ARBUSCULAR MYCORRHIZAL (AM) FUNGAL DIVERSITY AND GROWTH STUDIES IN MANGROVE PLANT SPECIES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT for THE DEGREE of

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IN THE DEPARTMENT OF BOTANY
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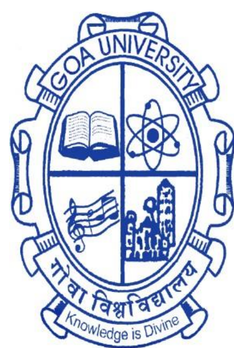

By

SANKRITA S. GAONKAR
GOA UNIVERSITY
TALEIGAO
GOA

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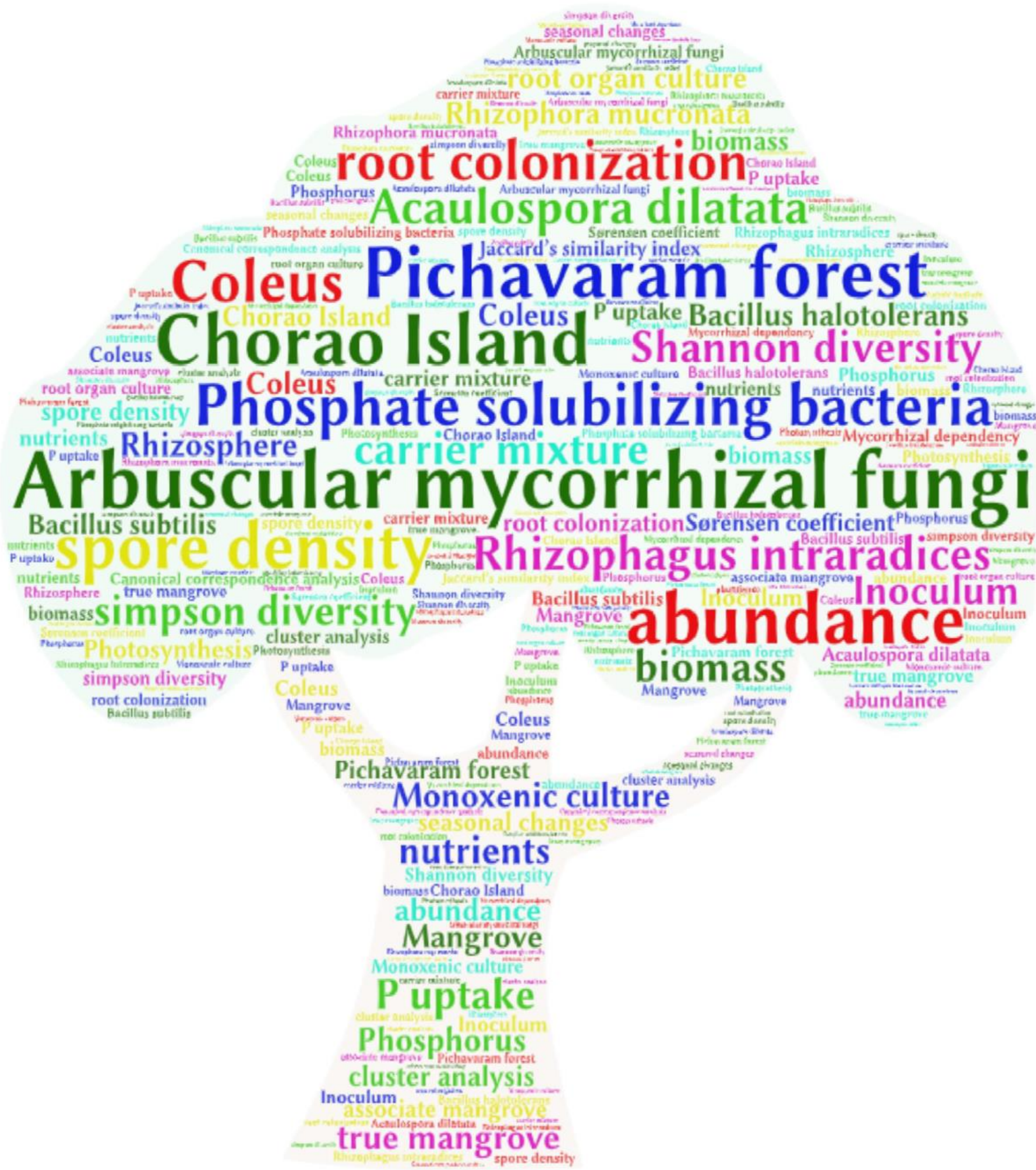

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Phosphorus
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cluster analysis
Inoculum
associate mangrove
true mangrove
spore density

DECLARATION

I, **Sankrita Shankar Gaonkar** hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau.
Date : 07.06.2021

Sankrita S. Gaonkar

CERTIFICATE

I hereby certify that the above Declaration of the candidate, Sankrita Shankar Gaonkar is true and the work was carried out under my supervision.

Prof. Bernard F. Rodrigues
Department of Botany,
Goa University

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Chapter 1: Introduction

1.1 Mangrove ecosystem

Mangroves form a plant community growing in saline habitats of tropical and subtropical regions. The term 'mangrove' describes both the ecosystem and the plants that have adapted to tolerate extreme tides, fluctuating salinity, high temperatures, and low oxygen (Arunprasath and Gomathinayagam 2014; Hogarth 2015). Mangrove plants are classified into two subgroups *viz.,* true- and associate- mangrove plants. True mangroves inhabit the intertidal zones, while associate mangroves occupy the landward fringes of mangrove habitats or (Alongi 2014) terrestrial marginal zones (Wu et al. 2008). Based on salt tolerance, true mangroves are considered halophytes while their associates are glycophytes (Wang et al. 2010b). These forests are most diverse and productive tropical ecosystems in the World (Kathiresan 2000). They serve as breeding and nurturing sites for not only marine organisms but also for terrestrial ones (Igulu et al. 2014; Alongi 2012). Mangrove ecosystem is known as 'carbon sinks' where C is decomposed and exported to neighbouring habitats (Alongi 2012). These forests also provide economic benefits in the form of food sources, timber, fuel, and medicine (Alongi 2002). Besides all these ecological and economic services, they play a major role in offering protection against natural calamities such as tsunami, cyclones, and tidal bores (Alongi 2008; Alongi 2014). Anthropological pressure such as aquaculture, mining, and overexploitation of timber, fuelwood, fodder, and other non-wood forest products (NWFPs) and climate change (sea level rise) constitute key threats for the degradation of mangrove habitats (Ellison and Zouh 2012).

The mangrove areas of India account for about 3% of the World's total mangrove vegetation, comprising of three diverse zones *viz.,* East coast, West coast, and Island territories. Sundarbans, in the West Bengal is the World's largest mangrove forest (2,136 km^2) located on the east coast of India. About 60% of Indian mangroves present on the east coast, 27% on the west coast, and 13% on Andaman and Nicobar Islands (Singh et al. 2012). Mangrove covers approximately 2539 ha of Goa's total land area of 370,200 ha. A total of 178 ha of thick mangrove area at Chorao, Goa, has been declared a Reserved Forest under the Indian Forest Act, 1927 to protect and conserve the system. Later in 1988, the area was declared as a Bird Sanctuary (Hisham et al. 2013).

Pichavaram mangrove forest is known to be the world's second-largest mangrove forest (Mariappan et al. 2016) with *Avicennia marina* and *Rhizophora* species being predominant (Kathiresan 2000). The Pichavaram mangrove forest is situated between Vellar estuary (North) and Coleroon estuary (South) (Srivastava et al. 2012) on the Coromandal coast (Bay of Bengal Sea Board) (Lingan, et al. 1999). It receives three types of waters *viz.,* neritic, brackish, and freshwater from the Bay of Bengal, Vellar-Coleroon estuaries, and irrigation and main channel of Coleroon river respectively (Kathiresan 2000). It covers an area of about 400 hectares and has many islands separated by intricate waterways (Arunprasath and Gomathinayagam 2014). The southern region of Pichavaram forest is covered with mangrove vegetation whereas, the northern region comprises mainly of mud-flats (Kathiresan 2000).

1.2 Arbuscular mycorrhizal (AM) fungi

Arbuscular mycorrhizal fungi are obligate symbionts belonging to the phylum Glomeromycota having a ubiquitous worldwide distribution in various ecosystems (Redecker et al. 2000b). In this association, the fungus receives sugars from the plant while facilitating the plant uptake of nutrients (Schüßler et al. 2007). It is estimated that around 90% of higher plants form this type of association (Loccoz et al. 2015). Janse (1897) named the intra-matrical spores as 'vesicles' and Gallaud (1905) named the intercellular structures 'arbuscules'. Accordingly, the name 'vesicular-arbuscular mycorrhiza' was determined which persisted until recently (Goltapeh et al. 2008). However, species belonging to the family Gigasporaceae (*Scutellospora* and *Gigaspora*) do not produce vesicles and hence the name 'arbuscular mycorrhiza' persisted (Smith and Read 2008).

1.3 Significance of AM fungi in mangroves

Various biotic and abiotic factors such as tidal inundation, soil type, microbe activity in soil, plant species, litter production, and decomposition control the availability of nutrients to mangrove plants. Nitrogen (N) and phosphorus (P) are the nutrients that limit plant growth in mangroves (Reef et al. 2010). Being highly immobile, P is adsorbed by soil particles, forming a phosphate-free zone around plant roots (Bolduc 2011) and thus unavailable for plant use. Therefore, organisms that mobilize P play an important role in plant growth. Arbuscular mycorrhizal fungi help in plant nutrition especially P (Aggarwal et al. 2012 (Willis et al. 2013). Extraradical hyphae of AM fungi can penetrate beyond the

P depletion zone thereby extending the absorption area of the host roots for the uptake of P (Xie et al. 2014).

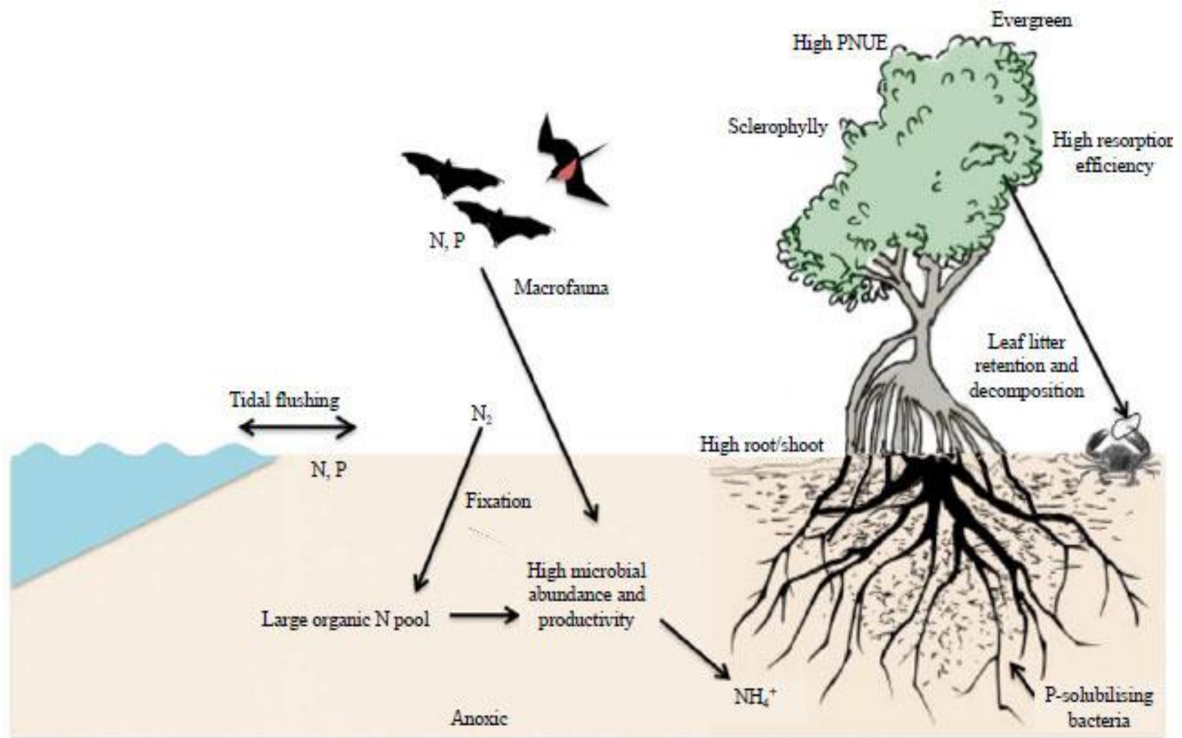


Fig. 1.1: Diagrammatic representation of various events in mangrove ecosystem (https://scialert.net/fulltextmobile/?doi=jest.2016.198.207)

It has been suggested AM fungi play a marginal role in wetland ecosystems due to the anaerobic conditions that decrease fungal activity (Šraj-Kržič et al. 2006). However, recent studies have shown that AM fungi can colonize the roots of wetland plants (Radhika and Rodrigues 2007), increasing nutrient uptake and photosynthetic activity, and therefore the diversity and productivity of mangrove ecosystems (Wang et al. 2010a). According to (Wang et al. 2011), AM fungi obtain oxygen from the root aerenchyma of mangrove plants during flooded conditions. Soil salinity also affects AM fungal spore germination, root colonization, and hyphal growth. However, many AM fungal species are salinity tolerant (Aggarwal et al. 2012).

Several studies have been carried out to investigate AM fungal status in various Indian mangrove habitats (Sengupta and Chaudhuri 2002; (Shalini et al. 2006; Kumar and Ghose 2008; Sridhar et al. 2011).

1.4 Origin and evolution of AM fungi

Fossil records resembling AM fungal spores appeared as early as the Silurian and Ordovician (440-410 million years ago) (Redecker et al. 2000b) as plants started land colonization. Whereas, *Glomus*-like spores were found in plant axes and decaying plant material from Rhynie Chert flora date back to approximately 400 million years ago (Kidston and Lang 1921). Research on AM fungal fossil records revealed the structures like intercellular hyphae, arbuscules, and vesicles in the protosteles of the sporophyte of *Aglaeophyton major*. Previously, AM associations were also discovered in cyanobacterial symbiosis *Geosiphon* as well as in many existing liverworts (Selosse 2005). Their perseverance indicates their coherent strategies to recompense the lack of spore germination and to allow the individuals and community to survive (Giovannetti 2002). The AM fungi exhibit low host specificity which shows their strategy to contact with a wider host range. Furthermore, the mycelial anastomoses during pre-symbiosis and symbiosis with compatible mycelia, forming an extensive hyphal network suggest their mechanism to increase the chance of contacting host roots (Giovannetti 2001).

Fossil records from late carboniferous deposits exposed various gymnosperm fossils with AM fungal symbiotic structures. The best-preserved plant species is *Amyelon radicans* which shared similar AM fungi of living gymnosperm (Smith and Read 2008). *Antarcticycas*, a plant from Triassic flora found in Antarctica exhibited septate as well as aseptate hyphae and other structures resembling arbuscules and vesicles (Phipps and Taylor 1996). (Redecker et al. 2000a) have documented spores from the Ordovician period similar to existing Glomalean spores, indicating probable associations with primitive non-vascular plants.

1.5 Taxonomy or AM fungi

Initial phases of AM fungal taxonomy merely dependent on a couple of morphological characters *viz.* sporocarp. Later, after the discovery of single spores, the wet sieving and decanting method Gerdemann and Nicolson (1963) was used for the extraction of AM fungal spores, and these extracted spores were further used for identification (Kehri et al. 2018).

Primary phase of taxonomy – the first-ever AM fungi discovered was *Endogone* sp. by Link (1809). Later, Tulsane and Tulsane (1845) described two species of *Glomus viz., G. microcarpus* and *G. macrocarpus* which were subsequently shifted to genus *Endogone* by

Tulsane and Tulsane (1851) due to similarity in the type of spores. Berkeley and Broome (1873) found the genus *Sclerocystis* which formed spores in compact sporocarps. These two genera were placed in a single-family Endogonaceae. Thaxter (1922) incorporated four genera in Endogonaceae *viz., Endogone, Glaziella, Sclerocystis,* and *Sphaeroceras.* The first mycorrhizal colonization was observed by Mosse (1953) in strawberry plants which were inoculated with *Endogone* sp. that was later named *Endogone mosseae* (now *Funneliformis mosseae*) (Kehri et al. 2018).

Intermediate phase of taxonomy – the very first key for the identification of AM fungal spores was prepared by Mosse and Bowen (1968). It included seven genera (*Glomus, Sclerocystis, Acaulospora, Gigaspora, Endogone, Glaziella,* and *Modicella*) with 44 species in the Endogonaceae family. The genus *Glomus* was then separated from *Endogone* (Kehri et al. 2018). As *Glaziella* and *Modicella* did not form AM fungal associations, they were later deleted from the Endogonaceae family (Trappe 1982; Gibson et al. 1986).

In 1979, Ames and Schneider described the genus *Entrophospora* with the species *E. infrequence* in Endogonaceae. It showed similar features of *Acaulospora* forming sporiferous saccule. However, the location of the spore on the neck and not on the side of the neck was the key feature of *Entrophospora* formation (Kehri et al. 2018). Later, Walker and Sanders (1986) defined the new genus *Scutellospora* which was separated from *Gigaspora* (defined by Gerdemann and Trappe 1974) due to the presence of 'germination shield' in *Scutellospora* while it was absent in *Gigaspora.*

Morton and Benny (1990) positioned arbuscule producing mycorrhizae in order Glomales (now Glomerales) with three families *viz.,* Glomeraceae, Acaulosporaceae, and Gigasporaceae. The Glomeraceae and Acaulosporaceae were differentiated from Gigasporaceae by the formation of vesicles that are not produced by Gigasporaceae.

Due to the uncertain position of AM fungi in the order Endogonales (Gerdemann and Trappe 1974) and Glomerales (Morton and Benny 1990), Cavalier-Smith (1998) placed all AM fungi in a new class Glomeromycetes.

Molecular taxonomy – Morton and Redecker (2001) described two novel families *viz.* Archaeosporaceae and Paraglomaceae based on morphological, biochemical, and molecular data. Oehl and Sieverding (2004) documented four new species and positioned

them in the genus *Pacispora*. (Schüßler and Walker 2010) propounded a classification having single class Glomeromycetes with four orders, 11 families, and 18 genera.

However, Oehl et al. (2011) proposed a new classification where phylum Glomeromycota was divided into three classes *viz.,* Glomeromycetes, Archaesporomycetes, and Paraglomeromycetes with five orders, 14 families and 29 genera. Further, Goto et al. (2012) proposed a new classification formed based on both morphological and molecular studies introducing a new family Intraornatosporaceae with two new genera *Intraornatospora* and *Paradentiscutata*.

Recently, (Redecker et al. 2013) proposed a new classification and rejected the splitting of the phylum Glomeromycota by Oehl et al. (2011) into three classes (**Fig. 2**).

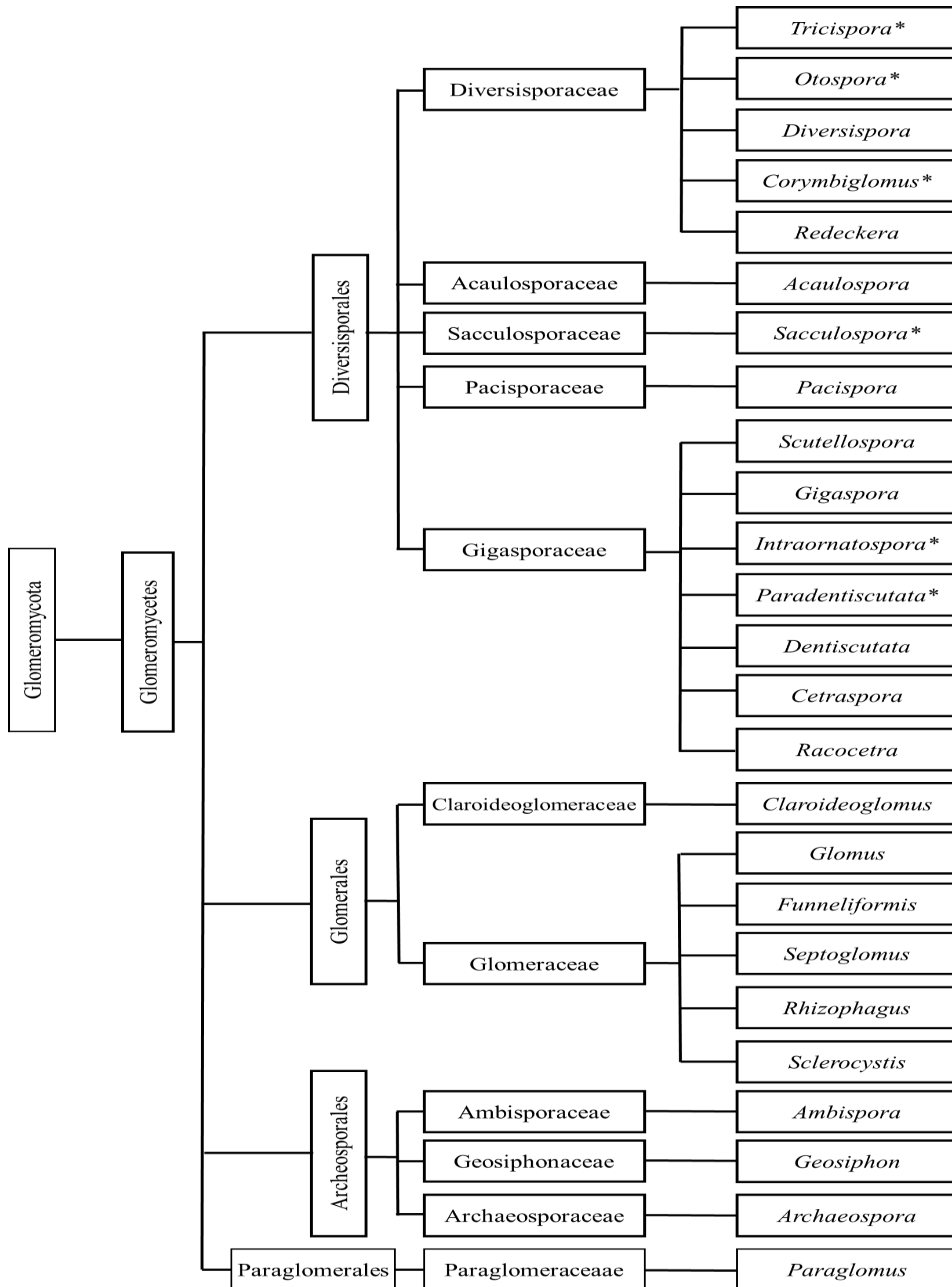


Fig. 1.2: Consensus classification of AM fungi by Redecker et al. 2013. (* designates the uncertain position of genera).

1.6 Development of AM fungi

Arbuscular mycorrhizal fungi colonize the host roots by undergoing series of complex morphogenetic changes such as spore germination, hyphal differentiation, appressorium formation, root penetration, intercellular growth, arbuscule formation, and nutrient transport (Giovannetti 2000). The morphological stages of development vary, depending on plant species. For a successful interaction, the signaling is established before the physical contact between the symbionts. Plant root exudates contain the compounds 'strigolactones' which stimulate hyphal branching and facilitate contact with the host plant (Navazio et al. 2020). Successful recognition is followed by the formation of appressorium (hyphopodium) on the root epidermal layer (Gadkar et al. 2001). The fungus produces hydrolytic enzymes which help in the degradation of the host cell wall. The action of hydrostatic pressure by the hyphal tip allows penetration (Bonfante and Perotto 1995). Within 4-5 hours after the formation of fungal hyphopodium, the plant cell forms a prepenetration apparatus (PPA). The plant nucleus travels towards the vicinity of the contact site (Genre et al. 2005). Subsequently, the reorganization of the endoplasmic reticulum, cytoskeleton, and polarization of microfilaments takes place. Next, the nucleus migrates towards the cortex forming a 'transcellular tunnel' which allows hyphal penetration (Siciliano et al. 2007). With the commencement of symbiosis, mycelia grow within and outside the roots in the soil, thus eventually causing the formation of multinucleate spores on the hyphal tips (Shah 2014).

Intraradical hyphae

Development: After penetration through epidermal cells, intra-radical hyphae start branching in the outer cortex initiating the development of other AM fungal structures within the host root (Peterson et al. 2004).

Functions: The conversion of much of C into triglycerides takes place in intra-radical hyphae (Siddiqui and Pichtel 2008). The persistence of these hyphae in decaying root pieces in the soil serves as an inoculum for the colonization of new host roots.

Arbuscules

Development: The intra-radical hyphae penetrate and spread in the cortex region forming highly branched structures named arbuscules. Arbuscules are ephemeral structures degenerating within 4-5 days after formation (Brundrett et al. 1985).

Arum-type: A branch of an intra-cellular or inter-cellular hypha penetrates the wall of cortical cells forming intricate tree-like branches. Mostly, they occupy the inner cortex adjoining endodermis and vascular tissue. The host-derived plasma membrane surrounding these arbuscules is called a 'periarbuscular membrane' (PAM). This membrane separates arbuscules from host cell cytoplasm which helps in the transfer and temporary storage of mineral nutrients and sugars (Peterson et al. 2004; Harrison 2005; Ramos et al. 2008).

Paris-type: This type of arbuscules is generally formed in plants with no inter-cellular spaces in their roots. This results in the presence of only intra-cellular hyphae. These hyphae develop coils with lateral branches collectively known as arbusculate coils.

The branched structure of arbuscules increases the surface area of the plant cell thereby enhancing nutrient uptake. The exchange of both sucrose and phosphates occurs in the periarbuscular membrane (van Aarle et al. 2005).

Intra-radical vesicles

The swelling of hyphal tips or lateral branches develops into vesicles. These are formed either inside the cell or in intercellular spaces of the root. Depending upon the fungal species, vesicles are of variable shapes like ovoid, lobed, or box-shaped (Smith and Read 2008). Abundant vesicles are formed towards the end of the host growing season. Matured vesicles are filled with lipid bodies and numerous nuclei. Vesicles of some AM species are also known to shelter bacteria (Peterson et al. 2004).

Vesicles act as storage organs storing lipids about 58% of their dry mass and also acts as chlamydospores.

Auxiliary cells

Auxiliary cells are produced exclusively by species belonging to the family Gigasporaceae. These are globose-shaped clusters of varying colour and ornamentation formed on the lateral branches of extra-radical mycelium. Ornamentation on the wall is used as a taxonomic character for AM fungal identification. The auxiliary cells in *Gigaspora* species are echinulate, while they are knobby in *Scutellospora* species (Bentivenga and Morton 1995).

The function of auxiliary cells remains speculative. However, various studies have predicted that they might support the storage of lipids due to the presence of high lipid content (Jabaji-Hare 1988) or in reproduction (Pons and Pearson 1985). De Souza and Declerck (2003) implied a potential role of auxiliary cells in C storage which can be used

for spore germination and development of hyphae. In an *in vitro* experiment conducted by Declerck et al. 2004, auxiliary cells of *Scutellospora reticulata* showed hyphal regrowth but failed to colonize the root.

Extra-radical mycelium

Terms like 'runner hyphae or absorptive hyphal networks' are used to describe extraradical mycelium (Dodd et al. 2000). After primary colonization, these hyphae assist in serving as a source of inoculum for colonizing root systems of the same or different plants (Smith and Read 2008).

The extraradical hyphae help in the uptake of nutrients from the soil and translocate them to the host roots. The highly ramified structure of these hyphae increases surface area for nutrient transfer. Hyphae can grow over long distances away from the nutrient depletion zone for the absorption of water and nutrients.

1.7 Stages of AM life cycle

Spore dormancy

Spore dormancy assists the AM fungal species to thrive in adverse environmental conditions. A dormant spore is the one that is unable to germinate when exposed to physiochemical conditions supporting the germination of similar spores, called quiescent spores (Giovannetti et al. 2010). The breaking of dormancy by storage is described by several authors. (Gazey et al. 1993) demonstrated breaking of spore dormancy in *Acaulospora laevis* by germinating them after storage of six months. Whereas, some of the other species of *Acaulospora* could overcome dormancy after two months of storage at 23˚C in soil (Douds and Schenck 1991).

Dormancy is sometimes considered to be a mechanism to synchronize spore germination with the root growth and suitable environments for colonization in temperate regions (Tommerup 1985). All AM species do not exhibit spore dormancy. Koske and Gemma 1996 reported spores of *Gigaspora gigantea* collected all over the year from dune habitats could germinate in a day after inoculation. As limited information is available on spore dormancy, the understanding of the whole phenomenon remains unclear.

Triggers for spore germination

Spores of different AM species germinate differently. Most of the species belonging to Glomeraceae germinate through hyphal attachments. They can either produce many germ tubes (*Rhizophagus clarus*) or a single one (*F. mosseae* and *F. caledonium*). In *G.*

viscosum, the spore germinates by producing bulbous swelling at the broken end of the hyphae (Godfrey 1957; Walker et al. 1995). Whereas, in the species of Acaulosporaceae and Gigasporaceae the germ tubes emerge via spore wall (Mosse 1970); Siqueira et al. 1985). Besides, germination in *Scutellospora* spores occurs through the germination shield (Walker and Sanders 1986).

The molecular signals triggering spore germination are poorly understood. Based on the evidence, it is shown that the quality and source of the exudate have a vital role to play in triggering spore germination. For example, exudate from *Brassica* spp. (non-mycorrhizal plant), could not stimulate germination (Giovannetti et al. 1993). The occurrence of pre-symbiotic signals between the host plant and the fungus was demonstrated by Mosse and Hepper 1975. Nagahashi and Douds 2000 designed an *in vitro* based experiment in *Gigaspora* species to purify and identify the signaling compound. Later, Buee et al. 2000 carried out semi-purification of active fraction from the exudate of carrot roots. Later, Akiyama et al. 2005 purified and identified the germination factor from *Lotus japonicas* as 5-deoxy-strigol. The compound is a secondary metabolite belonging to the 'strigolactone' family. Moreover, environmental factors such as pH, temperature, nutrient content, host plant, and soil microbes influence spore germination (Siqueira et al. 1985; Mayo et al. 2018). Strigolactones were identified in the 1970s as compounds released from the plant roots that can germinate seeds of parasitic plants. However, since AM fungi are far more ancient than parasitic angiosperms, these rhizosphere signals facilitated by strigolactones must probably have first used for AM symbiosis and later exploited by parasitic plants to sense their host (Rochange 2010).

Growth of pre-symbiotic mycelium

Succeeding germination, hyphae follow straight, linear growth-producing regular, right-angled branches. Hyphae consist of thick walls and are aseptate with numerous nuclei. Cytoplasm, as well as nuclei, migrate in the hyphae. The hyphae then elongate forming a mycelial network (Giovannetti 2010). To develop various inter-cellular structures and to establish successful colonization, AM hyphae have to form contact with the surface of root epidermal cells of the host. At the entry point, the growing hyphae form appressorium attaching to the cuticle of the host roots (Giovannetti et al. 1993). During the contact, hypha can form more than one entry point. Appressoria are multi-nucleate possessing small vacuoles. Hyphal sources initiating the colonization could be either germinating

spores, prevailing hyphae in the soil attached to roots, or hyphae growing from colonized root fragments that were remained in the soil as plants died (Peterson et al. 2004).

In the absence of host-derived signals, the hyphae undergo programmed growth arrest retaining long-term viability and ability to regerminate and colonize the living host (Goltapeh et al. 2008). Correspondingly, the capability of AM fungi to form anastomoses with self-compatible hyphae signifies their fundamental strategy for a wider range of symbiosis with the host plants (Giovannetti 2001).

1.8 Arbuscular mycorrhizal P uptake

Phosphorus (P) is a vital nutrient for plant growth but is a limiting factor in most habitats (Bucher 2007). It is present in the soil as inorganic (Pi) and organic (Po). Inorganic P is sequestered by cations like Fe, Al at lower pH levels and by Ca at higher pH which are insoluble forms. This results in a reduction of sequestered phosphate mobility thus making P unavailable to plants (Smith and Read 2008).

Mycorrhizal plants possess two pathways of nutrient uptake *viz.,* direct pathway in which nutrients from the rhizosphere are taken up by epidermal cells and the mycorrhiza-associated pathway which functions via AM fungal partners in AM plants (Smith et al. 2003). AM fungi help their host in the uptake of P, N, Cu, Zn, etc. However, it is suggested that P acquisition occurs at higher levels (Harrison et al. 2010). Non-mycorrhizal plants solely depend upon direct uptake by Pi transporters that are expressed in the epidermal cells while functioning of both the pathways take place in AM plants wherein Pi transporters are expressed in a cortical cell of colonized roots (Javot et al. 2006). Phosphate transporter genes (Pht1) get activated at the commencement of colonization by extra-radical hyphae of AM fungi (Karandashov and Bucher 2005; Bucher 2007; Javot et al. 2006). The transporters involved in the Pi transfer are H^+ symporters whose function is regulated by the H^+ gradient released by H^+-ATPase in the plasma membrane (Ferrol et al. 2002a). After P uptake by extra-radical hyphae, a substantial quantity of polyphosphates is synthesized. Besides, some amount of these polyphosphates are stored in fungal vacuoles (Dexheimer et al. 1996). It is suggested that the polyphosphates are hydrolyzed by phosphatases confined in the intra-radical hyphal vacuoles (Tisserant et al. 1993). Based on the earlier explanations (Rosewarne et al. 1999); (Ferrol et al. 2002b; Buee et al. 2000), it can be inferred that peri-arbuscular membrane (PAM) plays a vital role in delivering phosphate to cortical cells of their host plant (Ferrol et al. 2002a).

1.9 Benefits of mycorrhiza

Arbuscular mycorrhizal fungi play an extensive role in the growth and development of their host plants even under extreme environmental conditions (Hemalatha et al. 2010). In environments that are distinguished by various biotic and abiotic stresses, the AM plants can thrive better than non-mycorrhizal plants. Hence, AM fungi can promote inter- and intra-specific competitions then favouring mycorrhizal plants (Genre et al. 2005). An individual plant can be colonized by several AM fungi and *vice versa*, bringing about common mycorrhizal networks (CMN) (Jakobsen and Hammer 2015). The interconnections between plant communities can expand stability as weaker plants could gain nutrient supply through CMN at the cost of stronger individuals that entertain CMN (Van der Heijden and Horton 2009).

Nutrient uptake – the association of plants with their fungal partners can establish an enhanced uptake of nutrients such as P, Cu, Zn, S, Mg, Mn, Fe, etc. that are essential for their growth. Also, they are known to help in N transport taken from organic matter to the host (Leigh et al. 2008). It has been proved that the increase in C supply often upturns the absorption of P by the AM fungi and transfer it to their host (Smith and Read 2008).

Stress tolerance – AM fungi are known to offer an ecological competitive benefit to their host plants in enabling survival and improved plant growth under environmental stress conditions such as temperature, pH, moisture, salinity, etc. (Mohammadi et al. 2011). They can also improve the response of a plant to water scarcity by enhancing the uptake of water from the soil by hyphal extensions (Entry et al. 2002). Nevertheless, it is evident from previous studies that, AM fungi can uphold plant salinity tolerance by various mechanisms such as improving uptake of nutrients (Evelin et al. 2012), by regulating the plant physiology (Chang et al. 2018), etc.

Reducing soil erosion and leaching of nutrients – AM fungi are capable of modifying the soil structure by developing ramified hyphal networks that entangle and bind soil particles together forming stabilized aggregates of soil (Leifheit et al. 2014). Collectively, this results in increased water holding capacity that assists in better plant growth besides enhanced nutrient uptake (Chen et al. 2018). Correspondingly, it is known that AM fungi help in the reduction of nutrient leaching by sequestration of nutrients in soil aggregates and by absorption of soil nutrients (Clark and Zeto 2008; George 2000).

1.10 Interaction of AM fungi with other rhizosphere microbes

Mycorrhizal symbiosis is not just a bipartite association between the fungus and plant but AM fungi also interact with the other associated microorganisms (Tarkka and Frey-Klett 2008). These microorganisms have a mutual impact on each other forming a zone called 'mycorrhizosphere' (Frey-Klett and Garbaye 2005). Some of the bacteria that can support the growth of mycorrhiza are known as 'mycorrhiza helper bacteria' (MHB) (Fitter and Garbaye 1994). Furthermore, AM fungi also interact with phosphate solubilizing bacteria (PSBs) by taking up the released P ions that are solubilized from the insoluble form of P by these bacteria (Rodríguez and Fraga 1999).

1.11 Phosphate solubilizing bacteria

As phosphate ions have a negative charge, they can easily form insoluble complexes with aluminium and iron in acidic and calcium in calcareous soils (Khan et al. 2007). Soil microbes can solubilize and mineralize insoluble P into available form thus contributing towards better plant growth (Bhattacharya and Jha 2012). The inundation of the mangrove ecosystem with saline water for longer periods form unfavourable conditions for microbial growth that are important in nutrient mineralization (Shalini et al. 2006).

Phosphate solubilizing bacteria (PSB) are considered to be the most active microorganism assisting in the favourable supply of P to the plants (Solanki et al. 2018). *Bacillus* and *Pseudomonas* form the important genera of PSBs (Khan et al. 2010). Bacterial solubilization of P takes place by excretion of organic acids and their hydroxyl and carboxyl groups help in the chelation of phosphate bound cations (Khan et al. 2007). These organic acids are presumed to solubilize insoluble phosphate to soluble form (orthophosphate) thereby increasing its availability for plants (Vazquez et al. 2000). Gluconic acid is the most common among all the organic acids to solubilize mineral phosphates. Gram-negative bacteria directly oxidizes glucose to gluconic acid (Alori et al. 2017). The mineralization of organic P (phytate, phospholipids, nucleic acids, and phosphoric esters) by PSBs occurs due to the production of phosphatases either acid or alkaline (Rodríguez and Fraga 1999).

Various soil factors can influence the transformation of organic and inorganic P. PSBs from several extreme environments (saline, nutrient deficient, high-temperature ranges) have greater efficiency to solubilize phosphate than those in moderate environments (Zhu et al. 2011). Apart from P solubilization, PSBs provide other benefits to the plants such as

better seed germination, photosynthesis, tolerance to environmental stresses, disease resistance, sequestration of Fe through siderophore production and production of plant hormones (Sharma and Baishya 2017; Adesemoye and Kloepper 2009).

1.12 Monoxenic culture of AM fungi

Monoxenic culture technique is an advanced, powerful, and promising tool for the production of contamination-free inoculum of AM fungi. Wide numbers of AM fungal species have been successfully cultured monoxenically by root organ culture (ROC) using Ri T-DNA transformed roots of various host species. The root organ culture method provides extensive spore production in a small space and within a short period, thus increasing the spore load to be inoculated in the field influencing the production of agricultural and horticultural crops (Srinivasan et al. 2014). Factors such as pH, temperature, moisture, minerals, and organic nutrients play roles in spore germination and germ tube growth (Clark and Zeto 2008).

Only a few AM fungal species belonging to Glomeraceae and Gigasporaceae and single species belonging to Acaulosporaceae have been successfully cultured on ROC (Rodrigues and Rodrigues 2013). Ever since the 1980s, progress in the development of monoxenic methods and the media used for the cultivation of AM fungi on ROC has been limited (Abdellatif et al. 2019). Scientists have modified White's medium to produce modified Strullu Romand (MSR) medium (Strullu and Romand 1986; Declerck et al. 1998) and minimal (M) medium (Bécard and Fortin 1988). A new medium i.e. IH medium comprising of palmitic acid was developed for the better monoxenic culture of AM fungi (Ishii 2012). Trépanier et al. (2005) suggested that palmitic acid serves as an essential constituent for the production of AM fungal lipids.

Ri T-DNA transformed roots have been efficiently employed in recent decades to prepare the dual culture of AM fungi and host roots. A naturally obtained genetic transformation of plants using *Agrobacterium rhizogenes* Conn. results in the formation of hairy roots. The modifications in their hormones, allow them to grow profusely on the artificial media (Fortin et al. 2002).

AM fungal inocula containing spores (extra-radical), colonized fragments of root or isolated vesicles can be used for their monoxenic cultivation (Rodrigues and Rodrigues 2013). However, some of the AM fungal species producing no vesicles (Gigasporaceae) have been cultured using spores (Fortin et al. 2002).

The only study on the occurrence and diversity of AM fungi in mangroves of Goa was reported by (D'Souza and Rodrigues 2013a; 2013b). However, the location investigated in the present study has never been subjected to similar investigations previously. It is also proposed to explore the transformation of AM fungal diversity associated with the mangrove plants of the Pichavaram forest which were earlier reported as non-mycorrhizal.

To understand the ecology of the habitat and to develop conservation strategies, it is necessary to measure the biodiversity associated with the habitat. Therefore, the present study was conducted to quantify the AM fungal diversity and to identify dominant AM fungal species in mangroves of Chorao Island and Pichavaram forest. Also, using bioinoculants to investigate their effects on the growth and biomass of selected mangrove plant species and to discuss the potential application of bioinoculants in the recovery and revegetation of the mangrove ecosystem. The present study proposes the following objectives:

a. To identify the AM fungal diversity in mangrove plant species found in Chorao Island.
b. Preparation of trap and pure cultures.
c. Preparation of monoxenic cultures of dominant AM species.
d. Isolation, identification, and activity of phosphate solubilizing bacteria (PSB).
e. Mass multiplication and preparation of inocula.
f. Screening of efficient AM species for selected mangrove plant species.

Chapter 2: Review of literature

AM fungal diversity studies in mangroves.

Authors	Site/host plant	Inference/major findings
Sengupta and Chaudhuri 2002	Ganges river estuary, India	Rhizosphere soils of 31 species of true- and associate- mangrove plants were investigated for AM fungal associations. They reported that the colonization rates varied among species and their situation of occurrence, being highly colonized in dry and less saline mangrove sites.
Gupta et al. 2002	Bhitarkanika, Orissa, India	A study of 12 mangrove and 18 non-mangrove plants was carried out. The maximum colonization was shown by *Heritiera fomes*. The colonization was absent in herbaceous mangrove plants.
Shalini et al. 2006	Nicobar Island, India	Five *Glomus* species were recovered from the mangrove rhizosphere of Great Nicobar Island. They concluded that the colonization of aerenchymatous cells signifies the role of mangrove plants in providing oxygen to AM fungi in anoxic conditions.
Kumar and Ghose 2008	Sundarban mangroves, West Bengal, India	The rhizosphere soil of 15 true- and one associate-mangrove plant from three different inundation types was analyzed to examine the status of AM fungi. Forty-four AM species belonging to six genera *viz. Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis,* and *Scutellospora* were recovered. *Glomus*

		mosseae showed the highest frequency. They concluded that the host plant had a greater impact on the percent colonization and spore density than that of the inundation period.
Wang et al. 2011	Zhuhai mangrove area, China	Amplification of SSU-ITS-LSU of AM fungal colonized roots of three mangrove plant species across a tidal gradient was conducted. A total of 23 phylotypes of AM fungi were obtained, out of which 22 belonged to Glomeraceae and one Acaulosporaceae. They suggested that the duration of flooding has an impact on the diversity of AM fungi.
Sridhar et al. 2011	South west coast, India	The rhizosphere soil of eight mangrove plant species from the Netravathi mangrove forest was evaluated for the presence of AM fungi. An associate mangrove (*Derris trifolium*) showed the highest root colonization as well as maximum spore density. They inferred that the soil factors such as pH and salinity have an impact on root colonization.
Balachandran and Mishra 2012	Western coast, Maharashtra, India	AM fungi and glomalin content were assessed in the rhizosphere soils of heavy metal polluted areas of mangrove forests in Mumbai, Thane, and Raigad. Permissible levels of Ni, Pb, and Cr were present at the studied site. Root colonization and spore density of AM fungi were high at all the polluted sites.

		The correlation between glomalin and heavy metal content was significantly positive, which confirmed that the glomalin helps in the sequestration of heavy metals.
D'Souza and Rodrigues 2013	Rivers - Terekhol, Chapora, Mandovi, Zuari, Sal, Talpona and Galgibag, Goa, India	A Survey of 17 mangrove species from seven rivers of Goa was performed to investigate AM fungal associations. *Excoecaria agallocha* recorded the highest root colonization, whereas the least colonization was observed in *Avicennia marina*. Twenty-eight AM fungal species belonging to the genus *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora,* and *Entrophospora* were recovered. The study indicates the dominance of two AM fungal species *viz., Glomus intraradices* and *Acaulospora laevis*.
D'Souza and Rodrigues 2013	Rivers – Terekhol and Zuari, Goa, India	Effect of season on the diversity AM fungi in three mangrove plant species *viz. Acanthus ilicifolius, Excoecaria agallocha,* and *Rhizophora mucronata* from two different locations were examined. The maximum number of AM fungal spores and species was recorded during the pre-monsoon season, indicating that the season had a profound effect on AM fungal diversity.
Wang et al. 2014a	Qi'Ao mangrove forest, China	Molecular sequencing of each spore morphotype isolated from the mangrove rhizosphere and the roots of semi-mangrove plant species was carried out.

		Eleven new sequences from spores and 172 from the roots were derived. They concluded that the composition of AM fungal genera in semi-mangrove habitat was similar to those present in intertidal zones of mangrove habitats.
Hu et al. 2015	Mangrove forest, Southern China	They explored the occurrence of AM fungi in the rhizosphere of *Aegiceras corniculatum* and *Acanthus ilicifolius*. This study revealed that the available soil P and salinity are influencing factors for the development of AM in mangroves.
Gupta 2016	Bhitarkanika, Orissa, India	Assessment of AM fungal diversity in various salinity zones was carried out at 16 sites of Bhitarkanika mangrove forest. The maximum number of AM species was recovered from less saline zones. Genus *Glomus* was found to be dominant in all the salinity zones.
Gopinathan et al. 2017a	Muthupet mangrove area, Tamil Nadu, India	The occurrence of AM fungi in the rhizosphere of *Avicennia marina* was investigated. A total of 14 AM fungal species were isolated, with *Glomus* being the dominant genus.

Monoxenic culture of AM fungi.

Authors	AM species	Inference/major findings
Declerck et al. 2000	*Rhizophagus proliferus*	The association of *R. proliferus* with transformed *Daucus. carota*roots were obtained on Modified Strullu and Romand (MSR) medium. The sporulation was initiated one week after the preparation of dual cultures.
Gadkar and Adholeya 2000	*Gigaspora margarita*	An *in vitro* culture was established with *G. margarita* and transformed roots of *D. carota* on Minimal (M) medium to examine the growth and physiology of the fungal spore. Mostly single spores were formed in 18-20 months old cultures.
Karandashov et al. 2000	*Funneliformis caledonium*	The spores of *F. caledonium* were grown in dual culture with transformed roots of *D. carota* on M medium (pH 6.5). the spores were produced after 2-3 days of contact (within 1-3 weeks after spore germination) with the roots.
Dalpé and Declerck 2002	*Acaulospora rehmii*	The spores of *A. rehmii* were grown monoxenically on a Petriplate containing MSR medium with the transformed roots of *D. carota*.
Bi et al. 2004	*Sclerocystis sinuosa*	They established monoxenic culture of *S. sinuosa* using transformed roots of *D. carota* (carrot) on M medium. The sporocarps were formed after four months.
Kandula et al. 2006	*Scutellospora calospora*	This study reports the cultivation of *S. calospora* spores on MSR medium using ROC of *D. carota*. Only four

		spores were formed eight months after the preparation of dual culture.
Eskandari and Danesh 2010	*Rhizophagus intraradices*	An experiment was performed to study the life cycle of *R. intraradices* using the root organ culture of *D. carota* on MSR medium. The sporulation occurred 25 days after contact with the roots.
Bidondo et al. 2012	*Gigaspora decipiens*	A successful *in vitro* culture of *G. decipiens* was obtained using transformed roots of *D. carota*. The sporulation occurred after five months of inoculation on M medium.
Costa et al. 2013	*Gigaspora decipiens* and *Rhizophagus clarus*	An *in vitro* experiment for the verification of temperature and pH effect on the sporulation of *G. decipiens* and *R. clarus* was conducted using the transformed roots of *D. carota* on M medium. The sporulation increased at 22 °C and decreased at 28 °C and 32 °C. *G. decipiens* showed the highest sporulation at pH 6.5, whereas in *R. clarus* sporulation was higher at pH 4.0.
Rodrigues and Rodrigues 2015	*Funneliformis mosseae*	A monoxenic culture of *F. mosseae* spores was successfully established on MSR medium using *Linum usitatissimum*. The colonization occurred five days after co-cultivation. The spores produced showed 83% of viability.

Phosphate solubilizing bacteria (PSB) in mangroves.

Authors	Site	Inference/major findings
Vazquez et al. 2000	Laguna de Balandra, California, Mexico	They isolated 13 PSB isolates from two mangrove plant species *viz., Avicennia germinans,* and *Laguncularia racemosa*. The results indicated that *Vibrio proteolyticus* was the most active PSB isolate.
Ravikumar et al. 2007	Manakudi mangroves, Tamil Nadu, India	Diversity studies of phosphobacteria in the soil as well as in root samples of Manakudi mangroves. The number of phosphobateria was higher in roots than that in soil samples. A total of nine species of phosphobacteria belonging to seven genera were isolated, which were found to be sensitive to heavy metals (Hg and Zn). The P solubilizing activity was decreased with increased concentrations of heavy metals.
Subhashini and Kumar 2014	Corangi mangroves, Andhra Pradesh, India	15 strains of P solubilizing *Streptomyces* sp. were isolated from rhizosphere soil of *Ceriops decandra* on ISP-5 medium. St-3 was found to be the most efficient P solubilizing strain, which solubilized a maximum of 48.28 µg/mL of inorganic P at 30ºC with 3% of NaCl in the growth medium.
Behera et al. 2016	Mahanadi river delta, Odisha, India	In this study, a total of 48 strains of PSBs were isolated from

		mangrove soil on NBRIP medium belonging to genera *Pseudomonas, Bacillus*, *Alcaligens, Klebsiella, Serratia, Azotobacter*s, and *Micrococcus.* The P solubilizing ability ranged from 8.21 to 48.70 µg/mL.
Behera et al. 2017a	Mahanadi river delta, Odisha, India	A strain of PSB was isolated from mangrove soil on NBRIP medium, which was further identified as *Serratia* sp. Maximum 44.84 µg/mL of P was solubilized with a decrease in pH from 7.0 to 3.15.
Behera et al. 2017b	Mahanadi river delta, Odisha, India	A PSB identified as *Alcaligenes faecalis* was isolated from mangrove soil of Mahanadi delta on NBRIP medium supplemented with tricalcium phosphate. The P solubilizing activity was found to be 48 µg/mL, with a decrease in pH of the medium from 7.0 to 3.2. Organic acids such as oxalic acid, citric acid, malic acid, succinic acid, and acetic acid were detected in broth culture. Alkaline phosphatase activity was found to be 93.7 µg/mL.

Screening of efficient AM fungal species for mangrove plant species.

Authors	Site	Inference/major findings
Wang et al. 2010	Pearl River, South China Host plant – *Sonneratia apetala*	The AM fungal symbiosis in two mangrove swamps was examined and reported six AM fungal species (*Glomus* and *Acaulospora*). Also, a greenhouse experiment was performed using *S. apetala* as a host plant. It was reported that AM inoculated plants had better growth and biomass with improved levels of N, P, and K.
Wang et al. 2014b	Futian Nature Reserve of Shenzhen, South China Host plant – *Kandelia obovata* and *Aegiceras corniculatum*	The effect of municipal sewage discharge on the extent of AM fungal and mangrove plant symbiosis was estimated first by the construction of two mangrove belts and secondly by a pot-based experiment. *A. corniculatum* showed greater intensities of AM colonization. The vesicles and arbuscules had an inhibitory effect, whereas hyphae were more tolerant of wastewater discharge.
Xie et al. 2014	*Kandelia obovata*	They evaluated the effect of AM fungi and P supply on soil phosphatases, plant growth, and nutrient uptake in host plant *K. obovata*. The P supply (KH_2PO_4) enhanced the height and biomass of the plant, thereby partly inhibiting the activity of acid and alkaline phosphatases. In contrast, inoculation of plants with AM fungi increased root strength and plant

		biomass, controlled phosphatase activities, and increased nutrient uptake.
D'Souza and Rodrigues 2016	*Ceriops tagal*	An experiment was conducted to study the effect of three AM fungi *viz., Rhizophagus clarus, R. intraradices,* and *Acaulospora laevis* on the growth of *C. tagal*. The study revealed that *R. clarus* is the most efficient AM fungi, which increased the biomass of the selected plant.

Chapter 3: To identify the AM fungal diversity in mangrove plant species.

3.1: INTRODUCTION

Mangroves are rich and diverse in living resources and hence increase the economic and ecological value of the ecosystem (Kathiresan 2000). The mangrove–ecosystem has become a vital element for many conservation and environmental issues (Gopinathan et al. 2017b). Mangroves show substantial tolerance to salinity, inundation, and nutrient stress. However, they have degenerated drastically all over the world, mainly due to nutrient limitations (Xie et al. 2014). Hence, protecting and reconstructing the mangrove ecosystem has become a global concern (Krauss et al. 2008). Several geophysical and geomorphologic processes *viz.,* salinity, sulfide, pH, nutrients, light, space, and hydroperiod control mangrove productivity (Twilley 2009). Islands are considered to be crucial habitats to perform ecological studies (Walter 2004), which might sometimes connect to the mainland contributing to species sharing (Triantis et al. 2012).

Various AM fungal species colonizing the roots of different plant species play a crucial role in the regeneration, diversity, and distribution of plant communities (Nandi et al. 2014). They are known to maintain plant diversity and contribute to ecological processes (Francis and Read 1994). AM fungi play a significant role in soil nitrogen (N) and carbon (C) cycles and also helps in the reduction in plant uptake of phytotoxic heavy metals (Willis et al. 2013). It increases plant productivity, diversity, and enhances the plant resistance to biotic and abiotic stresses (Ijdo et al. 2011). It has been recommended that mixed communities of AM fungi have a more significant effect on plant growth than on individual species (Alkan et al. 2006).

Limited studies have been carried out on AM fungal diversity in Island environments (Schmidt and Scow 1986; Trufem 1990; Koske and Gemma 1996; Shalini et al. 2006; Stürmer et al. 2013). Thus, investigation of AM fungal occurrence and distribution in such environments would expand the knowledge about biogeographical patterns of these fungi, particularly in poorly explored habitats of the tropical region (Rodríguez-Echeverría et al. 2017). Therefore, in the present chapter, the quantification of AM fungal diversity and identification of dominant AM fungal species in true- and associate-mangroves of Chorao Island and Pichavaram forest was initiated.

3.2: MATERIALS AND METHODS

3.2.1: Study sites

For the study on the diversity of AM fungi in mangrove habitat, the two sites *viz.,* Chorao Island, Goa (**Plate 3.1**), and Pichavaram mangrove forest, Tamil Nadu (**Plate 3.2**), were selected.

Chorao Island (15° 32’ N, 73° 52´ E): it is located on the West Coast of India in the Mandovi River at an elevation of 8 m AMSL (**Fig. 3.1**). The total area of the Island is 423.75 ha which has a mangrove cover of about 250 ha and has an average annual rainfall of approximately 2500 mm (https://www.spectrumtour.com/south-india-tourism/chorao-island-goa.htm). The Island is divided by creeks and backwaters with continuous tidal variations and is formed from a confluence of the Mandovi River and its tributary, the Mapusa River (Sappal et al. 2014). The mangrove flora of the Island is represented by 17 plant species belonging to 10 families with *Rhizophora mucronata, Avicennia marina, Sonneratia alba,* and *Excoecaria agallocha* being dominant.

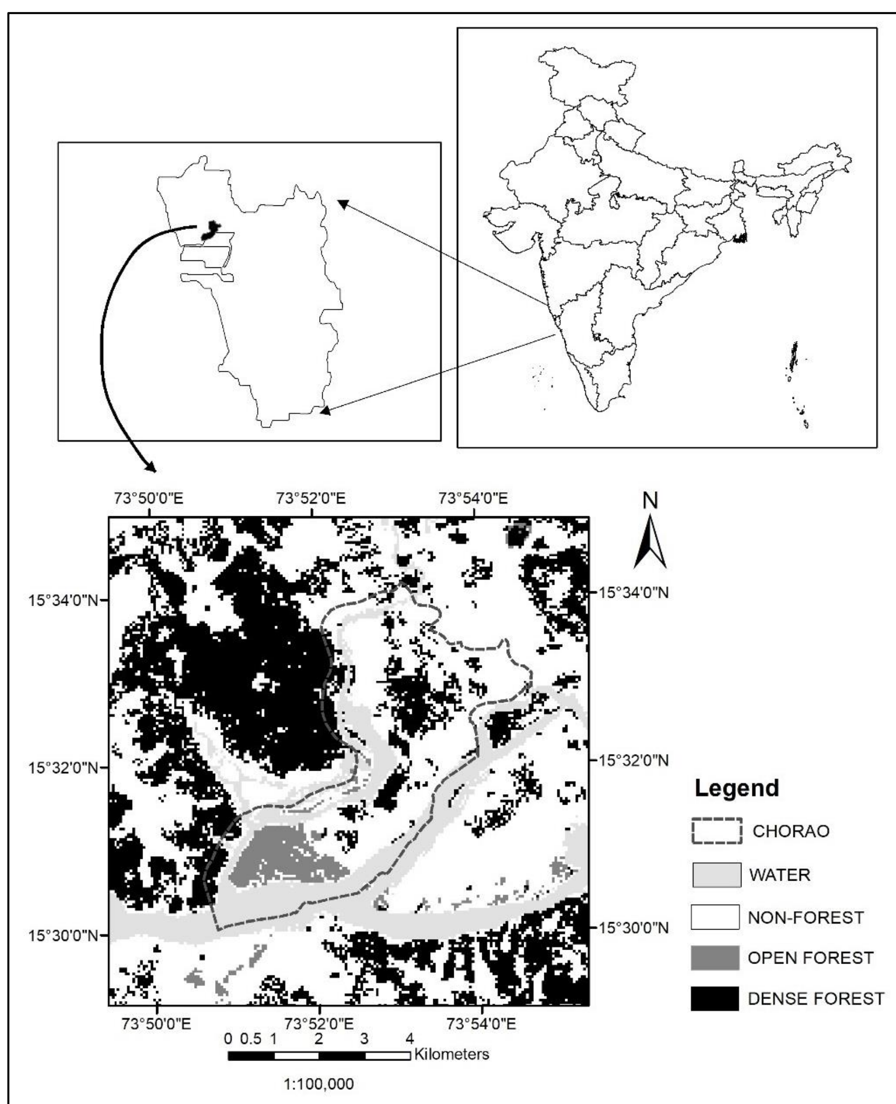


Fig. 3.1: Map showing Chorao Island.

Pichavaram mangrove forest (11° 29' N, 79° 46' E): it is situated on the southeast coast of India. It is a mangrove swamp located in the Vellar-Coleroon estuarine complex. The total area of the Pichavaram forest is 1100 ha traversed by 51 islets (Kathiresan 2000). About 241 ha of the entire forest is occupied by dense mangrove cover (Arunprasath and Gomathinayagam 2014). The average annual rainfall is 1310 mm (Selvam et al. 2003).

The plant and soil samples were collected from three mangrove sites of Pichavaram forest *viz.,* Pichavaram extension (PE), Pichavaram Reserved Forest (PRF), and Killai Reserved Forest (KRF) (**Fig. 3.2**).

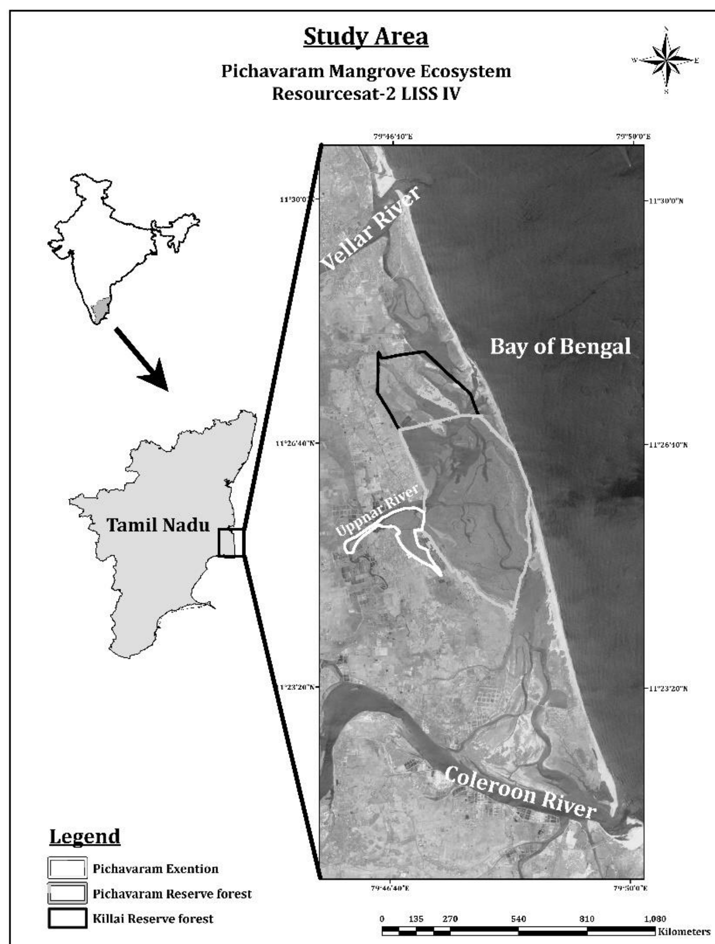


Fig. 3.2: Map of Pichavaram forest showing the sampling locations.

3.2.2: Sample collection

In the present study, 17 and 18 mangrove species from Chorao Island and Pichavaram forest respectively, were investigated (**Plate 3.3 to 3.7**). At Chorao Island, 11 species were true mangroves, while six were mangrove associates, which belonged to 10 families. Whereas, at Pichavaram forest, nine each were true- and associate- mangroves that belonged to 12 different families. Rhizosphere samples were collected from the depth of 0-30 cm using soil corer (5 cm diameter). During the collection, roots of the trees were traced by digging and removed to ensure that the collected roots belong to the same plant species. Three rhizosphere soil samples were collected from each plant species, placed in separate sealed bags, labeled, and brought to the laboratory. These three samples of each plant species were then thoroughly mixed to form a composite sample. The roots were separated from adhering soil, washed, and used for estimation of AM colonization. Each composite

sample was divided into two parts, one part for AM spore isolation, enumeration, and identification, the other as inoculum to prepare trap cultures.

To investigate the seasonal dynamics of AM fungal diversity in the west and east coast of India, the soil samples were collected from both Chorao Island and Pichavaram forest. The years arranged into three seasons are different for the east and west coasts. The seasonal months for the east coast are June to September (Pre-monsoon), October to December (Monsoon), and January to May (Post-monsoon). Whereas, for the west coast, it is February to May (Pre-monsoon), June to October (Monsoon), and November to January (Post-monsoon).

Soil and root samples of three common plants *viz., Avicennia marina, Bruguiera cylindrica,* and *Excoecaria agallocha* from both the sites were collected during all three seasons. The method used for sample collection was the same as described above.

3.2.3: Soil analyses

For this analysis, soil samples were randomly collected in triplicates from true- and associate- mangrove areas of Chorao Island and three sites of Pichavaram Forest and were analyzed separately. Also, the soil samples were collected during different seasons from the two sites. Soil texture was analyzed by the pipette method (Folk 1974). Soil pH and electrical conductivity (EC) were measured with pH meter (LI 120 Elico, India) and Conductivity meter (CM-180 Elico, India), respectively, in soil water suspension (1:2 ratio). Organic carbon (OC) was estimated by Walkley and Black (1934) method through oxidizing it using potassium dichromate in acidic medium and titrating the residual dichromate against ferrous ammonium sulphate (FAS), available nitrogen (N) was estimated by oxidative hydrolysis of liberated ammonia using $KMnO_4$, absorbing it on boric acid and titrating against standard acid (Subbiah and Asija 1956). Available P was extracted with 1.5% Dickman and Bray's reagent and determined by colorimetry (Bray and Kurtz 1945). The available potassium was extracted with 1N ammonium acetate and estimated by flame photometry (Hanway and Heidel 1952). Available zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) were extracted using DTPA (diethylene triamine penta acetic acid) extractant with soil: reagent ratio of 1:2 (Lindsay and Norvell 1978) and quantified using atomic absorption spectrophotometer (AAS) (nova 400P, Analytik Jena, Germany).

3.2.4: AM fungal root colonization

Fifty root pieces (secondary and tertiary roots) approximately one-centimeter long were cleared in 10% KOH at 90 °C for 90 minutes, acidified in 5 N HCl, and stained with 0.05% Trypan blue overnight (Phillips and Hayman 1970). Stained roots were then mounted on glass slides in polyvinyl alcohol Lacto-glycerol (PVLG) and examined using a bright-field Olympus BX41 research microscope. A root segment was considered mycorrhizal if it showed the presence of hyphae/hyphal coils, arbuscules/arbusculate coils, and/or vesicles. The intensity of total colonization (TC), root length containing hyphae (HC %), arbuscules (AC %), and vesicles (VC %) were quantified using the Magnified intersections method (McGonigle et al. 1990). Percent AM root colonization was estimated using the following formula:

$$\text{Percent colonization} = \frac{\text{Number of root segments colonized}}{\text{Total number of root segments observed}} \times 100$$

3.2.5: Isolation and identification of AM fungal spores

Spores from rhizosphere samples (n=3) and trap cultures were isolated using the wet sieving and decanting method (Gerdemann and Nicolson 1963), where 100 g of soil sample was suspended in 1 L of tap water in a beaker. The mixture was stirred for 10-15 seconds and kept undisturbed for approximately 30 seconds, to settle the heavier particles. The soil water mixture was decanted through the stacked sieves with the coarse sieve on top and a fine sieve at the bottom. The range of sieves used was 60 μ, 100 μ, 150 μ, and 240 μ. The suspension from each sieve was collected separately in the beaker. It was then filtered separately through Whatman No. 1 filter paper using a funnel. The filter papers were then placed in the Petri plate, and the spores were isolated under the stereomicroscope. Intact, non-parasitized healthy spores were quantified using the modified method of Gaur and Adholeya (1994). The spores were then mounted on glass slides in PVLG and examined under a bright-field Olympus BX41 research microscope (40x, 100x, and 400x). Spore morphology, wall characteristics, dimensions, and other relevant data were observed for the identification of the AM spores. The spore characters were compared with the descriptions given by Rodrigues and Muthukumar (2009), Blaszkowski (2012), and the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (invam.wvu.edu). Names and epithets of AM fungal species were followed

according to the recommendation of Schüßler and Walker (2010) and Redecker et al. (2013).

3.2.6: Data analysis

Relative abundance (RA) was evaluated using the formula: RA = (Number of spores of a species or genus / Total number of spores in all soil samples) x 100, while isolation frequency (IF) was derived by using the formula: IF = (Number of soil samples possessing spores of a particular species / Total number of soil samples analyzed) x 100.

Following formulae were used to calculate the Shannon-Wiener diversity index (H) (Shannon and Weaver 1948) and Simpson`s diversity index (D) (Simpson 1949):

$H = - \Sigma (pi \ln pi)$

$D = 1-[\Sigma n(n-1)/N(N-1)]$

(Where *pi* is the proportion of individual species that contributes to the total number of individuals, *n* is the number of individuals of a given species and N is the total number of individuals in a community).

Species evenness was estimated as (Σ (H) = H'/H' max) where, H'max = lnS, S = total number of species in the community (richness). Jaccard's similarity index (JI) was calculated pair-wise between mangrove plant species based on the presence or absence of each AM fungal species (Jaccard 1912).

JI (%) = $(c \div a + b + c) \times 100$, where 'c' stands for the number of species occurring in both hosts, 'a' is the number of species unique to the first host and 'b' is the number of species unique to the second host.

All data were statistically analyzed using SPSS v 22 software. To compare the soil parameters between true- and associate- mangroves, a paired sample t-test was performed. Pearson's correlation coefficient was calculated to evaluate the relationships between root colonization and spore density, isolation frequency, and relative abundance, and spore density and species richness. To understand the distribution of AM fungal species among true- and associate-mangrove plants, cluster analysis (Bray-Curtis similarity) was performed using PRIMER v 6.0.

To study the relationship between the relative abundance (RA) of AM genus and soil parameters at two different sites during three different seasons, Canonical correspondence analysis (CCA) was performed using Multivariate Statistical Package (MVSP) v 3.1.

3.3: RESULTS AND DISCUSSION

1) Diversity of AM fungi in mangroves of Chorao Island and Pichavaram mangrove forest:

3.3.1: Physico-chemical properties of mangrove soils

Chorao Island: Results of texture analysis showed that the soil from true mangrove areas is clayey silt, while associate mangrove areas have a nearly equal proportion of sand, silt, and clay (**Fig. 3.3**). In mangrove forests, sedimentation of clay particles takes place as these forests are enclosed and protected environments with low-energy waters (Hossain and Nuruddin 2016). Analyses of chemical properties of the estuarine soils at Chorao revealed acidic nature. All nutrients showed low availability, with P being the least available nutrient. This low nutrient availability, along with increased salinity, appears to be responsible for causing stress, thereby affecting plant growth, especially in the true mangroves. Except for the OC and K, all the other soil parameters varied significantly between true- and associate- mangroves (**Table 3.1**). The results of the t-test are presented in **Table 3.2.** The *p* values indicate the significant differences ($p<0.05$) between the soil parameters in two types of mangroves. The parameters, such as EC, OC, N, Cu, and Fe showed significant differences between true- and associate- mangroves. The negative t-values indicate that the mean values of pH and P are higher in associate mangrove plants.

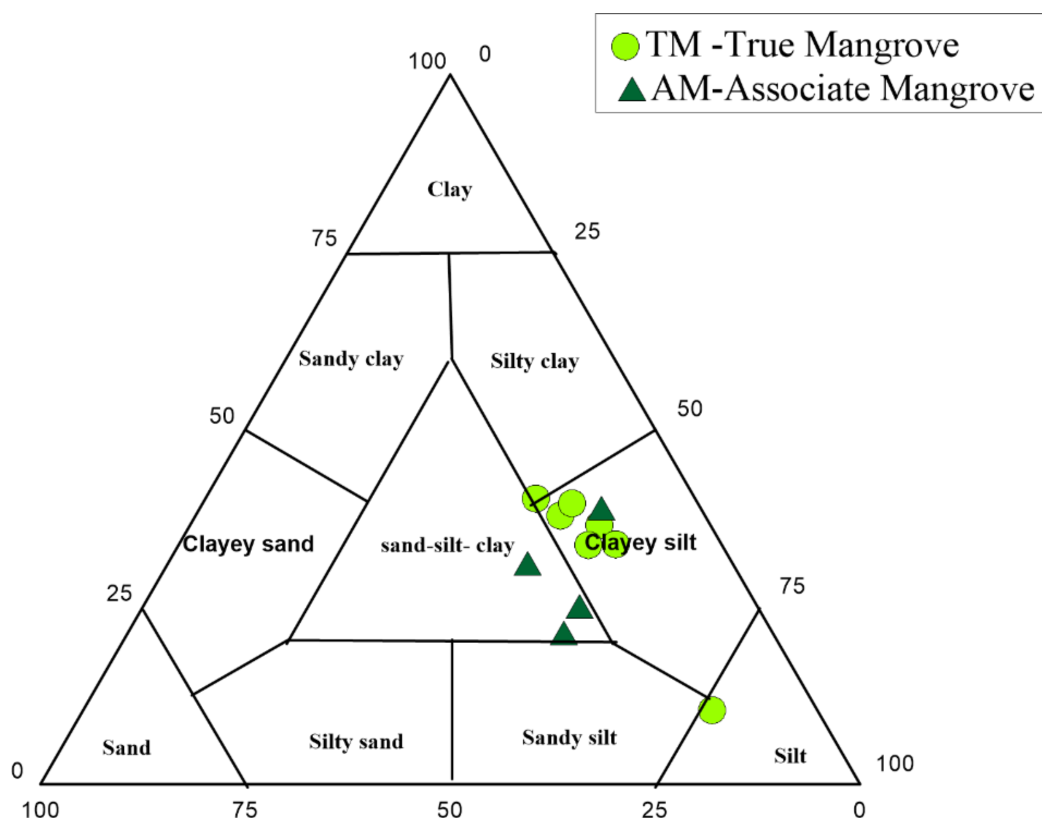


Fig 3.3: Ternary diagram of sand-silt-clay percentages of Chorao Island.

Table 3.1: Chemical properties of Chorao mangrove soils.

Parameters	True mangrove soil	Mangrove associate soil
pH	5.87 ± 0.59	5.65 ± 0.57
EC (dS/m)	8.95 ± 0.99	3.9 ± 0.10
OC (%)	2.81 ± 0.35	1.07 ± 0.13
N (g/kg)	0.073 ± 0.01	0.067 ± 0.01
P (g/kg)	0.007 ± 0.004	0.051 ± 0.03
K (g/kg)	0.231 ± 0.03	0.263 ± 0.04
Zn (ppm)	2.011 ± 0.40	1.834 ± 0.37
Cu (ppm)	0.50 ± 0.13	0.297 ± 0.07
Fe (ppm)	343.1 ± 3.43	266.9 ± 2.67
Mn (ppm)	2.28 ± 0.76	2.24 ± 0.75

Note: All values are mean of three readings; ± = Standard error; EC= Electrical conductivity; OC= Organic carbon.

Table 3.2: Paired sample t-test to compare soil parameters between true- and associate-mangrove plants.

Pairs of variables	t	df	P (2-tailed)
Pair 1 $pH_1 - pH_2$	-.653	2	0.581
Pair 2 EC_1- EC_2	5.435	2	0.032
Pair 3 OC_1- OC_2	5.125	2	0.036
Pair 4 N_1- N_2	20.000	2	0.002
Pair 5 P_1- P_2	-1.070	2	0.397
Pair 6 K_1- K_2	0.000	2	1.000
Pair 7 Zn_1- Zn_2	2.147	2	0.165
Pair 8 Cu_1- Cu_2	6.289	2	0.024
Pair 9 Fe_1- Fe_2	6.803	2	0.021
Pair 10 Mn_1- Mn_2	2.308	2	0.147

Note: 1 stand for True mangrove; 2 stands for associate mangrove.

Pichavaram forest: Soil chemical properties are presented in **Table 3.3.** Soils of the Pichavaram forest are neutral to slightly alkaline (6.9 – 7.6). Electrical conductivity (EC) ranged from 4.47 – 5.0 dS/m. The site was low in available nutrients, especially P. The flow of water causes the leaching of soil nutrients (Gandaseca et al. 2016), and up to 95% of the available P is removed in a short time making P the most thoroughly leached element (Oelkers and Jones 2008). A variety of biotic and abiotic factors *viz.,* inundation, soil type, soil microbes, plant species, litter production, and decomposition control the availability of nutrients in the mangrove ecosystem (Reef et al. 2010). The Pichavaram mangrove ecosystem consists of small Islands that experience micro and diurnal tides (Selvam et al. 2003). The frequency and period of tidal inundation are determined by topographic factors such as elevation, which subsequently affects the salinity and soil nutrient availability resulting in complex patterns of nutrient demand and supply (Reef et

al. 2010). Furthermore, increased soil salinity decreases the availability of major nutrients such as N, P, and K due to their precipitation and variation in nutrient metabolism (Evelin et al. 2011). AM fungal structures may bind or eliminate NaCl, thereby conferring salt tolerance to the plants (Kaldorf et al. 1999).

Table 3.3: Chemical properties of Pichavaram mangrove soils.

Soil Parameters	PE	PRF	KRF
pH	7.6 ± 0.84	7.0 ± 0.78	6.9 ± 0.77
EC (dS/m)	4.47 ± 0.56	4.76 ± 0.60	5.0 ± 0.63
N (g/kg)	0.035 ± 0.005	0.037 ± 0.006	0.032 ± 0.005
P (g/kg)	0.019 ± 0.004	0.019 ± 0.005	0.021 ± 0.007
K (g/kg)	0.095 ± 0.02	0.147 ± 0.04	0.138 ± 0.03
Fe (ppm)	15.63 ± 1.74	15.52 ± 1.72	14.55 ± 1.62
Mn (ppm)	7.94 ± 0.10	7.31 ± 0.91	6.12 ± 0.76
Zn (ppm)	0.85 ± 0.12	0.96 ± 0.14	0.48 ± 0.07
Cu (ppm)	1.77 ± 0.30	1.97 ± 0.33	2.63 ± 0.43

Note: PE= Pichavaram extension; PRF= Pichavaram Reserve Forest; KRF= Killai Reserve Forest.

3.3.2: AM fungal colonization, spore density, and species diversity

Chorao Island: AM fungal colonization was observed in the roots of all the mangrove plant species examined (**Plate 3.8, 3.9**). Roots of different plant species exhibited arbuscular and/or vesicular colonization. Maximum root colonization was recorded in *Thespesia populnea* (97.5%), an associate mangrove species, while the least root colonization was recorded in *Avicennia marina* (20%). Hyphal colonization (aseptate hyphae or mycelia are formed by AM fungi and can be differentiated from endophytic hyphae, which are septate) was dominant in *T. populnea*. During the study, vesicles were recorded in all the plant species analyzed, whereas arbuscules were rarely encountered in true mangroves **(Table 3.4).**

In this study, associate mangrove plants exhibited higher AM colonization than true mangroves. (Wang et al. 2014), reported similar observations in semi-mangrove communities in China. Gupta et al. (2002) reported the absence of AM colonization in three associate mangrove species *viz., A. ilicifolius, A. aureum,* and *D. heterophylla* while considerably high colonization in these plant species were recorded in the present study. Earlier studies have demonstrated that the intensity of colonization is higher in drier areas (Wang et al. 2010a). Therefore, in our study, increased colonization rates in associate mangrove plants could be due to their distribution in the landward areas of mangrove habitat.

Roots of associate mangrove plant species had a high percentage of TC, HC, and VC and recorded arbuscules in more plant species compared to true mangroves. Hence, their scantiness in true mangroves could be due to their sensitivity towards inundated and saline environments (Wang et al. 2010b). Zhao (1999) suggested that factors such as host species, phenology, mycorrhizal dependency, dormancy, and changes in soil conditions contribute to the variation in AM fungal colonization and spore density. However, *Derris* species showed the least AM species richness, as observed in an earlier study by D'Souza and Rodrigues (2013a). Sridhar et al. (2011) however, recorded high species richness in the same plant species and suggested that AM species richness is dependent on the host plant. Also, incompatibility between AM fungal species and the host plant (host preference), and environmental conditions (environmental preference) may result in lesser species richness (He et al. 2002; Jansa et al. 2002; Trejo et al. 2013).

Table 3.4: Percent root colonization in true and associate mangrove species of Chorao Island.

Plant species	Family	TC (%)	HC (%)	AC (%)	VC (%)
True mangroves					
Aegiceras corniculatum (L.) Blanco	Myrsinaceae	70.28 ± 9.23^{bc}	68.09 ± 6.40^{c}	nd	61.21 ± 6.80^{bc}
Avicennia officinalis L.	Acanthaceae	41.00 ± 1.00^{efg}	34.77 ± 0.35^{fg}	nd	23.15 ± 1.39^{ef}
Avicennia marina (Forssk.) Vierh.	Acanthaceae	20.00 ± 2.89^{h}	13.25 ± 4.42^{j}	nd	6.08 ± 1.01^{h}
Bruguiera cylindrica (L.) Blume	Rhizophoraceae	30.99 ± 7.47^{fgh}	21.59 ± 4.31^{i}	nd	11.45 ± 2.29^{gh}
Ceriops tagal (Perr.) C.B. Rob.	Rhizophoraceae	58.20 ± 7.66^{cde}	57.12 ± 5.25^{d}	35.42 ± 5.06^{b}	51.66 ± 6.31^{d}
Excoecaria agallocha L.	Euphorbiaceae	74.00 ± 1.00^{bc}	59.16 ± 7.95^{d}	41.9 ± 4.66^{b}	47.50 ± 8.00^{d}
Kandelia candel (L.) Druce	Rhizophoraceae	35.16 ± 7.43^{fgh}	29.84 ± 3.73^{gh}	nd	22.40 ± 5.6^{ef}
Rhizophora apiculata Blume	Rhizophoraceae	49.08 ± 0.92^{defg}	33.85 ± 4.23^{fg}	nd	9.23 ± 1.84^{gh}
Rhizophora mucronata Lam.	Rhizophoraceae	42.93 ± 11.09^{efg}	37.46 ± 5.35^{ef}	nd	12.27 ± 1.75^{gh}
Sonneratia alba Sm.	Lythraceae	31.04 ± 7.77^{gh}	24.08 ± 3.01^{hi}	nd	16.36 ± 2.73^{fg}
Sonneratia caseolaris (L.) Engl.	Lythraceae	50.34 ± 6.42^{def}	43.57 ± 3.00^{e}	nd	26.57 ± 1.50^{e}
Mangrove associates					
Acanthus ilicifolius L.	Acanthaceae	69.21 ± 0.79^{bc}	63.81 ± 3.80^{cd}	38.69 ± 4.30^{b}	54.76 ± 7.48^{cd}
Acrostichum aureum L.	Pteridaceae	44.34 ± 5.66^{efg}	40.13 ± 5.73^{ef}	18.65 ± 3.10^{c}	29.54 ± 3.28^{e}
Clerodendrum inerme (L.) Gaertn.	Lamiaceae	75.00 ± 10.41^{bc}	69.03 ± 7.67^{bc}	36.92 ± 4.62^{b}	64.36 ± 7.15^{b}
Derris heterophylla (Willd.) K. Heyne	Fabaceae	85.00 ± 4.08^{ab}	75.38 ± 0.50^{b}	nd	48.82 ± 5.05^{d}
Pongamia pinnata (L.) Pierre	Leguminosae	64.10 ± 5.90^{cd}	63.93 ± 7.10^{cd}	nd	48.91 ± 5.43^{d}
Thespesia populnea (L.) Sol. ex Corrêa	Malvaceae	97.50 ± 2.04^{a}	89.42 ± 6.84^{a}	58.78 ± 6.53^{a}	78.59 ± 4.99^{a}

Note: All values are mean of three readings; ± = Standard error; Values in the same column not sharing the same letters are significantly different ($P \leq 0.05$); nd = not detected; TC, HC, AC, VC = Root length containing total colonization, hyphae, arbuscules, and vesicles respectively.

The maximum spore density was recorded in *Ceriops tagal* (138 spores/100g of soil) and the minimum in *Acrostichum aureum* (20 spores/100g of soil). *Kandelia candel* recorded the highest AM fungal species richness with 13 spore morphotypes. A total of 31 AM fungal species belonging to 9 genera were recorded. *Acaulospora* (13 spp.) was the dominant genus followed by *Glomus* (6), *Gigaspora* (4), *Rhizophagus,* and *Funneliformis* (2 spp. each), *Claroideoglomus, Sclerocystis, Entrophospora,* and *Scutellospora* (1 sp. each) with the species number given in parenthesis. *Acaulospora dilatata* was the dominant species found occurring in 13 plant species **(Table 3.5).** The study revealed the dominance of *Acaulospora dilatata*. Similar observations have been recorded earlier by D'Souza and Rodrigues (2013a), who reported the dominance of genus *Acaulospora* in acidic mangrove soils of Goa. Giovannetti et al. (2010) observed that genus *Acaulospora* is predominant in low pH soils (< 6.0). It has been reported that species of *Glomus* are found commonly in neutral to slightly alkaline soils (Kumar and Ghose, 2008), while species of *Gigaspora* and *Scutellospora* are dominant in sandy soils (Lee and Koske 1994).

The low AM fungal spore density was recorded in rhizosphere soils of both true and associate mangroves. This conforms with an earlier study by Kumar and Ghose (2008). Salinity and tidal currents may be responsible for low spore density in the mangrove environment (Wang et al. 2014). Balachandran and Mishra (2012) however, reported high spore density and root colonization in heavy metal polluted mangroves sites.

The isolation of 31 AM species from 17 mangrove species indicated that the site is diverse in AM species (**Plate 3.10 to 3.14**). High environmental heterogeneity in mangrove habitats may assist in higher AM diversity (Fabian et al. 2018). Flooding has been identified as the cause of heterogeneity and dynamics of these ecosystems (Simões et al. 2013) that might affect the distribution of AM fungi.

Table 3.5: Spore density (SD) and diversity of AM fungi at Chorao Island.

Plant species	*Spore density	AM fungal species
True mangroves		
Aegiceras corniculatum	105 ± 2.50[b]	AcDi, AcLa, AcRe, AcMy, GiGi
Avicennia officinalis	34 ± 9.61[efghi]	AcGi, AcDel, ClEt, FuGe, FuMo, RhIn
Avicennia marina	39 ± 3.00[efgh]	AcDi, FuGe, RhFa, RhIn
Bruguiera cylindrica	30 ± 7.51[fghi]	AcDi, AcUn, AcLa, FuGe, RhFa, RhFn, ScRu
Ceriops tagal	138 ± 10.84[a]	AcDi, AcFo, AcMe, AcUn, FuGe, FuMo, GlFl, RhFa, RhIn, ScRu
Excoecaria agallocha	57 ± 10.90[c]	AcDi, AcSc, AcUn, AcBi, FuGe, FuMo, GiAl, RhFa, RhIn
Kandelia candel	24 ± 3.38[hi]	AcDi, AcSc, AcUn, AcDe1, AcDe2, FuGe, FuMo, GlMa, GlFl, ScSi, RhFa, *Scutellospora* sp. (unidentified), *Entrophosphora* sp. (unidentified).
Rhizophora apiculata	36 ± 11.24[efgh]	AcDi, AcSc, AcNi, FuGe, GiAl, RhFa, RhIn
Rhizophora mucronata	96 ± 10.14[b]	FuGe, GlRa, RhFa, RhIn, ScRu
Sonneratia alba	58 ± 9.5[defg]	AcDi, AcSc, AcUn, FuGe, GlTo, RhFa
Sonneratia caseolaris	94 ± 10.00[de]	AcDi, AcSc, AcRe, FuGe, RhFa, *Scutellospora* sp. (unidentified).
Mangrove associates		
Acanthus ilicifolius	26 ± 3.46[ghi]	AcDi, AcFo, AcSc, AcLa, AcNi, FuGe, GlMa, RhFa, RhIn, ScRu, *Entrophospora* sp. (unidentified).
Acrostichum aureum	20.00 ± 8.00[i]	AcDi, AcSc, AcBi, AcDe2, AcRe, FuGe, GlMu,
Clerodendrum inerme	129 ± 5.51[a]	AcDi, AcFo, AcLa, GlMa, RhFa
Derris heterophylla	59.50 ± 2.50[cd]	AcUn, AcNi, RhFa
Pongamia pinnata	37.50 ± 7.50[efgh]	AcFo, AcSc, AcUn, AcSp, GiMa, GiDe
Thespesia populnea	47.67 ± 1.67[def]	AcDi, AcDe2, AcRe, AcSp, FuGe, GlMu, RhFa

Note: * Spores/100g of soil. All values are mean of three readings; ± = Standard error; Values in the same column not sharing the same letters are significantly different ($P \leq 0.05$). **AM species:** AcDi = *Acaulospora dilatata,* AcFo = *A. foveata,* AcMe = *A. mellea,* AcSc = *A. scrobiculata,* AcUn = *A. undulata,*AcDe1 = *A. denticulata,* AcLa = *A. laevis,* AcBi = *A. bireticulata,* AcDe2 = *A. delicata,* AcNi = *A. nicolsonii,* AcRe = *A. rehmii,* AcMy = *A. myriocarpa,* AcSp = *A. spinosa,* ClEt = *Claroideoglomus etunicatum,* FuGe = *Funneliformis geosporum,* FuMo = *F. mosseae,* GlMa = *Glomus macrocarpum,* GlTo = *G. tortuosum,* GlFl = *G. flavisporum,* GlMu = *G. multicaule,* GlRa = *G. radiatum,* GiMa = *Gigaspora margarita,* GiGi = *Gi. gigantea,* GiDe = *Gi. decipiens,* GiAl = *Gi. albida,* RhFa = *Rhizophagus fasciculatus,* RhIn = *R. intraradices,* ScRu = *Sclerocystis rubiformis,* ScSi = *S. sinuosa*

Pichavaram mangrove forest: AM fungal colonization was observed in roots of all the mangrove plant species sampled. Maximum root colonization was recorded in *Salicornia brachiata* (93.54%) at KRF and least in *Avicennia marina* (22.08%) at PRF.

Maximum spore density was recorded in *Ceriops decandra* (270/100 g of soil) at PE and minimum in *Rhizophora mucronata* (8 spores/100 g of soil) at PRF **(Table 3.6).** Parameters such as inoculum density, root structure, genetic compatibility between host and AM fungi, edaphic factors, and soil microbes affect AM colonization rates and spore density (Zangaro et al. 2013; Sivakumar 2013). Likewise, the AM fungal community structure is affected by the tide level (Wang et al. 2011). This could be one of the reasons for maximum and minimum spore density in *C. decandra* and *R. mucronata,* respectively. Since the inundation level of the former plant is lower than the latter (Batool et al. 2014) at the study site. Inhibition of AM colonization seldom take place due to increased P level occurs in the wetland ecosystem (Kelly et al. 2004). However, higher rates of AM colonization in the present study could be attributed to low levels of P at the site. The study revealed variation in root colonization and spore density. According to Hildebrandt et al. (2001), the intensity of AM colonization is not the same during the plant life cycle, and hyphae bundled with spore strings could be patchily distributed in the soil, which may lead to more spore counts in a single soil sample.

Since all the mangrove plants examined in the present study from Pichavaram were mycorrhizal, this study contradicts the study of Mohankumar and Mahadevan (1986) who reported no AM association in Pichavaram mangroves, while Lingan et al. (1999) reported AM colonization in eight mangrove plants from the same site. It is interesting to observe that halophytes belonging to the family Chenopodiaceae are non- or poorly- mycorrhizal (Juniper and Abott 1993; Aliasgharzadeh et al. 2001; Wilde et al. 2009). However, the present study reveals clear evidence that species of Chenopodiaceae (now Amaranthaceae) family *viz., Salicornia, Anthrocnemum,* and *Suaeda* showed a high degree of AM colonization among all the other mangrove plants which are in accordance with Hildebrandt et al. (2001) and Yinan et al. (2017).

Table 3.6: Percent root colonization (RC), spore density (SD) in Pichavaram mangroves.

Plant species		PE		PRF		KRF	
		Percent Colonization	*Spore density	Percent Colonization	*Spore density	Percent Colonization	*Spore density
True mangroves							
Aegiceras corniculatum (L.) Blanco	Myrsinaceae	65.9 ± 0.8[abc]	79.0 ± 2.5[cd]	nd	nd	nd	nd
Avicennia marina (Forssk.) Vierh.	Acanthaceae	27.6 ± 1.0[f]	89.0 ± 7.0[c]	22.1 ± 5.4[f]	64.0 ± 4.5[e]	40.2 ± 4.2[c]	87.0 ± 7.5[c]
Avicennia officinalis L.	Acanthaceae	76.5 ± 1.5[a]	92.0 ± 4.5[c]	51.0 ± 1.0[cd]	124.0 ± 9.0[a]	nd	nd
Brugueira cylindrica (L.) Blume	Rhizophoraceae	54.4 ± 0.58[bcde]	30.0 ± 8.0[f]	63.8 ± 8.8[bc]	105.0 ± 1.0[bc]	nd	nd
Ceriops decandra (Griff.) W.Theob.	Rhizophoraceae	72.5 ± 7.5[ab]	270.0 ± 1.0[a]	27.5 ± 2.5[f]	90.0 ± 3.5[cd]	nd	nd
Excoecaria agallocha L.	Euphorbiaceae	77.0 ± 13.0[a]	60.0 ± 2.0[e]	22.7 ± 0.8[f]	79.0 ± 5.0[de]	90.0 ± 3.3[a]	96.0 ± 5.0[c]
Lumnitzera racemosa Willd.	Combretaceae	70.2 ±11.9[ab]	142.0 ± 8.0[b]	65.0 ± 3.0[b]	114.0 ± 1.5[ab]	nd	nd
Rhizophora apiculata Blume	Rhizophoraceae	37.5 ± 2.5[ef]	17.0 ± 0.5[f]	45.0 ± 5.0[de]	104.0 ± 4.5[bc]	nd	nd
Rhizophora mucronata Lam.	Rhizophoraceae	nd	nd	32.7 ± 0.7[ef]	8.0 ± 1.0[f]	nd	nd
Associate mangroves& salt marshes							
Arthrocnemum indicum (Willd.) Moq.	Amaranthaceae	nd	nd	nd	nd	65.4 ± 3.9[b]	38.0 ± 5.5[e]
Clerodendrum inerme (L.) Gaertn.	Lamiaceae	nd	nd	nd	nd	88.5 ± 3.9[a]	127.0 ±6.0[b]
Salicornia brachiata Miq.	Amaranthaceae	nd	nd	nd	nd	93.5 ± 0.2[a]	30.0 ± 3.5[e]
Ipomoea pes-caprae (L.) R. Br.	Convolvulaceae	nd	nd	nd	nd	55.0 ± 7.5[b]	102.0 ± 6.5[c]
Calamus sp.	Aracaceae	nd	nd	88.5 ± 3.9[a]	71.0 ± 11.5[e]	nd	nd
Salvadora persica L.	Salvadoraceae	42.3 ± 3.9[def]	77.0 ± 6.5[cd]	nd	nd	nd	nd
Sesuvium portulacastrum (L.) L.	Aizoaceae	50.0 ± 3.9[cde]	22.0 ± 0.5[f]	nd	nd	nd	nd
Suaeda monoica Forssk. ex J.F.Gmel.	Amaranthaceae	nd	nd	nd	nd	88.5 ± 3.9[a]	67.0 ± 5.5[d]
Suaeda maritima (L.) Dumort.	Amaranthaceae	58.0 ± 3.5[bcd]	71.0 ± 3.5[de]	nd	nd	89.0 ± 2.7[a]	161.0 ±8.0[a]

Note: * indicates spores/100g of soil; Data are means of three replicates; ± standard error; Values in each column followed by different letters are significantly different at $p < 0.05$; nd= not detected.

A total of 21 AM fungal species belonging to seven genera were recovered. Of these, 8 species belonged to the genus *Acaulospora,* one to *Entrophospora,* two to *Funneliformis,* three to *Gigaspora,* two to *Glomus,* three to *Rhizophagus,* and two to *Sclerocystis*. *Rhizophagus fasciculatus* was found to be dominant at PE and PRF, whereas *Funneliformis geosporum* was dominant at KRF **(Table 3.7).**

Species belonging to Acaulosporaceae and Glomeraceae are capable of adapting to extreme environmental conditions and a wide range of soil pH resulting in their predominance in varied habitats (Öpik et al. 2013). Moreover, these species form different types of propagules (hyphae, vesicles, and spores) to establish root colonization, while Gigasporaceae species are propagated by spores (Hart and Reader 2002).

Table 3.7: Diversity of AM fungal species in mangroves of Pichavaram forest.

Species	PE	PRF	KRF
True mangroves			
Aegiceras corniculatum	AcPo, AcUn, FuGe, RhFa, RhIr	nd	nd
Avicennia marina	RhFa, RhIr	*Entrophospora* sp., RhFa	AcUn, FuGe, RhFa
Avicennia officinalis	AcDi, AcUn, *Entrophospora* sp., RhFa, RhIr	AcDi, AcNi, *Entrophospora* sp., R RhFa, RhIr	nd
Brugueira cylindrica	AcDi, *Entrophospora* sp., FuGe, RhFa, RhIr	AcDi, AcNi, *Entrophospora* sp., GlMi, RhFa, RhIr	nd
Ceriops decandra	*Entrophospora* sp., FuGe, RhFa, RhIr	RhFa, RhIr	nd
Excoecaria agallocha	*Entrophospora* sp., GiAl, RhFa, RhIr	AcFo, AcUn, *Entrophospora* sp., FuGe, RhFa	AcDi, AcUn, FuGe, GiAl
Lumnitzera racemosa	*Entrophospora* sp., RhFa	AcDi, *Entrophospora* sp., FuGe, RhFa, RhIr	nd
Rhizophora apiculata	AcDi, AcRe, AcSc, AcUn, *Entrophospora* sp., FuGe, RhFa	*Entrophospora* sp RhFa, RhIr	nd
Rhizophora mucronata	nd	*Entrophospora* sp., RhIr	nd

Mangrove associates and salt marshes			
Arthrocnemum indicum	nd	nd	AcUn, FuGe, GiAl, RhFa
Clerodendrum inerme	nd	nd	AcDi, AcUn, FuGe, GiDe, GiMa, RhIn, ScPa, ScRu
Salicornia brachiata	nd	nd	AcDi, FuGe, GlAg
Ipomoea pes-caprae	nd	nd	AcDi, AcUn, FuGe
Calamus sp.	nd	AcDi, AcUn, FuGe	nd
Salvadora persica	AcUn, FuGe	nd	nd
Sesuvium portulacastrum	*Entrophospora* sp., FuGe, RhFa	nd	nd
Suaeda monoica	nd	nd	AcUn, FuGe, ScRu
Suaeda maritima	AcPo, AcSp, *Entrophospora* sp., FuGe, RhFa	nd	AcDi, AcUn, FuGe, FuMo, RhFa

Note: nd= not detected; AM species: AcDi = *Acaulospora dilatata,* AcFo = *A. 46enticu,* AcNi = *A. nicolsonii,* AcPo = *A. polonica,* AcRe = *A. rehmii,* AcSc = *A. scrobiculata,* AcSp = *A. spinosa,* AcUn = *A. undulata, Entrophospora* = Unidentified, FuGe = *Funneliformis geosporum,* FuMo = *F. mosseae,* GiAl = *Gigaspora albida,* GiDe = *G. decipiens,* GiMa = *G. margarita,* GlAg = *Glomus aggregatum,* GlMi = *G. microcarpum,* RhFa = *Rhizophagus fasciculatus,* RhIn = *R. intraradices,* RhIr = *R. irregulare,* ScPa = *Sclerocystis pachycaulis,* ScRu = *S. rubiformis.*

3.3.3: AM fungal distribution and diversity indices

Chorao Island: Shannon-Wiener diversity (*H'*) and Simpson`s dominance index (*D*) was highest in *K. candel* and lowest in *D. heterophylla* (**Fig. 3.4**). Species evenness was highest in *A. marina* and was least in *C. tagal* and *E. agallocha.* Maximum species richness was recorded in *K. candel* (**Fig. 3.5).**

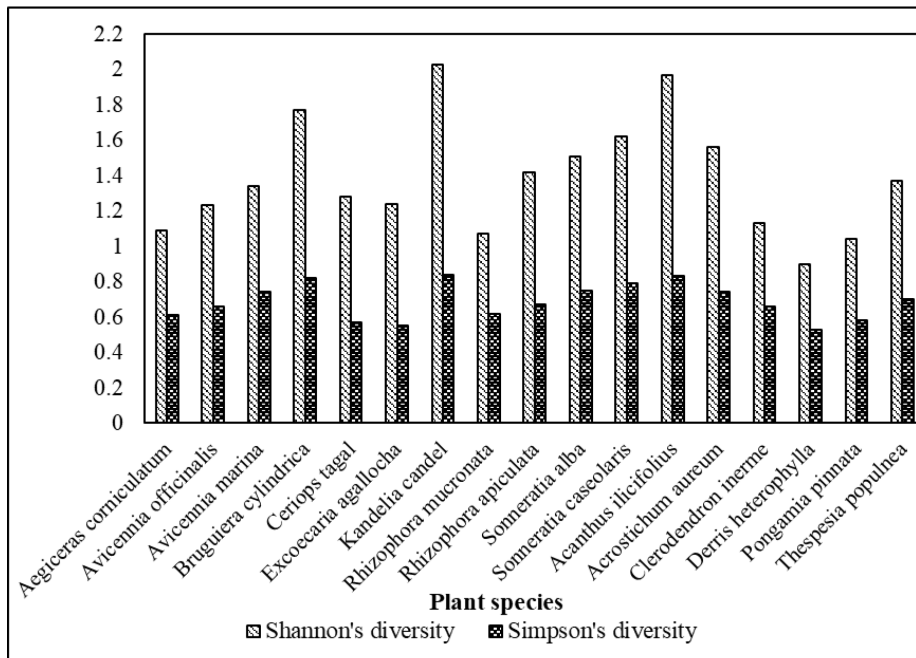


Fig. 3.4: Shannon and Simpson`s diversity indices of AM fungi at Chorao Island.

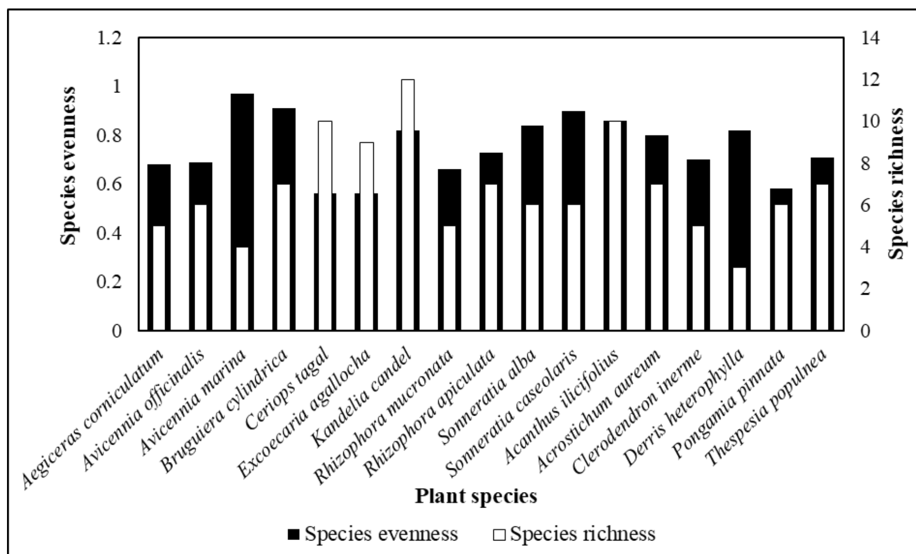


Fig. 3.5: Species evenness and species richness of AM fungi at Chorao Island.

Acaulospora dilatata recorded the highest relative abundance (RA) while the least was recorded for *C. etunicatum, G. tortuosum, G. radiatum, Gi. margarita* and *S. sinuosa.* Maximum isolation frequency (IF) was observed for *A. dilatata* and minimum for *A.*

mellea, A. myriocarpa, C. etunicatum, G. tortuosum, G. radiatum, Gi. margarita, Gi. gigantea, Gi. decipiens and *S. sinuosa* **(Table 3.8)**.

The genus *Gigaspora* recorded low relative abundance compared to other AM genera. Species belonging to Gigasporaceae predominates in sandy soils, especially dunes (Day et al. 1987; Lee and Koske 1994). The soil at Chorao Island is less sandy, which could have resulted in a lower abundance of the species of genus *Gigaspora.*

Table 3.8: Relative abundance (RA) and isolation frequency (IF) of AM fungal species at Chorao Island.

AM species	RA (%)	IF (%)
Acaulospora dilatata Morton	30.69	82.35
Acaulospora bireticulata Trappe & Janos	4.66	23.53
Acaulospora mellea Spain & Schenck	0.10	5.88
Acaulospora scrobiculata Trappe	4.28	47.06
Acaulospora undulata Sieverd.	4.56	41.18
Acaulospora denticulata Sieverd. & Toro	0.24	11.76
Acaulospora laevis Gerd. & Trappe	2.24	23.53
Acaulospora bireticulata Rothwell & Trappe	0.28	11.76
Acaulospora delicata Walker, Pfeiff. &Bloss	1.00	17.65
Acaulospora nicolsonii Walker, Reed & Sanders	3.94	17.65
Acaulospora rehmii Sieverd. & Toro	1.35	23.53
Acaulospora myriocarpa Spain, Sieverd. & Schenck	5.87	5.88
Acaulospora spinosa Walker & Trappe	0.07	11.76
Claroideoglomus etunicatum (Becker & Gerd.) Walker & Schüßler	0.03	5.88
Entrophospora sp. (unidentified)	0.41	11.76
Funneliformis geosporum (Nicolson & Gerd.) Walker & Schüßler	12.88	76.47
Funneliformis mosseae (Nicolson & Gerd.) Walker & Schüßler	1.42	23.53
Glomus macrocarpum Tul. & Tul.	0.72	17.65
Glomus tortuosum Schenck & Sm.	0.03	5.88
Glomus flavisporum (Lange & Lund) Trappe & Gerd.	0.07	11.76

Glomus multicaule Gerd. & Bakshi	0.03	11.76
Glomus radiatum (Thaxt.) Trappe & Gerd.	0.10	5.88
Gigaspora margarita Becker & Hall	0.03	5.88
Gigaspora gigantea (Nicolson & Gerd.) Gerd. & Trappe	0.03	5.88
Gigaspora decipiens Hall & Abbott	0.21	5.88
Gigaspora albida Schenck & Sm.	0.07	11.76
Rhizophagus fasciculatus (Thaxt.) Gerd. & Trappe	0.17	76.47
Rhizophagus intraradices (Schenck & Sm.) Walker & Schüßler	19.92	47.06
Sclerocystis rubiformis Gerd. & Trappe	4.14	23.53
Sclerocystis sinuosa Gerd. &Bakshi	0.31	5.88
Scutellospora sp. (unidentified)	0.14	11.76

Species richness showed non-significant (P>0.05) correlation with Simpson's dominance index (r=0.376) and with species evenness (r= 0.061) and a significant correlation with Shannon's diversity index (r=0.744, P<0.001). However, species evenness showed a significant correlation with Shannon diversity (r=0.683, P<0.01) and Simpson's dominance index (r=0.747, P<0.01). A significant correlation was observed between Shannon and Simpson's diversity indices (r=0.816, P<0.01).

A significant correlation existed between RA and IF (r= 0.899, P<0.001). In contrast, no correlation (P>0.05) was observed between spore density and root colonization (r=0.277). Similarly, spore density and species richness had a negative correlation (r=-0.193) that was not significant (P>0.05).

The AM fungal species similarity index was highest for *A. officinalis* and *A. marina* (66.67%), and *Aegiceras corniculatum* showed less similarity with most of the plant species (**Fig 3.6**). Moreover, in the present study, a high AM species similarity (up to 66.67%) indicated broad dispersal of AM species. Similar observations were recorded in an earlier study in Nethravathi mangroves where the AM species similarity ranged from 12.1% to 55% (Sridhar et al. 2011). Movement of AM fungal species from adjoining terrestrial habitats to intertidal zones of mangrove ecosystem (Wang et al. 2014) could be the cause of similarity in genus and species composition in true mangroves that inhabit intertidal zone areas and associate mangrove plants from the landward periphery of mangrove habitat.

	AO	AM	BC	CT	EA	KC	RA	RM	SA	SC	AI	AA	CI	DH	PP	TP
AC	10.00	12.50	20.00	7.14	7.69	5.88	9.09	0.00	10.00	22.22	14.29	20.00	25.00	0.00	0.00	20.00
	AO	66.67	44.44	33.33	36.36	26.67	30.00	22.22	20.00	20.00	21.43	18.18	10.00	0.00	0.00	18.18
		AM	57.14	40.00	44.44	21.43	57.14	50.00	42.86	42.86	36.36	22.22	28.57	16.67	0.00	37.50
			BC	54.55	45.45	25.00	40.00	50.00	44.44	30.00	50.00	16.67	33.33	25.00	8.33	27.27
				CT	46.15	35.29	30.77	36.36	33.33	23.08	40.00	13.33	25.00	18.18	14.29	21.43
					EA	37.50	60.00	27.27	50.00	36.36	33.33	33.33	7.69	20.00	15.38	23.08
						KC	25.00	12.50	35.71	35.71	26.32	25.00	20.00	14.29	11.76	25.00
							RA	33.33	44.44	44.44	50.00	27.27	20.00	25.00	8.33	27.27
								RM	22.22	22.22	33.33	9.09	11.11	14.29	0.00	20.00
									SA	50.00	30.77	30.00	22.22	28.57	20.00	30.00
										SC	30.77	44.44	22.22	12.50	9.09	44.44
											AI	20.00	45.45	16.67	13.33	20.00
												AA	9.09	0.00	8.33	40.00
													CI	14.29	10.00	20.00
														DH	12.50	11.11
															PP	8.33

Fig. 3.6: Jaccard's similarity index (%) of AM fungi among the mangrove plant species at Chorao Island.

Plant species: *Aegiceras corniculatum* (AC), *Avicennia officinalis* (AO), *Avicennia marina* (AM), *Bruguiera cylindrica* (BC), *Ceriops tagal* (CT), *Excoecaria agallocha* (EA), *Kandelia candel* (KC), *Rhizophora mucronata* (RM), *Rhizophora apiculata* (RA), *Sonneratia alba* (SA), *Sonneratia caseolaris* (SC), *Acanthus ilicifolius* (AI), *Acrostichum aureum* (AA), *Clerodendrum inerme* (CI), *Derris heterophylla* (DH), *Pongamia pinnata* (PP) and *Thespesia populnea* (TP).

Pichavaram mangrove forest:

Highest Relative abundance (RA) and isolation frequency (IF) of AM species were recorded in *R. fasciculatus* at PE and PRF and *F. geosporum* at KRF, respectively **(Fig. 3.7, 3.8)**. A significant positive correlation was found between RA and IF at all the three sites (r = 0.939, $p < 0.01$ at PE; r = 0.748, $p < 0.05$ at PRF and r = 0.829, $p < 0.01$ at KRF). The AM species *viz., A. dilatata, A. undulata, Entrophospora* sp., and *R. irregulare* showed low relative abundances but were widely distributed with high isolation frequencies. There was no significant correlation between spore density and root colonization at all three sites. Genera-wise, the highest RA was recorded in *Rhizophagus* at PRF, and the highest IF was recorded in *Acaulospora* and *Funneliformis* (**Fig. 3.9**).

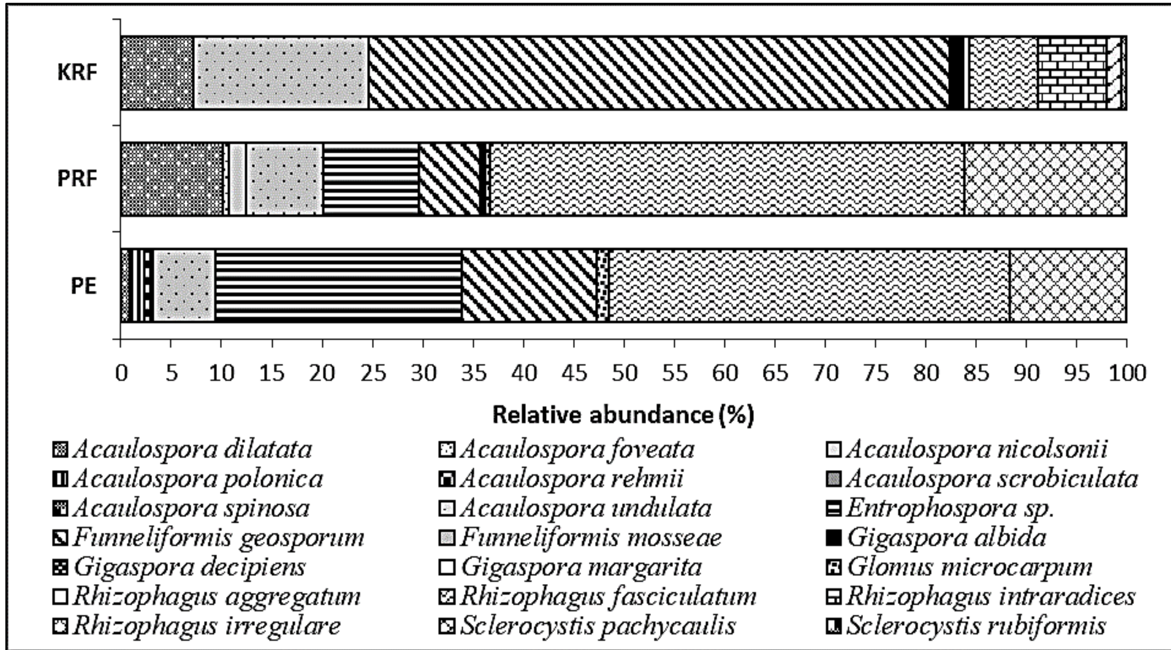


Fig. 3.7: Relative abundance of AM fungal species at Pichavaram Forest. PE= Pichavaram extension; PRF= Pichavaram reserve forest; KRF= Killai reserve forest.

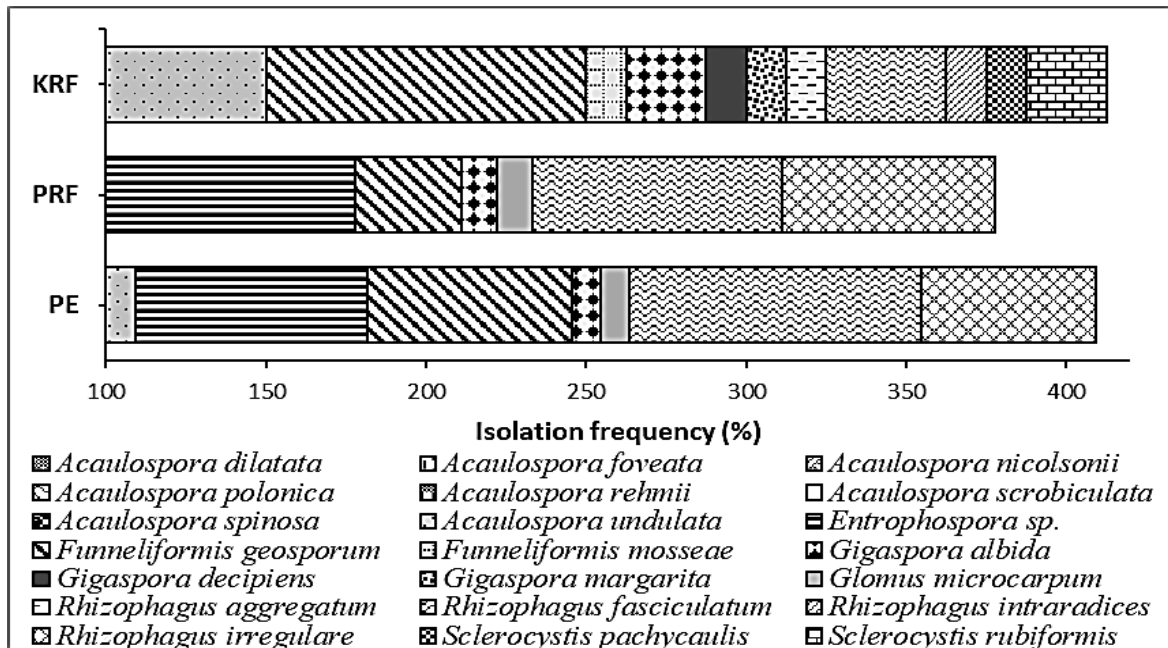


Fig. 3.8: Isolation frequency of AM fungal species at Pichavaram Forest. PE= Pichavaram extension; PRF= Pichavaram reserve forest; KRF= Killai reserve forest.

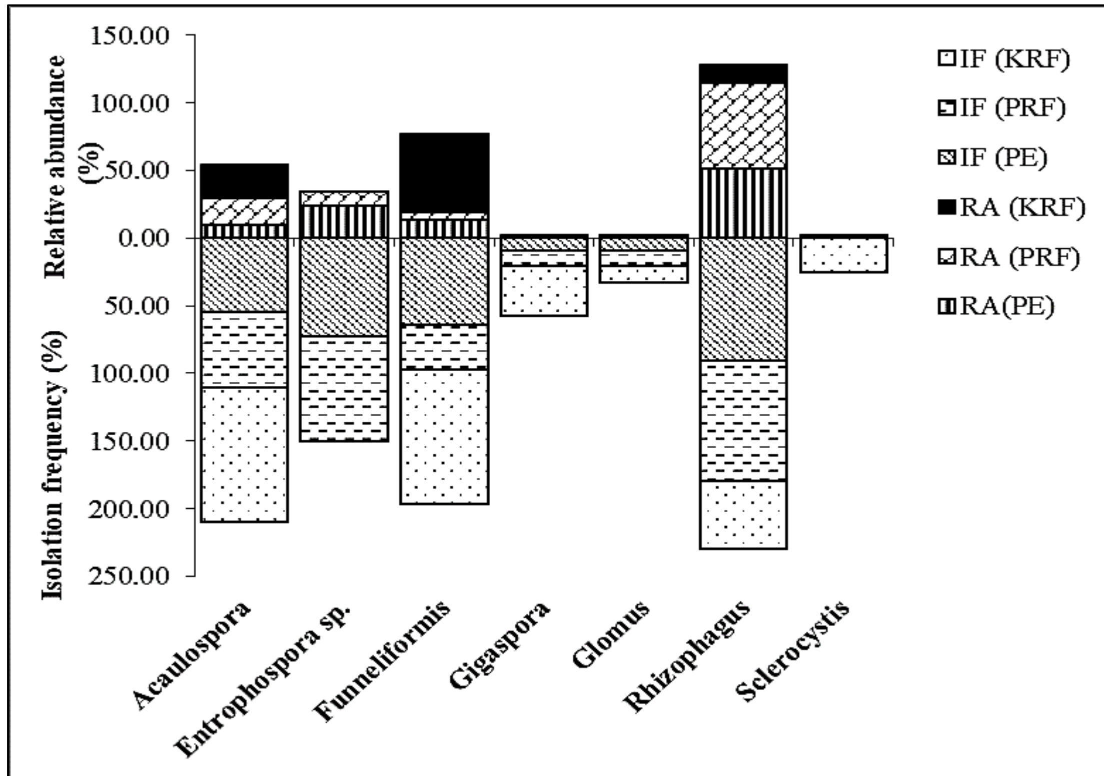


Fig. 3.9: Genera-wise relative abundance and isolation frequency of AM fungi at Pichavaram Forest. PE= Pichavaram extension; PRF= Pichavaram reserve forest; KRF= Killai reserve forest; IF= isolation frequency; RA= relative abundance.

Electrical conductivity was found to be comparatively high at KRF, which could be due to the influx of saltwater from the Bay of Bengal. It can be assumed that the dominance of *F. geosporum* at KRF indicates its tolerance to relatively higher salinity. This is following the study of Wilde et al. (2009).

The AM fungal diversity was high at PE, whereas the distribution was more uniform at PRF (**Fig. 3.10a, b, c).** The variation in the sporulation ability of various AM fungal species results in the unevenness of spore distribution (Bever et al. 1996). When comparing the similarity of AM fungi and plant species investigated between the three sites, it was observed that Sørensen's similarity coefficient of AM fungal community, as well as plant community, was higher between PE and PRF (0.73 and 0.70 respectively) (**Fig. 3.10d**). This indicates that the vegetation influences determining the AM community structure. This could be due to the dependency of AM fungal spore formation, distribution, and development on plant diversity in the natural ecosystem (Zhang et al. 2004).

Moreover, several factors such as climatic factors, spatial and temporal variation, vegetation, nutrient availability, host-preference, and differential sporulation ability of AM species can influence the distribution and community structure of AM fungi (Husband et

al. 2002; Muthukumar and Udaiyan 2002; Renker et al. 2005). The occurrence of recovered AM species from the saline rhizosphere of Pichavaram confirms their tolerance to high salinity levels.

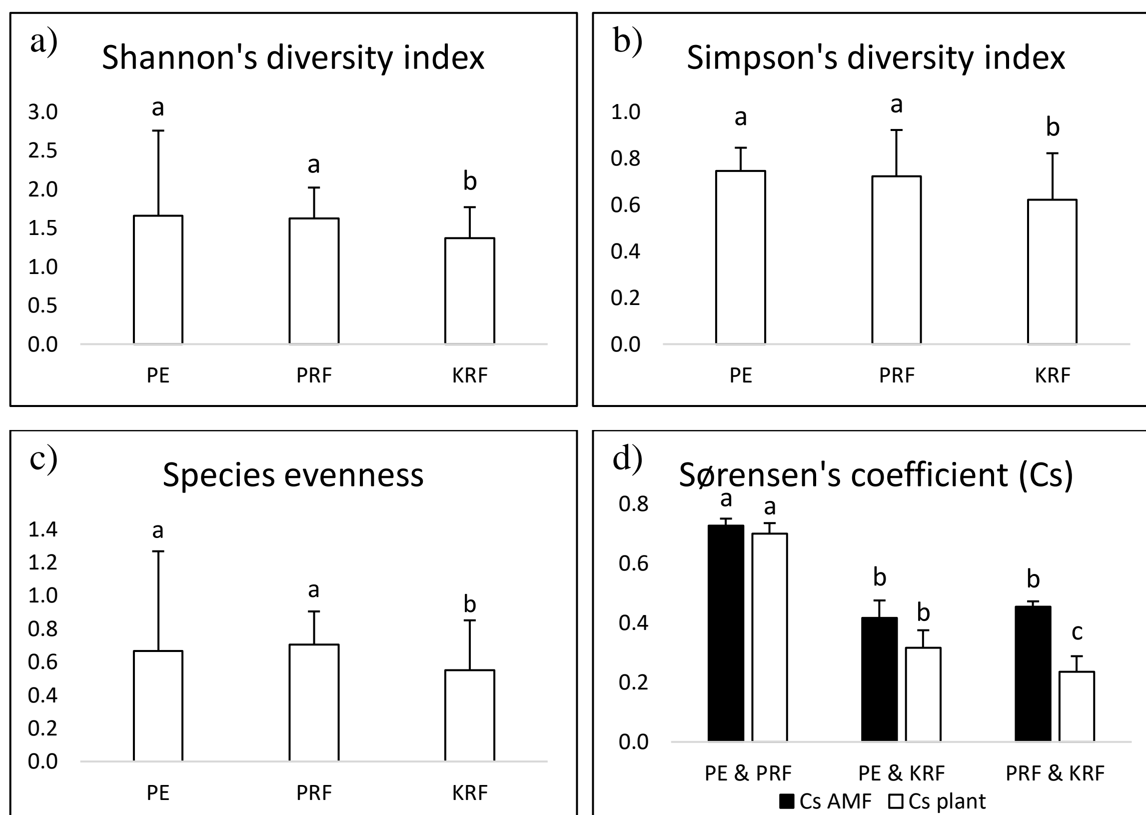


Fig. 3.10: Diversity measurements of AM fungal communities at Pichavaram Forest.

3.3.4: Cluster analysis

Cluster analysis was performed based on the RA of AM fungal species in true- and associate-mangrove plants. All the plants were grouped into four clusters *viz.,* Cluster I, Cluster II, Cluster III, and Cluster IV at a similarity level of 26%. *Aegiceras corniculatum* was not a part of any of the clusters. Cluster III was subdivided into two sub-clusters at 53% similarity. Cluster I showed *A. undulata* as a representative species with a similarity of 28.10%. In Cluster II, IIIb, and IV, *A. dilatata* showed the maximum similarity (20.33%, 21.41%, and 23.26%, respectively). Whereas, *R. fasciculatus* showed the highest similarity level of 32.69% in Cluster IIIa (**Fig. 3.11**).

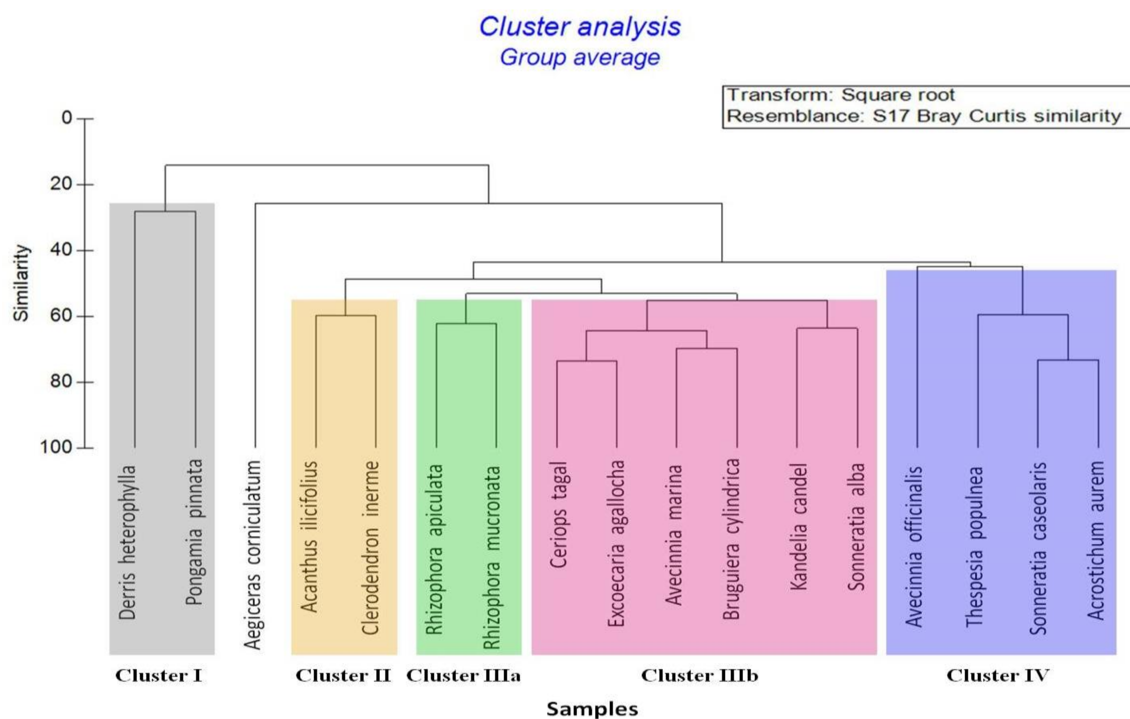


Fig. 3.11: Cluster analysis showing the similarity in the abundance of AM fungal species among true- and associate-mangrove plants at Chorao Island.

2) Seasonal variations in AM fungal communities in three mangrove plant species of East and West coast of India:

3.3.5: Chemical properties of soils

The chemical properties of soils from study sites are depicted in **Table 3.9.** The study revealed that soils of Chorao Island are acidic, whereas Pichavaram soils are almost neutral. Both the sites exhibited high levels of EC during the pre-monsoon season and were least during monsoon. Higher EC values during the pre-monsoon also could be attributed to higher rates of evaporation while during the monsoon season, the rainfall and influx of freshwater from the land are known to causes a decrease in salinity (Prabu et al. 2008). At Pichavaram, freshwater inflow from Vellar and Coleroon rivers reduces salinity in the monsoon (Kathiresan 2000). Phosphorus levels were low at both sites. A concentration of P reduces with increased salinity (Prasad et al. 2006). Iron content in Chorao soils is higher than that in Pichavaram soils. A higher concentration of Fe at Chorao could be due to the incidence of mining activities in the Mandovi basin (Nayak 1998).

Table 3.9: Soil chemical properties at the two sites during different seasons.

Parameters	Pre-monsoon		Monsoon		Post-monsoon	
	Chorao	Pichavaram	Chorao	Pichavaram	Chorao	Pichavaram
pH	5.3 ± 0.53^{bc}	6.8 ± 0.68^{a}	4.9 ± 0.49^{d}	6.9 ± 0.69^{a}	5.1 ± 0.51^{bc}	6.2 ± 0.62^{ab}
EC (mS/cm)	15.6 ± 1.74^{a}	16.0 ± 1.78^{a}	1.9 ± 0.21^{d}	8.7 ± 0.96^{c}	14.1 ± 1.57^{ab}	9.6 ± 1.06^{c}
OC (%)	1.3 ± 0.16^{ab}	0.2 ± 0.03^{c}	2.4 ± 0.30^{a}	1.0 ± 0.13^{ab}	2.0 ± 0.24^{a}	1.0 ± 0.12^{ab}
N (g/kg)	0.1± 0.03^{a}	0.03 ± 0.01^{ab}	0.1 ± 0.04^{a}	0.05 ± 0.02^{ab}	0.1 ± 0.03^{a}	0.07 ± 0.03^{a}
P (g/kg)	0.05 ± 0.02^{a}	0.04 ± 0.01^{a}	0.05 ± 0.01^{a}	0.07 ± 0.03^{a}	0.05 ± 0.00^{a}	0.04 ± 0.02^{a}
K (g/kg)	1.4 ± 0.35^{a}	0.8 ± 0.30^{ab}	1.2 ± 0.37^{a}	0.9 ± 0.20^{ab}	1.5 ± 0.23^{a}	0.7 ± 0.16^{ab}
Fe (ppm)	112.5 ± 4.50^{c}	15.9 ± 8.72^{f}	218.1 ± 9.45^{ab}	63.5 ± 0.63^{d}	236.3 ± 2.54^{a}	41.1 ± 1.64^{de}
Mn (ppm)	43.6 ± 4.36^{a}	19.3 ± 3.96^{d}	39.6 ± 4.20^{ab}	24.5 ± 1.93^{c}	42.0 ± 2.45^{a}	25.3 ± 2.53^{c}
Zn (ppm)	22.1 ± 2.45^{a}	3.4 ± 0.24^{bc}	2.2 ± 0.65^{cd}	3.4 ± 0.38^{bc}	5.9 ± 0.38^{b}	4.5 ± 0.49^{bc}
Cu (ppm)	0.7 ± 0.23^{a}	0.3 ± 0.12^{a}	0.4 ± 0.23^{a}	0.3 ± 0.09^{a}	0.7 ± 0.11^{a}	0.3 ± 0.10^{a}

Note: All values are mean of three readings; ± = Standard error; EC= Electrical conductivity; OC= Organic carbon. Values in the same row not sharing the same letters are significantly different ($P \leq 0.05$).

3.3.6: AM fungal root colonization and spore density

The roots of all the plant species showed AM fungal colonization during all the seasons. This suggests the dependency of the mangrove plant species on AM fungi throughout the year. At Chorao, root colonization rates were generally high during pre- and post-monsoon seasons. Whereas, at Pichavaram, all the plant species studied showed differential patterns of colonization rates in different seasons. The highest percentage of root colonization was recorded in *E. agallocha* at Chorao Island, while the lowest was recorded in *A. marina* at Pichavaram in the post-monsoon season (**Fig. 3.12).**

Spore density during pre-monsoon ranged from 22-83 spores, 53-124 spores in monsoon, and 39-162 spores/100g of soil in the post-monsoon season **(Fig. 3.13)**. The spore population was high during post-monsoon and was least during pre-monsoon season. At Chorao, *Acaulospora* was dominant whereas, at Pichavaram, *Rhizophagus* was the dominant genus.

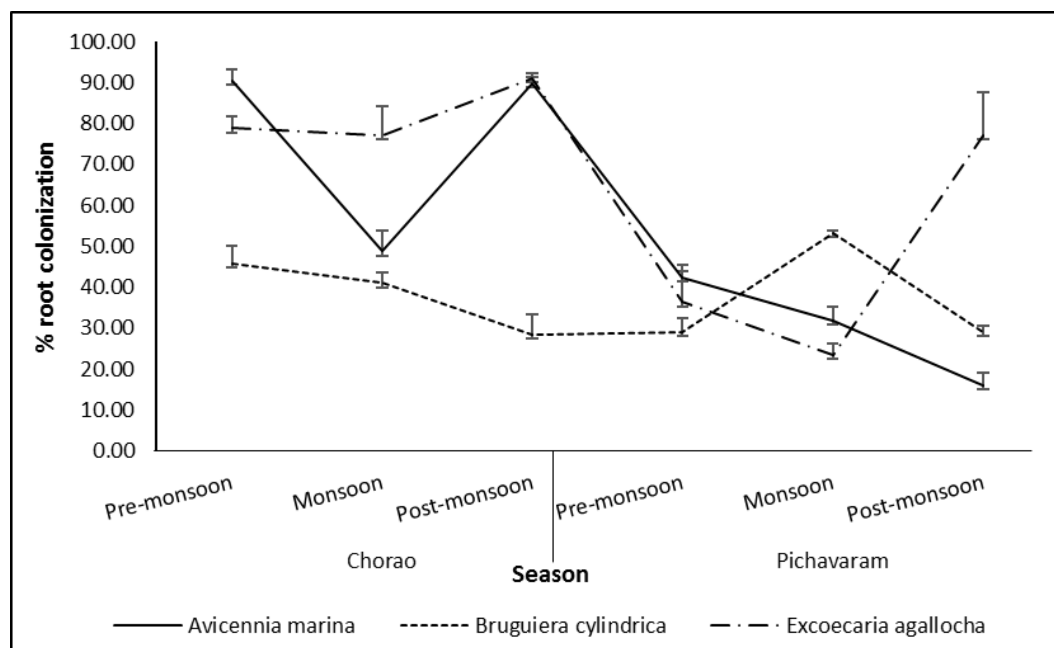


Fig. 3.12: Seasonal variations in AM root colonization.

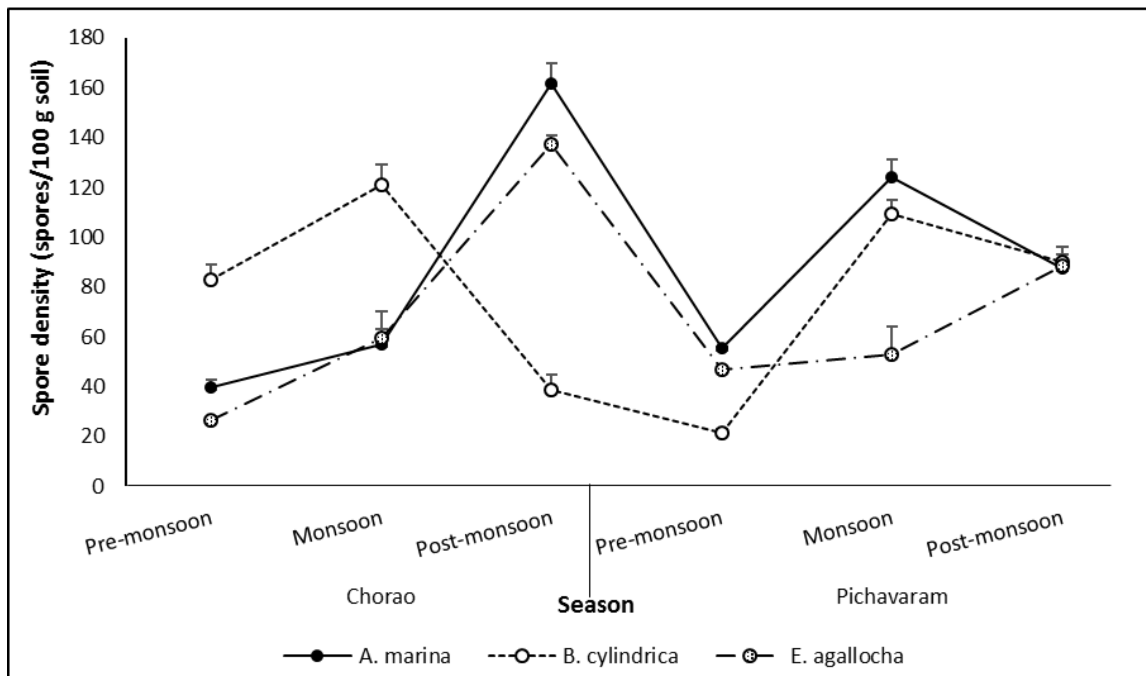


Fig. 3.13: Seasonal variations in AM spore density.

In the present study, variation in root colonization and spore density was observed both in species and seasons. The study also revealed low AM root colonization levels in monsoon, whereas earlier studies have shown humidity favouring AM spore germination, thereby resulting in increased root colonization during the rainy season (Mirdhe and Laxshman 2011; Nandi et al. 2014). However, it has been well demonstrated that seasonality, host plant, as well as soil factors, influence AM colonization and sporulation (D'Souza and Rodrigues 2013; Sigüenza et al. 1996). Moreover, a fungus can colonize at different levels when associated with different plant species (Smith and Read 2008).

Variation in AM colonization at the two sites could be attributed to different phenological patterns of the plant species studied. Enhanced plant growth during vegetative and at the fruiting stage leads to high metabolic activity and, in turn, to greater nutrient demand. The AM structures like hyphae, arbuscules, and vesicles are the storage and nutrient uptake sites (Su et al. 2011), thereby controlling the colonization rates in their host plants during different growing seasons. The other factors, such as soil microbes (Dauber et al. 2008), host species (Klironomos 2003), and host preference (Lugo et al. 2003), also may affect colonization. Similar seasonal patterns in root colonization and spore numbers were observed in an earlier study by Oliveira and Oliveira (2005). They suggested that the water

content affects sporulation in AM fungi. Lower spore density in the present study during pre-monsoon could be due to less water content during this season.

3.3.7: AM species diversity and RA

A total of 19 AM species belonging to four families and seven genera were identified throughout the study in different sites, seasons, and host plants. At both sites, Glomeraceae accounted for the highest number of species, followed by Acaulosporaceae. Only one species belonging to Entrophosporaceae was recovered from both sites. Out of 19 AM species, four species *viz., A. undulata, Entrophospora* sp.*, F. geosporum* and *R. fasciculatus* were recorded in almost all the seasons at both sites. High AM diversity was observed during pre-monsoon at both the sites, which also presented the highest value of evenness at Chorao as well as in Pichavaram. Species of Acaulosporaceae were most abundant at Chorao, whereas at Pichavaram, Glomeraceae presented higher abundance. Season-wise results of RA at the different study sites are represented in **Fig. 3.14.** The soil pH could be the cause for the prevalence of Acaulosporaceae at Chorao and Glomeraceae at Pichavaram, with the soils being acidic and neutral, respectively. Acaulosporaceae species are often abundant in acidic soils, whereas those of Glomeraceae are known to be present in neutral soils (Abbott and Robson 1991).

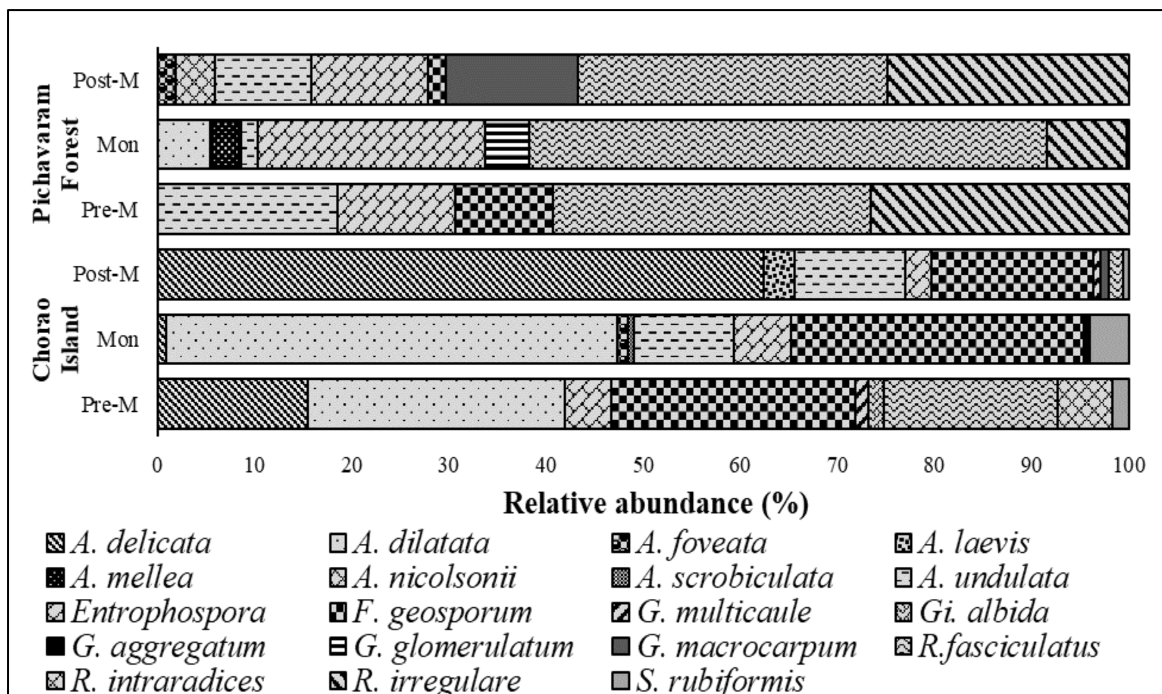


Fig. 3.14: Seasonal variation in relative abundance (%).

To know the probable correlation between AM fungal abundance and soil parameters in different seasons, Canonical correspondence analysis (CCA) was performed. The arrows in the CCA plot indicate relative significance affecting the community, whereas the angle between variables indicates a degree of correlation (**Fig. 3.15**). The CCA variable scores and biplot scores for soil variables are presented in **Table 3.10** and **Table 3.11,** respectively. The Eigenvalues of the first and second axes were 0.496 and 0.054, respectively. The cumulative percentage of the variance of genera was 82.98% and 8.98% on the first and second axes, respectively. The variables of soil parameters such as OC, N, Mn, Zn, Cu, and Fe had a significant impact on the abundance of *Acaulospora, Funneliformis, Gigaspora,* and *Sclerocystis*. Whereas, *Rhizophagus, Glomus,* and *Entrophospora* were largely governed by EC with lesser effects of pH.

Table 3.10: Canonical correspondence analysis variable scores.

Biological variables	Axis 1	Axis 2
Ac	0.640	0.120
En	-0.587	-0.098
Fn	0.708	-0.283
Gi	0.747	-0.011
Gl	-0.633	0.921
Rh	-0.794	-0.076
Sc	0.951	-0.435
Eigenvalue	0.496	0.054
Variation %	82.980	8.984
Cumulative %	82.980	91.964

Note: Ac = *Acaulospora*, En = *Entrophospora*, Fn = *Funneiformis*, Gi = *Gigaspora*, Gl = *Glomus*, Rh = *Rhizophagus*, Sc = *Sclerocystis.*

Table 3.11: Biplot scores for soil variables.

Soil variables	Axis 1	Axis 2
pH	-0.948	-0.193
EC	-0.218	0.175
OC	0.835	0.230
N	0.819	0.465
P	-0.111	-0.164
K	0.860	-0.046
Mn	0.238	-0.172
Fe	0.664	0.157
Zn	0.925	0.194
Cu	0.892	0.108

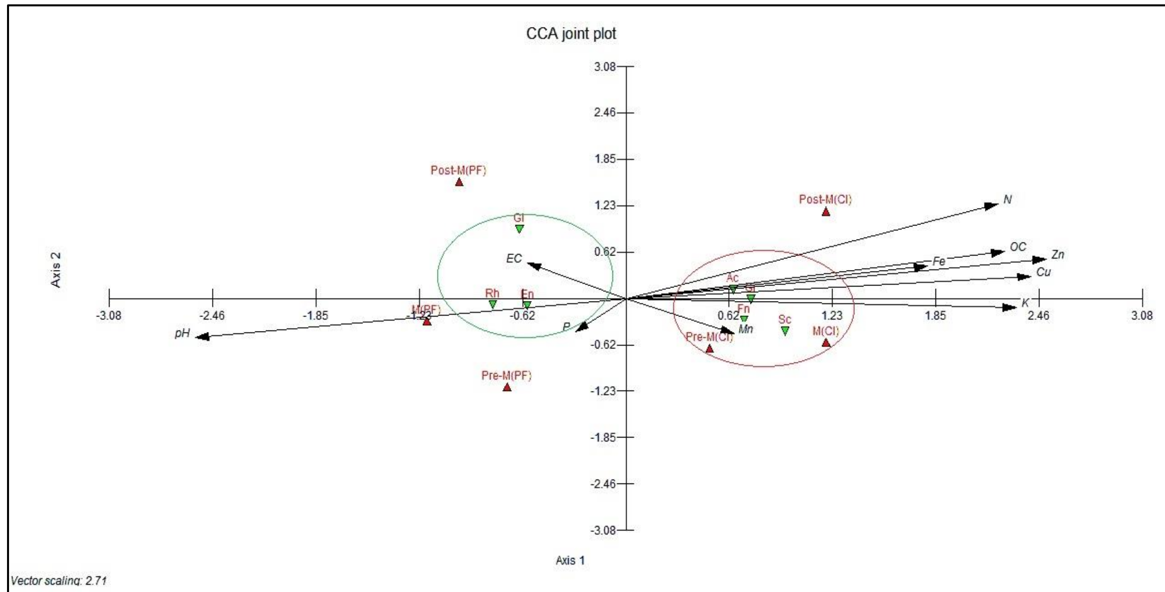


Fig. 3.15: Canonical correspondence analysis (CCA) of the relationship between AMF genera and soil variables during three seasons (Pre-M – Pre-monsoon, M – Monsoon, Post-M – Post-monsoon) in two mangrove sites (CI – Chorao Island, PF – Pichavaram forest).

3.4: CONCLUSION

The present study revealed non-uniform distribution and community structure of AM fungi were associated with different true and associate mangrove plants that varied significantly. Also, appreciable diverse forms of AM species in the study area support the conclusion that these species can tolerate high salinity. The identification of abundant and recurrent AM species among the mangrove plants, ascertain the fact that these species could be used efficiently in the re-establishment of mangrove habitats. This is the first study to explore AM symbiosis in 18 mangroves (true- and associate- mangrove) plants of Pichavaram forest, Tamil Nadu.

The seasonal study showed variation in AM fungal symbiosis among the East and West coast of India. The colonization rates were much lower at Pichavaram in the pre-monsoon season compared to Chorao. Our study also indicates the occurrence of seasonal patterns, though one is inversely expressed with a greater number of spores in monsoon season and greater root colonization in the dry seasons. The predominance of *Acaulospora* at Chorao and *Rhizophagus* at Pichavaram indicates their adaptation towards two different ecological conditions of the mangrove forest.

Such AM fungal diversity studies could be suitable for the assessment of AM fungal role in maintaining plant diversity during the conservation and restoration of various natural ecosystems.

Chapter 4: Preparation of Trap and Pure cultures

4.1 INTRODUCTION

AM fungi are known to influence the composition and productivity of the plant communities (Klironomos 2000). The distinctive role of these fungi has been demonstrated in agricultural plants and the plants growing in the various natural ecosystems (Piotrowski et al. 2004). AM fungi need to be cultured using a host plant to produce inocula containing hyphal fragments, colonized root, and/or healthy spores (Shah 2014). The spores isolated from the field soil could sometimes be dead or non-viable. The morphological characters of such spores change due to the root pigments, temperature, soil moisture, and other microbial activities within the rhizosphere (https://invam.wvu.edu/methods/culture-methods/trap-culture). Thus, soil samples collected from the field can be used to prepare 'trap cultures. Employing a suitable host plant would increase the spore production, which in turn is used for the preparation of pure or monospecific cultures (Rodrigues and Rodrigues 2014). Monospecific culture consists of a single species that is raised by isolating the desired spores of an AM fungal species from the trap culture (Shah 2014).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of trap cultures

Multiplication of AM fungal spores was accomplished by following the modified trap culture method of Morton et al. (1993) (**Plate 4.1**). Rhizosphere sample, along with the roots, was mixed with autoclaved sand in a 1:1 ratio. This mixture was added to 15 cm plastic pots which, were already wiped with 95% alcohol. *Plectranthus scutellarioides* (Coleus) was used as the catch plant. The Coleus cuttings were first washed with tap water and then with detergent water. The cuttings were then rinsed in sterile water. Three to four cuttings were planted per pot and, the pots were kept in the polyhouse for the establishment of colonization and subsequent sporulation. The plants were regularly watered when required. Hoagland's solution (minus P) was added at an interval of 15 days. Watering was stopped after 90 days, allowing the plants to dry, after which the shoot portion was cut off at the soil surface. The root segments of the *Coleus* plants were checked for colonization using the Trypan blue method (Phillips and Hayman 1970). On ensuring that the roots were colonized, the soil from each pot was separately placed in zip-lock polythene bags, labeled, and stored in the refrigerator at 4˚C.

4.2.2 Preparation of monospecific cultures

The AM fungal spores from trap cultures were extracted by wet sieving and decanting method (Gerdemann and Nicolson 1963). The extracted spores were identified, washed with autoclaved distilled water, and used to set up monospecific cultures. The substrate was prepared by mixing autoclaved sand: soil in the ratio of 1:1. This mixture was then transferred to plastic pots (15 cm). The extracted spores of single AM fungal species along with the filter paper were placed 2-3 cm deep in the pots. Three to four cuttings of *Plectranthus scutellarioides* were planted per pot. The pots were maintained for 90 days in the polyhouse and watered twice a week. After every 15 days, Hoagland solution (Hoagland and Arnon 1950) without P was added to the pots. The plants were allowed to dry after 90 days, and later the soil was analyzed for the spores.

4.3 RESULTS AND DISCUSSION

Monospecific cultures of 17 out of 35 AM fungal species recovered from the two sites were propagated in pot cultures. These included *Acaulospora dilatata, A. foveata, A. scrobiculata, A. undulata, A. delicata, A. rehmii, A. myriocarpa, A. spinosa, Entrophospora* sp., *Funneliformis geosporum, F. mosseae, Gigaspora decipiens, Gi. albida, Rhizophagus fasciculatus, R. intraradices, R. irregulare,* and *Sclerocytis rubiformis.* All the cultured AM fungal species recorded root colonization and produced an adequate number of spores to be used as inoculum. All these live cultures are maintained in the polyhouse of the Goa University Arbuscular Mycorrhizal Culture Collection (GUAMCC). The same cultures were used as inoculum to carry out further studies.

Trap cultures are usually prepared for trapping as many indigenous AM fungal species as possible. Also, some of the species can be trapped from the live colonized roots of the field plants (Shah 2014). A variety of substrates can be used either in pure or mixed form for the mass multiplication of AM fungi. However, sandy soil is commonly used for soil-based cultures (Douds and Schenck 1990) as the size of the substrate particles plays an important role in drainage and aeration and hence influencing sporulation (Gaur and Adholeya 2000). Although soil-based cultivation of AM fungal species is the cheap and most widely adopted system, it does not assure the absence of redundant contaminants even after a strict sanitization process is applied (Ijdo et al. 2011).

Chapter 5: Preparation of monoxenic cultures of dominant AM species

5.1 INTRODUCTION

Cultivation of AM fungi in monoxenic conditions started way back in the 1950s (Mosse 1959). The conventional method for the production of AM fungi is root organ culture (St Arnaud et al. 1996), which utilizes gelling agents like Phytagel, Gelgro, or agar (Gadkar et al. 2006). Such dual cultures have also been prepared using liquid media as a substitution of solid media (Joner et al. 2000). However, the liquid cultures are unreliable as they are challenging to maintain, and hence they have not been widely used (Gadkar et al. 2006). White (1943), Butcher and Street (1964), and Butcher (1980) developed the ROC technique by using synthetic media supplemented with vitamins and carbohydrate sources. Mosse and Hepper (1975) performed pioneering work and established monoxenic culture of AM fungi (*F. mosseae*) using transformed roots of *Lycopersicum esculentum* and *Trifolium pretense. Agrobacterium rhizogenes* causes the natural genetic transformation of plants producing hairy roots (Mathur and Vyas 2007). This produces Ri T-DNA transformation of the plant tissues resulting in profuse growth of roots on artificial media (Tepfer 1989). The first successful culture of AM fungus using hairy roots was accomplished by Mugnier and Mosse (1987). The better growth potential of transformed roots makes them adapt to diverse experimental conditions (Tepfer 1989). In several cases, fungal inoculums like spores or intra-radical propagules (colonized root fragments or vesicles) for the preparation of monoxenic culture have been used. However, AM fungal species (Gigasporaceaae and Scutellosporaceae) that do not produce vesicles are cultured using spores (Budi et al. 1999). For every AM fungal propagules, the right selection and sterilization procedure are the key steps for the establishment of monoxenic culture (Diop 2003).

5.2 MATERIALS AND METHODS

5.2.1 Extraction of AM fungi

AM fungal propagules *viz.,* spores, and colonized root fragments were extracted from monospecific cultures using the wet sieving and decanting method (Gerdemann and Nicolson 1963).

In vitro culture of AM fungi on Ri T-DNA transformed roots

5.2.2 Disinfection of AM fungal propagules

Firstly, extracted propagules (**Plate 5.1**) were rinsed with autoclaved distilled water. These propagules were surface-sterilized according to procedure modified from (Bécard and Fortin 1988). The concentration of the sterilizing agent and sterilization period varied based on the type of propagules and size of the spore. Isolated propagules were first disinfected in Sodium hypochlorite, followed by rinsing with sterile distilled water. The spores were then sterilized in an antibiotic solution, streptomycin sulfate.

5.2.3 Germination of disinfected AM fungal propagules

Surface sterilized propagules were transferred to Petri plates containing solid MSR (Modified Strullu and Romand) medium modified by Declerck et al. (1998) solidified with gellan gum clarigel (medium without sucrose). Petri plates were incubated in an inverted position in the dark at 27 °C. The pH and sucrose content were manipulated to attain maximum germination.

5.2.4 Establishment of dual culture

Germinated spores along with the media plug were picked up and placed in the vicinity of actively growing Ri T-DNA transformed roots of either Chicory (*Cichorium intybus*) or Linum (*Linum usitatissimum*) (**Plate 5.2**) to establish a dual culture. The Petri plates were incubated in an inverted position in the dark at 27 °C.

5.2.5 Estimation of AM fungal colonization in transformed roots

Estimation of AM fungal colonization in transformed roots was performed by following the trypan blue staining method of Phillips and Hayman (1970). The root bits were examined under a bright-field Olympus BX41 research microscope (40x, 100x, and 400x). Micrographs were imaged by using Nikon Digital Sight DS-U3 digital camera.

5.3 RESULTS AND DISCUSSION

5.3.1 *In vitro* germination of spores

In this study, AM fungal propagules such as extracted spores and colonized root segments were used to initiate Monoxenic cultures. Previous studies report that the species belonging to Gigasporaceae possess spores as the most effective propagules while that in

Acaulosporaceae and Glomeraceae can induce germination through spores as well as colonized root segments (Brundrett et al. 1999; Klironomos and Hart 2002).

Germination on MSR medium (minus sucrose) was achieved for nine AM fungal species *viz., Rhizophagus intraradices, Rhizophagus clarus, Rhizophagus fasciculatus, Funneliformis mosseae, Acaulospora spinosa, Gigaspora albida, Gigaspora decipiens, Dentiscutata scutata,* and *Racocetra gregaria* (**Plate 5.3, 5.4**). In the present study, we report for the first time germination of *A. spinosa* and *D. scutata* under Monoxenic conditions. The germination time varied from species to species. Also, the sterilization time differed from species to species depending upon the size of the spores (**Table 5.1**). During the pre-symbiotic phase, spores of AM fungi can germinate and develop germ tubes by using the stored materials (D'Souza et al. 2013). Several factors *viz.,* pH, temperature, light, nutrients, moisture, and substrate affect germination and germ tube growth under monoxenic conditions (Clark 1997; Maia and Yano-Melo 2001). Spores are among the propagules of AM fungi that germinate under suitable conditions developing an extra-radical mycelium. However, this mycelium ceases growth within 20 days after germination in the absence of host root (Giovannetti 2000).

In *Gi. albida* and *Gi. decipiens*, the multiple germ tubes were formed from the wall of a single spore. The development of multiple germ tubes in *Gigaspora* species could be due to their response towards the stimulatory substances within the growing environment, numerous nuclei near the wall, and genetic makeup of the spore (De Souza et al. 2005).

Table 5.1: Sterilization and *in vitro* germination of AM fungal spores.

AM species	Sodium hypochlorite (NaClO)(µl) + Time (min)	Streptomycin sulfate (%) + (2 min)	Germination time (days)
Rhizophagus intraradices	150 + 5	0.02%	1-3
Rhizophagus clarus	150 + 5	0.02%	6
Rhizophagus fasciculatus	70 + 3	0.02%	2
Funneliformis mosseae	250 + 5	0.05%	26
Acaulospora spinosa	100 + 4	0.02%	45
Gigaspora albida	350 + 5	0.05%	5-10
Gigaspora decipiens	350 + 5	0.05%	4
Dentiscutata scutata	250 + 5	0.05%	6
Racocetra gregaria	250 + 5	0.05%	4

5.3.2 Formation of primary structures in Gigasporaceae species

Following the establishment of dual culture, hyphae extended throughout the Petri plate, developing dichotomously branched ramifications known as branched absorbing structures (BAS) or arbuscule-like structures (ALS) (**Plate 5.5**). BAS were hyaline, thin-walled hyphal networks that became septate at maturity. The auxiliary cells began to form within 10 days after spore germination, which occurred concurrently on one or both sides of runner hyphae. Their colour ranged from pale yellow to dark brown in *Gigaspora* species and had a spherical shape with an ornamented surface, while those in *Scutellospora* were hyaline and knobby.

It has been indicated in earlier studies that BAS assists in the mineral nutrition of the host by taking up P, N, and few other nutrients actively (Bago et al. 1998) and transferring them to the host plant. It is well-known that Gigasporaceae species do not produce vesicles, but they form auxiliary cells on extra-radical hyphae (Dodd et al. 2000). In the present study, the total number of auxiliary cells ranged from 5 to 10 per plate, which was not enough to initiate spore production. In an experiment conducted by De Souza and Declerck (2003), it was observed that the formation of over 600 to 700 auxiliary cells resulted in average production of 56 spores per plate. Furthermore, they implied that the production of these

structures in large numbers perhaps helps in C storage, which can be used as energy sources for spore germination and mycelia development. This explanation was supported by the observations of Jabaji-Hare (1988), which detected high lipid content in auxiliary cells indicating its storage function.

In the present study, sporulation in Gigasporaceae species was not established even after the manipulation of culture media. Declerck et al. (2004) suggested that the formation of a single spore needs a C source from at least 19 auxiliary cells. Also, these species have a long vegetative phase and extended process of spore development (Kandula et al. 2006).

5.3.3 *In vitro* colonization in *Gigaspora decipiens*

In the case of *G. decipiens,* the germinated spore initiated contact with transformed Linum roots and established colonization *in vitro* within 12 days. The formation of BAS on medium indicates the better absorption of nutrients from the culture medium as these structures enhance the contact between the substrate and the fungus (Bago et al. 1998). Moreover, the formation of BAS in the soil increases the acquisition of nutrients and water by stabilizing the soil structure by improving the porosity of the soil Costa et al. 2013). BAS has a similar life span as arbuscules. The apoptotic process in these structures could be the reason for their rapid degradation (Mathur and Vyas 2007).

A variety of carbohydrates present in the medium may obstruct recognition sites on hyphae and cell walls of the host. This could prevent germ tubes from locating host roots (Allen 1992). Successful establishment of AM fungal culture in monoxenic conditions also depends on the physiological framework of the host root as roots even from the same breed respond differently when grown under the same conditions. Therefore, culture parameters such as explant selection, the orientation of the Petri plate during incubation (horizontal, inverted, or vertical), and the regularity of subculture must be optimized for each breed (Mathur and Vyas 2007).

5.3.4 *In vitro* sporulation in *Rhizophagus intraradices*

In *R. intraradices*, a germ tube emerged through the colonized root segments on MSR medium (without sucrose). The germination was initiated within three days after inoculation developing multiple germ tubes from the cut ends of root segments. Multiple entry points were seen following the colonization in the transformed roots of Chicory. After 2-3 weeks of the co-culture, a mycelial network developed that composed of runner hyphae with BAS/ALS. BAS were thin-walled, dichotomously branched, hyaline hyphal

networks close to the root zone. Sporulation was observed within 30 days of dual culture preparation. The bulging of spores was observed, which were then expanded beyond the juvenile stage. The progressive development of the spores led to a change in colour from hyaline to reddish-brown. The spores were filled with several lipid droplets. The formation of both intercalary and terminal spores was observed. Typically, spores formed were in clusters, while some of them were found singly around root segments. The average size of the spores was 40 µm. The total number of spores produced varied from 40 to 50 per plate (**Plate 5.6**).

The emergence of multiple germ tubes serves as a supplementary survival strategy of AM fungal spores to increase the possibility of symbiosis (Costa et al. 2013). Monoxenically produced spores of *R. intraradices* were viable, which was confirmed by sub-culturing them on fresh MSR medium in the vicinity of transformed Chicory roots after 16 weeks of initial sporulation. The sub-cultured spores colonized the roots and formed intercalary and terminal spores. Sporulation was observed within one month upon culturing.

An earlier study by Mosse (1988) indicates that the acidic pH of the medium inhibited the development of *R. intraradices* and its hyphae grew only after increasing the pH. However, in the present study, the acidic pH of the MSR medium (5.5) did not affect the germination and formation of colonization units in *R. intraradices*. This implies that early events in the developmental stages of AM fungi *viz.,* spore germination, germ tube growth, recognition, and contact with host determine the prospects of symbiosis. Moreover, these stages rely on environmental conditions.

In the present study, it was noted that the sporulation did not follow a typical three-phase pattern (lag, exponential, and plateau) generally observed in most biological species including AM fungi (Bago et al. 1998) while the spores were produced within several days. This could be a natural characteristic of the fungal species or due to an influence of various culture conditions on the fungus (Karandashov et al. 2000).

Trypan blue staining of excised transformed roots indicated 83% of colonization with intra-radical hyphae extending through the intercellular spaces. Vesicles were produced densely in several root fragments (**Plate 5.7**) however, arbuscules were not observed. In the study conducted by Mosse and Hepper (1975), it was revealed that arbuscules formed by *F. mosseae* and *Gi. margarita* in the older roots under monoxenic conditions appeared stumpy and remained vestigial having few fine branches. They suggested that in such

symbiosis, the key feature is swollen intercellular hyphae. In the present study, most of the intra-radical hyphal extensions were intracellular. Bi-directional protoplasmic flow and hyphae connecting colonizing units to the primary hyphae were observed. This indicates that the colonizing units are essential for the exchange of nutrients, even in the lack of arbuscules (Karandashov et al. 2000).

5.4 CONCLUSION

Monoxenic culture technology is a potent tool for the establishment of AM fungal associations experimentally. Although it is an artificial technology, it offers an experimental approach for understanding the biology and behaviour of AM fungi to a greater extent. Besides, this system could be used to increase the sporulation efficiency and for the mass production of inocula aseptically. Petri dish culture is a simple and efficient method of mass spore production as compared to various other techniques. The fungal propagules are contaminant-free and are easy to harvest. Factors influencing optimal production can be easily identified and modulated in monoxenic cultures.

Even after successive sub-culturing of *R. intraradices* using colonized transformed roots, the spore production remained constant, thus indicating the high potential of intra-radical structures to sustain species over more extended periods.

Chapter 6: Isolation, identification, and activity of phosphate solubilizing bacteria (PSB)

6.1: INTRODUCTION

Phosphorus (P) is a vital element for plant growth and development making about 0.2% of plant dry weight (Azziz et al. 2012). In soil, a very low concentration of P is present in available form. Elements like Fe, Al, and Ca immobilize available inorganic P by forming insoluble metal phosphates (Sharma and Baishya 2017). Various microbes especially bacteria are capable of solubilizing P and are used as biofertilizers in agriculture (Shrivastava and Kumar 2015) (**Fig. 6.1**). For plants to overcome P deficiency, PSB can play a vital role in supplying P to plants in a sustainable and eco-friendly manner (Khan et al. 2007). Apart from phosphate solubilization, PSB can sequester Fe by producing siderophores, produce plant hormones *viz.,* auxins, cytokinins, and gibberellins (Adesemoye and Kloepper 2009). Moreover, these bacteria can increase the rate of seed germination, photosynthetic rate, leaf area, root growth, yield, biomass, tolerance to abiotic stress, and delayed senescence (Adesemoye and Kloepper 2009). Phosphate solubilizing bacteria can help the plant to tolerate the inhibiting effects of environmental stresses such as drought, flooding, heavy metals, and salinity by the production of organic acids. These organic acids can solubilize inorganic P, cell wall degrading enzymes like chitinase, hydrogen cyanide, antibiotics, and siderophores. These factors lead to better seed germination and vigour of the plants (Patel et al. 2011). The release of H^+ to the outer surfaces as a substitute for cation or ATPase is also an alternative possibility for the solubilization of mineral phosphate other than the release of organic acids (Rodríguez and Fraga 1999). Moreover, organic acids form cation complexes on the mineral surface of soil thus obstructing the P absorption on it (Bianco and Defez 2010).

Besides PSB, the symbiotic relationship of the plant with AM fungi can assist in the availability of P. Arbuscular mycorrhizal association to enhance the uptake of water and nutrients (P, N, and some micronutrients (Smith and Read 2008). Furthermore, AM fungi can influence the diversity of the bacterial population in the rhizosphere (Toljander et al. 2005). AM fungi can absorb P available in the soil but are unable to extract P from insoluble phosphates (Antunes et al. 2007). However, they can efficiently translocate the P solubilized by PSB to their host plant (Villegas and Fortin 2002).

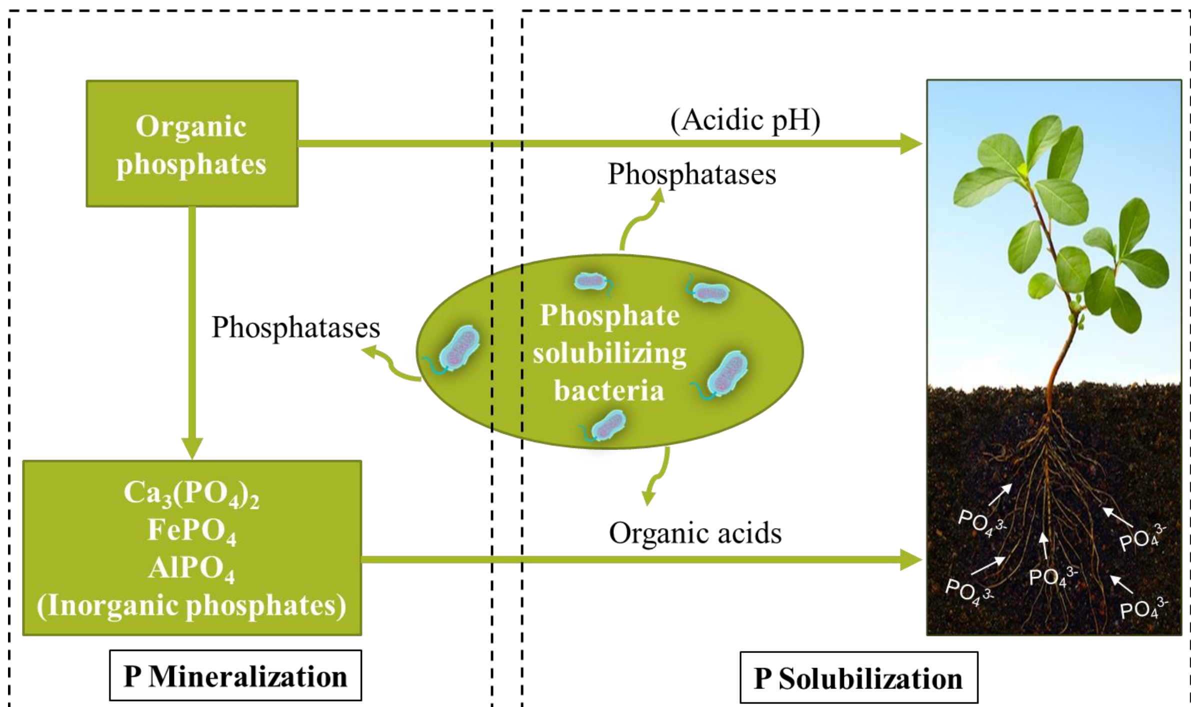


Fig. 6.1: Schematic diagram of soil phosphorus mineralization and solubilization by phosphate solubilizing bacteria.

Phosphate solubilizing microorganisms are the sole source of phosphate solubilization in saline soils of wetland ecosystems (Teymouri et al. 2016). Therefore, it is a fundamental approach to isolate and analyze their activity that would consequently contribute to accomplishing P demands and the growth of mangrove plants.

6.2: MATERIALS AND METHODS

6.2.1: Collection of rhizosphere and root samples

Rhizosphere and root samples from true- and associate- mangrove species *viz., Excoecaria agallocha,* and *Clerodendrum inerme* respectively were collected. For each plant species, three subsamples were collected in sealed bags. The roots were separated from the rhizosphere sample for the estimation of AM root colonization. Further, these samples were air-dried and used for isolation of PSB and AM spores.

6.2.2: Soil analyses

The rhizosphere samples of *E. agallocha* and *C. inerme* were analyzed for various chemical properties such as pH, electrical conductivity (EC), organic carbon (OC), N, P, and K. The procedures used for the analyses are the same as described in chapter 3.

6.2.3: Assessment of AM root colonization

AM fungal root colonization was assessed in the root segments of the above plants by using the Trypan blue staining technique (Phillips and Hayman 1970) as described in chapter 3.

6.2.4: Isolation and identification of AM fungi

AM fungal spores were isolated from the soil using the wet sieving and decanting method (Gerdemann and Nicolson 1963) and identified as described in chapter 3.

6.2.5: Isolation and purification of PSB

PSB was isolated using the serial dilution plate method on Pikovskaya (PKV) agar (Roychowdhury et al. 2015). Three dilutions (10^{-1}, $10^{-2,}$ and 10^{-3}) were selected for inoculation. 0.1 mL of culture was spread plated on Pikovskaya agar plate from selected dilutions separately. Plates were incubated at 30 °C for 48 hours. Distinct bacterial colonies based on morphological appearance and halo zone formation were selected and streaked on fresh PKV agar plates. Selected colonies were purified with repeated streaking and maintained on Nutrient agar slants at 4 °C for further analysis.

6.2.6: Gram staining

Gram staining of the PSB pure cultures was performed using Himedia K001-1KT gram stains-kit following the procedure given by the manufacturer.

6.2.7: Biochemical characterization of PSB

Preliminary morphological and biochemical characterization of purified isolates of PSB was performed by standard methodologies as described in 'Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1994). Tests such as Gram staining, IMViC, catalase, nitrate reduction, and sugar utilization test (glucose, cellobiose, mannitol, sucrose, arabinose, lactose, xylose, maltose) were carried out.

6.2.8: Molecular characterization of bacterial isolate

Molecular characterization of PSB isolates was done by sequencing of 16s rRNA. Genomic DNA was extracted using NucleoSpin® Tissue Kit (Macherey-Nagel) by following the instructions given by the manufacturer. The quality of extracted DNA was checked by running 5 µL of DNA on agarose gel electrophoresis set at 75V until the

migration of bromophenol dye front to the bottom of the gel. The resultant DNA bands were visualized in a UV transilluminator (GeNei).

6.2.9: Amplification of 16S rRNA gene

The 16S rRNA gene of PSB isolates was amplified in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using primers 16S-RS-F (CAGGCCTAACACATGCAAGTC) and 16S-RS-R (GGGCGGWGTGTACAAGGC).

The PCR amplification was carried out with the following components:

Reaction mixture	**Quantity (µL)**
2X Phire Master Mix	5.0
D/W	4.0
Forward Primer	0.25
Reverse Primer	0.25
DNA	1.0

Following temperature transitions were used:

Temperature (˚C)	**Time**
95	5 min
95	30 sec
60	40 sec
72	60 sec
72	7 min
4	∞

The thermal cycler was programmed for 35 cycles with one cycle for the first step of denaturation and 35 cycles for steps 2-4.

6.2.10: Agarose gel electrophoresis of PCR product

The PCR products were resolved by electrophoresis using 1.2% agarose gels in 0.5X TBE buffer stained with 0.5 µg/mL ethidium bromide. 1 µL of 6X loading dye was mixed with 4 µL of PCR products and was loaded. A 2-log DNA ladder (NEB) was used as a marker. The gel was run 75V for 1-2 h. The gels were viewed under UV transilluminator (Genei) and the image was captured using the Gel documentation system (Bio-Rad).

6.2.11: ExoSAP-IT Treatment

ExoSAP-IT consisting of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP) were used to remove unwanted primers and dNTPs from a PCR product. A 5 µL of PCR product was mixed with 0.5 µL of ExoSAP-IT and incubated at 37 ˚C for 15 min followed by enzyme inactivation at 85 ˚C for 5 min.

6.2.12: Sequencing of 16s rRNA gene

Sequencing of ExoSAP treated PCR product was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) by following manufacturer's instructions.

The Sequencing PCR mix consisted of the following components:

Reaction mixture	**Quantity (µL)**
D/W	6.6
5X Sequencing Buffer	1.9
Forward Primer	0.3
Reverse Primer	0.3
Sequencing Mix	0.2
ExoSAP treated PCR product	1.0

Sequencing PCR amplification profile

Temperature (˚C)	**Time**
96	2 min
96	30 sec
50	40 sec
60	4 min
40	∞

6.2.13: Sequence analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and editing of the sequences were performed using Geneious Pro v 5.5 (Drummond et al. 2010). The homology to the closest bacterial species having maximum similarity (98-100%) was obtained in the National Center for Biotechnology Information (NCBI). The forward and reverse sequences were aligned and

edited using BioEdit v. 7.2.5 sequence alignment editor. The sequences were deposited in the NCBI gene bank. The 16S RNA sequences were used to build a phylogenetic tree using Molecular Evolutionary genetics analysis software (MEGA-X) (Tamura et al. 2004). Bootstrap test with 1000 replicates was performed using the Neighbor-joining method to find out the relationship with the closest species.

6.2.14: Qualitative estimation of phosphate solubilization

The bacterial cultures having 10^8 CFU/mL were spotted on Pikovskaya's-Bromophenol blue (PKV-BPB) agar medium (0.5% Calcium phosphate) using an inoculation loop. The plates were maintained at 30 ± 2^0C. The colony diameter and halo zone around it was measured at the end of 2, 5, and 7 days after inoculation (DAI). Phosphate solubilizing efficiency was measured using the following formula (Kundu et al. 2009).

$$\text{Solubilization efficiency} = \frac{\text{Diameter of solubilization zone} - \text{colony diameter}}{\text{Colony diameter}} \times 100$$

6.2.15: Quantitative estimation of phosphate solubilization

Phosphate solubilization was estimated quantitatively using the Vanadomolybdate phosphoric yellow colour method (Jackson 1973). Twenty-four hours old bacterial culture CFU/mL (500 µl) was inoculated in 100 mL of PKV broth (0.5% tricalcium phosphate) in 250 mL of Erlenmeyer flask. The flasks were maintained at 30 ± 2^0 C for 15 days shaking at 100 rpm. Uninoculated medium served as control. On the 5th, $10^{th,}$ and 15th day, 10 mL culture suspension from each flask was centrifuged at 10,000 rpm for 10 min. From this, 5 mL of clear culture suspension was made up to 50 mL with sterile distilled water. One mL of the above filtrate was pipetted into a 50 mL volumetric flask and 2.5 mL of Barton's reagent was added, finally making up the volume with distilled water. The resultant yellow colour developed was measured in UV-visible spectrophotometer (Shimadzu model UV-2450) at 430 nm after 10 min. 2.5 mL Barton's reagent made up to 50 mL with distilled water served as the reagent blank. A standard curve was prepared by using potassium dihydrogen phosphate (KH_2PO_4) and the amount of solubilized P was calculated using a standard graph. The pH of the medium was recorded at the end of the experiment using a pH meter (LI 120 Elico, India).

6.2.16: Determination P solubilization under salt stress

The determination of P solubilization by PSB_2 under salt stress was examined in Pikovskaya's agar medium amended with 0, 0.1, 1, 3, and 5% (w/v) NaCl.

6.3: RESULTS AND DISCUSSION

6.3.1: Soil properties

Chemical properties of rhizosphere samples of *E. agallocha* and *C. inerme* are presented in **Table 6.1.** Rhizosphere pH was acidic for both plant species. However, EC was notably high in the rhizosphere of *E. agallocha*. Low EC in *C. inerme* could be due to its occurrence in landward areas (Kathiresan and Bingham 2001). The phosphorus (P) level in *C. inerme* was lower than that of *E. agallocha*. This could be attributed to the fact that former plant species being associated mangrove is an inhabitant of landward zones of mangrove ecosystem (Wu et al. 2008). The concentration of labile P across a mangrove forest reduces with declined tidal height becoming limited towards the landward zones (Behera et al. 2014).

Table 6.1: Chemical properties of mangrove plant rhizosphere.

Soil properties	*E. agallocha*	*C. inerme*
pH	4.9 ± 1.6	5.3 ± 0.7
EC (dS/m)	14.1 ± 0.9	9.5 ± 1.1
OC (%)	2.4 ± 0.8	1.3 ± 0.4
N (g/kg)	0.02 ± 0.01	0.03 ± 0.01
P (g/kg)	0.052 ± 0.02	0.005 ± 0.001
K (g/kg)	0.27 ± 0.1	0.35 ± 0.08

Note: All values are mean of three readings; ± = standard error; EC = electrical conductivity; OC = organic carbon

6.3.2: AM fungal association

The percent root colonization, spore density, and AM fungal diversity are presented in **Table 6.2**. *Clerodendrum inerme* showed the highest root colonization. Five AM fungal species were isolated from the rhizosphere of *E. agallocha* and *C. inerme* belonging to five different genera.

Although it is well known that AM fungi help in the uptake of nutrients, their efficiency of nutrient mobilization decreases in the mangrove ecosystem due to flooded conditions (Hackney et al. 2000). However, if the P is made easily accessible for the exploring hyphae, they can assimilate and transfer it to the plants. The PSB can assist in the

solubilization of mineral phosphates thus making them available for uptake by AM fungi (Toro et al. 1998). Hence, the PSB acts as 'mycorrhiza helper bacteria' (Tarkka and Frey-Klett, 2008).

Table 6.2: Percent root colonization, spore density, and diversity of AM fungal species.

Plant name	Root colonization (%)	Spore density (spores/100g of soil)	AM fungal diversity
Excoecaria agallocha	92.86 ± 7.15	58 ± 5.5	*A. dilatata, F. geosporum, Entrophospora* sp., *R. fasciculatus*
Clerodendrum inerme	100 ± 0.00	42 ± 4.0	*A. dilatata, F. geosporum, Entrophospora* sp., *R. fasciculatus, G. glomerulatum*

6.3.3: Isolation and identification

Two phosphate solubilizing bacterial species were isolated from the two mangrove species (*E. agallocha* and *C. inerme*) of Chorao Island which formed a halo zone on Pikovskaya agar medium (**Plate 6.1**). The bacterial isolates were named PSB_1 and PSB_2. The results of morphological and biochemical tests are presented in **Table 6.3 and Plate 6.2, 6.3**. Based on morphological and biochemical analyses, the two PSB isolates were tentatively identified as *Bacillus* sp. Moreover, molecular analysis of 16S RNA of these isolates revealed the close similarity of PSB_1 with *B. subtilis* and PSB_2 with *B. halotolerans* and their gene bank accession number obtained was MW365313 and MW365314, respectively. The close relation of the two PSB isolates with other bacterial strains of the NCBI database is depicted in the phylogenetic tree (**Fig. 6.2**). The bacterial species *B. subtilis* has been already reported as efficient phosphate solubilizers (Audipudi et al. 2012; Maheswar and Sathiyavani 2012; Abhijith et al. 2017). There is only a single report indicating phosphate solubilization by *B. halotolerans* (Slama et al. 2019). However, this bacterial strain was isolated from the dunes of the Mediterranean Sea.

Table 6.3: Morphological and Biochemical characterization of PSB.

Test	PSB_1	PSB_2
Cell shape	rod	rod
Motility test	motile	motile
Gram staining	positive	positive
Catalase	-	-
IMViC test		
Indole production	-	-
Methyl red	-	+
Voges–Proskauer	-	-
Citrate utilization (Simmons)	+	+
Nitrate reduction	+	+
Urease	+	-
Carbon source utilization		
Glucose	-	+
Cellobiose	-	+
Mannitol	-	+
Sucrose	-	-
Arabinose	-	+
Lactose	-	+
Xylose	-	+
Maltose	-	+

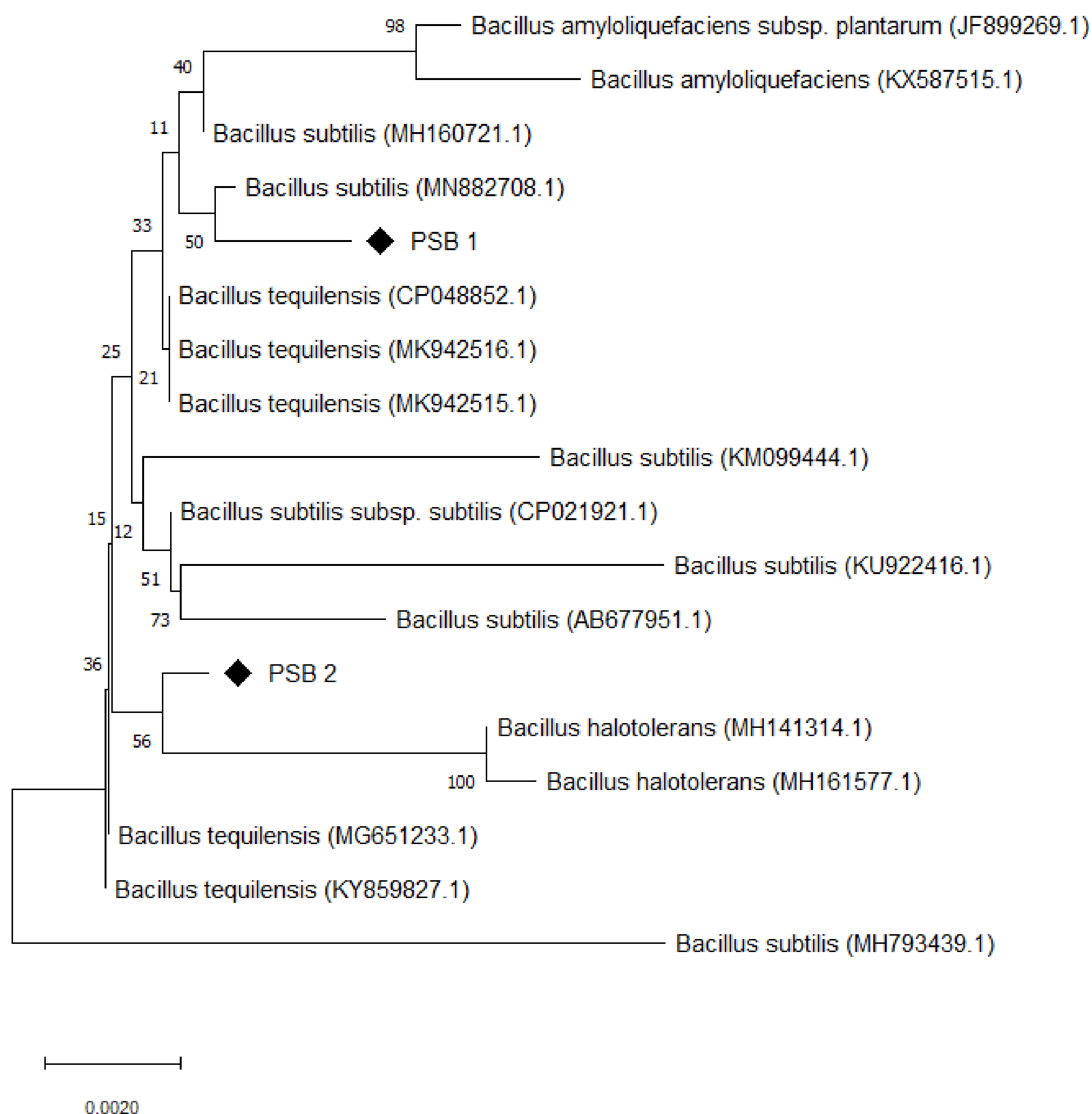


Fig. 6.2: Dendrogram showing the phylogenetic position of PSB_1 and PSB_2 with other bacterial strains.

6.3.4: Qualitative estimation of P solubilization.

Both the bacterial isolates showed the ability to solubilize P by the formation of a halo/clear zone around the colony (**Plate 6.4**). Bacterial isolates produced a yellow halo zone on blue PKV-BPB agar plates. The qualitative estimation of phosphate solubilization was calculated based on the size of the halo zone. PSB_2 showed higher efficiency with a solubilization efficiency of 195.54% while PSB_1 was less efficient with 92.36% after 7 days of inoculation (**Fig 6.3**).

It is well known that the solubilization of mineral phosphates by PSB occurs due to the production of organic acids (Kim et al. 1997). The hydroxyl and carboxyl groups of these organic acids chelate the cations bound to phosphates thus converting it into available form (Sagoe et al. 1998). The formation of a yellow zone around the colonies depicts the drop in the pH of the medium as a result of organic acid production by the bacterial isolates (Tiwari et al. 2018). Bromophenol blue is a pH indicator that changes its colour as the pH of the medium decreases (Behera et al. 2017a). Therefore, it helps in the easy visual screening of phosphate solubilization (Mehta and Nautiyal 2001). However, the solid medium-based screening is not reliable as some of the earlier studies reported that the bacterial isolates which were unable to form halo zone on solid medium, could solubilize P in broth medium (Das 1963; (Louw and Webley 1959). Hence, the solubilization of phosphate by bacterial isolates was further assessed on PKV broth.

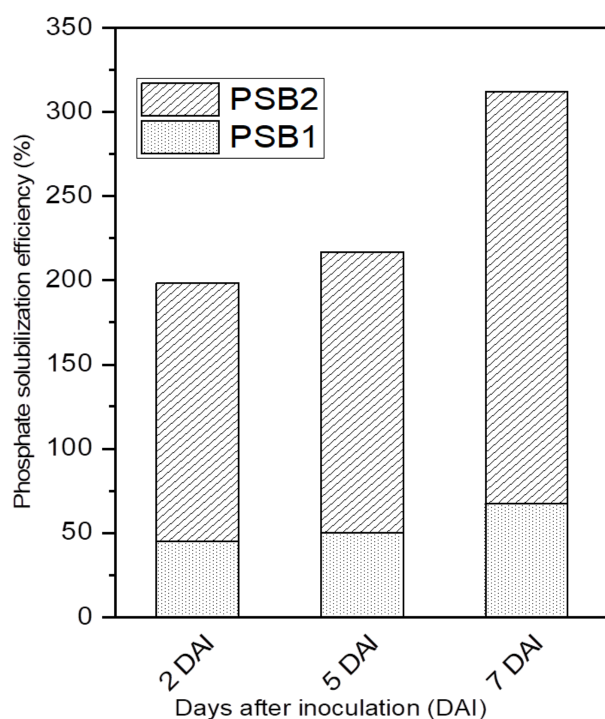


Fig 6.3: Bacterial phosphate solubilization on PKV-BPB agar medium.

6.3.5 Quantitative estimation of P solubilization.

Solubilization of tricalcium phosphate (0.5%) was estimated using PKV broth every fifth day for 15 days (**Plate 6.5**). The standard graph for quantitative phosphate solubilization is presented in **Fig. 6.4**. The solubilization of tricalcium phosphate occurred due to the production of organic acids by the bacterial strains which was confirmed by a significant drop in the pH of the liquid medium from 7.0 to 2.9 (**Fig. 6.5 a, b).** The amount of P

solubilized in the medium ranged from 1.43 to 37.05 µg/mL. In PSB_1, the pH of the medium remained constant after 10 days of incubation. The maximum phosphate solubilization was recorded by PSB_2 which showed the highest concentration of soluble P (37.05 µg/mL) which is equivalent to 37.05 mg/L. The reverse relationship between pH and soluble P content specifies the acidification of the medium due to the organic acid secretion which enables phosphate solubilization (Behera et al. 2017a). Secretion of organic acids e.g. carboxylic acid leads to ionization of $Ca_3(PO_4)_2$ (Mohammadi 2012).

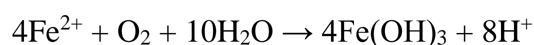
Comparable to the present study, Dastager and Damare (2013) isolated phosphate-solubilizing actinobacteria from the sediments of Chorao Island. They reported phosphate solubilization in the range of 89.3 to 164.1 µg/mL. They further explained that a large amount of phosphates gets precipitated due to the abundance of cations in the pore waters of the mangrove ecosystem. Hence, PSB act as an important source to make available soluble P for the mangrove species.

In the present study, both the bacterial isolates were found to be *Bacillus* species. However, the P solubilization kinetics differed between the two isolates. These results are following the findings of previous studies that reported variation in solubilized P content by *Bacillus* sp. recovered from lead-rich soil (Park et al. 2011) and alluvial soils of Gangetic plains (Tiwari et al. 2018).

Similar to the present observations, several studies have reported P solubilization by bacterial isolates from other mangrove ecosystems. Five bacterial species belonging to genera *Pseudomonas* and *Azotobacter* and two *B. subtilis* strains were isolated from Chollangi mangrove forest on the east coast of India (Audipudi et al. 2012). These strains could solubilize 20 – 400 mg/L of mineral phosphate. Kathiresan and Selvam (2006) isolated 24 PSB isolates from the rhizosphere of *R. mucronata* in the Vellar estuary on the south-eastern coast of India. They reported solubilized P in the range of 0.012 – 0.141 mg/L. Behera et al. (2016) reported soluble P content of 8.21 to 48.70 mg/L by PSB species belonging to genera *Pseudomonas, Bacillus*, *Alcaligens, Klebsiella, Serratia, Azotobacter,* and *Micrococcus*. These species were isolated from the mangroves of Mahanadi river delta, Odisha. In another study, Behera et al. (2017a) isolated *Serratia* sp. from Mahanadi river delta with a phosphate solubilizing efficiency of 44.84 mg/L. Teymouri et al. (2016) quantified phosphate solubilization potential of three PSB species (*Bacillus, Pseudomonas,* and *Acinetobacter*) isolated from the rhizosphere of *A. marina*

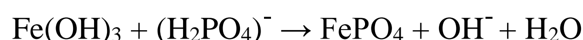
from Qeshm Island, Iran. These species could solubilize P in the range of 215 – 356 mg/L. A strain of *Alcaligenes faecalis* isolated from Mahanadi river delta solubilized 48 mg/L of phosphate at 144 hours of incubation (Behera et al. 2017b). The highest amount of phosphate solubilization by PSB isolated from the mangrove ecosystem was reported by Vazquez et al. (2000). They isolated six PSB species from the mangrove rhizosphere of Mexico and reported maximum solubilization of P (480 mg/L) by *Vibrio proteolyticus*. *Pseudomonas* and *Bacillus* are described as the most efficient phosphate solubilizers (Igual et al. 2001).

The adaptation to the anaerobic conditions of the mangrove ecosystem is accomplished by the formation of aerial roots for the gas exchange (Colmer 2003). The movement of oxygen in the surrounding sediment causes oxidation of Fe^{2+} which is precipitated as Fe oxides (immobile) resulting in the generation of H^+ ions and subsequently makes the root zone acidic (Begg et al. 1994).



$$4Fe^{2+} + O_2 + 10H_2O \rightarrow 4Fe(OH)_3 + 8H^+$$

This process leads to the trapping of P as $FePO_4$ (Silva and Sampaio, 1998).



$$Fe(OH)_3 + (H_2PO_4)^- \rightarrow FePO_4 + OH^- + H_2O$$

Although phosphates occur as insoluble precipitates of $Ca_3(PO_4)_2$, $FePO_4$, or $AlPO_4$ in soil, only $Ca_3(PO_4)_2$ is used as P source in phosphate solubilization screening protocols since Fe and Al phosphates could not show solubilization activity on indicator plates (Liu et al. 2015).

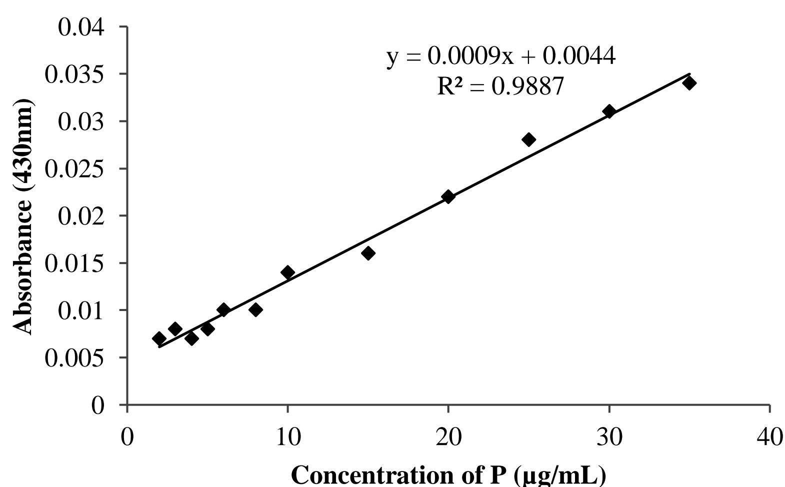


Fig. 6.4: Standard graph for quantitative estimation of Phosphorus.

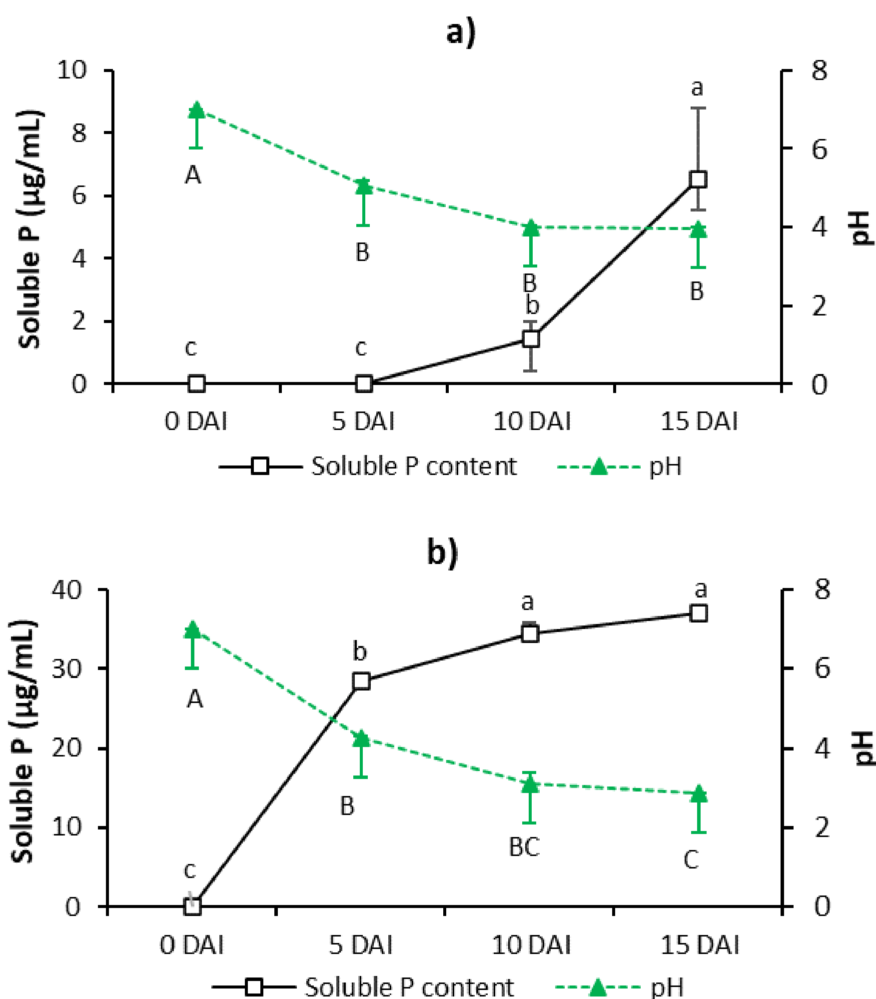


Fig. 6.5: Tri-calcium phosphate solubilization and drop of pH in Pikovskaya broth, a) PSB_1; b) PSB_2. DAI=days after incubation.

6.3.6: P solubilization under salt stress

The bacterial isolate PSB_2 was tested for its ability of P solubilization under the different concentrations of NaCl in Pikovskaya's agar medium. The results of P solubilization efficiency are presented in **Fig 6.6.** The solubilization efficiency increased with an increase in the incubation period irrespective of NaCl concentration. The solubilization percentage was found to be maximum on 12 days after inoculation (DAI) at 0.1 and 1% NaCl (59.05 and 60.44% respectively) concentration. The solubilization efficiency for all NaCl concentrations (except 1%) decreased after the 12 DAI. However, the solubilization efficiency of PSB_2 was significantly high even in higher concentrations of NaCl as the bacterial strain was isolated from highly saline mangrove habitats. Similar results have been reported by several authors (Zhu et al. 2011; Srinivasan et al. 2012; Patil 2014). This

adaptation of bacterial isolate to hypersaline conditions could be due to the synthesis of suitable solutes or aggregation of K against NaCl to overcome salt stress. Therefore, these PSB isolates have the genetic capability to solubilize P even at high salinity (Srinivasan et al. 2012).

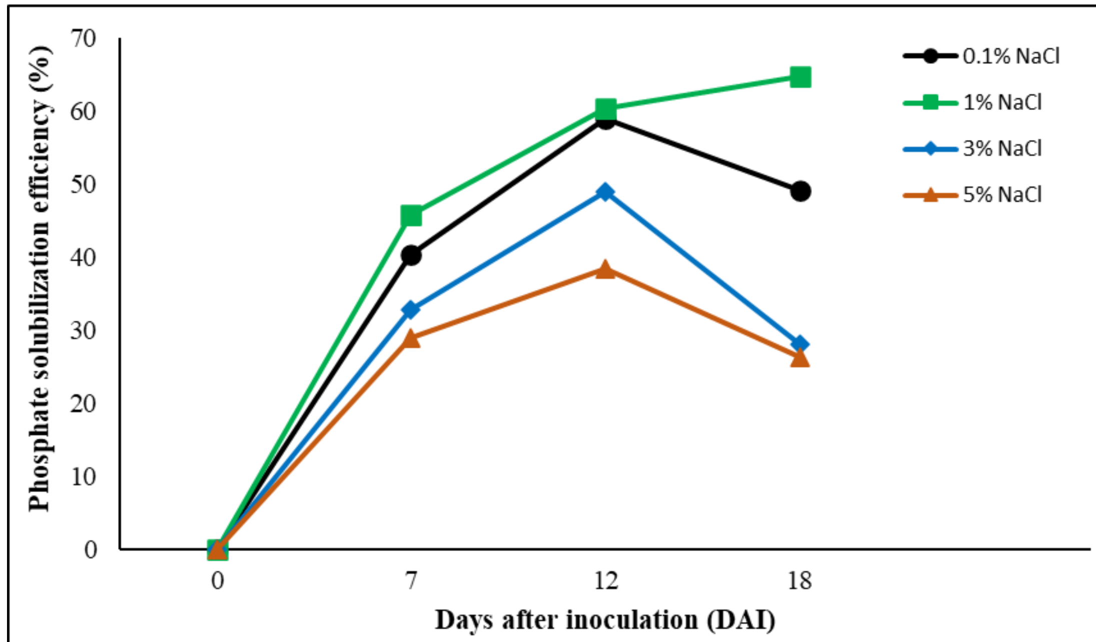


Fig. 6.6: Tri-calcium phosphate solubilization under salt stress.

6.4: CONCLUSION

The present study demonstrates the occurrence of PSB and AM fungi in the rhizosphere of two mangrove plant species from Chorao Island. The interaction of PSB and AM fungi has great potential to boost P requirements in plants. Two species of PSB were isolated *viz., B. subtilis* and *B. halotolerans* and were screened for phosphate solubilization activity. The latter solubilized more P than the former. Hence, we infer that *B. halotolerans* can be used as a bio-inoculant to improve the growth of mangrove seedlings. This is the first study wherein *B. halotolerans* has been isolated from mangrove habitat and for phosphate solubilization activity. Further investigation on the salt tolerance of this bacterial strain would be of great importance.

Chapter 7: Mass multiplication and preparation of inocula.

7.1 INTRODUCTION

Microbial inoculants supplying N and P have a crucial role in accomplishing the nutrient needs of the crops and agroforestry in drylands (Rafi et al. 2019). These bioinoculants are useful in reducing fertilizer input. Bacterial inoculants containing PSB are known to improve plant growth when the inoculated bacteria are well established in the rhizosphere. The deficiency of P can be overcome by inoculating the target plants with PSB (Bakhshandeh et al. 2015). Apart from the nutrient status of soil, PSBs as bio-inoculants also play an important role in retaining the soil structure and unfold a new horizon for improved plant growth (Ingle and Padole 2017).

Presently, AM fungal inocula are applied in horticulture and other field cultivation (Berruti et al. 2015). Most of the AM fungal inocula used in commercial cultivation are developed from transformed root cultures (Kokkoris and Hart 2019). AM fungi associated with plant roots in nature which fluctuate every day and seasonally (Lippu 1998). Whereas, in the case of transformed root culture, the flow of nutrients is continuous and constant (Fortin et al. 2002), thus supporting the unrestricted growth of AM fungi. In natural plants, most of the C is supplied to the shoot part hence allocating limited C to the root symbionts. While in the case of ROC, there are no C limitations. Such conditions promote better spore production (Rosikiewicz et al. 2017). It is well evident that symbiotic association between AM fungi and their host plants increases the bioavailability of key nutrients and restores soil fertility, diminishing the harmful impact of chemical fertilizers on the environment (Rashid et al. 2016). Despite the AM fungal enormous potential to enhance plant growth, the obligate biotrophic nature of these fungi has complicated the development of cost-effective methods to produce superior quality AM fungal inocula (Ijdo et al. 2011). Inoculum production can be carried out in pots of various materials or sizes under controlled or semi-controlled conditions (Ijdo et al. 2011). A variety of substrates *viz.,* peat (Ma et al. 2007), perlite, glass (Lee and George 2005), vermiculite, compost (Douds et al. 2006), soilrite (Mallesha et al. 1992) and calcinated clay (Plenchette et al. 1982) have been used for the mass multiplication of AM fungi. Also, the use of several organic amendments *viz.,* chitin, and humic substances (Gryndler et al. 2003 and 2005) are reported to influence AM fungal growth. Organic waste from animals and plants *viz.,* charcoal, farmyard manure, composts, soybean meal, corncobs, wheat bran, and press mud

are also considered as excellent carrier materials (Herrmann and Lesueur 2013; Wang et al. 2015; Araujo et al. 2020) for inoculum production. It can be possible to formulate carrier combinations using a mixture of soil, husk, compost, bark, and peat (Herridge et al. 2008) as well. Besides, soilless techniques *viz.,* aeroponic, hydroponic, and nutrient film technique (NFT) has experimented that are less bulky and not easily prone to contaminations (Malusá et al. 2012).

This study intends to highlight the preparation of carrier formulation for effective inoculum production having an increased number of propagules.

7.2 MATERIALS AND METHODS

7.2.1 Extraction of monoxenically produced AM fungal propagules

The monoxenically produced propagules of *R. intraradices* were extracted following the method of Cranenbrouck et al. (2005) under the laminar airflow. A media plug along with the spores and extra-radical hyphae was cut and placed in the empty sterile Petri plate. Already prepared 25 mL of 0.1 M citrate buffer was filtered using 0.22 µm syringe-driven Membrane Filter (Millex ®- GS) and poured in the Petri plate. To dissolve the clerigel in the media plug, the Petri plate was agitated gently. The separated AM fungal propagules were then rinsed with sterile distilled water.

7.2.2 Extraction of carrier-based AM fungal propagules

Propagules of *A. dilatata* were extracted by the wet sieving and decanting method (as described in Chapter 3).

7.2.3 Preparation of carrier mixture

The carrier mixture was prepared by using vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1 as formulated by Rodrigues and Rodrigues (2017). The materials used for the carrier mixture were autoclaved at 121°C for 1 hour on two successive days. The chemical analyses of the carrier materials were done by the standard methods as described in Chapter 3.

7.2.4 Preparation of AM fungal inocula in the carrier

AM fungal inocula of *R. intraradices* and *A. dilatata* were prepared using pots. 15 cm deep plastic pots were filled with the carrier mixture. The extracted propagules of the species were placed 2-3 cm deep in the pots. Shoot cuttings (3 to 4) of the Coleus plant were

planted per pot. The pots were kept in the polyhouse for 90 days. Hoagland's solution without P was added to the plants after every 15 days. After 90 days the culture along with the roots was air-dried and stored in plastic bags at room temperature for further use.

7.2.5 Assessment of root colonization

Assessment of AM fungal colonization in Coleus roots was performed by following the Trypan blue staining method of Phillips and Hayman (1970) after 90 days of growth as described in chapter 3.

7.2.6 Preparation of PSB inoculum

The bacterial isolate PSB_2 was selected for subsequent screening studies as this isolate showed high P solubilization efficiency. The PSB_2 starter was prepared by culturing the bacterial strain in Nutrient broth (NB) and incubating at 100 rpm at 30 °C. The cells were harvested at the end of five days by centrifugation of the culture at 8,000 rpm for 10 min. The cell pellet was resuspended in sterile distilled water making the final concentration to 10^8 CFU/mL.

7.3 RESULTS AND DISCUSSION

7.3.1 Physico-chemical properties of carrier materials

Physico-chemical characters of the carrier materials are depicted in **Table 7.1.** All the carrier materials showed variation in characteristics. The wood ash was alkaline which could be due to the presence of alkali and alkaline metal oxides (Demeyer et al. 2001). Organic C and N content were higher in cow dung powder. All the carrier materials showed lower levels of P. Vermiculite is an inert micaceous material that has been heated to 1000-1100 °C (Verdonck et al. 1980). This could be the reason that the vermiculite showed lesser amounts of nutrients analysed. The N content in wood ash is negligible which could be because the wood N gets converted to inorganic compounds of N *viz.,* NH_3, NO_x, and N_2 during the burning of wood (Misra et al. 1993).

Table 7.1: Chemical properties of carrier materials.

Parameters	Vermiculite	Cow dung powder	Wood powder	Wood ash
pH	6.08 ± 0.6	6.01 ± 0.7	5.42 ± 1.0	11.19 ± 1.1
EC (mS)	0.17 ± 0.1	0.02 ± 0.01	0.93 ± 0.5	0.04 ± 0.02
OC (%)	0.82 ± 0.4	4.52 ± 1.1	2.24 ± 0.7	2.21 ± 0.4
N (g/kg)	0.3 ± 0.2	6.8 ± 0.8	1.3 ± 0.4	0.01 ± 1.2
P (g/kg)	0.09 ± 0.04	0.12 ± 0.06	0.44 ± 0.1	0.22 ± 0.07
K (g/kg)	0.07 ± 0.03	1.32 ± 0.3	0.08 ± 0.02	2.01 ± 0.4

Note: All values are mean of three readings; ± = Standard error; EC= Electrical conductivity; OC= Organic carbon.

7.3.2 Colonization potential of monoxenically produced spores in carrier materials

Root colonization with AM fungal structures *viz.,* hyphae, arbuscules, and vesicles were observed in the Trypan blue stained root bits of coleus plant by the monoxenically produced *R. intraradices* spores and pot culture-based *A. dilatata* spores. Plants inoculated with *R. intraradices* resulted in 89.28% root colonization, while it was 63.63% in *A. dilatata* inoculated plants.

Rodrigues and Rodrigues (2017) in the carrier formulation experiment reported 100% colonization by monoxenically produced spores of *R. intraradices* and *F. mosseae* in *Eluesine coracana* using the carrier formulation of vermiculite, cow dung powder, wood powder, and wood ash (20:8:2:1). Several culture techniques *viz.,* pot culture, hydroponic culture, aeroponic culture, and root organ culture have been applied for the production of AM fungal inoculum (Bhowmik et al. 2015). With the advancement in techniques, the preparation of AM fungal inocula should have a commercial application (Sharma et al. 2017). Hence, the selection of suitable substrate for the mass multiplication of AM fungi holds an important challenge. One of the most efficient methods of propagating clean inocula in limited space is ROC (Stockinger et al. 2009). However, the use of waste substrates *viz.,* rice straw, and chickpea husk along with conventional substrates (soil-sand mixture) is an economical and most preferred method for inoculum production of AM fungi (Kadian et al. 2018). Yet, the product is bulky for transportation and application in the field. Gradually, inert substrates *viz.,* vermiculite, perlite, biochar, or a mixture of these

have replaced the conventional substrates (Khaliq et al. 2002). It is necessary to screen the inocula obtained in pots before application in the field so that it can be tested whether inocula can colonize the plant roots effectively, propagate in the substrate, and can enhance plant growth (Tanwar et al. 2013).

7.3.3 AM fungal and PSB inocula

The mass propagated AM fungal inoculum of *A. dilatata* produced 70 spores/100 g of soil while that of *R. intraradices* produced 92 spores/100 g of soil. The cell count of PSB_2 inoculated on the NA medium was 30 x 10^8 CFU/mL.

The key feature of inoculants contributing to high-quality inocula is the increased viable cell count capable of enhanced activity of the selected microbe (Ben Rebah et al. 2002). Desirable characters for an inoculant strain entail its genetic stability, competence ability with indigenous microorganisms, ability to display its effect on the target plant, and to persist in hostile environmental conditions (Herrmann and Lesueur 2013). Douds et al. (2010) produced inoculum of several AM fungal species using vermiculite, perlite, or horticultural potting media as diluents of compost and obtained higher propagule numbers in vermiculite based media. They suggested that the plate-like structure of vermiculite facilitates its water holding capacity thereby providing a suitable environment for the growth and persistence of AM fungal hyphae.

Bacterial inoculants are generally cultivated in liquid broth to produce higher population levels having media composition and culture conditions directly relating to the nature of the specific strain (Herrmann and Lesueur 2013). The carrier materials used in the present study are organic except for vermiculite. In addition to providing macro- and micro-nutrients, these materials can increase substrate porosity and improve water retention ability (Rodrigues and Rodrigues 2017).

7.4 Conclusion

In the present study, an attempt was made to employ an economical and efficient substrate medium using the optimal concentration of carrier materials to achieve maximum spore production of the selected AM fungal and bacterial strains. Both the AM fungal species selected for the investigation could adequately colonize and sporulate in the carrier formulation used. However, the development of a rapid and highly efficient culture system remains a major challenge for commercialization.

Chapter 8: Screening of efficient AM species for selected mangrove plant species.

8.1 INTRODUCTION

Mangrove ecosystem is the climax construction of hydrohalophytes occupying estuarine intertidal zones of tropical and subtropical regions (Lugo and Snedaker 1974). They act as natural shelterbelts and maintain ecological balance by providing habitat and food sources for the biological species. This ecosystem is fragile and eco-sensitive as they connect upland terrestrial and estuarine ecosystems (Xie et al. 2014). Although the plants growing in such an ecosystem shows substantial tolerance to salinity, inundation, and nutrient limitations, they have degenerated severely all over the world primarily due to nutrient stress especially P deficiency (Xie et al. 2014). Studies show that there is a 35% of reduction in mangrove forests of the world in the previous 20 years of the 20th century (Valiela et al. 2001). This resulted in the global consensus of protection and revegetation of mangrove forests (Krauss et al. 2008).

It is well known that several species of bacteria and fungi residing in the rhizosphere contain a functional relationship with plants exerting beneficial effects on plant biomass (Vessey 2003). AM fungi are a vital component of rhizosphere microbial communities which form a mutualistic symbiosis with terrestrial (Smith and Read 2008) and wetland plants (Tawaraya et al. 2003). They act as a living bridge between the soil and their host plant that absorb nutrients from the soil and transfer them to the plant. This symbiosis considerably promotes P uptake of plant and the improvement of P nutrition can boost other functions (Cozzolino et al. 2010). Indication of P solubilizing microorganisms (PSM) occurrence dates back to 1903 (Khan et al. 2007). Bacteria are considered more effective in P solubilization than fungi (Afzal and Bano 2008). P solubilization potential of PSB is 1 to 50%, whereas, in the case of P solubilizing fungi, it is only 0.1 to 0.5% (Chen et al. 2006).

Inoculation of plants with bio-inoculants to enhance the growth of plants is centuries old (Bashan et al. 2014). Bio-inoculants consist of a consortium of different types of microbes, which are capable of converting nutritionally essential elements from unavailable to available form through natural processes (Vessey 2003).

Based on the literature survey, no reports are indicating combined effects of AM fungi and P solubilizing bacteria on the growth, biomass, and nutrient uptake of mangrove plants.

Hence the present study was conducted to examine the influence of single and dual microbial inoculation on plant growth, biomass, and P nutrition of *R. mucronata*.

8.2 MATERIALS AND METHODS

8.2.1 Experimental design

The experiment comprised of the following eight treatments:
Treatment 1 (T_1): Control (Uninoculated)
Treatment 2 (T_2): *R. intraradices*
Treatment 3 (T_3): *A. dilatata*
Treatment 4 (T_4): *R. intraradices* + *A. dilatata*
Treatment 5 (T_5): *R. intraradices* + PSB 2
Treatment 6 (T_6): *A. dilatata* + PSB 2
Treatment 7 (T_7): *R. intraradices* + *A. dilatata* + PSB 2
Treatment 8 (T_8): PSB_2
All the treatments with 3 replicates were arranged in randomized block design.
Matured viviparous propagules of *R. mucronata* of similar size were collected from Chorao Island and brought to the laboratory. After surface sterilization in 0.01% mercuric chloride ($HgCl_2$) solution for 15 min followed by rinsing in 4-5 times in sterile distilled water, the undamaged propagules of *R. mucronata* with intact Testa were selected for planting. These propagules were then placed in the plastic 'plant grow bags' (15 cm in diameter, 35 cm in depth). The bags were filled with 1.5 kg sterilized sand (pH 7.8, P content of 10.6 mg/kg). Bacterial culture was applied using a syringe filled with 10 mL of bacterial cell suspension (30 x 10^8 CFU/mL) and pouring it in the vicinity of the propagules. For the AM fungal treatments, 10 g of inoculum was added to the soil in the vicinity of the propagules. The bags were kept in the shade net of the Department of Botany, Goa University under natural conditions for ten months (**Plate 8.1**).

8.2.2 Assessment of AM fungal colonization in roots

To confirm AM colonization in the inoculated plants, root segments of *R. mucronata* were collected from three randomly selected pots of each treatment at 305 (DAI). Trypan blue staining method (Phillips and Hayman, 1970) as described in chapter 3 was employed.

8.2.3 Measurement of plant growth and biomass

Various growth parameters *viz.,* plant height, stem diameter, number of branches, number of leaves, leaf area, petiole length, internode length, and root length were recorded on the 305th day after inoculation. Plant height (above ground) was measured using a measuring

tape before harvesting the plants. For the measurement of leaf area and petiole length, 3rd leaf from the top was selected. Leaf area was measured using a graph paper method.

Fresh and dry weights of leaf, stem, and root were recorded separately. The roots and other plant parts were rinsed with water thoroughly to remove the debris. Fresh weights of leaves, stems, and roots were recorded. The total plant dry biomass and root to shoot ratio was determined after drying the samples at 90 °C to constant weight (96 h).

8.2.4 Mycorrhizal dependency

Mycorrhizal dependency was proposed by Gerdemann (1975) to determine the significance of the association between the host plant and fungus. It is defined as the extent to which a plant is dependent on the mycorrhizal association to achieve its maximum growth or yield at a given level of soil fertility. The mycorrhizal dependency of a given plant would also vary by the fungal partner and the environmental conditions (Estaún et al. 2010).

Mycorrhizal dependency (MD) was calculated based on the formula given by Plenchette et al. 1983).

$$\text{MD}\ (\%) = \frac{\text{dry weight of mycorrhizal plant} - \text{dry weight of non mycorrhizal plant}}{\text{dry weight of mycorrhizal plant}} \times 100$$

8.2.5 Analysis of leaf pigments

Both the chlorophyll pigments (Chl $_a$ and Chl $_b$) absorb light in the blue and red spectral regions. The absorbance of photons at two different wavelengths of light i.e., at 663 nm and 645 nm is specific for Chl $_a$ and Chl $_b$ respectively.

The third pair of leaves from the top was taken for the estimation of pigments. 0.1 g fresh leaf tissue was ground in a mortar using 10 mL of 80% acetone. The homogenate was filtered using a double layer of muslin cloth and the extract was collected in a centrifuge tube. The absorbance of the extract was read at 645, 663, and 470 nm. The quantitative estimation of chlorophyll *a*, chlorophyll *b*, total chlorophyll was done using Arnon's equation (Arnon 1949).

Chl $_a$ (mg g^{-1}) = [(12.7 X A_{663}) – (2.6 X A_{645})] X acetone (mL) / leaf tissue (mg)

Chl $_b$ (mg g^{-1}) = [(22.9 X A_{645}) – (4.68 X A_{663})] X acetone (mL) / leaf tissue (mg)

Total Chlorophyll = Chl $_a$ + Chl $_b$

8.2.6 Estimation of plant P

The dried samples of leaf, stem, and root were analyzed for P content. Samples were ground into a fine powder using mortar and pestle. One gram of powdered sample was weighed and added to a 250 mL conical flask to which 10 mL of conc. HNO_3 was added. After mixing the contents, the funnel was placed on the flask and kept in a closed chamber overnight for pre-digestion. After pre-digestion, 10 mL of conc. HNO_3 and 3 mL of $HClO_4$ was added and the flask was placed on a hot plate inside the fume hood chamber. The hot plate was heated at 100 °C and continued the digestion until contents became colourless and dense white fumes appeared (**Plate 8.2**). The contents were reduced to 2-3 mL before removing the flask from the hot plate. After cooling, about 30 mL of distilled water was added to the flask and the contents were filtered through Whatman filter paper into a 100 mL volumetric flask. The final volume was made up to the mark using distilled water.

The P content of digested plant sample was determined colorimetrically by the vanadomolybdo-phosphoric yellow colour method (Chapman and Pratt 1982). Hyphae contribution was calculated using the formula given by Kothari et al. (1991).

Hyphae contribution (HC%) = [(P uptake of whole mycorrhizal plant - P uptake of the whole nonmycorrhizal plant) ÷ P uptake of the whole mycorrhizal plant] × 100

8.2.7 Statistical analysis

The experiment was set down as a completely randomized block design with three replicated of each treatment. All the data sets were statistically analyzed using SPSS v16.0. One-way analysis of variance (ANOVA) followed by S-N-K test at $p \leq 0.05$ was performed to find out differences between means. Correlation analysis of the parameters was carried out by Pearson's correlation test at $p \leq 0.01$ and $p \leq 0.02$.

8.3: RESULTS AND DISCUSSION

8.3.1 Root colonization

As depicted in **Table 8.1**, no AM colonization was observed in T_1 (uninoculated) and T_8 (PSB inoculated). AM fungal structures were recorded in all the AM inoculated plants (**Plate 8.3**). The root colonization in the AM inoculated plants ranged from 43.75 to 91.45%. Treatment 7 showed significantly higher root colonization than the other treatments ($p < 0.05$). These results are following the previous studies which reported an increase in root colonization of *Zea mays* (Wu et al. 2004) and *Helianthus tuberosus*

(Nacoon et al. 2020) on dual inoculation with PSB *Bacillus megaterium* and two AM species *viz., R. intraradices* and *Funneliformis mosseae* and PSB *Klebsiella variicola* with two strains of AM fungi *viz., Glomus multisubtensum* and *R. intraradices,* respectively.

Inoculation with PSB increased the colonization by both *R. intraradices* and *A. dilatata*. It is well known that rhizobacteria act as 'mycorrhiza helper bacteria' by improving AM colonization in plant roots (Fitter and Garbaye 1994). However, the mechanism involved in the stimulation of AM colonization by these bacteria is not well known. It has been assumed that the production of amino acids, vitamins, and hormones by bacteria may be responsible for this interaction (Barea et al. 1997).

Table 8.1: Percentage root colonization of *R. mucronata* seedlings.

Treatments	RC (%)
T1	0 ± 0^{f}
T2	60.3 ± 1.1^{d}
T3	43.8 ± 2.3^{e}
T4	68.2 ± 1.8^{bc}
T5	73.3 ± 0.7^{b}
T6	65.7 ± 2.7^{cd}
T7	85.7 ± 1.2^{a}
T8	0 ± 0^{f}

Note: Data are means of three replicates. ± standard error. Values in each column followed by different letters are significantly different at $p < 0.05$.

8.3.2 Plant vegetative growth

All the vegetative parameters of *R. mucronata* varied significantly in comparison to the control treatment (**Fig. 8.1**). Plant height was maximum in T_7 (*R. intraradices* + *A. dilatata* + PSB_2) compared to other treatments. However, the root length was significantly higher ($p < 0.05$) in all the inoculated plants compared to control (**Plate 8.4**). Besides, AM fungal inoculum in combination with PSB_2 showed a better impact on all the growth parameters of the inoculated plants. Similar results were recorded by Nacoon et al. (2020) who investigated the influence of co-inoculation on plant growth of *H. tuberosus* with AM fungi and PSB.

Studies on the screening of AM fungal species on the growth and biomass of mangrove plants are very scarce. Wang et al. (2010) recorded the positive effect of AM species (*F. geosporum, F. mosseae, R. intraradices,* and *Glomus aggregatum*) on the growth and

nutrient uptake of *Sonneratia apetala*. Xie et al. (2014) reported that the inoculation of *Kandelia obovata* seedlings with the consortium of four AM fungi (*F. geosporum, R. intraradices, Claroideoglomus claroideum,* and *C. etunicatum*) increased the plant biomass and uptake of N and P. In comparison, D'Souza and Rodrigues (2016) inoculated propagules of *Ceriops tagal* with three AM fungal species *viz., Rhizophagus clarus, R. intraradices,* and *Acaulospora laevis*. They concluded that *R. clarus* exhibited a greater influence on the growth and biomass of the plants. The effectiveness of AM fungi to promote nutrient uptake and plant growth varies with different species and isolates (Bagyaraj 1992) in having different developmental mechanisms (Hart and Reader 2002), physiological modifications (George 2000). There is a single report on the use of *B. halotolerans* as a biofertilizer in coriander crops that reported increased growth (Jiménez-Gómez et al. 2020).

8.3.3 Plant biomass and mycorrhizal dependency

The biomass of *R. mucronata* with dual treatment involving AM and PSB was significantly greater than the uninoculated plants (**Table 8.2**). The treatments with a consortium of *R. intraradices, A. dilatata,* and PSB_2 (*B. halotolerans*) (T_7) significantly enhanced the total biomass of *R. mucronata* ($p < 0.05$) (**Fig. 8.2**). The root to shoot ratio significantly increased under treatments 5 and 8 ($p < 0.01$). Positive effects of co-inoculation with AM fungi and PSB on root length have been reported earlier (Kavatagi and Lakshman 2014). They suggested that the enhanced root growth is due to the impact of inoculation with AM fungi and PSB on the modification of root morphology.

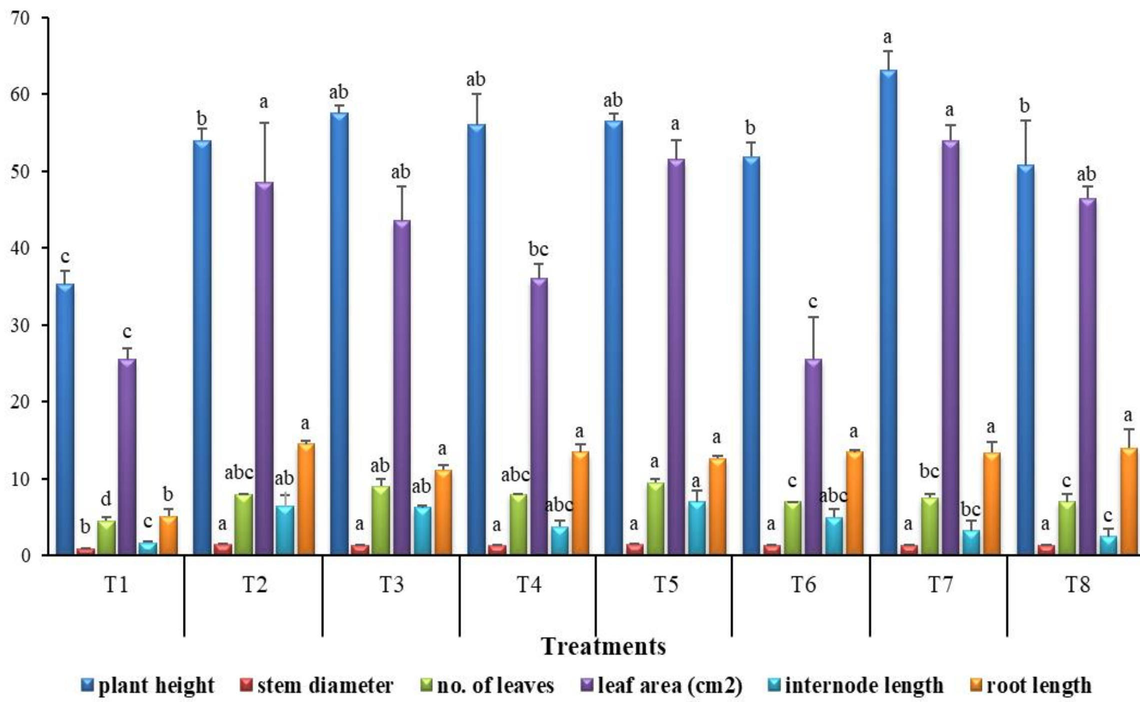


Fig. 8.1: Effect of inoculation on growth of *R. mucronata* seedlings. Values in each column presented with different lowercase or uppercase letters are significantly different at p<0.05.

Table 8.2: Biomass of *R. mucronata* seedlings under bio-inoculant treatments.

Treatments	Stem		Leaf		Root		Total plant dry biomass (g)
	Wet weight (g)	Dry weight (g)	Wet weight (g)	Dry weight (g)	Wet weight (g)	Dry weight (g)	
T1	35.25 ± 0.3^{c}	13.5 ± 1.4^{cd}	5.65 ± 0.6^{c}	1.25 ± 0.2^{d}	3.65 ± 0.2^{d}	$1.5^{c} \pm 0.3^{c}$	16.25 ± 1.3^{c}
T2	36.65 ± 4.6^{c}	12.25 ± 1.3^{d}	12.4 ± 3.1^{ab}	3.05 ± 0.7^{bc}	10.25 ± 1.6^{abc}	3.53 ± 0.5^{b}	18.83 ± 1.1^{bc}
T3	54.5 ± 7.2^{b}	18.6 ± 0.5^{abc}	16.35 ± 2.4^{a}	3.0 ± 0.9^{bc}	9.73 ± 2.0^{abc}	2.53 ± 0.5^{bc}	24.13 ± 1.9^{ab}
T4	59.75 ± 6.3^{ab}	19.23 ± 1.9^{ab}	12.5 ± 0.7^{ab}	2.9 ± 0.1^{bc}	7.0 ± 0.4^{bcd}	2.25 ± 0.3^{bc}	24.38 ± 1.7^{ab}
T5	50.05 ± 5.5^{bc}	16.5 ± 1.7^{bcd}	17.0 ± 2.1^{a}	4.68 ± 0.3^{a}	13.25 ± 0.1^{a}	6.15 ± 0.1^{a}	27.33 ± 1.9^{a}
T6	44.4 ± 5.2^{bc}	14.7 ± 1.6^{bcd}	9.45 ± 0.2^{bc}	2.15 ± 0.0^{cd}	5.85 ± 2.7^{cd}	3.13 ± 0.9^{b}	19.98 ± 2.5^{bc}
T7	71.58 ± 4.9^{a}	22.6 ± 3.2^{a}	15.85 ± 3.1^{ab}	4.25 ± 0.3^{ab}	8.25 ± 1.2^{bc}	2.85 ± 0.1^{bc}	29.70 ± 3.5^{a}
T8	48.7 ± 3.7^{bc}	15.7 ± 1.0^{bcd}	15.43 ± 2.0^{ab}	3.33 ± 0.5^{abc}	10.35 ± 0.0^{ab}	5.4 ± 0.1^{a}	24.43 ± 0.6^{ab}

Note: Data are means of three replicates. ± standard deviation Values in each column followed by different letters are significantly different at $p < 0.05$.

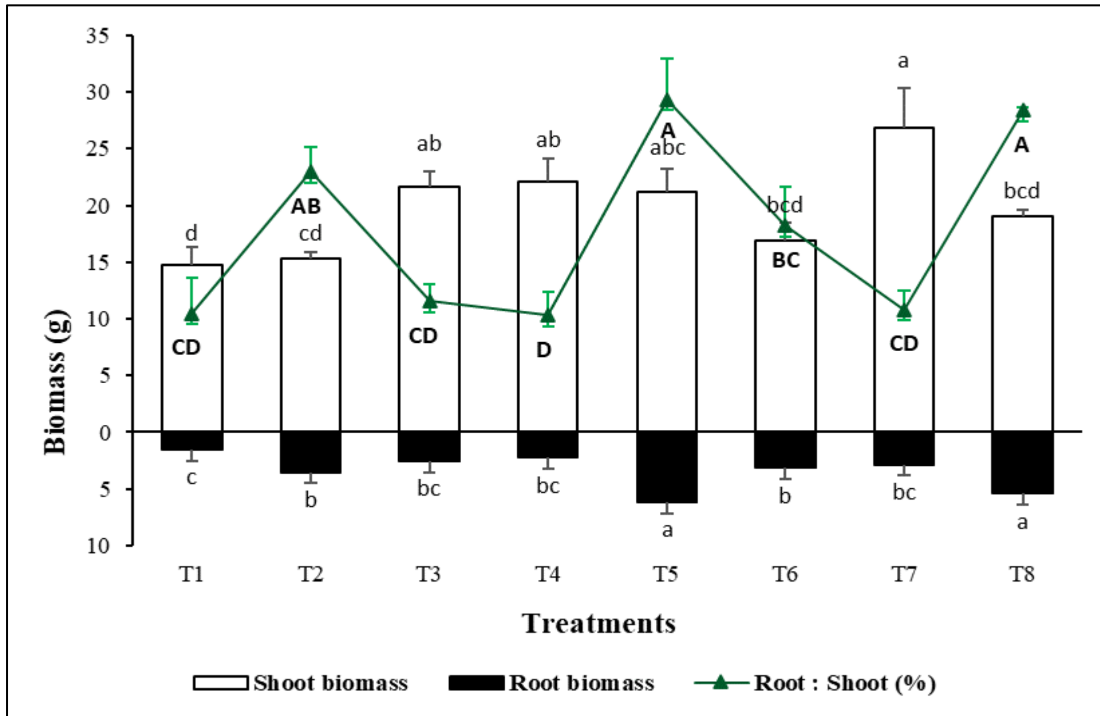


Fig. 8.2: Effect of inoculation on aboveground and belowground biomass and root to shoot ratio of *R. mucronata.* Values in each column presented with different lowercase or uppercase letters are significantly different at p < 0.05.

The mycorrhizal dependency of *R. mucronata* varied significantly with different treatments ($p < 0.05$) (**Fig. 8.3**). The MD values remained as low as 13.78% in treatment 2 and reached a maximum of 45.02% under treatment 7.

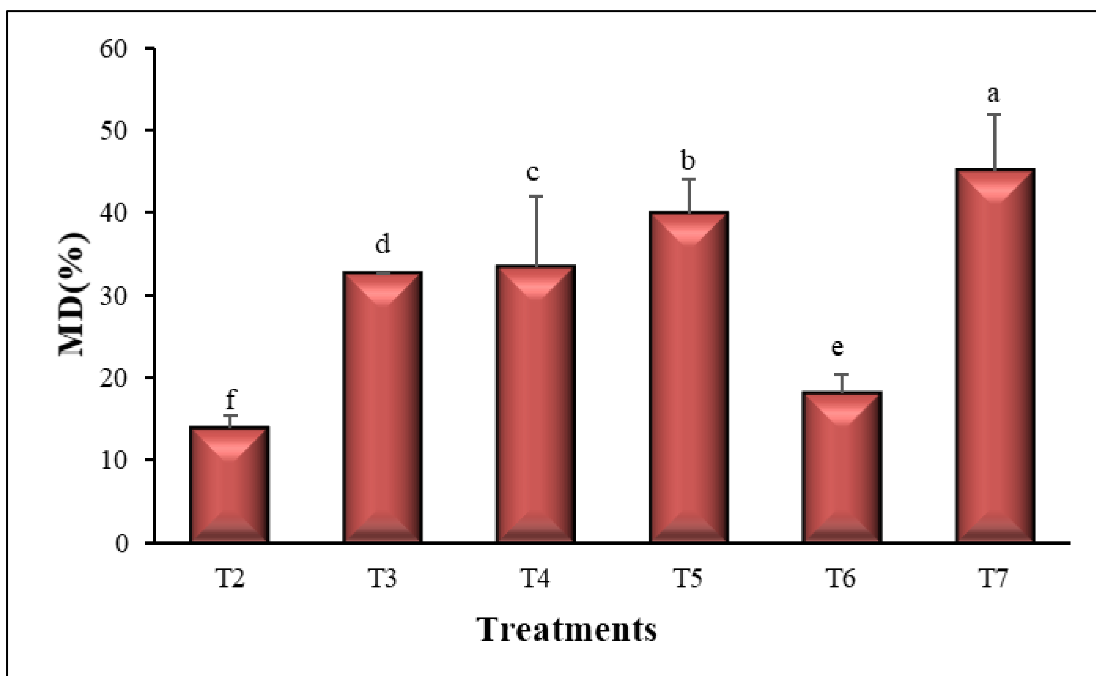


Fig. 8.3: Mycorrhizal dependency (MD) in AM inoculated plants. Values in each column presented with different letters are significantly different at p < 0.05.

The low level of available P in the substrate soil could have increased the root biomass in treatments 5 and 8 as the combination of PSB and *R. intraradices* would have assisted for an efficient P solubilization and mobilization, respectively. A previous study by Naidoo (2009) on *Avicennia marina* demonstrated that the seedlings invested more resources to increase root biomass in nutrient-deficient conditions. However, the present study showed plant biomass was significantly higher in inoculated plants than that of uninoculated or control plants. This confirms an enhanced uptake of nutrients by inoculated plants resulting in better plant growth (Kothari et al. 1990).

The values of MD indicated dual inoculation of plants with AM and PSB could produce greater biomass of *R. mucronata* than AM fungi alone. The lower MD values of *A. dilatata* inoculated plants could be due to suppression of growth with consumption of carbohydrates by AM species (Xie et al. 2014).

8.3.4 Chlorophyll content

In plants, chlorophyll molecules absorb light energy which is used to carry out photosynthesis (Wu et al. 2018). In the present study, a significant difference was recorded in the concentration of chlorophyll *a*, chlorophyll *b,* and total chlorophyll amongst all the treatments ($p < 0.05$) (**Fig. 8.4**). Overall, T_7 showed significantly higher contents of total chlorophyll ($p < 0.05$). These results are consistent with previous observations (Sheng et al. 2008; Wu et al. 2015; Gavito et al. 2019). Analysis of plant chlorophyll content is the key index for the assessment of photosynthesis (Zhu et al. 2012).

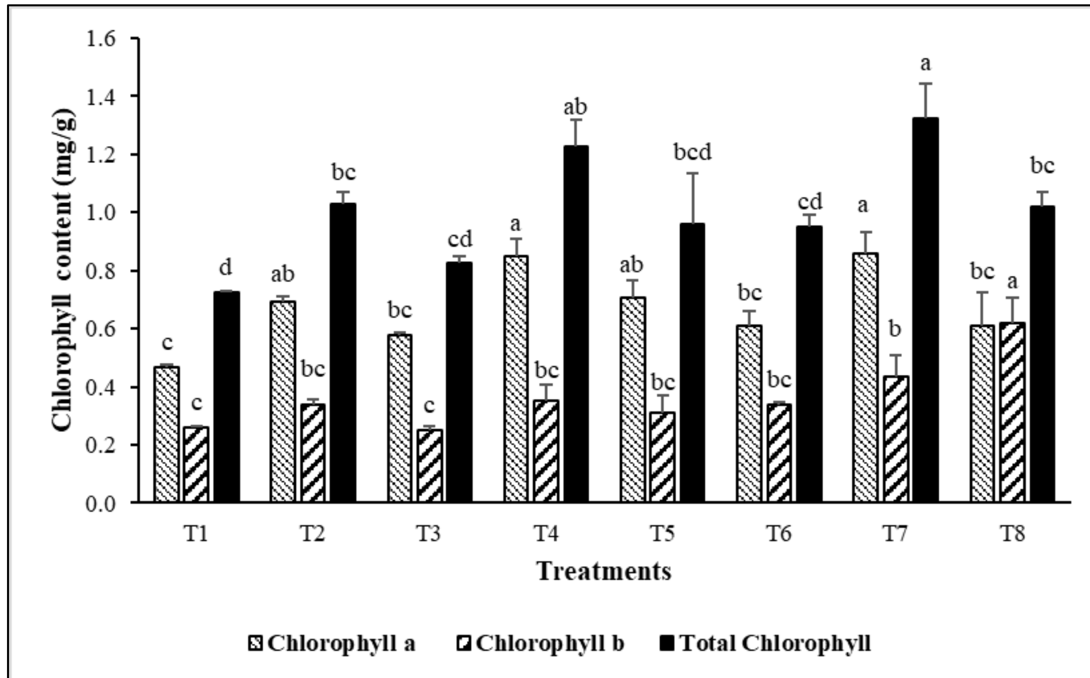


Fig. 8.4: Effect of inoculation on leaf pigments in *R. mucronata.* Values in each column presented with different letters are significantly different at $p < 0.05$.

In the present study positive effects of AM fungi and PSB on the elevation of chlorophyll content and hence photosynthesis was observed. Wu et al. (2019) reported increased chlorophyll concentration and photosynthetic activity in *Camellia oleifera* plants inoculated with PSB. The rate of photosynthesis is commonly higher in mycorrhizal plants compared to nonmycorrhizal plants (Amaya-Carpio et al. 2009; Zhu et al. 2012) which is assumed to be due to enhanced plant nutrition, especially P (Augé et al. 2016). Besides, AM fungal metabolism greatly depends on C supply which is derived from host photosynthesis (Douds et al. 2000).

8.3.5 P content of *R. mucronata*

A significant difference in leaf, stem, and root P of *R. mucronata* was found among the various treatments ($p < 0.05$) (**Fig. 8.5**). The total content of P in T_7 (14.7 µg/g) was significantly higher. Similar results were recorded in *Zea mays* inoculated with AM fungi and rhizobacteria (Wu et al. 2005).

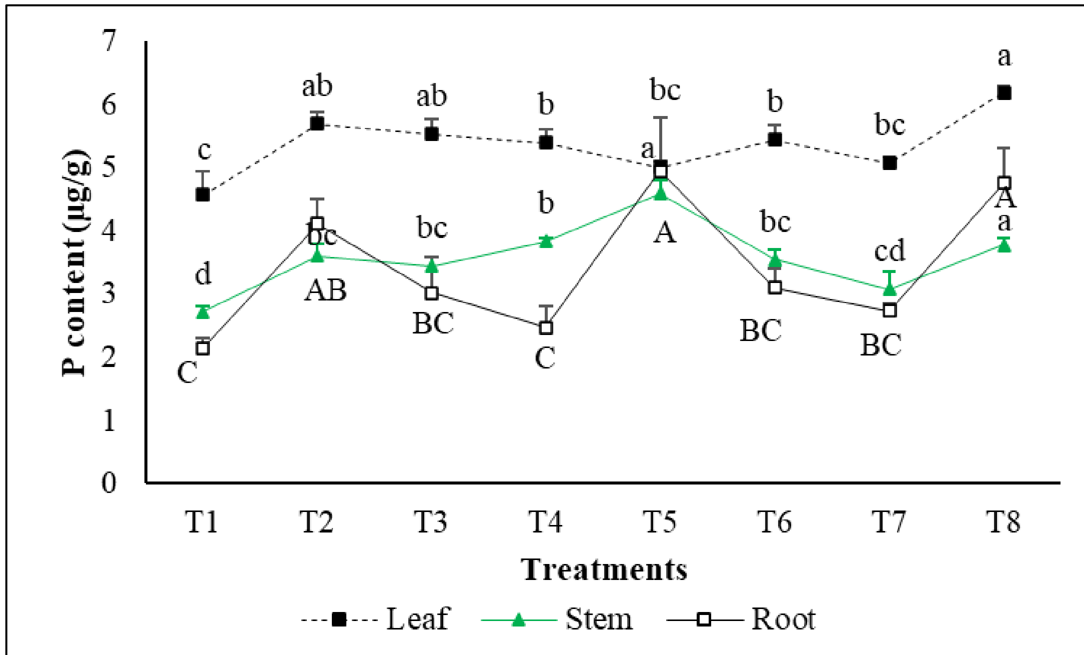


Fig. 8.5: P content in inoculated *R. mucronata* plants. Values in each line followed by different uppercase and lowercase letters are significantly different at $p < 0.05$.

Phosphorus is a vital nutrient being involved in various metabolic functions in plants and also it is a major component of crucial plant molecules (Bashan et al. 2013). Increased P content in T_5 can be attributed to the increased root length, as P uptake is known to increase with root length (Jungk and Claassen 1989). Such morphological changes in root resulting in longer and branched root systems are efficacious in nutrient acquisition (D'Souza and Rodrigues 2017). Besides, it is well evident that the P content of plants elevates when inoculated with PSB (Shrivastava et al. 2018) as these bacteria are capable of solubilizing insoluble P thereby resulting in improved P uptake and plant growth. The synergistic relationship between AM fungi and PSB enables AM fungi to translocate P that is solubilized by PSB to their host plant (Piccini and Azcon 1987).

AM fungal hyphae contribution (HC) to the total plant acquisition was found to be maximum in treatment 5 and 7 while it was least in treatment 4 (**Fig. 8.6**). AM fungal HC is the transfer of P by AM fungi from outer compartments to their host plant (Kothari et al. 1991).

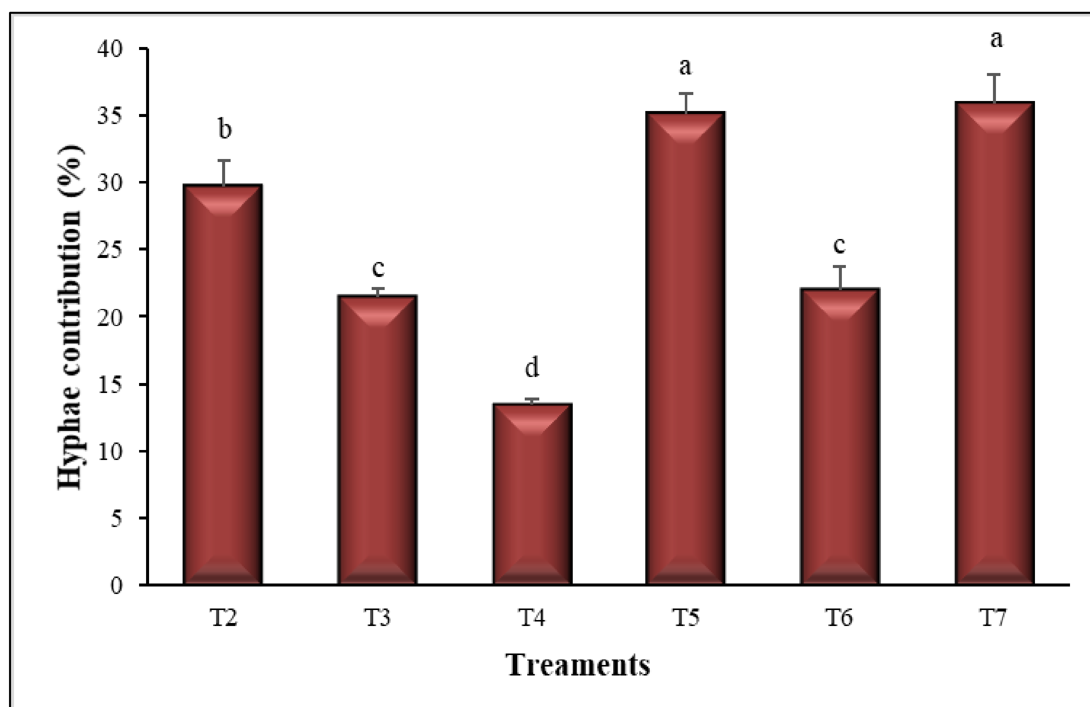


Fig. 8.6. Hyphae contribution (HC) in AM inoculated plants. (Values in each line followed by different lowercase letters are significantly different at $p < 0.05$).

8.3.6 Correlation analysis

The results of Pearson's correlation analysis are presented in **Table 8.3**. Total biomass was positively correlated with MD values; root P was positively correlated with RC and total chlorophyll. Leaf P content was positively correlated with total chlorophyll content.

Table 8.3: Pearson's correlation coefficients between different parameters in bio-inoculant treatments of *R. mucronata*.

	Total biomass	RC	Leaf P	Stem P	Root P	Total Chl
Total biomass	-	0.432	0.112	0.421	0.264	0.215
MD	0.772*	0.806	0.196	0.309	0.517	0.215
RC	0.432	-	0.513	0.409	0.765*	0.578
Total Chl	0.215	0.578	0.802**	0.378	0.637*	-

Note: MD = mycorrhizal dependency, RC = root colonization, Total Chl = total chlorophyll.

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

8.4 CONCLUSION

Based on the synergistic relationship of mangroves with AM fungi and PSB, the screening experiment was conducted to study the impact of these bio-inoculants on the growth and nutrient enhancement in *R. mucronata* plants. The present study is the first to use *B. halotolerans* as a bioinoculant for the investigation of mangrove plant growth. Also, this is the first study to evaluate the co-inoculation effects of AM fungi and PSB on the growth, biomass, and nutrient uptake of mangrove plants. The study revealed positive effects of dual inoculation on the growth promotion of *R. mucronata*. The interaction between AM fungi and PSB assist in the biogeochemical cycling of immobile nutrients especially P. The use of such microbial consortium may be a promising strategy to increase the growth and biomass of plants in the environmental restoration program. The present study is a step in that direction. As mangroves act as coastal and estuarine bio shields against harsh conditions, further research needs to be carried out in the field to determine the microbial function in the reconstruction of the mangrove ecosystem.

Chapter 9: Summary

Mangroves are a highly productive wetland ecosystem serving as a bio-shield of coastline. They are rich and diverse in living resources and hence increase the economic and ecological value of the ecosystem. Besides, the mangrove ecosystems have become a focus of conservation and environmental issues. However, they have been degraded drastically all over the world, mainly due to nutrient limitations (P deficiency) and human interference. Arbuscular mycorrhizal (AM) fungi are the 'hidden heroes' of nutrient-deficient soil (especially P) which helps in increased uptake of nutrients and improves stress tolerance in exchange for carbon.

To establish conservation strategies, it is important to explore the ecological framework of the habitat. The mangrove patch of about 178 ha at Chorao Island has been declared as Reserved Forest, while Pichavaram has the World's second-largest mangrove forest. However, both these forests have not been well explored for the presence of AM fungal occurrence. Accordingly, an attempt was made to investigate the AM fungal diversity and to record the dominant AM fungal species associated with the mangrove plants of Chorao Island and Pichavaram Forest (which were earlier reported as non-mycorrhizal). Further, some of the AM species recovered from the mangrove ecosystem were monoxenically cultivated. Correspondingly, the screening studies were carried out to examine the effect of the AM bio-inoculant on the growth and nutrient uptake of *R. mucronata*.

Physico-chemical analyses of Chorao soils revealed that the soil texture of true mangrove areas was clayey silt while that of associate mangrove areas have equivalent amounts of sand, silt, and clay. Chorao soils were acidic whereas the soils of Pichavaram forest were alkaline. Both the sites were low in available nutrients.

AM fungal association in different plant species was exhibited by the presence of hyphal, arbuscular, and/or vesicular colonization. At Chorao Island, the highest AM root colonization was reported in *Thespesia populnea*. Overall, associate mangroves showed higher AM colonization than true mangroves. Spore density was maximum in the rhizosphere of a true mangrove *Ceriops tagal* and the highest AM species richness was recorded in *Kandelia candel* with 13 spore morphotypes.

At Pichavaram forest, rhizosphere and root samples of mangrove plants were collected from three different sites *viz.,* Pichavaram extension (PE), Pichavaram Reserved Forest (PRF), and Killai Reserved Forest (KRF). The highest AM root colonization was recorded

in *Salicornia brachiata* at KRF, while the AM spore density was maximum in *Ceriops decandra* at PE.

A total of 31 AM species belonging to 9 genera with *Acaulospora dilatata* being the dominant species were recovered from Chorao Island. While 21 AM species belonging to seven genera were recovered from the Pichavaram forest. At PE, *Rhizophagus fasciculatus* was the dominant AM species whereas, *Funneliformis geosporum* was dominant at KRF. Diversity indices were highest in *K. candel* at Chorao Island while at Pichavaram, it was maximum at site PE. Jaccard index showed the greatest AM fungal similarity between *Avicennia officinalis* and *A. marina* from Chorao Island. Cluster analysis was performed based on AM fungal relative abundance (RA) in true- and associate-mangrove plants of Chorao Island. It indicated the four clusters of all the plants at a 26% similarity level.

Comparative studies on seasonal variation in AM fungal diversity were carried out among the three common mangrove species *viz., A. marina, Bruguiera cylindrica,* and *Excoecaria agallocha* from Chorao Island and Pichavaram forest. Results of seasonal studies revealed the highest percent root colonization in *E. agallocha* at Chorao Island in the post-monsoon season. The spore density was high during post-monsoon at both sites. Nineteen AM species belonging to four families were recovered from both the sites, in different host plants during different seasons. Of the 19 AM species, four AM species *viz., A. undulata, Entrophospora* sp., *F. geosporum,* and *R. fasciculatus* were recorded in nearly all the seasons from both the sites. Canonical correspondence analysis (CCA) of AM fungal abundance and soil parameters indicated that a significant effect of organic carbon (OC), N, Mn, Zn, Cu, and Fe on the abundance of *Acaulospora, Funneliformis, Gigaspora,* and *Sclerocystis.* Whereas, *Rhizophagus, Glomus,* and *Entrophospora* experienced the greater effect of electrical conductivity (EC).

The study represented the influence of several edaphic factors on variation in diversity, colonization, and spore density of mangrove plants from Chorao Island and Pichavaram forest. Monospecific cultures of 17 (out of 35) AM fungal species were successfully prepared using *Plectranthus scutellarioides* (L.) R. Br. as a catch plant. Following are the names of AM species: *Acaulospora dilatata, A. foveata, A. scrobiculata, A. undulata, A. delicata, A. rehmii, A. myriocarpa, A. spinosa, Entrophospora* sp., *Funneliformis geosporum, F. mosseae, Gigaspora decipiens, Gi. albida, Rhizophagus fasciculatus, R. intraradices, R. irregulare,* and *Sclerocytis rubiformis.*

Some of these AM species were further multiplied using modified methods of monoxenic technique. Nine AM species *viz., Rhizophagus intraradices, Rhizophagus clarus, Rhizophagus fasciculatus, Funneliformis mosseae, Acaulospora spinosa, Gigaspora albida, Gigaspora decipiens, Dentiscutata scutata,* and *Racocetra gregaria* could germinate on MSR (Modified Strullu and Romand) medium. Germination of *A. spinosa* and *D. scutata* was reported for the first time. A dual culture of *Gi. decipiens* with transformed Linum roots and *R. intraradices* with transformed roots of Chicory were established monoxenically. Germinated spores of *Gi. decipiens* colonized the transformed roots of Linum. However, no sporulation was observed. While in the case of *R. intraradices*, sporulation occurred within 30 days after the preparation of dual culture.

Two PSB (PSB_1 and PSB_2) isolated from the two mangrove plants *viz., E. agallocha* and *Clerodendrum inerme* belonged to the genus *Bacillus*. Based on the biochemical and molecular analysis, PSB_1 was identified as *B. subtilis* while PSB_2 was identified as *B. halotolerans*. Qualitative and quantitative estimation of phosphate solubilization was tested which indicated *B. haltolerans* was more efficient in solubilizing the mineral phosphate.

To prepare inocula for screening studies, two AM species *viz., R. intraradices* and *A. dilatata* were mass multiplied using a carrier formulation given by Rodrigues and Rodrigues (2017). The carrier materials used for mass production included vermiculite: cow dung powder: wood powder: wood ash in the ratio of 20:8:2:1. Also, the inoculum of PSB_2 was prepared in Nutrient broth with the final concentration of bacterial inoculum having 10^8 CFU/mL.

Studies were carried out to examine the effect of bio-inoculants on the growth and P uptake of *Rhizophora mucronata* seedlings. An experiment with 8 treatments of single and dual inoculation involving AM fungal species *Rhizophagus intraradices, Acaulospora dilatata,* and PSB *Bacillus halotolerans* inoculating the propagules of *R. mucronata* was set in the polyhouse. The study revealed positive effects of dual inoculation with AM fungi and PSB in the growth promotion of *R. mucronata*. Various parameters *viz.,* AM fungal colonization, plant growth, biomass, leaf pigments, and P contents were analyzed. Maximum AM fungal root colonization was recorded in treatment 8 (*R. intraradices* + *A. dilatata* + PSB). Treatment 7 (*R. intraradices* + *A. dilatata*) significantly promoted plant height. However, *R. intraradices* inoculum in combination with PSB recorded a better impact on all the growth parameters. Aboveground and belowground plant biomass was higher in treatment 7 and treatment 8, respectively. Chlorophyll and carotenoids content

was significantly higher in treatment 8. A dual inoculation of *R. intraradices* and *B. halotolerans* (treatment 5) and combined inoculation of all the three bio-inoculants (treatment 8) resulted in increased uptake of P. The total P content in treatment 5 and treatment 8 reached a maximum of 14.6 and 14.7 µg/g, respectively. The use of such a microbial consortium may be a promising strategy to increase plant growth and biomass in environmental restoration programs.

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- Gaonkar S and Rodrigues BF. 2020. Diversity of arbuscular mycorrhizal (AM) fungi in mangroves of Chorao Island, Goa, India. Wetlands Ecol. Manage. 28:765–778.
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- Gaonkar S and Rodrigues BF. 2021. Arbuscular mycorrhizal fungal status in mangroves of Pichavaram Forest, Tamil Nadu, India. Trop Ecol 62:538–548.

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1. Gaonkar S and Rodrigues BF. 2015. Diversity of Arbuscular Mycorrhizal (AM) fungi from mangroves of Chorao Island, Goa. In: Asian Mycological Congress. Co-organized by Department of Botany, Goa University, Goa, Asian Mycological Association (AMA), and Mycological Society of India (MSI) from 7th-10th October 2015 (Poster).
2. Gaonkar S and Rodrigues BF. 2016. Diversity of Arbuscular Mycorrhizal (AM) fungi from mangroves of Pichavaram forest, Tamil Nadu, India. In: National Symposium on Challenges to Plant Pathologists under Changing Disease Scenario. Co-organized by Department of Plant Pathology, Punjab Agricultural University, Ludhiana and Department of Botany, Goa University, Goa from 5th-7th October 2016 (Poster).
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4. Gaonkar S and Rodrigues BF. 2017. Seasonal Variations and Distribution of Arbuscular Mycorrhizal (AM) Fungi in Mangroves of Chorao Island, Goa. In: National conference on Mangrove ecosystems, organized by Mangrove Society of India and NIO, Goa from 26th-27th July 2017 (Oral).
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Plate 3.1: Mangrove habitat at Chorao Island

Plate 3.2: Mangrove habitat at Pichavaram Forest

Plate 3.3: Mangrove species: a. *Aegiceras corniculatum* (L.) Blanco.; **b.** *Avicennia officinalis* L.; **c.** *Avicennia marina* (Forssk.) Vierh.; **d.** *Bruguiera cylindrica* (L.) Blume; **e.** *Ceriops decandra* (Griff.) W. Theob.; **f.** *Ceriops tagal* (Perr.) C.B. Rob.

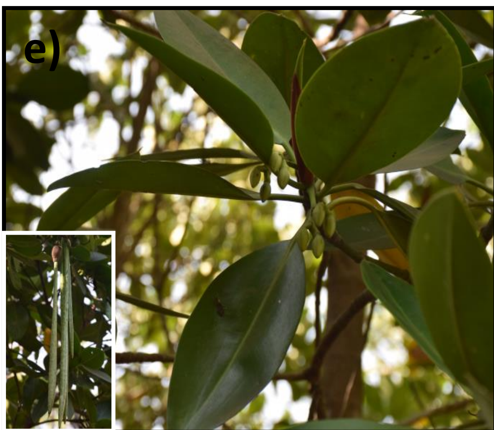


Plate 3.4: Mangrove species: a. *Excoecaria agallocha* L.; **b.** *Kandelia candel* (L.) Druce; **c.** *Lumnitzera racemosa* Willd.; **d.** *Rhizophora apiculata* Blume.; **e.** *R. mucronata* Lam.; **f.** *Sonneratia alba* Sm.

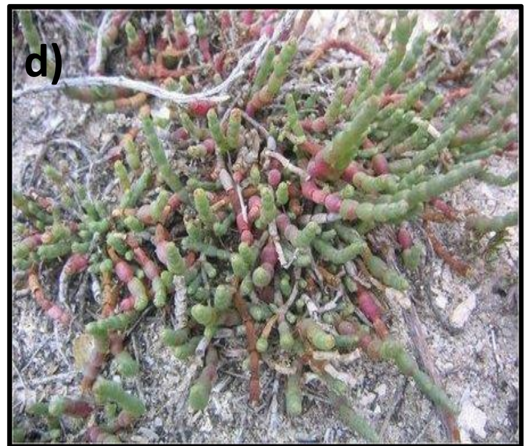


Plate 3.5: Mangrove species: a. *Sonneratia caseolaris* (L.) Engl.; **b.** *Acrostichum aureum* L.; **c.** *Acanthus ilicifolius* L.; **d.** *Arthrocnemum indicum* (Willd.) Moq.; **e.** *Clerodendrum inerme* (L.) Gaertn.; **f.** *Derris heterophylla* (Willd.) K. Heyne

Plate 3.6: Mangrove species: a. *Ipomoea pes-caprae* (L.) R. Br.; **b.** *Pongamia pinnata* (L.) Pierre; **c.** *Salicornia brachiata* (Willd.) Paul G.Wilson.; **d.** *Salvadora persica* L.

Plate 3.7: Mangrove species: a. *Sesuvium portulacastrum* (L.) L.; **b.** *Suaeda maritima* Forssk. ex J.F.Gmel.; **c.** *Thespesia populnea* (L.) Sol. ex Corrêa.

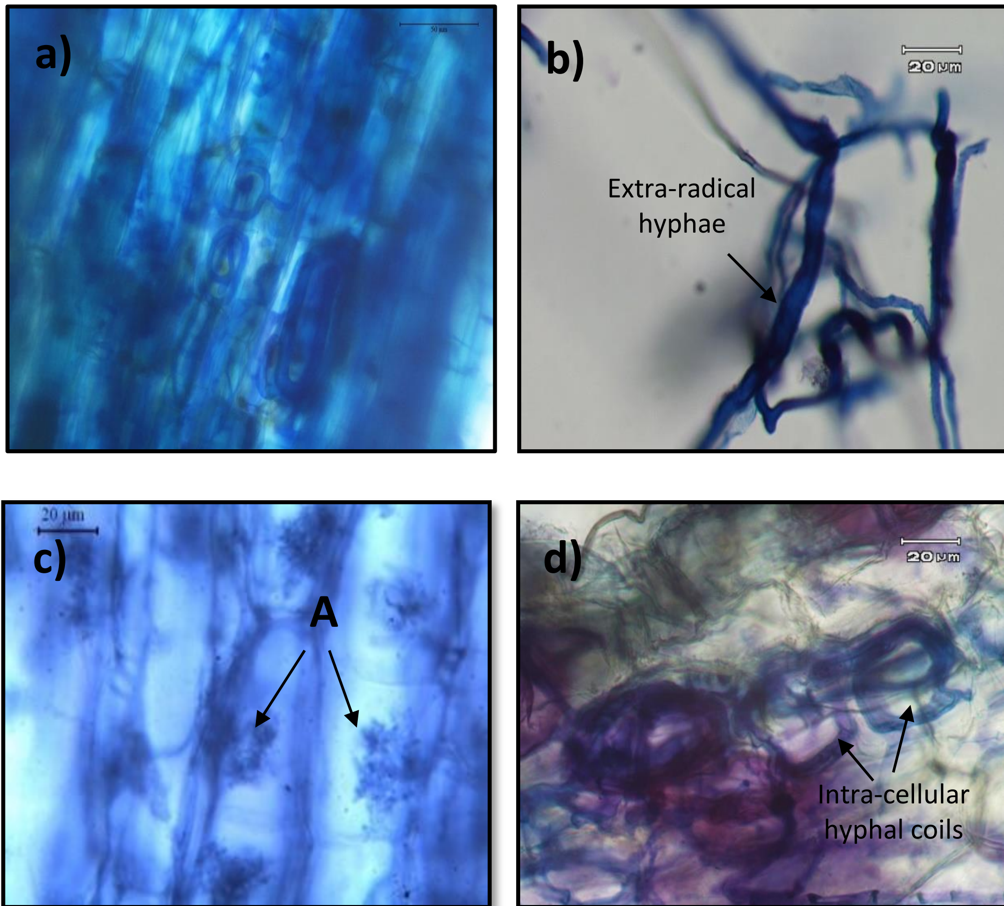


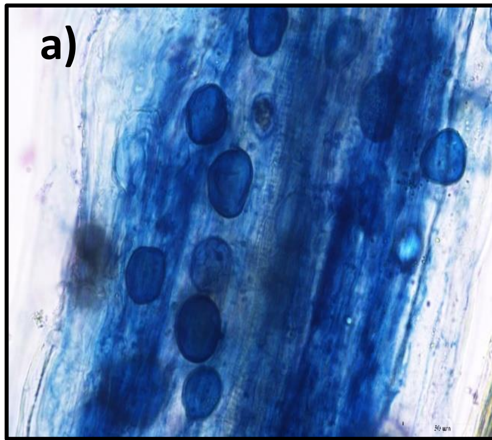
Plate 3.8: Intra- and extra-radical structures of AM fungi in roots.

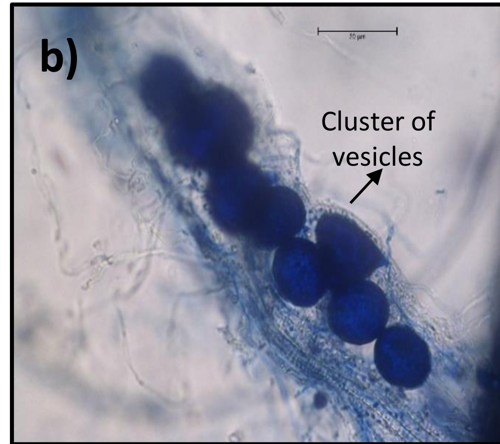
a. Hyphal coils in roots of *Avicennia marina* (Forssk.) Vierh.

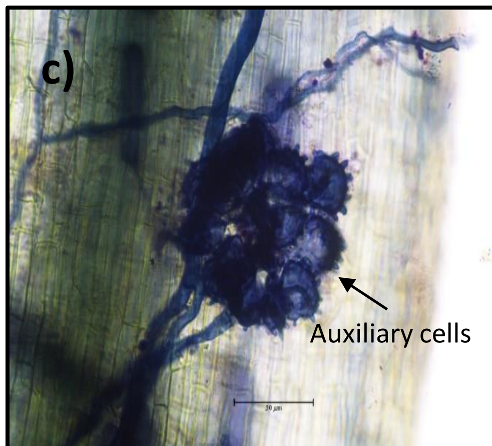
b. Extra-radical hyphae in *Ceriops tagal* (Perr.) C.B. Rob.

c. *Arum*-type of arbuscular colonization in roots of *E. agallocha* L.

d. *Paris*-type of arbuscular colonization in roots of *C. tagal* (Perr.) C.B. Rob.







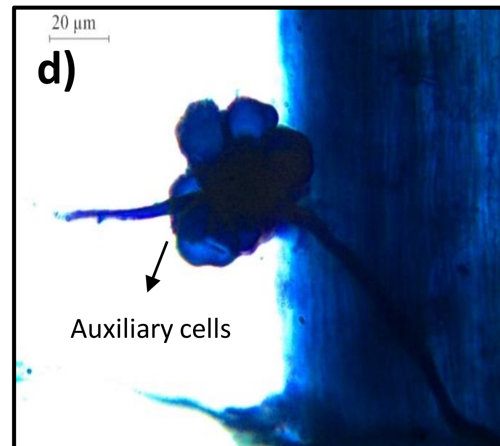


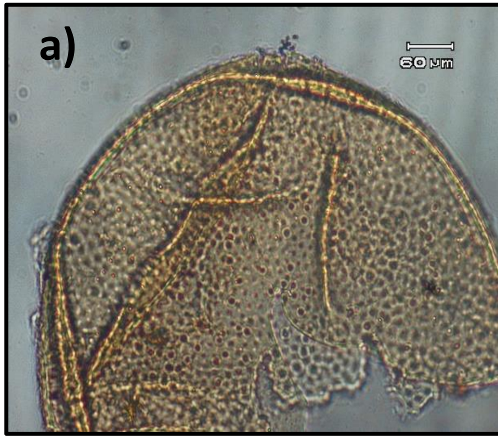
Plate 3.9: Intra- and extra-radical structures of AM fungi in roots.

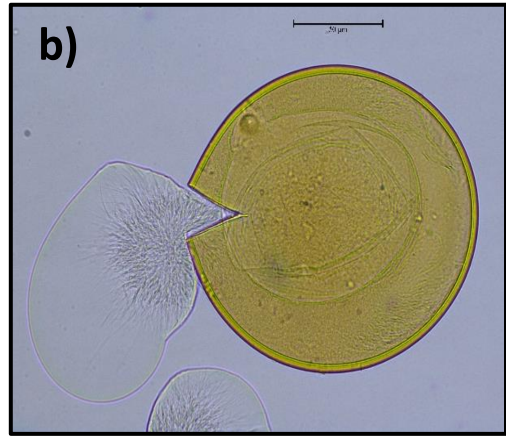
a. Vesicular colonization in roots of *Clerodendrum inerme* (L.) Gaertn.

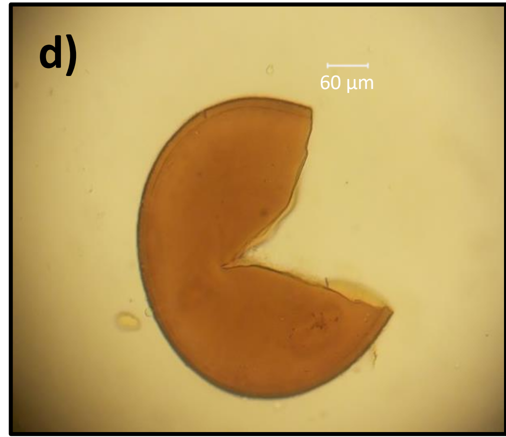
b. Cluster of globose vesicles in roots of *Avicennia officinalis* L.

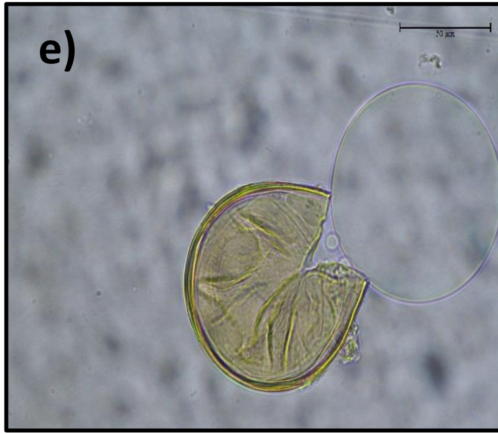
c. Spiny/papillate auxiliary cells in roots of *Salicornia brachiata* (Willd.) Paul G.Wilson.

d. Knobby auxiliary cells in roots of *S. caseolaris* (L.) Engl.









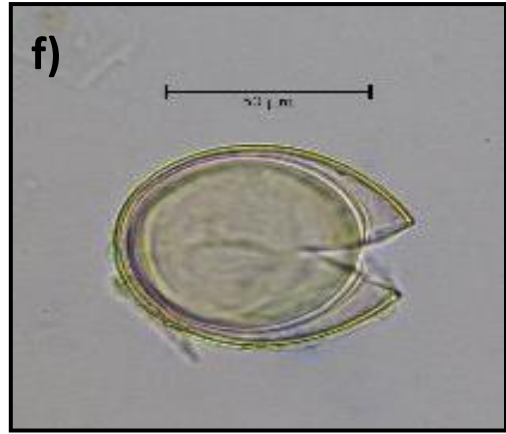


Plate 3.10: AM fungal species: a. *Acaulospora bireticulata* Rothwell & Trappe.; **b.** *A. dilatata* Morton.; **c.** *A. foveata* Trappe & Janos.; **d.** *A. laevis* Gerd. & Trappe.; **e.** *A. mellea* Spain & Schenck; **f.** *A. polonica* Błaszk.

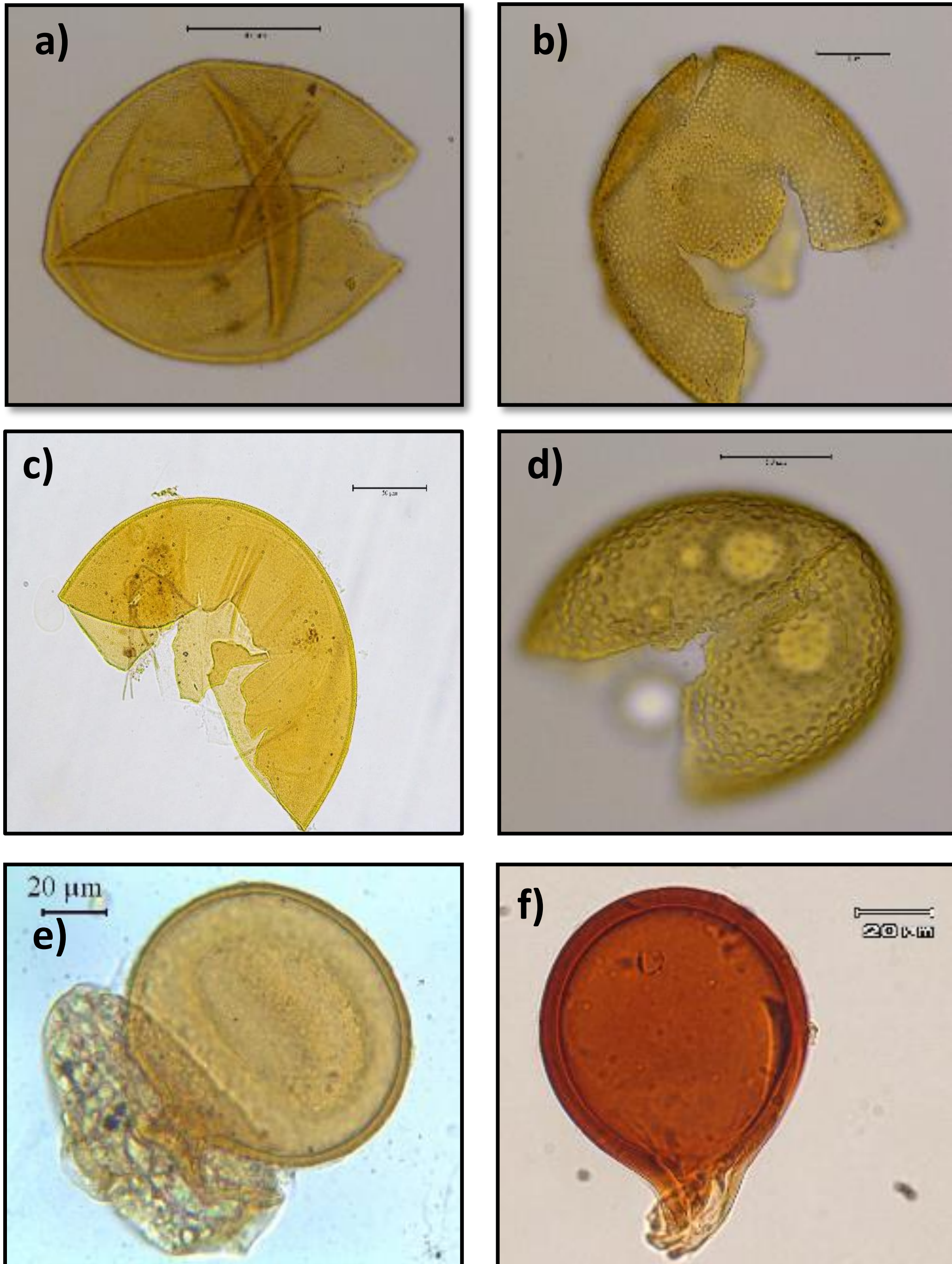


Plate 3.11: AM fungal species: a. *Acaulospora rehmii* Sieverd. & Toro.; **b.** *A. scrobiculata* Trappe.; **c.** *A. spinosa* Walker & Trappe.; **d.** *A. undulata* Sieverd.; **e.** *Entrosphospora* sp.; **f.** *Funneliformis geosporum* (Nicolson & Gerd.) Walker & Schüßler.

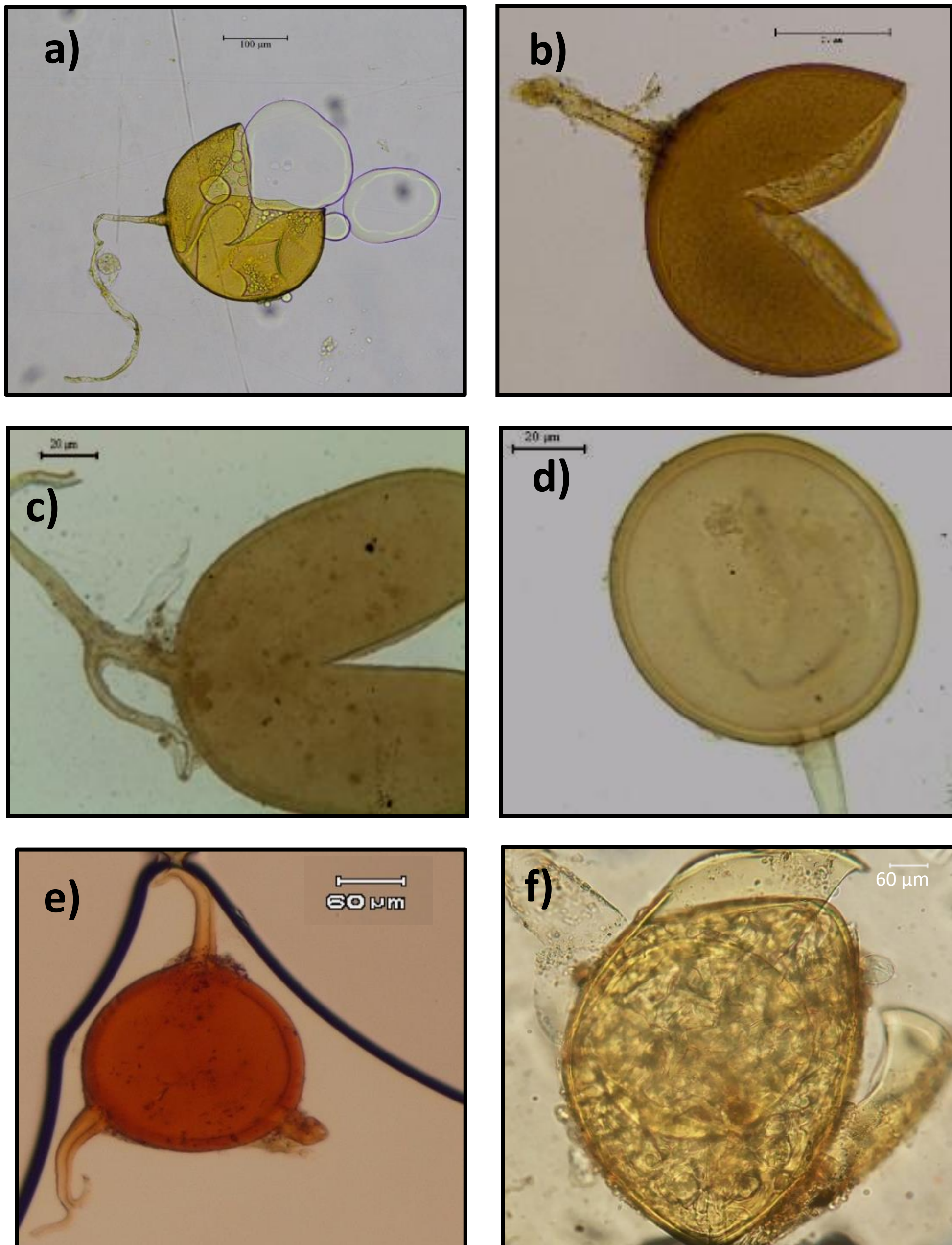
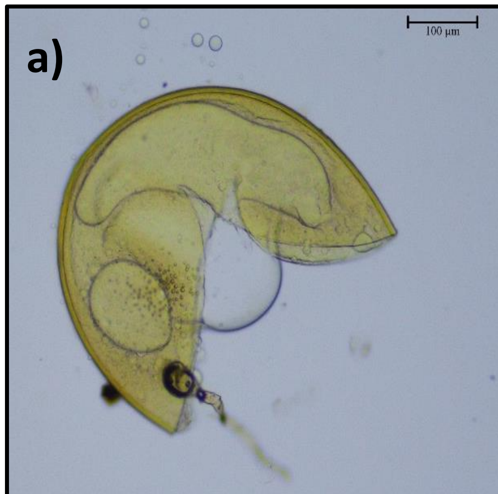
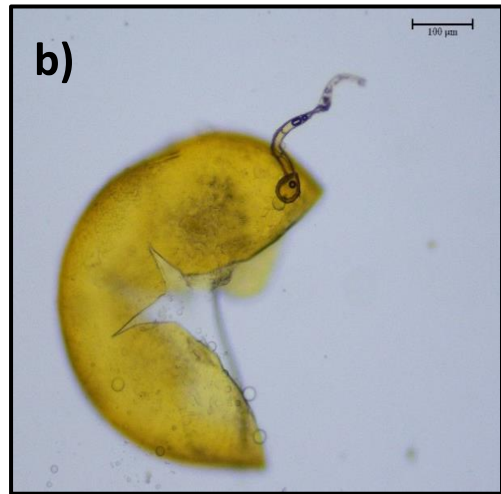
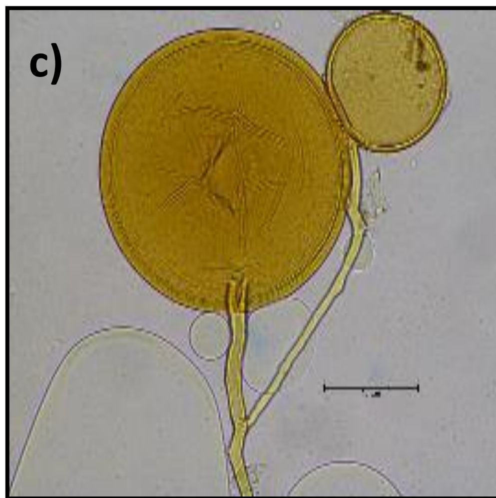


Plate 3.12: AM fungal species: a. *Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler.; **b.** *Glomus macrocarpum* Tul. & Tul.; **c.** *G. flavisporum* (Lange & Lund) Trappe & Gerd.; **d.** *G. microcarpum* Tul. & Tul.; **e.** *G. multicaule* Gerd. & Bakshi.; **f.** *G. tortuosum* Schenck & Sm.







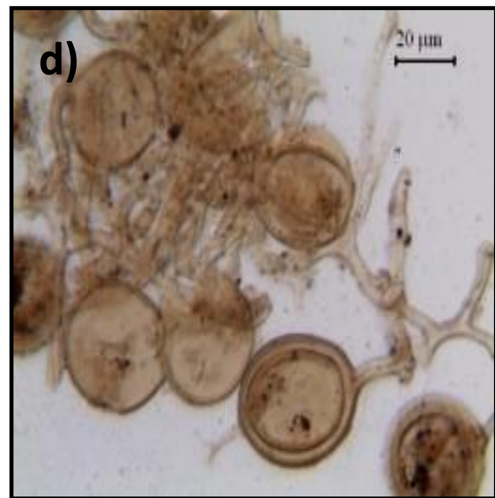


Plate 3.13: AM fungal species: a. *Gigaspora albida* Schenck & Sm.; **b.** Broken spore of *Gi. decipiens* Hall & Abbott.; **c.** Spore of *Rhizophagus fasciculatus* (Thaxt.) Walker & A. Schüßler.; **d.** Spore cluster of *R. intraradices* (Schenck & Sm.) Walker & Schüßler.

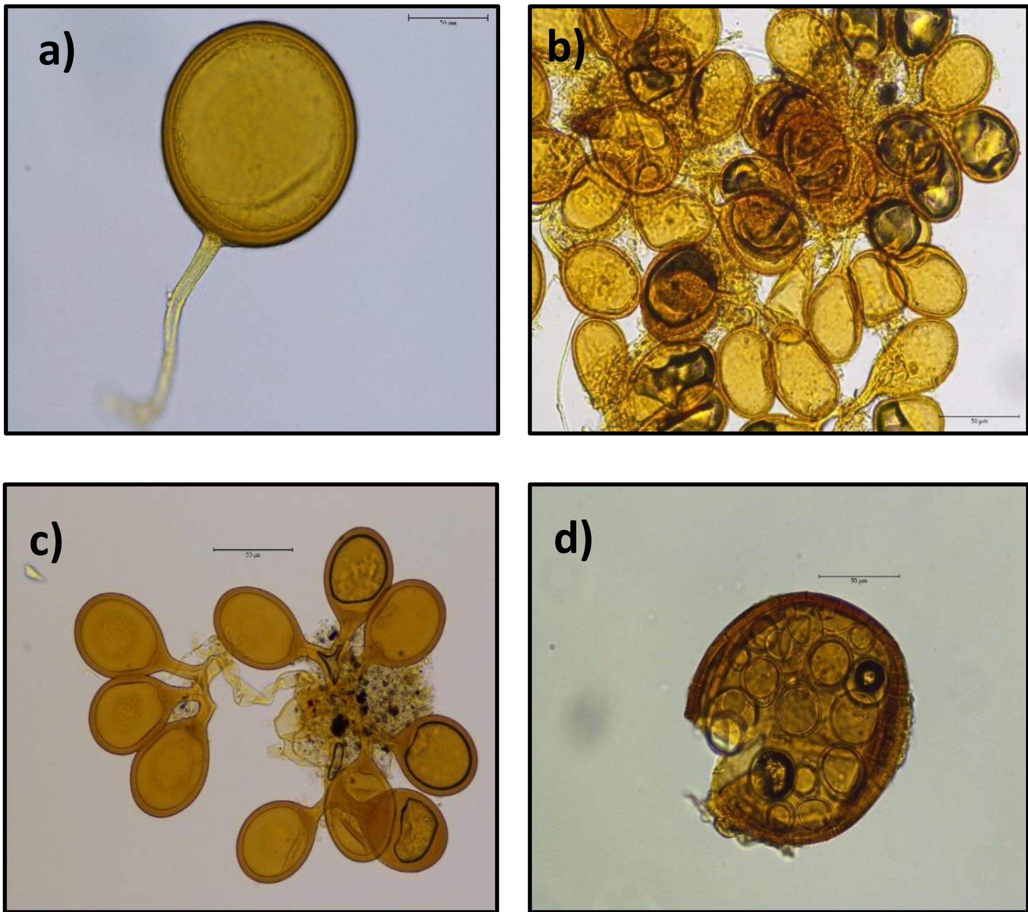


Plate 3.14: AM fungal species: a. *Rhizophagus irregulare* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler.; **b.** *Sclerocystis pachycaulis* Wu & Chen.; **c.** *S. rubiformis* Gerd. & Trappe.; **d.** Spores in spore syndrome.

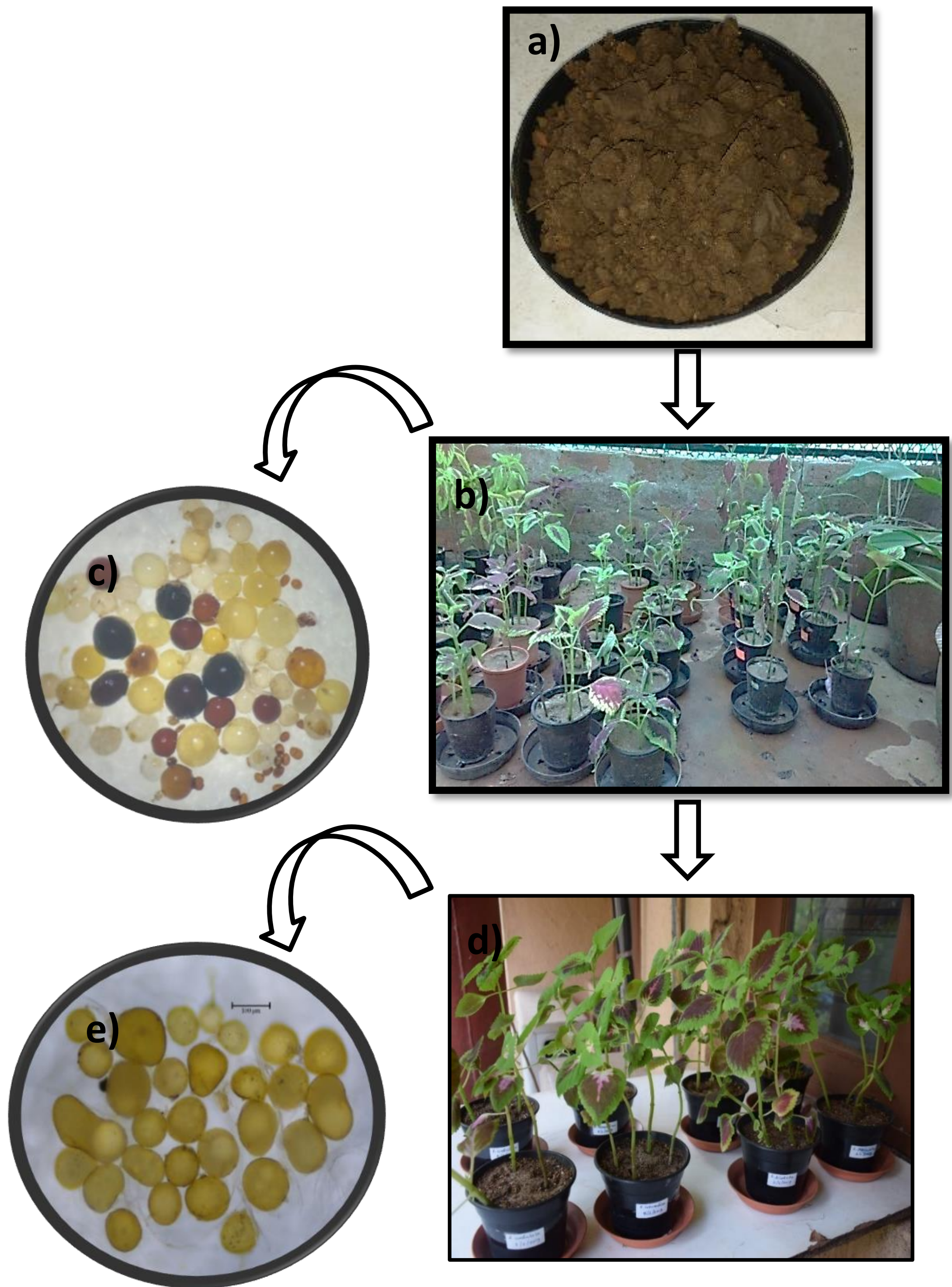


Plate 4.1: Trap and monospecific cultures: a. Rhizosphere sample; **b.** Trap cultures; **c.** Spores from trap cultures; **d.** Monospecific cultures; **e.** Spores from monospecific cultures.

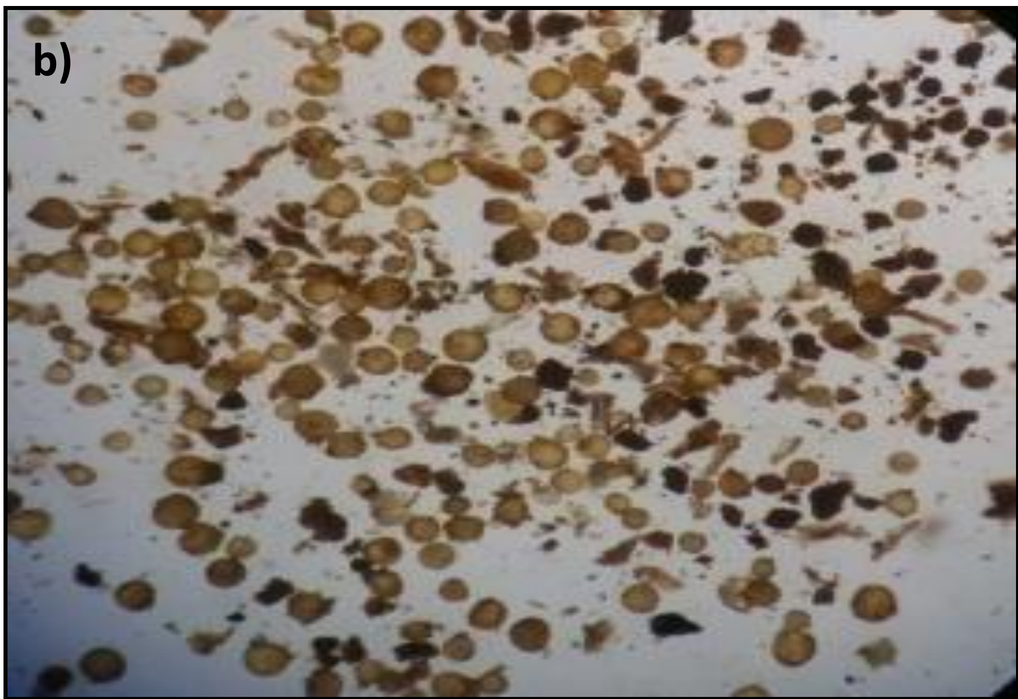


Plate 5.1: Propagules used for monoxenic cultures: a. Colonized root fragments; **b.** spore isolated from monospecific cultures.

Plate 5.2: Ri T-DNA transformed roots growing on MSR medium.: a. Linum *(Linum usitatissimum* L.); **b.** Chicory *(Cichorium intybus* L.).

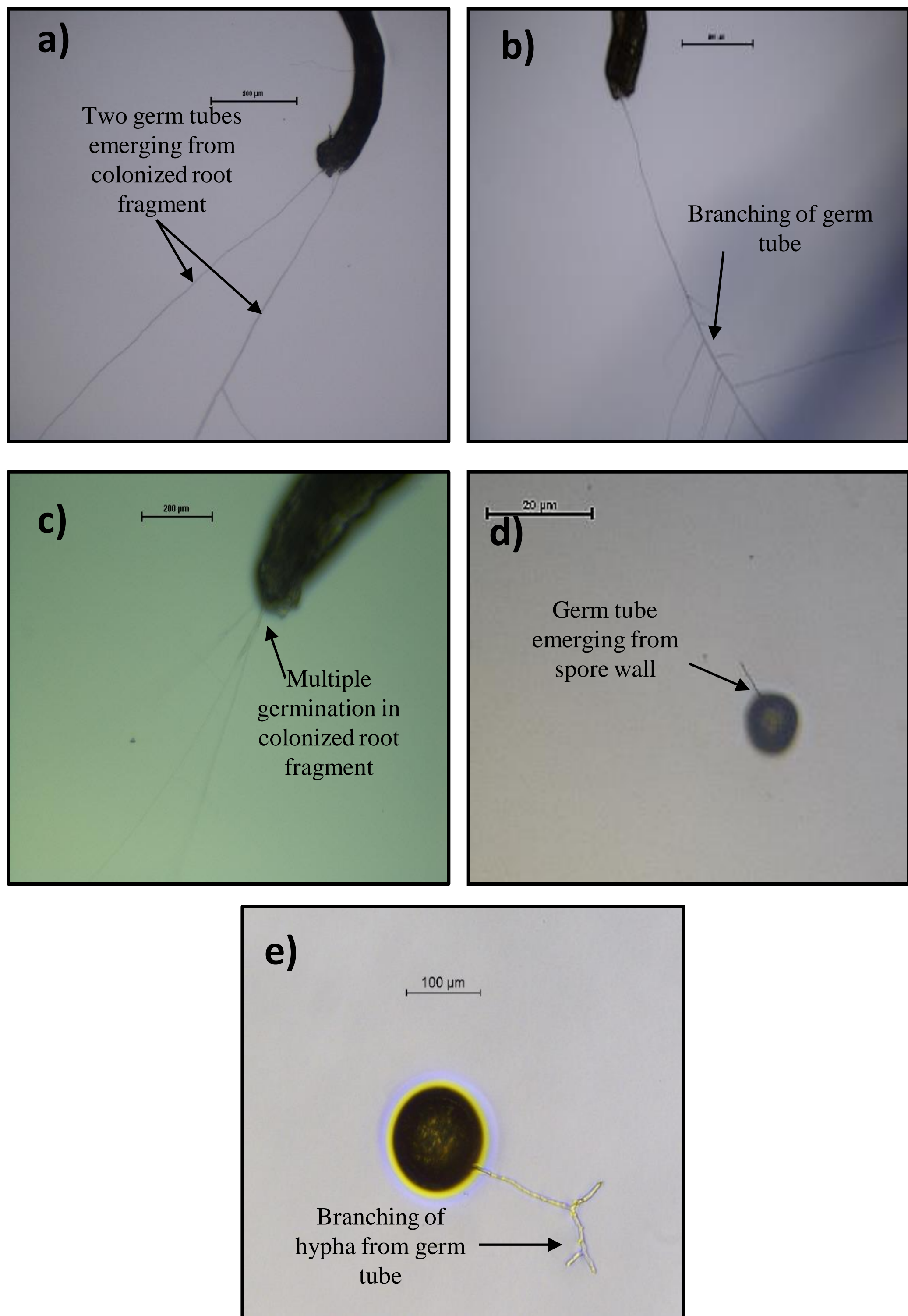
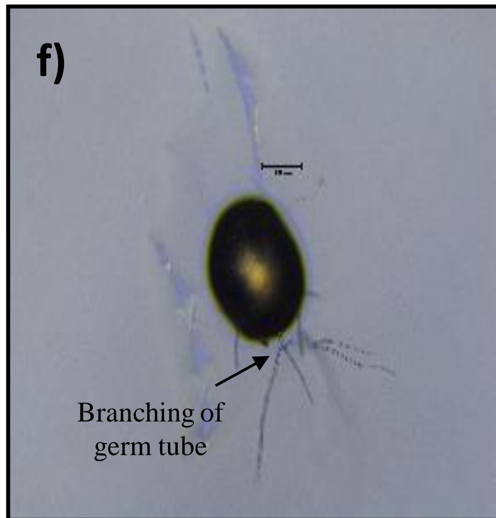
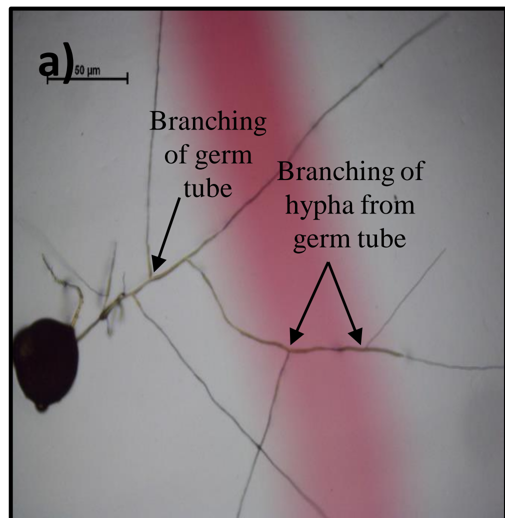
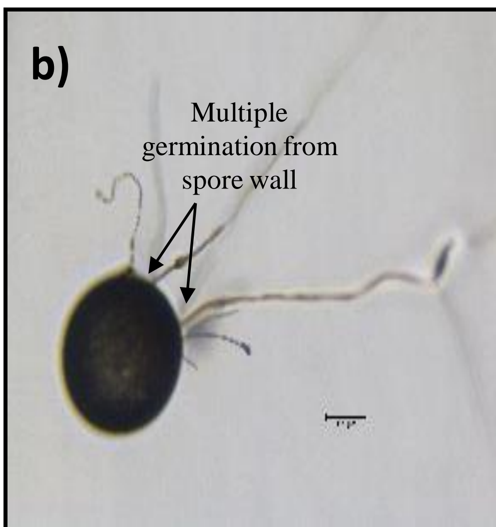


Plate 5.3: AM fungal propagule (colonized roots and spores) germination on MSR (-sucrose) medium: a. *Rhizophagus intraradices*; **b.** *Rhizophagus clarus*; **c.** *Rhizophagus fasciculatus*; **d.** *Funneliformis mosseae*; **e.** *Acaulospora spinosa*; **e.** *Gigaspora albida*







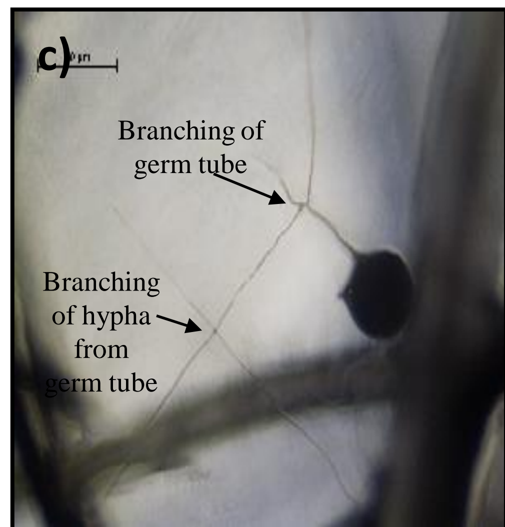


Plate 5.4: AM fungal propagule (colonized roots and spores) germination on MSR (-sucrose) medium: a. *Gigaspora decipiens;* **b.** *Scutellospora scutata*; **c.** *Racocetra gregaria*

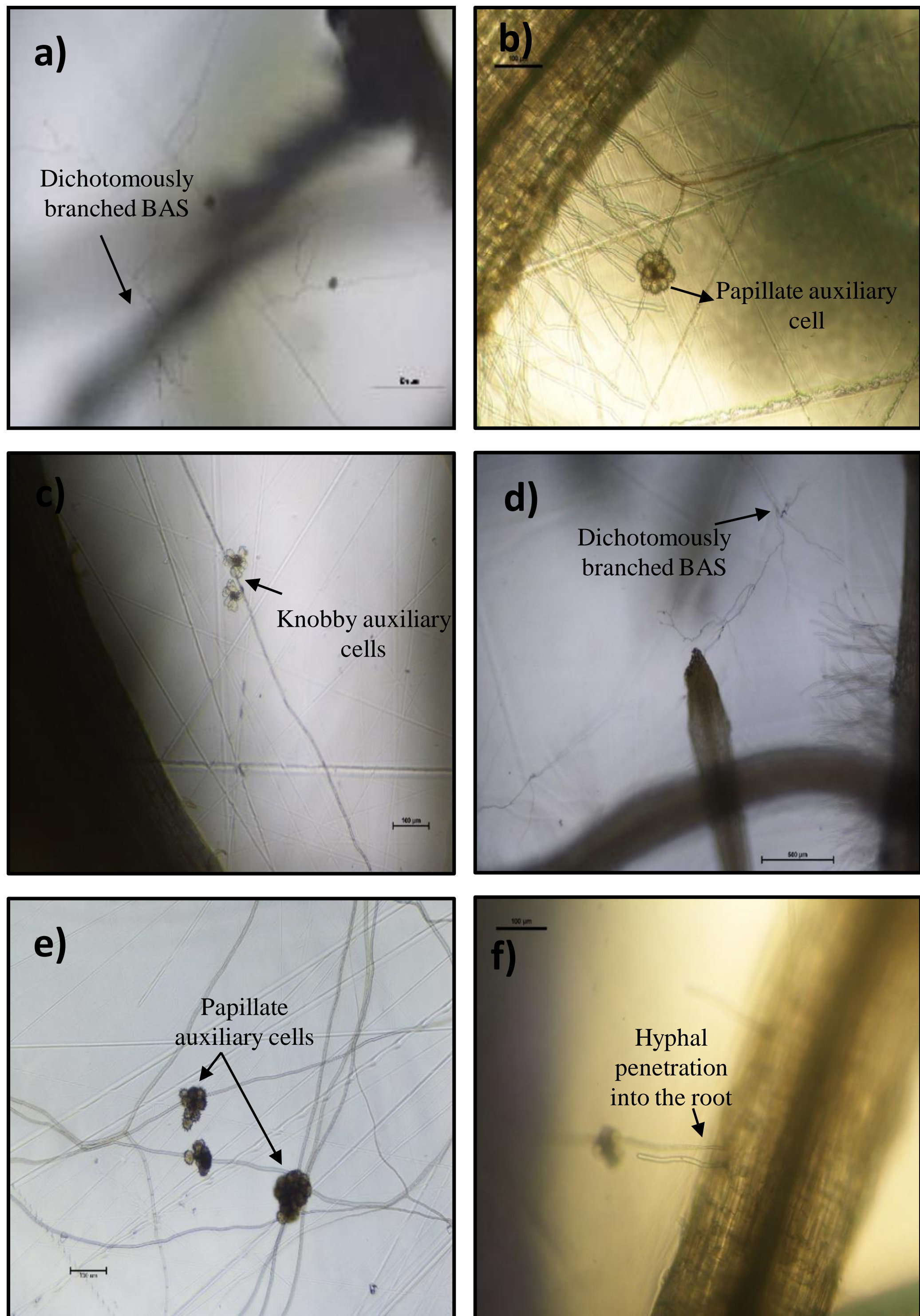


Plate 5.5: Monoxenic culture of AM species with transformed roots: a. Branched absorbing structures (BAS) in *Gigaspora albida*; **b.** Auxiliary cells in *Gigaspora albida*; **c.** Auxiliary cells in *Scutellospora scutata*; **d.** BAS in *Gigaspora decipiens*; **e.** Auxiliary cells in *Gigaspora decipiens*; **f.** Colonization of transformed Linum roots by *Gigaspora decipiens.*

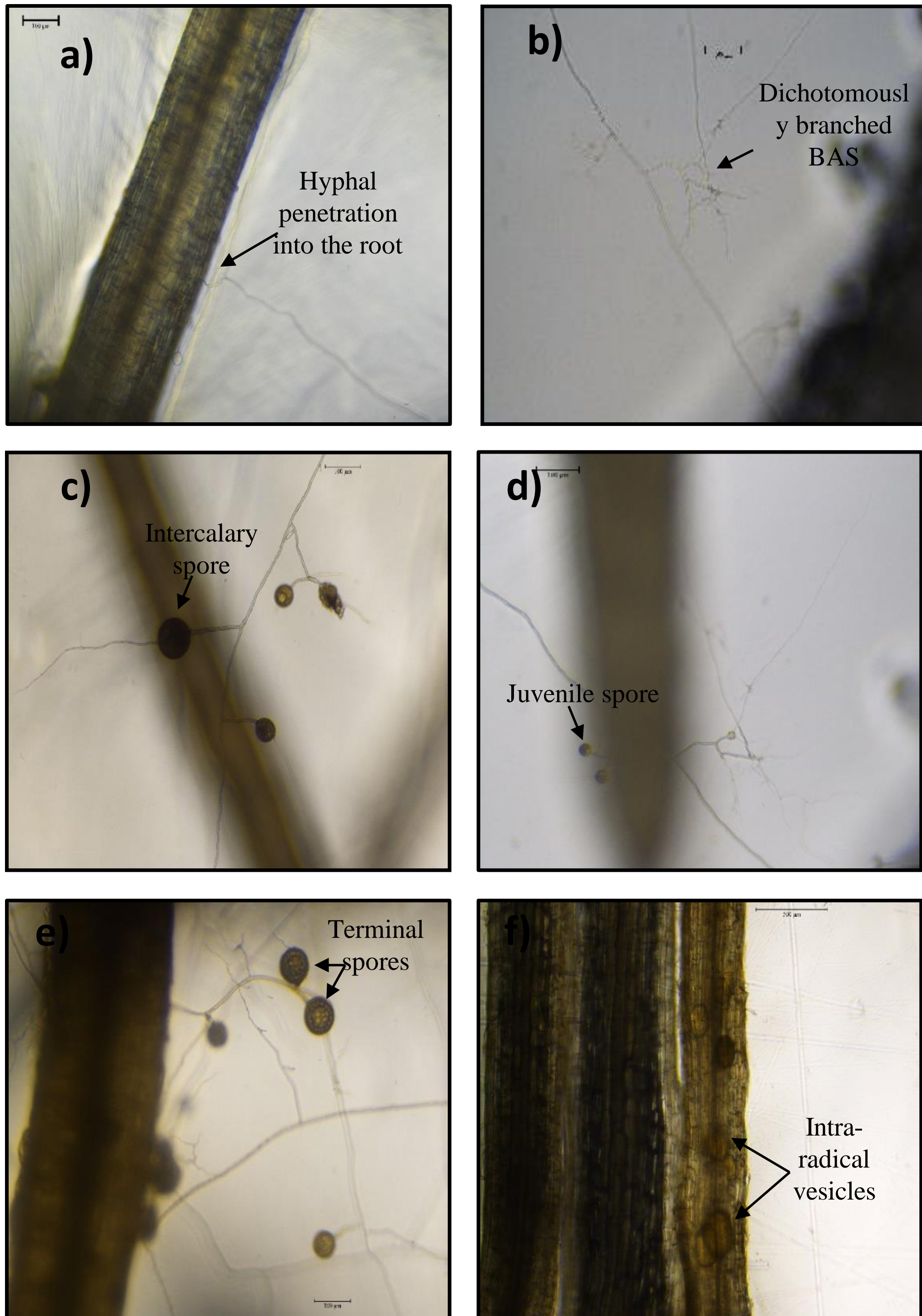


Plate 5.6: Monoxenic culture of *Rhizophagus intraradices* with transformed Chicory roots: a. Contact with roots; **b.** Branched absorbing structures; **c.** Intercalary spores; **d.** Juvenile spores; **e.** Terminal spores; **f.** Intra-radical vesicles in roots.

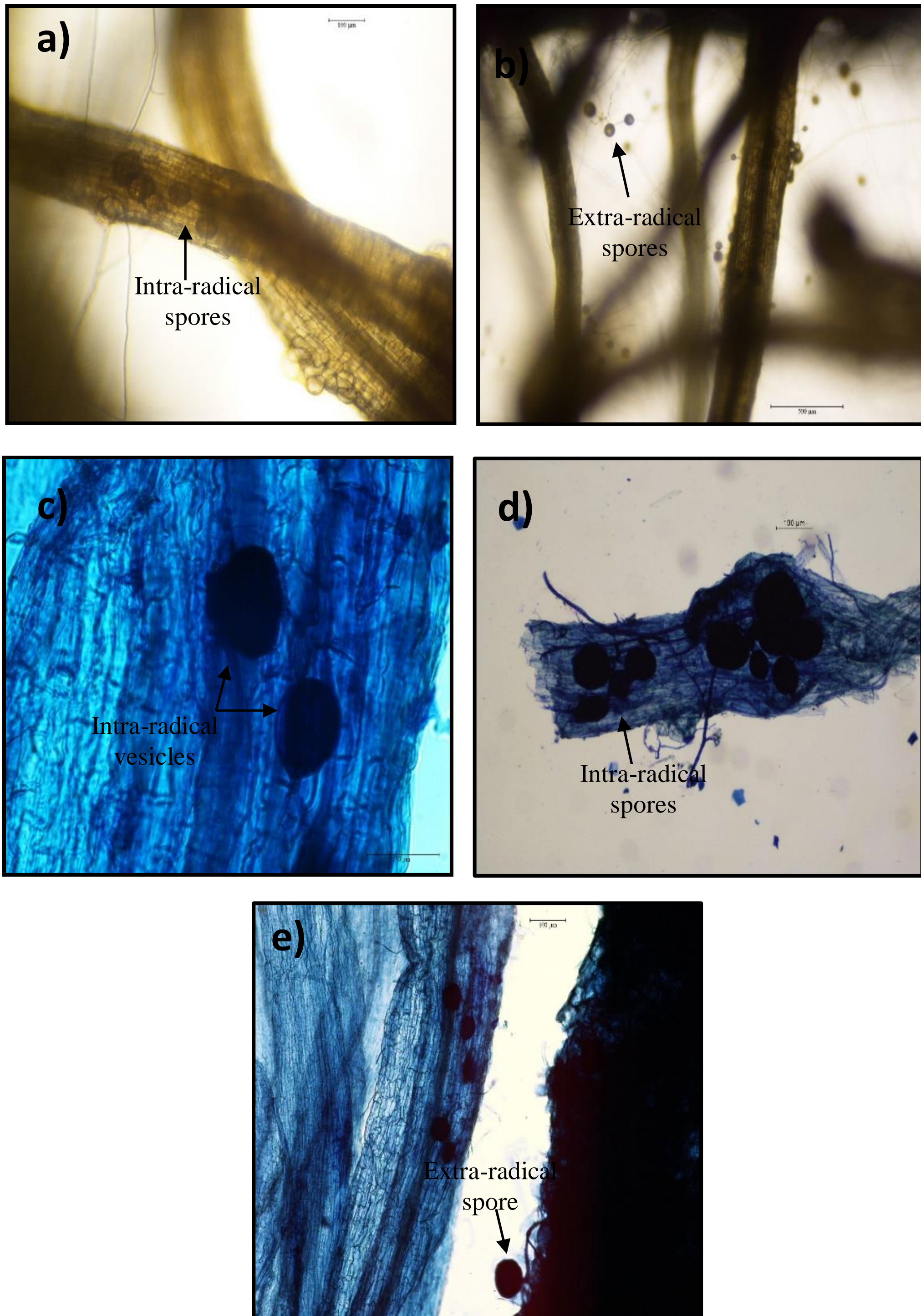


Plate 5.7: Monoxenic culture of *Rhizophagus intraradices* with transformed Chicory roots: a. Intra-radical sporulation ; **b.** Extra-radical sporulation; **c.** Vesicular colonization in stained roots; **d.** Intra-radical sporulation in stained roots; **e.** Extra-radical sporulation in stained roots.

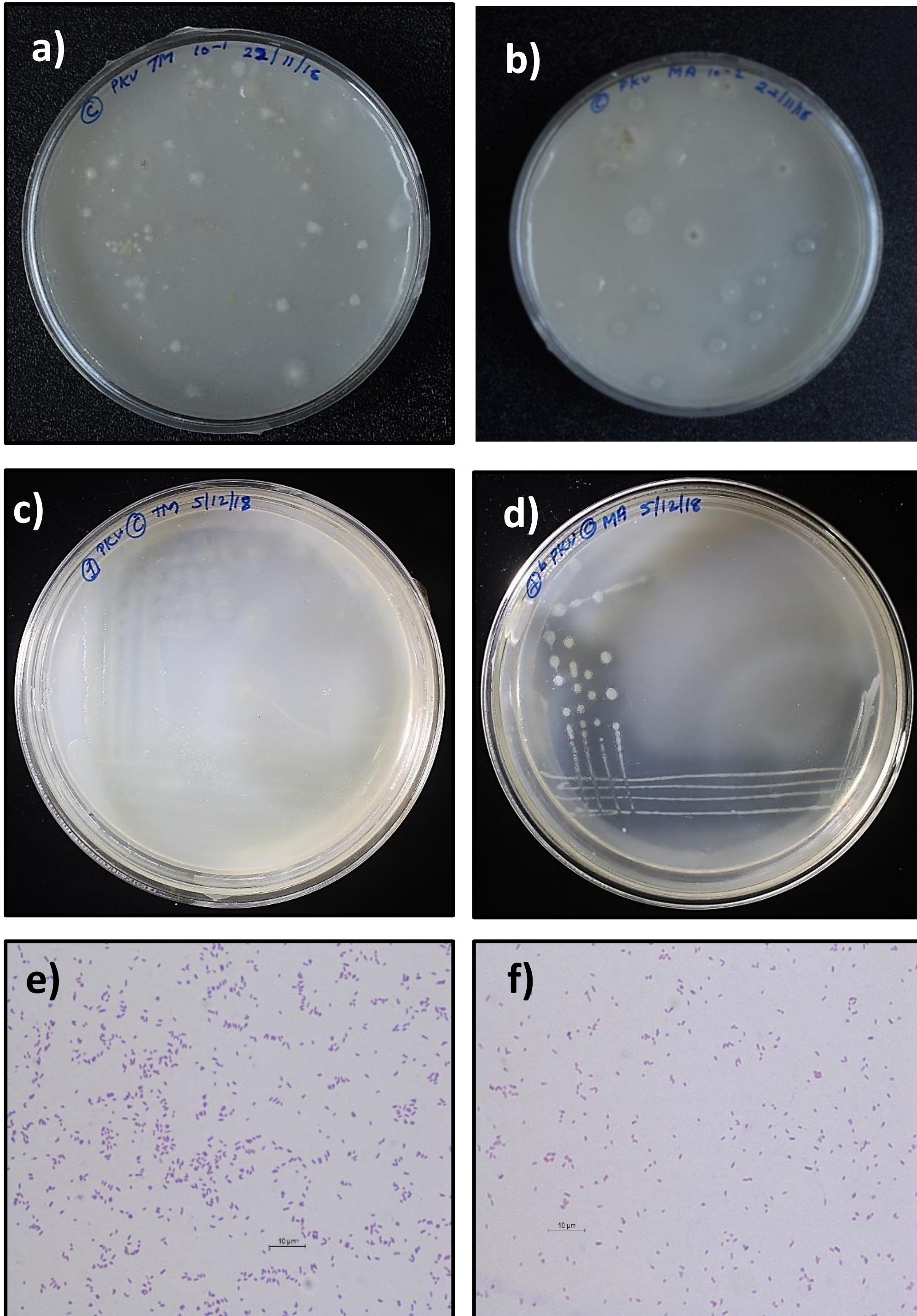


Plate 6.1: Isolation and gram staining of phosphate solubilizing bacteria (PSB): a. Bacterial colonies from *Excoecaria agallocha*; **b.** Bacterial colonies from *Clerodendrum inerme*; **c.** Pure culture of PSB_1; **d.** Pure culture of PSB_2; **e.** Gram staining of PSB_1; **f.** Gram staining of PSB_2.

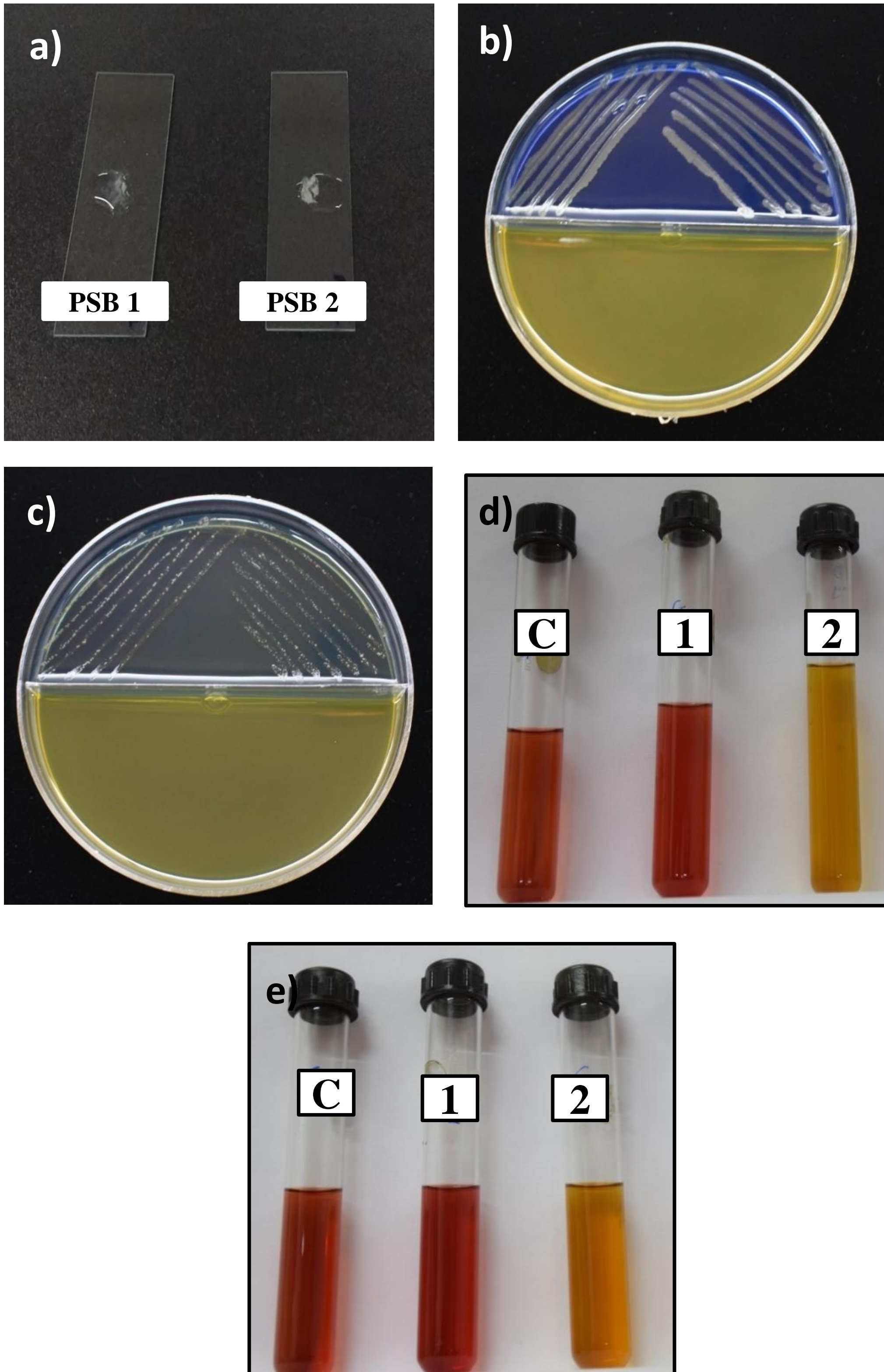


Plate 6.2: Biochemical tests of PSB: a. Catalase test; **b.** Citrate utilization test ((PSB_1); **c.** Citrate utilization test of (PSB_2); **d.** Glucose test; **e.** Cellobiose test;

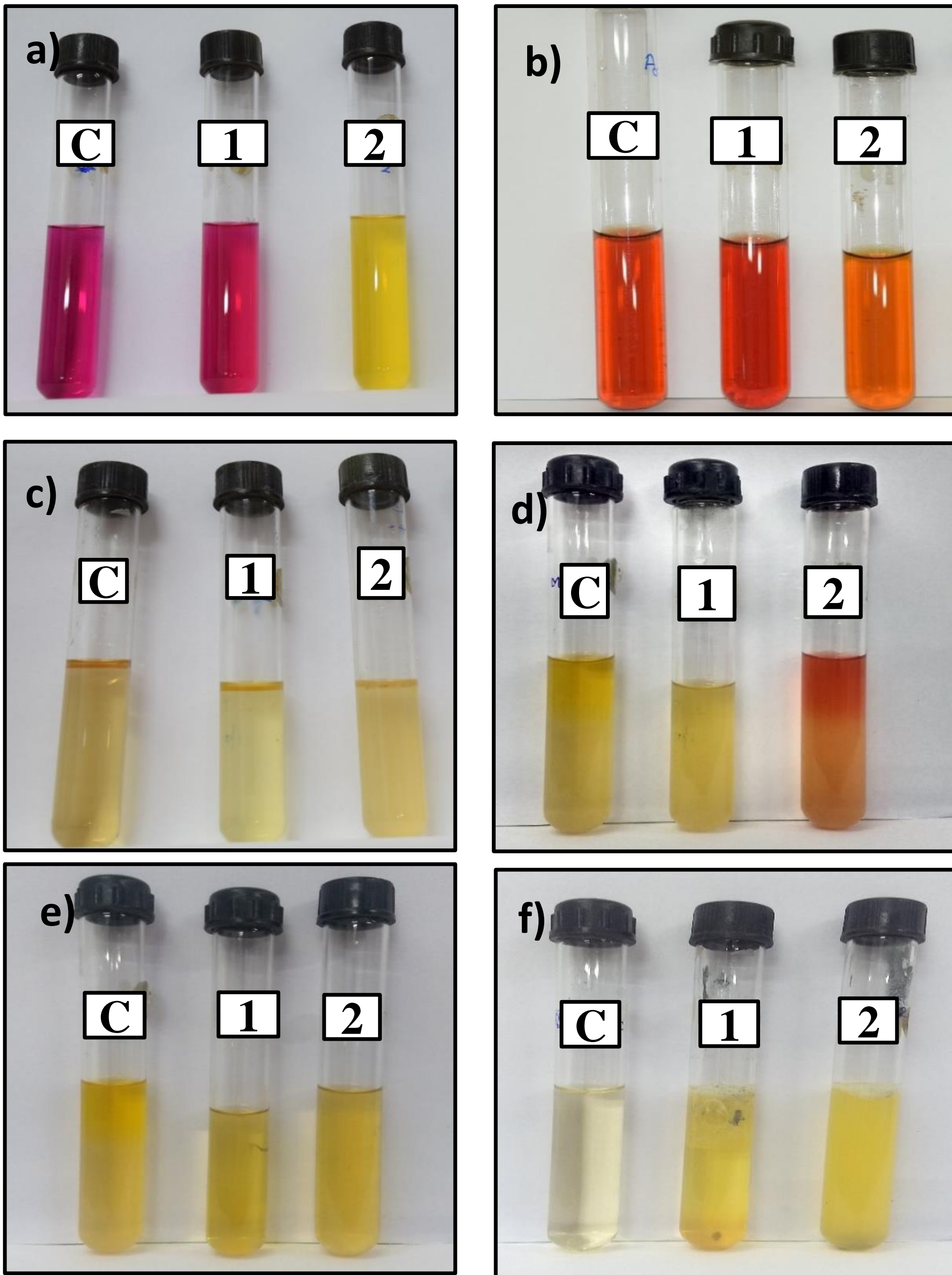


Plate 6.3: Biochemical tests of PSB: a. Mannitol test; **b.** Arabinose test; **c.** Indole test; **d.** Methyl red test; **e.** Voges test; **f.** Nitrate reduction test.

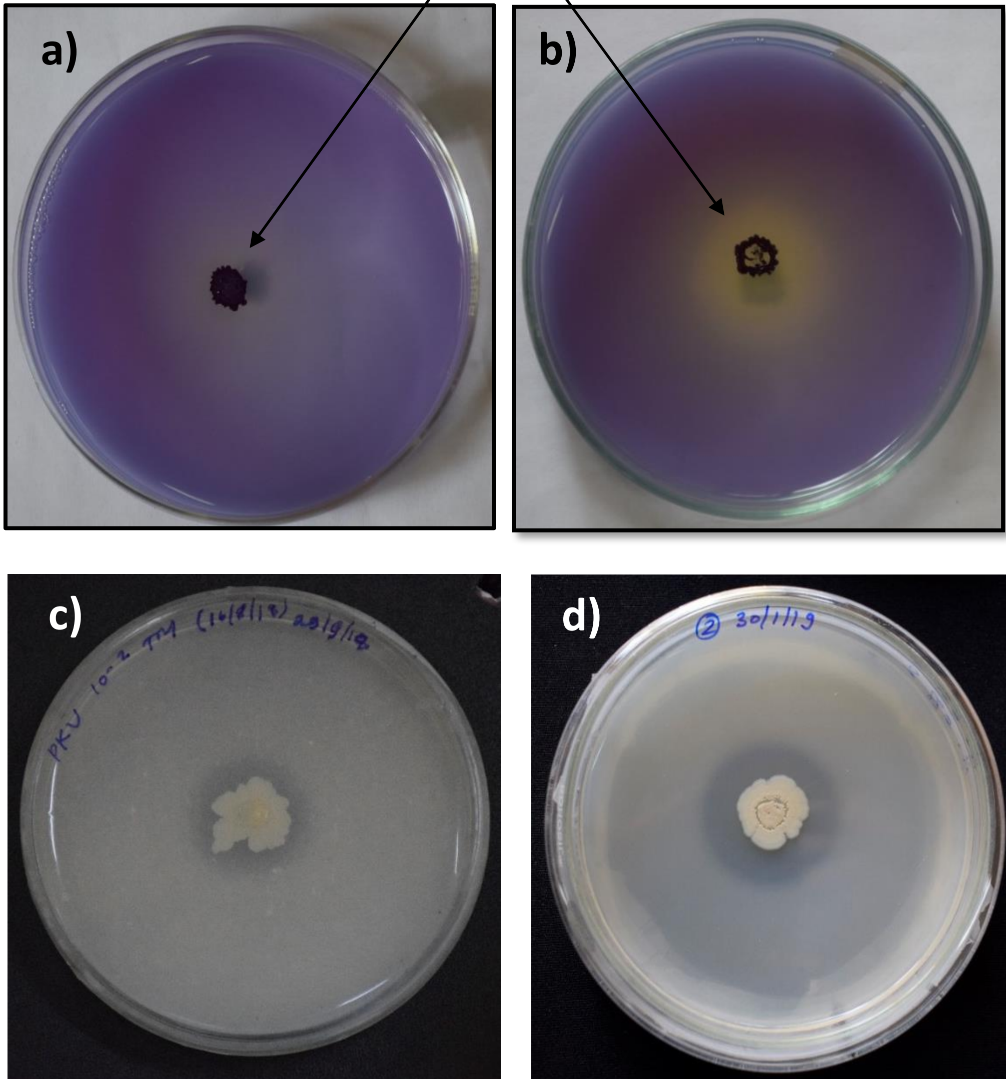


Plate 6.4: Qualitative analysis of phosphate solubilization: a. PSB_1 on Pikovskaya's-Bromo phenol blue (PKV-BPB) agar medium; **b.** PSB_1 on (PKV-BPB) medium; **c.** PSB_1 on Pikovskaya (PKV) medium; **d.** PSB_2 on PKV medium.

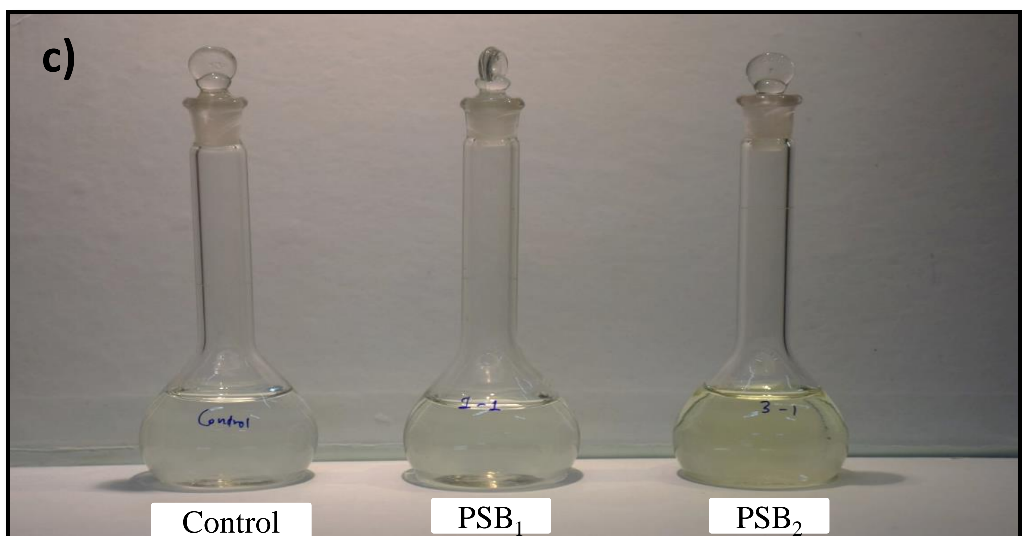
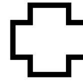


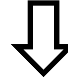
Plate 6.5: Quantitative analysis of phosphate solubilization: a. Inoculated bacterial cultures on rotary shaker; **b.** Standard solutions; **c.** Development of yellow colour in bacterial isolates.

AM fungal inoculum in carrier

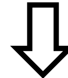
Bacterial inoculum in Nutrient broth



Propagules of *Rhizophora mucronata*



One propagule planted per bag



Arrangement of eight treatments with three replicates in the shed net

Plate 8.1: Screening experiment in *Rhizophora mucronata* Lam.

Plate 8.2: Sample digestion of *Rhizophora mucronata* plants: a. Reduction of acid content at high temperature; **b.** Appearance of white fumes after complete digestion of the sample.

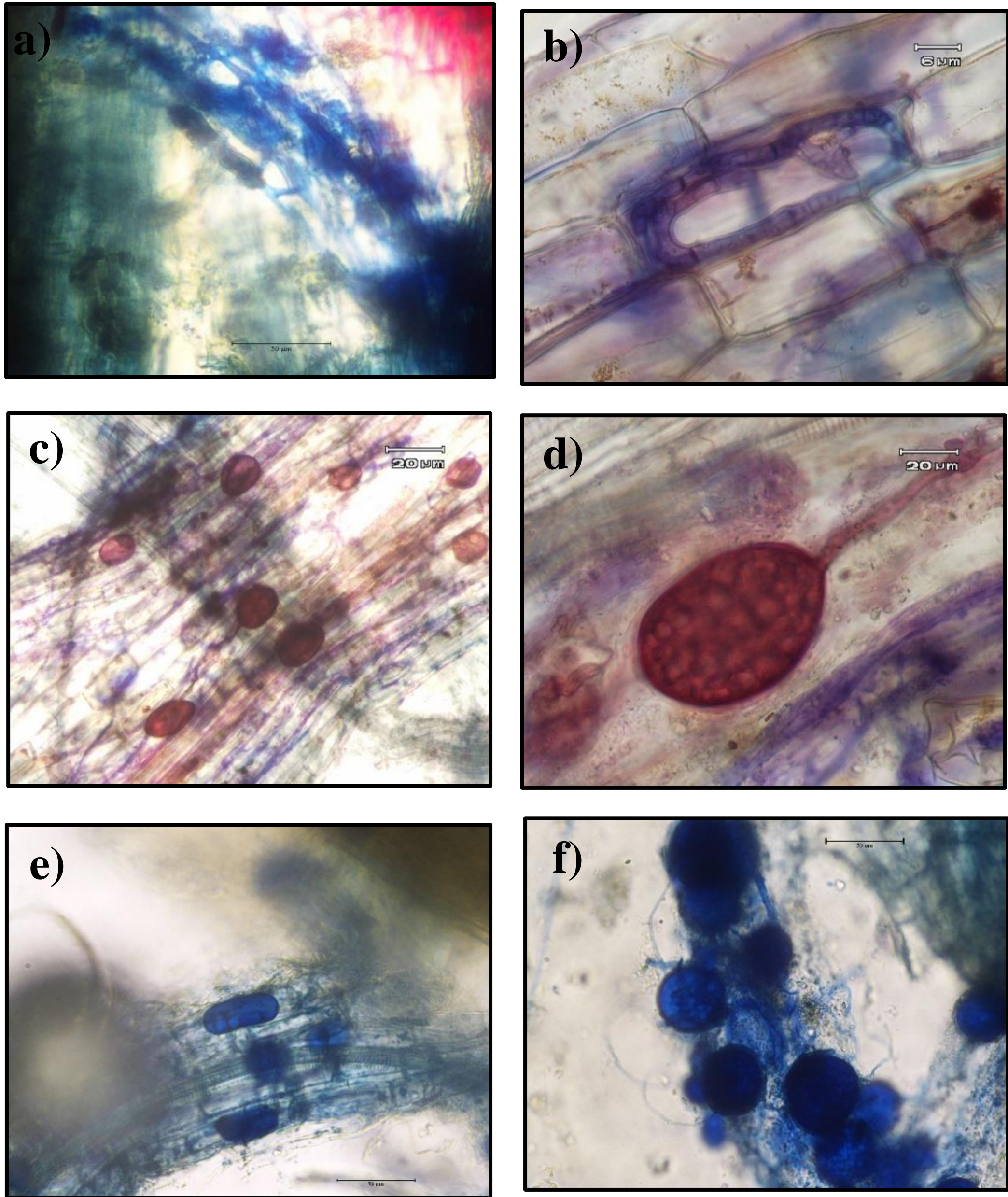
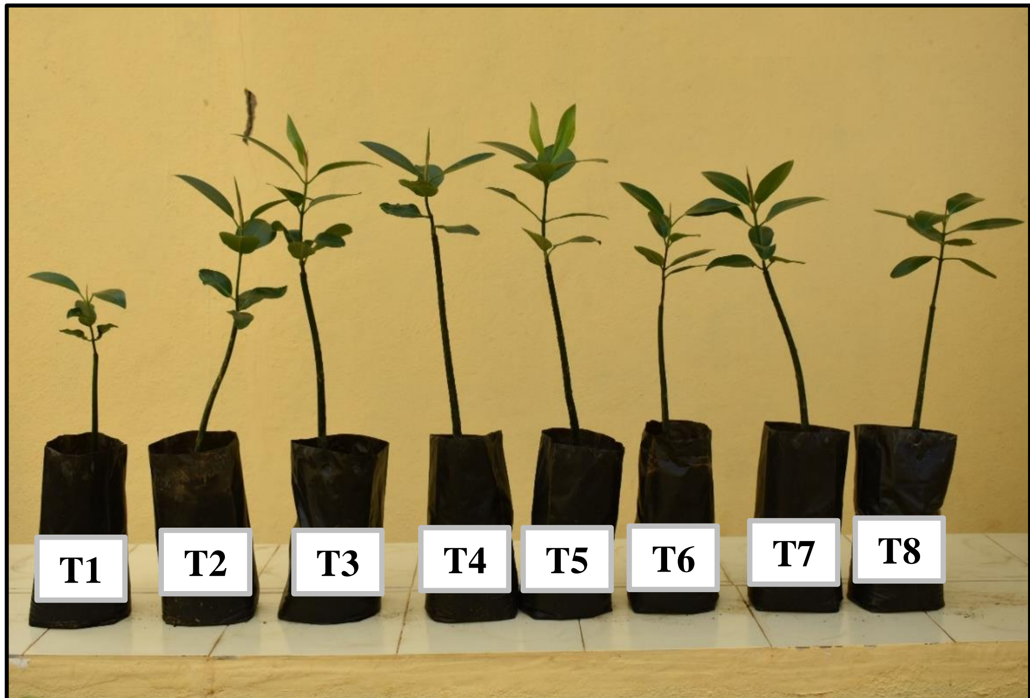


Plate 8.3: Root colonization in AM inoculated *Rhizophora mucronata* plants: a. Arbuscular colonization by *A. dilatata*; **b.** Hyphal coil; **c.** Vesicular colonization by *Rhizophagus intraradices*; **d.** Enlarged view of a vesicle; **e.** Vesicular colonization by *Acaulospora dilatata*; **f.** Extra-radical spores of *Rhizophagus intraradices*



T1 T2 T3 T4 T5 T6 T7 T8

Plate 8.4: Effect of inoculation (AM fungi and PSB) on the growth of *Rhizophora mucronata*: T_1: Control (Uninoculated); **T_2:** *R. intraradices*; **T_3:** *A. dilatata*; **T_4:** *R. intraradices* + *A. dilatata*; **T_5:** *R. intraradices* + PSB_2; **T_6:** *A. dilatata* + PSB_2; **T_7:** *R. intraradices* + *A. dilatata* + PSB_2; **T_8:** PSB_2 (*B. halotolerans*)

Scope and Limitations of AMF Biofertilizer Production

*Sankrita S Gaonkar and B F Rodrigues**

Introduction

Arbuscular mycorrhizal (AM) fungi are soil-borne microbes belonging to phylum Glomeromycota that form a symbiotic association with roots of higher plants. Hyphae colonize their host roots and form a mycelial network in the rhizosphere to facilitate nutrient uptake, especially P (Rodrigues and Rodrigues 2014), and in turn acquire photosynthates from the host plant. Around 90% of vascular plants form AM association (Smith and Read 2008). Plant genes and signal molecules enable hyphal entry and development of the fungus in the plant (Parniske 2008). The extra-radical mycelium extends several centimetres beyond the depletion zone absorbing nutrients that are transported to host roots (Khan *et al.* 2000). These fungi play an important role in agriculture, forestry, and horticulture by increasing crop yield, health, and resistance to stress by reducing the cost of agrochemicals (Johansson *et al.* 2004). Occurrence of AM symbiosis is dated back to >460 million years ago (Read *et al.* 2000). Based on the spore morphology, approximately 240 AM fungal taxa belonging to order Glomales have been described (Schubler and Walker 2010; Kruger *et al.* 2012), although molecular analysis data shows that the actual number of AM fungal taxa can be much higher (Vandenkoornhuyse *et al.* 2002).

Culture Techniques for AM Fungal Inoculum

Various cultivation techniques of AM fungal inoculum production have been attempted in the last few decades. Sand/soil- and substrate-based production techniques, substrate-free culture techniques (hydroponics and aeroponics), and *in vitro* cultivation methods have been attempted in the large-scale production of AM fungi. Several parameters must be taken into consideration for the culture of AM fungi, such as controlled or semi-controlled conditions in greenhouses, AM fungal species, the host plant, substrate, and amendments.

Substrate-Based Production System

Conventional production of AM fungi is commonly achieved by the cultivation of host plants and their symbionts in a soil- or sand-based substrate (substrate-based production system). The inoculum to initiate production consists of dried root fragments or colonized root fragments, AM spores, sporocarps, and fragments of hyphae. When spores are extracted from the soil and used as inoculum directly they tend to have very low viability or may even be dead or parasitized. To overcome this, initially, the rhizosphere soil is used to prepare a 'trap culture' using a suitable host plant. This increases the number of viable spore propagules for further isolation, multiplication, and production of monospecific cultures. The pure culture inoculum thus produced consists of spores, colonized root fragments, and AM hyphae of a single species.

Selection of host plant is based on numerous criteria, such as plants exhibiting a short life cycle, rapid growth, adaptation to the prevailing growing conditions, and ready colonization by a range of AM fungal species. A large quantity of roots should also be produced in a relatively short period, and resistance to pest and diseases common to the inocula production environment.

A range of plant species, such as *Zea mays* (corn), *Allium cepa* (onion), *Arachis hypogaea* (peanut), *Paspalum notatum* (bahia grass), *Pueraria phaseoloides* (kudzu), coleus (*Plectranthus scutellarioides*), ragi (*Eleusine coracana*), etc., have been used as hosts with encouraging results.

Various substrates, such as soil, sand, peat, vermiculite, perlite, calcinated clay, and compost have been used to propagate AM fungi (Ijdo *et al.* 2011). Addition of different organic amendments also influences AM fungal colonization. Chitin and humic substances increase colonization levels (Gryndler *et al.* 2003; Gryndler *et al.* 2005). Manipulation of nutrient content has a further impact on AM fungal propagule production (Douds and Schenck 1990). The substrate-based culture technique is the most widely used method for AM fungal production as it requires a relatively little less technical support, is cheap, is the least artificial, and a large set of AM fungal species can be cultured (Ijdo *et al.* 2011). Conversely, the sand/soil-based systems have certain disadvantages such as the presence of unwanted contaminants, even with good phytosanitary care, fewer viable spores than *in vitro* system, and parasitized spores.

Substrate-Free Production System

Substrate-free cultivation systems, such as hydroponic and aeroponic have also been used for the

Department of Botany, Goa University, Goa 403 206, India.
* Corresponding author, Email: felinov@gmail.com

multiplication of AM fungi wherein a continuous flow or mist of nutrient solution is provided for the plant and the symbionts. Although this system offers the advantage of providing inoculum which is free from attached substrate particles, a disadvantage has been that the nutrient solution is prone to microbial contamination and algal growth (Elmes and Mosse 1984).

Monoxenic Culture System

The first attempt to culture AM fungi monoxenically dates back to the late 1950s (Mosse 1959). Thereafter, tremendous progress has been made for the mass production of AM fungi using Ri T-DNA transformed roots (Mugnier and Mosse 1987). Different *in vitro* culture techniques have been derived such as the bicompartment system wherein AM fungal mycelia and spore are produced free from roots (St-Arnaud *et al.* 1996), and manipulation of culture medium to induce sporulation (Becard and Piche 1992). These developments have enabled studies in spore ontogeny (Pawlowska *et al.* 1999), sporulation dynamics (Declerck *et al.* 2001), 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 (Dupre de Boulois *et al.* 2005) and isolation of contaminant-free spores for molecular analysis (Pawlowska and Taylor 2004). A wide number of AM fungal species belonging to Glomeraceae and a few Gigasporaceae have been successfully cultured in the root organ culture (ROC) system.

Species, such as *Acaulospora rehmii* (Dalpe and Declerck 2002), *Gigaspora rosea* (Bago *et al.*1998c), *Gi. margarita* (Miller-Wideman and Watrud 1984; Diop *et al.* 1992; Gadkar and Adholeya 2000), *Gi. gigantea* (Gadkar *et al.* 1997), *Gi. decipiens* (Fernandez Bidondo *et al.* 2012), *Glomus etunicatum* (Schreiner and Koide 1993), *G. versiforme* (Diop *et al.* 1994; Declerck *et al.* 1996), *G. deserticola* (Mathur and Vyas 1995), *G. fistulosum* (Nuutila *et al.* 1995; Gryndler *et al.* 1998), *G. clarum* (De-Souza and Berbara 1999; Rodrigues and Rodrigues 2012), *Funneliformis caledonius* (Hepper 1981; Karandashov *et al.* 2000), *F. geosporus* (Declerck *et al.* 1998), *F. mosseae* (Douds 1997; Rodrigues and Rodrigues 2015), *Rhizophagus irregularis* (Chabot *et al.* 1992; St-Arnaud *et al.* 1996), *R. fasciculatus* (Declerck *et al.* 1998), *R. proliferus* (Declerck *et al.* 2000) and *Sclerocystis sinuosa* (Bi *et al.*2004) have been successfully cultured *in vitro.*

Culture media such as minimal (M) medium (Becard and Fortin 1988) and modified Strullu Romand (MSR) medium (Strullu and Romand 1986, modified by Declerck *et al.* 1998) are often used to culture AM fungi. The growth of germ tube is inhibited by the presence of sucrose in MSR medium. Healthy germination of AM fungal spores in MSR medium without sucrose was achieved by D'Souza *et al.* (2013). During the pre-symbiotic phase, AM spore use the reserve materials from propagules for the germination and growth of germ tubes (Clark 1997). Root Organ Culture (ROC) was first developed by White (1943), followed by development of further Ri T-DNA transformed roots of different plant species, *viz.*, clover (*Trifolium*) (Mosse and Hepper 1975), bindweed (*Convolvulus sepium*) (Tepfer and Tempe 1981), onion (*Allium cepa*), tomato (*Solanum lycopersicum*) (Strullu and Romand 1986, 1987), carrot (*Daucus carota*) (Mugnier and Mosse 1987), strawberry (*Fragaria x ananassa*), chicory (*Cichorium intybus*) (Boisson-Dernier *et al.* 2001), barrel medic (*Medicago truncatula*) (Fontaine *et al.* 2004) and linum (*Linum usitatissimum*) (Rodrigues and Rodrigues 2015).

Fungal inocula such as isolated spores or propagules from intra-radical phase (colonized root fragments and isolated vesicles) of AM fungi can be used to initiate monoxenic cultures (Rodrigues and Rodrigues 2015). The culture established needs to be maintained by continuous sub-culturing, transferring the mycorrhizal roots onto fresh medium (St-Arnaud *et al.* 1996). Under aseptic conditions, AM symbiosis with the transformed roots takes place by development of extra-radical mycelium which is often accompanied by formation of arbuscule-like structures (ALS) (Bago *et al.* 1998a) or branched absorbing structures (BAS) (Bago *et al.*1998b). These structures are probably nutrient-exchange sites between the fungus and its host (Diop 2003). Sporulation in AM fungi differs between species as well as between isolates of the single species and is related to spore size (Declerck *et al.* 2001).

The most important advantage offered by *in vitro* cultivation system is the absence of undesirable organisms. Contamination by other undesirable microorganisms can occur, however, during the establishment of culture process or during the later stages of culture maintenance. This type of system can be used for the large-scale production of AM fungi consisting of high-quality inoculum with minimum space. Also, the factors influencing optimum production can be easily detected and controlled, and harvesting time can be determined. The maintenance of a successfully established culture is easily achieved by sub-culture and maintaining the plates in dark condition. As a disadvantage, the *in vitro*-grown AM fungal diversity is lower than that under-pot culture system (Rodrigues and Rodrigues 2013). Furthermore, the *in vitro* production is expensive, requiring skilled technicians and sophisticated

laboratory equipment to carry out the whole process in sterile and controlled conditions (Ijdo *et al. 2011*). Further studies are in progress to identify and eliminate contaminants in established cultures.

AM Fungi as Biofertilizers

It has been observed that AM fungal inoculation provides beneficial results in plant growth both in controlled and open-field conditions. AM fungi have been confirmed to show better performance in terms of plant growth and yield characteristics. This would make the AM fungal technology more suitable to sustainable cropping systems (Berruti *et al.* 2016). Khan *et al.* (2008) reported that the inoculation of a single or dual AM fungi increased the growth and nutrient uptake of *Medicago sativa* which resulted in the increased dry weight of shoot and root. Bhat *et al.* (2010) studied the effect of AM fungi and *Rhizobium* on green gram (*Vigna radiata*) and reported a significant effect on nodulation, yield, crude protein content, and NPK content in grain. Various further studies have proved that AM fungi are an effective resource when used as biofertilizers with no adverse environmental effect.

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ORIGINAL PAPER

Diversity of arbuscular mycorrhizal (AM) fungi in mangroves of Chorao Island, Goa, India

Sankrita Gaonkar · B. F. Rodrigues

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Abstract For a desirable understanding of diversity and species composition of arbuscular mycorrhizal (AM) fungi, in true and associate mangrove plants, 17 true mangrove and their associate species belonging to ten families were assessed from Chorao Island, Goa, India. Maximum AM root colonization was recorded in *Thespesia populnea* and minimum in *Avicennia marina.* Rhizosphere soils of *Ceriops tagal* showed highest and that of *Acrostichum aureum* showed the least spore density. The results showed that the associate mangrove species were highly mycorrhizal compared to true mangrove plants. Our study recorded greater diversity involving thirty-two AM fungal species belonging to nine genera viz., *Acaulospora*, *Claroideoglomus*, *Entrophospora*, *Funneliformis*, *Gigaspora*, *Glomus*, *Rhizophagus*, *Sclerocystis*, and *Scutellospora*. *Acaulospora* was the dominant genus and *A. dilatata* was the dominant AM fungal species. *Acaulospora dilatata* was the most common AM species in both true and associate mangrove plants, revealing its wider adaptability.

Keywords Phosphorus · Colonization · Spore density · Diversity · Salinity · Jaccard's similarity index

S. Gaonkar (✉) · B. F. Rodrigues
Department of Botany, Goa University, Panjim, Goa 403 206, India
e-mail: sankrita002@gmail.com

Introduction

Mangroves are woody shrubs or trees forming intertidal forests in tropical or sub-tropical regions. They are adapted to a hostile environment tolerating extreme tides, fluctuating salinity, high temperatures, and low oxygen (Hogarth 2015). These forests are among the World's most diverse and productive tropical ecosystems (Kathiresan 2000). Mangrove plants are classified into two subgroups: true- and associate- mangrove plants. True mangroves are restricted to the intertidal zones, while associate mangroves grow on the landward fringes of mangrove habitats or terrestrial marginal zones (Wu et al. 2008). Based on salt tolerance, true mangroves are considered as halophytes while their associates are glycophytes (Wang et al. 2010b).

The mangrove areas of India account for about 3% of the World's total mangrove vegetation, 4639 km^2 that are comprised of three distinct zones, East coast habitats, West coast habitats, and Island territories. Sundarbans, the World's largest mangrove forest (2136 km^2) is in West Bengal on the east coast of India. About 60% of the mangroves of India occur on the east coast, 27% on the west coast, and 13% on Andaman and Nicobar Islands (Singh et al. 2012). Of Goa's total land area of 370,200 ha, the mangrove covers approximately 2539 ha. A total of 178 ha of prime mangrove area at Chorao, Goa has been declared a Reserved Forest under the Indian Forest

Act, 1927 to protect and conserve the system. In 1988 the area was further declared a Bird Sanctuary (Nagi et al. 2014).

Various biotic and abiotic factors such as tidal inundation, soil type, microbe activity in the soil, plant species, litter production, and decomposition control the availability of nutrients to mangrove plants. Nitrogen (N) and phosphorus (P) are the nutrients that limit growth in mangroves (Reef et al. 2010). Firstly, being highly immobile, P is adsorbed by carbonate compounds thus making it unavailable for plant use (Kothamasi et al. 2006). Secondly, the available P is used by the growing plants forming a phosphate-free zone around the plant roots (Smith and Read 2008). Therefore, organisms that mobilize P play an important role in plant growth. Arbuscular mycorrhizal (AM) fungi are obligate symbionts belonging to phylum Glomeromycota having a ubiquitous worldwide distribution in various ecosystems (Redecker et al. 2000). They help in plant nutrition especially P (Aggarwal et al. 2012; Willis et al. 2013). Extraradical hyphae of AM fungi can extend beyond the P depletion zone thereby enlarging the absorption area of the host roots for the uptake of P (Xie et al. 2014).

It has been suggested AM fungi play a marginal role in wetland ecosystems due to the anaerobic conditions that decrease fungal activity (Krazic-Sraj et al. 2006). However, recent studies have shown that AM fungi can colonize the roots of wetland plants (Radhika and Rodrigues 2007), increasing nutrient uptake and photosynthetic activity, and therefore the diversity and productivity of mangrove ecosystems (Wang et al. 2010a). According to Wang et al. (2011), AM fungi obtain oxygen from the root aerenchyma of mangrove plants during flooded conditions. Soil salinity also affects AM fungal spore germination, root colonization, and hyphal growth. However, some of the AM fungal species are salinity tolerant (Aggarwal et al. 2012).

Most of the studies on AM fungal association in the estuarine ecosystem emphasize on the plants from intertidal zones (Sengupta and Chaudhuri 2002; Wang et al. 2010a; Kumar and Ghose 2008; Sridhar et al. 2011). A literature survey indicates that no studies are comparing the AM fungal distribution in true- and associate-mangroves. The only study on the occurrence and diversity of AM fungi in mangroves of Goa (India) was reported by D'souza and Rodrigues (2013a, b). However, the location investigated in the present study has never been subjected to similar investigations previously. The vital role of AM fungi in modulating the primary and secondary succession of plants, especially in low nutrient ecosystems such as coastal regions is well known (Karthikeyan and Selvaraj 2009). To understand the ecology of the habitat, and to develop conservation strategies, it is necessary to measure the biodiversity associated with the habitat. Therefore, the present study was initiated to quantify the AM fungal diversity and to identify dominant AM fungal species in true- and associate-mangroves of Chorao Island.

Materials and methods

Study site and sample collection

Chorao Island (15° 32′50.7″ N, 73° 52′45.8″ E) is located on the West Coast of India in the Mandovi River at the elevation of 8 m AMSL (Fig. 1). The total area of the Chorao Island is 423.75 ha which has a mangrove cover of about 250 ha. and has an average annual rainfall of approximately 2500 mm (https://www.spectrumtour.com/south-india-tourism/chorao-island-goa.htm). The Island is divided by creeks and backwaters with continuous tidal variations and is formed from a confluence of Mandovi River and its tributary the Mapusa river (Sappal et al. 2014). The mangrove flora of the Island is represented by 17 plant species belonging to 10 families with *Rhizophora mucronata*, *Avicennia marina*, *Sonneratia alba*, and *Excoecaria agallocha* being dominant. In the present study, all the 17 mangrove species were investigated. Of these, 11 were true mangroves while six were mangrove associates. Soil and root samples were collected during the period from March to May 2015 and 2016. During the collection, roots of the trees were traced by digging and removed to ensure that the collected roots belong to the same plant species. The roots were separated from adhering soil, washed gently under tap water, and used for estimation of AM colonization.

Soil samples were collected from the depth of 0–30 cm using soil corer (5 cm diameter) of 1 m length. Three rhizosphere soil samples were collected from each plant species, placed in separate Ziploc bags, labeled and brought to the laboratory. These three samples of each plant species were then

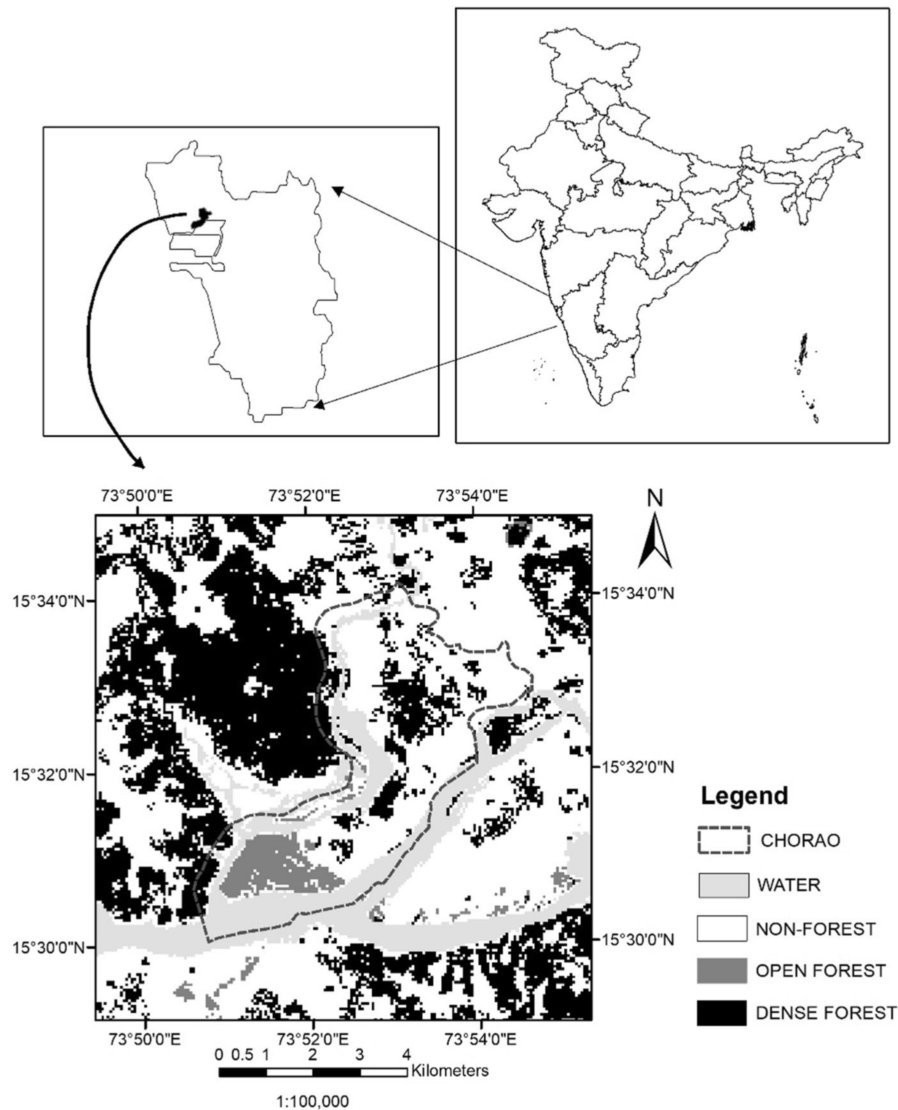

Fig. 1 Map showing study area

thoroughly mixed to form a composite sample. The rhizosphere soil was divided into two parts, one part for AM spore isolation, enumeration, and identification, the other as inoculum to prepare trap cultures.

Soil analyses

The soils from true- and associate- mangrove areas were separately analyzed for their physical and chemical properties. Soil pH and electrical conductivity (EC) was measured in soil water suspension (40% w/v) using pH meter (LI 120 Elico, India) and conductivity meter (CM-180 Elico, India) respectively. Soil organic carbon (OC) was detected by Walkley and Black (1934) rapid titration method. Available soil N was estimated using the method proposed by Subbiah and Asija (1956). The Bray and Kurtz method (1945) was used to determine available P. Available potassium (K) was estimated by ammonium acetate method (Hanway and Heidel 1952) using Atomic absorption spectrophotometer (AAS) (nova 400P, Analytik Jena, Germany). Available Zinc (Zn), Copper (Cu), Manganese (Mn), and Iron (Fe) were determined by DTPA-$CaCl_2$-TEA

(diethylenetriaminepentaacetic acid-Calcium Chloride-triethylamine) method of Lindsay and Norvell (1978) using AAS. Soil texture was analyzed by the pipette method (Folk 1968). For this analysis, soil samples were randomly collected from true- and associate- mangrove areas within the study site and were analyzed separately.

AM fungal root colonization

Fifty root pieces (secondary and tertiary roots) approximately 1 cm long were cleared in 10% KOH at 90 °C for 90 min, acidified in 5 N HCl and stained with 0.05% Trypan blue overnight (Phillips and Hayman 1970). Stained roots were then mounted on glass slides in polyvinyl alcohol lacto-glycerol (PVLG) and examined using a bright-field Olympus BX41 research microscope. A root segment was considered mycorrhizal if it showed the presence of hyphae/hyphal coils, arbuscules/arbusculate coils, and/or vesicles. The intensity of total colonization (TC), root length containing hyphae (HC%), arbuscules (AC%), and vesicles (VC%) was quantified using the magnified intersection method (McGonigle et al. 1990).

Isolation and identification of AM fungal spores and preparation of trap cultures

Spores from composite soil samples (n = 3) and trap cultures were isolated using wet sieving and decanting method (Gerdemann and Nicolson 1963). Intact, non-parasitized healthy spores were estimated using the modified method of Gaur and Adholeya (1994). The spores were then mounted on glass slides in PVLG and examined under a bright-field Olympus BX41 research microscope (40×, 100×, and 400×). Spore morphology, wall characteristics, dimensions, and other relevant data were observed for the identification of the AM spores. The spore characters were compared with the descriptions given by Rodrigues and Muthukumar (2009), Blaszkowski (2012), and International Collection of Vesicular Arbuscular Mycorrhizal Fungi (invam.wvu.edu). Names and epithets of AM fungal species were followed according to the recommendation of Schüßler and Walker (2010) and Redecker et al. (2013).

To propagate and recover AM spores that were not encountered in field soils, trap cultures were prepared mixing rhizosphere soil and sterile sand (1:1) in the pots and maintained for 6 months. Coleus (*Plectranthus scutellarioides*) was used as the catch plant. The culture was harvested at the end of the sixth month.

Data analysis

Spore density is the number of spores per 100 g of soil. Relative abundance (RA) was evaluated using the formula: RA = (Number of spores of a species or genus/Total number of spores in all soil samples) × 100, while isolation frequency (IF) was derived by using the formula: IF = (Number of soil samples possessing spores of a particular species/Total number of soil samples analyzed) × 100.

Following formulae were used to calculate Shannon–Wiener diversity index (H) and Simpson's diversity index (D):

$$(1)\quad H = -\sum (pi \ln pi)$$

$$(2)\quad D = 1 - \left[\sum n(n-1)/N(N-1)\right]$$

where *pi* is the proportion of individual species that contributes to the total number of individuals, *n* is the number of individuals of a given species and N is the total number of individuals in a community.

Species evenness was estimated as (Σ (H) = H′/H′ max) where, H′max = lnS, S = total number of species in the community (richness). Jaccard's similarity index was calculated using the formula: JI (%) = (c ÷ a + b + c) 100, where 'c' stands for the number of species occurring in both hosts, 'a' is the number of species unique to the first host and 'b' is the number of species unique to the second host.

All data were statistically analyzed using SPSS (Version 22) software. To compare the soil parameters between true- and associate- mangroves, a paired sample t-test was performed.

Pearson's correlation coefficient was calculated to evaluate the relationships between root colonization and spore density, isolation frequency, and relative abundance and spore density and species richness. Jaccard's similarity index (JI) was calculated pairwise between mangrove plant species based on the presence or absence of each AM fungal species

(Jaccard 1912). To understand the distribution of AM fungal species among true- and associate-mangrove plants, cluster analysis (Bray–Curtis similarity) was performed using PRIMER v. 6.0.

Results

Physico-chemical properties of soils

Results of texture analysis showed that the soil from true mangrove areas is clayey silt, while associate mangrove areas have a nearly equal proportion of sand, silt, and clay (Fig. 2). Physico-chemical properties of the estuarine soils at Chorao revealed acidic nature. All nutrients showed low availability, with P being the least available nutrient (Table 1). This low nutrient availability along with increased salinity appears to be responsible for causing stress thereby affecting plant growth especially in the true mangroves. The results of the t-test are presented in Table 2. The p values indicate the significant differences ($p < 0.05$) between the soil parameters in two types of mangroves. The parameters such as EC, OC, N, Cu, and Fe showed significant differences between true- and associate-mangroves. The negative t-values indicate that the mean values of pH and P are higher in associate mangrove plants.

Table 1 Physico-chemical analyses of mangrove soils

	True mangrove soil	Mangrove associate soil
pH	5.87 ± 0.59	5.65 ± 0.57
EC (ds/m)	8.95 ± 0.99	0.90 ± 0.10
OC (%)	2.81 ± 0.35	1.07 ± 0.13
N (g/kg)	0.073 ± 0.01	0.067 ± 0.01
P (g/kg)	0.007 ± 0.004	0.051 ± 0.03
K (g/kg)	0.231 ± 0.03	0.263 ± 0.04
Zn (ppm)	2.011 ± 0.40	1.834 ± 0.37
Cu (ppm)	0.50 ± 0.13	0.297 ± 0.07
Fe (ppm)	343.1 ± 3.43	266.9 ± 2.67
Mn (ppm)	2.28 ± 0.76	2.24 ± 0.75

All values are mean of three readings

± = standard error; EC = electrical conductivity; OC = organic carbon

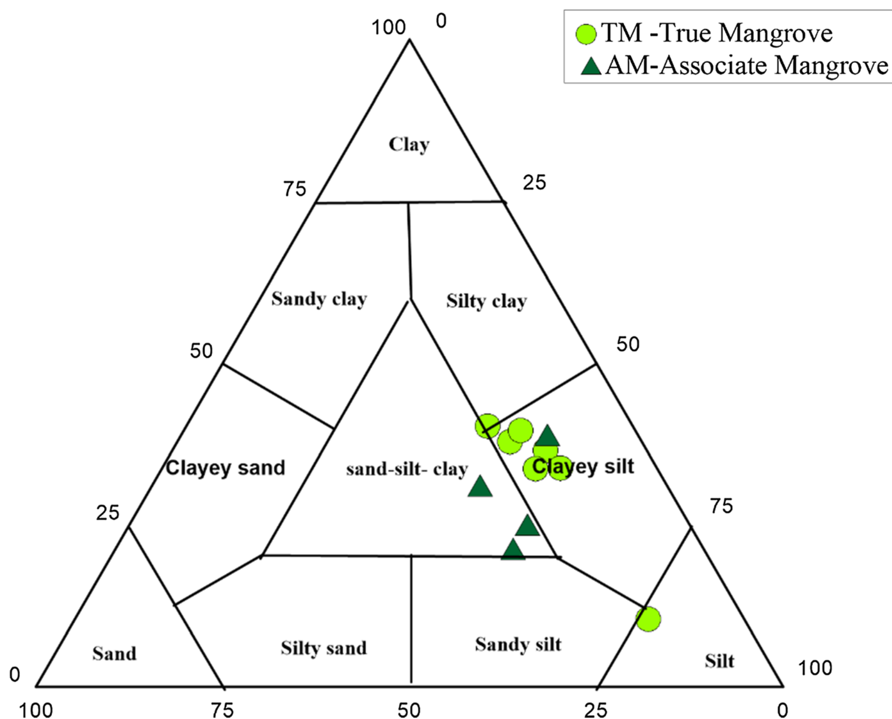

Fig. 2 Ternary diagram of sand–silt–clay percentages

Table 2 Paired sample t-test to compare soil parameters between true- and associate-mangrove plants

Pairs of variables	t	df	P (2-tailed)
Pair 1 pH_1–pH_2	− 0.653	2	0.581
Pair 2 EC_1–EC_2	5.435	2	0.032
Pair 3 OC_1–OC_2	5.125	2	0.036
Pair 4 N_1–N_2	20.000	2	0.002
Pair 5 P_1–P_2	− 1.070	2	0.397
Pair 6 K_1–K_2	0.000	2	1.000
Pair 7 Zn_1–Zn_2	2.147	2	0.165
Pair 8 Cu_1–Cu_2	6.289	2	0.024
Pair 9 Fe_1–Fe_2	6.803	2	0.021
Pair 10 Mn_1–Mn_2	2.308	2	0.147

1 stand for true mangrove; 2 stands for associate mangrove

AM fungal colonization, spore density, and species diversity

AM fungal colonization was observed in the roots of all the mangrove plant species examined. Roots of different plant species exhibited the presence of arbuscules and/or vesicles. Maximum root colonization was recorded in *Thespesia populnea* (97.5%), an associate mangrove species, while the least root colonization was recorded in *Avicennia marina* (20%). Hyphal colonization (aseptate hyphae or mycelia are formed by AM fungi and can be differentiated from endophytic hyphae which are septate) was dominant in *T. populnea*. During our study, vesicles were recorded in all the plant species analyzed whereas arbuscules were rarely encountered in true mangroves (Table 3).

The maximum spore density was recorded in *Ceriops tagal* (138 spores/100 g of soil) and the minimum in *Acrostichum aureum* (20 spores/100 g of soil). *Kandelia candel* supported the greatest AM fungal species richness with 13 spore morphotypes. A total of 31 AM fungal species belonging to 9 genera were recorded (Fig. 3). *Acaulospora* (13 spp.) was the dominant genus followed by *Glomus* (6), *Gigaspora* (4), *Rhizophagus* and *Funneliformis* (2 spp. each), *Claroideoglomus*, *Sclerocystis*, *Entrophospora* and *Scutellospora* (1 sp. each). *Acaulospora dilatata* (818 spores of total 2568 spores of all the AM species) was the dominant species found occurring in 13 plant species (Table 4).

AM fungal distribution and diversity indices

Shannon–Wiener diversity (H') and Simpson's dominance index (D) was highest in *K. candel* and lowest in *D. heterophylla* (Fig. 4**).** Species evenness was highest in *A. marina* and was least in *C. tagal* and *E. Agallocha*. Maximum species richness was recorded in *K. candel* (Fig. 5).

Acaulospora dilatata recorded the highest relative abundance (RA) while the least was recorded for *C. etunicatum, G. tortuosum, G. radiatum, Gi. margarita* and *S. sinuosa*. Maximum isolation frequency (IF) was observed for *A. dilatata* and minimum for *A. mellea, A. myriocarpa, C. etunicatum, G. tortuosum, G. radiatum, Gi. margarita, Gi. gigantea, Gi. decipiens* and *S. sinuosa* (Table 5).

Species richness showed non-significant ($P > 0.05$) correlation with Simpson's dominance index ($r = 0.376$) and with species evenness ($r = 0.061$) and a significant correlation with Shannon's diversity index ($r = 0.744$, $P < 0.001$). However, species evenness showed a significant correlation with Shannon diversity ($r = 0.683$, $P < 0.01$) and Simpson's dominance index ($r = 0.747$, $P < 0.01$). A significant correlation was observed between Shannon and Simpson's diversity indices ($r = 0.816$, $P < 0.01$).

A significant correlation existed between RA and IF ($r = 0.899$, $P < 0.001$). In contrast, no correlation ($P > 0.05$) was observed between spore density and root colonization ($r = 0.277$). Similarly, spore density and species richness had a negative correlation ($r = -0.193$) that was not significant ($P > 0.05$).

The AM fungal species similarity index was highest for *A. officinalis* and *A. marina* (66.67%) and *Aegiceras corniculatum* showed less similarity with most of the plant species (Table 6).

Cluster analysis

Cluster analysis was performed based on the RA of AM fungal species in true- and associate-mangrove plants. All the plants were grouped into four clusters viz., Cluster I, Cluster II, Cluster III, and Cluster IV at a similarity level of 26%. *Aegiceras corniculatum* was not a part of any of the clusters. Cluster III was subdivided into two sub-clusters at 53% similarity. Cluster I showed *A. undulata* as a representative species with a similarity of 28.10%. In Cluster II, IIIb, and IV, *A.* dilatata showed the maximum similarity

Table 3 Arbuscular mycorrhizal colonization in true and associate mangrove species

	Family	TC (%)	HC (%)	AC (%)	VC (%)
True mangroves					
Aegiceras corniculatum (L.) Blanco	Myrsinaceae	70.28 ± 9.23^{bc}	68.09 ± 6.40^{c}	nd	61.21 ± 6.80^{bc}
Avicennia officinalis L.	Acanthaceae	41.00 ± 1.00^{efg}	34.77 ± 0.35^{fg}	nd	23.15 ± 1.39^{ef}
Avicennia marina (Forssk.) Vierh.	Acanthaceae	20.00 ± 2.89^{h}	13.25 ± 4.42^{j}	nd	6.08 ± 1.01^{h}
Bruguiera cylindrica (L.) Blume	Rhizophoraceae	30.99 ± 7.47^{fgh}	21.59 ± 4.31^{i}	nd	11.45 ± 2.29^{gh}
Ceriops tagal (Perr.) C.B. Rob.	Rhizophoraceae	58.20 ± 7.66^{cde}	57.12 ± 5.25^{d}	35.42 ± 5.06^{b}	51.66 ± 6.31^{d}
Excoecaria agallocha L.	Euphorbiaceae	74.00 ± 1.00^{bc}	59.16 ± 7.95^{d}	41.9 ± 4.66^{b}	47.50 ± 8.00^{d}
Kandelia candel (L.) Druce	Rhizophoraceae	35.16 ± 7.43^{fgh}	29.84 ± 3.73^{gh}	nd	22.40 ± 5.6^{ef}
Rhizophora apiculata Blume	Rhizophoraceae	49.08 ± 0.92^{defg}	33.85 ± 4.23^{fg}	nd	9.23 ± 1.84^{gh}
Rhizophora mucronata Lam.	Rhizophoraceae	42.93 ± 11.09^{efg}	37.46 ± 5.35^{ef}	nd	12.27 ± 1.75^{gh}
Sonneratia alba Sm.	Lythraceae	31.04 ± 7.77^{gh}	24.08 ± 3.01^{hi}	nd	16.36 ± 2.73^{fg}
Sonneratia caseolaris (L.) v	Lythraceae	50.34 ± 6.42^{def}	43.57 ± 3.00^{e}	nd	26.57 ± 1.50^{e}
Mangrove associates					
Acanthus ilicifolius L.	Acanthaceae	69.21 ± 0.79^{bc}	63.81 ± 3.80^{cd}	38.69 ± 4.30^{b}	54.76 ± 7.48^{cd}
Acrostichum aureum L.	Pteridaceae	44.34 ± 5.66^{efg}	40.13 ± 5.73^{ef}	18.65 ± 3.10^{c}	29.54 ± 3.28^{e}
Clerodendrum inerme (L.) Gaertn.	Lamiaceae	75.00 ± 10.41^{bc}	69.03 ± 7.67^{bc}	36.92 ± 4.62^{b}	64.36 ± 7.15^{b}
Derris heterophylla (Willd.) K. Heyne	Fabaceae	85.00 ± 4.08^{ab}	75.38 ± 0.50^{b}	nd	48.82 ± 5.05^{d}
Pongamia pinnata (L.) Pierre	Leguminosae	64.10 ± 5.90^{cd}	63.93 ± 7.10^{cd}	nd	48.91 ± 5.43^{d}
Thespesia populnea (L.) Sol. ex Corrêa	Malvaceae	97.50 ± 2.04^{a}	89.42 ± 6.84^{a}	58.78 ± 6.53^{a}	78.59 ± 4.99^{a}

All values are mean of three readings; ± = standard error; Values in the same column not sharing the same letters are significantly different ($P \leq 0.05$)

nd = not detected; TC, HC, AC, VC = root length containing total colonization, hyphae, arbuscules, and vesicles respectively

(20.33%, 21.41%, and 23.26% respectively). Whereas, *R. fasciculatus* showed the highest similarity level of 32.69% in Cluster IIIa (Fig. 6).

Discussion

As AM fungi are ubiquitous and ecologically significant symbionts of plants, they are affected by various biotic and abiotic factors (Liu et al. 2012). Mangrove soils of Chorao Island are acidic and silty clay. In mangrove forests, sedimentation of clay particles takes place as these forests are enclosed and protected environments with low-energy waters (Hossain and Nuruddin 2016). The study revealed the dominance of *Acaulospora dilatata*. Similar observations have been recorded earlier by D'souza and Rodrigues (2013a) who reported the dominance of genus *Acaulospora* in acidic mangrove soils of Goa. Giovannetti et al. (2010) observed that genus *Acaulospora* is predominant in low pH soils (< 6.0). Species of *Glomus* are more common in neutral to slightly alkaline soils (Kumar and Ghose 2008), while species of *Gigaspora* and *Scutellospora* are dominant in sandy soils (Lee and Koske 1994).

In the present study, most of the soil nutrient levels (including available P) in both true and associate mangroves were low. It is commonly reported that AM colonization and soil P are negatively correlated (Wang et al. 2010a). Hence, AM fungi are known to thrive better in nutrient deficient soil especially P (Hindumathi and Reddy 2011; Sridhar et al. 2011). Besides they play an important role in plant community development and enhancing plant nutrient acquisition in nutrient deficient soils of mangroves (Sridhar et al. 2011). In mangrove plants, root hairs are absent or poorly developed which limits the absorption of nutrients (Tomlinson 1986) and this feature would make plants possibly mycotrophic for nutrient uptake (Baylis 1975).

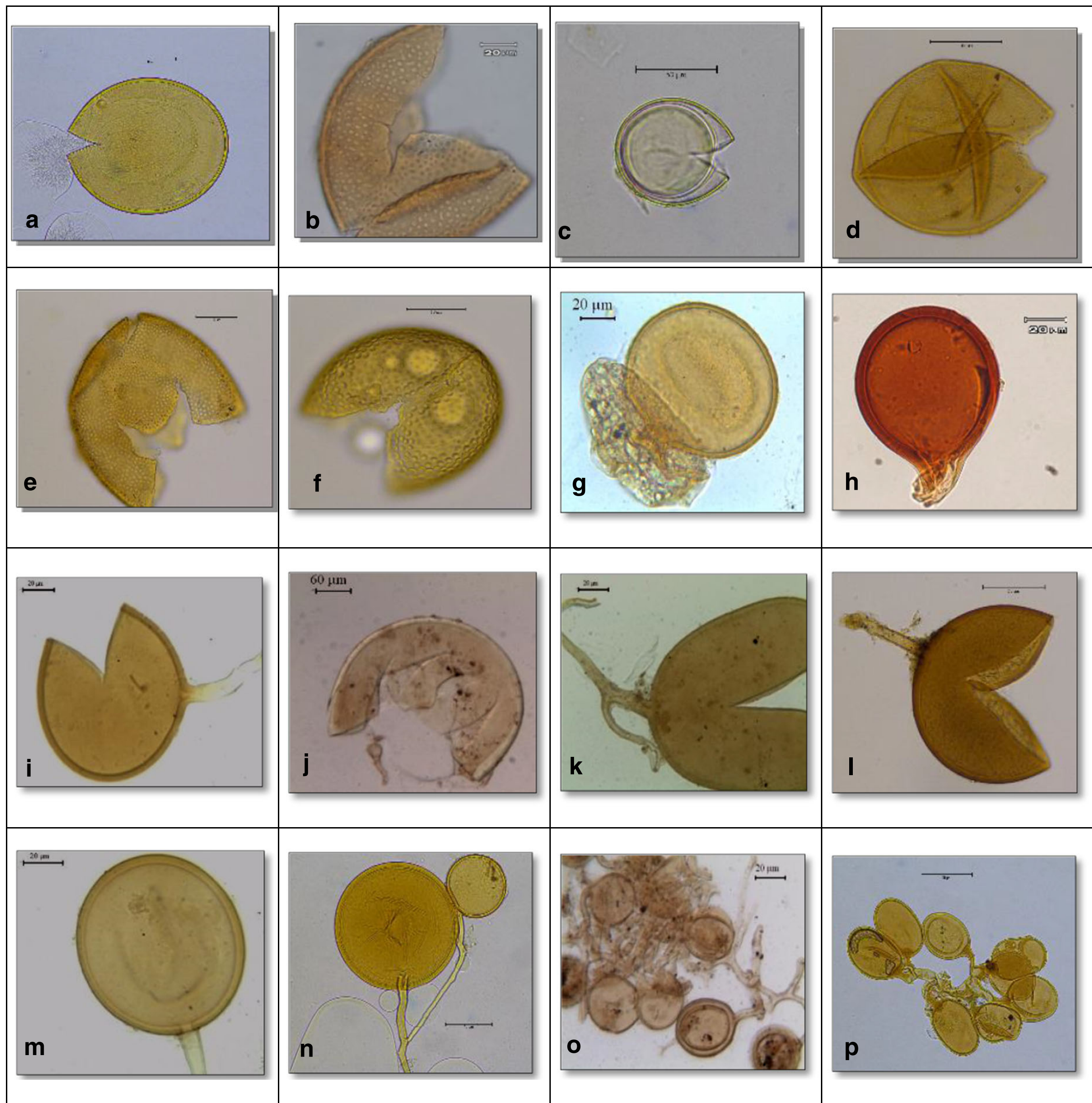

Fig. 3 Arbuscular mycorrhizal fungal species. **a** *Acaulospora dilatata*; **b** *Acaulospora foveata*; **c** *Acaulospora polonica*; **d** *Acaulospora rehmii*; **e** *Acaulospora scrobiculata*; **f** *Acaulospora undulata*; **g** *Entrophospora* sp.; **h** *Funneliformis geosporum*; **i** *Funneliformis mosseae*; **j** *Gigaspora albida*; **k** *Glomus flavisporum*; **l** *Glomus macrocarpum*; **m** *Glomus microcarpum*; **n** *Rhizophagus fasciculatus*; **o** *Rhizophagus intraradices*; **p** *Sclerocystis rubiformis*

In this study, associate mangrove plants exhibited higher AM colonization than true mangroves. Wang et al. (2014), reported similar observations in semi-mangrove communities in China. Gupta et al. (2002) reported the absence of AM colonization in three associate mangrove species viz., *A. ilicifolius, A. aureum,* and *D. heterophylla* while considerably high colonization in these plant species was recorded in the present study. Earlier studies have demonstrated that the intensity of colonization is higher in drier areas (Wang et al. 2010a, b). Therefore, in our study increased colonization rates in associate mangrove plants could be due to their distribution in the landward area of mangrove habitat.

Table 4 Spore density (SD) and diversity of arbuscular mycorrhizal fungi

	SD (spores/100 g of soil)	AM fungal species
True mangroves		
Aegiceras corniculatum	105 ± 2.50[b]	AcDi, AcLa, AcRe, AcMy, GiGi
Avicennia officinalis	34 ± 9.61[efghi]	AcGi, AcDel, ClEt, FuGe, FuMo, RhIn
Avicennia marina	39 ± 3.00[efgh]	AcDi, FuGe, RhFa, RhIn
Bruguiera cylindrica	30 ± 7.51[fghi]	AcDi, AcUn, AcLa, FuGe, RhFa, RhFn, ScRu
Ceriops tagal	138 ± 10.84[a]	AcDi, AcFo, AcMe, AcUn, FuGe, FuMo, GlFl, RhFa, RhIn, ScRu
Excoecaria agallocha	57 ± 10.90[c]	AcDi, AcSc, AcUn, AcBi, FuGe, FuMo, GiAl, RhFa, RhIn
Kandelia candel	24 ± 3.38[hi]	AcDi, AcSc, AcUn, AcDe1, AcDe2, FuGe, FuMo, GlMa, GlFl, ScSi, RhFa, *Scutellospora* sp. (unidentified), *Entrophosphora* sp. (unidentified)
Rhizophora apiculata	36 ± 11.24[efgh]	AcDi, AcSc, AcNi, FuGe, GiAl, RhFa, RhIn
Rhizophora mucronata	96 ± 10.14[b]	FuGe, GlRa, RhFa, RhIn, ScRu
Sonneratia alba	58 ± 9.5[defg]	AcDi, AcSc, AcUn, FuGe, GlTo, RhFa
Sonneratia caseolaris	94 ± 10.00[de]	AcDi, AcSc, AcRe, FuGe, RhFa, *Scutellospora* sp. (unidentified)
Mangrove associates		
Acanthus ilicifolius	26 ± 3.46[ghi]	AcDi, AcFo, AcSc, AcLa, AcNi, FuGe, GlMa, RhFa, RhIn, ScRu, *Entrophospora* sp. (unidentified)
Acrostichum aureum	20.00 ± 8.00[i]	AcDi, AcSc, AcBi, AcDe2, AcRe, FuGe, GlMu,
Clerodendrum inerme	129 ± 5.51[a]	AcDi, AcFo, AcLa, GlMa, RhFa
Derris heterophylla	59.50 ± 2.50[cd]	AcUn, AcNi, RhFa
Pongamia pinnata	37.50 ± 7.50[efgh]	AcFo, AcSc, AcUn, AcSp, GiMa, GiDe
Thespesia populnea	47.67 ± 1.67[def]	AcDi, AcDe2, AcRe, AcSp, FuGe, GlMu, RhFa

All values are mean of three readings; ± = standard error; Values in the same column not sharing the same letters are significantly different ($P \leq 0.05$)

AM species: AcDi = *Acaulospora dilatata*, AcFo = *A. foveata*, AcMe = *A. mellea*, AcSc = *A. scrobiculata*, AcUn = *A. undulata*, AcDe1 = *A. denticulata*, AcLa = *A. laevis*, AcBi = *A. bireticulata*, AcDe2 = *A. delicata*, AcNi = *A. nicolsonii*, AcRe = *A. rehmii*, AcMy = *A. myriocarpa*, AcSp = *A. spinosa*, ClEt = *Claroideoglomus etunicatum*, FuGe = *Funneliformis geosporum*, FuMo = *F. mosseae*, GlMa = *Glomus macrocarpum*, GlTo = *G. tortuosum*, GlFl = *G. flavisporum*, GlMu = *G. multicaule*, GlRa = *G. radiatum*, GiMa = *Gigaspora margarita*, *GiGi* = *Gi. gigantea*, GiDe = *Gi. decipiens*, GiAl = *Gi. albida*, RhFa = *Rhizophagus fasciculatus*, RhIn = *R. intraradices*, ScRu = *Sclerocystis rubiformis*, ScSi = *S. sinuosa*

Roots of associate mangroves plant species had a high percentage of TC, HC, and VC and recorded arbuscules in more plant species compared to true mangroves. Hence, their scantiness in true mangroves could be due to their sensitivity towards inundated and saline environments (Wang et al. 2014). Zhao (1999) suggested that factors such as host species, phenology, mycorrhizal dependency, dormancy, and changes in

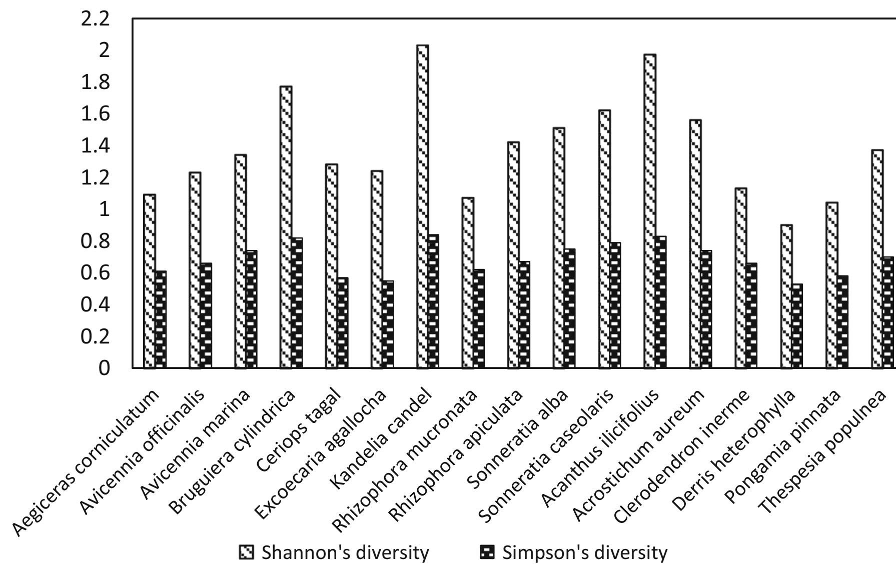

Fig. 4 Shannon and Simpson's diversity indices of arbuscular mycorrhizal fungi

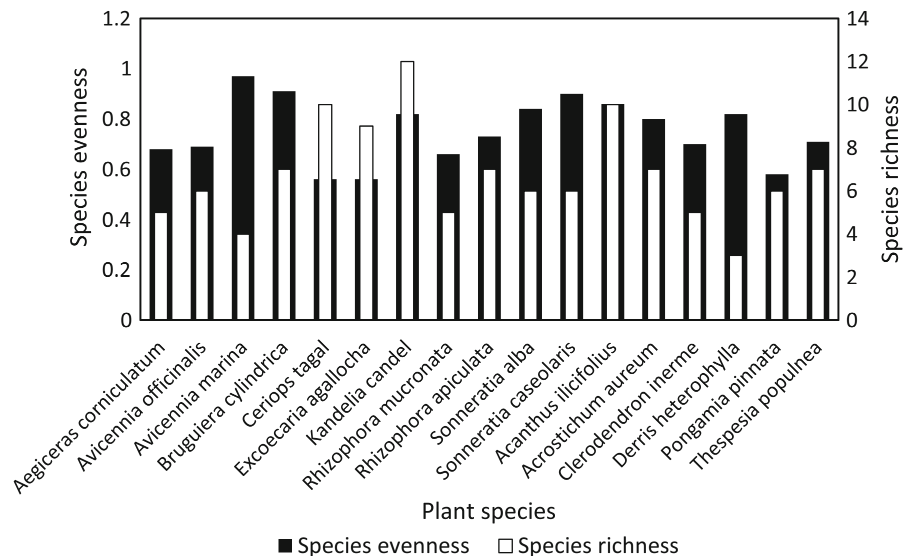

Fig. 5 Species evenness and species richness of arbuscular mycorrhizal fungi

soil conditions contribute to the variation in AM fungal colonization and spore density. However, *Derris* species showed least AM species richness as observed in an earlier study by D'souza and Rodrigues (2013a). Sridhar et al. (2011) however recorded high species richness in the same plant species and suggested that AM species richness is dependent on a host plant. Also, incompatibility between AM fungal species and the host plant (host preference), and environmental conditions (environmental preference) may result in lesser species richness (He et al. 2002; Jansa et al. 2002, 2014; Trejo et al. 2013).

In the present study, low AM fungal spore density was recorded in rhizosphere soils of both true and associate mangroves. This is in conformity with an earlier study by Kumar and Ghose (2008). Salinity and tidal currents could be responsible for low spore density in the mangrove environment (Wang et al. 2014). Balachandran and Mishra (2012) however reported high spore density and root colonization in heavy metal polluted mangroves sites.

Table 5 Relative abundance (RA) and isolation frequency (IF) of arbuscular mycorrhizal fungi

	RA (%)	IF (%)
Acaulospora dilatata Morton	30.69	82.35
Acaulospora foveata Trappe & Janos	4.66	23.53
Acaulospora mellea Spain & Schenck	0.10	5.88
Acaulospora scrobiculata Trappe	4.28	47.06
Acaulospora undulata Sieverd	4.56	41.18
Acaulospora denticulata Sieverd. & Toro	0.24	11.76
Acaulospora laevis Gerd. & Trappe	2.24	23.53
Acaulospora bireticulata Rothwell & Trappe	0.28	11.76
Acaulospora delicata Walker, Pfeiff. &Bloss	1.00	17.65
Acaulospora nicolsonii Walker, Reed & Sanders	3.94	17.65
Acaulospora rehmii Sieverd. & Toro	1.35	23.53
Acaulospora myriocarpa Spain, Sieverd. & Schenck	5.87	5.88
Acaulospora spinosa Walker & Trappe	0.07	11.76
Claroideoglomus etunicatum (Becker & Gerd.) Walker & Schüßler	0.03	5.88
Entrophospora sp. (unidentified)	0.41	11.76
Funneliformis geosporum (Nicolson & Gerd.) Walker & Schüßler	12.88	76.47
Funneliformis mosseae (Nicolson & Gerd.) Walker & Schüßler	1.42	23.53
Glomus macrocarpum Tul. & Tul.	0.72	17.65
Glomus tortuosum Schenck & Sm.	0.03	5.88
Glomus flavisporum (Lange & Lund) Trappe & Gerd.	0.07	11.76
Glomus multicaule Gerd. & Bakshi	0.03	11.76
Glomus radiatum (Thaxt.) Trappe & Gerd.	0.10	5.88
Gigaspora margarita Becker & Hall	0.03	5.88
Gigaspora gigantea (Nicolson & Gerd.) Gerd. & Trappe	0.03	5.88
Gigaspora decipiens Hall & Abbott	0.21	5.88
Gigaspora albida Schenck & Sm.	0.07	11.76
Rhizophagus fasciculatus (Thaxt.) Gerd. & Trappe	0.17	76.47
Rhizophagus intraradices (Schenck & Sm.) Walker & Schüßler	19.92	47.06
Sclerocystis rubiformis Gerd. & Trappe	4.14	23.53
Sclerocystis sinuosa Gerd. & Bakshi	0.31	5.88
Scutellospora sp. (unidentified)	0.14	11.76

In the present study, a significant positive correlation between RA and IF of AM species was observed indicating that the species producing more spores have a wide distribution, while those producing fewer spores have small geographic ranges (Dandan and Zhiwei 2007).

The genus *Gigaspora* recorded low relative abundance compared to other AM genera. Species belonging to Gigasporaceae predominates in sandy soils especially sand dunes (Day et al. 1987; Lee and Koske 1994). The soil at Chorao Island is less sandy which could have resulted in a lower abundance of the species of genus *Gigaspora.*

In the present study, a total of 31 AM species were recovered from 17 mangrove species indicating that the site is diverse in AM species. High environmental heterogeneity in mangrove habitats may assist in higher AM diversity (Fabián et al. 2018). Flooding has been identified as the cause of heterogeneity and dynamics of these ecosystems (Simoes et al. 2013) that might affect the distribution of AM fungi.

Moreover, in the present study, a high AM species similarity (up to 66.67%) indicated broad dispersal of AM species. Similar observations were recorded in an earlier study in Nethravathi mangroves where the AM species similarity ranged from 12.1 to 55% (Sridhar

Table 6 Jaccard's similarity index (%) of arbuscular mycorrhizal fungi among the mangrove plant species

	AO	AM	BC	CT	EA	KC	RA	RM	SA	SC	AI	AA	CI	DH	PP	TP
AC	10.00	12.50	20.00	7.14	7.69	5.88	9.09	0.00	10.00	22.22	14.29	20.00	25.00	0.00	0.00	20.00
AO		66.67	44.44	33.33	36.36	26.67	30.00	22.22	20.00	20.00	21.43	18.18	10.00	0.00	0.00	18.18
AM			57.14	40.00	44.44	21.43	57.14	50.00	42.86	42.86	36.36	22.22	28.57	16.67	0.00	37.50
BC				54.55	45.45	25.00	40.00	50.00	44.44	30.00	50.00	16.67	33.33	25.00	8.33	27.27
CT					46.15	35.29	30.77	36.36	33.33	23.08	40.00	13.33	25.00	18.18	14.29	21.43
EA						37.50	60.00	27.27	50.00	36.36	33.33	33.33	7.69	20.00	15.38	23.08
KC							25.00	12.50	35.71	35.71	26.32	25.00	20.00	14.29	11.76	25.00
RA								33.33	44.44	44.44	50.00	27.27	20.00	25.00	8.33	27.27
RM									22.22	22.22	33.33	9.09	11.11	14.29	0.00	20.00
SA										50.00	30.77	30.00	22.22	28.57	20.00	30.00
SC											30.77	44.44	22.22	12.50	9.09	44.44
AI												20.00	45.45	16.67	13.33	20.00
AA													9.09	0.00	8.33	40.00
CI														14.29	10.00	20.00
DH															12.50	11.11
PP																8.33

AC = *Aegiceras corniculatum*, AO = *Avicennia officinalis*, AM = *Avicennia marina*, BC = *Bruguiera cylindrica*, CT = *Ceriops tagal*, EA = *Excoecaria agallocha*, KC = *Kandelia candel*, RM = *Rhizophora mucronata*, RA = *Rhizophora apiculata*, SA = *Sonneratia alba*, SC = *Sonneratia caseolaris*, AI = *Acanthus ilicifolius*, AA = *Acrostichum aureum*, CI = *Clerodendrum inerme*, DH = *Derris heterophylla*, PP = *Pongamia pinnata*, TP = *Thespesia populnea*

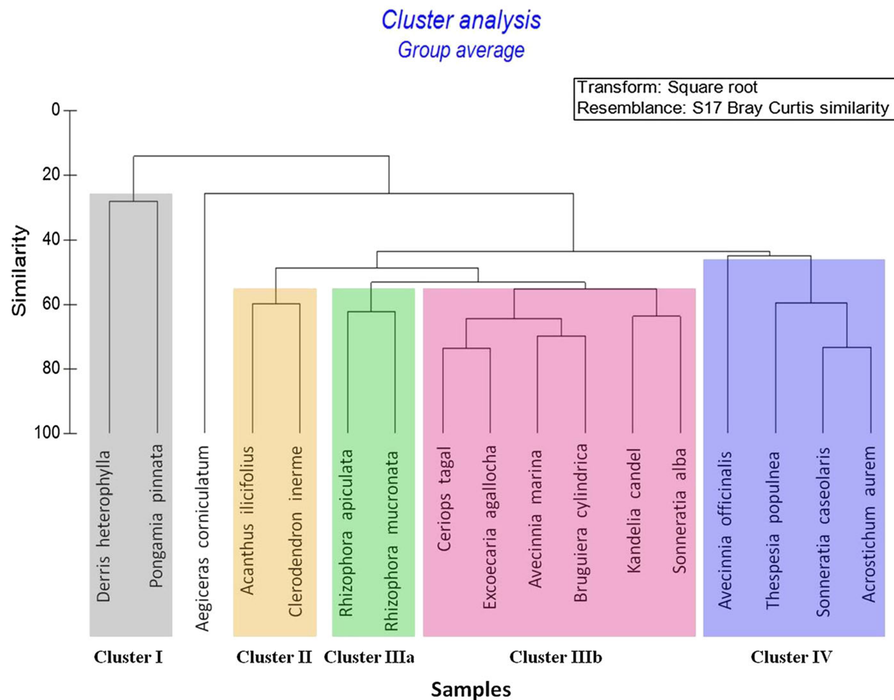

Fig. 6 Cluster analysis showing similarity in the abundance of AM fungal species among true- and associate-mangrove plants

et al. 2011). Movement of AM fungal species from adjoining terrestrial habitats to intertidal zones of mangrove ecosystem (Wang et al. 2014) could be the cause of similarity in genus and species composition in true mangroves that inhabit intertidal zone areas and associate mangrove plants from the landward periphery of mangrove habitat.

Our study revealed non-uniform distribution and community structure of AM fungi associated with different true and associate mangrove plants that varied significantly. Also, appreciable diverse forms of AM species in the study area support to conclude that these species can tolerate high salinity. The identification of abundant and recurrent AM species among the mangrove plants, ascertain the fact that these species could be used efficiently in re-establishment of mangrove habitats. Further research requires a meticulous study of seasonal effects as well as soil parameters on AM fungal growth in the mangrove environment.

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RESEARCH ARTICLE

Arbuscular mycorrhizal fungal status in mangroves of Pichavaram Forest, Tamil Nadu, India

Sankrita Gaonkar[1] · B. F. Rodrigues[1]

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Abstract
The diversity and species distribution of arbuscular mycorrhizal (AM) fungi were analyzed in mangrove patches of the Pichavaram Forest. The AM fungal colonization, spore density, and diversity indices in 18 species of true- and associate-mangrove were investigated. Soil analysis results indicated low levels of available nutrients, especially phosphorus (P), with soil being neutral to slightly alkaline, having high electrical conductivity. Some of the earlier reported non-mycorrhizal plant families also showed AM symbiosis with a high degree of root colonization. All the species at the three sites explored showed significant variation in AM fungal root colonization and spore density. The salt marsh species *Salicornia brachiata* showed the highest degree of root colonization. Overall 21 AM species belonging to seven genera were recovered from the mangroves of Pichavaram Forest. Statistical analysis showed that relative abundance and isolation frequency of AM fungal species were positively correlated. Among AM fungi, species belonging to the family Glomeraceae were dominant. Our results indicated that AM fungal communities differ with the change in soil conditions within the short distances among the habitats.

Keywords AM fungal diversity indices · Isolation frequency · Phosphorus · Relative abundance · Soil electrical conductivity · Sørensen's coefficient · Spore density

Introduction

Mangrove is a highly productive wetland ecosystem occupying the marine intertidal zone in tropical and sub-tropical regions. Mangroves are rich and diverse in living resources and hence increase the economic and ecological value of the ecosystem (Kathiresan 2000). However, there are several causes of mangrove destruction, hypersalinity being one of the causes at Pichavaram (Bhatt and Kathiresan 2011) and Sundarbans (Selvam et al. 2002). Besides, the mangrove ecosystems have become a focus of conservation and environmental issues (Gopinathan et al. 2017).

Mangroves show substantial tolerance to salinity, inundation, and nutrient stress. However, they have been degraded drastically all over the world, mainly due to nutrient limitations (P deficiency) and human interference (Xie et al. 2014). Studies have shown that the P availability is low in mangrove ecosystems as it is absorbed and co-precipitated within carbonate-dominated environments, thus limiting the growth of mangrove plants (Lovelock et al. 2004). Hence, the protection and restoration of mangrove ecosystems have become a global concern (Krauss et al. 2008).

Pichavaram Forest is known to be the world's second-largest mangrove forest (Mariappan et al. 2016) after Sundarbans with *Avicennia marina* and *Rhizophora* species being predominant (Kathiresan 2000). It is situated between Vellar estuary (North) and Coleroon estuary (South) (Srivastava et al. 2012) on the Coromandal coast (Bay of Bengal Sea Board) (Lingan et al. 1999). It receives three types of waters viz., neritic, brackish, and freshwater from the Bay of Bengal, Vellar-Coleroon estuaries, and irrigation and main channel of Coleroon river, respectively (Kathiresan 2000).

Arbuscular Mycorrhizal (AM) fungi are the 'hidden heroes' of nutrient-deficient soil, especially P (Hindumathi and Reddy 2011), which helps in increased uptake of mineral nutrients and improve stress tolerance in exchange for carbon (Smith and Read 2008). The saline and anaerobic conditions of the mangrove rhizosphere limit the occurrence of AM fungi in these environments (Wang et al. 2010). Various AM fungal species colonizing the roots of different plant

✉ Sankrita Gaonkar
sankrita002@gmail.com

[1] Department of Botany, Goa University, Panjim, Goa 403206, India

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species play a crucial role in the regeneration, diversity, and distribution of plant communities (Nandi et al. 2014).

To establish conservation strategies, it is important to explore the ecological framework of the habitat. Accordingly, the objective of this investigation was to study the AM fungal diversity and to record the dominant AM fungal species associated with the mangrove plants of Pichavaram Forest. The present study also investigates the effect of various soil parameters on the distribution of AM fungi.

Materials and methods

Study site and sample collection

Pichavaram Forest (11°20'–11°30' N; 79°45'–79°55' E) is situated on the southeast coast of India. It is a mangrove swamp located in the Vellar-Coleroon estuarine complex. The total area of the Pichavaram Forest is 1100 ha, traversed by 51 islets (Kathiresan 2000). About 241 ha of the entire forest is occupied by dense mangrove cover (Srivastava et al. 2012; Arunprasath and Gomathinayagam 2014). The average annual rainfall is 1310 mm (Selvam et al. 2003).

In the present study, 18 plant species with each of nine true- and associate-mangroves belonging to 12 families were investigated. The 18 plants were randomly selected based on the ease of soil collection. The rhizosphere samples were collected from three mangrove sites of Pichavaram Forest viz., Pichavaram extension (PE), Pichavaram Reserved Forest (PRF), and Killai Reserved Forest (KRF) (Fig. 1).

The collection of soil and root samples was carried out from November 2015 to October 2016. The root system of each plant was dug up to trace and collect the roots belonging to that plant.

Three rhizosphere soil samples were collected from each plant species, placed in separate sealed bags, labeled, and brought to the laboratory. These three samples of each plant species were then separately mixed to form a composite sample. The roots were separated from adhering soil, washed gently under tap water, and used for assessment of AM colonization.

Each composite soil sample was divided into two parts, one for AM spore isolation, enumeration, and identification, and the other as inocula to prepare trap cultures.

Soil analyses

To carry out the soil analyses triplicates of rhizosphere samples (0–15 cm) from three different estuarine regions of the study site were collected in separate sealed bags, brought to the laboratory, and were air-dried. To measure soil pH and electrical conductivity (EC), a soil–water suspension (40% w/v) was prepared. The pH was measured using a pH meter (LI 120 Elico, India), and the EC was measured using a conductivity meter (CM-180 Elico, India). Available Nitrogen (N) and P were measured using the methods of Subbiah and Asija (1956) and Bray and Kurtz (1945), respectively. For the detection of soil available potassium (K), the ammonium acetate method (Hanway and Heidel 1952) was employed. The determination of available Zinc (Zn), Copper (Cu), Manganese (Mn), and Iron (Fe) was done by the DTPA-$CaCl_2$-TEA method of Lindsay and Norvell (1978) using an atomic absorption spectrophotometer (Nova 400P, Analytik Jena, Germany).

Assessment of root colonization

Approximately one-centimeter-long root segments were hydrolyzed in 10% KOH at 90 °C for 90 min, followed by acidification in 2 N HCl for 5 min. Root segments were then stained overnight with 0.05% Trypan blue (Phillips and Hayman 1970). After staining, root segments were mounted on glass slides using polyvinyl alcohol Lacto-glycerol (PVLG) as a mountant and examined under a Brightfield Olympus BX41 research microscope (40×, 100× and 400×). A root segment was considered mycorrhizal if it showed the presence of hyphae, arbuscules, and/or vesicles.

Percent AM root colonization was estimated using the following formula:

$$\%\ \text{colonization} = (\text{Number of root segments colonized} \div \text{Total number of root segments observed}) \times 100$$

Isolation, identification, and spore density of AM fungi

For the identification of AM fungal species, spores from rhizosphere samples (n = 3) and trap cultures were isolated using a wet sieving and decanting method (Gerdemann and Nicolson 1963). Spore morphology, wall characteristics, dimensions, and other relevant data were observed for the identification of the AM spores. The spore characters were compared with the descriptions given by Schenck and Perez (1990), Rodrigues and Muthukumar (2009), and the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM). Revised binomials and epithets of AM fungal species were followed according to the reference of Schüßler and Walker (2010) and Redecker et al. (2013). Intact, healthy

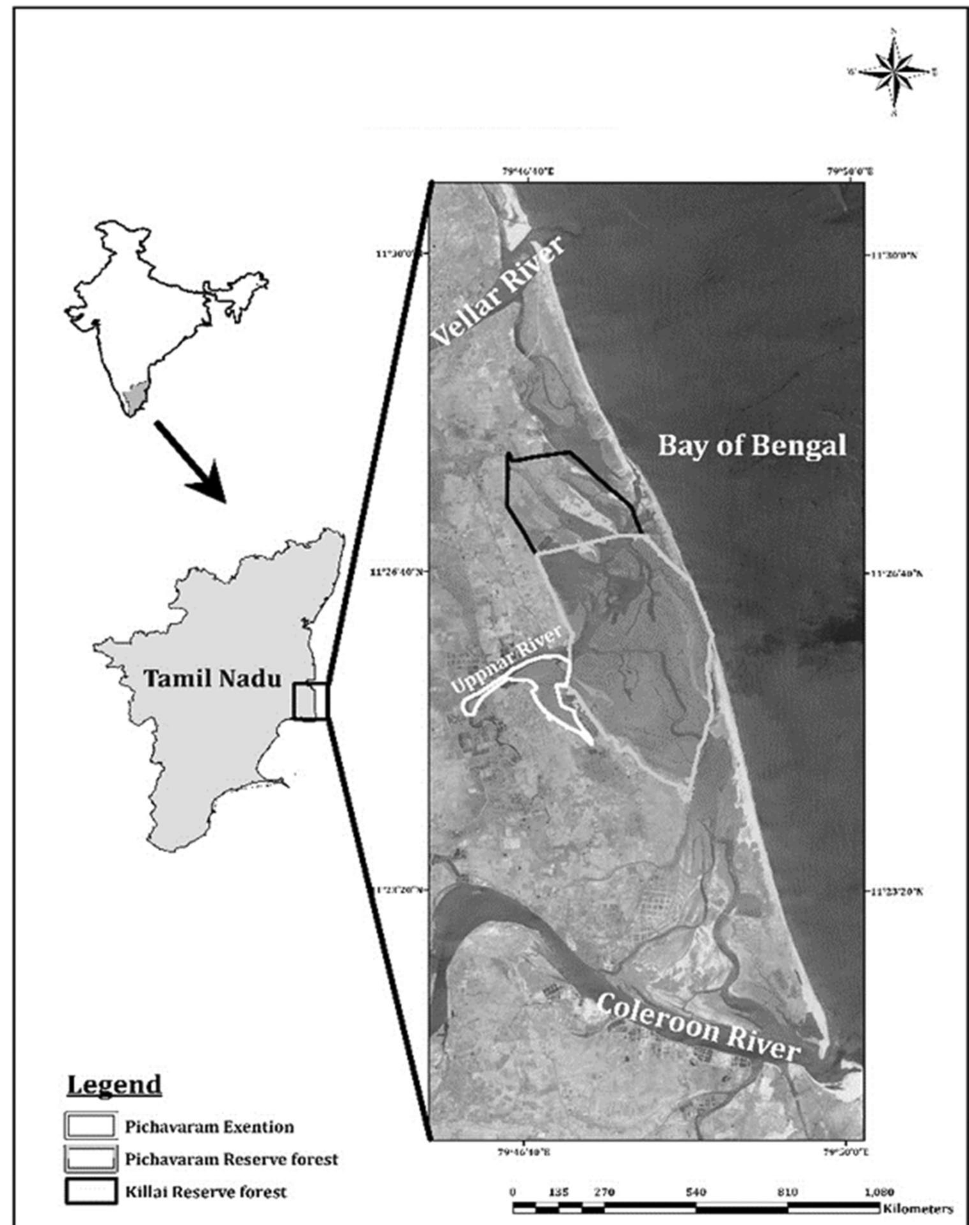

Fig. 1 Map of Pichavaram Forest showing study areas

spores from rhizosphere samples were selected for the estimation of spore density by the modified method of Gaur and Adholeya (1994).

Preparation of trap cultures

Trap cultures were prepared by following the modified trap culture method of Morton et al. (1993) to multiply AM fungal spores using the substrate mixture of rhizosphere and sterile sand (1:1) in the pots. Coleus (*Plectranthus scutellarioides*) was used as the catch plant. Coleus cuttings were first washed with tap water and then with detergent water. The cuttings were then rinsed in sterile water. Three to four cuttings were planted per pot and, the pots were maintained for six months in the polyhouse (27 °C, 63% relative humidity) for the establishment of colonization and subsequent sporulation. The plants were watered twice a week, and Hoagland's solution (Hoagland and Arnon, 1950) without P was added every 20 days. The cultures were harvested at the end of the 6th month and the spores were used for identification.

Data analysis

To quantify diversity, Simpson's diversity index, Shannon diversity index, species evenness, isolation frequency,

Table 1 Soil physico-chemical properties of the study sites

Soil Parameters	PE	PRF	KRF
pH	7.6 ± 0.8[a]	7.0 ± 0.8[ab]	6.9 ± 0.8[b]
EC (mS/cm)	5.1 ± 0.6[ab]	4.3 ± 0.6[b]	6.7 ± 0.6[a]
N (g/kg)	0.04 ± 0.005[a]	0.04 ± 0.01[a]	0.03 ± 0.01[a]
P (g/kg)	0.02 ± 0.004[a]	0.02 ± 0.01[a]	0.02 ± 0.01[a]
K (g/kg)	0.1 ± 0.02[a]	0.2 ± 0.04 [a]	0.1 ± 0.03[a]
Fe (ppm)	15.6 ± 1.7[a]	15.5 ± 1.7[a]	14.6 ± 1.6[b]
Mn (ppm)	7.9 ± 0.1[a]	7.3 ± 0.1[b]	6.12 ± 0.1[c]
Zn (ppm)	0.9 ± 0.1[a]	1.0 ± 0.1[a]	0.5 ± 0.1[b]
Cu (ppm)	1.8 ± 0.3[b]	2.0 ± 0.3[ab]	2.6 ± 0.4[a]

Data are means of three replicates; ± standard error

PE Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest

Values in the same row not sharing the same letters are significantly different ($P \leq 0.05$)

relative abundance, and species richness were calculated in PRIMER v. 6.0 using the following formulae:

$$\text{Relative abundance (\%)} = (\text{No. of spores of a species per genus} \div \text{Total no. of spores in all soil samples}) \times 100$$

$$\text{AMF species richness (SR)} = \text{Number of AM species per soil sample.}$$

The diversity of AM fungi in plant species was assessed based on the Shannon- Wiener index of diversity (H) (Shannon and Weaver 1949) and Simpson`s index of dominance (D) (Simpson 1949),

$$\text{Shannon index (H)} = -\sum (\text{pi ln pi})$$

(where *pi* is the proportions of individual that species *i* contributes to the total number of individuals)

$$\text{Simpson's index (D)} = 1 - \left(\sum n(n-1)/N(N-1)\right)$$

(where *n* is the number of individuals of a given species, and N is the total number of individuals in a community).

The evenness (E) indicates the distribution of individuals within species of AM fungi in plant species.

$$\text{Species evenness } \left[\sum (\text{H})\right] = \text{H}'/H'\text{max}$$

(where $H'\text{max} = lnS$, S = total number of species in the community).

All data were statistically analyzed using SPSS (Statistical Package for the Social Sciences) (Version 22) software. A one-way ANOVA was done to test the variation in spore density and root colonization among plant species. Pearson's correlation coefficient was calculated to evaluate the relationships between root colonization and spore density and isolation frequency and relative abundance. The similarity between the plant species was estimated by calculating Sørensen's similarity coefficient based on the presence or absence of each AM fungal species (Dandan and Zhiwei 2007).

Results and discussion

Soil properties

Soil physico-chemical properties are presented in Table 1. Soils of Pichavaram Forest are neutral to slightly alkaline (6.9–7.6). The alkaline pH at PE could be due to the inflow of a high amount of freshwater from the Coleroon River (Sahua and Kathiresan 2019). Soil electrical conductivity (EC) ranges were between 4.47 and 5.0 mS/cm. The higher EC value at KRF may be attributed to less influx of freshwater and vegetation cover. In contrast, lower soil EC is apparent at other sites due to freshwater input from irrigation and Coleroon River and thick forest canopy, which decreases evapotranspiration (Ranjan et al. 2010).

All the sites were low in available nutrients, especially P. This may be explained by the flow of water causes the leaching of soil nutrients (Gandaseca et al. 2016), and P is a highly leached element (Oelkers and Jones 2008).

Nutrients in mangrove ecosystems are controlled by a variety of biotic and abiotic factors viz., inundation, soil type, soil microbes, plant species, litter production, and decomposition (Reef et al. 2010). The Pichavaram mangrove ecosystem consists of small Islands that experience micro- and diurnal-tides (Selvam et al. 2003). The frequency and period of tidal inundation are determined by topographic factors such as elevation, which subsequently affects the salinity and soil nutrient availability, resulting in complex patterns of nutrient demand and supply (Reef et al. 2010). Furthermore, increased soil salinity decreases the availability of major nutrients such as N, P, K due to their precipitation and variation in nutrient metabolism (Evelin et al. 2009). Salinity affects N metabolism by interfering in uptake and reduction of NO_3^- and correspondingly in protein synthesis (Frechill et al. 2001). In saline soils, P becomes unavailable to the plants due to the precipitation of phosphate ions with Ca^{2+}, Mg^{2+}, and Zn^{2+} (Azcón-Aguilar et al. 1979).

When salt concentration in the soil increases, plants absorb more Na^+ which results in a reduction of K^+ uptake. This facilitates the competition between Na^+ and K^+ ions for the binding sites of cellular functions (Blaha et al. 2000).

The AM fungal structures may bind or eliminate NaCl, thereby conferring salt tolerance on the plants (Kaldorf et al. 1999).

Colonization and spore density

AM fungal colonization was observed in the roots of all the mangrove plant species sampled. Maximum root colonization was recorded in *Salicornia brachiata* (93.54%) at KRF and least in *Avicennia marina* (22.08%) at PRF. The low colonization in *A. marina* could be attributed to its occurrence in inundated areas. According to the earlier studies, AM fungi exhibit low tolerance to hypoxic conditions as they are aerobic microbes (Allaway et al. 2001; Wang et al. 2011). Previous publications have suggested that hyphal networks are initiators of colonization (Smith and Read 1997). High levels of soil salinity in salt marshes have been observed to reduce the extra-radical mycelia growth (Carvalho et al. 2003). However, in the present study, *S. brachiata* showed a high degree of colonization despite a stressful environment.

Maximum spore density was recorded in *Ceriops decandra* (270/100 g of soil) at PE and minimum in *Rhizophora mucronata* (8 spores/100 g of soil) at PRF (Table 2). Parameters such as inoculum density, root structure, genetic compatibility between host and AM fungi, edaphic factors, and soil microbes affect AM colonization rates and spore density (Zangaro et al. 2013; Sivakumar 2013). Likewise, AM fungal community structure is affected by the tide level (Wang et al. 2011). This could be one of the reasons for maximum and minimum spore density in *C. decandra* and *R. mucronata,* respectively, since the inundation level of the former plant is lower than the latter (Batool et al. 2014) at the study site. Inhibition of AM colonization seldom take place due to increased P level occurs in the wetland ecosystem (Kelly et al. 2004). However, higher rates of AM colonization in the present study may be attributed to low levels of P at all the sites investigated. The study revealed variation in root colonization and spore density. According to Hildebrandt et al. (2001), the intensity of AM colonization is not the same during the plant life cycle, and hyphae bundled with spore strings could be patchily distributed in the soil, which might lead to more spore counts in a single soil sample.

All the mangrove plants examined in the present study from Pichavaram were mycorrhizal. This contradicts the study of Mohankumar and Mahadevan (1986) who reported no AM association in Pichavaram mangroves, while Lingan et al. (1999) reported AM colonization in eight mangrove plants from the same site. It is interesting to observe that halophytes belonging to the family Chenopodiaceae are considered non- or poorly mycorrhizal (Juniper and Abbott 1993; Aliasgharzadeh et al. 2001; Wilde et al. 2009). However, the present study reveals clear evidence that species of Chenopodiaceae (now Amaranthaceae) family viz., *Salicornia brachiata, Arthrocnemum indicum, Suaeda maritima,* and *S. monoica* showed a high degree of AM colonization compared to all the other mangrove plants which is in accordance with Hildebrandt et al. (2001) and Yinan et al. (2017).

Diversity and distribution

A total of 21 AM fungal species belonging to seven genera were recovered. Among them, eight species belonged to the genus *Acaulospora,* one to *Entrophospora,* two to *Funneliformis,* three to *Gigaspora,* two to *Glomus,* three to *Rhizophagus,* and two to *Sclerocystis*. *Rhizophagus fasciculatus* was found to be dominant at PE and PRF, whereas *Funneliformis geosporum* was dominant at KRF (Table 3). The predominance of *F. geosporum* at KRF could be due to the dominance of salt marshes at the site, as *F. geosporum* is often dominant in salt marshes (Carvalho et al. 2004). Similar observations were reported by d'Entremont et al. (2018) in salt marshes of Minas Basin, Nova Scotia. They have also suggested that *F. geosporum* has been found globally and is one of the most halotolerant AM fungal species.

AM fungal species richness differed significantly. Goomaral et al. (2013) suggested that the AM fungal diversity and community composition are affected by the host plant. This could be because the structure and functioning of different host plants vary (Chen et al. 2012). High environmental heterogeneity could be another reason behind the high AM fungal richness observed in the present study. Flooding has been verified as the chief source for heterogeneity in wetlands (Simões et al. 2013).

The highest relative abundance (RA) and isolation frequency (IF) of AM species were recorded in *R. fasciculatus* at PE and PRF and *F. geosporum* at KRF, respectively (Figs. 2, 3). A significant positive correlation was found between RA and IF at all the three sites ($r=0.94$, $P<0.01$ at PE; $r=0.75$, $P<0.05$ at PRF and $r=0.83$, $P<0.01$ at KRF). Some of the AM species, such as *A. dilatata, A. undulata, Entrophospora* sp, and *R. irregulare* showed low relative abundances but were widely distributed with high isolation frequencies. There was no significant correlation between spore density and root colonization at all three sites. In terms of genera, the highest RA was recorded in *Rhizophagus* at PRF, and the highest IF was recorded in *Acaulospora* and *Funneliformis* (Fig. 4).

Table 2 Percent root colonization (RC), spore density (SD) in Pichavaram mangroves

Plant Name	PE		PRF		KRF	
	Percent Colonization	*Spore density	Percent Colonization	*Spore density	Percent Colonization	*Spore density
True mangroves						
Aegiceras corniculatum (L.) Blanco (Myrsinaceae)	65.9 ± 0.8^{abc}	79.0 ± 2.5 ^{cd}	nd	nd	nd	nd
Avicennia marina (Forssk.) Vierh. (Acanthaceae)	27.6 ± 1.0^{f}	89.0 ± 7.0^{c}	22.1 ± 5.4^{f}	64.0 ± 4.5^{e}	40.2 ± 4.2^{c}	87.0 ± 7.5^{c}
Avicennia officinalis L (Acanthaceae)	76.5 ± 1.5^{a}	92.0 ± 4.5^{c}	51.0 ± 1.0 ^{cd}	124.0 ± 9.0^{a}	nd	nd
Brugueira cylindrica (L.) Blume (Rhizophoraceae)	54.4 ± 0.58^{bcde}	30.0 ± 8.0^{f}	63.8 ± 8.8^{bc}	105.0 ± 1.0^{bc}	nd	nd
Ceriops decandra(Griff.) W.Theob (Rhizophoraceae)	72.5 ± 7.5^{ab}	270.0 ± 1.0^{a}	27.5 ± 2.5^{f}	90.0 ± 3.5 ^{cd}	nd	nd
Excoecaria agallocha L (Euphorbiaceae)	77.0 ± 13.0^{a}	60.0 ± 2.0^{e}	22.7 ± 0.8^{f}	79.0 ± 5.0^{de}	90.0 ± 3.3^{a}	96.0 ± 5.0^{c}
Lumnitzeraracemosa Willd (Combretaceae)	70.2 ± 11.9^{ab}	142.0 ± 8.0^{b}	65.0 ± 3.0^{b}	114.0 ± 1.5^{ab}	nd	nd
Rhizophora apiculata Blume (Rhizophoraceae)	37.5 ± 2.5^{ef}	17.0 ± 0.5^{f}	45.0 ± 5.0^{de}	104.0 ± 4.5^{bc}	nd	nd
Rhizophora mucronata Lam (Rhizophoraceae)	nd	nd	32.7 ± 0.7^{ef}	8.0 ± 1.0^{f}	nd	nd
Associate mangroves & salt marshes						
Arthrocnemum indicum (Willd.) Moq (Amaranthaceae)	nd	nd	nd	nd	65.4 ± 3.9^{b}	38.0 ± 5.5^{e}
Clerodendrum inerme (L.) Gaertn (Lamiaceae)	nd	nd	nd	nd	88.5 ± 3.9^{a}	127.0 ± 6.0^{b}
Salicornia brachiata Miq (Amaranthaceae)	nd	nd	nd	nd	93.5 ± 0.2^{a}	30.0 ± 3.5^{e}
Ipomoea pes-caprae (L.) R. Br (Convolvulaceae)	nd	nd	nd	nd	55.0 ± 7.5^{b}	102.0 ± 6.5^{c}
Phoenix paludosa Roxb. (Aracaceae)	nd	nd	88.5 ± 3.9^{a}	71.0 ± 11.5^{e}	nd	nd
Salvadora persica L (Salvadoraceae)	42.3 ± 3.9^{def}	77.0 ± 6.5 ^{cd}	nd	nd	nd	nd
Sesuvium portulacastrum (L.) L (Aizoaceae)	50.0 ± 3.9^{cde}	22.0 ± 0.5^{f}	nd	nd	nd	nd
Suaeda monoica Forssk. ex J.F.Gmel. (*Amaranthaceae)*	nd	nd	nd	nd	88.5 ± 3.9^{a}	67.0 ± 5.5^{d}
Suaeda maritima (L.) Dumort (Amaranthaceae)	58.0 ± 3.5^{bcd}	71.0 ± 3.5^{de}	nd	nd	89.0 ± 2.7^{a}	161.0 ± 8.0^{a}

Data are means of three replicates; ± standard error

PE Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest, *nd* plant not detected at the site

[a]Indicates spores/100 g of soil

Values in each column followed by different letters are significantly different at $P < 0.05$

Table 3 Occurrence of AM species in Pichavaram mangroves

Plant Name	PE	PRF	KRF
True mangroves			
Aegiceras corniculatum	*A. po, A. un, F. geo, R. fas, R. irr*	nd	nd
Avicennia marina	*R. fas, R. irr*	*Entrophospora* sp., *R. fas*	*A. un, F. geo, R. fas*
Avicennia officinalis	*A. di, A. un, Entrophospora* sp., *R. fas, R. irr*	*A. di, A. ni, Entrophospora* sp., *R. fas, R. irr*	nd
Brugueira cylindrica	*A. di, Entrophospora* sp., *F. geo, R. fas, R. irr*	*A. di, A. ni, Entrophospora* sp., *G. mic, R. fas, R. irr*	nd
Ceriops decandra	*Entrophospora* sp., *F. geo, R. fas, R. irr*	*R. fas, R. irr*	
Excoecaria agallocha	*Entrophospora* sp., *G. alb, G. mic, R. fas, R. irr*	*A. fo, A. un, Entrophospora* sp., *F. geo, R. fas*	*A. di, A. un, F. geo, G. alb*
Lumnitzera racemosa	*Entrophospora* sp., *R. fas*	*A. di, Entrophospora* sp., *F. geo, R. fas, R. irr*	nd
Rhizophora apiculata	*A. di, A. re, A. sc, A. un, Entrophospora* sp., *F. geo, R. fas*	*Entrophospora* sp., *R. fas, R. irr*	nd
Rhizophora mucronata	nd	*Entrophospora* sp., *R. irr*	nd
Mangrove associates and salt marshes			
Arthrocnemum indicum	nd	nd	*A. un, F. geo, G. alb, R. fas*
Clerodendrum inerme	nd	nd	*A. di, A. un, F. geo, G. dec, G. mar, R. int, S. pac, S. rub*
Salicornia brachiata	nd	nd	*A. di, F. geo, G. agg*
Ipomoea pes-caprae	nd	nd	*A. di, A. un, F. geo*
Phoenix paludosa	nd	*A. di, A. un, F. geo*	nd
Salvadora persica	*A. un, F. geo*	nd	nd
Sesuvium portulacastrum	*Entrophospora* sp., *F. geo, R. fas*	nd	nd
Suaeda monoica	nd	nd	*A. un, F. geo, S. rub*
Suaeda maritima	*A. po, A. sp, Entrophospora* sp., *F. geo, R. fas*	nd	*A. di, A. un, F. geo, F. mos, R. fas*

nd not detected, AM species: *A. di Acaulospora dilatata, A. fo A. foveata, A. ni A. nicolsonii, A. po A. polonica, A. re A. rehmii, A. sc A. scrobiculata, A. sp A. spinosa, A. un A. undulata, Entrophospora* sp. Unidentified, *F. geo Funneliformis geosporum, F. mos F. mosseae, G. alb Gigaspora albida, G. dec G. decipiens, G. mar G. margarita, G. agg Glomus aggregatum, G. mic G. microcarpum, R. fas Rhizophagus fasciculatus, R. int R. intraradices, R. irr R. irregulare, S. pac Sclerocystis pachycaulis, S. rub S. rubiformis, PE* Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest

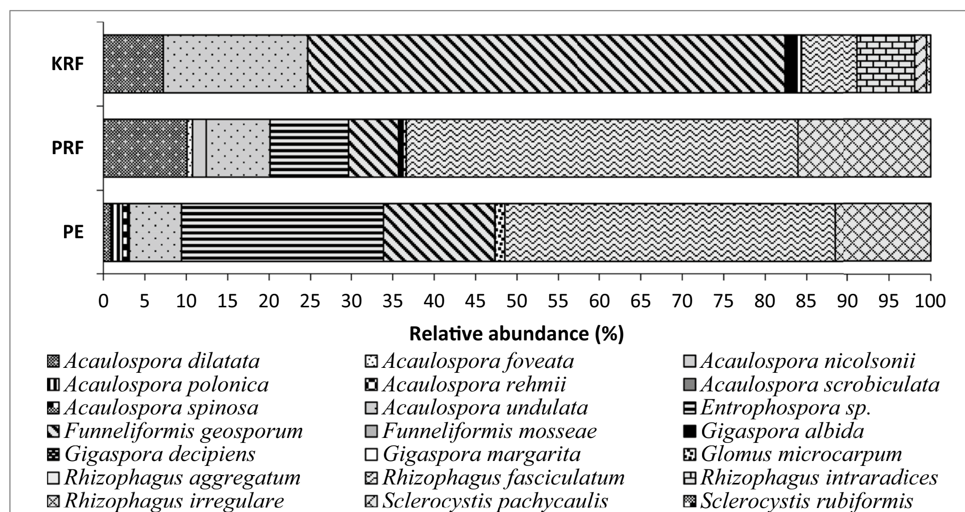

Fig. 2 Relative abundance of AM fungal species. *PE* Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest

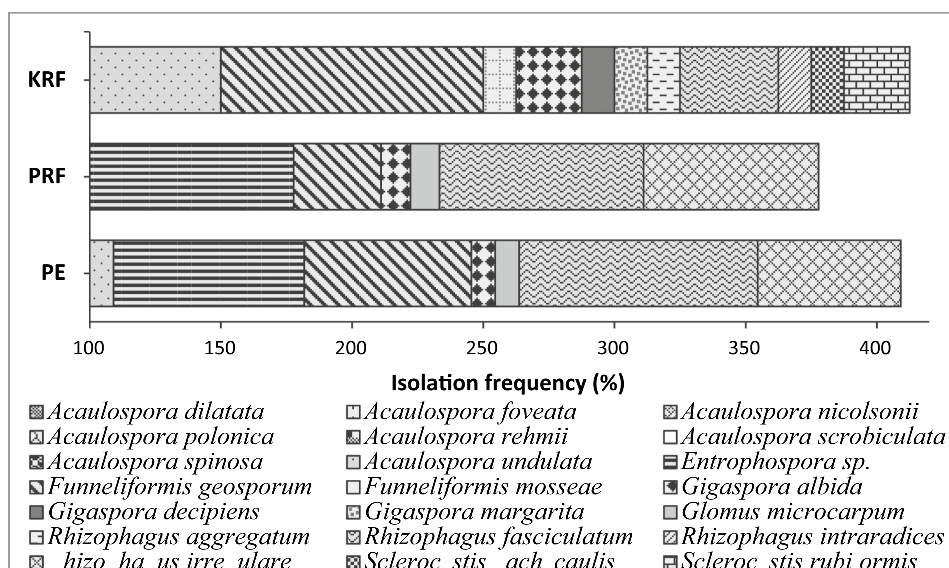

Fig. 3 Isolation frequency of AM fungal species. *PE* Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest

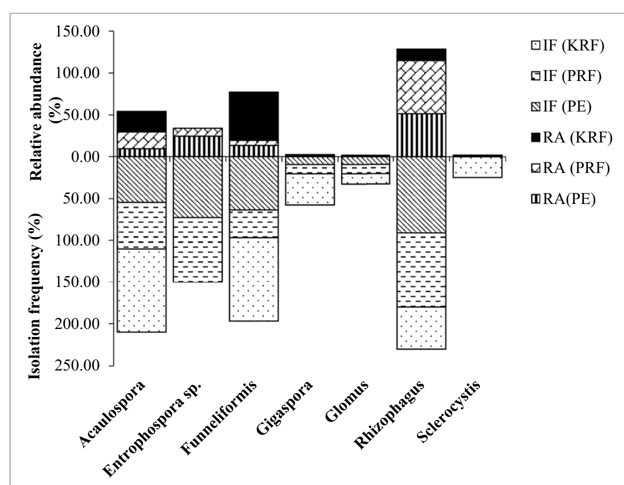

Fig. 4 Genera-wise relative abundance and isolation frequency of AM fungi. *PE* Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest, *IF* isolation frequency, *RA* relative abundance

The AM fungal diversity and distribution were high at PE and PRF, (Fig. 5a–c). The dissimilarity in the sporulation ability of various AM fungal species results in the unevenness of spore distribution (Bever et al. 1996). When comparing the similarity of AM fungi and plant species investigated between the three sites, it was observed that Sørensen's similarity coefficient of AM fungal community, as well as plant community, was higher between PE and PRF (0.73 and 0.70 respectively) (Fig. 5d). This indicates that the vegetation influences in determining the AM community structure, which may be due to the dependency of AM fungal spore formation, distribution, and development on plant diversity in the natural ecosystem (Zhang et al. 2004).

Moreover, several factors such as climatic factors, spatial and temporal variation, vegetation, nutrient availability, host-preference, and differential sporulation ability of AM species can influence the distribution and community structure of AM fungi (Husband et al. 2002; Muthukumar and Udaiyan 2002; Renker et al. 2005). The occurrence of recovered AM species from the saline rhizosphere of Pichavaram verifies their tolerance to high salinity levels.

Conclusion

The AM fungal diversity studies appear to be suitable for understanding the relationship between AM fungi and plant species, within the context of the restoration of various natural ecosystems. This is the first study to explore AM symbiosis in 18 mangroves (true- and associate- mangrove) plant species of Pichavaram Forest. The results of this study also indicate that the recovered AM fungal species from mangroves have the greater potential to assist plants in salt tolerance and hence may have a strong influence on the distribution of plants in saline soils. An important finding in our study is the presence of AM colonization in roots of plants belonging to the family Chenopodiaceae, which was otherwise considered to be a non-mycorrhizal family. Further investigation is needed to check the AM fungal behaviour for different seasons and different phenological stages of the host plant along with tidal effects.

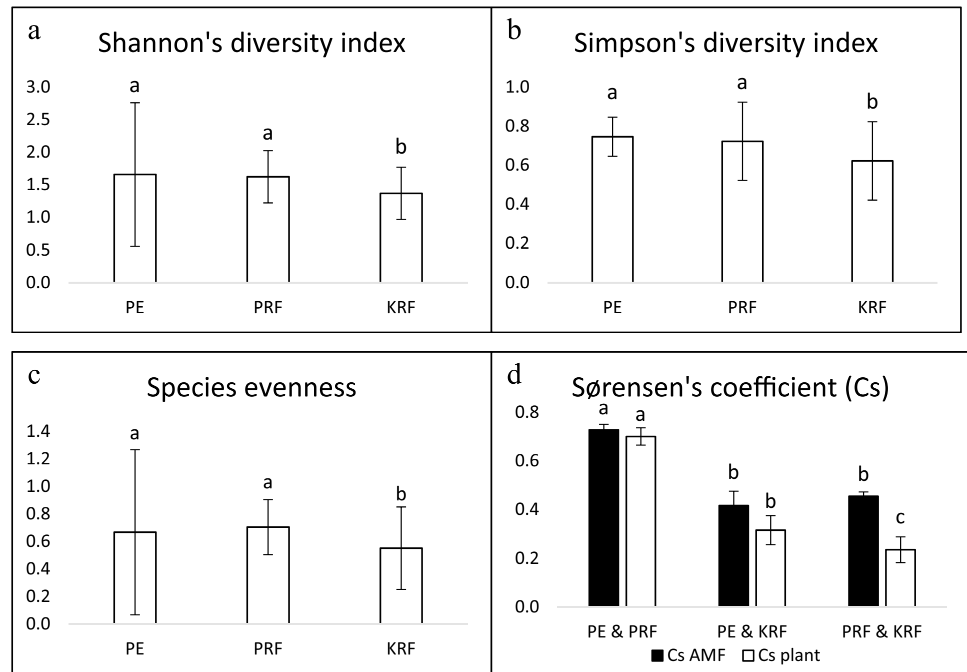

Fig. 5 Diversity measurements of AM fungal communities. *PE* Pichavaram extension, *PRF* Pichavaram reserve forest, *KRF* Killai reserve forest. Values in each column presented with different letters are significantly different at $P \leq 0.05$

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