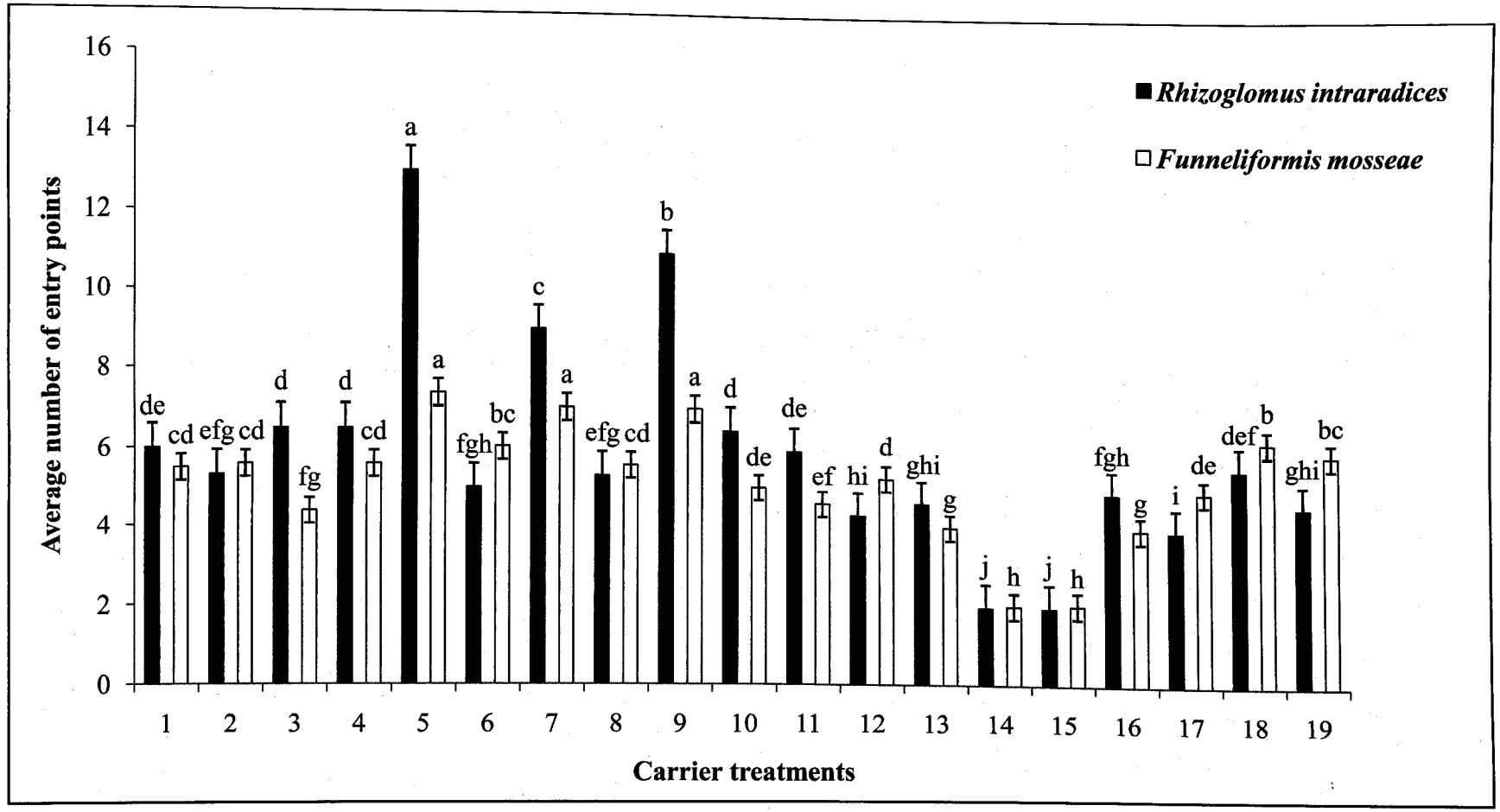


Fig. 6.3: Average number of entry points formed in roots of *Eleusine coracana* Gaertn. plants inoculated with *in vitro* produced propagules of *Rhizoglomus intraradices* and *Funneliformis mosseae* in different carrier treatments.

Legend: Values are means of six replicates ± standard deviation. Bars not sharing the same letters are significantly different ($P \leq 0.05$).

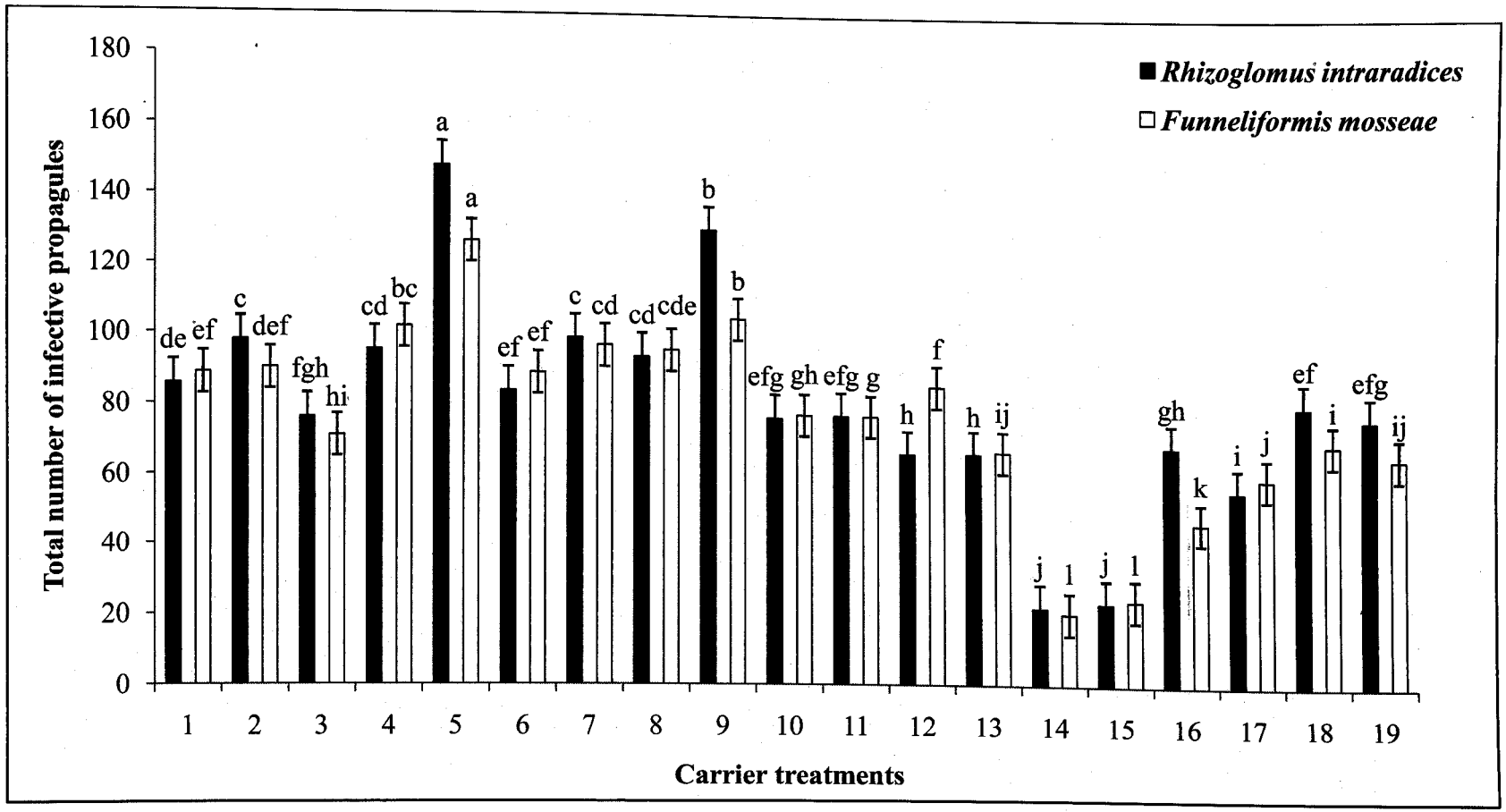


Fig. 6.4: Total number of infective propagules (IP) formed in roots of *Eleusine coracana* Gaertn. plants inoculated with *in vitro* produced propagules of *Rhizoglomus intraradices* and *Funneliformis mosseae* in different carrier treatments.

Legend: Values are means of six replicates ± standard deviation. Bars not sharing the same letters are significantly different ($P \leq 0.05$).

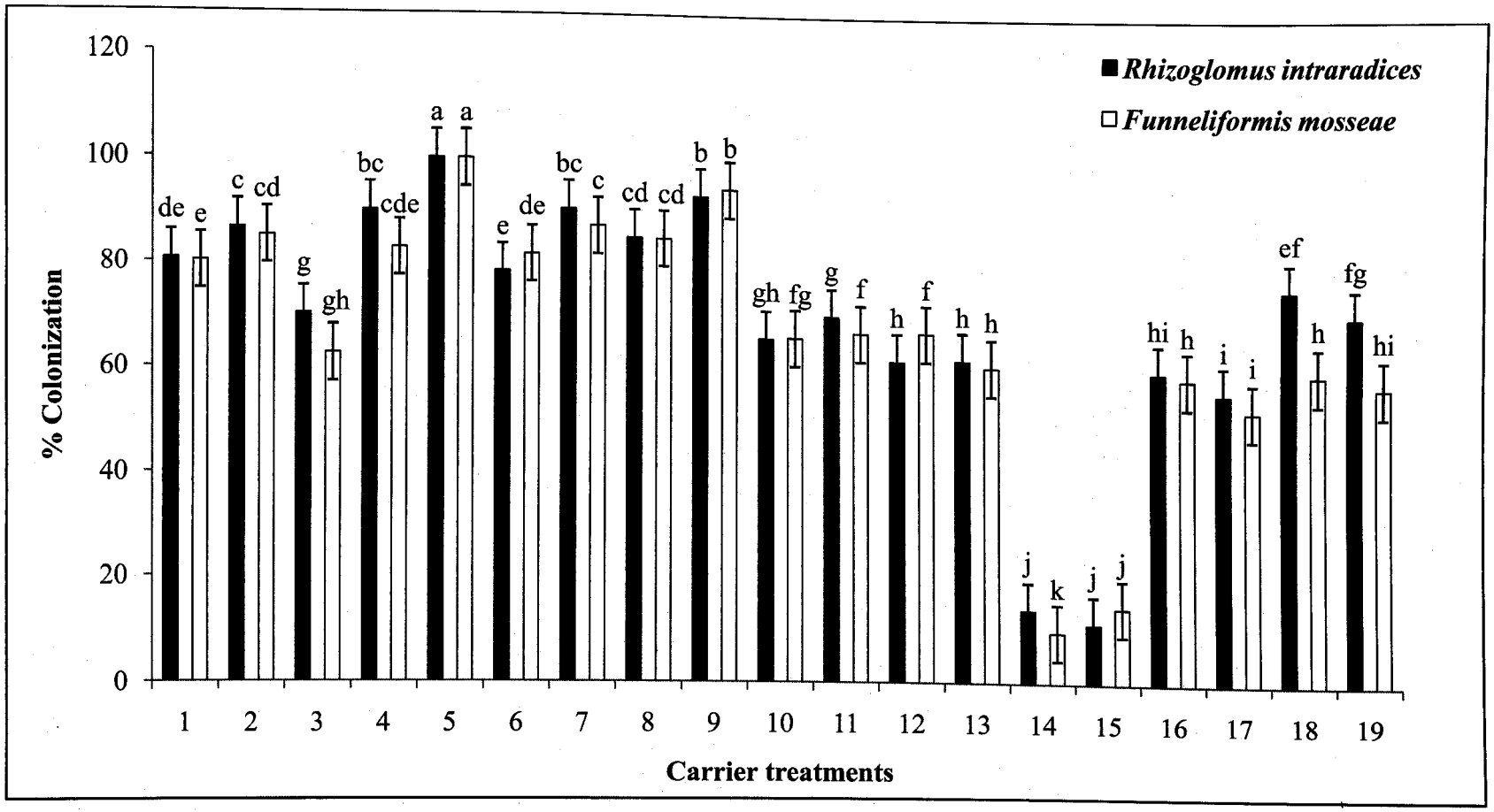


Fig. 6.5: Percent colonization formed in roots of *Eleusine coracana* Gaertn. plants inoculated with *in vitro* produced propagules of *Rhizoglomus intraradices* and *Funneliformis mosseae* in different carrier treatments.

Legend: Values are means of six replicates ± standard deviation. Bars not sharing the same letters are significantly different ($P \leq 0.05$).

Plate 6.5: Micrographs of root colonization in *Eleusine coracana* Gaertn. by monoxenically produced spores of *Rhizoglomus intraradices* and *Funneliformis mosseae* in optimum carrier formulation (treatment 5).

a) Arbuscular colonization by *Rhizoglomus intraradices*.

b) Intra-radical spores of *Rhizoglomus intraradices*.

c) Spore of *Rhizoglomus intraradices* isolated from optimum carrier formulation.

d) Arbuscular colonization by *Funneliformis mosseae*.

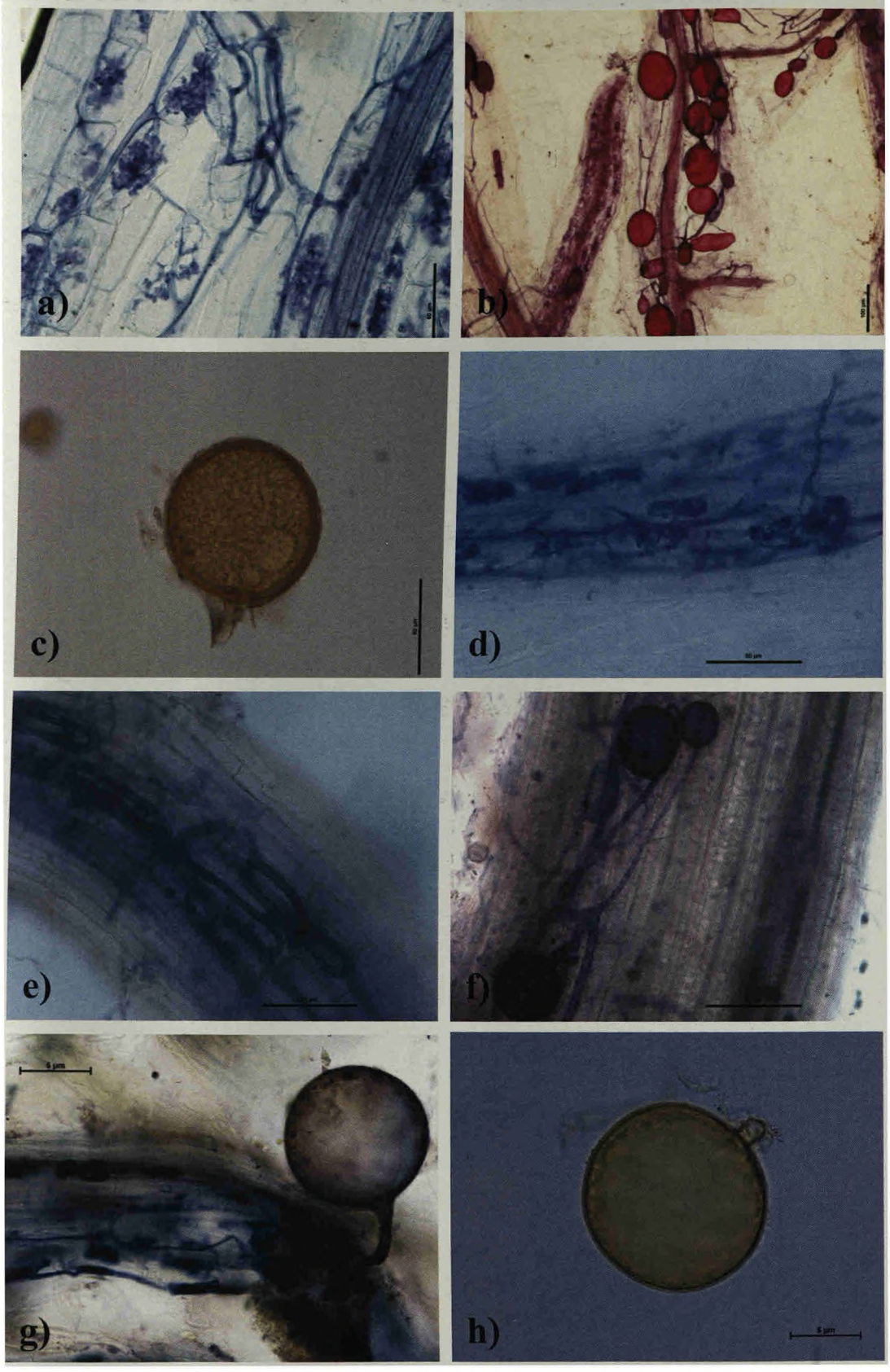
e) Hyphal coils formed by *Funneliformis mosseae*.

f) Vesicular colonization by *Funneliformis mosseae*.

g) Extra-radical spore of *Funneliformis mosseae*.

h) Spore of *Funneliformis mosseae* isolated from optimum carrier formulation.

Plate 6.5



(Gerdemann and Nicolson, 1963) and estimated by a modified method of Gaur and Adholeya (1994) as described in Chapter 3. The total spores obtained per gram of inoculum of *R. intraradices* were 279 spores 100 g^{-1} while for *F. mosseae* 300 spores 100 g^{-1} were obtained. Moisture content of treatment 5 was also estimated as per Fertilizer (Control) Order, 1985 (Ministry of Agriculture, Government of India 2009) which revealed moisture content of 10.3 % by mass.

Physico-chemical parameters of treatment 5 were analyzed. Physico-chemical parameters of treatment 14 and 15, which showed the least AM fungal colonization overall were also analyzed **(Table 6.5)**.

ANOVA was calculated to compare the effect of the carrier treatments on percent colonization by AM fungal species. ANOVA revealed that the effect of carrier treatment on percent colonization by both the AM species was significantly greater as compared to other treatments, F (18, 95) = 106.01, $P \leq 0.05$ for *R. intraradices* and F (18, 95) = 152.7, $P \leq 0.05$ for *F. mosseae* **(Table 6.6)**.

A Pearson product-moment correlation coefficient was computed to assess the relationship between the infective propagules and percent colonization by both the AM species. There was a positive correlation between the two variables [r = 0.926, n = 19, $P \leq 0.01$] for *R. intraradices* and [r = 0.978, n = 19, $P \leq 0.01$] for *F. mosseae*. Overall, a strong positive correlation between the infective propagules and percent colonization was observed in both the AM species **(Fig. 6.6, Fig. 6.7)**.

Table 6.5: Physico-chemical parameters of carrier formulations (treatments 5, 14, 15).

Treatments	pH	E.C. m.mhos/ cm	Macro-nutrients			Micro-nutrients (ppm)				
			O C %	P Kg/Ha	K Kg/Ha	Zn	Fe	Mn	Cu	B
5	8.20	1.70	2.55	371.70	2360.00	3.75	7.44	20.74	1.70	5.30
14	9.60	1.20	0.30	229.90	3946.00	58.40	1.74	153.70	303.90	30.42
15	9.20	0.60	0.32	130.40	3472.00	8.17	3.00	101.60	15.62	8.79

Table 6.6: Analysis of variance for percent colonization of *Eleusine coracana* Gaertn. inoculated with *in vitro* produced AM fungal inocula under different carrier treatments.

		Rhizoglomus intraradices				*Funneliformis mosseae*			
Source	***df***	***SS***	***MS***	***F***	***P***	***SS***	***MS***	***F***	***P***
Between	18	59503.949	3305.775	106.090	≤ 0.05	59931.998	3329.555	152.678	≤ 0.05
Within	95	2960.222	31.160			2071.726	21.808		
Total	113	62464.171				62003.724			

Legend: *df* degrees of freedom, *SS* sum of squares, *MS* mean square.

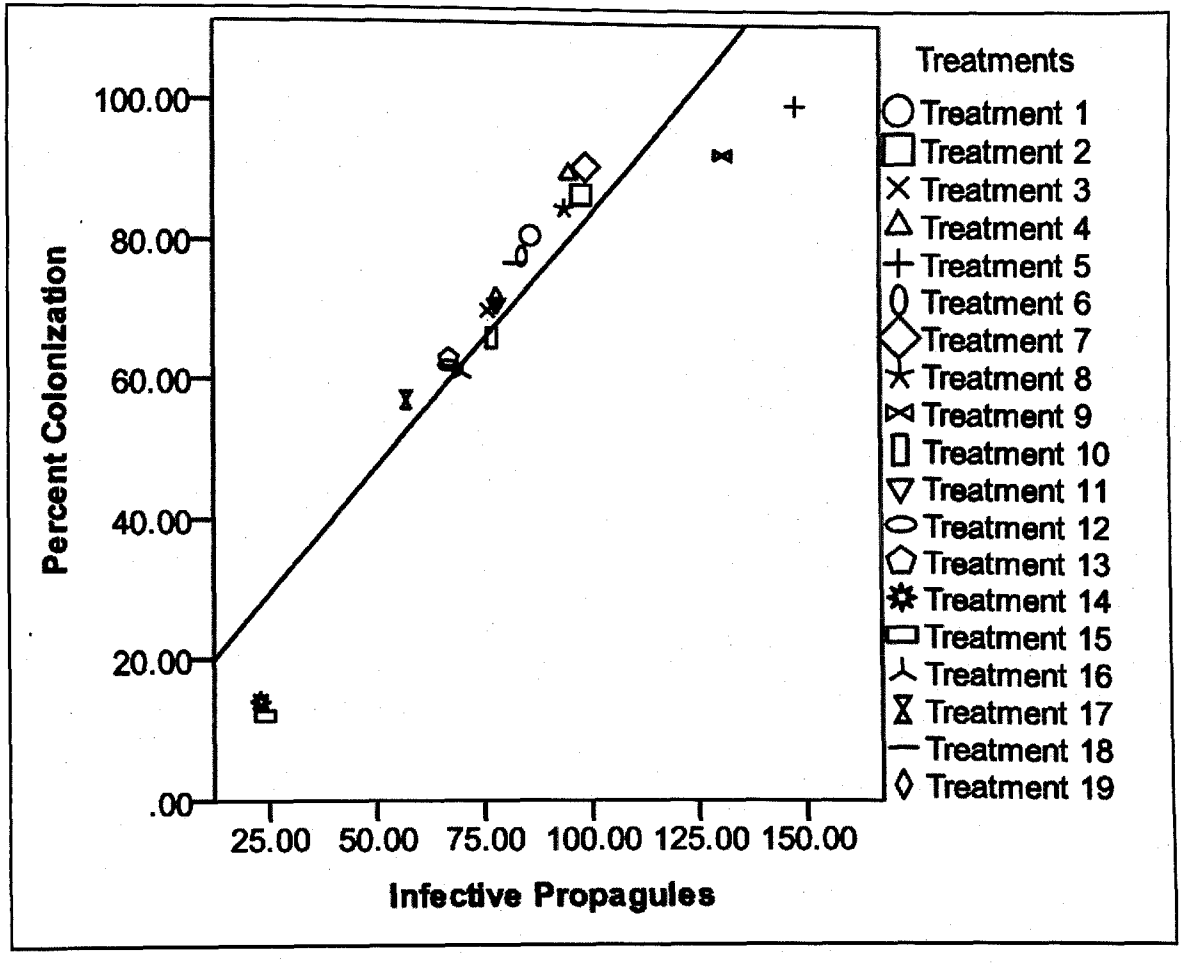


Fig. 6.6: Correlation between infective propagules and percent colonization by *Rhizoglomus intraradices*.

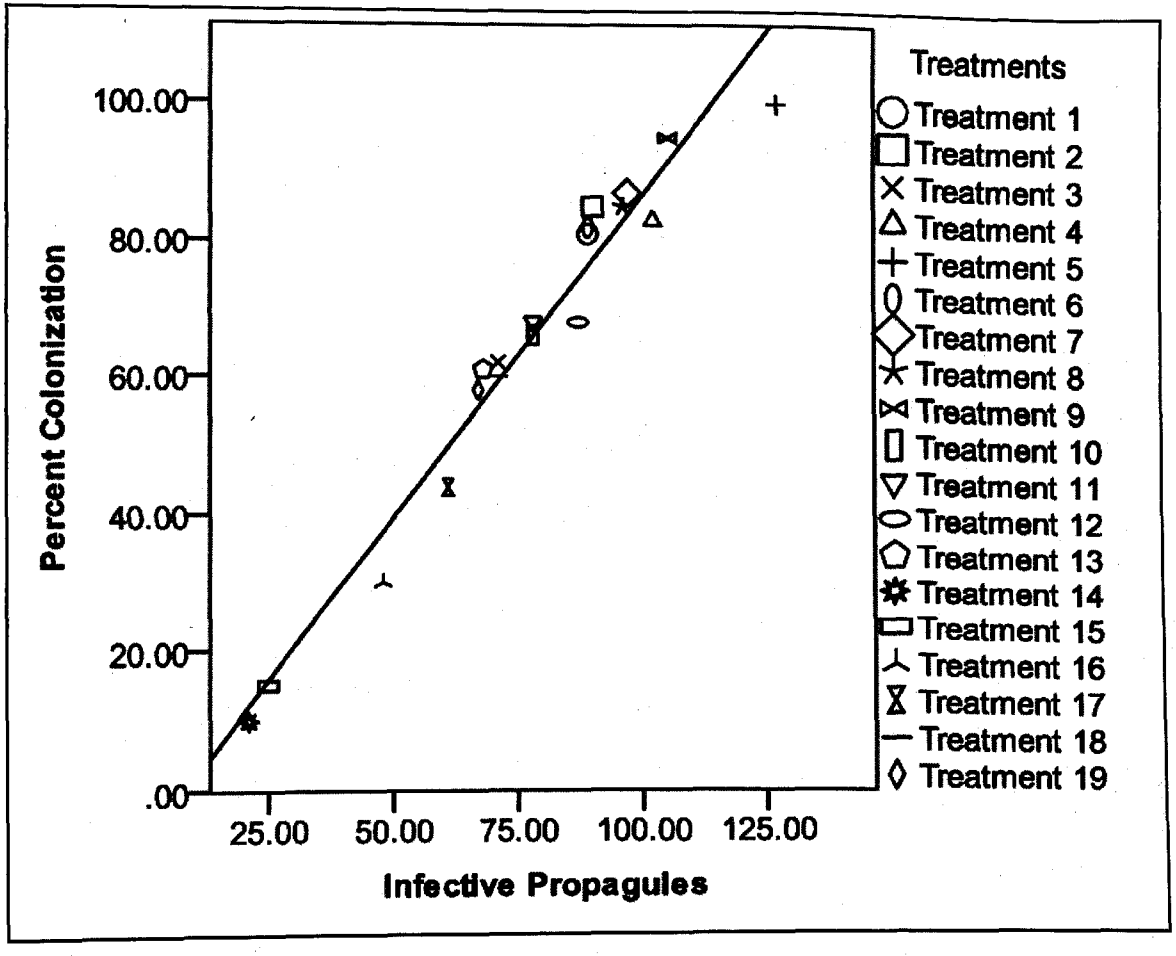


Fig. 6.7: Correlation between infective propagules and percent colonization by *Funneliformis mosseae*.

6.4: DISCUSSION

In the present study, we observed that respiring monoxenically produced AM spores rapidly reduced TTC and accumulated highly coloured formazan in the cytoplasm visible as distinct red colouration. We also observed that older AM spores required higher concentrations of TTC and/or longer incubation periods to achieve maximum red colour reaction. Spore colour reaction by vital dye staining is dependent on incubation time, stain concentration, AM species and spore age (Walley and Germida, 1995). In our study it was observed that the non-viable spores remained unstained and maximum red colour reaction was achieved within 48-72 h with relatively stable colouration. An and Hendrix (1988) reported that viable spores of *F. mosseae* stained bright red in MTT whereas dead spores stained blue or remained unstained and maximum red colour reaction was achieved within 40-72 h. Walley and Germida (1995) suggested that obtaining maximum colour reaction is related to the ease with which the tetrazolium salt can reach the site of dehydrogenase activity. Spore wall thickness and related wall permeability for stain penetration and or delay in enzymatic reduction due to metabolic activity can also account for staining variation (Sutherland and Cohen, 1983; Walley and Germida, 1995). In our study approximately 82 % of *F. mosseae* and 92 % of *R. intraradices* spores were viable as assessed by vital dye staining. Vimard et al. (1999) reported 96.4 % of *in vitro* produced *R. intraradices* spores displayed viability with MTT. The significance of the vital dye-staining technique is largely dependent on its capability to distinguish spores which possess the potential for germination and subsequent host plant colonization. Walley and Germida (1995) reported that spores in which metabolic activity has reduced, the germination and colonization are metabolically improbable and thus such spores are of little practical importance.

In the present study, it was observed that *in vitro* produced spores of both the AM species retained maximum germination potential up to 28 weeks *i.e.* fully matured spores at 197 days old and therefore these spores had been selected for preparation of carrier based inocula. The test for germination potential of monoxenically produced spores by inoculation on fresh MSR media provides better accuracy into the viability of the spores in addition to vital dye staining.

Our study recorded the presence of hyphae, arbuscules, vesicles and extra-radical spores indicating successful colonization of *P. scutellarioides* by monoxenically produced spores of *R. intraradices* and *F. mosseae* grown in sterilized vermiculite. Vimard et al. (1999) reported colonization in *Allium porrum* L. (leek) by monoxenically produced spores of *R. intraradices* grown in sterilized soil mix and calcined montmorillonite clay.

Several researchers have proposed different methods for production of AM fungal inocula in soil based cultures as well as carrier based inocula (Adholeya, 2003). Soil-less techniques, such as monoxenic culture have the advantage of being less bulky, less sensitive to contamination, more concentrated and more uniform than soil cultures, and the propagules can be easily harvested (Okon and Hadar, 1987; Malusá et al., 2012). More recently, methods based on transformed root organ cultures feasible for implementation on a commercial scale have been developed that allow production of large numbers of propagules in a limited space (St-Arnaud et al., 1996; Dalpé and Monreal, 2004; Adholeya et al., 2005). Yet commercially, mycorrhizal inoculum is still produced mainly *via* the conventional method where host plants are grown under controlled conditions using sand/soil as the substrate for mass production of the inoculum in pots, bags, or beds for large-scale application.

Present study represents the first attempt to develop a suitable carrier formulation comprising of vermiculite, cow dung powder, wood powder and wood ash for mass multiplication of *in vitro* produced AM fungal inocula. Both the *in vitro* produced AM fungal species (*R. intraradices* and *F. mosseae*) responded more positively to treatment 5 comprising of carrier formulation (vermiculite: cow dung powder: wood powder: wood ash) in the ratio of 20:8:2:1 through 100 % colonization highlighting the positive interaction between AM, carrier formulation and host plant. Organic amendments are rich in nutrients and their positive influence on AM fungal root colonization has been reported earlier (Gaur and Adholeya, 2002; Gryndler et al., 2005; Perner et al., 2006; Douds et al., 2010; Saranya and Kumutha, 2011; Tanwar et al., 2013). Addition of organic residues to the substrate is known to increase AM fungal sporulation hence leading to increased inoculum production (Gaur and Adholeya, 2005; Silva et al., 2005; Douds et al., 2006; Coelho et al., 2014). Douds et al. (2010) successfully produced AM fungal inoculum in compost mixed with vermiculite, perlite, or horticultural potting media and observed that the propagule numbers were greatest in vermiculite based media. They suggested that the laminar sheets of vermiculite create favourable conditions for growth and persistence of AM fungal hyphae since similar spore populations and colonization of roots among the three media amendments were observed. The carrier formulation developed in our study offers several other benefits such as the carrier materials with the exception of vermiculite are organic in nature besides providing macro- and micro-nutrients, offer increased substrate permeability and improved water retention. In addition they help in maintaining the inoculum potential of *in vitro* produced AM fungal propagules.

Physico-chemical characterization of treatment 5 revealed that although the treatment had high concentration of nutrients especially P, it did not affect AM colonization,

Bolan and Robson (1984) reported significant effects of increased P supply resulting in increased formation of mycorrhizal structures. They also observed that addition of P increased both root growth and the percentage of root length colonized by AM fungi. If AM fungal isolates are produced in organic substrate with high P levels it is likely that the isolates will be more adapted to conditions of high P (Silva, 2006; Coelho et al., 2014). However, the overall least AM fungal interaction was observed in treatments 14 and 15. This may be attributed to the absence of cow dung and wood powder in the treatments. The physico-chemical characterization of treatments 14 and 15 revealed high levels of K, Zn, Mn, Cu and B as compared to the optimum carrier formulation (treatment 5). High concentrations of Zn, Mn, Cu, B and K have been shown to suppress spore germination, root colonization and mycelial growth of AM fungi (Gildon and Tinker, 1981; Moreira and Siqueira, 2002; Luis et al., 2006; Ortas and Akpinar, 2006; Motha et al., 2014).

In the present study, a strong positive correlation between the numbers of infective propagules and percent colonization was observed. The importance of entry points for the development of mycorrhizal structures within the roots and ensuing overall effectiveness of AM fungi is well known (Smith and Read, 2008). After spore germination, the AM fungal hyphae grows towards the host plant roots (Vierheilig et al., 1998a; Sbrana and Giovanetti, 2005), followed by penetration into the root cortical cells and leading to formation of intra-radical structures. Scervino et al. (2005b, 2007) reported a close relationship between the number of entry points and the degree of colonization. In our study as observed in treatment 5, if entry points within the roots are high, percent colonization will also be correspondingly high and therefore can strongly influence the beneficial effect of AM symbiosis on plant growth.

6.5: CONCLUSION

The value of an inoculum production method depends upon its ability to stimulate sporulation and maintain high inoculum infectivity and efficiency (Gianinazzi and Vosátka, 2004) when re-inoculated along with carrier formulation. Thus, from the tested *in vitro* produced isolates, both *R. intraradices* and *F. mosseae* presented such characteristics.

The present study showed that the monoxenically produced spores of *R. intraradices* and *F. mosseae* were highly viable and colonized the host plant successfully without any contamination and along with the carrier material the mycorrhizal association or the inoculum potential was enhanced. Considering the carrier formulation *i.e.* the type of carrier materials used, their physico-chemical attributes and the proportions incorporated, the organic carrier formulation prepared proved suitable for mass production of *in vitro* produced AM fungal inocula. This suggests that the carrier based *in vitro* produced AM fungal inocula developed may be suitable for large scale production of an effective bio-fertilizer. However, further research is needed to test the efficacy of the developed carrier formulation using other AM fungal species and plants.

CHAPTER 7

To maximize the shelf life of the *in vitro* prepared inoculum. (Objective 5)

7.1: INTRODUCTION

The results of Chapter 6 imply that the developed carrier formulation was able to maintain high inoculum infectivity and efficiency of the *in vitro* produced isolates of *Rhizoglomus intraradices* and *Funneliformis mosseae*. Considering the carrier formulation (treatment 5 *i.e.* vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1), the type of carrier materials used, their physico-chemical properties and the proportions incorporated proved that the organic carrier formulation prepared was suitable for mass production of *in vitro* produced AM fungal inocula.

However, for any bio-inoculant to be used within agronomical practices, the carrier formulation should primarily retain the viability of a large population of the incorporated inoculant microorganism during long-term storage period (Malusá and Vassilev, 2014). A good carrier should assure a sufficient shelf life of at least 2-3 months at room temperature (Herrmann and Lesueur, 2013). Non-availability of good quality and appropriate carrier materials can affect shelf life of the beneficial microbial inoculants. Drying process, moisture content, storage conditions plus storage temperature are also important determinants of the shelf life of microbial inoculants or formulations and can affect their activity pre- or post-application (Connick et al., 1996; Kannaiyan, 2000; Larena et al., 2003; Hong et al., 2005; Friesen et al., 2006).

In general, fungi can be preserved and stored as per the following three procedures described by Smith and Onions (1994): 1) continuous growth method which reduces the need for sub-cultivation such as storage of the culture on growth media in the refrigerator, freezer, under oil or water, 2) drying by air or with silica gel and freeze-drying, and 3) reduction of available water in the cells by dehydration (freeze-drying)

or freezing (cryopreservation) at low temperature or in liquid nitrogen, which results in suspension of metabolism.

Monoxenic cultivation of AM fungi is currently the most promising technique to produce pure contaminant-free inocula (Ijdo et al., 2011). However, *in vitro* cultures are only successful for a limited number of AM species, and maintenance is done essentially via sub-cultivation (Plenchette et al., 1996; Strullu et al., 1997; Declerck et al., 1998). But, the risk of contaminations during sub-cultivation is not excluded as well as the loss of infectivity after several successive sub-cultures (Plenchette et al., 1996). Moreover, sub-cultivation is difficult or even unreported for some AM species, impact on genetic stability through successive generations is unknown (Declerck et al., 2005) and, genetic and physiological changes overtime cannot be prevented (Douds and Schenck, 1990; Plenchette et al., 1996; Declerck and Van Coppenolle, 2000). Thus, a method to maintain the viability, purity and stability of monoxenically produced AM fungal isolates over long-term storage periods is needed.

Therefore, this chapter is aimed at maximizing the shelf life of the *in vitro* prepared inocula. Thus this chapter is divided into two parts *viz.,* I) assessment of infectivity potential of *in vitro* prepared inocula in carrier formulation during storage, II) re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions.

7.2: MATERIALS AND METHODS

7.2.1: I) Assessment of infectivity potential of *in vitro* prepared inocula in carrier formulation during storage:

Assessment of infectivity potential of stored *in vitro* prepared AM fungal inocula in carrier formulation was carried out to observe the effects of carrier formulation on maximizing the shelf life of the *in vitro* prepared inocula over different storage temperatures.

7.2.1.1: Extraction of monoxenically produced AM propagules

Extraction of monoxenically produced AM fungal propagules from MSR media was carried out using method by Cranenbrouck et al. (2005) as described in Chapter 5. The extracted spores along with attached extra-radical mycelium were then used for storage studies.

7.2.1.2: Storage of inocula

The inocula of the AM species *Rhizoglomus intraradices* and *Funneliformis mosseae* containing colonized transformed root fragments with extra-radical hyphae and spores was mixed along with optimum carrier formulation (treatment 5) consisting of vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1 (64.51 % : 25.80 % : 6.45 % : 3.22 %) and stored at different temperatures *viz.*, 4°C, 25°C and room temperature (28-30°C) to assess the viability. Inocula of the two AM fungal species were stored separately in zip-loc polythene bags **(Plate 7.1 a)**. Care was taken to ensure the absence of free moisture on the inside of the bags.

Plate 7.1: Assessment of infectivity potential of *in vitro* prepared inocula in carrier formulation upon storage.

a) Storage of inocula in optimum carrier formulation (treatment 5).

b) Infectivity tests of the inocula using *Plectranthus scutellarioides* (L.) R.Br. as the host.

Plate 7.1

7.2.1.3: Infectivity tests

Infectivity tests of the inocula were carried out at the end of every 2nd month after storage for 2, 4, and 6 months, by incorporating 10 g of the carrier based inoculum containing approximately 1200 infective propagules into 500 g of sterilized sand in 15 cm plastic pots and using cuttings of *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) as the host plant **(Plate 7.1 b)**. Each treatment consisted of six replicates. The pots were maintained for a period of 3 months in the shade net of Department of Botany, Goa University, under natural conditions of light, temperature and humidity (4000-6000 lux light intensity, 32°C/25°C day/night, RH 80-90 %) for the establishment of AM symbiosis.

7.2.1.4: Processing of root segments for AM fungal colonization

Assessment of AM colonization in roots of *P. scutellarioides* was carried out by using Trypan blue staining technique after 3 months of growth (Phillips and Hayman, 1970) as described in Chapter 3. The infectivity of the stored inocula was ascertained by the same procedure at every 2nd month up to the 6th month.

7.2.1.5: Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using Root Slide method (Read et al., 1976) as described in Chapter 3.

7.2.1.6: Statistical Analysis

The experimental data was subjected to one-way analysis of variance (ANOVA) followed by Tukey post-Hoc pairwise comparison test. Statistical Package for Social Sciences (SPSS) (ver. 22.0 Armonk, NY: IBM Corp.) was used for all statistical analyses.

7.2.2: II) Re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions:

Assessment of re-germination potential of *in vitro* produced spores of *R. intraradices* and *F. mosseae* from carrier based inocula to *in vitro* conditions was carried out one time to observe the efficiency of carrier formulation in maintaining the vigour of *in vitro* produced spores.

7.2.2.1: Extraction of AM fungal propagules

Extraction of AM propagules (spores and colonized root fragments) from the carrier formulation was carried out by Wet Sieving and Decanting technique (Gerdemann and Nicolson, 1963) as described in Chapter 3.

7.2.2.2: AM fungal propagule disinfection process

The disinfection process was modified from Mosse (1959), Mertz et al. (1979), Daniels and Menge (1981), and Bécard and Fortin (1988). Isolated propagules were first rinsed twice with sterilized distilled water after which they were disinfected. The propagules were then disinfected with 250-400 µl sodium hypochlorite (NaClO) for 3-5 min. After disinfection, the propagules were rinsed three times with sterilized distilled water and treated with antibiotic solution (Streptomycin 0.02 % + Gentamycin 0.01 %) for 10 min.

7.2.2.3: Germination of disinfected propagules

Disinfected AM propagules were then inoculated on Modified Strullu-Romand (MSR) minus sucrose media and Petri plates were incubated in the dark at 27°C in an inverted position.

All microscope observations were made using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x), and Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom). Micrographs were imaged by Nikon Digital Sight DS-U3 digital camera, and were not digitally edited.

7.3: RESULTS

7.3.1: I) Infectivity potential of *in vitro* prepared inocula in carrier formulation during storage

Upon storage the infectivity potential of *in vitro* prepared inocula of both the AM fungal species *viz., R. intraradices* and *F. mosseae* were observed to be optimum at 25°C when compared to other storage temperatures (4°C and room temperature) used in the study. AM fungal colonization levels of 93.33-89.27 % for *R. intraradices* and 89.32-85.58 % for *F. mosseae* were observed at 25°C indicating that the inocula did not lose infectivity potential even after 6 months of storage. ANOVA comparing the effect of storage at the three different temperatures on percent colonization by *in vitro* prepared inocula in carrier formulation revealed that the percent colonization was significantly greater where propagules had been stored at 25°C **(Fig. 7.1, Table 7.1)**.

7.3.2: Re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions

It was observed that spores of *R. intraradices* germinated 2-10 days after plating while spores of *F. mosseae* germinated 10-15 days after plating **(Plate 7.2 a-d)**, and 100 % germination was recorded indicating that spores of both the AM species were viable enough to re-germinate when cultured back to *in vitro* conditions.

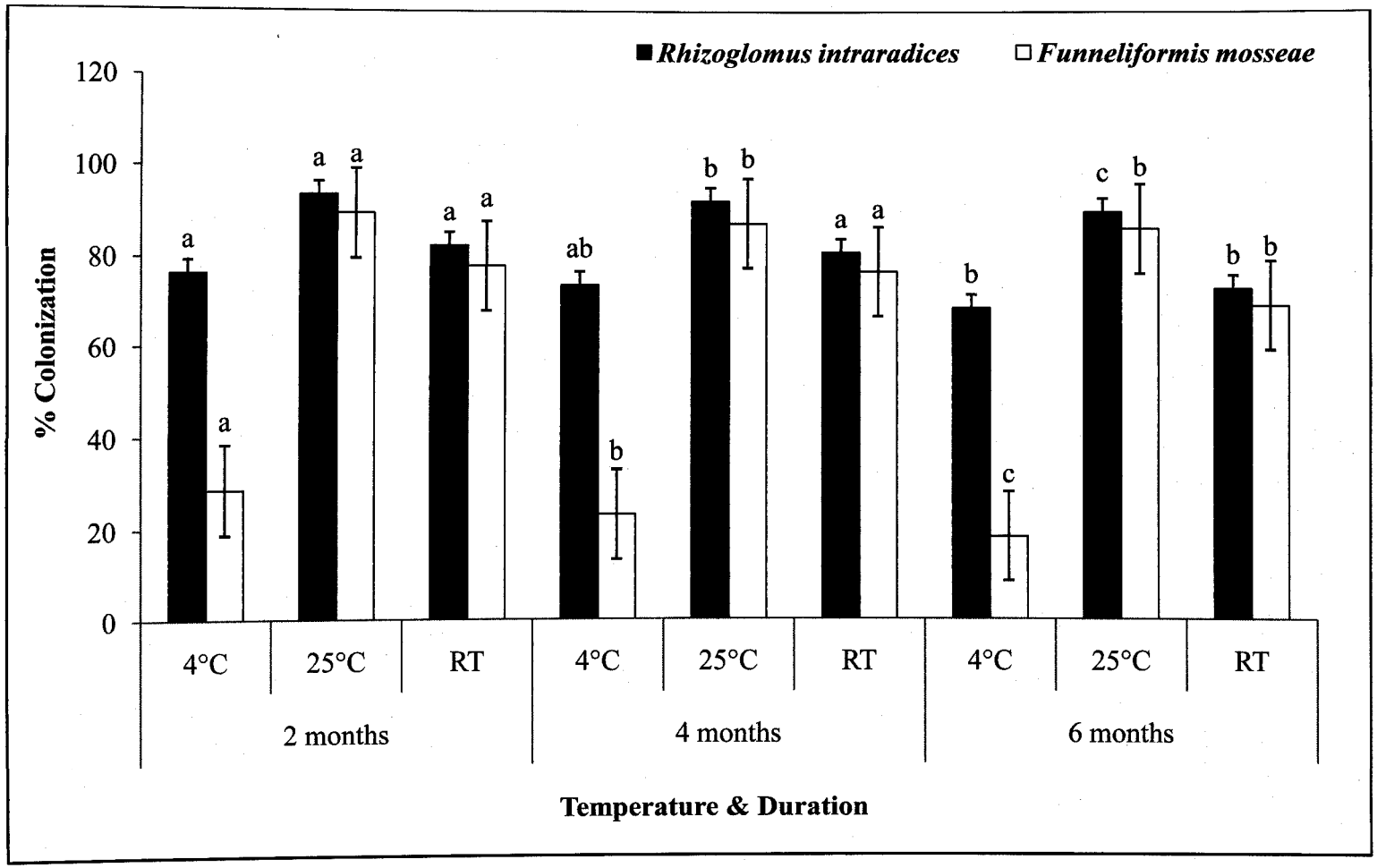


Fig. 7.1: Percent root colonization in *Plectranthus scutellarioides* (L.) R.Br. by *in vitro* produced AM fungal inocula stored at different temperatures. Legend: Values are means of six replicates ± standard deviation. Bars not sharing the same letters are significantly different ($P \leq 0.05$). RT = Room Temperature (28-30°C).

Table 7.1: Analysis of variance for percent colonization of *Plectranthus scutellarioides* (L.) R.Br. inoculated with *in vitro* produced AM fungal inocula stored at different temperatures.

Temperature			*Rhizoglomus intraradices*				*Funneliformis mosseae*			
	Source	***df***	***SS***	***MS***	***F***	***P***	***SS***	***MS***	***F***	***P***
4°C	**Between**	2	203.473	101.736	5.971	0.02*	319.062	159.531	4.455	0.03*
	Within	15	218.938	17.039			537.104	35.807		
	Total	17	422.411				856.167			
25°C	**Between**	2	49.681	24.840	18.849	0.00**	43.942	21.971	16.118	0.00**
	Within	15	19.768	1.318			20.447	1.363		
	Total	17	69.449				64.388			
RT	**Between**	2	311.924	155.962	16.967	0.00**	265.577	132.789	5.324	0.01**
	Within	15	137.881	9.192			374.130	24.942		
	Total	17	449.805				639.708			

Legend: *df* degrees of freedom, *SS* sum of squares, *MS* mean square; **Significant at $P \leq 0.01$ and $P \leq 0.05$, *Significant at $P \leq 0.05$; RT = Room Temperature (28-30°C).

Plate 7.2: Re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions.

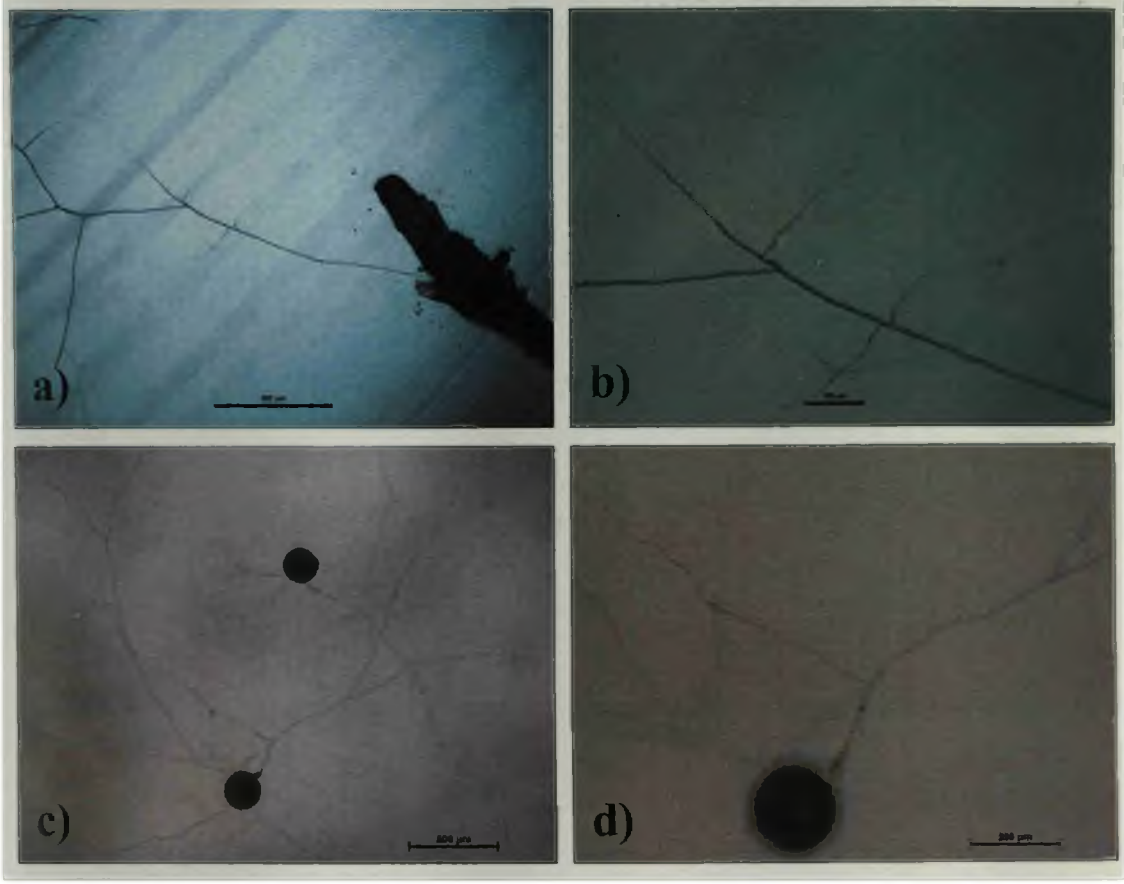
a) *In vitro* germination of colonized root fragment of *Rhizoglomus intraradices*.

b) Enlarged view of branching of hypha from germ tube emerging from colonized root fragment of *Rhizoglomus intraradices*.

c) *In vitro* spore germination of *Funneliformis mosseae*.

d) Enlarged view of *in vitro* spore germination of *Funneliformis mosseae*.

Plate 7.2



7.4: DISCUSSION

In the present study, *in vitro* prepared inocula in carrier formulation were tested for their infectivity potential during storage. Upon storage the infectivity potential of *in vitro* prepared inocula of both the AM fungal species *viz., R. intraradices* and *F. mosseae* were observed to be optimum at 25°C when compared to other storage temperatures (4°C and room temperature) used in the study. AM fungal colonization levels of 93.33-89.27 % for *R. intraradices* and 89.32-85.58 % for *F. mosseae* were observed at 25°C indicating that the inocula did not lose infectivity potential even after 6 months of storage. Louis and Lim (1988) showed that the germination ability of a tropical isolate of *R. clarum* was enhanced after 3-6 months of dry storage at 25-30°C. Kuszala and Gianinazzi-Pearson (2011) preserved propagules of AM fungal isolates belonging to *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora* extracted from 6-11 months old pot cultures, in osmosed water at 4°C, at ambient temperature, at +27°C or at +37°C. AM fungal propagules are commonly stored at 4-5°C in dried pot culture soil (Siqueira et al., 1985). At the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM), most of the AM fungal propagules produced in pot cultures are stored in dried soil or substrate at 4°C for different time periods depending on the genus. A number of studies have demonstrated that the preservation of AM fungal propagules in soil, saturated salt solutions, alginate beads and osmosed water by drying or cold storage was optimum at 4°C (Ferguson and Woodhead, 1982; Mugnier and Mosse, 1987; Tommerup, 1988; Strullu and Plenchette, 1991; Kuszala et al., 2001; Wagner et al., 2001). This was, however, contrary to our findings. The longer viability at low temperature could be attributed to lower metabolic rate of the propagules. Our results however indicate that 25°C was optimum storage temperature for the carrier based AM fungal inocula which can be considered as a feasible option.

It has been reported that the higher maintenance of viability of AM fungal inocula in substrates containing vermiculite than in substrate containing only sand could be due to better aeration and water retention capacity (Santana et al., 2014). The viability and efficiency of the inocula can be maintained for several months at 20-25°C, but the inocula should be kept in its packaging and must be partially dried (Berruti et al., 2014). It is important to stress that the inocula must be dried before storage (Hung and Sylvia, 1988; Sylvia, 1994). If in a humid substrate the viability of inocula declines with storage (Sylvia and Jarstfer, 1992). It has been reported that the sources of inoculum based in colonized roots stored in water or moistened vermiculite retained its infective potential only for 2 months (Sieverding, 1991).

The AM species used in our study *viz., R. intraradices* and *F. mosseae* occur in a wide range of biomes and therefore it is possible that different isolates of these species from different geographical regions may exhibit different survival strategies adapted to that particular environment, and hence may exhibit viability in a range of temperatures.

In our study, it was further observed that spores of *R. intraradices* germinated 2-10 days after plating and spores of *F. mosseae* germinated 10-15 days after plating, and no spore dormancy was observed indicating that spores of both the AM species were viable, re-germinating when cultured back to *in vitro* conditions.

7.5: CONCLUSION

Considerable progress has been made over the last few years in the preservation of AM fungi. Some of the methods developed with pot- and *in vitro*-cultured isolates are adequate for short-term storage using standard infrastructures and equipment (sub-cultivation, preservation in alginate beads or in dried soil), while others can be applied

to long-term storage (cryopreservation or lyophilization) but require specific equipment and infrastructures (Lalaymia et al., 2014).

The results obtained in our study are very promising from the point of view of a possible commercial production of AM fungal inoculants. The *in vitro* produced inocula stored at 25°C remained viable up to 6 months in the organic carrier formulation composed of vermiculite, cow dung powder, wood powder and wood ash, therefore being recommended for use as bio-inoculants. However, further experiments may be needed to test the suitability of the carrier formulation developed for long-term preservation and reproducibility in other AM fungal species.

CHAPTER 8

To study the effect of *in vitro* produced carrier based bio-inocula on selected plant species suitable for revegetation of the sand dunes.

(Objective 6)

8.1: INTRODUCTION

Coastal sand dune ecosystems are fragile and dynamic environments consisting predominantly of sandy soils with low nutrient and organic matter content, are exposed to salt-spray, sand movement intermittent with foliage burial, a wide range of humidity and temperature, and strong winds (Maun, 2004; Rippley and Pammenter, 2008; Yamato et al., 2012). Coastal sand dune systems are of utmost ecological importance providing a variety of ecosystem services such as coastal protection, erosion control, water capture and purification, sustaining flora and fauna in addition to being used for tourism, recreation and research (Barbier et al., 2011). In spite of their vast importance, these ecosystems are severely affected by anthropogenic actions like mining, pedestrian traffic, urbanization, port and tourist activities, and deforestation which compromise and affect the local ecological communities (Emery and Rudgers, 2010).

Arbuscular mycorrhizal (AM) fungi are an essential functional group of soil microbiota in coastal dune habitats around the world providing numerous benefits to plants (van der Heijden et al., 1998; Greipsson, 2002; Gianinazzi et al., 2010; Johansen et al., 2015). However, their propagules may be rare or less in un-vegetated dune sites (Sylvia and Will, 1988). If the vegetation of an area is destroyed, the population of viable propagules of native AM fungi declines (Miller, 1979; Reeves et al., 1979). Stabilization or restoration of sand dunes by planting of vegetation has long been recognized as an effective means of slowing the inland movement of sand (Jagschitz and Bell, 1966; Hewett, 1970; Ranwell, 1972; Woodhouse, 1982). Studies have shown that the composition of AM fungal community affects the structure and functioning of plant communities, and plays a fundamental role in conservation and stabilization of sediments in ecosystems such as coastal sand dunes (de Assis et al., 2016).

As AM fungi are ubiquitous soil inhabitants a key link between plants and soil minerals, enhancing plant access to unavailable nutrient sources, especially insoluble P. The fungi have gained a growing interest as natural fertilizers (Gianinazzi et al., 1990; Gianinazzi and Vosatka, 2004; Fitter et al., 2011; Berruti et al., 2015). Many research studies have depicted the importance of AM fungal bio-inoculants for agriculture as well as for reforestation programs of degraded areas (Jarstfer and Sylvia, 1992; Caravaca et al., 2002; Douds et al., 2007; Ijdo et al., 2011; Souza et al., 2010). AM fungal bio-inoculants are tailored formulations consisting of propagules of individual strains or consortia of strains mixed with an inert carrier (Gentili and Jumpponen, 2006). A bio-inoculant product is best used when there is a reason to believe that indigenous AM fungal populations are low or native AM fungi are ineffective. It should be stressed that the mere presence of AM fungal isolate/isolates does not imply benefits to host plants (Adholeya et al., 2005). Although the presence of AM fungi is widespread, field experiments have shown that a further addition of AM fungi by inoculation can positively affect host plant root colonization and increase plant growth and productivity (McGonigle, 1988; Lekberg and Koide, 2005; Lehmann et al., 2012). The application of AM fungal bio-inoculants by seedling inoculation plays an important role in growth and establishment of the plants during field transplantation (Azcön-Aguilar and Barea, 1997). Transplantation of mycorrhizal nursery seedlings may prove advantageous on reclamation sites as it will facilitate better acquisition of limited soil nutrients (Jasper et al., 1989; Sylvia, 1990). However, the infectivity and efficiency of AM fungal isolates is not always related to the degree of root colonization (Corkidi et al., 2004) making it necessary to test the effectiveness of the bio-inocula for practicable relevance in agriculture, horticulture, forestry and environment-recovery programs.

Therefore, this chapter is aimed at evaluating the effectiveness of *in vitro* produced carrier based bio-inocula of two AM fungal species *viz., Rhizoglomus intraradices* (Schenck & Smith) Sieverding, Silva & Oehl, and *Funneliformis mosseae* (Nicolson & Gerdemann) Walker & Schüßler in stimulating growth and nutrient uptake of *Anacardium occidentale* L. (Anacardiaceae). *A. occidentale* is a deep rooted woody perennial tree thriving well in semi-arid conditions with mild resistance to drought and salinity, and dominant in the backshores (hind shore dunes) on the Goa coast (Dessai, 1995). The species was thus considered suitable for revegetation strategies of sand dune ecosystems and hence used in the present study.

8.2: MATERIALS AND METHODS

8.2.1: Soil analysis

Analysis of physico-chemical properties of sand was carried out at the beginning of the experimental setup. Composite soil sample from study site Varca (S4) (Grid Ref. 15°13'58.68"N, 73°55'31.95"E) was air-dried and sieved to 2 mm to remove larger soil particles. The sample was then submitted to Government of Goa Agricultural Department, Soil Analysis Laboratory, Margao, Goa for chemical analysis. Analyses followed procedures laid out by Singh, Chhonkar and Dwivedi (2005), pH was measured by pH meter (Elico L1 120) in a 1:2 ratio soil water solution, Electrical Conductivity was measured by conductivity meter (Elico CM 180) in a 1:5 ratio soil suspension. Organic Carbon (OC) was analyzed by Walkley and Black (1934) titration method. Available Phosphorus (P) was estimated by Bray and Kurtz (1945) method. Available potassium (K) was estimated by ammonium acetate method from Hanway and Heidal (1952) using flame photometer (Systronic 3292). Available micro-nutrients

zinc (Zn), iron (Fe), manganese (Mn), copper (Cu) were quantified by DTPA-$CaCl_2$-TEA method from Lindsay and Norvell (1978) using atomic absorption spectrophotometer (AAS-EC Element AS AAS 4139), and boron (B) was assessed by hot water soluble method from Berger and Truog (1939).

8.2.2: Experimental design

The experiment comprised of the following six treatments:

- Treatment 1 (T_1): Control sterilized sand
- Treatment 2 (T_2): Control unsterilized sand
- Treatment 3 (T_3): *R. intraradices* inoculum + sterilized sand
- Treatment 4 (T_4): *R. intraradices* inoculum + unsterilized sand
- Treatment 5 (T_5): *F. mosseae* inoculum + sterilized sand
- Treatment 6 (T_6): *F. mosseae* inoculum + unsterilized sand

The six treatments with eight replicates per treatment were arranged in a randomized block design.

Two kilograms of sand was dispensed in pots (21.5 cm diameter) for all treatments (T1 to T6). For treatments (T1, T3, T5) containing sterilized sand, sand was sterilized in hot air oven at 180°C for 3 days. For AM treatments (T3, T4, T5, T6), 300 g of *in vitro* produced inoculum (600 spores g^{-1} + extra-radical hyphae + colonized root fragments) mixed along with carrier formulation consisting of vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1 (64.51 % : 25.80 % : 6.45 % : 3.22 %) was added. Seeds of *A. occidentale* (local variety) were soaked overnight in sterilized distilled water and then sown in all the pots (2-3 seeds per pot later retaining one seedling per pot). Pots were maintained for a period of six months (183 days after sowing) in the shade net of Department of Botany, Goa University, under natural

conditions of light, temperature and humidity (4000-6000 lux light intensity, 32°C/25°C day/night, RH 80-90 %). Hoagland's solution (Hoagland and Arnon, 1950) minus P was added after 20 days interval. Various fungal, plant growth and chemical parameters were recorded **(Plate 8.1 a-e)**.

8.2.3: Processing of root segments for AM fungal colonization

The root systems of *A. occidentale* from five randomly selected pots (per treatment) were assessed for AM fungal colonization at 45 and 183 days after sowing (DAS) by using Trypan blue staining technique (Phillips and Hayman, 1970) as described in Chapter 3.

8.2.4: Estimation of percent root colonization

Estimation of percent root colonization by AM fungi was carried out using Root Slide method (Read et al., 1976) as described in Chapter 3.

8.2.5: Extraction of AM fungal spores

Extraction of AM fungal spores was carried out by Wet Sieving and Decanting technique (Gerdemann and Nicolson, 1963) at 183 days after sowing (DAS) as described in Chapter 3.

8.2.6: Quantification of spore density of AM fungi

Quantification of AM spore numbers was carried out by a modified method of Gaur and Adholeya (1994) as described in Chapter 3.

8.2.7: Plant vegetative growth parameters

Plant growth (height, basal stem diameter, number of branches, leaf number, leaf area, petiole length, internode length, and root length) was recorded. Leaf area and petiole

Plate 8.1: Pictorial representation of experimental design in *Anacardium occidentale* L.

Plate 8.1

Inocula in optimum carrier formulation

Seeds of *Anacardium occidentale* L.
(soaked overnight in sterilized water)

2-3 seeds sown per pot later retaining one seedling per pot

Six treatments with eight replicates per
treatment were arranged in a
randomized block design

Anacardium occidentale L. plant
(183 DAS)

length were recorded from the 3rd leaf from top. A meter ruler was used to measure the plant height from base (above ground) to the tip of the main shoot. Leaf area was recorded on a graph sheet and measured. All the parameters were recorded from three randomly selected replicates per treatment at 183 DAS.

8.2.8: Plant above- and below-ground biomass measurement

Fresh and dry weights of stem, leaf and root were recorded. Total plant dry biomass and root to shoot ratio were also measured on dry weight basis (oven dried at 70°C, 72 h until reaching a constant weight). All the measurements were recorded from three randomly selected replicates per treatment at 183 DAS.

8.2.9: Plant aboveground (shoot) nutrient analyses

Macro- and micro-nutrients were analyzed from three replicates per treatment separately at 183 DAS, wherein stem and leaf samples were rinsed with distilled water and air dried. Air dried samples were then dried to constant weight in oven at 70°C for 72 h. Samples were ground using mixer grinder to obtain fine powder which was then placed on butter paper and stored in dry condition in zip-loc bags until analyses. Nutrient analyses were carried out at Soil Sciences Laboratory, Natural Resource Management Section, ICAR-CCARI, Ela, Old Goa; Directorate of Agriculture, Soil Testing Laboratory, Ela, Old Goa; and Department of Chemistry, Goa University. Nutrient analyses [phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and boron (B)] followed procedures laid out by Singh, Chhonkar and Dwivedi (2005).

8.2.9.1: Sample digestion

For the release of mineral elements from plant tissues, wet oxidation method was adopted wherein powdered shoot material was digested using di-acid mixture (concentrated nitric acid and 60 % perchloric acid). The procedure for which is described as follows: 1 g of dry powdered shoot material was added to a 250 mL conical flask after which 10 mL of conc. HNO_3 was added and a funnel was placed on the flask. The flask was kept overnight at a covered place or chamber for pre-digestion. After pre-digestion when the shoot material was no longer visible, 10 mL of conc. HNO_3 and 3 mL of $HClO_4$ was added, and the flask was kept on a hot plate in acid-proof digestion chamber with a fume exhaust system. The hot plate was heated at 100°C for first one hour and then the temperature was raised to 300°C. The acid contents were reduced to 2-3 mL by heating at the same temperature and without allowing the contents to dry up. The digestion was continued till the contents became colourless and only white dense fumes appeared. The flasks were then removed from the hot plate and allowed to cool. The contents from the flasks were filtered through Whatman No. 42 filter paper into a 100 mL volumetric flask and the total volume was made to 100 mL with distilled water. This aliquot was then used for determination of P, K, Ca, Mg, Na, Fe, Zn, Mn, Cu and B concentrations.

The concentration of P was determined from the aliquot by vanadomolybdophosphoric yellow colour method (Chapman and Pratt, 1982). K and Na were determined by Flame Photometry (Microcontroller Flame Photometer, Labtronics LT-671). Ca, Mg, Fe, Zn, Mn and Cu were quantified using Atomic Absorption Spectrophotometer (Analytikjena novAA 400 P). B was determined by azomethine-H method (Gupta, 1967; John et al., 1975).

Carbon (C), nitrogen (N) and sulphur (S) were measured from the dried powdered shoot material using CHN Elemental Analyzer (Elementar Variomicro Cube CHNS Analyser) with Sulfanilamide as the standard.

8.2.10: Mycorrhizal dependency

Mycorrhizal dependency is defined as the degree to which a plant is dependent on the mycorrhizal association to attain its maximum growth or yield at a given level of soil fertility (Gerdemann, 1975). It is based on determining the extent of growth increase attributed to mycorrhizal association in sterilized soil (Norris et al., 1992; Mukerji, 1996). Mycorrhizal dependency of a given plant species can differ based on the AM species being studied, plant species involved and soil P availability (Schenck et al., 1974; Plenchette et al., 1983).

Mycorrhizal dependency (MD) was calculated by the formula given by Menge et al. (1978).

MD % = shoot dry weight of mycorrhizal plant ÷ shoot dry weight of non-mycorrhizal plant × 100

8.2.11: Mycorrhizal efficiency index

It has been reported that the introduction of mycorrhizal fungi can improve plant growth in unsterilized soil containing native endophytes. Mycorrhizal efficiency index enables the assessment of growth improvement brought about by mycorrhizal inoculation in unsterile soil consisting of indigenous AM fungi (Norris et al., 1992). It is also useful in assessing the extent to which the introduced AM fungus competes with indigenous AM fungi to bring about plant growth response (Mehrotra, 2005).

Mycorrhizal efficiency index (MEI) was calculated by the formula given by Bagyaraj et al. (1994).

MEI % = weight of inoculated plant – weight of un-inoculated plant ÷ weight of inoculated plant × 100

8.2.12: Statistical analysis

The experimental data was subjected to one-way analysis of variance (ANOVA) followed by Tukey's HSD test at $P \leq 0.05$. Parameters were correlated using Pearson's correlation coefficient at $P \leq 0.01$ and $P \leq 0.05$. Statistical Package for Social Sciences (SPSS) (ver. 22.0 Armonk, NY: IBM Corp.) was used for all statistical analyses.

All microscope observations were made using bright field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40x, 100x, 400x, 1000x), and Olympus stereo microscope SZ2-ILST (10 × 4.5 zoom). Micrographs were imaged by Nikon Digital Sight DS-U3 digital camera, and were not digitally edited.

8.3: RESULTS

8.3.1: Soil analysis

Results of physico-chemical properties of sand medium revealed that the pH was alkaline **(Table 8.1)**. Electrical Conductivity (E. C.) was in normal range. Organic carbon percentage, available P concentrations and available K concentrations were low. Micro-nutrients *i.e.* Zn, Fe, Mn, Cu, and B were present in low-high levels (0.00-1.30 ppm).

Table 8.1: Physico-chemical properties of sand at the beginning of the experiment.

pH	E. C. m.mhos/ cm	Macro-nutrients			Micro-nutrients (ppm)				
		O C %	P Kg/Ha	K Kg/Ha	Zn	Fe	Mn	Cu	B
8.5	<1	0.13	21.9	80	0.00	1.18	0.26	0.03	1.30

Legend: Values are derived from a composite sample.

8.3.2: Root colonization and spore density

Results of root colonization and spore density in *A. occidentale* are shown in **Table 8.2.** Root colonization (hyphal, arbuscules, vesicles, intra- and extra-radical spores) was observed in all AM inoculated treatments (T3, T4, T5 and T6) except in the control treatments (T1 and T2) at 45 DAS **(Plate 8.2 a-f).** The magnitude of percent root colonization varied significantly among the AM treatments, and with the two studied AM species inocula. Root colonization was highest with *R. intraradices* when recorded at 45 DAS (89.0 %) and at 183 DAS (79.8 %) in treatment 3 (*R. intraradices* inoculum + sterilized sand). The control treatments (un-inoculated plants) showed absence of, or little, root colonization. The study revealed that there was a reduction in percent root colonization at 183 DAS when compared to percent root colonization at 45 DAS in all AM treatments.

AM spore density was maximum in treatment 3 (206.6 100 g^{-1} soil) at 183 DAS. Least spore density was recorded in control with unsterilized sand *i.e.* treatment 2 (11.8 100 g^{-1} soil).

8.3.3: Plant vegetative growth response

A statistically significant positive response of *A. occidentale* to AM treatments was observed in growth characteristics *viz.,* plant height, stem diameter, number of branches, number of leaves, leaf area, petiole length, internode length, and root length as compared to control treatments **(Table 8.3, Plate 8.3 a).** Among the AM treatments, treatment 3 (*R. intraradices* inoculum + sterilized sand) recorded a significant increase in all the growth characteristics of *A. occidentale.*

Table 8.2: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on root colonization and spore density in *Anacardium occidentale* L.

Treatments	Percent root colonization (at 45 DAS)	Percent root colonization (at 183 DAS)	Spore density 100 g^{-1} soil (at 183 DAS)
T_1	$0.00^{e} \pm 0.00$	$0.00^{c} \pm 0.00$	$0.00^{c} \pm 0.00$
T_2	$0.00^{e} \pm 0.00$	$7.40^{c} \pm 5.59$	$11.80^{c} \pm 6.45$
T_3	$89.00^{a} \pm 9.62$	$79.80^{a} \pm 15.15$	$206.60^{a} \pm 38.82$
T_4	$76.00^{b} \pm 8.94$	$58.40^{b} \pm 9.07$	$99.20^{b} \pm 10.84$
T_5	$60.00^{c} \pm 7.07$	$49.60^{b} \pm 22.30$	$90.20^{b} \pm 8.13$
T_6	$46.00^{d} \pm 10.25$	$38.00^{b} \pm 22.46$	$79.80^{b} \pm 45.55$

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Values are means of five replicates ± standard deviation. Values in the same column not sharing the same superscript are significantly different at $P \leq 0.05$; DAS = Days after sowing.

Plate 8.2: Micrographs of root colonization in *Anacardium occidentale* L. by monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae*.

a) Arbuscular colonization in *Anacardium occidentale* at 45 DAS.

b) Vesicular colonization in *Anacardium occidentale* at 45 DAS.

c) Vesicular colonization in *Anacardium occidentale* by *Rhizoglomus intraradices* at 183 DAS.

d) Vesicular colonization in *Anacardium occidentale* by *Funneliformis mosseae* at 183 DAS.

e) Extra-radical spores of *Rhizoglomus intraradices* at 183 DAS.

f) Extra-radical spore of *Funneliformis mosseae* at 183 DAS.

Plate 8.2

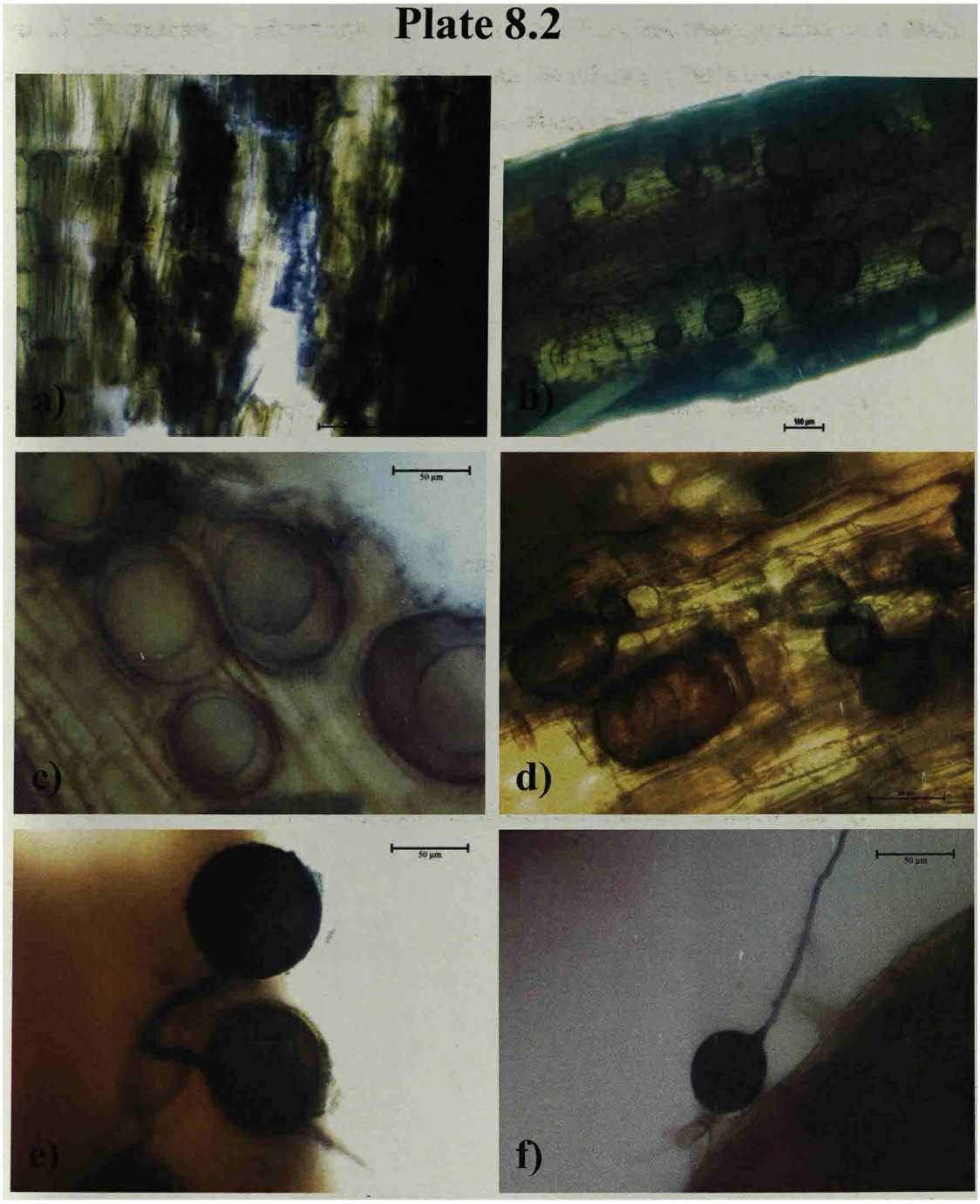


Table 8.3: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on vegetative growth response in *Anacardium occidentale* L. at 183 DAS (days after sowing).

Treatment	Plant height (cm)	Stem diameter (cm)	Number of branches plant^{-1}	Number of leaves plant^{-1}	Leaf area (cm^2)	Petiole length (cm)	Internode length (cm)	Root length (cm)
T_1	$27.3^{c} \pm 4.88$	$1.33^{b} \pm 0.15$	$0.00^{c} \pm 0.00$	$17.00^{c} \pm 2.64$	$31.00^{d} \pm 4.35$	$0.63^{b} \pm 0.23$	$0.66^{c} \pm 0.55$	$23.13^{b} \pm 7.55$
T_2	$27.5^{c} \pm 8.23$	$1.72^{b} \pm 0.41$	$0.66^{c} \pm 0.57$	$18.66^{c} \pm 3.78$	$31.00^{d} \pm 4.35$	$0.66^{b} \pm 0.20$	$0.66^{c} \pm 0.15$	$23.60^{b} \pm 3.96$
T_3	$58.27^{a} \pm 2.37$	$3.40^{a} \pm 0.28$	$4.66^{a} \pm 1.52$	$46.00^{a} \pm 10.81$	$113.33^{a} \pm 13.20$	$1.36^{a} \pm 0.11$	$2.00^{a} \pm 0.60$	$54.56^{a} \pm 6.87$
T_4	$42.25^{b} \pm 9.69$	$2.95^{a} \pm 0.41$	$3.00^{b} \pm 0.00$	$36.33^{ab} \pm 4.16$	$68.00^{bc} \pm 4.00$	$1.10^{a} \pm 0.00$	$1.50^{ab} \pm 0.20$	$51.66^{a} \pm 7.23$
T_5	$54.15^{a} \pm 5.91$	$2.88^{a} \pm 0.77$	$2.00^{b} \pm 0.00$	$33.33^{b} \pm 6.65$	$81.33^{b} \pm 34.19$	$1.23^{a} \pm 0.15$	$1.66^{a} \pm 0.15$	$50.00^{a} \pm 2.50$
T_6	$38.68^{b} \pm 2.48$	$2.91^{a} \pm 0.54$	$0.66^{c} \pm 0.57$	$28.0^{bc} \pm 3.46$	$44.33^{cd} \pm 13.79$	$1.06^{a} \pm 0.35$	$1.06^{bc} \pm 0.11$	$47.03^{a} \pm 8.37$

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Leaf area, petiole length: mean of 3rd leaf from top. Values are means of three replicates ± standard deviation. Values in the same column not sharing the same superscript are significantly different at $P \leq 0.05$.

Plate 8.3: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on vegetative growth response in *Anacardium occidentale* L. at 183 DAS (days after sowing).

a) *Anacardium occidentale* L. plants with un-inoculated and AM inoculated treatments.

T_1: Control sterilized sand

T_2: Control unsterilized sand

T_3: *Rhizoglomus intraradices* inoculum + sterilized sand

T_4: *Rhizoglomus intraradices* inoculum + unsterilized sand

T_5: *Funneliformis mosseae* inoculum + sterilized sand

T_6: *Funneliformis mosseae* inoculum + unsterilized sand


T1
T2
T3
a)

Plate 8.3

8.3.4: Total plant above- and below-ground biomass

Shoot and root weights (fresh and dry weights) varied significantly between the treatments. Total plant dry biomass in all AM treatments was significantly greater than control treatments at 183 DAS, with maximum in treatment 3 (*R. intraradices* inoculum + sterilized sand) (19.7 g) followed by treatment 5 (*F. mosseae* inoculum + sterilized sand) (15.8 g) and least in treatment 1 (Control sterilized sand) (3.47 g). Root to shoot ratios were significantly greater in all AM treatments compared to control treatments **(Table 8.4)**. Biomass partitioning between roots and shoots was significantly greater in all AM treatments as compared to control treatments, with greater shoot biomass than root **(Fig. 8.1)**. The magnitude of growth response of *A. occidentale* varied with the two AM species. The overall effect of AM treatments was observed as a significant increase of biomass towards above-ground (shoot) growth.

8.3.5: Plant aboveground (shoot) nutrient content

AM colonization facilitated greater uptake of macro- and micro-nutrients in AM treatments as compared to control treatments. Treatment 3 (*R. intraradices* inoculum + sterilized sand) had significantly greatest effect in enhancing growth and nutrient acquisition of *A. occidentale* compared to other AM treatments and control treatments **(Table 8.5, Table 8.6)**.

8.3.6: Mycorrhizal dependency and Mycorrhizal efficiency index

A significant variation was observed in mycorrhizal dependency and mycorrhizal efficiency index of *A. occidentale* in the different treatments. High MD and MEI values were recorded in treatment 3 (*R. intraradices* inoculum + sterilized sand) followed by treatment 5 (*F. mosseae* inoculum + sterilized sand) **(Fig. 8.2)**.

Table 8.4: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on total above- and below-ground biomass of *Anacardium occidentale* L. at 183 DAS (days after sowing).

Treatment	Stem		Leaf		Root		Total plant dry biomass (g)	Root to shoot ratio ($g\ g^{-1}$)
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)		
T_1	$6.16^{c} \pm 1.45$	$1.36^{c} \pm 0.45$	$7.73^{c} \pm 3.27$	$1.70^{c} \pm 0.15$	$4.40^{c} \pm 0.69$	$0.40^{d} \pm 0.01$	$3.47^{c} \pm 2.01$	$0.13^{b} \pm 0.02$
T_2	$6.23^{c} \pm 2.53$	$2.40^{c} \pm 1.05$	$8.83^{c} \pm 1.16$	$1.86^{c} \pm 0.23$	$4.60^{c} \pm 1.30$	$0.56^{d} \pm 0.05$	$4.83^{c} \pm 2.82$	$0.13^{b} \pm 0.01$
T_3	$14.56^{a} \pm 0.23$	$7.26^{a} \pm 0.87$	$26.43^{a} \pm 1.59$	$8.83^{a} \pm 0.40$	$10.63^{a} \pm 1.32$	$3.60^{a} \pm 0.51$	$19.70^{a} \pm 8.05$	$0.22^{a} \pm 0.04$
T_4	$13.16^{a} \pm 0.20$	$6.16^{a} \pm 0.32$	$19.76^{b} \pm 6.83$	$6.66^{b} \pm 0.65$	$9.36^{ab} \pm 1.98$	$2.80^{b} \pm 0.20$	$15.63^{ab} \pm 6.30$	$0.21^{a} \pm 0.00$
T_5	$12.66^{a} \pm 1.02$	$6.13^{a} \pm 0.37$	$20.36^{b} \pm 1.64$	$6.93^{ab} \pm 0.40$	$8.30^{b} \pm 0.30$	$2.73^{b} \pm 0.11$	$15.80^{ab} \pm 6.69$	$0.20^{a} \pm 0.00$
T_6	$8.86^{b} \pm 1.32$	$4.33^{b} \pm 0.32$	$15.33^{b} \pm 1.98$	$6.43^{b} \pm 2.73$	$7.16^{b} \pm 0.90$	$2.16^{c} \pm 0.32$	$12.93^{b} \pm 6.40$	$0.21^{a} \pm 0.07$

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Values are means of three replicates ± standard deviation. Values in the same column not sharing the same superscript are significantly different at $P \leq 0.05$.

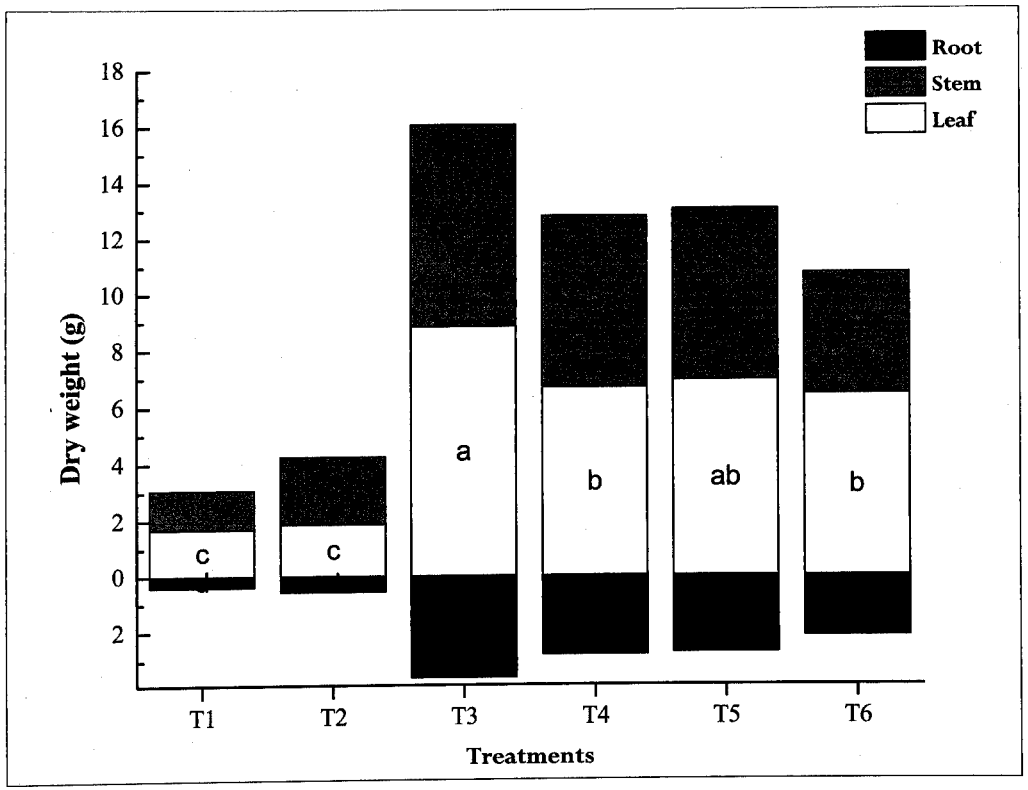


Fig. 8.1: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on above- and below-ground biomass partition of *Anacardium occidentale* L. at 183 DAS (days after sowing).

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *R. intraradices* + sterilized sand; T_4: *R. intraradices* + unsterilized sand; T_5: *F. mosseae* + sterilized sand; T_6: *F. mosseae* + unsterilized sand. Values are means of three replicates. Different letters between treatments are statistically significant at $P \leq 0.05$.

Table 8.5: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on macro-nutrient content of *Anacardium occidentale* L. at 183 DAS (days after sowing).

Treatments	C (%)	N (%)	P (%)	K (%)	S (%)	Ca (%)	Mg (%)
T_1	44.41	2.40	$0.12^{b} \pm 0.00$	$0.15^{b} \pm 0.00$	0.15	$0.21^{d} \pm 0.02$	$0.11^{b} \pm 0.00$
T_2	44.73	2.59	$0.66^{a} \pm 0.49$	$0.17^{b} \pm 0.06$	0.15	$0.24^{cd} \pm 0.01$	$0.11^{b} \pm 0.00$
T_3	47.00	3.09	$0.95^{a} \pm 0.02$	$0.25^{a} \pm 0.00$	0.33	$0.46^{a} \pm 0.05$	$0.42^{a} \pm 0.18$
T_4	45.12	3.05	$0.83^{a} \pm 0.02$	$0.24^{a} \pm 0.00$	0.32	$0.38^{b} \pm 0.04$	$0.12^{b} \pm 0.01$
T_5	45.25	2.98	$0.87^{a} \pm 0.06$	$0.23^{a} \pm 0.00$	0.26	$0.37^{b} \pm 0.03$	$0.33^{a} \pm 0.19$
T_6	45.16	2.84	$0.76^{a} \pm 0.03$	$0.23^{a} \pm 0.00$	0.24	$0.29^{c} \pm 0.03$	$0.12^{b} \pm 0.00$

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Values are means of three replicates ± standard deviation. Values in the same column not sharing the same superscript are significantly different at $P \leq 0.05$. C, N and S values are derived from a composite sample prepared from three sub-samples.

Table 8.6: Effect of monoxenically produced carrier based bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae* on micro-nutrient content of *Anacardium occidentale* L. at 183 DAS (days after sowing).

Treatments	Na (%)	Fe ($\mu g\ g^{-1}$)	Zn ($\mu g\ g^{-1}$)	Mn ($\mu g\ g^{-1}$)	Cu ($\mu g\ g^{-1}$)	B ($\mu g\ g^{-1}$)
T_1	$0.25^{e} \pm 0.01$	$129.96^{c} \pm 33.12$	$11.80^{c} \pm 0.81$	$8.34^{b} \pm 0.78$	$13.81^{c} \pm 1.14$	$184.30^{c} \pm 67.23$
T_2	$0.27^{d} \pm 0.01$	$137.74^{bc} \pm 19.51$	$14.99^{c} \pm 1.16$	$12.81^{b} \pm 0.40$	$13.96^{c} \pm 1.33$	$215.90^{bc} \pm 57.21$
T_3	$0.43^{a} \pm 0.00$	$249.56^{a} \pm 39.25$	$74.26^{a} \pm 4.59$	$31.72^{a} \pm 20.20$	$56.77^{a} \pm 5.83$	$322.96^{a} \pm 53.22$
T_4	$0.36^{b} \pm 0.18$	$204.89^{ab} \pm 51.46$	$26.10^{b} \pm 4.56$	$29.54^{a} \pm 0.58$	$46.97^{ab} \pm 9.93$	$247.83^{abc} \pm 22.98$
T_5	$0.36^{b} \pm 0.18$	$213.76^{a} \pm 46.05$	$27.45^{b} \pm 1.46$	$23.45^{ab} \pm 1.17$	$40.29^{b} \pm 6.04$	$302.56^{a} \pm 31.67$
T_6	$0.30^{c} \pm 0.00$	$210.16^{a} \pm 35.62$	$26.64^{b} \pm 3.11$	$18.43^{ab} \pm 1.94$	$37.80^{b} \pm 7.59$	$284.16^{ab} \pm 4.99$

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Values are means of three replicates ± standard deviation. Values in the same column not sharing the same superscript are significantly different at $P \leq 0.05$.

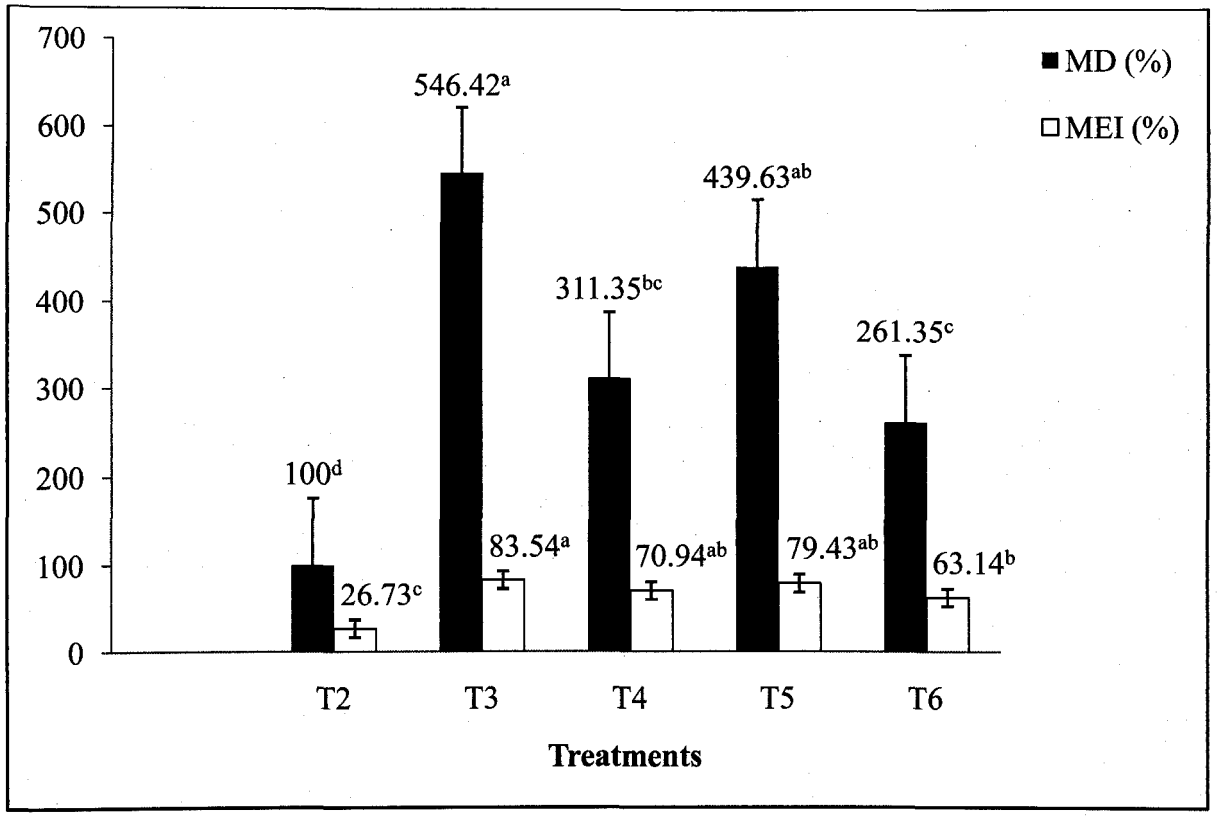


Fig. 8.2: Mycorrhizal dependency (MD) and mycorrhizal efficiency index (MEI) of *Anacardium occidentale* L. in different treatments.

Legend: T_1: Control sterilized sand; T_2: Control unsterilized sand; T_3: *Rhizoglomus intraradices* + sterilized sand; T_4: *Rhizoglomus intraradices* + unsterilized sand; T_5: *Funneliformis mosseae* + sterilized sand; T_6: *Funneliformis mosseae* + unsterilized sand.

Values are means of three replicates ± SE. Values above the bars not sharing the same superscript are significantly different at $P \leq 0.05$.

A strong positive correlation was observed between root colonization and mycorrhizal dependency, mycorrhizal efficiency index, spore density, shoot (N, P, K), root to shoot ratio, and total plant dry biomass **(Table 8.7)**.

8.4: DISCUSSION

In the present study, physico-chemical analysis of the sand medium revealed that the pH was alkaline and macro- and micro-nutrient contents were low. Soil pH has immense significance for plant growth as it influences nutrient mobilization and accessibility (Marschner, 1995). AM colonization and extra-radical mycelial growth are known to be affected by soil pH (van Aarle et al., 2002). AM species have variable pH optima for spore germination and root colonization (Hayman and Tavares, 1985). For most AM species it ranges between pH 5.0 and 8.0 (Maun, 2009). The root colonization levels can increase with increasing soil pH. This can be attributed to differential response of different AM species or a greater ability of the species or strains present to colonize host plant roots (Robson and Abbott, 1989; Coughlan et al., 2000). The variation in root colonization can also be attributed to the host-mediated changes in the pH of rhizosphere (Ananthakrishnan et al., 2004). It has been reported that most *Glomus* and *Acaulospora* species occur at pH 6.1 or higher (Tiwari et al., 2008). Medeiros et al. (1994) observed that as pH increased, root colonization also increased. They also reported that root colonization levels were low at pH 4.0 and high at pH 5.0, 6.0 and 7.0. As observed in our study the alkaline soil pH (8.5) did not have any negative effect on AM colonization and percent root colonization was as high as 89 %.

We conducted the experiment to evaluate the effect of *in vitro* produced carrier based bio-inocula of two AM fungal species *viz., R. intraradices* and *F. mosseae* in

Table 8.7: Correlation co-efficient (r) among important parameters as influenced by AM fungal inoculation in *Anacardium occidentale* L. at 183 DAS (days after sowing).

Parameters	RC (%)	MD (%)	MEI (%)	Spore density ($100\ g^{-1}$ soil)	Shoot N (%)	Shoot P (%)	Shoot K (%)	Root to shoot ratio ($g\ g^{-1}$)	Total plant dry biomass (g)
RC (%)	-	0.954**	0.935**	0.963**	0.967**	0.836*	0.955**	0.928**	0.988**
MD (%)	0.954**	-	0.950**	0.932**	0.935**	0.869*	0.914*	0.874*	0.968**
MEI (%)	0.935**	0.950**	-	0.850*	0.980**	0.938**	0.978**	0.939**	0.968**
Total plant dry biomass (g)	0.988**	0.968**	0.968**	0.933**	0.976**	0.853*	0.977**	0.959**	

Legend: ** Significant at $P \leq 0.01$, * Significant at $P \leq 0.05$.

RC = root colonization, MD = mycorrhizal dependency, MEI = mycorrhizal efficiency index.

stimulating growth and nutrient uptake of *A. occidentale*. It is known that cashew and many other tropical tree crops (*e.g.* coffee, cacao, citrus) are responsive to AM colonization (Janos, 1987; Alexander, 1988; Sieverding, 1991; Smits, 1992). All the AM treatments showed significant increase in root colonization and spore density compared to control treatments. Root colonization levels were high at 45 DAS in all AM treatments but a decrease in root colonization levels was observed at 183 DAS. At the beginning of AM fungal life cycle, germination of AM propagules and colonization of host plant roots occurs rapidly for providing water and mineral nutrients for plants. However, during completion of AM fungal life cycle, *i.e.* by sporulation occurring at the end of harvest, plants require less nutrients and low root colonization levels can be observed (Hindumati and Reddy, 2011). In our study, the control treatments showed least root colonization and spore density compared to AM treatments which may have been due to the presence of less infective AM population in the field sand.

In the present study, all vegetative growth parameters were significantly greater in AM treatments than in controls. Ananthakrishnan et al. (2004) reported significant increase in shoot length, internode number, number of leaves, stem diameter, root length, and root number of cashew by *Rhizoglomus fasciculatum* compared to un-inoculated control. Lakshmipathy et al. (2000) reported significant increase in plant height, stem girth and total biomass of cashew rootstock when inoculated with *Acaulospora laevis* and *F. mosseae* compared to un-inoculated plants. Jaiswal (2002) reported a significant positive response of *A. occidentale* to AM inoculation *i.e.* with *Glomus macrocarpum* and *R. intraradices*, observing a significant increase in stem girth, number of leaves and dry matter through AM inoculation. Gaonkar (2002) reported a significant increase in biomass of *A. occidentale* when inoculated with *R. intraradices*. It has been reported that different species and strains of AM fungi show variation in their ability to enhance

plant nutrient uptake and growth (McGraw and Schenck, 1980; Bagyaraj, 1992). However, these experiments were carried using pot cultured AM fungal inocula, whereas in our study, the experiment was carried out using *in vitro* produced carrier based AM inocula. Declerck et al. (2002) reported the potential benefits of *in vitro* produced spores of *R. intraradices* in micro-propagated banana (*Musa* spp. c.v. Grand Naine) plantlets growing in a pasteurized (100°C) and non-pasteurized Vertisol, wherein they directly introduced the AM inoculum (400 spores per plant) in the planting hole prior to planting the plantlets in pots. They recorded that mycorrhizal plants had larger shoot and root dry weight, and P content than the non-mycorrhizal plants. In our study, a significant increase in root to shoot ratios (the shoot biomass increased over the root) was observed in all AM treatments compared to control treatments. It was observed that there was as a significant modification of biomass towards shoot growth in the AM treatments. A relatively lower root biomass in mycorrhizal plants compared to non-mycorrhizal plants indicates an efficient transport of soil minerals and water by AM fungi leading to better shoot growth (Kothari et al., 1990).

In the present study, a significant increase in macro- and micro-nutrient content was observed in AM treatments compared to control treatments indicating that AM colonization improved nutrient acquisition and accumulation. Lakshmipathy et al. (2000) reported significant increase in P uptake of cashew rootstock inoculated with *Acaulospora laevis* and *F. mosseae* compared to un-inoculated plants. Mycorrhizal fungi especially *R. intraradices* and *F. mosseae* (both formerly *Glomus* species) are the most widely used AM species in agriculture (Krüger et al., 2012). Both of these AM species have been shown to increase P uptake in various crop plants (Barea et al., 1983; Douds et al., 2007; Antunes et al., 2009; Williams et al., 2012; Cozzolino et al., 2013).

P concentration in plants is an important indicator of mycorrhizal activity and its concentration positively affects plant metabolic processes such as cell division, expansion, and formation and movement of carbohydrate (Marschner, 1995). Another important advantage of using *Glomus* species as bio-inoculants is in their ability to survive and propagate well through root fragments colonized with intra-radical structures or vesicles formed earlier than the spores, which helps them in adapting to different soil conditions (de Souza et al., 2005).

In the present study, mycorrhizal dependency and mycorrhizal efficiency index of *A. occidentale* varied significantly among the different treatments. Treatment 3 (*R. intraradices* inoculum + sterilized sand) and treatment 5 (*F. mosseae* inoculum + sterilized sand) showed highest MD and MEI values followed by treatment 4 (*R. intraradices* inoculum + unsterilized sand) and treatment 6 (*F. mosseae* inoculum + unsterilized sand) whereas least MD and MEI values were recorded in control treatments. The high MD and MEI values along with increased plant tissue nutrient content indicates efficiency of the two studied AM species for enhancing growth of *A. occidentale* under sterilized and unsterilized sand conditions.

In the present study, a positive correlation between root colonization and mycorrhizal dependency, mycorrhizal efficiency index, spore density, shoot (N, P, K), root to shoot ratio, total plant dry biomass was observed indicating the effectiveness of the two AM species in enhancing growth, biomass and nutrient uptake of *A. occidentale*. These AM species are known to be beneficial to *Zea mays* L., *Capsicum annuum* L., *Prunus cerasifera* Ehrh., *Olea europaea* L., and many other plant species, even under stressful conditions (Berta et al., 1995; Eom et al., 2000; Bi et al., 2003; Estaun et al., 2003; Douds and Reider, 2003; Wu et al., 2005).

8.5: CONCLUSION

Our work demonstrates that the *in vitro* produced carrier based bio-inoculum of two AM fungal species *viz., R. intraradices* and *F. mosseae* was efficient in stimulating growth and nutrient uptake of *A. occidentale* grown in sterilized and unsterilized sand. Pre-inoculation of *A. occidentale* can result in better performance under field condition as the plant is already mycorrhizal when planted out. The present study affirms the expedience of two species of carrier based *in vitro* produced bio-inocula in improving seedling growth, biomass and nutrient uptake of *A. occidentale*. However, the survival and effectiveness of AM fungal inocula may vary depending upon the AM species involved in the study, the effectiveness of native AM fungal populations, the fertility or nutritional conditions of the soil, and agricultural practices involved. The AM species tested in our study are commonly recognized as 'generalist' species which are found in all ecosystems (Öpik et al., 2010) and have the ability to interact with many different plant species. Some plant-fungus combinations can perform better than others however. Thus, the efficacy or responsiveness of the *in vitro* produced carrier based bio-inocula needs to be tested with other plants.

The *in vitro* produced carrier based bio-inocula could be a valuable tool leading to strategic improvements in agriculture, horticulture, forestry and environmental recovering programs whilst being more ecologically sustainable. Further studies are needed to improve the knowledge of application and incorporation of these beneficial AM fungal symbionts into sustainable practices and understanding their effectiveness with different plants and soil conditions.

CHAPTER 9

Summary

Arbuscular mycorrhizal (AM) fungal communities positively affect the establishment and functioning of plant communities, and play a critical role in conservation and stabilization of coastal sand dune ecosystems. AM fungal bio-inoculants may be considered for the benefit of agriculture and environment recovery by revegetation.

As high amounts of AM fungi can be produced monoxenically, an attempt was made to produce a suitable organic carrier for *in vitro* produced AM fungal bio-inocula that might be effectively applied in revegetation strategies of degraded sand dune ecosystems.

The present investigation was devised to evaluate and identify AM fungal diversity from sand dunes, mass multiply the isolated AM species as monospecific cultures, and develop an efficient protocol of *in vitro* culture technique for dominant AM species from sand dune ecosystem. The development of a viable monoxenically produced inoculum using suitable carrier for re-inoculation was attempted. The shelf life of *in vitro* prepared inoculum was studied. The efficacy of *in vitro* produced carrier based bio-inocula in relation to plant growth has been investigated, and the application and integration of the *in vitro* produced carrier based AM fungal bio-inocula for sustainable restoration of degraded sand dunes has been recommended.

The main findings of the present investigation are summarized below:

The AM fungal status of dominant plant species from 12 different coastal sand dune sites of Goa was evaluated. AM fungal association with sand dune plants was characterized by the presence of intra- and extra-radical aseptate hyphae, hyphal coils, *Arum*- and *Paris*-type arbuscules, vesicles, intra- and extra-radical spores, and auxiliary cells (knobby and spiny/papillate). This indicated a dependency of dune vegetation on

beneficial effects of AM symbiosis. The taxonomy of the isolated AM species was studied. A total of 22 AM fungal species belonging to 8 genera were recorded. These included *Acaulospora delicata*, *A. scrobiculata*, *A. bireticulata*, *A. rehmii*, *A. elegans*, *A. nicolsonii*, *A. foveata*, *A. dilatata*, *Rhizoglomus fasciculatum*, *R. intraradices*, *R. manihotis*, *R. clarum*, *Funneliformis geosporum*, *F. mosseae*, *Claroideoglomus claroideum*, *Glomus macrocarpum*, *Gigaspora albida*, *Gi. gigantea*, *Gi. ramisporophora*, *Racocetra gregaria*, *Scutellospora pellucida* and *S. scutata*. *Acaulospora scrobiculata* was the most abundant AM species recorded from all 12 sites. The study depicted a variation in AM fungal root colonization, spore density, spore abundance and species richness suggesting a variable influence of several ecological factors which may play a significant role in shaping AM populations.

Eight out of 22 AM fungal species were successfully multiplied as monospecific cultures using cuttings of *Plectranthus scutellarioides* (L.) R.Br. as host. These included *A. scrobiculata*, *A. delicata*, *A. rehmii*, *F. geosporum*, *F. mosseae*, *Gi. albida*, *Ra. gregaria* and *R. intraradices*.

Efficient protocol was developed of *in vitro* culture technique for dominant AM species from sand dune ecosystem. *In vitro* spore germination was recorded in eight AM species *viz.*, *C. claroideum*, *R. manihotis*, *R. clarum*, *R. intraradices*, *F. mosseae*, *Gi. albida*, *Ra. gregaria*, and *A. scrobiculata*. Upon establishment of dual cultures, sporulation was observed in four AM species *viz.*, *R. clarum*, *R. intraradices*, *F. mosseae* and *A. scrobiculata*. Continuous cultures of *R. clarum*, *R. intraradices* and *F. mosseae* showing active growth of hyphae were obtained upon sub-culturing. The AM species with maximum potential for spore production using monoxenic culture system were identified and selected for mass production of pure and viable inocula. In the

present study, it was found that the percent spore germination, frequency of culturing (sporulation) and sub-culturing (re-growth) was greatest in *R. intraradices* and *F. mosseae*. Thus, these two AM species were screened for further studies.

Viability, germination and colonization potential of *in vitro* produced spores of *R. intraradices* and *F. mosseae* were evaluated. It was recorded that the spores from 90-197 days old cultures of both AM species were viable as assessed by vital dye staining. The monoxenically produced spores retained maximum germination potential up to 28 weeks and successfully colonized roots of *in vitro* established *P. scutellarioides* in sterilized vermiculite. An efficient organic carrier to sustain *in vitro* produced AM fungal propagules was formulated. Among the 19 treatments prepared, treatment 5 (vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1) was found to be optimal for both AM species. The *in vitro* produced propagules of both AM species were highly infective and efficient when re-inoculated along with the carrier formulation.

Shelf life of *in vitro* prepared inoculum was studied by assessing the infectivity potential of *in vitro* prepared inocula in optimum carrier formulation (treatment 5) during storage. The re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions was also examined. The *in vitro* produced inocula stored at 25°C remained viable up to 6 months in the organic carrier formulation. 100 % germination was recorded when the spores of both AM species were cultured back to *in vitro* conditions indicating high viability, and efficiency of the carrier formulation in maintaining vigour of *in vitro* produced propagules.

Study on the effect of *in vitro* produced carrier based bio-inocula of the two AM fungal species in stimulating growth and nutrient uptake of *Anacardium occidentale* L.

revealed a significant increase in growth, biomass and nutrient uptake in all AM treatments compared to un-inoculated controls. Treatment 3 (*R. intraradices* inoculum + sterilized sand) proved to be most effective in enhancing growth and nutrient acquisition of *A. occidentale*. A variation was observed in mycorrhizal dependency (MD) and mycorrhizal efficiency index (MEI) of *A. occidentale* in the different treatments. High MD and MEI values were recorded in treatment 3 (*R. intraradices* inoculum + sterilized sand) followed by treatment 5 (*F. mosseae* inoculum + sterilized sand). For greater transplantation success of *A. occidentale* or any other plant species, pre-inoculation with AM fungal inoculum is highly recommended. Therefore it was concluded that the developed carrier based *in vitro* produced AM fungal inocula may be suitable for large scale production of an effective bio-fertilizer.

The present study is relevant to agriculture, horticulture, forestry and environment-recovery programs. It promotes the use of an organic carrier formulation that facilitates the transfer to, multiplication of, and increase in efficacy of *in vitro* produced AM fungal propagules in the rhizosphere soils. In particular, it demonstrates an effective protocol in carrier based *in vitro* produced AM fungal bio-inocula for revegetation strategies of degraded sand dune ecosystems.

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Synopsis

Research Goal and Significance:

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil mutualists, forming obligate symbiosis with 90% of terrestrial vascular plant roots (Smith and Read, 2008; Young, 2015). These fungi have been included in the Phylum Glomeromycota and are considered as ancient eukaryotes originating >400 million years ago. Structures resembling spores and arbuscules of extant AM fungi have been found in fossil plants from the Rhynie chert formation (Remy et al., 1994). Currently, AM fungi are distributed in 11–14 families and 18–29 genera depending on the system of classification followed (Stürmer et al., 2013) with 270 described species (www.mycobank.org). As they are widespread in nature and occur at soil-plant interface, AM fungi are considered as a prime functional group of the soil microbiome due to the nutritional and non-nutritional benefits they offer to plant communities (Gianinazzi et al., 2010).

Coastal sand dunes are naturally fragile habitats due to the particular environmental characteristics they possess such as low levels of nutrients and organic matter, salt-spray, strong winds, sand movement, wide range of temperatures and humidity (Rippley and Pammenter, 2008; Yamato et al., 2012). These ecosystems are of great ecological significance as they provide a variety of ecosystem services such as coastal defense, erosion control, water capture and purification, supporting flora and fauna, carbon sequestration, besides being used for tourism, recreation and research purpose (Barbier et al., 2011). Although these habitats are of immense ecological importance, they are subjected to natural and anthropogenic (mining, pedestrian traffic, urbanization, port/tourist activities and deforestation) disturbances, which affect the

local ecology, and structure and stability of various above and below ground communities (Emery and Rudgers, 2010).

Sand dune ecosystems have been surveyed extensively for occurrence of AM fungi throughout the world: USA (Koske, 1987), Canada (Dalpé, 1989), Hawaii (Koske and Gemma, 1996), Australia (Peterson et al., 1985), Denmark (Błaszkowski and Czerniawska, 2011), Poland (Błaszkowski, 1994), Brazil (Stürmer and Bellei, 1994), Chile (Godoy and González, 1994), Mexico (Corkidi and Rincón, 1997a), Italy (Giovannetti and Nicolson, 1983), India (Kulkarni et al., 1997) and Japan (Abe et al., 1994). About 65 AM fungal species have been reported from sand dune ecosystems worldwide, representing 28% of the total AM species (Stürmer et al., 2010). Most of the dune vegetation distributed in embryonic dunes, mid shore dunes and hind shore dunes is heavily colonized by AM fungi.

AM fungi are essential for the colonization and proliferation of pioneer plant species of sand dunes (Koske and Polson, 1984). Dune vegetation benefits greatly from AM fungi by improved plant establishment, greater increase in total biomass and relative growth rate, faster colonization of bare patches, improved water relations and increased seed output (Corkidi and Rincón, 1997b). Major benefits of AM fungi to the host plants are more effective exploitation of soil minerals, better acquisition and transfer of organic soil nutrients, especially phosphorus, soil aggregation by extra-radical hyphae through glomalin production, protection from pathogenic organisms, enhanced tolerance to salinity, salt spray and water stress (Maun, 2009). Primary and secondary succession of plant species in coastal sand dunes is a process of change over time in plant community composition that is mediated by a change in structure and stability of substrate, organic matter, nutrient status, and microorganism complement of the soil. AM fungi play a key

role in facilitating the establishment, growth and reproduction of plant communities (Maun, 2009). Thus AM fungi are vital for sustainable management of these low nutrient ecosystems.

AM fungal inoculum production using *in vitro* culture technique outperforms the traditional soil based pot culture technique as it results in production of a large number of pure, viable and contamination-free propagules in a single Petri plate. Thus, the *in vitro* culture technique is more suitable for the mass production of high quality AM fungal inocula. Biofertilizers are normally developed as carrier based inoculants containing the effective microorganism; there is a necessity for development of a suitable carrier formulation for mass multiplication of *in vitro* produced AM fungal propagules to facilitate its use as carrier based bio-inocula.

The current study focuses on the potential of AM fungal inoculum production using *in vitro* culture technique to produce organic carrier based bio-inocula, and promote the use of carrier based *in vitro* produced AM fungal bio-inocula for re-vegetation strategies of degraded sand dunes. The thesis comprises of nine chapters that are listed below.

Chapter 1: Introduction

This chapter introduces the research objectives and highlights the potential of AM fungi from coastal sand dune systems and *in vitro* culture technique for development of carrier based inocula for sustainable restoration of degraded sand dunes. Details of coastal sand dunes of Goa along with the dominant plant species are briefly described. The significance and beneficial aspects of AM fungal diversity in coastal sand dunes is explained. Various methods employed for cultivation of AM fungal inocula are

described and the need for development of carrier based AM fungal inocula is emphasized.

Chapter 2: Review of Literature

In this chapter an update of research pertaining to the research objectives is highlighted. Previous studies on AM fungi pertaining to their occurrence, distribution and influence in coastal sand dune vegetation have been described. Finally, the research work on *in vitro* culture of AM fungi is reviewed.

Chapter 3: To identify the dominant AM fungal species from the sand dune ecosystem (Objective 1).

This chapter deals with the study of AM association and its diversity in sand dune vegetation of Goa. Continuous one time sampling of dominant plant species from dunes was carried out to assess colonization and species richness of AM fungi.

Methodology

- Rhizosphere soil and root samples of dominant plant species were collected from sand dunes of 12 different beaches of Goa.
- Extraction of AM fungal spores was carried out by Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963).
- Assessment of mycorrhizal colonization in roots was carried out by using Trypan blue staining technique (Phillips and Hayman, 1970).
- Estimation of percent root colonization by AM fungi was carried out using Root slide method (Read et al., 1976).

- Taxonomic identification of AM fungal spores by using the morphological and taxonomic criteria described in available literature was carried out (Schenck and Perez, 1990; Rodrigues and Muthukumar, 2009) and INVAM (invam.wvu.edu).
- AM species richness, Spore abundance and Relative abundance (RA %) of AM fungi (Beena et al., 2000) were determined.

Key findings

A variation in the distribution of AM species, spore density and extent of colonization in the rhizosphere soil samples from the study sites and plant species was observed. AM colonization was characterized by the presence of hyphae, hyphal coils, arbuscules, vesicles and auxiliary cells. A total of 22 AM fungal species belonging to 8 genera were recorded from the selected 12 study sites. The genus *Acaulospora* (8) was found to be dominant followed by the genus *Rhizoglomus* (4), *Gigaspora* (3), *Scutellospora* (2), *Funneliformis* (2), *Claroideoglomus* (1), *Glomus* (1), and *Racocetra* (1).

Chapter 4: To prepare pure culture inoculum using trap and pot cultures (Objective 2).

This chapter deals with preparation of pure cultures of isolated AM fungi.

Methodology

- Propagation of AM fungi by Trap culture method (Morton et al., 1993).
- Establishment of Monospecific (single species) cultures (Gilmore, 1968).

Key findings

Monospecific cultures of isolated AM species were prepared and mass multiplied using *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) as the host plant.

Chapter 5: To prepare and standardize the protocol for *in vitro* culture technique for dominant AM fungal species (Objective 3).

This chapter deals with standardizing an efficient protocol for *in vitro* culture of dominant AM species.

Methodology

- Extraction of AM spores (monospecific cultures) by Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963).
- Monoxenic culture establishment:

a) Disinfection of AM propagules (spores and colonized root fragments): Method modified from Bécard and Fortin, 1988; Mosse, 1959. 250-400µl sodium hypochlorite was used as the sterilizing agent for 3-5 minutes; concentration and sterilization time was found to vary from species to species.

b) Germination of disinfected AM propagules: 50 disinfected propagules (5 spores/Petri plate x 10 Petri plates) plated in triplicate were incubated in the dark at 27°C, the plates kept in inverted position.

c) Culture media for cultivation of AM propagules and transformed roots used was Modified Strullu-Romand (MSR) medium (Declerck et al., 1998).

d) Establishment of dual culture: Clarigel plugs with germinated AM propagules were associated with transformed Chicory (*Cichorium intybus* L.) and Linum (*Linum usitatissimum* L.) roots.

e) Continuous culture: Propagule re-association onto fresh MSR media.

- Assessment of AM fungal colonization in roots (Phillips and Hayman, 1970).
- Measurement of hyphal length of germinated spores using ocular micrometer was carried out after every alternate day.

Key findings

Spore germination was observed in 8 AM species *viz., Claroideoglomus claroideum, Rhizoglomus manihotis, R. clarus, R. intraradices, Funneliformis mosseae, Gigaspora albida, Racocetra gregaria,* and *Acaulospora scrobiculata.* Upon establishment of dual cultures, sporulation was observed in 4 AM species *viz., R. clarus, R. intraradices, F. mosseae* and *A. scrobiculata.* Continuous cultures of *R. clarus, R. intraradices,* and *F. mosseae* showing active growth of hyphae were obtained upon sub-culturing. It was found that in *R. intraradices* and *F. mosseae,* the percent spore germination, frequency of culturing (sporulation) and sub-culturing (re-growth) were high. Hence, these two AM species were screened for further studies.

Chapter 6: To develop viable inoculum using suitable carrier for re-inoculation (Objective 4).

This chapter is divided into four parts *viz.,* estimation of viability of *in vitro* produced spores, study of the germination potential of monoxenically produced spores, colonization potential of *in vitro* produced spores and selection of efficient carrier formulation.

The first part comprises of **estimating the viability of *in vitro* produced spores** of two AM species *viz., F. mosseae* and *R. intraradices* using Tetrazolium salt 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (TTC) as a vital stain.

Methodology

- Extra-radical hyphae and spores of both AM species were extracted from an *in vitro* propagating dual culture and were subjected to TTC test (An and Hendrix, 1988) to determine the viability.

- For isolation of monoxenically cultured AM propagules - a small piece of gel containing sufficient spores with extra-radical mycelia was dissolved in 25ml citrate buffer (0.01 M) under sterile conditions (Cranenbrouck et al., 2005) and subjected to TTC test.

Key findings

At 90 days old, the majority of spore primordia for both the AM species tested positive for viability with TTC and the level of viability remained unchanged throughout the spore maturation period (90-197 days old).

The second part comprises of **assessment of germination potential of monoxenically produced spores** of *R. intraradices* and *F. mosseae* for inoculum preparation using carrier material.

Methodology

- The monoxenically produced spores of *R. intraradices* and *F. mosseae* in different stages of development were inoculated on fresh MSR media to study their germination potential.

Key findings

The study revealed that *in vitro* produced spores of both the AM species retained maximum germination potential up to 28 weeks *i.e.* fully matured spores at 197 days old, and therefore were selected for inoculum preparation using carrier material.

The third part comprises of *in vitro* establishment of *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) seedlings to **test the colonization potential of *in vitro* produced spores**.

Methodology

- Seeds of *P. scutellarioides* were surface sterilized with sodium hypochlorite and inoculated on MSR media. Upon germination the seedlings were transferred to conical flasks containing sterilized vermiculite and were associated with *in vitro* produced spores of both AM species separately.
- For isolation of monoxenically cultured spores - a small piece of gel containing sufficient spores with extra-radical mycelia was dissolved in 25ml citrate buffer (0.01 M) under sterile conditions (Cranenbrouck et al., 2005).
- Assessment of root for AM colonization was carried out (Phillips and Hayman, 1970).
- Estimation of percent root colonization by AM fungi was carried out using Root slide method (Read et al., 1976).

Key findings

Presence of hyphae, arbuscules, vesicles and extra-radical spores indicated successful colonization of *P. scutellarioides* by monoxenically produced spores of *R. intraradices* and *F. mosseae.*

The fourth part comprises of **selection of efficient carrier for formulation of carrier based inocula.**

Methodology

- For carrier preparation, initially sterilized soil and vermiculite were used separately as base components supplemented with sterilized cow dung powder, wood powder and wood ash in different proportions. Treatments containing vermiculite as the base component showed maximum AM colonization compared to soil. Hence vermiculite was selected and used as base component for the carrier supplemented with sterilized

cow dung powder, wood powder and wood ash in different proportions resulting in 19 formulations/treatments in all.

- Carrier materials were sterilized by autoclaving for two consecutive days at 121°C for 2 h.
- Physico-chemical characterization of the carrier materials was carried out - pH, EC, OC (Walkley and Black, 1934), P (Bray and Kurtz, 1945), K (Hanway and Heidel, 1952). Available micronutrients *viz.,* Zn, Fe, Mn and Cu (Lindsay and Norvell, 1978), B (Berger and Truog, 1939).
- *In vitro* produced spores of *R. intraradices* along with colonized transformed chicory roots, and *F. mosseae* spores along with colonized transformed linum roots were used as inocula along with the carrier formulations and pre-germinated seeds of *Eleusine coracana* Gaertn. (Poaceae) being used as host.
- Assessment of colonization was carried out (Phillips and Hayman, 1970).
- Various parameters *viz.,* average number of entry points in 1cm root segment, root length, total number of infective propagules as per Fertilizer (Control) Order, 1985 (Ministry of Agriculture, Government of India 2009) and percent root colonization (Read et al., 1976) were determined.

Key findings

Among the various carrier formulations used, treatment 5 (containing vermiculite, cow dung powder, wood powder and wood ash in proportion of 20:8:2:1) was optimum and found suitable for mass production of AM fungal inocula. The monoxenically produced spores of *R. intraradices* and *F. mosseae* were viable and colonized the host plant successfully without any contamination. Along with carrier material, the inocula of both AM species exhibited enhanced inoculum potential with respect to percent root colonization, average number of entry points and total number of infective propagules.

Chapter 7: To maximize the shelf life of the *in vitro* prepared inoculum (Objective 5).

This chapter is divided into two parts *viz.,* assessment of infectivity potential of *in vitro* prepared inocula in carrier formulation upon storage and re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions.

The first part comprises of **assessment of infectivity potential of stored *in vitro* prepared AM fungal inocula in carrier formulation** to observe the effects of carrier formulation on maximizing the shelf life of the *in vitro* prepared inocula.

Methodology

- The inocula of both the AM species *viz., R. intraradices* and *F. mosseae* were stored for 6 months in optimum carrier formulation (treatment 5) at different temperatures *viz.,* 4°C, 25°C and room temperature 28-30°C.
- Infectivity tests were carried out bi-monthly after storage, by incorporating the inocula in sterilized sand and using *Plectranthus scutellarioides* (L.) R.Br. (Coleus) (Lamiaceae) as the host plant.
- Percent root colonization was determined (Read et al., 1976).

Key findings

The infectivity potential of inocula upon storage of both the AM fungal species *viz., R. intraradices* and *F. mosseae* was observed to be optimum at 25°C. High AM fungal colonization levels were observed at 25°C in both the AM species indicating that the inocula did not lose its viability even after 6 months in storage. This indicated that the inoculum carrier was suitable in maintaining the viability or infectivity of the *in vitro* produced AM fungal propagules under storage with no dormancy.

The second part comprises of **assessment of re-germination potential of *in vitro* produced spores** of *Rhizoglomus intraradices* and *Funneliformis mosseae* **from carrier based inocula to *in vitro* conditions** to observe the efficiency of carrier in maintaining vigour of *in vitro* produced spores.

Methodology

- Extraction of spores from the carrier formulation by Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963).
- Disinfection of spores with 250-400µl sodium hypochlorite for 3-5 minutes (method modified from Bécard and Fortin, 1988; Mosse, 1959).
- Disinfected spores were inoculated on MSR media and Petri plates were incubated in the dark at 27°C in an inverted position.

Key findings

Spores of both the AM species were viable enough to re-germinate when cultured back to *in vitro* conditions indicating that the carrier formulation was efficient in maintaining vigour of *in vitro* produced spores.

Chapter 8: To study the effect on selected plant species suitable for re-vegetation of the sand dunes (Objective 6).

A pot experiment was conducted to evaluate the effectiveness of *in vitro* produced carrier based bio-inocula of two AM fungal species *viz., Rhizoglomus intraradices* (Schenck and Smith) Sieverding, Silva and Oehl, and *Funneliformis mosseae* (Nicolson and Gerdemann) Walker and Schüßler in stimulating growth and nutrient uptake of *Anacardium occidentale* L. (Anacardiaceae) in sand dune soil. *Anacardium occidentale* is a woody perennial tree thriving well in semi-arid conditions with mild resistance to drought and salinity, and dominant in the backshores (hind shore dunes) of Goa coast

(Dessai, 1995). The species was considered suitable for re-vegetation strategies of sand dune ecosystems and therefore used in the present study.

Methodology

The experiment comprised of following six treatments:

- Treatment 1 (T_1): Control sterilized sand
- Treatment 2 (T_2): Control unsterilized sand
- Treatment 3 (T_3): *R. intraradices* + sterilized sand
- Treatment 4 (T_4): *R. intraradices* + unsterilized sand
- Treatment 5 (T_5): *F. mosseae* + sterilized sand
- Treatment 6 (T_6): *F. mosseae* + unsterilized sand
- Six treatments with eight replicates per treatment were arranged in a randomized block design.
- Analysis of physico-chemical properties of sand was carried out - pH, EC, OC (Walkley and Black, 1934), P (Bray and Kurtz, 1945), K (Hanway and Heidel, 1952), micronutrients *viz.,* Zn, Fe, Mn, Cu (Lindsay and Norvell, 1978) and B (Berger and Truog, 1939).
- Pots were maintained for a period of 6 months in the shade net of Department of Botany, Goa University. Plant growth (height, stem diameter, number of branches, leaf number, leaf area, petiole length, internode length, root length and total biomass) was recorded and Plant shoot nutrient content was analyzed.
- Percent root colonization (Read et al., 1976) and spore density (Gerdemann and Nicolson, 1963) were determined.
- Mycorrhizal dependency (MD) (Menge et al., 1978) and mycorrhizal efficiency index (MEI) (Bagyaraj et al., 1994) were calculated.

- The experimental data was analyzed with one-way analysis of variance (ANOVA) followed by Tukey's HSD test at $P \leq 0.05$ for separation of means. Parameters were correlated using Pearson's correlation at $P \leq 0.01$ and $P \leq 0.05$. Statistical Package for Social Sciences (SPSS) (ver. 22.0 Armonk, NY: IBM Corp.) was used for all statistical analyses.

Key findings

Root colonization and AM spore density was maximum in treatment 3 (*R. intraradices* + sterilized sand) when recorded at 45 DAS and at 183 DAS (days after sowing). A significant positive response of *A. occidentale* to AM treatments was observed in its growth characteristics as compared to control treatments. Among the AM treatments, treatment 3 (*R. intraradices* + sterilized sand) recorded a significant increase in all the growth characteristics of *A. occidentale*. Total plant dry biomass in all AM treatments was significantly greater than control treatments at 183 DAS, with maximum in treatment 3 (*R. intraradices* + sterilized sand) followed by treatment 5 (*F. mosseae* + sterilized sand) and least in treatment 1 (Control sterilized sand). Biomass partitioning between roots and shoots was significantly greater in all AM treatments as compared to control treatments. AM colonization facilitated greater uptake of nutrients in AM treatments as compared to control treatments. Treatment 3 (*R. intraradices* + sterilized sand) proved to be most effective in enhancing growth and nutrient acquisition of *A. occidentale*. High MD and MEI values were recorded in treatment 3 (*R. intraradices* + sterilized sand) followed by treatment 5 (*F. mosseae* + sterilized sand).

Chapter 9: Summary

In the current investigation *in vitro* culture of AM species from a sand dune ecosystem was attempted for the development of carrier based inocula for sustainable restoration

of degraded sand dunes. An efficient protocol for *in vitro* culture of dominant AM species was standardized. Viability, germination potential, and colonization potential of *in vitro* produced spores was studied. A suitable and efficient organic carrier for *in vitro* produced spores of AM fungi has been formulated. The infectivity potential of *in vitro* prepared inocula in carrier formulation upon storage and re-germination potential of *in vitro* produced spores from carrier based inocula to *in vitro* conditions were assessed. Plant growth response to *in vitro* produced carrier based bio-inocula was evaluated.

The main findings of the entire work are summarized as follows:

In the present study, with regard to *R. intraradices* and *F. mosseae,* the *in vitro* percent spore germination rate, frequency of culturing (sporulation) and sub-culturing (re-growth) was high. The inocula could survive long-term storage in the carrier, indicating that the carrier formulation was efficient in maintaining vigour of *in vitro* produced spores, indicating suitability for mass production of AM fungal inocula.

The *in vitro* produced carrier based bio-inocula of two AM fungal species *viz., R. intraradices* and *F. mosseae* was also effective in stimulating growth and nutrient uptake of *A. occidentale* in sand dune soil. This makes the two AM species used as best inoculum components.

Research work published:

- Rodrigues KM, Rodrigues BF (2015) Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum*: germination, colonization, and sporulation studies. Mycology: An International Journal on Fungal Biology 6 (1): 42-49. doi: 10.1080/21501203.2015.1024777.
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- Rodrigues KM, Rodrigues BF (2012) Monoxenic culture of AM fungus *Glomus clarum* using Ri T-DNA transformed roots. In: State Level Seminar on Recent Innovative

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Prof. B. F. Rodrigues

(Guide)

Kim Maria Rodrigues

Appendix

A. Publications:

- Rodrigues KM, Rodrigues BF (2017) Development of carrier based *in vitro* produced arbuscular mycorrhizal (AM) fungal inocula for organic agriculture. Annals of Advanced Agricultural Sciences 1 (1): 26-37.

- Rodrigues KM, Rodrigues BF (2015) Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum*: germination, colonization, and sporulation studies. Mycology: An International Journal on Fungal Biology 6 (1): 42-49. doi: 10.1080/21501203.2015.1024777.

- Rodrigues KM, Rodrigues BF (2013) *In vitro* cultivation of Arbuscular Mycorrhizal (AM) fungi. J. Mycol. Pl. Pathol. 43 (2): 155-168.

- D'Souza J, Rodrigues KM, Rodrigues BF (2013) Modified Strullu and Romand (MSR) medium devoid of sucrose promotes higher *in vitro* germination in *Rhizophagus irregularis*. J. Mycol. Pl. Pathol. 43 (2): 240-242.

- Rodrigues KM, Rodrigues BF (2015) Advances in Arbuscular Mycorrhizal (AM) Biotechnology. Pejaver MK, Mulgaonkar MS, Kolet MJ (Eds.) In: Proc. Nat. Conf. New and Emerging Trends in Bioinformatics and Taxonomy (NETBT-2015). pp: 33-36. ISBN: 978-81-923628-2-3.

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Development of Carrier Based *in Vitro* Produced Arbuscular Mycorrhizal (AM) Fungal Inocula for Organic Agriculture

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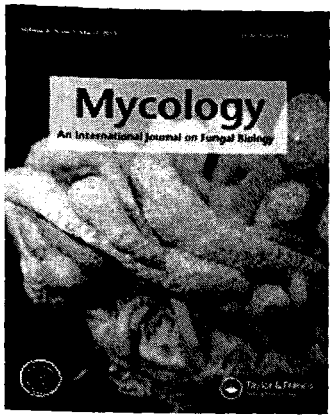
Abstract. Studies on the advantageous effects of arbuscular mycorrhizal (AM) fungi are providing new possible ways to exploit them as biofertilizers in sustainable agriculture. Many studies have described the potential of root organ culture (ROC) system for production of AM fungal inocula. However there is a need for development of a suitable carrier formulation to support *in vitro* produced AM fungal inocula when mixed with substrate, so as to enable the delivery of inocula in the rhizosphere. The aim of this study was to assess the performance of the organic carrier formulation consisting of vermiculite as the main component along with cattle manure, wood powder and wood ash in different proportions; and its ability to retain inoculum potential of the *in vitro* produced AM fungal propagules of *Rhizoglomus intraradices* and *Funneliformis mosseae*. Treatment 5 comprising of carrier formulation (vermiculite: cow dung powder: wood powder: wood ash) in the ratio of 20:8:2:1 was observed to be as the best carrier treatment for both the *in vitro* produced AM species. The *in vitro* produced propagules of both AM species were viable and effectively colonized the roots of *Eleusine coracana* Gaertn. The method established shows the efficiency of the carrier formulation in sustaining the inoculum potential of *in vitro* produced AM propagules for mass multiplication and possibility in application.

Keywords: *In vitro*, AM fungal propagules, carrier formulation, inoculum.

1 Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi forming mutualistic symbiosis with plant roots. AM fungal extra-radical hyphal network spreading extensively in the soil and acting as an extension to the host's root system in nutrient depletion zones has significant effects on overall host plant growth and development. Efficient exchange of nutrients is mediated via specialized structures within the root cortical cells (arbuscules). The basis of this symbiosis is the ability of AM fungi to form fine extra-radical hyphae in order to increase root-soil contact area as well as secrete enzymes/organic acids for improved nutrient acquisition [1]. In addition to improved uptake of soil minerals, other benefits ascribed to the host plant are improved water relations and disease resistance [2]. The beneficial effects of AM fungi on plant growth and nutrition have led to an increased use of AM fungal inoculum as biofertilizer [3]. Large scale AM fungal inoculum production is precluded due to their obligate biotrophic nature *i.e.* they must grow in symbiosis with living host plant roots in order to complete their life cycle and to produce infective propagules. AM fungal inoculum is presently produced in a variety of ways utilizing *in vitro*, greenhouse, or field-based methods [4-6]. The *in vitro* method comprises of monoxenic culture of sterilized AM fungal spores with Ri T-DNA transformed carrot roots [7]. The root organ culture (*in vitro*) system is preferred over the classical (pot/trap culture) method, permitting production of pure, viable, contamination free propagules in a smaller space.

AM fungal inoculum is commercially available in a variety of forms ranging from high concentrations of AM fungal propagules in carrier materials to potting media containing inoculum at low concentrations [8]. Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms [9]. A carrier is a delivery vehicle which is used to transfer live microorganism from an agar slant to the rhizosphere [10]. A suitable biofertilizer carrier should comprise of certain characteristic features *viz.*, it should be in powder or granular form, should support the growth and survival of the microorganism, should be able to release the functional microorganism easily into the soil, should have high moisture absorption and retention capacity, should have good aeration characteristics and pH



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Endomycorrhizal association of Funneliformis mosseae with transformed roots of Linum usitatissimum: germination, colonization, and sporulation studies

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Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum*: germination, colonization, and sporulation studies

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Spores of arbuscular mycorrhizal (AM) fungus *Funneliformis mosseae* (Nicolson & Gerdemann) Walker & Schuessler were cultured in association with transformed roots of *Linum usitatissimum* L. (Linaceae) for the first time on modified Strullu–Romand medium (pH 5.5) in monoxenic culture. Germ tubes emerged through the spore wall in 88% of spores after 5 days. Hyphal contact with transformed linum roots was observed 5 days after co-cultivation. *Paris*-type arbuscules and hyphal coils were seen. Extra-radical branched absorbing structures were common. Terminal and intercalary secondary spores were also formed. Spore viability when assessed using vital dye staining (MTT test) was 83%. Secondary spores that proved viable were subsequently transferred from *in vitro* to *in vivo* culture where *Arum*-type arbuscules, intra-radical intercellular hyphae, and extra-radical spores were observed. The procedure established shows potential in AM inoculum mass production and possibility in application.

Keywords: *Funneliformis mosseae*; monoxenic culture; transformed linum roots; MSR; *in vivo*

Introduction

The arbuscular mycorrhizal (AM) association is a symbiotic union between the fungi of phylum Glomeromycota and plant roots. The phylum forms a monophyletic group of obligate mycobionts (Schüssler et al. 2001). In order to overcome the obligate biotrophic nature, AM fungal propagules are cultured in association with Ri T-DNA transformed hairy roots (Root Organ Culture technique). An advantage of using root organ culture as a system is that both symbionts (AM and host root) are grown and easily maintained on a defined medium, allowing nondestructive *in vivo* observations over long periods of time (de Souza and Berbara 1999). The system has afforded studies on organization and development of mycelium (Bago et al. 1998a), sporulation dynamics (Declerck et al. 2001, 2004; Voets et al. 2009; Ijdo et al. 2011), and spore ontogeny (de Souza et al. 2005). Further potential uses of the system are the production of pure and concentrated inoculum, and contaminant-free fungal tissues for genetic and physiological studies. Utilizing excised roots of a number of host species and various media formulations, the system has been developed to culture glomalean fungi monoxenically (Mosse and Hepper 1975; Miller-Wideman and Watrud 1984; Mugnier and Mosse 1987a, 1987b; Bécard and Fortin 1988). A large number of AM fungal species and isolates have been successfully maintained and sporulated in association with Ri T-DNA transformed roots of carrot (*Daucus carota* L.) (Fortin et al. 2002).

Funneliformis mosseae has been reported colonizing excised roots of tomato (*Solanum lycopersicum* L.) and clover (*Trifolium pratense* L.) (Mosse and Hepper 1975) and Ri T-DNA transformed roots of bindweed (*Calystegia sepium* (L.) R.Br.) and carrot (Mugnier and Mosse 1987b; Douds 1997). Mugnier and Mosse (1987a) reported successful establishment of *F. mosseae* on Ri T-DNA transformed roots of *C. sepium* in a two-compartment system. Roots were grown in nutrient medium in one compartment, crossing into a water agar and peat medium in the second compartment where they were inoculated with pregerminated fungal spores. These authors further reported mycorrhizal dependence on the nitrogen (N) concentration of the nutrient medium in the root compartment for mycelium development. Douds (1997) manipulated buffer, pH and P levels to increase successful formation of mycorrhizae between carrot and *F. mosseae in vitro*. He reported that the cultures grew for 17–24 weeks before termination of the experiments, and new spores were not produced though the hyphae had spread throughout the Petri plates. Pawlowska et al. (1999) also reported absence of sporulation in *F. mosseae* while in dual culture. In our study, we report the successful establishment of endomycorrhizal association of *F. mosseae* with transformed roots of

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Mini Review

In vitro Cultivation of Arbuscular Mycorrhizal (AM) Fungi

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Abstract

Arbuscular mycorrhizal (AM) fungal association is essential for most of the vascular plants for their growth and survival. The obligate biotrophic nature of AM fungi makes this association intricate. Several attempts have been made for cultivation and mass inoculum production of this plant beneficial symbiosis. The use of root organ culture (ROC) technique has proved to be particularly successful. This technique has greatly influenced our understanding on various aspects of AM symbiosis. This article provides an update on the developments made in the *in vitro* AM fungal inoculum production and the range of AM fungal species being cultivated using ROC. The method by which ROC of AM fungi have been cultivated is described along with the culture media used, choice of transformed host root to study the interaction, selection and sterilization of different AM fungal propagules and the use of continuous cultures to preserve the colonizing potential of the *in vitro* produced AM fungal inoculum. The morphological features of AM fungal cultures developed under *in vitro* conditions and how these have improved our understanding on this symbiosis are also discussed. Some of the potential uses and limitations of this system are also highlighted.

Key words: arbuscular mycorrhizal fungi, root organ cultures, AM fungal propagules, extra-radical mycelium

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Introduction

Arbuscular mycorrhizal (AM) fungi are soil organisms with a worldwide distribution that form root symbiosis with most plant families. Their functional importance in natural and semi-natural ecosystems is commonly accepted with regard to enhanced plant productivity and diversity as well as increased plant resistance to biotic and abiotic stresses (Smith and Read 2008). Nowadays, these fungi are receiving attention because of their increasing range of application in agriculture, horticulture and forestry, as well as in environmental reclamation, increasing crop yield and health, and limiting the use of agrochemicals (Johansson et al 2004).

Arbuscular mycorrhizal fungal propagules exist as spores, living hyphae, isolated vesicles, mycorrhizal root segments or colonized soil (Diop et al 1994a). Root segments and spores isolated from open-pot culture (Gilmore 1968) of AM-inoculated plants have been the usual source of AM inoculum for research purposes (Ferguson and Woodhead 1982). However, this type of inoculum occupies a large space in production and is prone to contamination even with good phytosanitary care (Ames and Linderman 1978). Production of propagules under aseptic conditions remains one of the most promising methods of obtaining high quality pathogen-free inoculum that is required for research purposes.

The conventional method used to study the life cycle of AM fungi *in situ* is to associate them with root organ culture (ROC) (Fortin et al 2002). The establishment of *in vitro* ROC has greatly increased our understanding of various aspects of the AM symbiosis by allowing non destructive *in vivo* observations throughout the fungal life cycle and its potential for research and inoculum production is gaining importance. The cultivation of AM fungi in association with the Ri T-DNA transformed roots has enabled new possibilities in the study of the extra-radical mycelium of AM (Fortin et al 2002). In this mini review, an effort has been made to highlight various *in vitro* cultivation systems of AM fungi along with different hosts, culture media and types of AM fungal propagules used to initiate monoxenic cultures. The use of mycorrhizal ROC has allowed the elucidation of many aspects of this intimate symbiotic plant-fungal association. Although the host plant is replaced by Ri T-DNA transformed roots, the fungus is able to colonize and sporulate. The development of spores, morphologically and structurally similar to those produced in pot cultures, and 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. Thus the success 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.

System Description

Since Mosse and Hepper (1975) first established cultures of AM fungi using excised roots, tremendous improvements have been made in the use of Ri T-DNA transformed roots (Mugnier and Mosse 1987), in the manipulation of the culture media to induce sporulation

Short Communication

Modified Strullu and Romand (MSR) Medium Devoid of Sucrose Promotes Higher *in vitro* Germination in *Rhizophagus irregularis*

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Key words: Arbuscular mycorrhizal fungi, MSR medium, *Rhizophagus irregularis*

Citation: D'Souza J, Rodrigues KM and Rodrigues BF. 2013. Modified Strullu and Romand (MSR) medium devoid of sucrose promotes higher *in vitro* germination in *Rhizophagus irregularis*. *J Mycol Pl Pathol* 43(2): 240-242.

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts belonging to the phylum Glomeromycota (Schüßler et al 2001), that cannot complete their life cycles without establishing a functional symbiosis with a host plant. Spore germination ability has considerable impact on different host plant species (Pawlowska et al 1999) and capacity of the fungi to complete its life cycle relies on the production of adequate spores. Though the potential of AMF's plant growth promotion and biocontrol efficacy is well recognized, culturing and mass multiplication still remain a constraint in their popularization. A primary pre-requisite for germination of AM propagules (spores and root fragments) is hydro-metabolism activation (Dalpe et al 2005). The time required for spore germination of different genera may range from 2 to 90 d. Attempts to determine requirements for spore germination and germ tube growth on artificial media have met with variable success, probably due to variation in methodology, fungal species and the culture conditions employed.

In pre-symbiotic phase AM spores germinate and develop germtubes which grow using the reserve materials from propagules. Some components of the medium may interfere with attempts at synthesizing symbioses, either by directly inhibiting the fungus or by rendering the root less capable of supporting mycorrhiza formation. Many factors such as pH, temperature, moisture, mineral and organic nutrients (Clark 1997), substrate (Maia and Yano-Melo 2001) and flavanols (Bécard and Piché 1992) have been identified as playing important roles in initiating germination and germtube growth. However, systematic information about effects of sucrose concentration in the substrate on AMF spore germination is limited (Carr et al 1985; Dalpe et al 2005). The presence of various carbohydrates in the medium may result in the blocking of receptor/recognition sites on hyphae or host cell walls, which could prevent germ tubes and hyphae from locating host roots (Allen 1992). Anatomical changes in excised *Solanum lycopersicum L.* (tomato) root, in relation to the sucrose concentration in the culture medium, were also observed by Street and McGregor (1952). The purpose of this study was to evaluate the effect of sucrose on spore germination and germ- tube growth in *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) Walker & Schuessler.

Rhizophagus irregularis was originally isolated from rhizosphere of mangrove plant *Rhizophora apiculata* Blume from Sal estuary (Goa, India) and propagated using sieving in pot cultures with sterile sand as substrate and *Solenostemon scutellarioides* (L.) Codd (Coleus) as host. Subsequently, pure culture of *R. irregularis* was raised and maintained in a polyhouse using the same host. Identification was done based on spore morphological characteristics and the relevant literature (Blaszkowski et al 2008; Stockinger et al 2009).

Spores of *R. irregularis* were isolated from pure culture by wet sieving and decanting method (Gerdemann and Nicolson 1963) and selected spores were stored at 5C before sterilization. After surface sterilization, using micropipette, they were transferred to Petri plates containing a solution of 2% (w/v) streptomycin sulphate and stored overnight. Modified Strullu and Romand (MSR) medium (Declerck et al 1998) solidified with 5% clarigel, with and without sucrose was used as substrate.

An experimental design with 30 replicates was employed for two treatments. A single spore was inoculated in each Petri plate and incubated in an inverted position in dark at 26C. Germination was assessed every 4d up to 26d. Spores were considered to have germinated if a germtube was visible. Observation on hyphal growth was carried out after every 12 h. Pearson's correlation coefficient analysis was performed using WASP (Web Based Agricultural package) 2.0 ($P \leq 0.05$).

The present study is the first report on an inhibitory effect of sucrose on germtube growth of *R. irregularis* using MSR medium. Germinating spores produced germtubes that grew through the subtending hyphae. In the present study, *in vitro* germination in MSR medium without sucrose was observed 38h after inoculation and recorded 90% germination whereas in

Lead Lecture

Advances in Arbuscular Mycorrhizal (AM) Biotechnology

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Introduction

Arbuscular mycorrhizal (AM) fungi from the phylum Glomeromycota are ubiquitous soil borne microbial symbionts forming mutualistic associations with a majority of terrestrial plants. They facilitate uptake of nutrients (mostly immobile P) through their extra-radical mycelial network and provide other benefits to their host plants. These benefits can be physiological, nutritional and ecological and therefore exploiting and managing AM fungi has important consequences for both agricultural and natural ecosystems. Nowadays, they are increasingly considered in agriculture, horticulture, and forestry programs, as well as for environmental reclamation, to increase crop yield and health and to limit the application of agrochemicals (Gianinazzi *et al.*, 2002; Johansson *et al.*, 2004). However, the obligate biotrophic nature of AM fungi has complicated the development of cost-efficient large-scale production methods to obtain high-quality AM fungal inoculum. This is one of the reasons why their commercial exploitation is still in its infancy (Ijdo *et al.*, 2011).

The inoculum production systems for AM fungi are classified into *viz.*, the classical sand/soil or more advanced substrate-based production systems and the *in vitro* cultivation systems, which are based either on excised roots i.e. root organ cultures (ROC) or on whole autotrophic plants (Ijdo *et al.*, 2011).

Am Fungal Inoculum Production and Multiplication:

2.1: Inoculum Production

AM fungal inoculum has been utilized in agriculture, horticulture, landscape restoration, and site remediation for almost two decades (Hamel, 1996). In the early 1990s, researchers described multiple ways in which AM species management would be useful for sustainable systems, including agro-systems and restoration (Bethlenfalvay and Linderman, 1992; Pfleger and Linderman, 1994). In a long-term study comparing organic and conventional agriculture, Maeder *et al.* (2002) found that AM were stimulated in organic treatments, which was correlated to enhanced system health (faunal diversity, soil stability, and microbial activity) and to increased crop efficiency.

2.2: Sources of AM inoculum

AM fungi are obligate symbionts, growing only in association with a host plant. Current production systems therefore rely on soil-based systems (plots or pots), which are not sterile and are often contaminated with other AM species, and other microbes, including pathogens (Gianninazzi and Bosatka, 2004). Non-soil based approaches include *in vitro* systems involving the use of Ri T-DNA transformed plant root organs (genetically modified with *Agrobacterium rhizogens*) to grow on media under sterile conditions. These are much cleaner, but have a limited production capacity (Declerk *et al.*, 2005).

2.2.1: Soil based systems or pot cultures

Soil from the root zone of a plant hosting AM can be used as inoculum. Such inoculum is composed of dried root fragments or colonized root fragments, AM spores, sporocarps, and fragments of hyphae. Soil may not be a reliable inoculum unless one has some idea of the abundance, diversity, and activity of the indigenous AM species. Spores can be extracted from the soil and used as in-oculum but such spores tend to have very low viability or may be dead or parasitized. In such a case, soil sample can be taken to set up a 'trap culture' using a suitable host plant to boost the number of viable spore propagules for isolation, further multiplication and also to produce pure or monospecific cultures.

Pure cultures or monospecific cultures are obtained after a known isolate of AM and a suitable host are grown together in a me-dium (sterilized soil/sand) optimized for development of AM association and spore for-mation. It consists of spores, colonized root fragments, and AM hyphae.

2.2.2: Host plant species

The plant grown to host AM fungi in the inocu-lum production medium should be carefully selected. It should grow fast, be adapted to the prevailing grow-ing conditions, be readily colonized by AM, and pro-duce a large quantity of roots within a relatively short time (45–60 days). It should be resistant to any pests and diseases common in the inocula production environment.

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ARBUSCULAR MYCORRHIZAL (AM) FUNGI AND PLANT HEALTH

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Abstract

Arbuscular mycorrhizal (AM) fungi are ubiquitous in nature and represent the oldest and most widespread symbiosis with land plants thereby constituting a vital component of terrestrial ecosystems including horticulture and agro based ecosystems. AM fungi prominently facilitate in uptake of nutrients especially phosphorus (P) uptake in plants by the extra-radical mycorrhizal hyphae leading to better plant growth and development, but they can also perform several other functions that are equally beneficial. AM fungi improve nutrient cycling and soil quality by formation of soil aggregates thus controlling soil erosion by a better plant rooting capacity, influence plant biodiversity, help protect against pests and diseases, increase plant establishment and survival at seeding or transplanting, enhance flowering and fruiting, increase crop yield and quality, improve tolerance to drought and soil salinity, and improve the growth of plants in nutrient deficient soils or polluted environments. This study discusses AM fungal inoculum production and multiplication, role and applications of AM fungi in growth of agro-economically important plants including vegetable crops, fruit crop plants and ornamental plants.

Keywords

Mycorrhiza, inoculum, Phosphorus, agro-ecosystem, horticulture.

Introduction

Arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) are one of the beneficial soil borne microbial symbionts found in almost all habitats. They associate mutually with plant species by colonizing their roots and developing mycelial network in the rhizosphere to facilitate uptake of nutrients (mostly immobile P) and to provide other benefits to their host plants. As obligate symbionts, the arbuscular mycorrhizal association began more than 400 million years ago with the first land plants and both the partners have coevolved since then by obtaining sustainable net benefits. These benefits can be physiological, nutritional and ecological and therefore exploiting and managing AM fungi has important and sustainable consequences for both agricultural and natural ecosystems.

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ARBUSCULAR MYCORRHIZAL (AM) FUNGI FOR SUSTAINABLE AGRICULTURE

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Introduction

The term 'Mycorrhiza' was first introduced by Frank in 1885 and comprises of all symbiotic associations of soil-borne fungi with roots or rhizoids of higher plants. Allen (1991) described the fungal-plant interaction from a more neutral or microbially oriented aspect stating that 'Mycorrhiza is a mutualistic symbiosis between plant and fungus localized in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant'. The group of fungi and plants, which are involved in the interaction, determines the type of mycorrhiza they form (Molina *et al.* 1992).

Soil microorganisms have significant impact on soil fertility and plant health. Microbial symbionts including arbuscular mycorrhizal (AM) fungi form an essential component of the soil microbial community playing a key role in overall plant growth and development. In addition to increasing the absorptive surface area of their host plant root systems, the extra-radical hyphae of AM fungi provide an increased area for interactions with other microorganisms, and an important pathway for the translocation of energy-rich plant assimilates to the soil.

AM symbiosis is the oldest (>460 million years) and most widespread type of mycorrhizal association. It is estimated that 2,50,000 species of plants worldwide are mycorrhizal. The host plants include angiosperms, gymnosperms and pteridophytes (Read *et al.* 2000). Approximately 160 fungal taxa of the order Glomales (Glomeromycota) have been described on the basis of their spore morphology (Schussler *et al.* 2001), although recent molecular analyses indicate that the actual number of AM taxa may be much higher (Vandenkoornhuyse *et al.* 2002; Daniell *et al.* 2001).

During AM symbiosis, the fungal hyphae penetrate the root cortical cell walls by formation of appresoria leading to the development of intra-radical hyphal colonization and formation of arbuscules or coils that interface with the host cytoplasm (Smith and Read 1997). The highly branched arbuscules aid in metabolic exchanges between the plant and the fungus.

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10. Arbuscular Mycorrhizae in Sustainable Agriculture

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Soil microorganisms play an important role in major processes such as soil formation and nutrient cycling of the agro-ecosystem. They form an essential link between soil nutrient availability and plant productivity as they are directly involved in the cycling of nutrients through the transformation of organic and inorganic forms of nutrients. Arbuscular mycorrhizal (AM) fungi are ubiquitous soilborne mycobionts interacting physically with plants in the rhizosphere through the formation of intra-radical (hyphae, arbuscules, vesicles) and extra-radical (hyphae, spores) structures in the host root and having a positive influence on the host plant growth and development. They are one of the beneficial soilborne microbial symbionts and associate mutually with plant species by colonizing their roots and developing mycelial network in the rhizosphere to facilitate uptake of nutrients and to provide other benefits to their host plants thereby constituting a vital component of terrestrial ecosystems including horticulture and agro-based ecosystems. The benefits obtained through AM symbiosis can be physiological, nutritional, and ecological and therefore, exploiting and managing AM fungi has important and sustainable consequences for both agricultural and natural ecosystems. AM fungal inoculum has been utilized in agriculture, horticulture, landscape restoration, and site remediation for almost two decades.

AM fungi are capable of significantly improving plant phosphate, nitrogen, and sulfur acquisition and in turn receive plant carbon leading to completion of its life cycle. In addition, they provide numerous other benefits to their host plants including improved soil quality by formation of soil aggregates thus controlling soil erosion by a better plant rooting capacity, influence plant biodiversity, help protect against insect pests and diseases, increase plant establishment and survival at seeding or transplanting, enhance flowering and fruiting, increase crop yield and quality, and improve tolerance to drought and soil salinity.

Various abiotic and biotic factors influence the distribution, growth, and functioning of AM fungi. These include soil chemistry, e.g., pH, nutrient availability, and pesticides, climatic variables, e.g., temperature, light, and precipitation, and soil structure and stability. Biotic factors are primarily linked to the composition of the plant community, which strongly influences the diversity and assembly of AM fungal communities. Other biotic factors that have been shown to influence AM fungi are root predators, plant parasites, and herbivores. Many of these abiotic and biotic factors are

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Monoxenic Culture of AM fungus *Glomus clarum* using Ri T-DNA transformed roots

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Abstract:

In the present study indigenous isolate of *Glomus clarum* was successfully grown in monoxenic culture system using Ri T-DNA transformed chicory roots (*Cichorium intybus* L.). Surface-sterilized spores isolated from pure culture were used as a source of fungal inoculum for monoxenic culture. Spore germination was carried out on MSR medium. Various developmental features such as spore germination, development of extra-radical mycelium and sporulation in *G. clarum* as observed under *in vitro* conditions were recorded.

Introduction:

Arbuscular mycorrhizae (AM) are obligate symbionts and around 80% of plants are colonized by these fungi which belong to the phylum *Glomeromycota.* The co-existence of various AM fungal isolates in the soil results in healthy plant growth and helps in seedling establishment; nutrient cycling; conservation of soil structure; resistance to drought, temperature and salinity; and control of plant pathogens. Since AM fungi are obligate biotrophs, they are unable to complete their life cycle in the absence of a host plant (Azcón-Aguilar *et al.*, 1998). After contact with a suitable host, inter- and/or intracellular colonization of the root cortex occurs (Bonfante-Fasolo, 1984 and 1987; Smith and Smith, 1997) allowing the completion of the AM fungal life cycle. 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.

In vitro culture of AM fungi was achieved for the first time in the early 1960s

Arbuscular Mycorrhizal Fungi in Fruit Crop Production

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Chapter 1

Diversity of Arbuscular Mycorrhizal Fungi in Fruit Cropping System

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationship with more than 80% of terrestrial plants. AMF are reported to occur in almost all habitats. The diversity of AMF has significant ecological consequences because individual species or isolates vary in their potential to promote plant growth and adaptation to biotic and abiotic factors. AMF population in a given agro-ecosystem plays a crucial role in the structure of plant community and maintenance of ecosystem stability and development of sustainable agriculture. It is well known that many fruit crops are mycorrhizal. The species richness and diversity of AMF have been shown to be influenced by several soil factors and environmental adaptations indicating that the physiology and genetics of AMF along with their responses to the host and edaphic conditions regulates their diversity. This review summarizes the rich diversity of AMF associated with different fruit crops and indicates the need to identify indigenous AMF populations for their distinct roles in association with fruit crops so as to achieve maximum benefits of the mutualistic association.

B. Conference Presentations:

- Rodrigues KM, Rodrigues BF (2012) Monoxenic culture of AM fungus *Glomus clarum* using Ri T-DNA transformed roots. In: State Level Seminar on Recent Innovative Trends in Plant Sciences. Organized by Mahatma Phule Arts, Science and Commerce College, Panvel on 1st September 2012 (Poster).

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