Exploring *in vitro* root colonization in some Arbuscular Mycorrhizal (AM) Fungi from the dune vegetation of Goa

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By

SIDDHI RAMESH NAIK

22PO480032

255-649-031-353

20190210

Under the Supervision of

PROF. BERNARD F. RODRIGUES

School of Biological Sciences and Biotechnology

Botany discipline

GOA UNIVERSITY
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I hereby declare that the data presented in this Dissertation report entitled, **"Exploring *in vitro* root colonization in some Arbuscular Mycorrhizal (AM) Fungi from the dune vegetation of Goa"** is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Prof. Bernard F. Rodrigues and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations and experimental or other findings given the dissertation.

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PREFACE

Arbuscular mycorrhizal (AM) fungi exist in the rhizosphere of several vascular plants and have important roles in sustainable agriculture and agricultural ecosystem management. These fungi colonize host plants through spores, mycorrhizal roots, and extra-radical mycelia. There are obvious differences among fungal families and genera in life cycle and ecology. AM fungi colonize host plants and produce spores. Spore formation depends on factors such as seasonality, nutrient levels, and interaction with other soil microorganisms. There have been many efforts to get pure isolates of AM fungi, but most have faced problems and failed due to the biotrophic nature of these fungi. *In vitro* culturing of these fungi is very important, especially for studying host plant growth and taxonomic studies. Mosse first used the root tissue culture method to obtain a pure culture. Also, *in vitro* culturing of AM fungal species using transformed hairy carrot roots (*Daucus carota* L.) by Ri plasmid of *Agrobacterium rhizogenes* (Ricker) Conn. is another method. Several fungal species could be propagated *in vitro,* such as *Gigaspora margarita*, *Gi. gigantea*, *Rhizophagus fasciculatus*, *R. intraradices*, *Diversispora versiformis* and *Funneliformis mosseae.* It is possible to study molecular and biochemical aspects of AM symbiosis using the *in vitro* method. There have been studies on the *in vitro* culturing of these fungi isolated from the rhizosphere. The main purpose of this study is to examine the possibility of in vitro culturing the most prevalent mycorrhizal fungi using a transformed hairy roots system and to study the fungal life cycle under laboratory conditions.

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ABBREVIATIONS USED

Entity	Abbreviation
Aluminium	Al
Arbuscular mycorrhizae	AM
Arbuscules like structure	ALS
Branch absorbing structure	BAS
Calcium	Ca
Carbon	C
Cation exchange capacity	CEC
Common mycorrhizal network	CMN
Costal dune	CD
Costal sand dune	CSD
Goa University Arbuscular Mycorrhizal Culture Centre	GUAMCC
Iron	Fe
Lateral root development	LRD
Mespholine ethane sulphonic acid	MES
Minimal media	M
Modified Strullu Romand	MSR
Mycorrhizal helper bacteria	MHB
Nutrient film technique	NFT
Peri-arbuscular membrane	PAM
Phosphorous	P
Polyvinyl alcohol lacto glycerol	PVLG
Pre-penetrating apparatus	PPA
Relative abundance	RA
Root organ culture	ROC
Strigolactone	SL
Transformed root culture	TRC
White’s media	WM

ABSTRACT

A symbiosis between arbuscular mycorrhizal fungi (AM) and higher plants provides a wide scope for its use as a biofertilizer. Mass multiplication of monoxenic cultures, however, has always been a challenge. The use of transformed roots to establish monoxenic cultures of AM has been done in recent years but with a low success rate concerning spore production *in vitro*. The present study exhibits a successful attempt towards *in vitro* culturing and sporulation of *Gigaspora decipiens* (Hall & Abbott) and *Racocetra gregaria* (Schenck & Nicolson and Oehl, Souza & Sieverd) using transformed roots of *Dacus carota*. Also, in the present study the protocol for spore germination has been standardized by altering the media constituents, concentration of sterilizing agents, and time taken for sterilization of AM spores. Successful sporulation was achieved in *R. gregaria* for the first time.

CHAPTER 1: INTRODUCTION

1.1: DUNES

Dunes along the coast are characterized as mounds and narrow strips of sand with clearly defined borders set by the limits of sand transport both landward and towards the sea. With 6×106 km^2 of land area, dunes are extensively dispersed worldwide (Wasserstrom *et al*., 2017). According to Hesp (1991), because of the influence of the sea, the dune ecosystem is considered distinct and unique from inland dunes. Protecting the beach, natural ecosystems, and developed areas inland are the sea's natural barriers against wave action (Costas, 2022). Sand from the beaches that have dried out and been carried inland by wind activity accumulates to form coastal dunes. The most effective and affordable method of preventing coastline erosion is through dune systems. Sand is typically trapped by dune vegetation (Clowes and Comfort, 1987). According to Nayak *et al*. (2019), this is regarded as a specialized habitat with high salinity, low moisture, and low organic matter content conditions that are inhospitable to living things. Various plant, animal, and microbiological species that have evolved to thrive in these conditions can also live on dunes (Jayaprakashvel *et al*., 2014). Microbial communities, crucial for incorporating dunes, are highly prevalent in the rhizosphere, phyllosphere, and plants. These include groups of fungi, bacteria, and actinobacteria (Méndez *et al*., 2024). According to Garcia and Pintó *et al*. (2018), it is suggested that dunes are essential components of our seashore systems with inherent biodiversity values. Permeable sand materials, which serve as biocatalytic filters for a variety of materials carried by winds and currents, including dissolved and particulate organic matter generated from living and dead biomass of terrestrial and marine origin, make up the majority of the composition of coastal dunes (CDs) (de Beer *et al*., 2005).

According to Sarig (1999), coastal dunes are highly structured, dynamic natural systems constantly changing due to weather variations and geomorphological processes. Mc Garg (1972) suggested that coastal dunes are naturally occurring structures that operate as a barrier to the coastal zone by absorbing energy from wind, tide, and wave activity. Due to significant wind and wave action, these dynamic and disturbance-prone habitats constantly change in topography due to sand movement and physicochemical properties. The common characteristics include infertile soil, salty mist, salt spray, dryness, desiccation, low water retention, high light intensity, nutrient insufficiency, sand erosion, sand accumulation, and other transitional variables (Sarig, 1999). Such environmental pressures influence the dynamics and composition of the microbial community in addition to the pattern of plant and faunal diversity (Desai, 2005; Aureen *et al*., 2010); Muthukumar and Samuel, 2011; Gaonkar *et al*., 2012; Nayak *et al.*, 2013). According to Krumbein and Slack (1956), dunes are made up of four zones: the near shore, where waves and water currents play a role in sand movement; the foreshore, where waves and water currents play a significant role in sand transport; the backshore, where the wind plays an important role in sand movement, and breaking waves have the least influence; and the dunes, where wind action is primarily responsible for sand movement. Climate, ecological conditions, and the characteristics of the sediment all have an impact on how differently the dune systems are structured. An ample supply of sediment carried by the wind is a fundamental requirement for creating dunes, with vegetation stabilizing the sand deposited thereafter. Zones of vegetation tend to develop on the dunes based on the extent of exposure to coastal stress conditions. The pioneer zone, which extends landward from the debris line at the top of the beach in the vicinity of the frontal or fore dune, is closest to the sea (Labuz, 2005).

1.2: DIVERSITY OF DUNES

Investigating the microbiological diversity of coastal dunes is essential, especially on India's west coast (Shet and Garg, 2021). Investigating microbial communities is necessary to understand the diverse functions in the coastal dune ecosystem. Terrestrial and marine ecosystems in coastal dunes are created over many years as wind-blown sand becomes trapped by beach grasses and interacts dynamically with the tides (Poyyamoli *et al*., 2012). Among ecosystems, coastal dunes are among the least investigated. Coastal dunes are specialized environments with little vegetation influenced by salty sea mists. Coastal dunes offer a fascinating niche for studying microbial diversity (Shet and Garg, 2021). According to Ramarajan and Murugesan (2014), coastal dunes are among Earth's most productive and taxonomically rich ecosystems, making up about 8% of the planet's surface. According to Rodgers and Panwar (1988), coastal dunes are recognized as one of India's most significant biogeographical ecosystems.

India's coastline stretches over nine states and 2.1 million km² (Khoshoo, 1998). Goa is the smallest state in India, which contains an approximately 22.62 km expanse of dunes that face the Arabian Sea (Shet and Garg, 2021). There are two ways to look at the diversity of microorganisms in the dune ecosystem: bulk sand and plant-associated. Su *et al*. (2004) suggested that in dune ecosystems, soil carbon availability is limited by a scarcity of vegetation cover. Thus, the microbial biomass in CD habitats is impacted by the limited availability of soil carbon caused by a lack of substantial vegetation cover (Rajaniemi *et al*., 2009). According to Warren *et al*. (2002), variations in the structure and composition of plantations found on CD may impact the soil's microbial communities. Rhizosphere microorganisms coexist in the rhizosphere, the thin soil layer that immediately surrounds plant roots. This microflora is unique to plants in dunes, and it generally depends on the amount, density, composition, and diversity of exudates derived from plants and the influx of

mineral nutrients to roots (Warren and Zou, 2002). Plant-microbe interaction is essential for survival, *viz.*, AM fungi and *Rhizobium* bacteria in root nodules. The plant-microbes interaction helps the plants provide soil nutrients more effectively by promoting root growth, N uptake, P solubilization, mobilization, and resistance to plants against biotic and abiotic stress (Nayak *et al*., 2019). Park *et al*. (2007) suggested that in nutrient-less saline dunes, the successful regeneration of plants like *Ammophila arenaria* depends on symbiotic relationships between plants and microbes, such as N-fixing bacteria. According to Nayak *et al*. (2019), AM fungi improve plant nutrition and water uptake, shield plants from root herbivores and pathogens, and facilitate vegetation establishment in stressed situations. Rodrigues and Rodrigues (2019) suggested that this particular microbial group is crucial to stabilizing and restoring dunes because it forms a massive hyphae network and a protein called glomalin, which stops soil erosion by forming wind-resistant soil aggregates. Arun and Sridhar (2004) suggested that leguminous plants like *Casuarina* and *Canavalia*, which have nodules that create a symbiotic connection with N_2-fixing bacteria acting as N_2-fixers, predominate in coastal dunes. A special focus is needed on microorganisms that adapt to the harsh environmental conditions of CD, such as AM and rhizobia. Many bioactive secondary metabolites important to several industries, including pharmaceuticals and agriculture, are known to be produced by coastal dune organisms (Shet and Garg, 2021).

Vessey (2003) suggested that bio-inoculants, *viz.*, AM fungi, play an important role in naturally transforming the nutritionally necessary components from an unavailable state to an available state to the plants. Various anthropogenic activities and tourism growth harm coastal dunes. Pollution and contamination decrease coastal dune flora and fauna. One of the most significant issues that many nations deal with is oil spills in the ocean, which cause Tar ball deposition on coastal dunes. Suneel *et al*. (2015) noted tarball contamination in the coastal dunes of Goa. High molecular weight n-alkanes and polycyclic aromatic

hydrocarbons, frequently found in coastal regions worldwide, are present in these tar balls (Warnock, 2015). These issues affect the diversity of microbes, affecting biological diversity. According to Dessai (2005), they are divided into four types, depending on their distance from the coast, pH, and amount of flora.

A. An embryo dune has a pH of 8.5 and is parallel to the ocean. The pioneer plants growing on the embryonic dunes are *Ipomoea pes-caprae* (L.) R. Br., *Spinifex littoreus* (L), and other herbaceous species.

B. A fore dune with a pH of 7-8 is parallel to the first beach ridge and is just above the high tide line. The pioneer plants found growing are *Ammophila arenaria*, *Ipomea pes-caprae* (L.), and other grasses.

C. A yellow dune has a pH of 7.5 and a higher humus. The pioneer plants are *Calystegia soldanella*, *Eryngium maritimum*, and *Carex arenaria*, commonly found in yellow dunes.

D. A grey dune is on the landside, 50-100 meters from the embryo dune, with a pH range of 5-6, and is covered in various bushes.

E. A mature dune is 100 meters from the coast with a pH range of 4-5 and rich in humus. It is covered in an oak and pine climax flora.

Muthukumar and Samuel (2002) suggested that coastal dunes create a rhizome system by plants that emit root exudates, support the microbiota, and aid in cementing the sand particles together, playing a crucial function in the environment.

1.3: ARBUSCULAR MYCORRHIZAL (AM) FUNGI

Frank used the term "mykorrhiza" for the first time in 1885. AM fungi are obligate symbionts between common soil-borne fungi (Smith and Read, 2008), belonging to the phylum Glomeromycota (Schüßler and Walker, 2011) and host plant roots having a ubiquitous worldwide distribution in various ecosystems (Spatafora *et al.*, 2016). According to Finlay (2008), AM fungi provide nutrients to plants, especially P, and plants offer carbon (C) sources to fungi. These fungi are essential to the soil-plant interface because they live in mutualistic symbiosis with the roots of over 80% of vascular plants. They effectively facilitate the absorption of nutrients by their host plants, including those most inaccessible, like phosphorus, and increase the complementarities of nutritional resources. The final stages of the symbiotic mineral nutrient transfer are thought to take place in the peri-arbuscular membrane, a plant-derived membrane that envelops the arbuscule. Plant transporters in this membrane can transfer mineral nutrients from the peri-arbuscular apoplast to the cortical cell (Javot *et al*., 2011; Bapaume and Reinhardt, 2012). Besides nutrients, ensure greater tolerance to biotic and abiotic stresses (Sun *et al.,* 2018) and stabilize soils by forming stable soil aggregates (Rillig and Mummey, 2006; Leifheit *et al*., 2014, 2015; Rillig *et al*., 2015). According to Bender *et al.* (2014), AM fungi help to lower the emissions of N_2O, a significant greenhouse gas, which raises the possibility that they may be able to mitigate the effects of climate change. By improving plant N absorption and assimilation, AM fungi may be able to control N_2O emissions. This would reduce the amount of soluble N in the soil and, as a result, decrease denitrification (Bender *et al*., 2014). According to Schulz *et al*. (2013), AM fungi involvement in most biological changes, such as nutrient recycling, helps plant communities form soil microorganisms, which is extremely important. It is well known that AM fungi can withstand elevated metal concentrations in the soil and reduce heavy metal toxicity in the host plants (Göhre and Paszkowski, 2006; Lingua *et al.,* 2008; Cornejo *et al*.,

2013; Tamayo *et al.,* 2014; Meier *et al.,* 2015). For many years, the term "vesicular-arbuscular mycorrhizae" was used to describe these extensively branched haustoria that grow within the cells of the root cortex, known as arbuscules (Gallaud,1905) and the hyphal swelling that contains lipids in the root cortex, known as vesicles (Jansa *et al*.,2011). While vesicles are produced by 80% of AM fungi, the species in the Gigasporaceae family do not produce vesicles, but instead, they produce auxiliary cells. Therefore, the term "arbuscular mycorrhizae" was used. The Glomeromycota were divided into a single class (Schüßler and Walker, 2011), which included four orders, eleven families, and eighteen genera. This category comprises about 288 species with taxonomy descriptions (Öpik and Davison, 2016). According to Smith and Read (2008), the phylum is regarded as a monophyletic group and represents a primitive form of plant symbiosis. ITS1-5.8S-ITS2 (ITS), 28S (LSU), and 18S (SSU) are consensus sections spanning rRNA genes that form the basis of the most current taxonomy of Glomeromycota. This phylogenetic reconstruction of this classification was covered by Redecker *et al.* (2013) **(Fig 1.1)**.

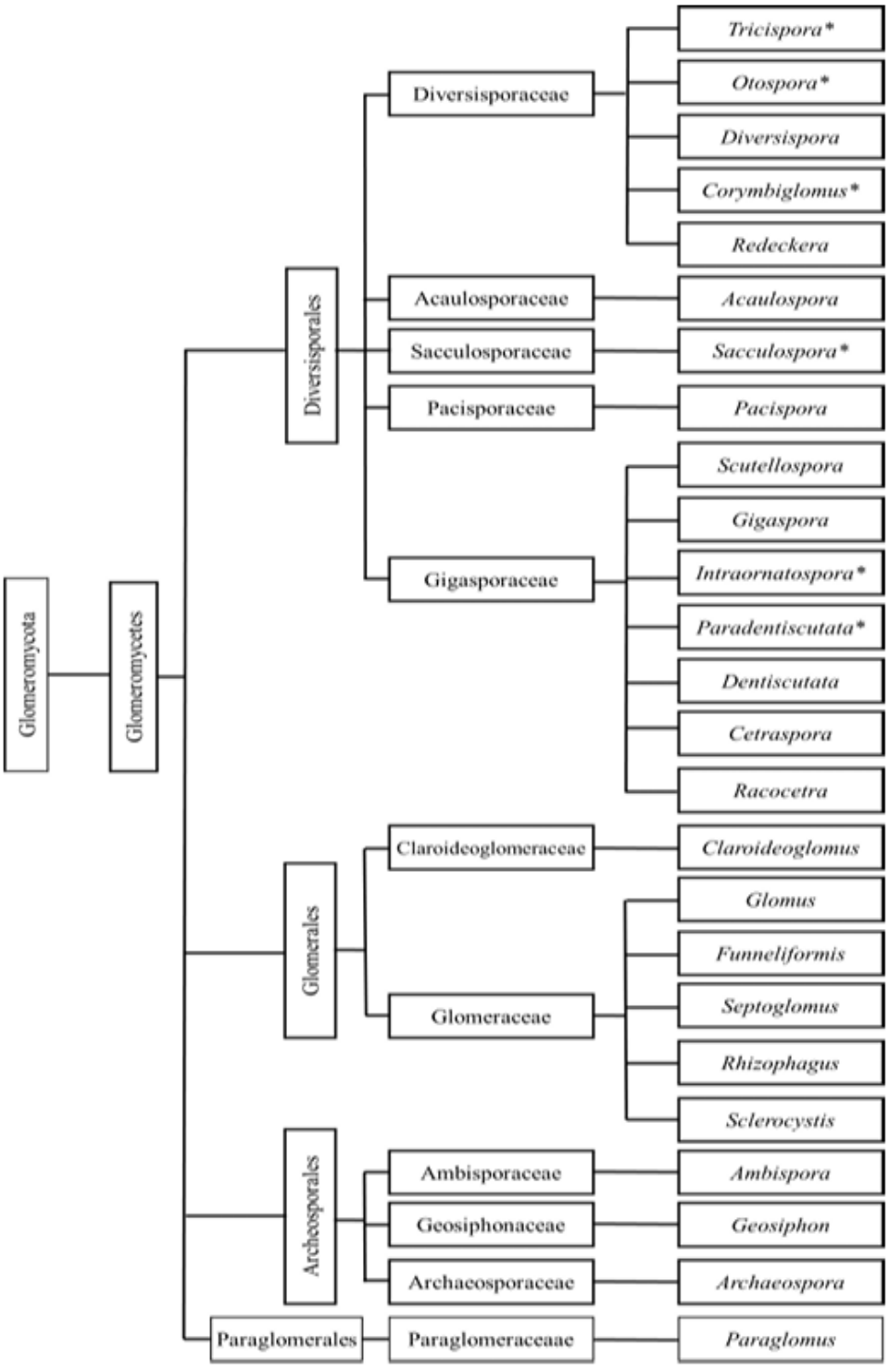

Fig. 1.1: Consensus classification of AM fungi by Redecker *et al*., 2013. (* designates the uncertain position of genera).

1.4: ORIGIN AND EVOLUTION OF AM FUNGAL SYMBIOSIS

AM connections linked to Glomeromycota have long been recognized (Remy *et al*., 1994; Taylor *et al.*, 2004). The 407-million-year-old Rhynie chert has the first direct fossil evidence of plant-fungal interactions (Trewin and Rice, 2004). Glomeromycota-affinity spores and hyphae were documented in layers dating back 460 million years, although these fossils were not connected to plants directly (Redecker *et al*., 2000). The first reliable evidence for arbuscules in the aerial axes of the ancient plant *Aglaophyton majus* and vesicles in *Rhynia gwynne-vaughanii* has been long known since (Boullard and Lemoigne, 1971; Karatygin *et al.*, 2006). In *Nothia aphylla*, spores, and intercellular vesicles were produced; however, no arbuscules were observed (Krings *et al.*, 2007). According to Pressel *et al*. (2010), AM colonization with liverworts and hornworts was also observed.

1.5: THE GROWTH AND DEVELOPMENT OF AM FUNGI

Arbuscular mycorrhizal (AM) fungi use a variety of intricate morphogenetic processes, including hyphal differentiation, spore germination, appressorium creation, root penetration, intercellular development, arbuscular production, and nutrient delivery, to colonize the host roots (Giovannetti, 2000). According to Navazio *et al*. (2020), different plant species have different morphological stages of development. For an encounter to succeed, the symbionts must establish their signaling before making physical contact. "Strigolactones," which are chemicals found in plant root exudates, are known to increase hyphal branching and facilitate interaction with the host plant. The development of appressorium (hypopodium) on the root epidermal layer occurs after successful recognition by (Gadkar *et al*., 2001). The fungus secretes hydrolytic enzymes that aid in the host cell wall's breakdown. Bonfante and Perotto (1995) state that permeation is made possible by the hyphal tip's hydrostatic pressure action. A pre-penetration apparatus (PPA) is formed by the plant cell 4-5 hours after the fungal

hyphopodium develops. The nucleus of the plant moves in the direction of the contact location (Genre *et al*., 2005). According to Siciliano *et al*. (2007), the endoplasmic reticulum, cytoskeleton, and microfilament polarisation undergo reorganization. Subsequently, the nucleus moves toward the cortex, creating a "transcellular tunnel" that permits inhalation. As symbiosis begins, mycelia proliferate in the soil inside and outside the roots, eventually developing multinucleate spores on the tips of the hyphae (Shah *et al*., 2014).

1.5.1: Intra radical hyphae

Advancement: Intra-radical hyphae begin to branch in the outer cortex after penetrating epidermal cells, producing further AM fungal structures within the host root (Peterson *et al*., 2004).

Role: A large portion of C is converted by intra-radical hyphae into triglycerides. These hyphae survive in the soil in decomposing root fragments, acting as an inoculum to colonize new host roots (Siddiqui and Pichtel, 2008).

1.5.2: Arbuscules

Advancement: Intra-radical hyphae enter the cortical region and proliferate there to create arbuscules, which are highly branched structures. According to Brundrett *et al*. (1985), arbuscules are transient structures that disappear four to five days after they form.

1.5.2.1: *Arum*-type: Inside cortical cells, a branch of intra- or inter-cellular hyphae penetrates the wall to generate complex branches that resemble trees. They are primarily found around vascular tissue and the endodermis in the inner cortex. A "peri arbuscular membrane" (PAM) is the plasma membrane formed from the host that envelops these arbuscules. Arbuscules and host cell cytoplasm are separated by this membrane, facilitating the transmission and short-term storage of glucose and mineral requirements (Peterson *et al*., 2004; Ramos *et al*., 2008; Harrison, 2012).

1.5.2.2: *Paris*-type: Typically, plants lacking intercellular gaps in their roots generate these kinds of arbuscules. As a consequence, only intracellular hyphae are visible. These hyphae create arbuscular coils, which consist of coils with lateral branches.

Arbuscules' branching structure enhances the plant cell's surface area, which improves nutrient uptake. The peri-arbuscular membrane facilitates the exchange of phosphates and sucrose (Van Aarle *et al*., 2009).

1.5.3: Intra-radical vesicles

Vesicles form from the swelling of hyphal tips or lateral branches. These develop in the root's intercellular gaps or inside the cell. Vesicles can have a variety of morphologies, such as ovoid, lobed, or box-shaped, depending on the type of fungus (Smith and Read, 2008). Vesicles proliferate as the host growth season comes to an end. Lipid bodies and many nuclei are present in matured vesicles. Certain AM species' vesicles are also known to be home to bacteria (Peterson *et al*., 2004). In addition to serving as chlamydospores, the vesicles store lipids at a rate of approximately 58% of their dry bulk (Smith and Read, 2008).

1.5.4: Auxiliary cells

Members of the Gigasporaceae family are the only species capable of producing auxiliary cells. These are colourful, globose-shaped clusters of extra-radical mycelium that grow on the lateral branches (Pedro *et al*., 2008). Wall ornamentation serves as a taxonomic trait for the identification of AM fungi. Meanwhile, the auxiliary cells in the *Scutellospora* species are knobby and spiny in the *Gigaspora* species (Bentivenga and Morton, 1996; Walker *et al*., 2018).

Auxiliary cell function is still up for debate. Nonetheless, because of their high lipid content, several studies have suggested that they may facilitate the storage of lipids (Jabaji, 1988) or reproduction (Pons and Pearson, 1985). According to de’Souza and Declerck (2003),

auxiliary cells help in C storage and may play a role in spore germination and hyphae formation. Declerck *et al*. (2004) conducted an *in vitro* experiment in which auxiliary cells of *Scutellospora reticulata* exhibited hyphal renewal but could not colonize the root.

1.5.5: Extra-radical hyphae

Extra radical mycelium is referred to as "runner hyphae" or "absorptive hyphal networks" (BAS) (Dodd *et al*., 2000). These hyphae help to act as a source of inoculum for colonizing the root systems of either the same or a different plant after initial colonization (Smith and Read, 2008). The nutrients are taken from the soil and transferred to the host roots via extra-radical hyphae. These hyphae's highly ramified shape improves the surface area for transmitting nutrients (Fei *et al*., 2022). Hyphae can absorb water and nutrients even when they are far from the zone of nutritional depletion (Jin *et al*., 2005). The extra-radical hyphae of AM fungi could serve as highways for P solubilizing bacteria, leading to the heterogeneous distribution of bacteria according to organic P availability and facilitation of P mineralization (Fei *et al*., 2020).

1.6: Phases of the AM life cycle

1.6.1: Spore dormancy

The AM fungi species may survive unfavourable environmental circumstances because of spore dormancy. When a spore is in an environment that promotes the germination of comparable quiescent spores, it becomes dormant (Giovannetti *et al*., 2010). According to Christian *et al*. (2014), the cold stratification process inhibited spore dormancy for 14 days in *Glomus intraradices*. Gazey *et al*. (1993) demonstrated that *Acaulospora laevis* spores can break their dormancy by germinating those following six months of storage. On the other hand, several other *Acaulospora* species emerged from dormancy after being stored in the

soil for two months at 23^0C (Douds and Schenck, 1991). According to Tommerup (1983), the dormancy period in wet soil was approximately six weeks for *Glomus caledonium* and *Glomus monosporum*, and 12 weeks for *Gigaspora calospora*. In temperate locations, dormancy is occasionally thought to serve as a mechanism to synchronize spore germination with root growth and favourable circumstances for colonization (Tommerup, 1985). *Gigaspora gigantea* spores collected year-round from dune habitats were found by (Koske and Gemma, 1996) to germinate within a day of inoculation. Not every AM species displays spore dormancy. The information about spore dormancy is limited; hence, less research has been done on the spore dormancy of AM fungi.

1.6.2: Triggers for spore germination

Most Glomeraceae and Acaulosporaceae species germinate via hyphal attachments, while spores of other AM species germinate in diverse ways. *Rhizophagus clarus* may produce many germ tubes, but *Funneliformis mosseae* and *Funneliformis caledonium* can produce one. In the *Glomus viscosum*, the spore germinates by producing a bulbous swelling at the hyphae's broken end. On the other hand, the germ tubes in the Gigasporaceae species emerge through the spore wall and germination shield (Mosse, 1975; Costa *et al*., 2013; Velip and Rodrigues, 2019). In *Glomus viscosum*, the spore germinates by producing a bulbous swelling at the hyphae's broken end Godfrey (1957); Walker *et al*., (1995). Furthermore, the germination shield facilitates spore germination in *Scutellospora* (Walker and Sanders, 1986). For instance, exudate from non-mycorrhizal plants such as Brassica species could not promote germination (Giovannetti *et al.*, 1993). Mosse and Hepper (1975) observed that pre-symbiotic signals occur between the fungus and the host plant. Nagahashi and Douds (2000) developed an *in vitro*-based experiment in the *Gigaspora* species to purify and identify the signalling chemical. Semi-purifying the active fraction from the carrot root exudate was done

by (Buee *et al*., 2000). 5-deoxy-strigol was shown to be the pure germination factor from *Lotus japonicas* (Akiyama *et al*., 2005).

Tania and Jose (2022) suggested that Strigolactones (SLs) from the plant side and Myc factors from the fungal side are the essential components for symbiotic communication. Furthermore, the host plant, pH, temperature, nutritional content, and soil microbes all affect the germination of spores (Siqueira *et al*., 1985; Mayo *et al*., 2018). The substances exuded from plant roots that can sprout parasite plant seeds were identified as strigolactones. As a branching factor, strigolactone has been demonstrated to cause fungal respiration, mitosis, enhanced expression and proliferation of mitochondrial genes, and germination of AM fungal spores (Buee *et al*., 2000; Tamasloukht *et al*., 2003). Recently, it has been demonstrated that 2-hydroxy tetradecanoic acid induces the first hyphal germ tube (Nagahashi *et al*., 2010; Nagahashi and Douds, 2011). According to Alexa and Maria (2014), a third class of AM fungus-stimulating substances was discovered, "cutin monomers" (ω-OH C16) fatty acids. Rochange (2010) explained that parasitic angiosperms are far more recent than AM fungi. Strigolactone-facilitated rhizosphere signals were initially employed for AM symbiosis before parasitic plants used them to sense their host.

1.6.3: Growth of pre-symbiotic mycelium

According to Giovannetti (2010), hyphae develop into uniform, right-angled branches that are straight and linear in growth after germination. Hyphae are septate with many nuclei and thick walls. Nuclei and cytoplasm move within the hyphae. After that, the hyphae lengthen to form a mycelial network. AM hyphae must contact the root epidermal cells to colonize a host and generate different intracellular structures successfully. The developing hyphae at the entrance point create an appressorium and adhere to the host roots' cuticle (Giovannetti *et al*., 1993). Hypha may establish many entrance points during the encounter. Appressoria are

multinucleate structures with tiny vacuoles. According to Peterson *et al.* (2004), hyphal sources that started colonization could be spores just beginning to germinate or hyphae growing from colonized root pieces that remained in the soil after the plants perished. According to Goltapeh *et al*. (2008), hyphae experience slow growth without host-derived signals, yet they can regerminate and colonize the living host and maintain long-term survival. A soil-germinated AM fungal spore can only survive for a few days on its lipid store before finding a host. A germinated spore will retract the hyphal cytoplasm for use in a subsequent attempt at germination if there is no host root; this process may occur because the spore is not receiving signals from plants (Logi *et al*., 1998; Smith and Read,2008).

Moreover, it has been demonstrated that MtENOD11 expression is a diffusible factor from the germination of AM fungal spores and stimulates lateral root development (LRF) in the *Medicago* plant. According to Giovannetti (2001), AM fungi's basic approach for a greater variety of symbiosis with the host plants is indicated by their ability to develop anastomoses with self-compatible hyphae. A third piece of evidence supporting AM diffusible signals is the ability of germinating spores to cause brief alterations in the cytosolic Ca^{2+} of plant cells before contact (Navazio *et al*., 2007).

1.7: P UPTAKE IN AM FUNGI

Despite being a necessary nutrient for plant growth, P is a limiting element in most ecosystems (Bucher, 2007). Both organic and inorganic forms of P can be found in the soil. At lower pH levels, insoluble forms of cations like iron (Fe) and aluminium (Al) sequester inorganic P, while calcium (Ca) does the same at higher pH levels. Plants cannot access P due to decreased sequestered phosphate mobility (Smith and Read, 2008). According to Smith *et al*. (2012), mycorrhizal plants have two different routes for absorbing nutrients: the direct pathway, which involves epidermal cells absorbing nutrients from the rhizosphere, and the

mycorrhiza-associated pathway, which works with AM fungal partners to help AM plants. AM fungi improve their host's absorption of P, N, Cu, Zn, and other elements. Harrison *et al.* (2010) state that P acquisition occurs at greater levels.

While both mechanisms function in AM plants, where Pi transporters are expressed in a cortical cell of colonized roots, non-mycorrhizal plants rely on direct uptake by Pi transporters expressed in the epidermal cells (Javot *et al*., 2006). When extra radical hyphae of AM fungus begin to colonize, phosphate transporter genes (Pht1) are activated (Karandashov and Bucher, 2005; Bucher, 2007; Javot *et al*., 2006). H^+ symporters, whose activity is controlled by the H^+ gradient generated by H^+-ATPase in the plasma membrane, are the transporters implicated in the Pi transfer (Ferrol *et al*., 2002). After extra-radical hyphae absorb P, a significant number of polyphosphates are produced. Also, fungus vacuoles contain some polyphosphates (Dexheimer *et al*., 1996). Phosphatases contained in intra-radical hyphal vacuoles are thought to be responsible for hydrolyzing the polyphosphates (Tisserant *et al*., 1993). Peri-arbuscular membrane (PAM) is essential for phosphate delivery to the cortical cells of the host plant, according to previous explanations (Rosewarne *et al*., 1999; Ferrol *et al*., 2002; Buee *et al*., 2000; Ferrol *et al*., 2002).

1.8: BENEFITS OF AM FUNGI

Even in harsh environments, AM fungi are essential for the growth and development of their host plants (Hemalatha *et al*., 2010). AM plants can outperform non-mycorrhizal plants in various biotic and abiotic stressors. Therefore, mycorrhizal plants can benefit from AM fungi's promotion of intra- and inter-specific contests (Genre *et al*., 2005). Multiple AM fungi can colonize a single plant and vice versa, forming common mycorrhizal networks (CMN) (Jakobsen and Hammer, 2015). Because weaker plants may receive more nutrients through CMN at the expense of stronger individuals that receive CMN, the links within plant

communities can increase stability (Van and Horton, 2009). They also significantly impact all terrestrial habitats, impacting the biotic, edaphic, and spatiotemporal domains (Willis *et al*., 2013). AM fungi's main job is to supply micronutrients and P, which is frequently a limited resource, to plants, particularly in nutrient-depleted adverse situations (Bolan, 1991; Clark and Zeto, 2000). Additionally, AM fungus helps to lessen the absorption of heavy metals that are phytotoxins (Göhre and Paszkowski, 2006), managing the invasion of roots (Newsham *et al*., 2009), reducing the host plants' water stress (Auge, 2001). Glomalin-mediated soil particle aggregation decreases insect herbivory (Bennett *et al*., 2009), boosts insect pollination and insect density of the trophic food web (Gange and Smith, 2005; Hoffmann *et al*., 2011), and increases soil community population and organization in structural patterns (Van *et al*., 2008; Rillig *et al*., 2006). AM fungi produce arbuscules, which aid in the exchange of inorganic minerals and C and P compounds (Li *et al*., 2016; Prasad *et al*., 2017), indicating that AM fungi are essential for the soil N and C cycles (Jones *et al*., 2009) and thus contribute significantly to terrestrial ecosystem C sinks (Wright and Upadhyaya, 1998).

1.8.1: Nutrient uptake: The relationship between plants and their fungal partners can lead to an increased intake of nutrients necessary for their growth, such as P, Cu, Zn, S, Mg, Mn, Fe, etc. Furthermore, according to Leech *et al*. (2008), they are known to assist in the transfer of N from organic matter to the host. It has been demonstrated that an increase in the availability of C frequently causes the AM fungus to absorb P oppositely, transferring it to its host (Smith and Read, 2008).

1.8.2: Stress tolerance: According to Mohammedi *et al*. (2011), AM fungi provide their host plants with an ecological competitive advantage that helps them survive and grow better in stressful environmental circumstances like temperature, pH, wetness, and salinity. Increasing water intake from the soil through hyphal extensions can also increase a plant's response to water scarcity (Entry *et al*., 2002). However, prior research has demonstrated that AM fungi

maintain plant salinity tolerance through various strategies, including enhanced nutrient uptake (Evelin *et al*., 2012) and physiological regulation (Chang *et al*., 2018).

1.8.3: Minimize nutrient loss and soil erosion: AM fungi can alter the soil structure by growing ramified hyphal networks that bind and entangle soil particles to create stabilized soil aggregates (Leifheit *et al*., 2014). Together, these lead to greater water retention capacity and improved nutrient uptake, which promote healthier plant growth (Chen *et al*., 2018). According to Clark and Zeto (2008) and George (2000), AM fungi contribute to a decrease in nutrient leakage by absorbing and storing nutrients in soil aggregates.

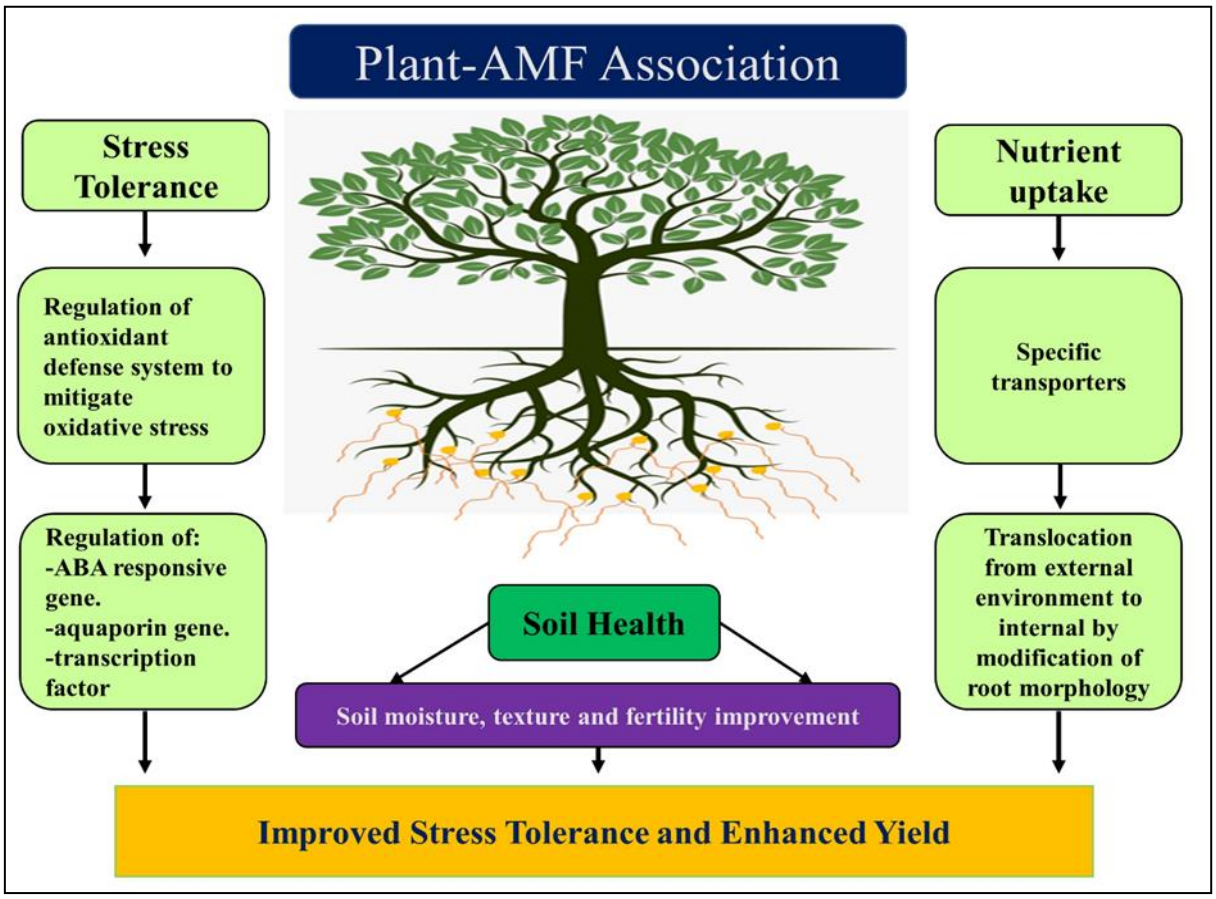

Fig. 1.2: Benefits of AM fungi Khaliq *et al.*, (2022)

1.9: AM FUNGI INTERACTION WITH OTHER RHIZOSPHERE MICROBES

In addition to the bipartite relationship between the fungus and the plant characterizing mycorrhizal symbiosis, AM fungi interact with other related microorganisms (Tarkka and Frey-Klett, 2008). According to Frey-Klett and Garbaye (2005), these microorganisms influence one another and form a zone known as the "mycorrhizosphere." "Mycorrhiza helper bacteria" (MHB) are some of the bacteria that can promote the growth of mycorrhiza (Fitter and Garbaye, 1994). Moreover, AM fungi interact with PSB's by absorbing the P ions after solubilizing the insoluble form of P (Rodríguez and Fraga, 1999).

2.0: MONOXENIC CULTURE OF AM FUNGI

The monoxenic culture technique is a sophisticated, potent, and promising approach to producing an AM fungal inoculum free of contamination. Using Ri T-DNA-transformed roots from various host species, many AM fungal species have been successfully cultivated monoxenically. In a short amount of time and space, the root organ culture method produces many spores, which increases the spore load that may be injected in the field and affects the development of horticultural crops (Srinivasan *et al*., 2014). Few AM fungal species from the Gigasporaceae and Glomeraceae families and just a few species from the Acaulosporaceae family have been effectively cultivated on root organ culture (ROC) (Rodrigues and Rodrigues, 2013). Since the 1980s, there has not been much advancement in the creation of monoxenic techniques or the media utilized to cultivate AM fungus on ROC (Abdellatif *et al*., 2019). White's medium has been altered by scientists to create a minimal (M) medium (Bécard and Fortin, 1988) and modified Strullu Romand (MSR) medium (Strullu and Romand, 1986; Declerck *et al*., 1998). To improve the monoxenic culture of AM fungus, a novel medium called HI medium that contains palmitic acid was created (Ishii, 2012). According to Trépanier *et al*. (2005), palmitic acid is necessary for synthesizing AM fungal

lipids. In recent decades, the efficient use of Ri T-DNA altered roots has been utilized to set up the dual cultivation of AM fungi and host roots. *Agrobacterium rhizogenes* Conn. is used to spontaneously alter plants genetically, resulting in the development of hairy roots. According to Fortin *et al*. (2002), the hormone changes enable them to develop rapidly on artificial substrates. For their monoxenic culture, AM fungal inoculum, including spores, colonized root fragments, or isolated vesicles, can be utilized (Rodrigues and Rodrigues, 2013). Nonetheless, spores have been used to cultivate certain AM fungal species (Gigasporaceae) that do not produce vesicles (Fortin *et al*., 2002).

The present study of AM inoculum production techniques depends on non-soil based including *in vitro*, *monoxenic*, and (ROC) systems that use plant root organs that have been genetically manipulated with *Agrobacterium rhizogenes* to produce Ri T-DNA transformed plant root organs that can grow on media in a sterile environment. Mugnier and Mosse (1987) invented the use of Ri-plasmid-modified root organ cultures for the growth of AM fungus. The growth of pioneer vegetation, which primarily affects the primary and secondary succession of plant life in coastal settings, is greatly aided by AM fungi. Natural and man-made disturbances can impact the stability and composition of dune plant communities in coastal dune systems. Because AM fungi connect many different plant species via their extensive underground mycelial web, they affect a habitat's ecology. Examining AM relationships in dune plant species and their distribution in the sandy soils is necessary to sustain these habitats due to their beneficial effects on terrestrial ecosystems. The conventional soil-based pot culture method is not preferred over the *in vitro* culture method for producing AM fungal inoculum. A Petri plate produces a sizable quantity of pure, viable, and contamination-free spores. The approach is more suited for creating high-quality inoculum because the sterile conditions keep out unwanted bacteria, and the cultures are regularly monitored. Realizing the possibility of producing AM spores by *in vitro* culture, the

research fills the multiplication gap. It boosts the effectiveness of AM fungal propagules grown *in vitro* conditions to revegetate ecosystems of degraded dunes.

The following goals guide the current work that is being attempted:

i. Isolation and identification of AM fungi species from the dune.

ii. Preparation of trap cultures.

iii. Isolation and identification of AM fungi species from trap cultures.

iv. Standardization of AM species mass multiplication through *in-vitro* method and *in-vivo* method.

CHAPTER 2: REVIEW OF LITERATURE

In-vitro culture of AM fungi

Sr. No.	AM species	Key findings	Reference
1.	*Glomus caledonium*	The first sporulation of *G. caledonium* in monoxenic culture was obtained using transformed roots of *D. carota*. In this experiment, spores were produced after four months of dual culture.	Hepper and Mosse, (1975)
2.	*Glomus sinuosum*	The study was carried out using Ri T-DNA transformed roots *D. carota* in monoxenic culture using a Minimal nutrient medium. The first 20 days are important for lateral root formation. After three days of spore germination, hyphae infect the root. After 4-6 months, a new sporocarp was developed. Each culture yielded around 47 sporocarps after 6 months.	Declerck *et al.*, (1988)

3.	*Glomus intraradices*	The study was carried out using an *in vitro* two-compartment system, *where G. intraradices were cultured on transformed roots of D. carota i*n an MSR medium. Mycorrhizal roots were present in one compartment, which contained sugar-containing media, whereas the other compartment had a sugar-free medium, which only supported mycelial development. This method shows a high rate of sporulation. These AM fungi show a negative correlation with *F. chrysantherni* conidia formation.	St-Arnaud *et al.,* (1995)
4.	*Glomus margarita*	In spores of *G. margarita*, several hyphae germinated from the wall of the spore near the subtending hyphae.	Karandashov *et al.,* (1999)
5.	*Rhizophagus proliferous*	The association of *R. proliferous* with transformed roots of *D. carota* was obtained on the (MSR) medium. The sporulation was initiated one week after the preparation of dual cultures.	Declerck *et al.,* (2000)

6.	*Glomus caledonium*	The *G. caledonium* was established in a dual culture with Ri T-DNA-transformed roots of *D. carota.* Spore germination was observed after six days. The spore germination shows the presence of 1-4 germ tubes. After 5-7 weeks, more than seven dual cultures are obtained, producing 180±300 mm spores. *G. caledonium* formed a Paris-type mycorrhiza on transformed carrot roots.	Karandashov *et al.,* (2000)
7.	*Funneliformis caledonium*	The spores of *F. caledonium* were grown in a dual culture with transformed roots of *D. carota* on a Minimal nutrient medium at pH 6.5. The spores were produced after 2-3 days of contact within 1-3 weeks after spore germination with the roots.	Karandashov *et al.,* (2000)
8.	*Gigaspora margarita*	An *in vitro* culture was established with *Gi. margarita* and transformed roots of *D. carota* on a Minimal nutrient (M) medium to examine the growth and physiology of the fungal spore. Single spores were mostly formed in 18 to 20-month-old cultures.	Gadkar and Adholeya, (2002)

9.	*Glomus intraradices* and *Glomus versiforme*	Flavonoids stimulate hyphal elongation in *G. intraradices* and *G. versiforme*. The presence of slime-forming bacteria identified *Paenibacillus validus* on surface sterilized spores of *G. intraradices* encourages sporulation. Sheared roots of *G. versiforme* and *G. intraradices* develop on a water agar medium and show a high sporulation rate without a living host partner in three months.	Fortin *et al.,* (2002)
10.	*Scutellospora reticulata*	An *in vitro* culture was established with *Sc. reticulata* and transformed roots of *D. carota* on an MSR medium. It shows the development of extraradical mycelium, auxiliary cells, lag-exponential plateau, and late decline phases. The extraradical mycelium biomass production is directly correlated with spore production.	Declerck *et al.,* (2003)
11.	*Sclerocystis sinuosa*	Using the transformed of *D. carota* roots on a minimal nutrient medium, a monoxenic culture of *S. sinuosa* is prepared. After four months, the new sporocarps developed.	Bi *et al.,* (2004)

12.	*Scutellospora calospora*	The first sporulation of *Sc. calospora* in monoxenic culture was obtained using Ri T-DNA transformed roots of *D. carota.*	Kandula *et al.,* (2006)
13.	*Rhizophagus intraradices*	An experiment was performed to study the life cycle of *Rh. intraradices* using the ROC of *D. carota* on an MSR medium. The sporulation occurred 25 days after contact with the roots.	Eskandari and Danesh, (2010)
14.	*Gigaspora decipiens*	A successful *in vitro* culture of *Gi. decipiens* was obtained using transformed roots of *D. carota*. The sporulation occurred after five months of inoculation on Minimal nutrient medium. Five successive generations (G1, G2, G3, G4, G5) of *Gi. decipiens* were obtained from the newly *in vitro* formed spores.	Bindondo *et al.,* (2012)
15.	*Rhizophagus clarus* and *Gigaspora decipiens*	The effects of pH and temperature on spore formation were carried out *in vitro* transformed root of *D. carota* on a Minimal nutrient medium. The spore germination in both species was observed after 5-6 days. Production of spores increases at 22^0C, whereas it decreases at temperatures 28^0C to 32^0C. At pH 6.5, *Gi.*	Costa *et al.,* (2013)

		decipiens produced the most spores, whereas *Rh. clarus* produced a high rate of sporulation at pH 4.0.	
16.	*Scutellospora reticulata*	The study used Ri T-DNA-transformed roots of *D. carota* on a Minimal nutrient medium. The development of mycelium and spore production. About 75% of the spores generated in the monoxenic culture germinated. The branched absorbing structures (BAS) and hyphal swellings (HS) are seen.	Dsouza and Declerck, (2013)
17.	*Rhizophagus irregularis*	The study used an *in vitro* culture of *Rh. irregularis* obtained by using transformed roots of *D. carota*. Spore germination was 90% in the MSR medium without sucrose at 38 hours, while 75% of spore germination in the MSR medium with sucrose germination was recorded 60 hours after 26 days. This confirms that sucrose delays the formation of appressorium.	D'Souza *et al.,* (2013)
18.	*Funneliformis mosseae*	A monoxenic culture of *F. mosseae* spores was successfully established on an MSR medium using *Linum usitatissimum*. The	Rodrigues and Rodrigues,

		colonization occurred five days after co-cultivation. The spores produced showed 83% of viability.	(2015)
9.	*Glomus intraradices*	The study was conducted under *in vitro* root colonization of *G.intraradices* species with and without sucrose in an MSR medium. The root colonization shows the presence of fungal mycelia, vesicles, arbuscules, and intraradical spores. However, vesicles and mycelia were more abundant structures in both media.	Danesh *et al.*, (2016)
20.	*Glomus proliferum*	Five arbuscular mycorrhizal (AM) fungi isolated from the rhizosphere of banana and sugarcane were successfully cultured *in vitro* with genetically transformed roots of *D. carota*. Vesicles constituted excellent sources of inoculum for the establishment of *in vitro* cultures and for the continuous culture of the species.	Declerck *et al.*, (2018)
21.	*Acaulospora rehmii*	The first successful *in vitro* growth of *A. rehmii* was observed in transformed roots of *D. carota* on MSR medium, resulting in the observation of extra-radical thin-	Dalpé and Declerck, (2019)

		walled hyphal swellings.	
22.	*Rhizophagus irregularis*	This study was carried out to assess the performance of hairy roots in the propagation of AMF in monoxenic cultures. The host, AM fungus, and time play roles in three-way interaction. Through stacked probability plots, they found *Nicotiana* is the poor host compared with the *D. carrot* and *Medicago* plant for *Rh. irregularis*.	Dane Goh *et al.*, (2022)
23.	*Gigaspora decipiens*	The present study demonstrates a successful attempt towards *in vitro* culturing and sporulation of *Gi. decipiens* in transformed roots of *Linum usitatissimum L. (Flax). In vitro* spore germination in *Gi. decipiens* was recorded within three days of plating on the MSR medium. BSA auxiliary cells are seen. Sporulation was observed after 50 to 55 days. On average, five spores were observed per Petri plate.	Velip and Rodrigues, (2021)
24.	*Gigaspora albida*	These findings provide valuable insights into the optimal conditions for spore germination in *Gi. albida*, highlighting	Maia and Yano-Melo, (2001)

		the importance of the water-agar medium and showcasing the temporal dynamics of germination across different substrates. The presence of auxiliary cells in the water-agar medium may indicate a specific interaction or response of the fungus to this substrate, which could be further explored in future research.	
25.	*Rhizophagus irregularis*	This study investigates the impact of two different cultivation systems for producing inoculum of AM fungi, specifically *Rh. irregularis*. The two cultivation methods are *in vivo* culture and *in vitro* culture. The study focused on evaluating the size of spores obtained from both cultivation methods and assessing the impact on plant growth.	Calvet *et al.*, (2013)
26.	*Gigaspora gigantea*	Solid media with a pH of 5.7 was determined to be optimal. They investigated the impact of secondary metabolites on the growth of transformed roots of tomato seedlings and the formation of *Gi. gigantea* propagules at pH 5.7 and 6.5. AM root colonization	Ellatif *et al.*, (2019)

		increases by catechin anhydrous.	
27.	*Rhizoglomus irregulare*	They used photomicrography and image processing to examine the morphology and growth characteristics of 14 isolates, representing a gradient of in vitro cultivation from 0 to >80 generations *in vitro*. They looked into the extent of trait variation across isolates of *Rh. irregulare* that develops in symbiotic growth. According to their research, AM fungal communities may be strongly influenced by intra-specific diversity from an ecological standpoint.	Kokkoris *et al*., (2019)
28.	*Funneliformis mosseae*	*In vitro*, mass production of AM fungal propagules was achieved by using transformed root cultures (TRC). In this study, they explore the possible effects of TRC propagation on AM fungal features, as well as how this could impact the usefulness of fungal traits. Lastly, they investigate if it is feasible to reverse TRC-induced domestication. They first investigate whether the domestication of AM fungus has genuinely occurred.	Kokkoris and Hart, (2019)

29.	*Glomus intraradices*	A successful in vitro culture of *G. intraradices* was obtained using transformed roots of *D. carota*. After three months of dark incubation, a Petri dish containing an average of 8500–9000 spores and a notable generation of widespread hyphal growth on MSR media were noted.	Srinivasan *et.al.*, (2014)
30.	*Gigaspora decipiens*	For the first time, *Gi. decipiens* was effectively cultivated *in vitro*. Over six years, five consecutive generations were produced from axenic spores. Stability, propagule viability, and morphological features were preserved.	Bidondo *et al*., (2012)

CHAPTER 3: ISOLATION AND IDENTIFICATION OF INDIGENOUS AM FUNGI SPECIES FROM DUNE VEGETATION

3.1: INTRODUCTION

Massive amounts of moving sand particles on the beach build up to form mounds of drifting sand, known as dunes. Because of their dynamic character, the structures of the dunes and beaches are constantly changing (http://www.goaenvis.nic.in). Three fundamental factors with complex interactions: wind, sand, and vegetation are necessary for the formation and construction of dunes (Carter, 1988).

Dunes are an important ecological feature for the coastal population. Due to the widespread distribution of AM fungi, soils, and plants directly impacts their taxonomy, functional diversity, and genetic diversity (Bever *et al*., 1996) and the significance of determining the diversity of AM communities (Lovelock and Ewel, 2005).

The inland flora that grows directly behind the beaches is known as "dune vegetation," which plays a crucial role in the construction of dunes because it acts as a windbreaker, forcing the wind to carry sand along its path. Thus, moving sand particles are captured and stabilized by dune vegetation. These plants have evolved to survive in a hostile habitat that includes a saline atmosphere, a mobile substrate, and frequent disturbance by moving sand grains (Paskoff, 1989).

Poaceae members are the primary plants in temperate dunes that stabilize coastal dunes (Read, 1989). In tropical regions, however, plant species from the Asteraceae, Convolvulaceae, Fabaceae, and Poaceae families help to stabilize coastal dunes (Devall, 1992; Kulkami *et al*., 1997).

Dunes serve as nature's barrier, source of beach sustenance, buffer against coastal erosion, replenish sand lost to wave and current energy and act as sand banks to maintain the sedimentary and dynamic balance of the coastal ecosystem, supporting a rich diversity of flora and fauna, providing an ideal setting for recreation, shielding the hinterlands from winds, and providing protection from coastal erosion (Oehl *et al*., 2003).

AM fungi are found in dunes along the coast worldwide (Sturmer and Bellei, 1994). Due to P deficiency, dunes provide an ideal environment for AM fungi to associate and grow with dune plants. The symbiotic relationship also increases stress tolerance (Gemma, 1995).

The stabilization of dunes is primarily achieved by AM fungus binding sand grains into stable aggregates and dune plants colonizing for improved nutrient uptake (Koske and Poison, 1984).

The importance of AM fungi for agriculture, horticulture (Jarstfer and Sylvia, 1992), reforestation programmes (Caravaca *et al*., 2002) is recognized. Many positive effects of AM fungal symbiosis on the general growth and development of plant communities are well known (Douds Jr *et al*., 2007; Souza *et al*., 2010; Ijdo *et al*., 2011).

According to Maun (2009), *Acaulospora, Gigaspora, Glomus*, and *Scutellospora* are the most abundant AM fungal genera in the dune ecosystem. Smith and Read (1997) explained that host plants and geographic location impact AM fungus species richness and spore density in dunes. The number of propagules, v*iz*., spores and colonized root fragments in the rhizosphere, soil, climate, host, and fungal species significantly impact AM fungal root colonization (Maun, 2009).

One of the main factors controlling the variety of plants and the efficiency of the dune environment is the diversity of AM fungus in the rhizosphere. Dune instability is caused by a decline in plant balance and productivity caused by the loss of AM fungi (Sridhar, 2009).

Kulkarni *et al*. (1997) reported that *Gi. ramisporophora*, *G. clarum*, and *R. gregaria* were the most common spore communities in *Ipomoea pes-caprae* (L.).

Koske and Gemma (1995) recorded *Sc. weresubiae* and *Sc. hawaiiensis* in American dunes. *Sacculospora felinovii* was first reported by Willis *et al.* (2016) from the West coast dunes of Goa. In the coastal dunes of Karnataka, *Sc. calospora* was the most prevalent AM fungus, followed *by Gi. margarita and Sc. pellucida* (D'Cunha and Sridhar, 2009). Khade and Rodrigues (2008) reported the dominance of the genus *Glomus* in their studies.

To successfully apply AM in the conservation and management of the dune ecosystem, it is crucial to comprehend the relationship between AM and dune vegetation and their distribution in sandy soils. Thus, the current study aims to assess to determine the diversity of AM species in the dune vegetation.

3.2: MATERIALS AND METHODS

3.2.1: Study site and plant species undertaken for the study:

Caranzalem Beach, situated at a latitude of 15.4665°N and longitude of 73.8048°E, was the study site. *Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae) was the plant species selected for the study.

3.2.2: Sample collection:

A continuous one-time sampling of random plant species from a chosen dune site was conducted to assess their AM status **(Plate 1)**. The roots and rhizosphere sand samples of *Ipomoea pes-caprae* (L.) R.Br. (Convolvulaceae) were collected from 0-25 cm depth, placed in *zip*-loc bags, labeled, and brought to the laboratory. While the sand samples were kept at 4^0C until processing, the roots were examined to quantify root colonization.

3.2.3: Soil analysis

The soil analysis was carried out using the methods outlined by Singh *et al*. (2005). The pH was measured using an "EUTECH" pH meter in a 1:2 ratio with a sand water solution**.**

3.2.4: Processing of root segments for AM fungal colonization

The Trypan blue staining procedure of Phillips and Hayman (1970), as outlined below, was used to assess the AM colonization in roots.

The roots were gently washed with tap water to remove any adhering dirt particles and then cut into 1 cm segments. After being cleaned, they were heated in an oven for 30-45 minutes after adding. Later, the root segments were acidified with 5N HC1 for 5 minutes and then stored overnight in 0.05% Trypan blue stain. Bright-field Olympus BX 41 and Nikon Eclipse E200 research microscopes (40X, 100X, 400X, 1000X) were used to analyze the stained root

segments for AM colonization. The micrographs were taken using digital cameras (Olympus DP 12-2 and Nikon Digital Sight DS-U3) without any digital editing. The root segments contained auxiliary cells, arbuscules, vesicles, hyphal coils, and intra- and extra-radical non-septate hyphae.

3.2.5: Calculating the percentage of roots colonized

The Root Slide method was used to estimate the percentage of roots colonized by AM fungus (Read *et al*., 1976). The root fragments stained with Trypan blue were embedded in PVLG (polyvinyl lacto-glycerol), and the following formula was used to determine if AM colonization was present or absent:

$$\text{Percent colonization} = \frac{\text{No. of root segments colonized}}{\text{Total no. of root segments}} \times 100$$

3.2.6: Preparation of Trypan blue stain

Requirements: Trypan blue - 0.05g, Lactic acid - 50ml, Glycerine - 10ml, Water - 40ml

Preparation: 0.05 g of Trypan blue powder was dissolved in 40 ml of autoclaved distilled water. To this mixture, 50 ml of lactic acid and 10 ml of Glycerine was added.

3.2.7: Isolation of AM fungal spores from rhizosphere soil samples

AM fungal spores were isolated using the Wet Sieving and Decanting Technique of Gerdemann and Nicolson (1963). A 100 g sample of fresh rhizosphere soil was placed in a beaker, and 200 ml of tap water was added. The sediment was allowed to settle after agitating the soil suspension with a glass rod for 10 to 20 seconds. The portion was poured through sieves placed 250 µm – 37 µm in descending order. The process was carried out twice for every sample to ensure maximum spore recovery. Residue from each of the sieves was collected and placed in separate beakers and filtered through Whatman No. 1 filter paper.

After that, the filter paper was placed in a Petri dish, and care was taken to keep it moist. Spores/sporocarps were recovered using an Olympus stereomicroscope SZ2-ILST (10 x 4.5 zoom) and were placed in small Petri plates.

3.2.8: Estimation of AM fungal spore density

The method of Gaur and Adholeya (1994), as given below was used to quantify the AM spores in the samples. Whatman No. 1 filter paper was folded into two halves, then into four equal halves by another fold. After opening the filter paper, two lines were drawn to represent four identical quadrats. When the filter paper was opened, two lines were drawn to represent four identical quadrats. Vertical lines were used to divide one-half of the filter paper into ten columns roughly 0.5 cm apart. Arrows were used to indicate the counting direction and to number the columns. After that, the filter paper was folded so the designated area got the aliquot during filtration, and the unmarked area stayed spore-free. After that, the filter paper was put on a Petri plate and examined using a stereomicroscope. The spores in each of the columns were counted, and the spore density was determined. Using a needle, intact spores were removed and placed in PVLG for taxonomic identification.

3.2.9: Preparation of polyvinyl alcohol lacto-glycerol (PVLG)

Requirements: Polyvinyl alcohol - 16.6g, Lactic acid – 100ml, Distilled water – 100ml, Glycerine - 10ml

Preparation- 16.6 g of PVLG was mixed in 100 ml of distilled water by heating at 90^0C. After cooling the mixture 100 ml of lactic acid and 10 ml of Glycerine were added.

3.2.10: Taxonomy-based spore identification

Spores that were uncontaminated, undamaged, and mounted in PVLG were employed for taxonomic classification. Using morphological criteria, original species protologues

described by Morton and Benny (1990), Schenck and Perez (1990), Rodrigues and Muthukumar (2009), Schüßler and Walker (2010), Redecker *et al*. (2013) and online species descriptions given by (International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi at the West Virginia University, USA (https://invam.wvu.edu/), arbuscular mycorrhizal fungi (Glomeromycota), *Endogone and complexips* species deposited in the Department of Plant Pathology, University of Agriculture in Szczecin, Poland (http://www.zor.zut.edu.pl/Glomeromycota/index.html), and (amf-phylogeny.com) were consulted for the identification of AM fungal spores

3.2.11: Determination of AM species spore abundance spore density and relative abundance

Spore abundance = $\dfrac{\text{Number of spores of a particular species}}{\text{100 g of soil sample}}$

Relative abundance (RA%) = $\dfrac{\text{Number of spores of particular species}}{\text{Total number of spores}} \times 100$

3.3: RESULTS

3.3.1: Soil analysis

The results of the soil analysis indicated that the soil texture was sandy, with a pH of 5.9.

3.3.2: AM fungi diversity

It was observed that *Ipomoea pes-caprae* (L.) R. Br. was the dominant plant species at the study site. Three genera, viz., *Scutellospora, Gigaspora,* and *Acaulospora,* were identified from the samples **(Plate 2)**.

3.3.3: Taxonomic identification of AM fungi

3.3.3.1: General description of genus

3.3.3.1.1: *Gigaspora* (Gerd. & Trappe): is a Greek word that means "giant spore," referring to the exceptionally larger spores. The sporocarp is unknown. Spores are colour, large, globose to sub-globose without ornamentations and three-layered spore walls. Spores develop blastically from a hyphal tip, which swells and becomes the "sporogenous cell." After the sporogenous cell reaches its full size, usually about 25-50 µm in most species, the spore begins to develop at the tip. The outer layer and the laminate layer develop simultaneously and often cannot be distinguished in juvenile spores without the assistance of Melzer's reagent. The laminae thicken and ultimately develops the warty inner layer, from which multiple germ tubes arise.

3.3.3.1.1.1: *Gigaspora decipiens* (Hall & Abbott): spore produced singly in the soil coloured ranging from White to cream, becoming yellow-brown with age or prolonged storage. Shape and size of the spore Globose to subglobose, 280-490µm.The spore wall is 34-47 µm thick with 11-15 equal sub laminations. Sporogenous cells are 51-63 µm wide **(Plate 3)**.

3.3.3.1.1.2: *Gigaspora albida* (Schenck & Smith): spore dull white to light greenish-yellow, spherical, 143-350 µm diameter. The spore wall is continuous, 4-12 µm thick, with 1-6 layers. The layer 1 is smooth, 1-2 µm thick, and layer 2-6 laminated. Germ tube produced directly from spore wall near the bulbous suspensor separating it from the spore content. Sporogenous cell hyaline to yellow, 24-36 µm diameter, attached to septate hypha with fine hyphal branches **(Plate 4)**.

3.3.3.1.2: *Scutellospora* (C. Walker & F. E. Sanders): is a Latin word that means "shielded" in Greek, "spore." Spore colourless to light colour, large, with or without ornamentations. Spores consist of a bilayered spore wall and two bilayered flexible inner walls. Germ tubes arise from a persistent, plate-like germination shield associated with the innermost flexible wall. Spores develop blastically from a hyphal tip, which swells and becomes the "sporogenous cell." After the sporogenous cell reaches its full size (usually about 25-50 µm in most species), the spore begins to develop at the tip. The outer layer and the laminate layer develop simultaneously and often cannot be distinguished in juvenile spores without the assistance of Melzer's reagent. The laminae then thicken, and the outer ornamentations develop. Inner walls develop, and the last stage is the formation of the germination shield.

3.3.3.1.2.1: *Racocetra gregaria* (Schenck & Nicolson) Oehl, Souza & Sieverd: spore reddish brown, globose to subglobose, 250-448 µm in diameter. The spore wall is composed of 4 layers. Layer 1: brown, 1-5 µm, including the closely packed warts on its outer surface. Warts are brown, 1-2 µm high with rounded tips (2-10 µm), closely appressed to layers 2 and 3. Layer 2: laminate, yellow, 3 µm thick. Layer 3: laminate, pale yellow, 5-13 µmthick. Layer 4: hyaline, membranous, 1-2 µm thick; Sporogenous cell 50-66 µm in diameter. The sporogenous cell wall consists of two hyaline layers (L1 and L2) that probably are present

continuous with the two layers of the spore wall, but only L2 is readily discernible at the level of the compound microscope. L2 is yellow-brown 4.0-6.3 µm thick near the spore and then thinning to 1.4-1.6 µm beyond the sporogenous cell **(Plate 5)**.

3.3.3.1.3: *Acaulospora* (Gerd. & Trappe): is a Greek word that means "spores without a stem" or sessile spores. This genus was originally defined by spores borne laterally from the neck of a pre-differentiated "sporiferous saccule." However, species previously in *Entrophospora* that show genetic relatedness to *Acaulospora* species are included in this genus because the "entrophosporoid" spore phenotype is a convergent trait. Spores are globose to ellipsoid, ranging from 40-400 µm in diameter. The surface of the spore wall may be ornamented with pits, projections, spines, or reticulations. The sporiferous saccule develops blastically from a hyphal tip. After the saccule has fully expanded, a spore begins to develop from the side of the subtending hypha, termed the "saccule neck." As the spore matures, the saccule loses its contents and eventually sloughs off so that it is often not attached to fully mature spores.

3.3.3.1.3.1: *Acaulospora scrobiculata* (Trappe): spore borne singly in the soil, hyaline to light brown, globose to sub-globose, occasionally irregular, 100-240 µm in diameter. Spore surface evenly pitted with depression 1-1.5×1-3 µm septate by ridges 2-4 µm thick at the mouth of depressions, circular to elliptical or occasionally linear to Y-shaped. The spore wall is composed of 4 layers. Layer 1: sub-hyaline to light greenish-yellow, 3-6 µm thick. Layer 2: adhering, smooth, hyaline, 0.2-0.5µm thick. Layer 3: hyaline, 0.5-1.0 µm thick. Layer 4: roughened, hyaline, 0.2-1.0 µm thick **(Plate 6)**.

3.3.4: Spore abundance:

In the present study, the AM Spore abundance was highest in *R. gregaria*, followed by *Gi. decipiens*, *Gi. albida* and *A. scrobiculata* **(Table 3.1) (Fig. 3.1)**.

Table 3.1: Spore abundance of AM fungal species.

AM species	**Spore abundance (%)**
R. gregaria	22.67±5.41
Gi. decipiens	13.00±4.47
Gi. albida	11.17±4.40
A. scrobiculata	7.83±3.49

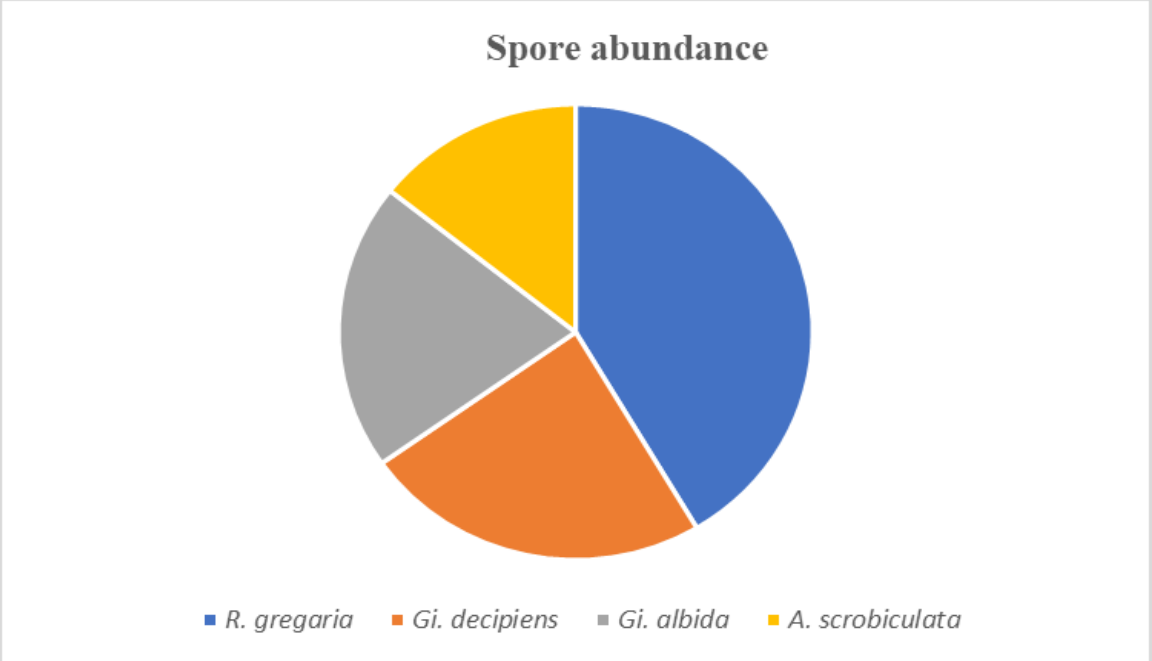

Fig. 3.1: Spore abundance of AM fungal species at the study site.

Table 3.2: Relative abundance (RA%) of AM fungal species

AM species	Relative abundance (RA%)
R. gregaria	41.46
Gi. decipiens	23.78
Gi. albida	20.43
A. scrobiculata	14.33

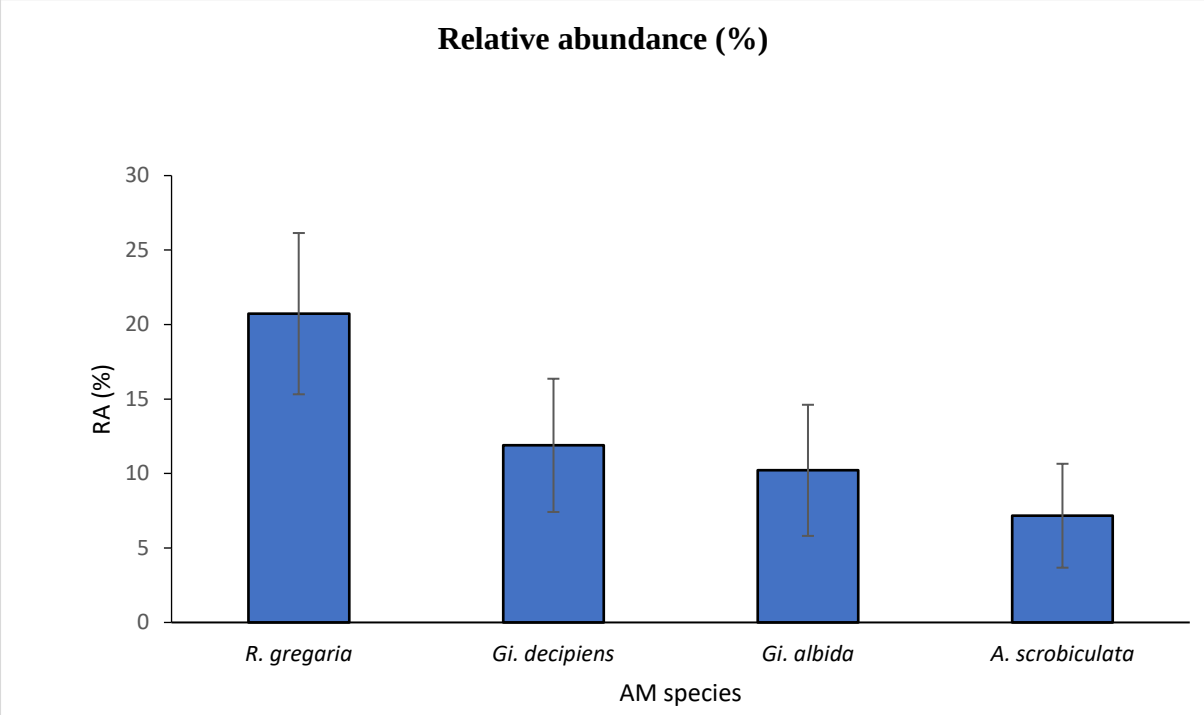

Fig. 3.2: Relative abundance (RA%) of AM fungal species at the study site.

3.3.5: Root colonization and spore density of AM fungi

In the present study, AM root colonization was recorded in *Ipomoea pes-caprae* (L.). The colonization revealed the presence of vesicles, **(Plate 7)** auxiliary cells being knobby and echinate, arbuscules, intra-radical spores, hyphal coils, hyphal swelling, and intra- and extra-radical hyphae **(Plate 8)**. The spore density in the rhizosphere soil of *Ipomoea pes-caprae* (L.) R. Br. was 109 spores/100g of soil.

3.4: Discussion

Smith and Read (2008) suggested that edaphic conditions are crucial for AM symbiosis. In the present study, the soil was sandy in texture. According to Rodrigues and Rodrigues (2017), there are major differences in the interaction between AM colonization and soil physio-chemical factors. A broad range of soil texture, pH, P, and salinity can affect AM fungal root colonization (Muthukumar and Udaiyan, 2002; Abdel Latef and Chaoxing, 2011; Chmura and Gucwa-Przepiora, 2012; Owens *et al*., 2012).

Degraded organic matter forms organic C in the soil. According to Micheni *et al.* (2004), it is essential for preserving the physical, chemical, and biological characteristics of the soil. According to Hodge *et al*. (2010), AM fungal mycelium is essential for breaking organic matter and absorbing nutrients from available sources. Numerous biotic and abiotic variables, including host plant species, pH, soil type, moisture, temperature, total C and N content, and season, influence the distribution of AM fungi (Boddington and Dodd, 2000; Carvalho *et al*., 2003).

AM fungi are essential to the diversity, dynamism, and structure of plant communities. Overcoming biotic and abiotic stressors results from plant nutrition, water uptake, and resistance to soil-borne and plant diseases. Growth and survival, uptake of soil nutrients,

particularly P and N nutrient cycling, play a significant part in the C and N cycle and support C sinks (Douds *et al*., 2005; Smith and Read, 2008; Turrini and Giovannetti, 2012).

The present study observed that the pH was acidic to neutral, ranging from 5.3 to 7.2. The members of the family Gigasporaceae are known to be dominant in sandy subtropical to tropical soils (Lekberg *et al.,* 2007). From the present study, it is observed that *R. gregaria* produced a large number of spores. This may be attributed to low soil acidity, sandy loam texture, and different agricultural site-specificity favouring spore germination and sporulation. Soil pH regulates AM spore germination (Maun, 2009). Also, AM fungal communities depending on micro-climatic changes, *viz*., soil pH, organic matter, and texture content (Ji *et al.*, 2012; Njeru *et al*., 2015). Lower soil pH recorded fewer AM fungal species (Mbogne *et al*., 2015). According to Pontes *et al*. (2017), the variation in sporulation may be attributed to biotic and abiotic factors. The soil pH is one of the key elements controlling spore germination and AM development (Maun, 2009). According to Hayman and Tavares (1985), the ideal pH range for spore germination, root colonization, and growth in AM species varies and is typically between 5.0 and 8.0 (Maun, 2009). While the majority of Glomeraceae and Acaulosporaceae members occur at pH 6.1 or above, and Gigasporaceae members reside in soils with pH 5.3 or lower (Tiwari *et al*., 2008). Rodrigues and Rodrigues (2017) suggested that variations in the pH of the soil can also lead to modifications in its chemical makeup.

According to Gaur and Kaushik (2011), mycorrhizal association also depends on the kind of soil, plant species, and AM species involved, as well as the accessible types of nutrients, particularly P. Additionally, AM fungi positively impact the uptake of micronutrients, which are essential for plant metabolic activities (Lehmann and Rillig, 2015). By promoting or inhibiting spore germination, root colonization, and mycelial growth, concentrations of

micronutrients significantly influence AM fungal communities (Moreira and Siqueira, 2002; Luis *et al*., 2006; Ortas and Akpinar, 2006; Motha *et al*., 2014; Alguacil *et al*., 2016).

In the current study, *Ipomoea pes-caprae* (L.) revealed typical AM fungal structures in the roots, indicating an active functional AM symbiosis. Rodrigues and Rodrigues (2017) reported maximum root colonization in *Ipomea pes-caprae* from Sinquerim dunes.

Micronutrient availability affects root colonization and spore germination by stimulating or inhibiting mycelial cell proliferation (Alguacil *et al*., 2016). According to Cwala *et al*. (2010) and Lehmann *et al*. (2014), AM fungi efficiently enhance plant growth and development by increasing macronutrient intake relative to micronutrients.

Variations in root colonization levels can be caused by various factors, including environmental conditions, habitat differences, and soil disturbance (Boddington and Dodd, 2000; Hindumathi and Reddy, 2011). According to Neeraj *et al*. (1991), under natural stress conditions, Amaranthaceae and other non-mycorrhizal families, including Cactaceae, Chenopodiaceae, Cyperaceae, and Juncaceae, have been reported to be mycorrhizal. According to Rodrigues and Rodrigues (2017), low soil nutrient levels in coastal habitats can cause dune plants to depend on mutualistic relationships, such as those formed by AM fungi. When these non-AM plants coexist with AM plants, mycorrhizal colonization is also seen in these groups (Miller *et al*., 1983).

In the present study *R. gregaria* was the dominant species, followed by *Gi. decipiens, Gi. albida* and *Acaulospora* sp. from the study site. The RA depends on host plant species and ecosystem regions (Radhika and Rodrigues, 2010; Torrecillas *et al*., 2012; Pontes *et al*., 2017). Willis (2013) identified four AM genera in dune vegetation at Morjim in North Goa, India: *Acaulospora, Gigaspora, Scutellospora*, and *Glomus.* According to Maun (2009), most of the AM fungal genera, including *Glomus, Gigaspora, Acaulospora,* and *Scutellospora*, are

found in coastal regions of the world. Mukerji and Kapoor (1986) reported that the genera *Gigaspora, Acaulospora,* and *Glomus* are associated with most plants in semi-arid and dry parts of India.

In general, members of the Acaulosporaceae and Glomeraceae genera produce more spores in the dune environment than members of the Gigasporaceae (Suresh and Nagarajan, 2010). This is because members of the Gigasporaceae spend more time in the vegetative phase (Desouza and Declarck, 2013). According to Jobim and Goto (2016), *A. scrobiculata* was the most prevalent AM species in coastal dunes worldwide. Radhika and Rodrigues (2010) suggested that the sample size affects the richness of AM species. The sampling depth can also impact the richness and composition of AM species (Tiwari *et al*., 2008). The diversity and richness of AM fungal species are also influenced by the kind of habitat, the surrounding environment, the plant community, and the climate (HelWubet *et al*., 2004; Maun, 2009). Eight species of *Acaulospora,* namely *A. bireticulata, A. delicata, A. dilatata, A. elegans, A. foveata, A. nicolsonii, A. rehmii,* and *A. scrobiculata,* were recorded in a study by Rodrigues and Rodrigues, (2017). According to Jaiswal (2002), the most common and abundant AM species in the coastal dune vegetation of Goa were *A. spinosa*, *A. scrobiculata*, *G. macrocarpum, Gi. margarita,* and *Sc. weresubiae*. Willis *et al.* (2013) reported the AM species *A. spinosa, A. scrobiculata, Gi. margarita,* and *R. gregaria* were dominant in the dune vegetation at Morjim in North Goa, India. Numerous factors, including initial spore counts, the soil's physico-chemical characteristics, the host plant's genotype, and the amount of vegetation cover, influence the spore population, which results from complex interactions between the fungus, plant, and habitat. These factors have a significant impact on the abundance of AM spores (Tiwari *et al*., 2008; Wang *et al*., 2013). The existence and quantity of suitable host plant species that permit the fungus to proliferate and sporulate are the primary factors influencing the incidence and abundance of an AM species (Mueller, 2011).

Small-sized spores of AM species found in arid environments belong to *Glomus*, *Acaulospora*, and *Entrophospora* (Stutz *et al*., 2000). Compared to large spore species, fast-growing, reproducing AM species with small spore sizes have a higher chance of surviving because they spread quickly and have sporulation patterns that are adaptive to changing environmental conditions (Dandan and Zhiwei, 2007; Yang *et al*., 2011). There are significant differences in the composition and volume of AM fungal taxa between and within sites because AM fungal species have a specific multi-dimensional niche that is influenced by the plant community present at a site as well as the physical and chemical composition of the soil at that particular site (Burrows and Pfleger, 2002; Ahulu *et al*., 2006).

3.5: CONCLUSION

The results of the present study indicated a positive AM fungal relationship with the vegetation found on dunes. This suggests that the dune vegetation depends on AM fungus for the symbiotic benefits that support dune ecosystem adaption. One of the essential elements sustaining plant biodiversity and ecosystem function is the diversity of AM fungi. Consequently, this indicates that environmental preservation plans must incorporate these helpful soil fungi to conserve dune habitats.

CHAPTER 4: PREPARATION OF TRAP AND MONOSPECIFIC CULTURES UNDER *IN VIVO* CONDITION

4.1: INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are soil-borne fungi that are widely distributed and interact with most plant species in a symbiotic and mutualistic manner (Smith and Read, 2008). Research indicates that AM fungi can enhance plant growth in the face of both biotic and abiotic stressors, even in unfavourable environmental conditions. AM fungi are often used in forestry, ecological restoration, and agriculture to increase crop output and soil health (Johansson *et al*., 2004). Due to their obligate biotrophic nature, these fungi cannot be produced economically on a large scale. Nonetheless, a practical technique for cost-effective, small-scale plant growth and land rehabilitation is the traditional approach of trap culturing (Gaur and Adholeya, 2002).

There are three primary categories of AM fungal production systems. (1) The more complex substrate-based production systems and the "traditional" sand and soil. These widely used technologies, for the most part, offer an affordable means of mass-producing AM fungal inoculum suitable for large-scale applications. (2) The development of substrate-free cultivation systems, often known as "real" hydroponics and aeroponics, which yield a comparatively clean (sheared) AM fungal inoculum. However, these production systems' greater costs have restricted their deployment to research and smaller-scale applications. (3) Whole autotrophic plants or excised roots known as "root organ cultures" (ROC) serve as the foundation for *in vitro* cultivation methods. These methods ensure the generation of pure AM fungus free of contaminants, notwithstanding their high cost.

AM cultures have been produced with a variety of host plant propagation techniques. The most widely used method is substrate-based. However, Dougs *et al*. (2006) claim that the

spores produced by this process have been contaminated with other microorganisms. According to Trejo-Aguilar *et al*. (2013), using a single host and long-term sub-culturing decreased the diversity of AM spores in trap culture.

For the large-scale production of AM fungus, host plants like *Zea mays* (corn), *Plectranthus scutellarioides* (coleus), *Allium* spp. (onion) and *Paspalum notatum* Flugge (Bahia grass) are frequently utilized. These plants have several benefits, including a short life cycle, sufficient root system growth, high levels of AM fungal colonization, and resistance to comparatively low P concentrations. The low sensitivity to infections, the yellow appearance of colonized roots as opposed to white, un-colonized roots like leek and maize, and a broad tolerance to temperature fluctuations are noteworthy traits (Millner and Kitt, 1992).

Using the C_4 *Bahia* grass, (Douds *et al*., 2005, 2006) emphasized that this tropical plant is frost-killed in the winter in temperate regions, which would promote AM fungus sporulation. The sporulation of AM fungal species, which is dependent on the host, is a crucial component in the formation of the inoculum. Gaur and Adholeya (2002) explained that the host plant species affected the quantity of infectious propagules generated. Although achieving a spore inoculum may not always require significant intra-radical colonization levels, they are crucial for forming a spore-root inoculum. In contrast, there has occasionally been evidence of a correlation between intra-radical colonization and extra-radical sporulation (Douds, 1994). This relationship has not always been observed, and it relies on the specific culture conditions and plant and fungus association (Hart and Reader, 2002). According to the INVAM website (http://invam.caf.wvu.edu), the number of spores in some pot cultures in their collection decreased over multiple propagation cycles. When this issue arises, they advise switching up the hosts.

The C_4 Sudan grass (*Sorghum sudanese*), a host regularly used by INVAM, should be replaced with the C_3 legume red clover (*Trifolium pratense*). Furthermore, Egerton-Warburton *et al*. (2007) showed that hyphal growth of Gigasporaceae species increased after N fertilization when associated with a C_3 host. In contrast, some *Glomus* species increased spore production after N fertilization when associated with a C_4 host. Burrows and Pfleger (2002) have previously shown that AM fungal species that produce large spores increase sporulation in conjunction with a greater variety of plant hosts. In contrast, the spore production of species that produce tiny spores varied according to the host.

AM fungi have been propagated and produced on a wide scale using a variety of pure or mixed substrates. Gryndler *et al*. (2003) noted that adding chitin had neutral to favourable impacts on the spore production of several *Glomus* species. The particle size of the substrate is known to affect proper drainage, humidity, and aeration (Gaur and Adholeya, 2000; Millner and Kit, 1992). Pre-treatment of the cultivation substrate is typically done to avoid contamination by unwanted soil microorganisms. Pots, containers, and bags can be treated with radiation, heat sterilization, or steam sterilization of the substrate (Douds *et al*., 2005, 2006; Gaur and Adholeya, 2002). Low-nutrient, mainly P environments are generally favourable for AM fungal colonization (Smith and Read, 2008).

It has frequently been observed that nutrient solutions with or without low P levels are advantageous for AM fungal root colonization and spore generation (Gaur and Adholeya, 2000). Millner and Kitt (1992) reported that using maize plants in a sand-based system found that 20 μM P was the ideal concentration for spore generation in *G. mosseae*, *G. etunicatum*, and *Gi. margarita*. Increased crop production was observed when *G. intraradices* was grown using maize in sand culture and fertilized with a nutrient solution devoid of P (Gaur and Adholeya, 2000). Lee and George (2005) used a nutrient film technique (NFT), where *G. mosseae* was grown on *Lactuca sativa* using a nutrient solution with high P levels and perlite

as the carrier substrate. Propagule production may also be impacted by the type of P used, such as organic phosphate, rock phosphate, or superphosphate. Superphosphate was found to be advantageous for *Gi. margarita, G. clarum, G. mosseae,* and *Gi. heterogama,* but to be detrimental for *G. etunicatum, G. macrocarpum,* and *Gi. gigantea* (Sylvia and Schenck, 1983).

The distribution of C to the roots and the light intensity (Furlan and Fortin, 1977) might indirectly affect AM fungal colonization and spore production. The properties of the substrate are intimately linked to soil properties like pH, cation exchange capacity (CEC), temperature (T), and water content (Lee and George, 2005). Millner and Kitt (1992) suggested using four-morpholine ethane sulfonic acid (MES) as a buffer in the nutritional solution to prevent pH variations. Water should be adequately available to host plants and AM fungus while preventing excess water and oxygen deficiency.

Adequate medium aeration in substrate-based hydroponics systems may mitigate the negative effects of water (White and Charvat, 1999). Dilution of the trap culture inoculum may favour varied AM species over the field during the continuous propagation cycle (Wang *et al*., 2008). A trap culture can support various AM species and is comprised of spores and colonized root parts as an inoculum. Schalamuk and Cabello (2010) reported that the Glomeraceae family multiplied more readily when the trap culture inoculum was used, whereas fewer spores from other families were obtained. As a result, host plants rotated regularly, which could lessen the loss of AM diversity in trap culture.

The AM spore development trap culture method is frequently employed to create an AM inoculum. The culture obtained from the trap culture method is not pure despite its similarities to other methods. To reduce the loss or viability of the AM spores, recently collected soil samples have been preserved using the trap technique (Brundrett *et al*., 1999).

Several methods are employed for AM proliferation in trap culture, depending on the host plant and predominant AM species. In addition to stimulating plant growth, AM protects plants from pathogen invasion and aids in their tolerance of a range of environmental stressors, including drought (Asrar and Elhindi, 2011), salinity (Abdel Latif and Elhindi, 2011; Navarro *et al*., 2012), and other environmental stressors (Nair *et al.*, 2014). According to Hajiboland *et al*. (2010), tomato plant development was enhanced by AM inoculation at a salinity of 5 dS m^{-1}m1. According to Wu *et al*. (2010), orange plants accumulate fewer Reactive Oxygen Species (ROS) under saline conditions than non-AM plants.

Mycorrhizal inoculation boosted *citrus* (Zou, 2009) and pepper (Kaya *et al.*, 2009) plant growth while decreasing the salt effect. The most common and conventional method for creating AM fungal inoculum is the *in vivo* substrate-based culture of AM in plants cultivated in containers. It is known to work well for large-scale production with little technical assistance. Although fragments of roots and spores can all initiate AM symbiosis, *in vivo* mycorrhizal inoculation treatments demonstrated a high risk of infection (Mosse, 1988).

AM fungi play a vital role in influencing the composition and productivity of plant communities (Klironomos, 2000). They facilitate nutrient exchange, enhanced nutrient absorption (Brundrett and Abbott, 2002), improved water relations, and disease resistance, thus impacting plant productivity and influencing ecosystem functioning (Simard and Austin, 2010). The complex interactions between AM fungi and plant communities are crucial for understanding the dynamics of terrestrial ecosystems and have implications for ecosystem management and restoration efforts (Piotrowski *et al*., 2004). Utilizing host plants to create inoculum containing colonized roots and healthy spores is a common practice in the cultivation of AM fungi (Ijdo *et al*., 2011).

To be successfully cultivated, AM fungi must have a symbiotic relationship with their host plants (Shah, 2014). Numerous elements, such as root pigments, temperature, soil moisture, and microbial activity in the rhizosphere, can influence the viability and physical characteristics of spores collected from field soil. It is possible to prepare trap cultures for AM fungal multiplication using soil samples that are collected from the field. The purpose of trap cultures is to propagate AM fungal propagules, such as spores and colonized roots (https://invam.wvu.edu/methods/culturemethods/trap-culture).

Using an appropriate host plant is a key strategy for increasing spore production and creating pure or monospecific cultures of AM fungi (Rodrigues and Rodrigues, 2014). Trap cultures of AM using maize root indicates that species of *Acaulospora* and *Glomus* are dominant producers of AM spores, and these include *A. mellea, A. scrobiculata, G. manohotis,* and *G. monosporum* (Caraka, 2023).

Gaonkar and Rodrigues (2019) reported using *Plectranthus scutellarioides* (coleus) as the catch plant for trap cultures is most suitable because it is easy to propagate, its transparent roots, and it has a short life cycle.

4.2: MATERIALS AND METHODS

4.2.1: Preparation of trap cultures

The rhizosphere dune soil sample collected from the study site was divided into two parts: one part is used for preparing trap cultures, and the other is used for isolation and taxonomic identification.

The dune soil was washed three to four times with tap water, air dried, and then sieved through a 60µm sieve. The sand was sterilized using an oven for three hours daily for three consecutive days at 180^0C. A mixture of rhizosphere dune soil and roots was used to prepare trap cultures.

The pots (11.5 cm × 111 cm) were submerged in soap water for a full day and then washed with tap water. Later, the pots were air-dried and later wiped with absorbent cotton using 100% alcohol. Non-absorbent cotton was used to plug the pot holes. Three to four cuttings initially washed with tap water and then rinsed in sterile distilled water were planted in each pot. The pots were maintained at 27^0C and 63% relative humidity in the polyhouse. The plants were watered as and when required.

Hoagland's solution (minus P) was added after every 15 days. After 45 days of growth, the Trypan blue staining method (Phillips and Hayman, 1970) was employed to confirm the colonization in the roots. Watering was stopped after 90 days, allowing the plants to dry, after which the shoot portion was cut off at the soil surface. The soil from each pot and the roots were collected in labeled *zip*-lock polyethylene bags and stored in the refrigerator at 4^0C.

4.2.2: Isolation of AM fungal spores and taxonomic identification

AM fungal spores were isolated using the Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963). The method of Gaur and Adholeya (1994) was used to quantify AM spores in the rhizosphere soil samples. The AM spores were identified as described previously in section 3.2.10.

4.2.3: Preparation of monospecific cultures

The AM fungal spores were isolated from trap cultures using the wet sieving and decanting technique (Gerdemann and Nicolson, 1963). Autoclaved sand to soil was used in a 1:1 ratio. Pots (15 cm dia.) were used to prepare the trap cultures. The extracted spores of a single AM fungal species were placed 2-3 cm below the soil to ensure colonization. Three to four coleus cuttings were planted in each pot. The pots are maintained in a polyhouse and watered as and when required. Hoagland's solution (minus P) was added every fortnightly. AM colonization in the roots was confirmed after 45 days of growth. Watering was stopped after 90 days, and the plants were allowed to dry. Later, the shoot portion was cut at the soil surface, and the soil and the roots were placed in the polyethylene bags and stored at 4^{o}C. A small amount of the soil sample was processed to extract AM fungal spores. Microscopic observations were carried out using bright-field microscopes (Olympus BX 41 and Nikon Eclipse E200). Photomicrographs were captured using Olympus DP 12-2 and Nikon Digital Sight DS-U3 digital cameras, which were not digitally modified.

4.3: RESULTS AND DISCUSSION

In the present study, two AM fungus species, *R. gregaria* and *Gi. decipiens* were isolated from the trap cultures **(Plate 9)**. Typically, trap cultures are set up to capture many native AM fungus species. Certain species can be captured using the live, colonized roots of field plants (Shah 2014). According to reports, continuous rhizosphere soil sample collection or the setting up of repeated trap cultures can be beneficial in determining the composition of AM species in natural environments (Stutz *et al*., 2000). To reduce spore loss or viability, freshly collected rhizosphere soil samples can be kept in trap culture (Brundrett *et al*., 1999). Nonetheless, a variety of factors can impact the composition and richness of AM fungi isolated from trap cultures, including differences in host root type and morphology, C biomass, and nutrient levels (Brundrett *et al*., 1999).

Several substrates can be utilized, either in their pure or combined forms, to facilitate the large-scale growth of AM fungus. On the other hand, sandy soil is frequently utilized for soil-based cultures (Douds and Schenck 1990), as sporulation is influenced by the size of the substrate particles, which also affects drainage and aeration (Gaur and Adholeya, 2000). However, according to Ijdo *et al*. (2011), even with a thorough sanitization procedure, the soil-based culture of AM fungal species was not guaranteed against free contaminants despite being the most affordable and commonly used technology.

4.4: CONCLUSION

The pot culture technique provides appropriate material for the identification of AM fungi and provides inoculum for *in vitro* studies. In the present study, the cultures of *R. gregaria* and *Gi. decipiens* were successfully developed. These cultures were further multiplied and were used for the *in-vitro* studies.

CHAPTER 5: PREPARATION AND STANDARDIZATION OF THE PROCEDURE FOR *IN VITRO* CULTURE OF AM FUNGAL SPECIES

5.1: INTRODUCTION

Natural soil-derived AM fungal spore propagules may be non-viable, empty, or parasitized, so trap cultures and pot cultures are the most frequently used methods for propagating AM fungal propagules. One uses the pot culture approach to cultivate, isolate, and preserve AM fungus (Gilmore, 1968). When using the pot culture approach, AM fungi are propagated according to the host plants for optimal colonization and sporulation. Depending on the soil water content, pH, salinity, temperature, P level, and amount of light, a single host plant species may impact mycorrhization.

On the other hand, the host plant must be mycotrophic. It should be resistant to plant pathogens, have a large root system, high photosynthetic efficiency, and phosphate acquisition capacity, and should be able to support the sporulation of all AM fungus species. Plants like *Zea mays* L. (com), *Allium cepa* L. (onion), *Arachis hypogaea* L. (peanut), *Stylosanthes* sp., *Paspalum notatum Floigge* (bahiagrass), and *Pueraria phaseoloides* (Roxb.) Benth. (Kudzu) are a few examples of often utilized host plants (http://invam.wvu.edu/methods/cultures/host-plant-choices).

The pot culture approach has proven to be the most popular way for multiplying AM fungal propagules. Even with stringent quality control procedures, contaminant-free inoculum is not guaranteed, even with this least artificial technique (Ijdo *et al*., 2011). As a result, the culture obtained may not be high in purity. The process also takes a lot of time and space, necessitating frequent evaluations of the monospecificity and viability of the inocula generated, making it more challenging to establish high-quality cultures (http://invam.wvu.edu). According to Thompson (1986) and Hung and Sylvia (1988), other

approaches are still being researched for creating an inoculum, such as hydroponic and aeroponic cultural methods.

Root organ culture (ROC) St. Arnaud *et al*. (1996) is the standard procedure for producing AM fungus. Gelling agents such as Phytagel, Gelgro, or agar are used in ROC (Gadkar *et al.*, 2006). Liquid media have also been used to create these dual cultures in place of solid medium (Joner *et al*., 2000). However, due to their difficulty in maintenance, liquid cultures are unreliable and have not been frequently used (Gadkar *et al*., 2006).

AM fungi can be established *in vitro* using the monoxenic culture, also known as root organ culture (ROC), technique, which is based on *Agrobacterium rhizogenes* Conn, transformed roots, and untransformed roots (Ijdo *et al*., 2011). AM fungal cultures can be kept fresh and viable longer when cultivated on a synthetic growth medium combined with altered roots.

Using altered *Lycopersicum esculentum* and *Trifolium pretence* roots, Mosse and Hepper (1975) conducted their studies and established a monoxenic culture of *F. mosseae*. Plants that produce hairy roots naturally undergo genetic change due to *Agrobacterium rhizogenes* (Mathur and Vyas, 2007). This leads to the plant tissues undergoing Ri T-DNA transformation, which causes the roots to grow profusely on artificial media (Tepfer, 1989).

Mugnier and Mosse (1987) successfully cultivated the AM fungus utilizing hairy roots for the first time. Transformed roots have a greater capacity for growth, allowing them to adapt to various experimental settings (Tepfer, 1989). The ROC technique was established by Butcher and Street (1964) and Butcher (1980) utilizing synthetic media that was enhanced with supplies of carbohydrates and vitamins.

Spores and colonized root fragments are examples of fungal inocula employed in several instances to prepare monoxenic cultures. Spores are used to cultivate AM fungus species in Gigasporaceae, but they do not produce vesicles (Budi *et al*., 1999). The correct selection and

sterilization process is essential for forming a monoxenic culture for any AM fungal propagules (Diop 2003).

According to Rodrigues and Rodrigues (2015), the ROC technique has several benefits, such as easy quantification of fungal growth, inoculum quality control, minimal space, and time requirements because the cultures are maintained under controlled growth chamber conditions, morphological observations of the fungal life cycle can be conducted without disturbing the system, and reliable cultures that produce contaminant-free cultures.

Despite its artificial nature, the mass inoculum production of AM fungus and physiological, molecular, and biochemical studies are effectively accomplished by using the in vitro approach. Some drawbacks of the monoxenic culture system include the need for specialized knowledge, specialized equipment, laminar airflow to maintain sterility, low sporulation levels for certain species, a large number of strains cultivated *in vitro*, challenges with maintaining continuous cultivation of certain strains, and changes in the organism and genome over time as a result of repeated culturing (http://invam.wvu.edu). Using the *in vitro* method, it is crucial to determine which species has the greatest potential for producing spores to increase the production of AM fungal inoculum (Declerck *et al*., 2001; Ijdo *et al*., 2011). The present work aims to establish and standardize the *in vitro* culture technique for two AM fungal species.

5.2: MATERIALS AND METHODS

5.2.1: Preparation of WM media for multiplication of Ri T-DNA transformed roots

Procedure:

Take 1000 ml distilled water in a 1L media bottle. To this, add 30 g of sucrose followed by 10 mL of Solutions I and II each, 5 mL of Solutions V, and 1 mL of Solutions III and IV each. Adjust pH to 5.5 by using 1N NaOH and 1N HCl. After adjusting the pH, add 2mL of Solution VI and 4 g of clerigel. Mix the content thoroughly using a magnetic stirrer and autoclave at 121^0C for 15 minutes. Once the media has been autoclaved, allow it to cool but not solidify. Pour the media into Petri plates and leave the plates on a sterile surface until WM media has solidified. Replace the lid of each Petri plate and store the plates in a laminar airflow (Media composition is as given in appendix).

5.2.2: Preparation of WM media for spore germination

Procedure:

Take 1000 ml distilled water in a 1L media bottle. To this, add 10 mL of Solutions I and II each, 5 mL of Solutions V, Add 1 mL of Solutions III and IV each. Adjust pH to 5.5 by using 1N NaOH and 1N HCl. After adjusting the pH, add 2mL of Solution VI and 4 g of clerigel. Mix the content thoroughly using a magnetic stirrer and autoclave at 121^0C for 15 minutes. Once the media has been autoclaved, allow it to cool but not solidify. Pour the media into Petri plates and leave the plates on a sterile surface until WM media has solidified. Replace the lid of each Petri plate and store the plates in a laminar airflow (Media composition is as given in the appendix).

5.2.3: Isolation and disinfection of AM fungal spores

The wet sieving and decanting method were used to isolate AM propagules (spores and colonized root fragments) from rhizosphere soil samples (Gerdemann and Nicolson, 1963). The isolated spores **(Plate 10, b)** were washed using autoclaved distilled water. The protocol adapted from Bécard and Fortin (1988) was followed to surface-sterilize the spores. Sodium hypochlorite was used first to disinfect the isolated spores. After this, they were rinsed twice with sterile distilled water. Later, the spores were sterilized using streptomycin sulphate.

5.2.4: Germination of disinfected AM spores

Surface sterilized spores were placed on Petri plates containing solid WM medium with clarigel, which was devoid of sucrose. Petri plates were incubated in an inverted position at 27^0C in the dark. The optimum germination was achieved by adjusting the pH and sugar concentration.

5.2.5: Establishment of dual culture

The media plug and germinated spore were carefully placed near the actively growing Ri T-DNA transformed roots of the *Daucus carota* to establish a dual culture. The Petri plates were then incubated in the dark at 27^0C in an inverted position **(Plate 11)**.

5.2.6: AM fungal colonization in transformed roots and establishment of continuous culture

The trypan blue staining method (Phillips and Hayman, 1970) was employed to check AM colonization in the transformed roots. The root bits were examined under a bright-field Olympus BX41 research microscope with magnifications of 40x, 100x, and 400x. Micrographs were captured using a Nikon Digital Sight DS-U3 digital camera.

Continuous cultures were established by placing the colonized root portion on a fresh WM media containing the Ri T-DNA roots. Also, the monoxenically produced propagules were extracted from the gel (WM media) using the method of Cranenbrouck *et al*. (2005). The extracted monoxenic spores and attached extra-radical mycelium were placed on a fresh WM media containing the Ri T-DNA roots.

5.3: RESULTS AND DISCUSSION

5.3.1: *In vitro* spore germination

The *in vitro* germinated AM fungal spores were used to initiate the monoxenic cultures. The spores of two AM fungal species viz., *R. gregaria* and *Gi. decipiens* were germinated on WM media (minus sucrose) at pH 5.6 supplemented with Gamborg solution **(Fig 5.1)**.

Monoxenic cultures can successfully be produced for a wide range of AM fungal species (Fortin *et al.,* 2002). According to Gaonkar and Rodrigues (2022), *A. spinosa* and *Sc. scutata* germinated for the first time in monoxenic cultures. According to Srinivasan *et al*. (2014), *G. intraradices* produced 8500-9000 spores per Petri dish under monoxenic conditions. For the first time, Rodrigues and Rodrigues (2017) reported *in vitro* germination of native species of *A. scrobiculata, Rh. manihotis, Gi. albida, R. gregaria,* and *C. claroideum.*
Klironomos and Hart (2002) suggested that members of Glomeraceae induce colonization using spores and colonized root fragments, while members of Gigasporaceae induce new colonization through spores.

In the present study, spores of *R. gregaria* germinated in 1-2 days, while spores of *Gi. decipiens* germinated in 3 days **(Table 5.1) (Plate 12 b & Plate 15 b)**. Velip and Rodrigues (2022) reported spore germination in *Gi. decipiens* after three days, while spores of *R. gregaria* required 4 to 6 days for germination. Rodrigues and Rodrigues (2017) reported

varying days for spore germination for *R. gregaria* (6), *C. claroideum* (19), *Rh. manihotis* (18), *Rh. clarum* (6-10), *Rh. intraradices* (5-8), *F. mosseae* (10-14), *Gi. albida* (2) and *A. scorbiculata* (23), with days required for germination given in parenthesis. Similarly, Gaonkar and Rodrigues (2019) reported varying days for spore germination for *Gi. decipiens* (4), *R. gregaria* (4), *R. intraradices* (1-3), *Rh. clarus* (6), *Rh. fasciculatus* (2), *F. mosseae* (26), and *A. spinosa* (45) with days required for germination given in parenthesis. Similarly, Velip and Rodrigues (2019) reported varying days for spore germination for *Gi. albida* (5-10), *Sc. scutata* (5–6), and *Rh. intraradices* (8-9), with days required for germination given in parenthesis. In another study, Prabhu and Rodrigues (2022) reported varying days for spore germination for *Gi. albida* (4), *Gi. decipiens* (2), *R. gregaria* (3), *Sc. calospora* (3), *Sc.heterogama* (4), and *Sc. scutata* (4), with days required for germination given in parenthesis. Koske (1981) recorded that spore of *Gi. gigantea* germinated after one day of incubation, while spores of *Gi. margarita* germinated after 72 hours on water agar.

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

AM species	**Sodium hypochlorite (NaClO)(µl) + Time (min)**	**Streptomycin sulphate (%) + (min)**	**Germination time (days)**
R. gregaria	165 + 7	0.05%	1-2
Gi. decipiens	210 + 9	0.05%	3

Legend: Number of spores observed = 50/species; Number of spores per plate=5/per plate

5.3.2: Hyphal growth and development

In the present study, spores of *R. gregaria* germinated through the germination shield, while spores of *Gi. decipiens* germinated through the spore wall. Rodrigues and Rodrigues (2017) demonstrated that there are two distinct processes of *in vitro* spore germination, *viz*., the direct mode, in which the germ tube emerges directly through the germination shield or spore wall, and the standard mode, in which the germ tube arises from the sub-tending hypha. Spores of *R. gregaria* and *Gi. decipiens* show the presence of multiple germ tubes due to a significant number of nuclei along the spore wall (Maia and Yano-Melo, 2001) and advantageous host compatibility (Maia *et al*., 2010). Giovannetti *et al*. (2010) reported that the germ tubes in *Acaulospora, Scutellopora,* and *Gigaspora* emerge directly through the spore wall. Kokkoris *et al*. (2019) explain different spore germination processes in Glomeraceae species. In *Rh. clarum*, several germ tubes may also emerge from the subtending hypha, while in *F. mosseae* and *C. caledonium*, a single germ tube arises from the subtending hypha. Rodrigues and Rodrigues (2015) recorded both types of *in vitro* spore germination in *F. mosseae* and *Rh. clarum*. Oehl *et al*. (2011) recorded that the glomoid spores of *Paraglomus* species *viz., Pa. occultum*, *Pa. brasilianum,* and *Pa. lacteum* directly germinate through the spore wall. Multiple germination is considered an extra survival strategy for the germinating spores to increase the possibility of successful contact and colonization of a host root.

In the present study, the hyphal growth was faster in *R. gregaria* than in *Gi. decipiens* (**Table 5.2)**. Rodrigues and Rodrigues (2017) reported varying hyphal lengths of *in vitro* germinated spores of *C. claroideum* (352.35 ± 45.46 µm), *Rh. manihotis* (413.10 ±57.50 µm), *Rh. clarum* (497.91 ± 59.75 µm), *Rh. intraradices* (573.07 ±67.17 µm), *F. mosseae* (291.6 ±70.42 µm), *Gi. albida* (200. 47 ± 37.45 µm) *R. gregaria* (81.46 ±59.72 µm), and *A. scrobiculata* (153.90

±52.49 µm), with values in parenthesis indicating the average hyphal length of the *in vitro* germinated spores.

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

AM species	Average hyphal length (cm)
R. gregaria	0.06 ± 0.03
Gi. decipiens	0.07 ± 0.03

Legend: Values are the mean of 5 readings; ± = Standard deviation.

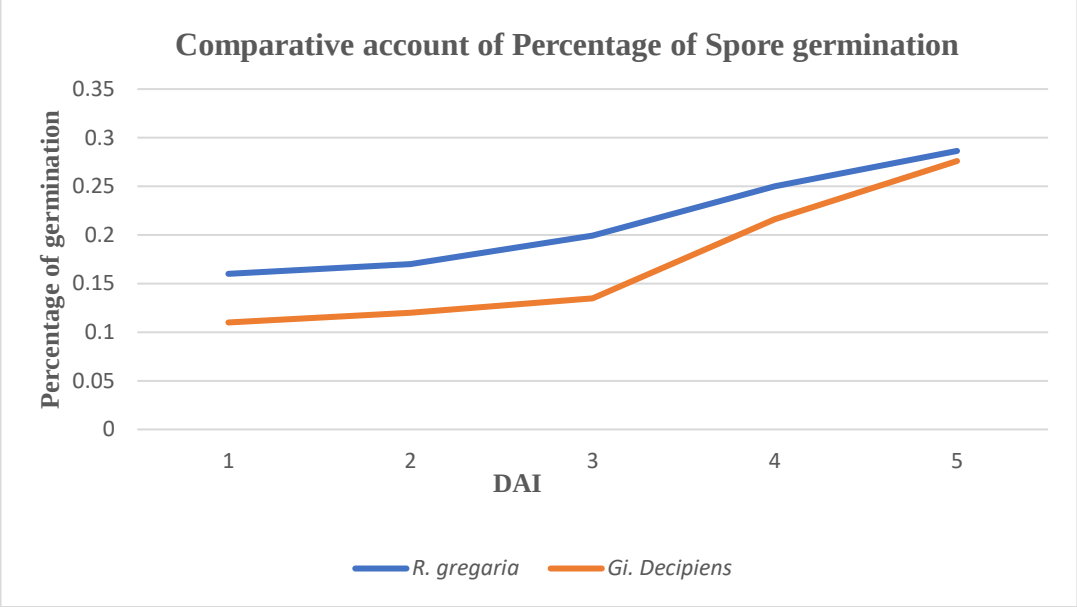

Fig. 5.1: Percent spore germination in *R. gregaria* and *Gi. decipiens* grown in *in vitro* conditions.

In the present study, *in vitro* spore germination and germ tube growth of *R. gregaria* and *Gi. decipiens* were influenced by spore age, media composition, and experimental conditions, viz., temperature, moisture, humidity, and other experimental errors. Clark (1997) and Maia and Yano-Melo (2001) reported that spore germination and germ tube growth were

influenced by many factors, including pH, temperature, light, nutrients, moisture, and substrate under in vitro conditions. Becard and Fortin (1988) and Sancholle *et al*. (2001) suggested that germ tube proliferation typically depends on spore reserves. Meier and Charvat (1992) suggested that the protoplasm contains all the organelles necessary to ensure germ tube development. According to Diop *et al*. (1994), de Souza and Berbara (1999), and Declerck *et al*. (2000), the germ tube grows as a runner hypha that penetrates the media by progressively branching into filaments with thinner diameters.

AM fungal spores can germinate in vitro in response to various culture conditions. However, the hyphae cannot grow extensively. Moreover, in the absence of a host root, the no-host signal is detected, and the germinated spores stop growing in 8 to 20 days (Mosse, 1959; Giovannetti et al., 1993; Schreiner and Koide, 1993; Logi *et al*., 1998).

Spore dormancy, viability, and sterilization are some of the elements that result in variations in the germination rate (Tommerup, 1983; Gemma and Koske, 1988; Juge *et al*., 2002; Bharadwaj *et al*., 2012). D'Souza *et al*. (2013) suggested that the MSR medium without sucrose induced appressorium development and increased spore germination activity *in vitro*.

St-Arnaud *et al*. (1995) reported that in an *in vitro* two-compartment system, one compartment devoid of sucrose promotes AM fungi mycelium development, and the other compartment with sucrose promotes the growth of transformed roots of *D. carota*. This technique demonstrates a high rate of sporulation in *G. intraradices.*

Sward (1981) reported that *Sc. fulgida* and *Sc. persica* spores germinate for two weeks in dark conditions at 24^0C. Costa *et al*. (2013) reported increased *in vitro* sporulation in *Gi. decipiens* at 22^0C. Velip and Rodrigues (2021) reported that spores of *Gi decipiens* germinate at 5.6 pH in MSR media. Costa *et al*. (2013) reported that spores of *Gi. decipiens* and *R. clarum* germinate at 6.5 and 4 pH in M media, respectively. Similarly, Karandashov *et al*.

(2000) reported that spores of *F. caledonium* germinate at 6.5 pH on M media. Ellarif *et al*. (2019) recorded that the spore of *Gi. gigantea* germinate at 5.7 pH on solid media.

5.3.3: Formation of primary structures in Gigasporaceae species

In the present study, upon establishment of dual culture, the hyphal growth occurred in the Petri plate, giving rise to dichotomously branched ramifications called Branched Absorbing Structures (BAS) or arbuscular-like structures (ALS) **(Plate 16 a).** BAS are hyaline, thin-walled hyphal networks that matured into septate structures. BAS or arbuscular-like structures (ALS), which are dichotomously branched ramifications, are characteristic features of Gigasporaceae (Gaonkar and Rodrigues, 2019). Chabot *et al*. (1992) suggested that the formation of BAS or ALS typically occurs together with the growth of extra-radical hyphae in sterile culture conditions.

Bidondo *et al.* (2012) demonstrated a phenomenon known as the "maternal effect". It is challenging to achieve first-generation sporulation under *in vitro* environments since AM strains are suited to the host plant and the natural ambient parameters of the isolation site. Members of the Gigasporaceae family are more challenging to sub-culture and propagate in monoxenic environments (Dalpe *et al.*, 2005). According to Ijdo *et al*. (2011), members of the Glomeraceae family allocate their resources to reproduction, thus making them more suited to *in vitro* environments, whereas members of the Gigasporaceae family invest their energy and resources in somatic growth, which allows them to grow and survive (D'souza and Declerck, 2013).

In the present study, the *in vitro* development of *Gi. decipiens* and *R. gregaria,* following the establishment of the dual culture, served as evidence for the development of auxiliary cells in monoxenic culture **(Plate 12 d & Plate 15 d).** The auxiliary cells appeared 15 days after dual culture on both sides of the runner hyphae in *R. gregaria* and *Gi. decipiens*. Prabhu and

Rodrigues (2022) reported varying days for *in vitro* auxiliary cell formation in *Gi. albida* (16), *Gi decipiens* (20), *R. gregaria* (20), *Sc. calospora* (23), *Sc. heterogama* (32) and *Sc. scutata* (44) with values in parenthesis indicating the number of days required for auxiliary cell formation. Gaonkar and Rodrigues (2019) reported that the auxiliary cells began to form 10 days after the spores germinated, either on one or both sides of the runner hyphae. Members of the Gigasporaceae family develop auxiliary cells on extra-radical hyphae rather than vesicles (Dodd *et al*., 2000). In the present study, *Gi. decipiens* produced spiny-type auxiliary cells, while *R. gregaria* produced knobby-type auxiliary cells **(Plate 12 d & 15 d)**. The auxiliary cells of the *Gigaspora* species were spherical and had an ornate surface, ranging in colour from pale yellow to dark brown, while those in the *Scutellospora* species were knobby and hyaline (Gaonkar and Rodrigues, 2019; Prabhu and Rodrigues, 2022; and Velip and Rodrigues, 2022)

In the present study, the total number of auxiliary cells ranged from 5 to 6 in *Gi. decipiens* and 10-15 per Petri plate in *R. gregaria*, insufficient to begin sporulation. De Souza and Declerck (2003) observed that developing 600 to 700 auxiliary cells produced an average of 56 spores per plate of *Sc. reticulata*. Gaonkar and Rodrigues (2019) suggested that the mass production of auxiliary cells would aid in storing C, which is needed as an energy source for mycelia development and spore germination.

In the present study, the sporulation was observed in *R. gregaria,* while in *Gi. decipiens,* only root colonization was recorded. According to Kandula *et al*. (2006), *Gigaspora* species exhibit a lengthy vegetative phase before sporulation.

5.3.4: *In vitro* colonization in *Gi. decipiens*

In the present study, spore germination occurred within three days after incubation on WM media devoid of sucrose. On the WM medium, echinate type of auxiliary cells were

observed. However, no BAS development was recorded. This could be due to delayed observation or spore reserves (Sancholle *et al.,* 2001). It was observed that AM hyphae successfully colonized the Ri T-DNA roots **(Plate 13)**. However, no sporulation has been observed to date. The trypan blue staining confirmed the AM root colonization in Ri T-DNA transformed roots of *D. carota* **(Plate 14)**.

Additionally, the development of BAS in the soil improves the soil's porosity, stabilizing the soil structure and facilitating the uptake of nutrients and water (Costa *et al*.,2013). Arbuscules and BAS have comparable lifespans. These structures rapidly degenerate, which may be due to the apoptotic process (Mathur and Vyas, 2007). The host hyphae and cell walls may have recognition sites blocked by various carbohydrates in the medium. This may hinder the ability of germ tubes to find host roots (Allen, 1992).

Even roots from the same breed react differently when grown under the same conditions. Therefore, the physiological makeup of the host root is essential for successfully establishing AM fungal culture in monoxenic environments. Consequently, each breed requires different cultural conditions, such as explant selection, the regularity of subculture, and the orientation of the Petri plate during incubation, such as horizontal, inverted, or vertical (Mathur and Vyas, 2007). Velip and Rodrigues (2019) reported successful colonization and sporulation in *Gi. decipiens*. According to Gadkar and Adholeya (2002), an *in vitro* culture of *Gi. margarita* was established using transformed roots of *D. carota* in a minimal nutrient (M) medium.

5.3.5: *In vitro* sporulation in *Racocetra gregaria*

In the present study involving *R. gregaria*, the spore germination process commenced within 1 to 2 days after inoculation, producing several germ tubes from the germ tube initials of the germination shield. The germ tubes grew and branched in the direction of the transformed

roots. The hyphal branching in the culture medium exhibited two growth patterns, *viz*., apical and lateral. The lateral branches showed the presence of septa **(Plate 17 a-b)**.

Again, in the present study, a mycelial network of runner hyphae with BAS/ALS emerged after two weeks of co-cultivation (**Plate 17 c-d)**. BAS are hyaline hyphal networks growing near the root zone with thin walls and dichotomously branched structures. Knobby-type auxiliary cells appeared after 15 days of establishment of dual culture **(Plate 16 b)**. These auxiliary cells are known to store nutrients from media required for hyphal growth and spore germination (Declerck *et al.*, 2004). The hyphae showed dense hyphal colonization in the transformed *D. carota* roots after 40 to 45 days of inoculation.

In the present study involving *R. gregaria*, a single spore was formed in an intercalary position on the sporogenous hyphae. According to Costa *et al.* (2013), in species of Giagasporacae, spore formation can occur alone or in a group, in terminal or intercalary positions on the sporogenous hyphae. Further, mycelial development from the germinated spore is highly prolific. Although auxiliary cells are rapidly occurring, sporulation takes several weeks (Fortin *et al.*, 2002).

The present study observed the first-time sporulation in *R. gregaria* after 50-55 days of growth **(Plate 18)**. This is a significant step in the monoxenic culture of AM fungi as it is essential to scale up AM fungal inoculum production (Declerck *et al.*, 2001; Ijdo *et al.*, 2011). The ability of the *R. gregaria* strain to sporulate within two months of inoculation is a highly promising step towards considering it for potential inoculum production. Owing to its high BAS formation, colonization intensity, sporulation, and faster re-growth *R. gregaria* can be regarded as an indigenous, stable, and homogenous monoxenic culture species. The species can be further used for mass multiplication and screening studies as biofertilizers. The trypan

blue staining confirmed the AM root colonization in Ri T-DNA transformed roots of *D. carota* **(Plate 19)**.

5.3.6: Continuous cultures

The colonized transformed Ri T-DNA root fragments in both the AM species were sub-cultured on fresh MSR medium. All the Petri plates inoculated with the colonized roots recorded growth after 30 days **(Plate 20)**. However, intra-radical structures and re-growth were sometimes challenging to achieve. Similar observations were recorded in earlier studies (Strullu and Romand, 1986; Fortin *et al*., 2002).

In vitro cultivation methods reduce the contamination risks and meet the demand for quality mass production on a commercial scale (Ijdo *et al.,* 2011). Generally, AM fungi should complete their life cycle with good sporulation and fungal-continuity *in vitro* culture conditions (Declerck *et al*., 2005). This would make the monoxenic culture a contamination-free, promising, rapid, and high-spore-producing method. AM root-organ culture technique has essential implications for producing AM inoculum for research and commercial purposes.

5.4: CONCLUSION

To study the life cycle of AM fungal species and the large-scale production of microbiologically high-quality and clean inoculum, the *in vitro* cultivation of AM fungi is essential. In the present study, the monoxenic cultures of *Gi. decipiens* and *R. gregaria* were successfully established in the transformed roots of *D. carota* as hosts on WM medium. Further, the culture produced BAS and spores, which are highly desirable for establishing mass production of pure and viable inoculum. The monoxenic method, over a short period and in limited space, resulted in extensive spore production. Therefore, the indigenous species of *R. gregaria* significantly facilitate monoxenic inoculum for bio-inoculum production.

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APPENDIX

Composition of Hoagland solution (-P)

Macronutrients	g/ml
KNO_3	10.11/100
$MgSO_4.H_20$	24.65/100
$Ca\ (NO_3)_2$	16.41/100
$MgCl_2$	20.33/100
$FeSO_4.7H_2O$	0.557/50
Na_2EDTA	0.745/50
Micronutrients	
H_3BO_3	0.715
$MnCl_2.H_2O$	0.452
$ZnSO_4.7H_2O$	0.055
$CaSO_4.5H_2O$	0.020
$NaMoO_4$	0.725

Dissolve in 500ml of distilled water

Composition of WM Media

Sr. No	Chemical name	g/L
1.	**Solution I Stock**	1L (100x)
a	Magnesium sulphate ($MgSO_4$-$7H_2O$)	73.1 g
b	Potassium nitrate (KNO_3)	8 g
c	Potassium chloride (KCL)	6.5 g
d	Potassium phosphate (NaH_2PO_4-H_2O)	1.9 g
e	Sodium phosphate (Na_2SO_4)	19.9 g
2.	**Solution II Stock**	1L (100x)
a	Calcium nitrate tetrahydrate	28.8g/L
3.	Solution III	250 mL (1000x)
a	Potassium iodide	0.1875 g/250mL
4.	**Solution IV Stock**	250 mL (1000x)
a	Manganese sulphate ($MnSO_4$-H_2O)	1.165 g
b	Zink sulphate ($ZnSO_4$-$7H_2O$)	0.6625 g
c	Boric acid H_3BO_3	0.375 g
d	Sulphate pentahydrate ($CuSO_4$-$5H_2O$)	Weight and dilute 0.65 g in 50mL water and take 2.5mL of this solution to the mix
e	Sodium molybdate dihydrate (Na_2MoO_4-$2H_2O$)	Weight and dilute 0.12 g in 100mL water and take 0.5mL of this solution to the mix.
5.	**Solution V Stock**	
a	Sodium iron EDTA (NaFe)	0.8 g/500 mL
6.	**Solution VI (Gamborg Vitamin) Stock**	mg/L
a.	Nicotinic acid	100g
b.	Myo-inositol	100g
c.	Thiamine hydrochloride	1g
d.	Pyridoxine hydrochloride	1g

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Plate 1 : a-b. *Ipomoea pes-caprae* (L.) R. Br. vegetation at Caranzalem beach

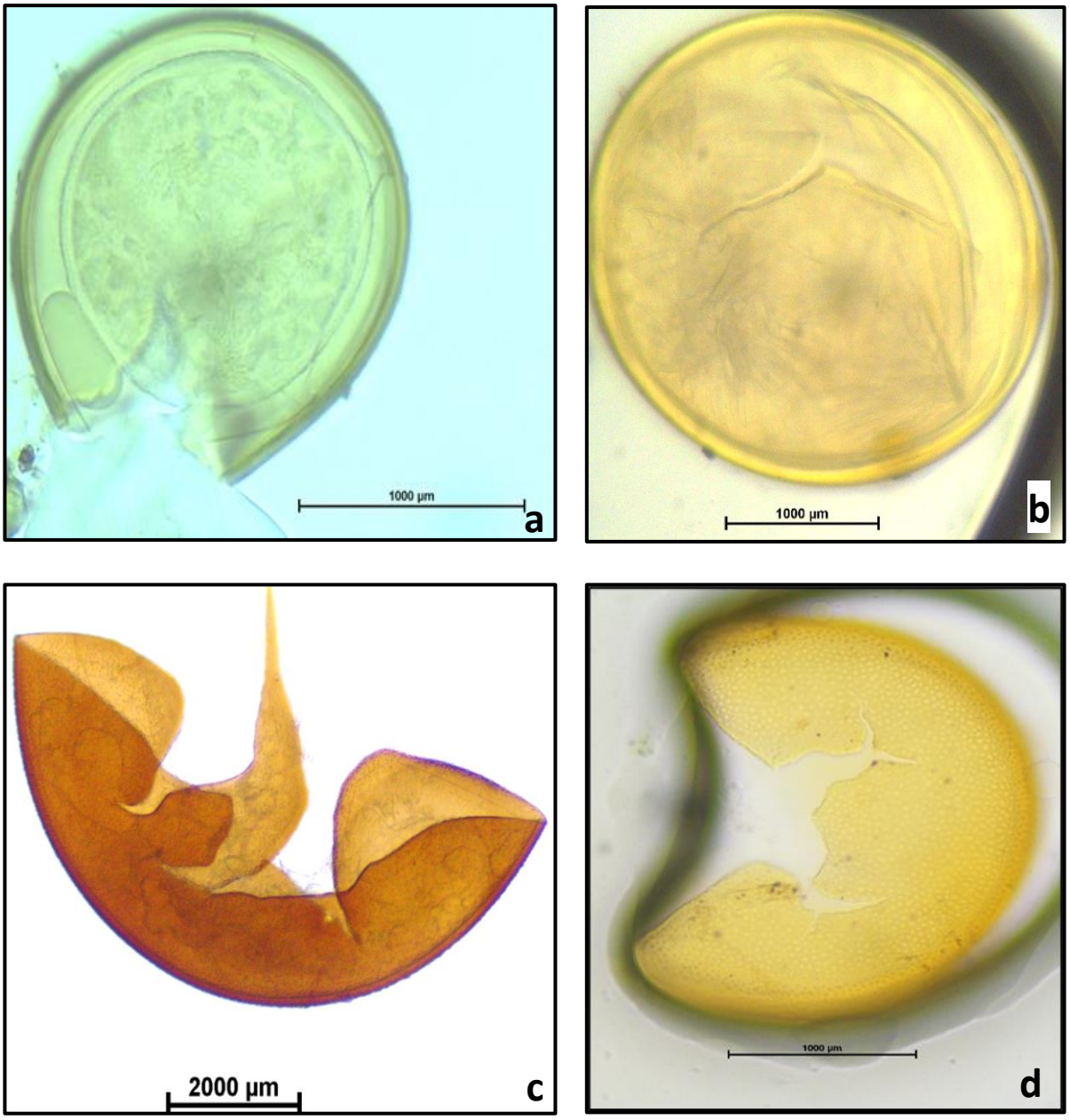


Plate 2 : AM fungal species: a. *Gigaspora albida*; **b.** *Gigaspora decipiens* **c.** *Racocetra gregaria* ***d.*** *Acaulospora scrobiculata.*

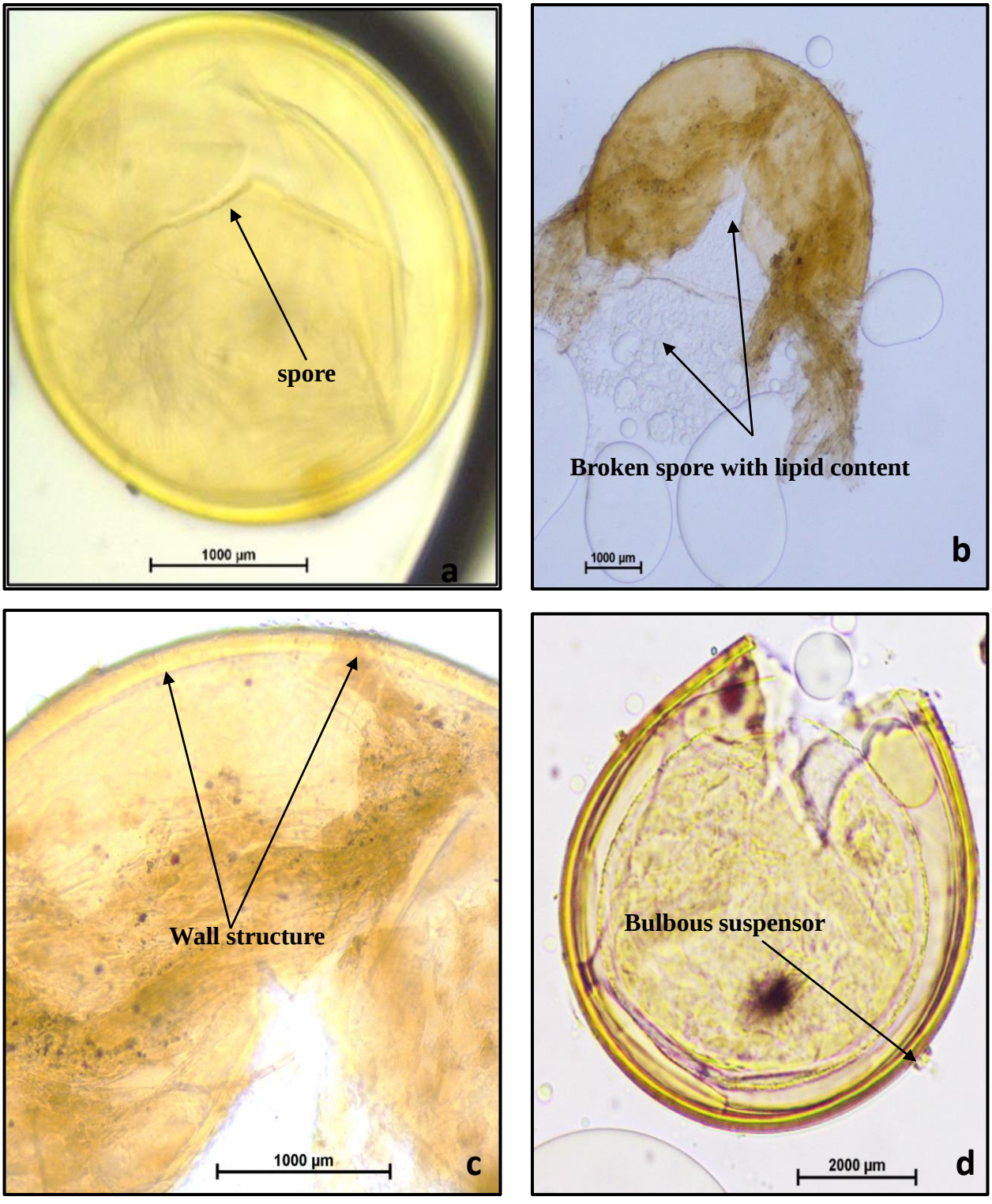


Plate 3 : AM fungal species extracted from selected study site describing morphological features: **a.** Spore of *Gi. decipiens* (Schenck & Smith); **b.** Broken spore with spore reserve; **c.** Wall structure; **d.** Sporogenous cell.

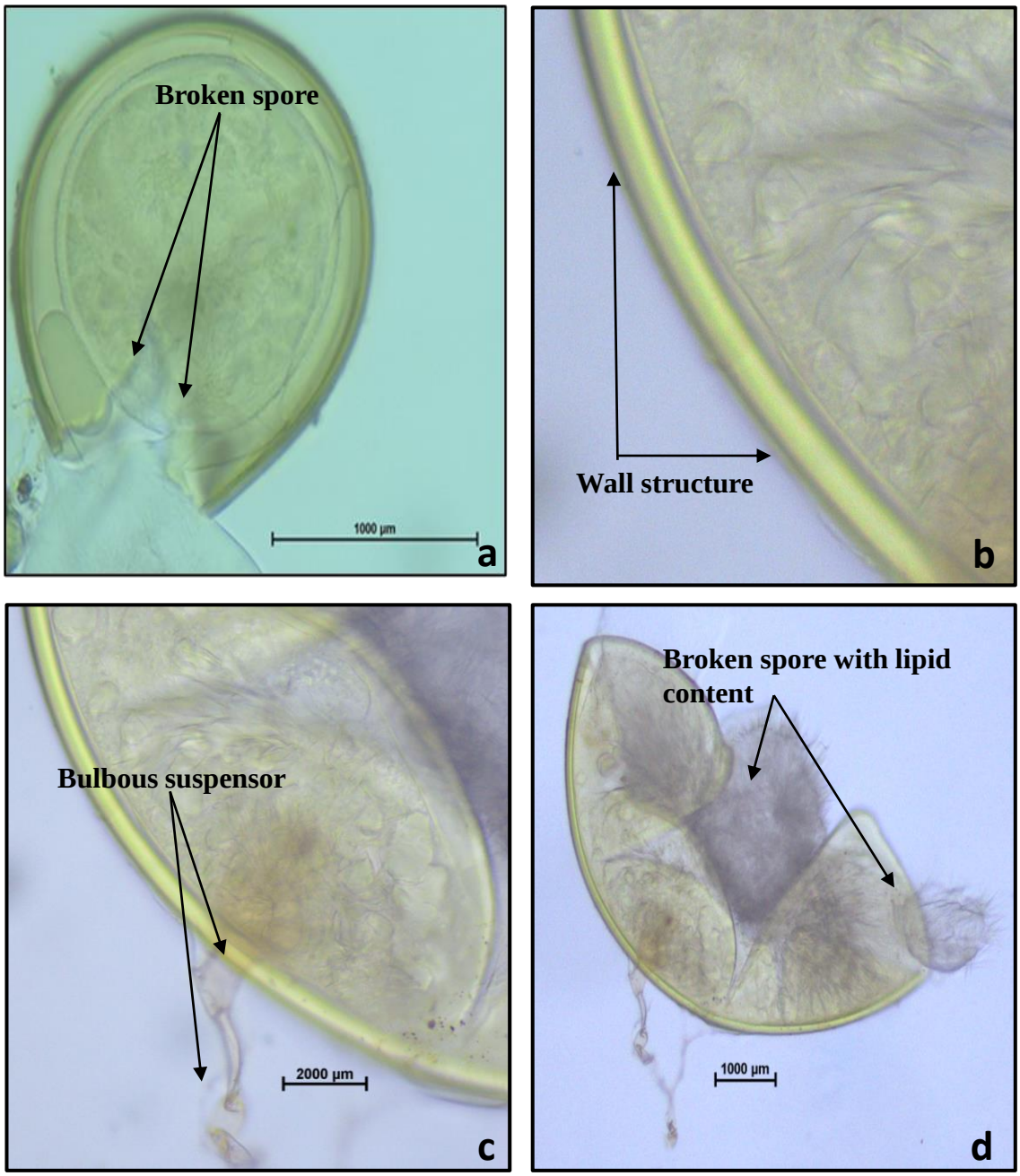


Plate 4: AM fungal species extracted from selected study site describing morphological features: a. Broken spore of *Gi. albida* (Schenck & Smith); **b.** Wall structure; **c.** Sporogenous cell; **d.** Spore content.

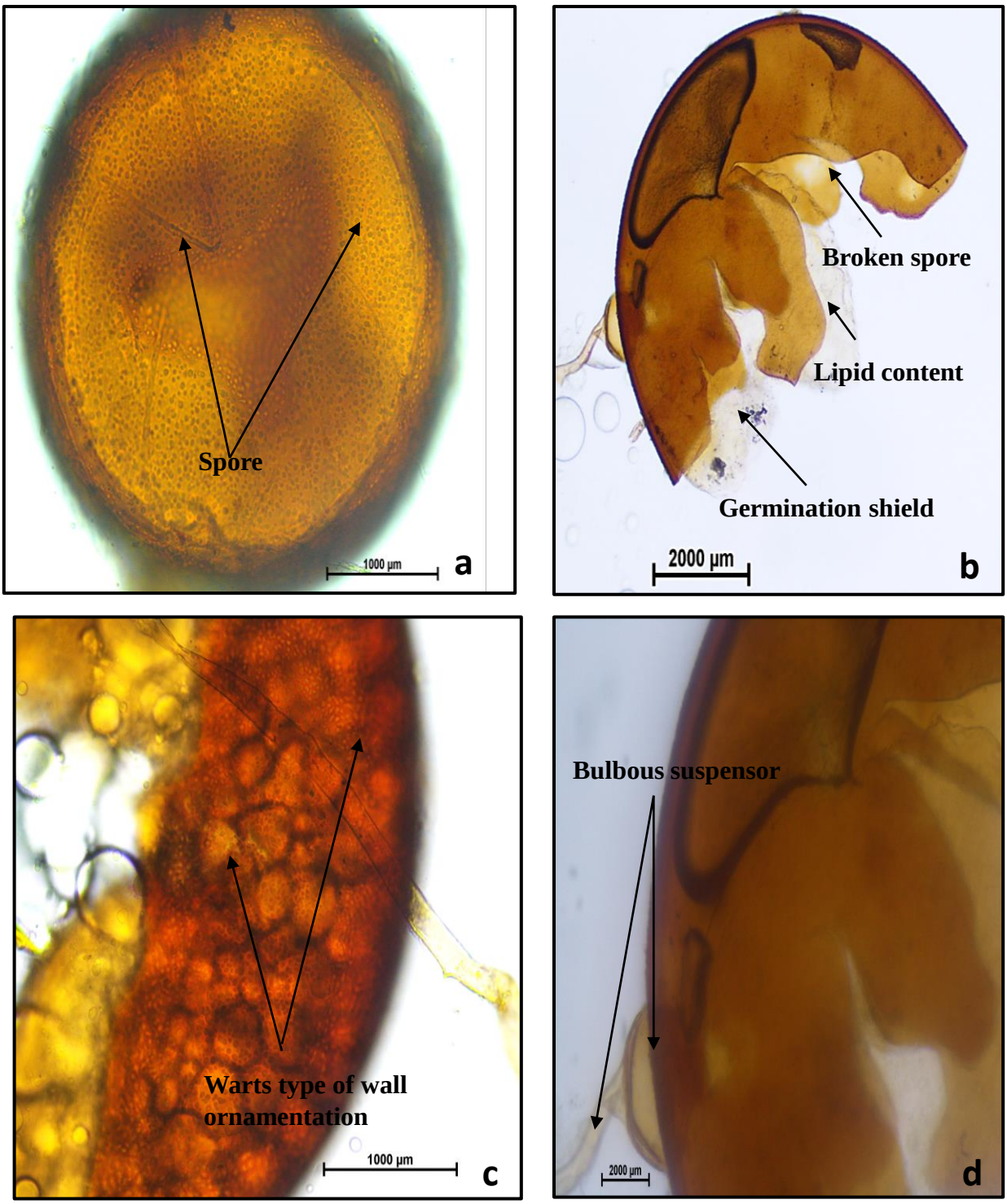


Plate 5: AM fungal species extracted from selected study site describing morphological features: **a.** Spore of *R. gregaria* ((Schenck & Nicolson; Oehl, Souza & Sieverd); **b.** Broken spore with spore reserve and germination shield; **c.** Wall structure; **d.** Sporogenous cell.

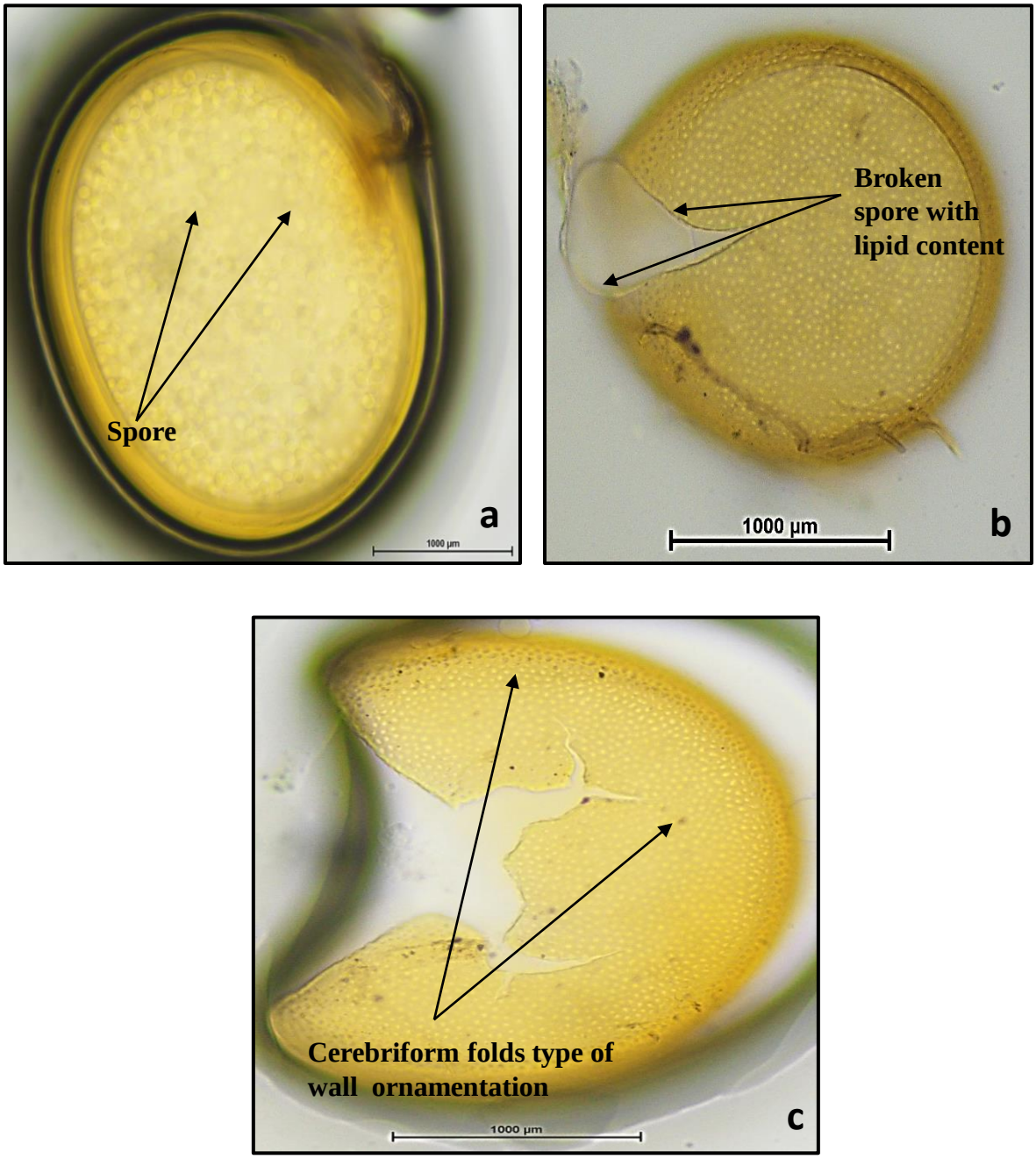


Plate 6 : AM fungal species extracted from selected study site describing morphological features: a. Spore of *Acaulospora scrobiculata* (Trappe); **b.** Broken spore with spore reserve; c. Wall structure.

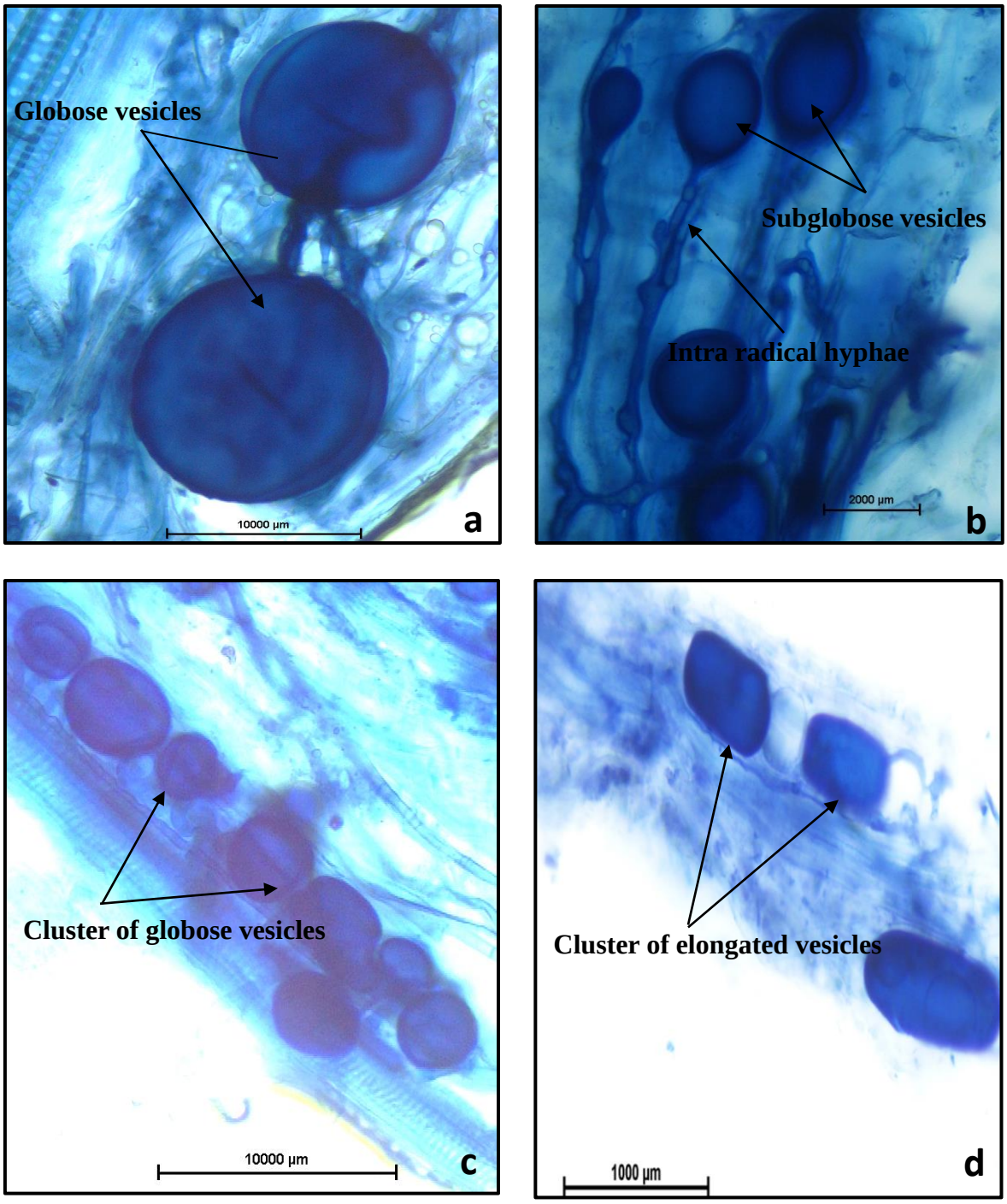


Plate 7: Vesicles in roots of *Ipomoea pes-caprae* (L.) R. Br. : a. Globose vesicles; **b**. Sub-globose vesicle; **c.** Cluster of globose vesicles; **d.** Cluster of elongated vesicles.

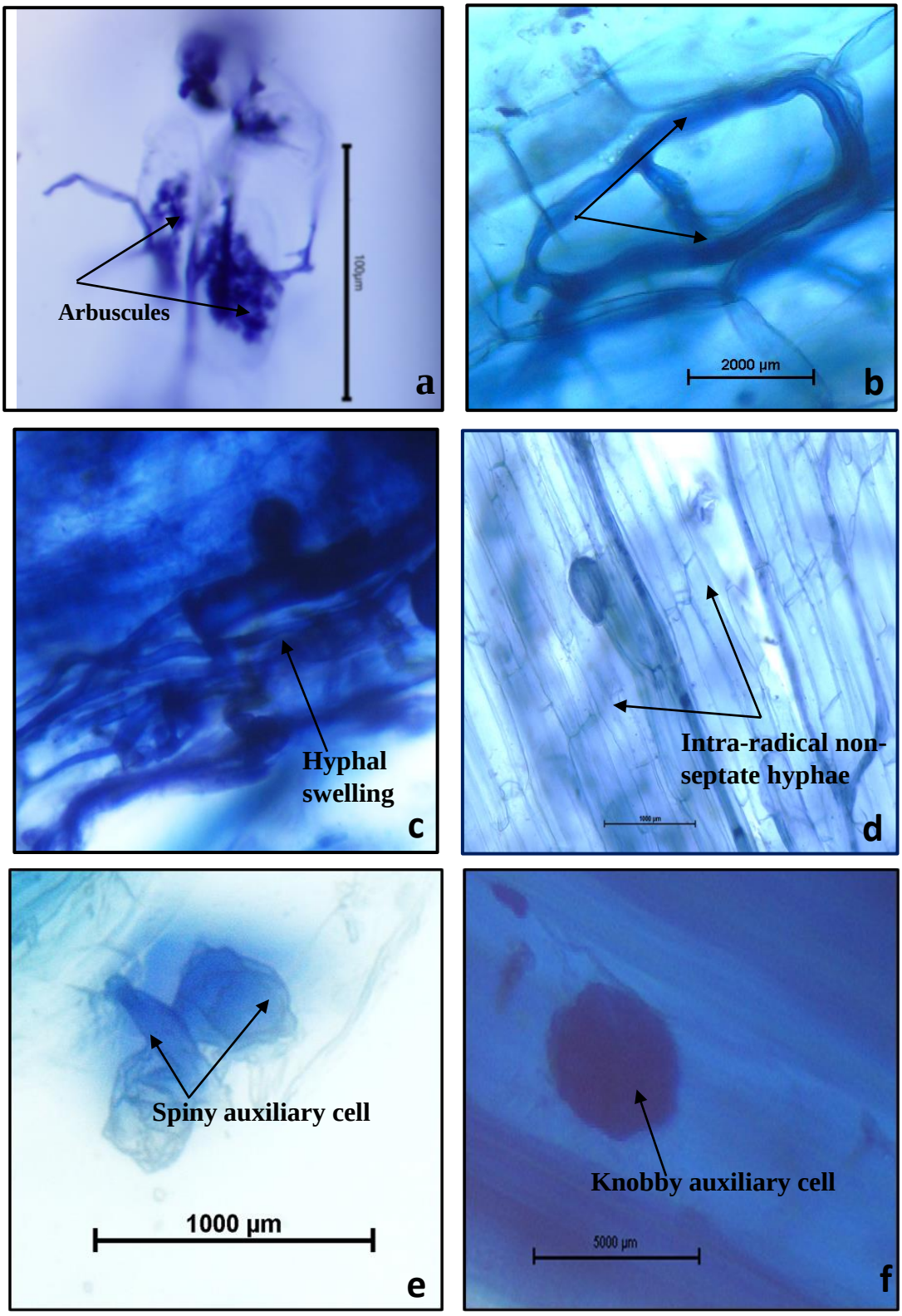


Plate 8: AM fungal structures in roots of *Ipomoea pes-caprae* (L.) R. Br. : a. *Arum*-type arbuscular colonization; **b.** Hyphal coils; **c.** Hyphal swelling **d.** Intra-radical non-septate hyphae; e. Spiny auxiliary cell **f.** Knobby auxiliary cell.

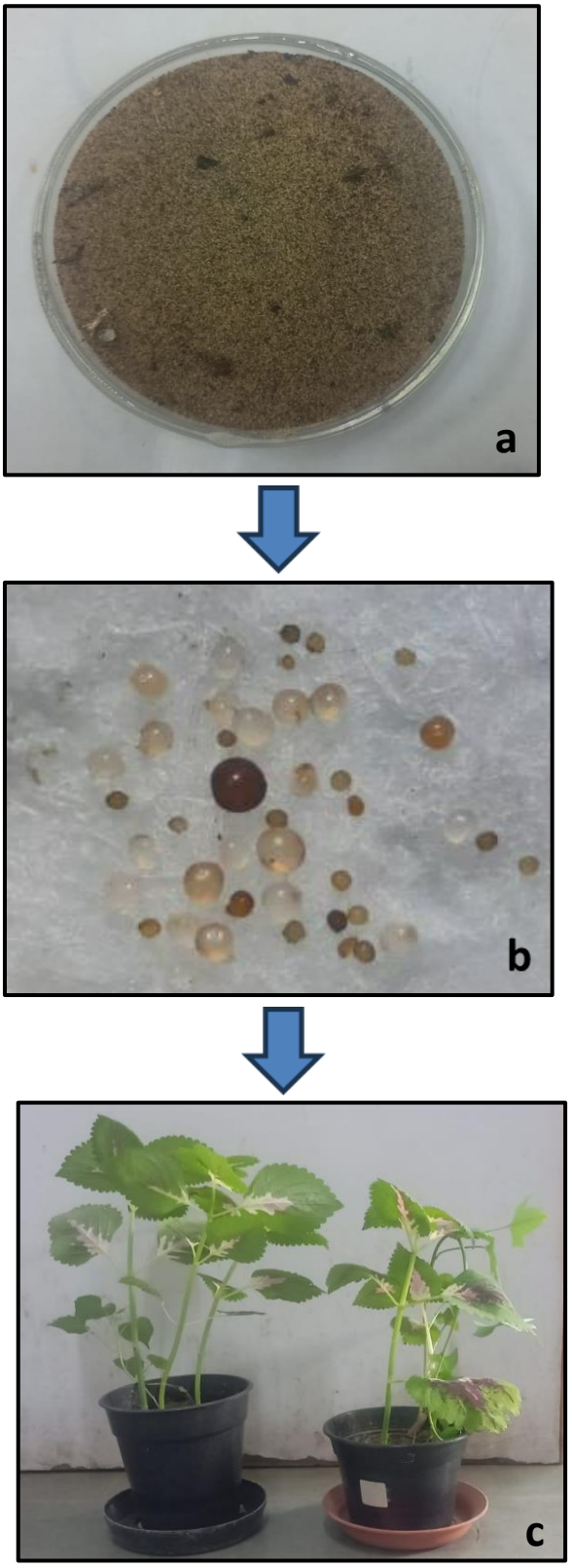


Plate 9: Mass multiplication of AM fungi using pot culture technique: a. sand sample from study site; **b.** Spores from a sample; **c.** trap cultures.

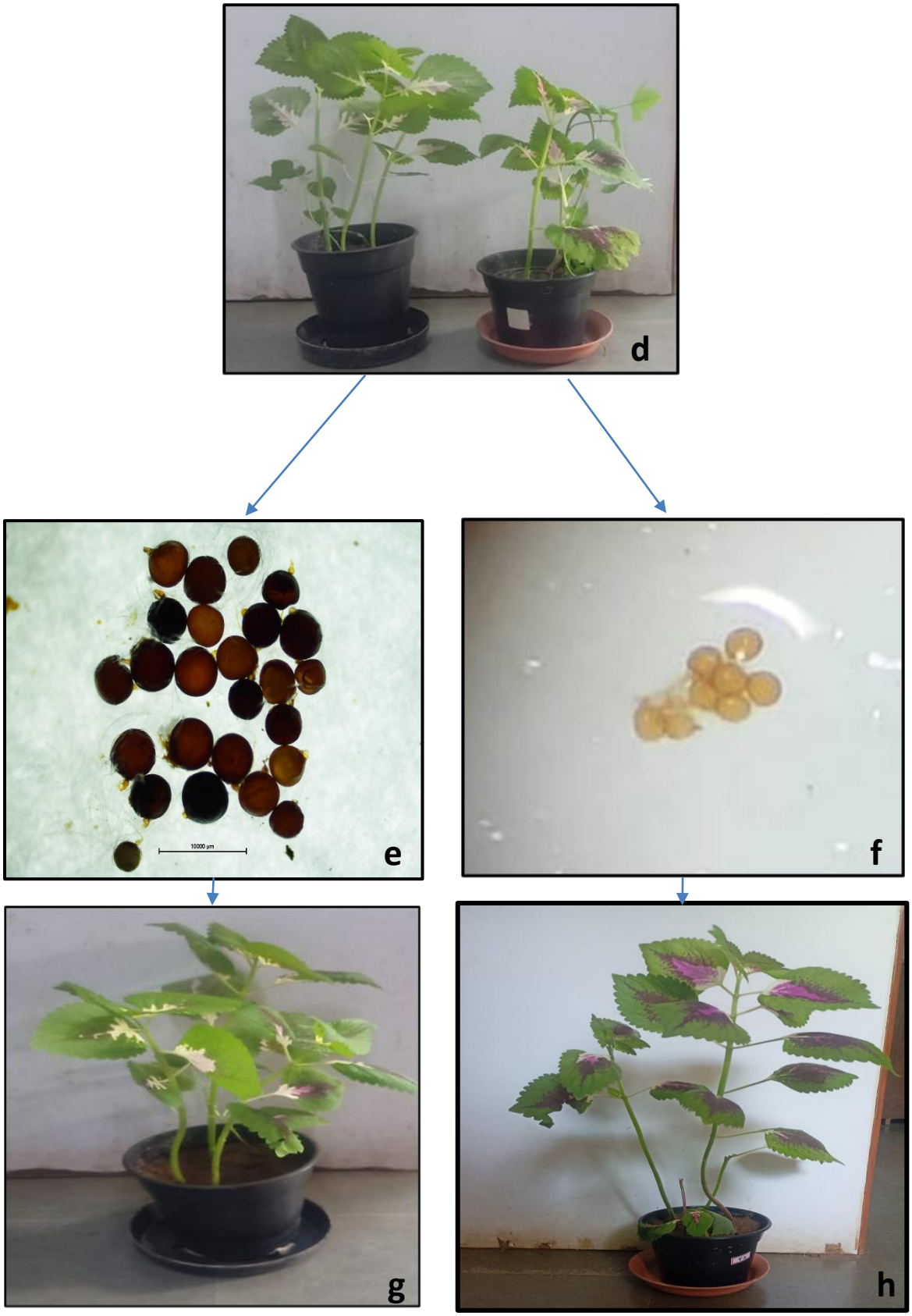
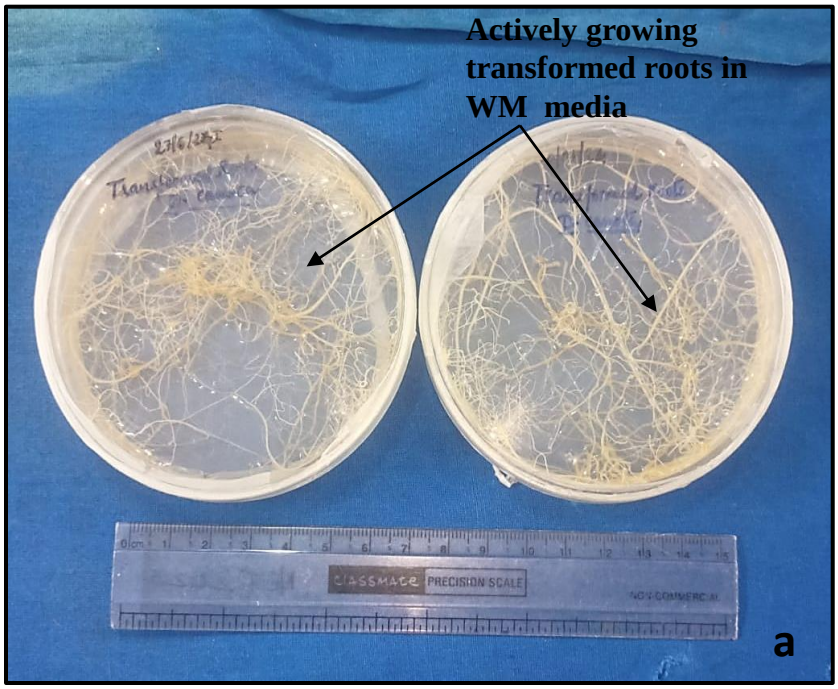


Plate 9: Mass multiplication of AM fungi using pot culture technique: d. trap culture; **e.** *Racocetra gregaria* spores; **f.** *Gigaspora decipiens* spores; **g.** Monospecific culture of *R. gregaria*; **h**. Monospecific culture of *Gi. decipiens*



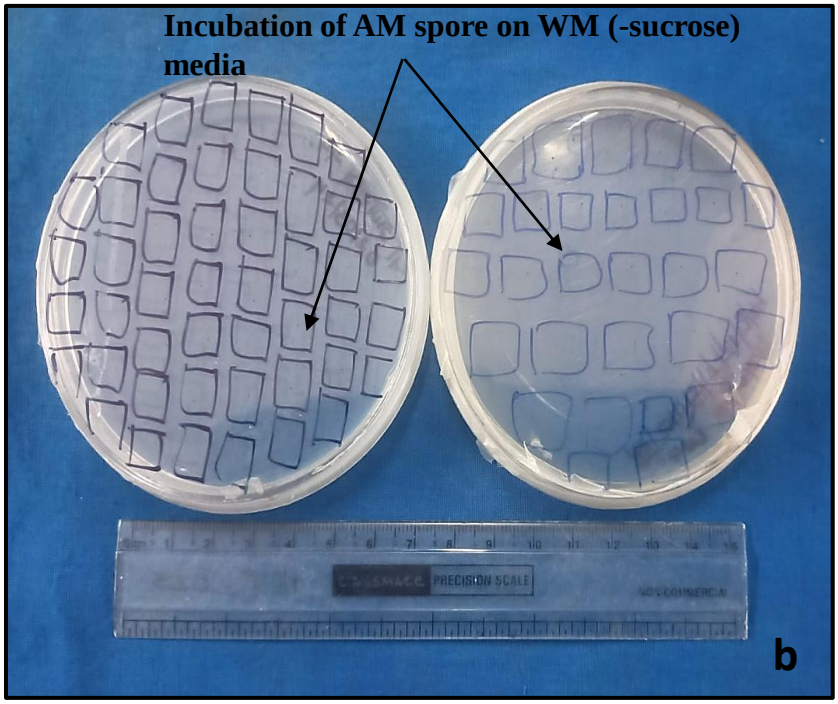
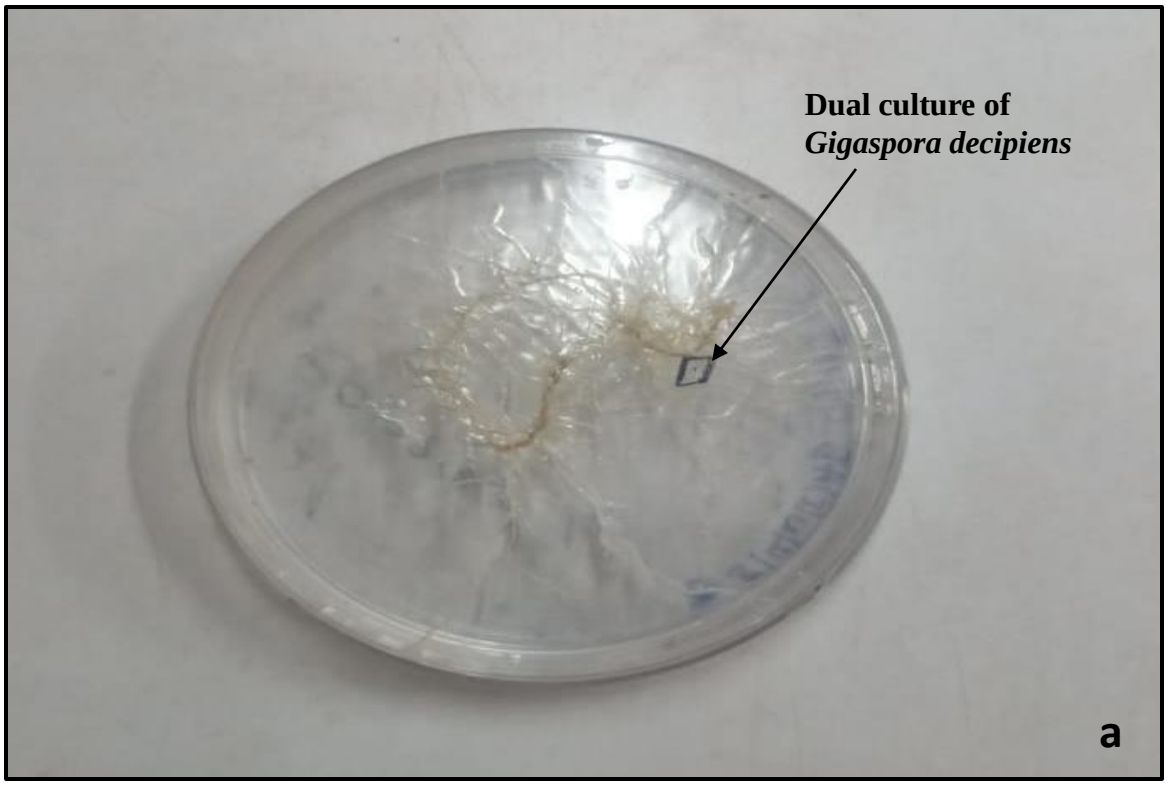


Plate 10: Germination media a: The actively growing transformed root of *Daucus carota* **b:** AM spores on WM (-sucrose) media.



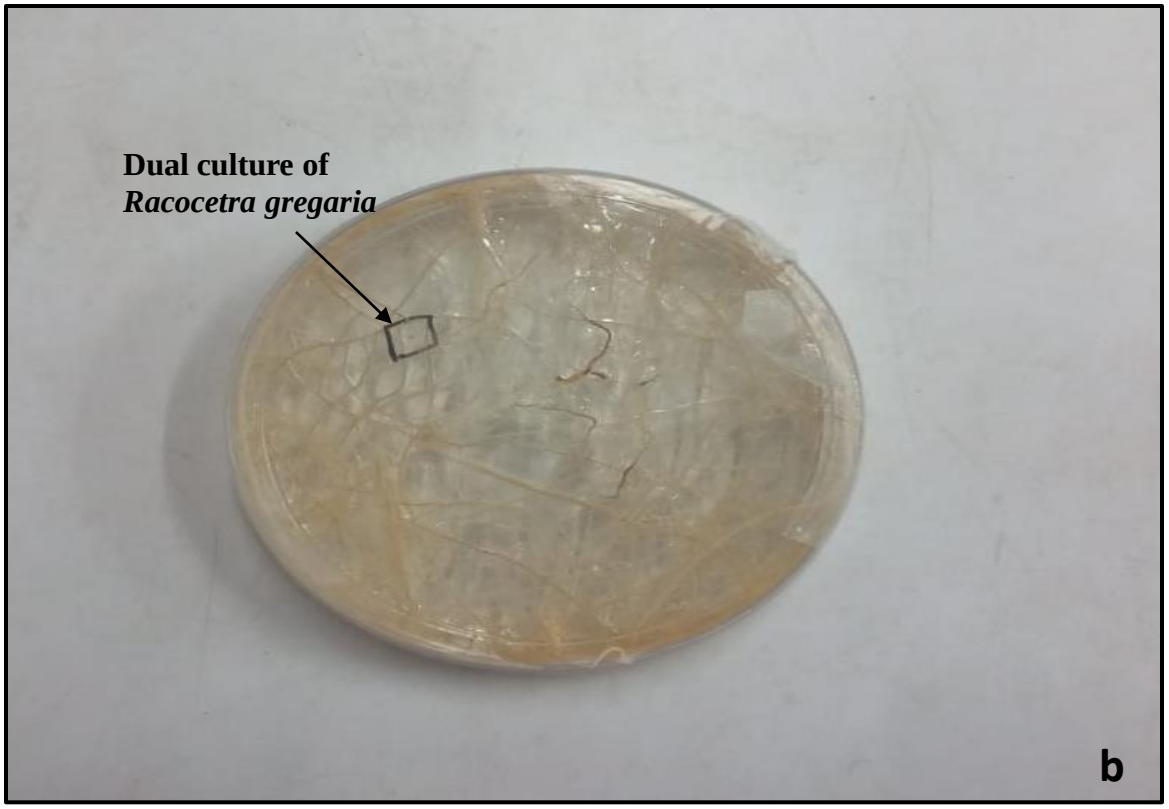


Plate 11 : Establishment of dual culture on WM media; a. Dual culture of *Gi. decipiens*; **b.** Dual culture of *R. gregaria.*

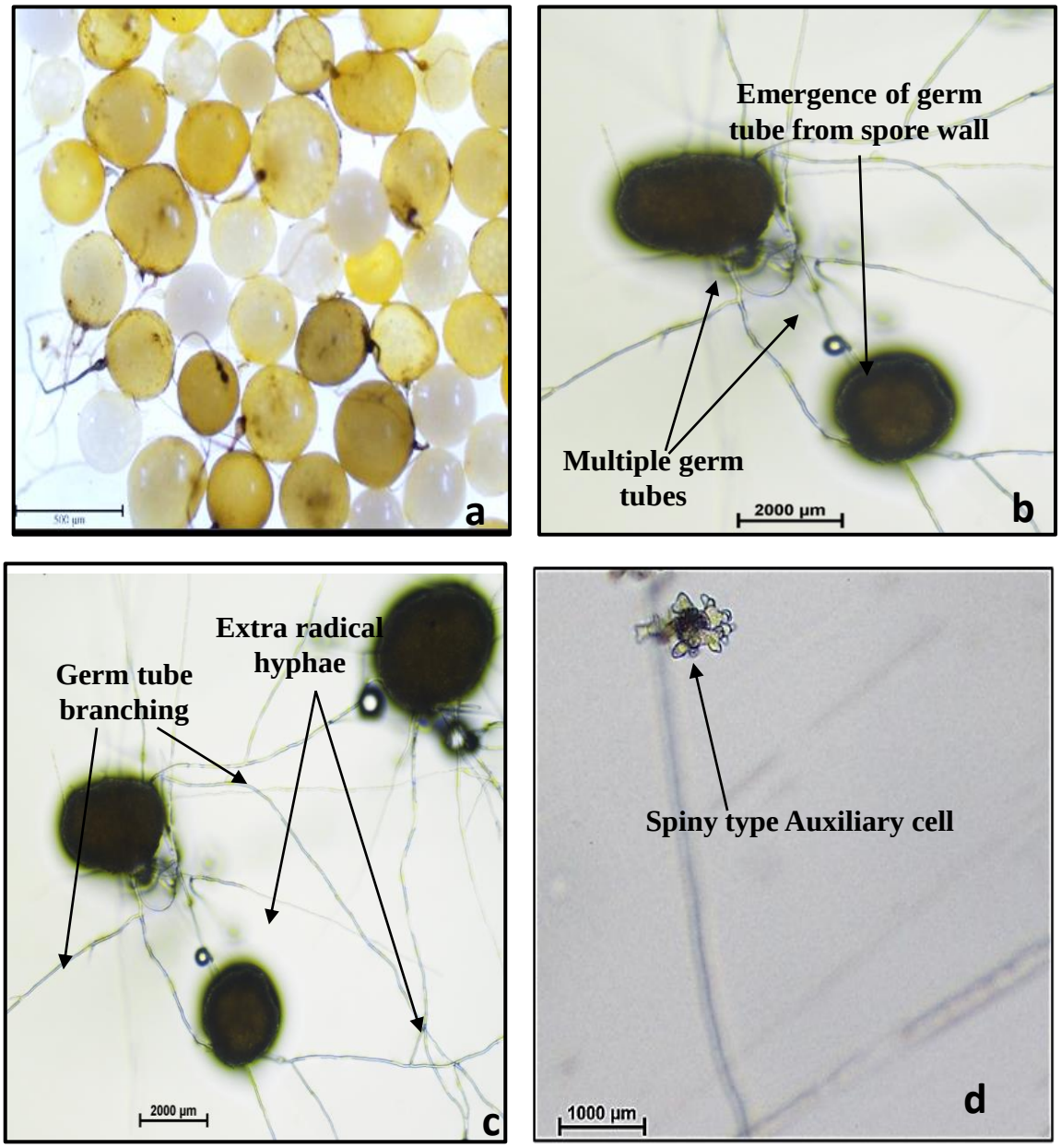


Plate 12: *In vitro* spore germination of *Gi decipiens* on WM (-sucrose) media : a. Isolated spores of *Gi. decipiens* **b.** Germination from spore wall; **c.** Extra radical hyphae and Hyphal branching; **d**. Spiny auxiliary cell.

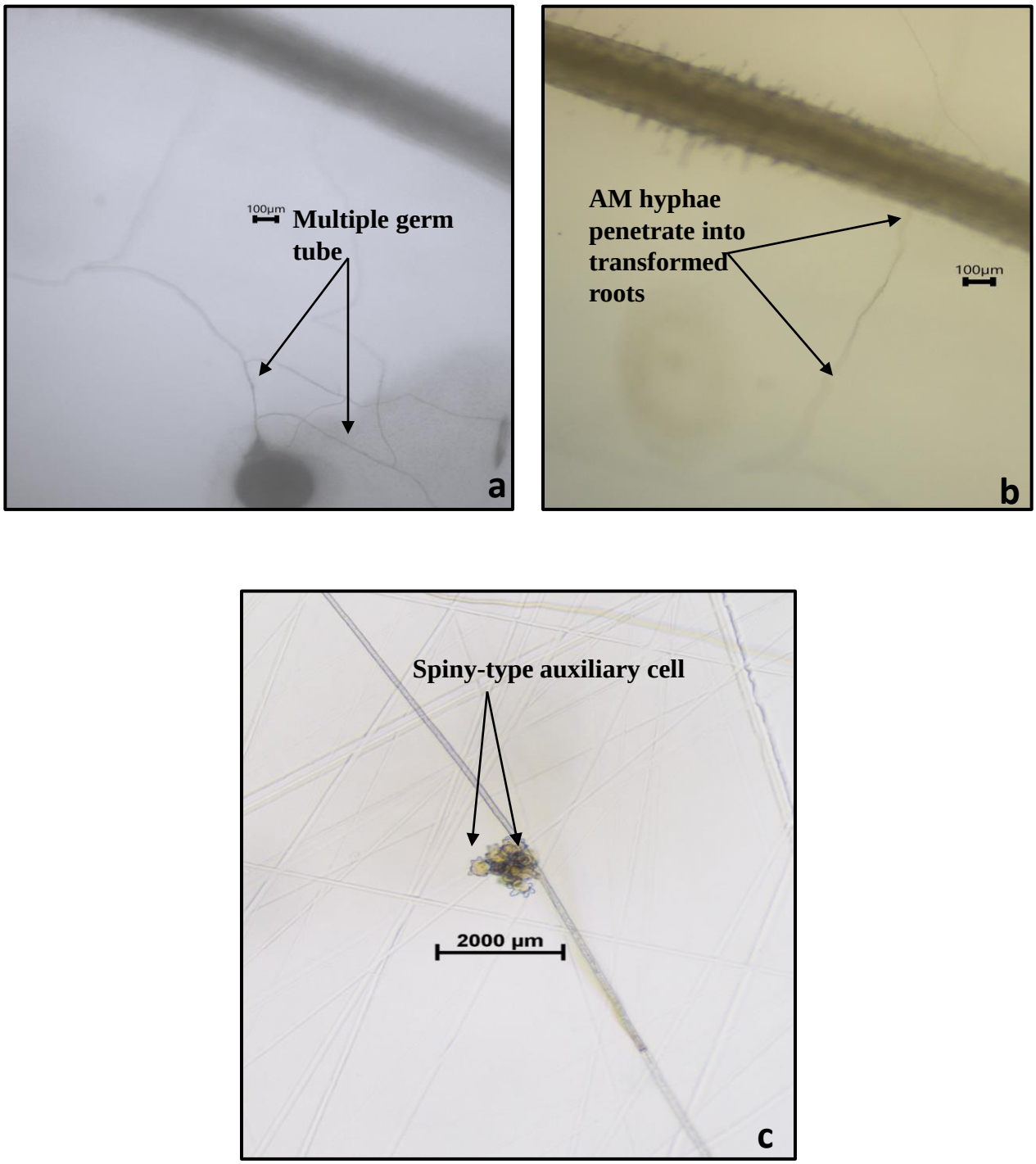


Plate 13 : Monoxenic culture of *Gi. decipiens* with Ri-TDNA transformed *D. carota* roots on WM media : a: Multiple germ tube; **b:** AM hyphae penetrate transformed roots; **c.** Spiny type auxiliary cell.

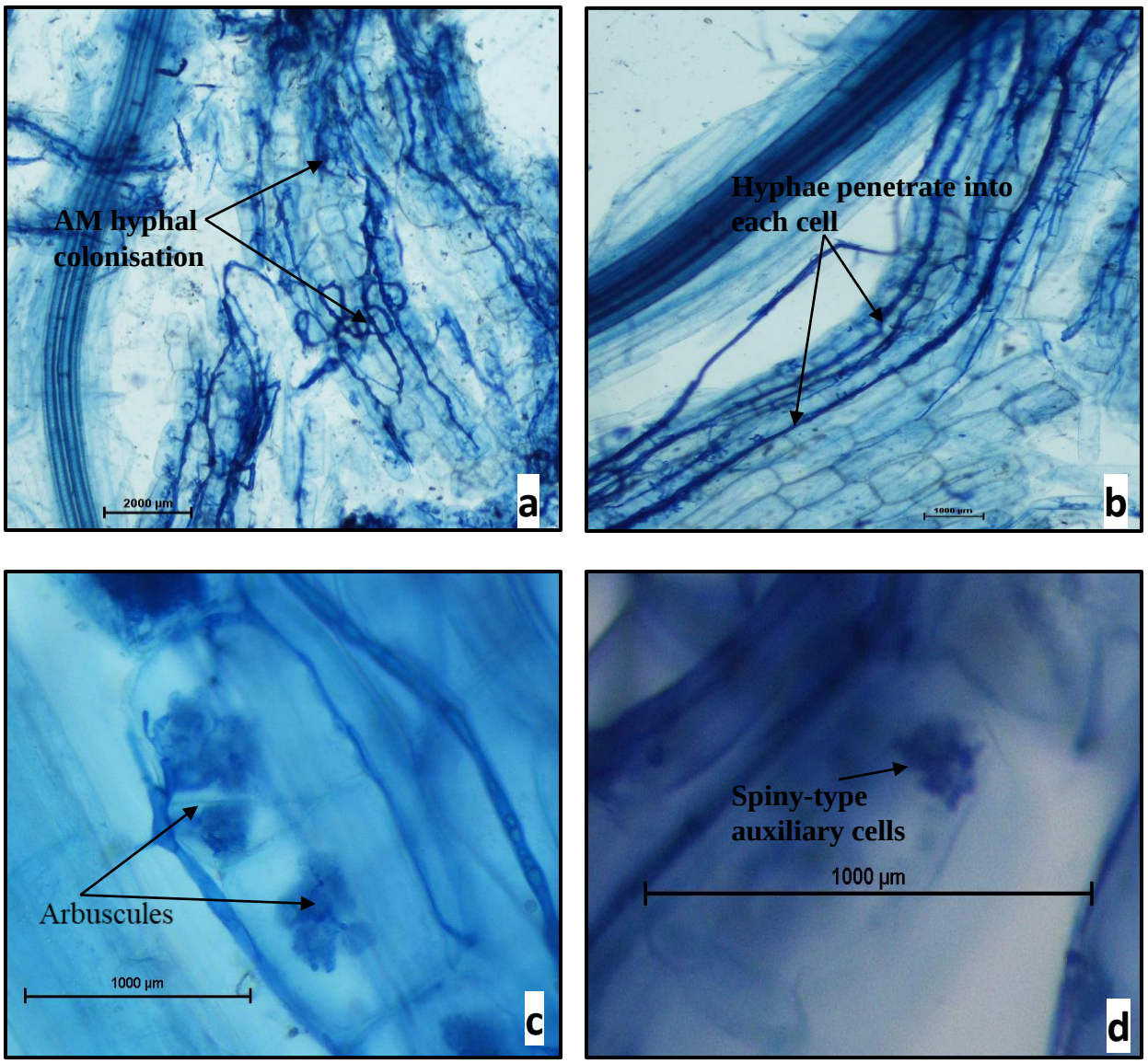


Plate 14: *In vitro* root colonisation of AM fungi in transformed roots of *D. carota* **a.** AM hyphal colonisation by *Gi. decipiens*; **b.** Hyphae penetrate cells of transformed roots ; **c.** Arbuscules; **d.** Spiny type auxiliary cells.

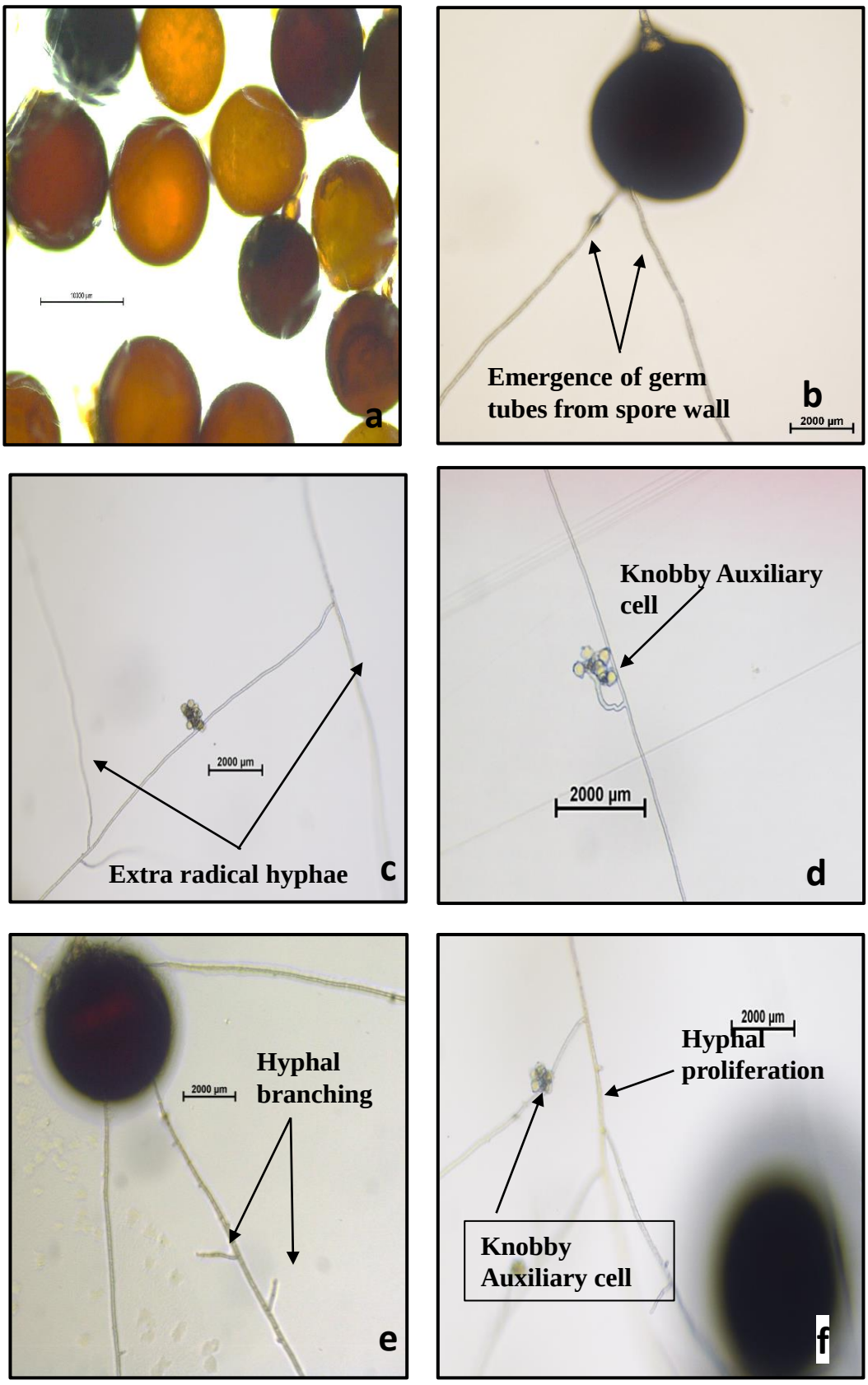


Plate 15: *In vitro* spore germination of *R. gregaria* on WM (-sucrose) media : a. Isolated spores of *R. gregaria*; **b.** Germination on WM (-sucrose) media; **c.** Extra-radical hyphae; d. Knobby auxiliary cell; e. Hyphal branching; **f**. Hyphal proliferation and Knobby auxiliary cell.

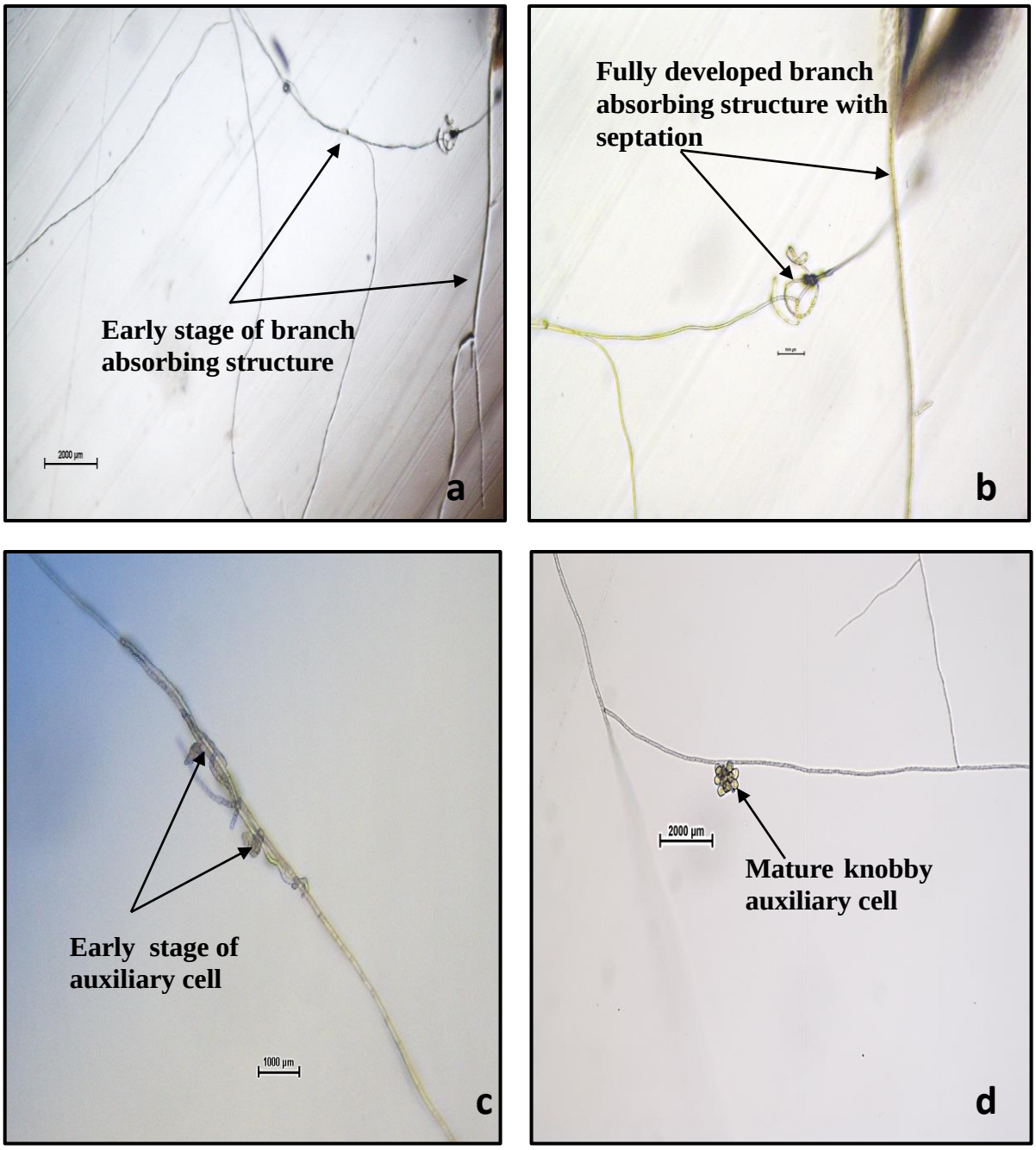


Plate 16: Monoxenic culture of *R. gregaria* with Ri T-DNA transformed *D. carota* roots on WM media : a: Early stage of branch absorbing structure; **b:** Fully developed branch absorbing structure with septation; **c:** Early stage of auxiliary cell formation; **d:** Mature knobby auxiliary cell.

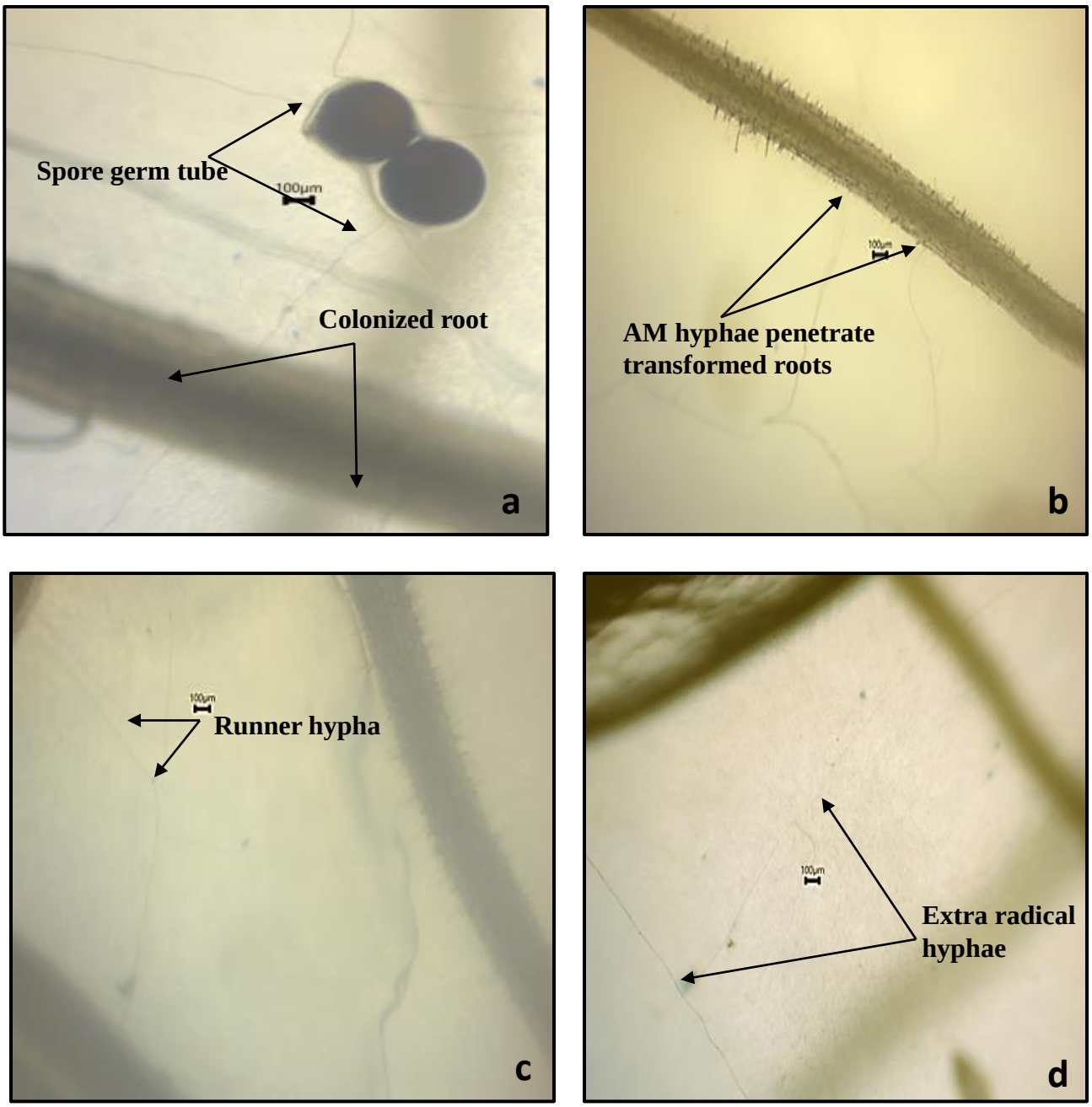


Plate 17: Monoxenic culture of *R. gregaria* with Ri T-DNA transformed *D. carota* roots on WM media : a: Multiple germ tubes colonized the transformed root; **b:** AM hyphae penetrate transformed roots; **c.** Runner hyphae; **d.** Extra-radical hyphae.

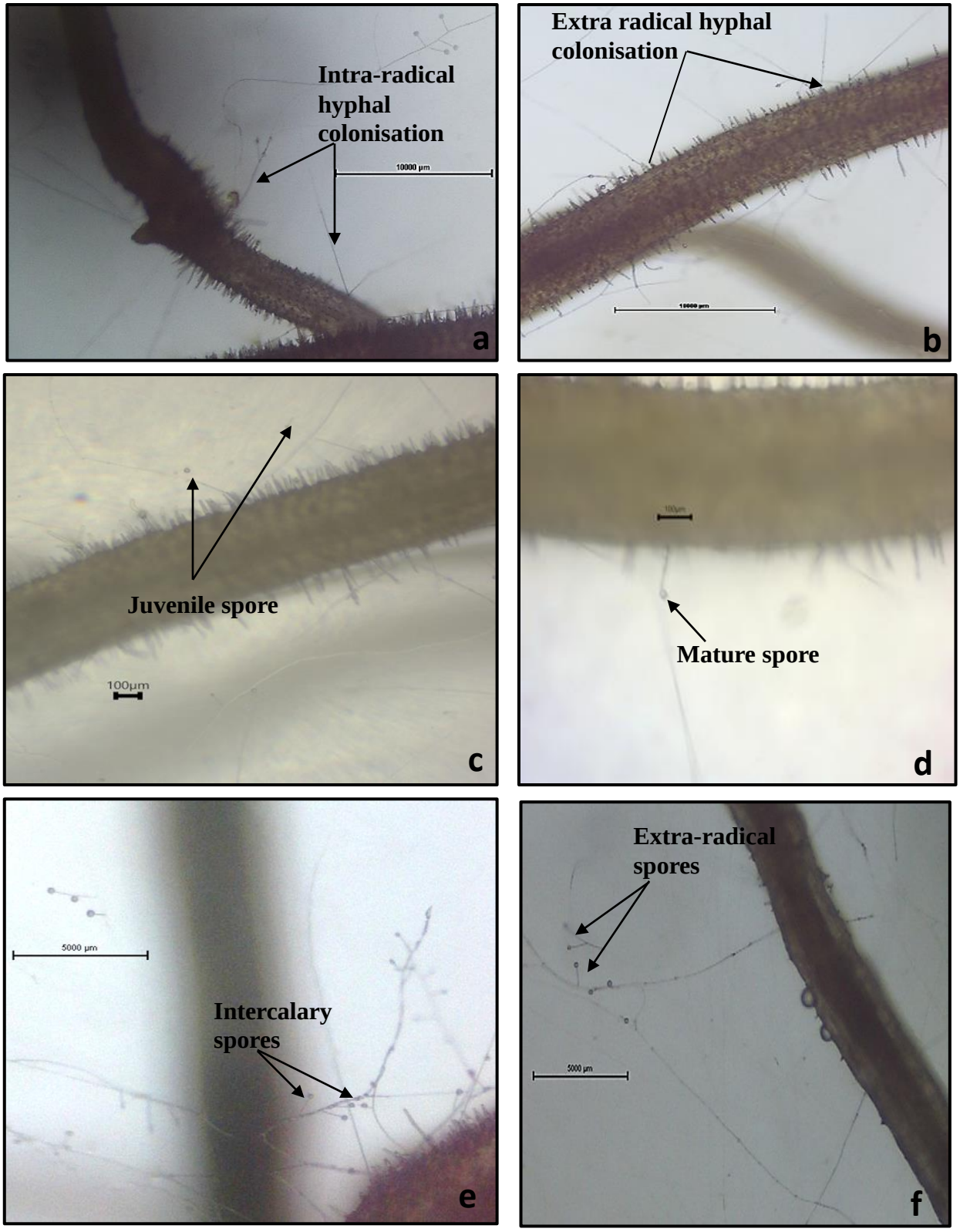


Plate 18: Monoxenic culture of *R. gregaria* with Ri T-DNA transformed *D. carota* roots on WM media: a. Intra-radical hyphal colonisation**; b.** Extra-radical hyphal colonisation**; c.** Juvenile spore**; d.** Mature spore**; e.** Intercalary spores**; f.** Extra-radical spores.

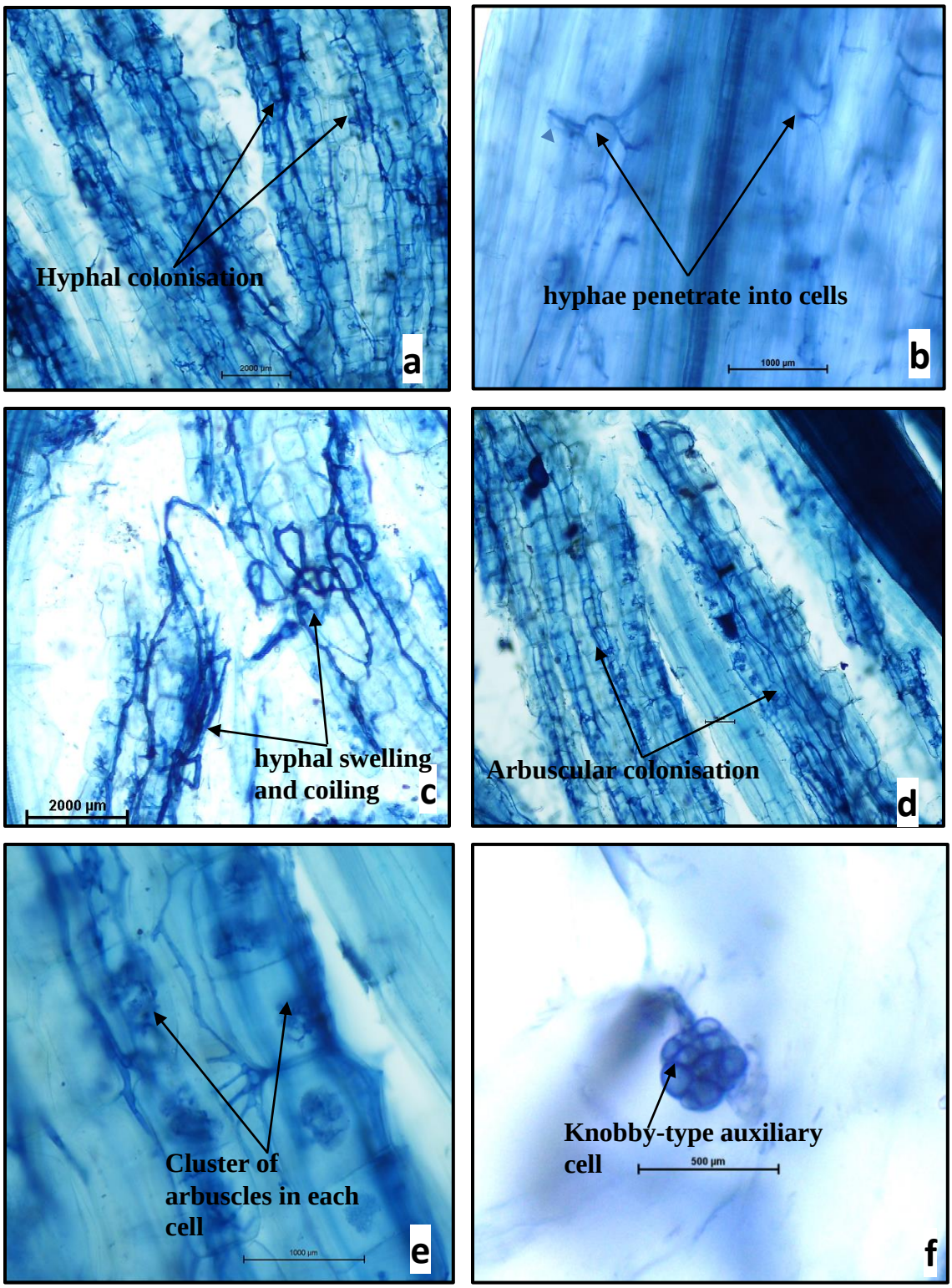
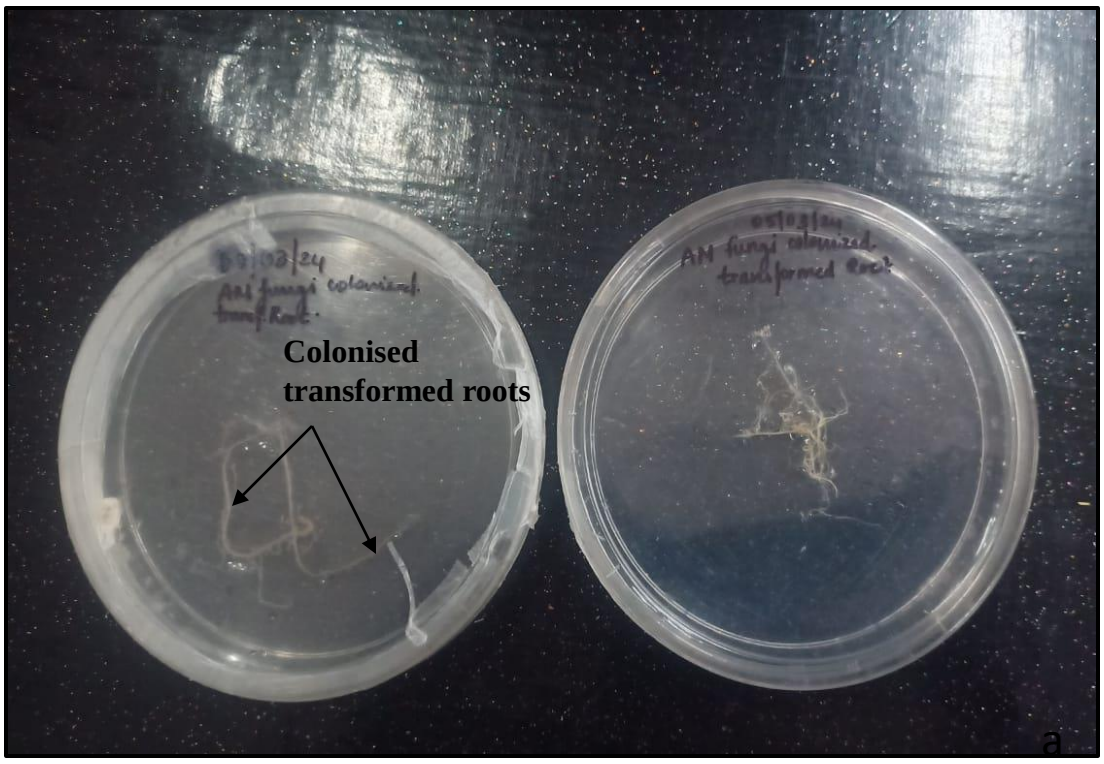


Plate 19: *In vitro* root colonisation of AM fungi in transformed roots of *D. carota*: a. Hyphal colonisation by *R. gregaria*; **b.** Hyphae penetrate cells of transformed roots; **c:** hyphal coiling and swelling; **d.** Arbuscular colonisation; **e.** Cluster of arbuscles in each cell; **f.** knobby type auxiliary cell.



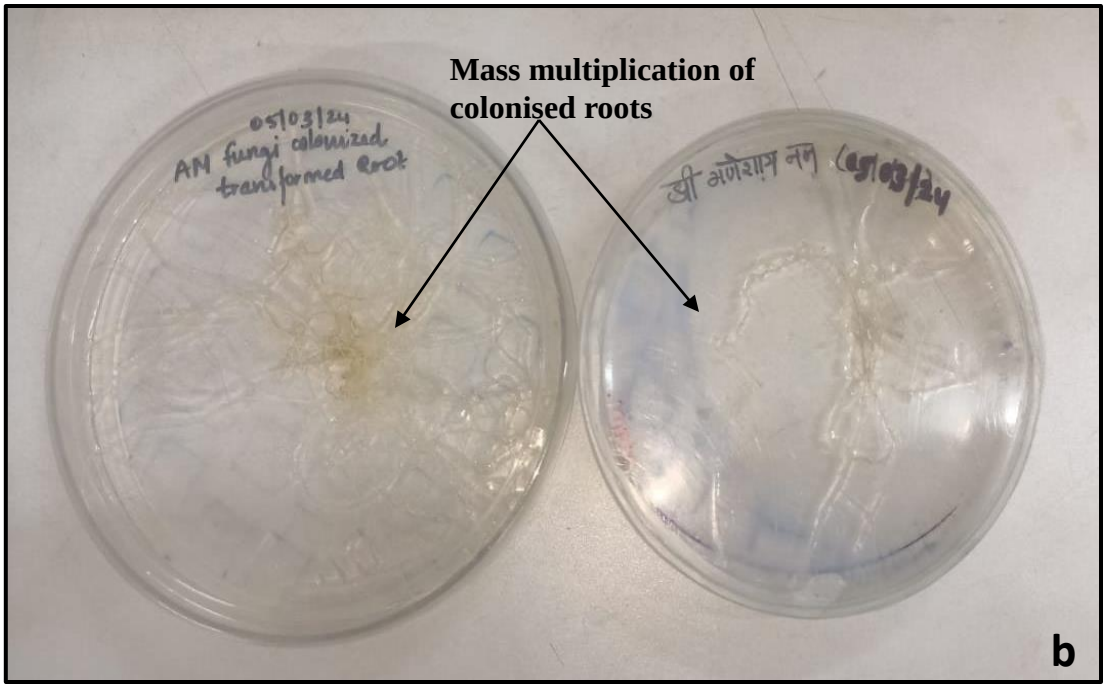


Plate 20: Establishment of continuous culture of AM fungi; a-b. Mass multiplication of colonised roots on WM media.