Cultivation of Arbuscular Mycorrhizal (AM) Fungal Spores *in vitro* using Root Organ Culture Technique

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I hereby declare that the data presented in this Dissertation report entitled, "Cultivation of Arbuscular Mycorrhizal (AM) Fungal Spores *in vitro* using Root Organ Culture Techniques" 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 Dr. 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 / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "Cultivation of Arbuscular Mycorrhizal (AM) Fungal Spores *in vitro* using Root Organ Culture Techniques" is a bonafide work carried out by Ms. Nifa Colaco under my supervision in partial fulfilment of the requirements for the award of the degree of (Masters of Science) in the Discipline (Botany) at the (School of Biological Sciences and Biotechnology), Goa University.

Prof. Bernard F. Rodrigues

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PREFACE

AM fungi form a symbiotic relationship with higher plant roots. They are considered to be essential for plant growth and nutrient cycling. These fungi help to establish plant communities, maintain coastal dune vegetation and stability and support revegetation. Hence the following work was chosen through my curiosity on AM fungi.

The aim of this study is to explore the morphological characteristics and colonization of AM fungi *in vitro* using transformed roots and to mass multiply it, which could be further used to obtain bio-fertilizers. With extensive experimentations, standardization and analysis, this study shows the successful colonization of AM fungi *in vitro*.

Hoping this work will serve as a valuable means to the mycorrhizal field of research.

NIFA COLACO

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Ms. Nifa Colaco

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Entity	**Abbreviation**
Arbuscular Mycorrhiza	AM
Root inducing transfer-deoxyribonucleic acid	Ri T-DNA
Root organ cultures	ROC
Sodium hypochlorite	NaClO
Modified Strullu-Romand	MSR
Modified White's medium	MW medium
Polyvinyl-Lacto-Glycerol	PVLG
Dibutylphthalate Polystyrene Xylene	DPX

Arbuscular Mycorrhizal (AM) fungal symbiosis with higher plants provides a wide scope for its use as biofertilizer. AM fungi are essential to soil microorganisms involved in plant community establishment. In coastal dunes they play a vital role in nutrient cycling and aggregation of sand particles. Hence there is a need to conserve and multiply these fungi. Mass multiplication of AM fungi *in vitro* has been a challenge. There are studies carried out on *in vitro* cultivation of AM fungi but not all show successful spore production. The present study exhibits an attempt towards *in vitro* culturing and sporulation of AM fungal species, *Gigaspora albida.* It dealt with the morphological identification, spore germination and colonization in Ri T-DNA roots of *Daucus carota* L. (carrot).

CHAPTER 01: INTRODUCTION

A large number of diverse microorganisms interact in different ways with each other in the ecosystem (Moënne-Loccoz *et al*., 2015). Soil microbes are of great significance, as they help in nutrient cycling and assisting the plant community establishment (Schulz *et al*., 2013).

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that form symbiotic associations with plant roots (Smith and Read, 2008). They are formed by bryophytes, pteridophytes, gymnosperms and angiosperms. These fungi belong to the phylum Glomeromycota (Schüßler *et al.,* 2001). The ancient forms of the symbiosis of this phylum show that almost 90% of extant plant species are mycorrhizal, and the interaction of a plant and a microbe plays a major role in the functioning of the plant and its community establishment (Moënne-Loccoz *et al*., 2015).

In soil, the fungus initially enters the cell wall of the host plant roots, then penetrates the cortical cells where it can form intra-radical structures (hyphae, arbuscules, and vesicles) and extra-radical structures (hyphae and spores). This association is a kind of mutualistic type, as it is bidirectional. The fungus helps the plant to acquire nutrients from the soil while the plant provides carbon sources to the fungi (Brundrett, 2009).

AM fungal diversity and dominance

AM fungal symbiosis is considered to be a tripartite relationship between the plant, fungus and soil (Kemaghan, 2005). In terrestrial ecosystems, AM fungi occur as mixed species, with certain species being dominant. Most of the AM species are found widespread and hence considered to be generalists (Öpik *et al*., 2006), while some species are restricted to only certain ecosystem types and are considered to be specialists (Oehl *et al*., 2007). AM species diversity is more distinct in undisturbed ecosystems than disturbed ecosystems as in undisturbed surroundings there is greater variability in terms of critical determinants. The diversity of AM

fungi can be affected by various factors like climatic and seasonal variations, edaphic conditions, host genotype, habitat type and vegetation cover, and habitat disturbance (Bhatia *et al*., 2013). The productivity of the plant is enhanced when there is a presence of AM symbiosis (Moora *et al.,* 2004). When the AM fungal diversity increases, there is a chance of higher plant productivity due to increased species richness (Finlay, 2008). Assessment of such AM fungal diversity is important if the benefits of such symbiosis are to be exploited. Knowledge of such species diversity in a functioning ecosystem is important to develop inoculum that can be provided for agricultural and horticultural crops to revegetate degraded ecosystems.

Morphology of AM fungi

The AM fungal spores have unique morphological and biochemical attributes. The spore wall formation occurs through a sporogenous hypha. It either forms at the tip of the sporogenous hypha, from inside the sporogenous hypha or from the side of the sporogenous hypha. Changes in the spore wall occur as the spore size increases i.e. it grows, thickens and differentiates. The spore wall layers can be permanent or impermanent. Inner walls are colourless, permanent structures present in AM fungi of some genera. The number of inner walls can range from 1-3, with the innermost wall being frequently called as a germinal wall (Blaszkowski, 2012). Spore germination can occur upon full differentiation of the innermost wall (Morton, 2002). The germ tubes arise from the pre-germination structures associated with the innermost wall. The pre-germination structures are called germination orb- it is formed by a centrifugally rolled hypha which is hyaline in colour. It is an impermanent structure that decomposes with time (seen in *Acaulospora* spp.), germination shield- these are formed by a coiled hypha and are generally elliptical, irregular plate-like, more or less flexible and they may be divided into 1-30 compartments which contain germ tube initial (seen in *Scutellospora, Racocetra, Pacispora*

spp.) or germination structure or germ layer- it is a semi-flexible layer from which the germ tube emerges (seen in *Ambispora appendicula*) (Blaszkowski, 2012).

Spores are formed in a highly ordered or loose arrangement around a hyphal plexus called the sporocarp, and the sporocarps may be surrounded by a loose or compact interwoven hyphal network called peridium. The spore arises from a point of attachment called the subtending hypha, which can be simple, recurved, constricted or swollen. The shape and the width of the hypha can vary within different genera and AM species (Gerdemann and Trappe, 1974).

There are three growth phases in the AM fungal life cycle: asymbiotic, pre-symbiotic and symbiotic. In the asymbiotic phase multiple, successive rounds of spore germination and retraction of nuclei and cytoplasm occur in the AM fungal development (Parniske, 2008). Spores germinate and germ tube growth occurs for about 2-3 weeks without any association with roots or root exudates under appropriate water and temperature conditions (Garg and Chandel, 2010). During the presymbiotic phase, the exploratory hyphal growth changes dramatically in the presence of signals derived from plant root exudates (strigolactones), inducing profuse hyphal branching, increased physiological activity and continued hyphal growth (Parniske, 2008). In the symbiotic phase, on penetrating the root surface, AM fungi have the ability to induce calcium oscillations in root epidermal cells and activate plant AM symbiosis-related genes (Kosuta *et al*., 2008). A synchronized sub-cellular development of the host plant cell and the AM fungus results in the formation of arbuscules. Repeated branching of the fungal hyphae forms the tree-shaped structure of arbuscules (Parniske, 2008). There are two morphologically distinct types of AM fungi, characterized by intraradical hyphal modifications within the root. The *Paris*-type, where the hyphal development is extensively intra-cellular in the form of hyphal coils formed within host root cortical cells, and the *Arum*-type, where the intra-radical hyphae spread intercellularly between the root cortical cells,

penetrating cells only to form tree-like structures (Smith and Smith, 1997). The *Paris*-type AM colonization is a characteristic of plants growing in low-nutrient and high-stress environments, while the *Arum*-type colonization is associated with fast growing plant species (Brundrett and Kendrick, 1990). However, the presence of both the types of colonization has also been noted in some plants (Kubota *et al.,* 2005).

The fungi produce vesicles that are terminal, globose to sub-globose structures which function as lipid storage bodies and fungal propagules/intra-radical spores (Dodd *et al*., 2000). They are formed by intercalary or terminal swellings of AM fungal hyphae in the inter- or intra-cellular areas of the root cortex (Javaid, 2009). The hyphae of the fungi also develop auxiliary cells which are clusters of thin-walled cells formed on the extra-radical hyphae. They allow partitioning of nutrients and nuclei prior to spore formation (Boddington and Dodd, 1999). Then the hyphae produce spores that are multinucleate single cells produced as terminal swellings on the tip of sporogenous hyphae continuous with extra-radical hyphae (Koske, 1985). Sometimes, they also arise inside sporogenous hyphae (intercalary spores), inside roots and rhizosphere soil (Sieverding, 1987).

The AM fungal mycelium is multinucleate and coenocytic (Dodd *et al*., 2000). The intra-radical mycelium undergoes constant development and re-organization within the root cells by forming arbuscules and vesicles (Dickson and Smith, 2001). The extra-radical mycelium spreads through the soil creating a network that subsequently associates with different plants (Puschel *et al*., 2007). The extra-radical mycelium connects the root systems of same or different plant species (Jasper *et al*., 1991).

Significance of AM fungi

It is assumed that soil microorganisms play a major role in soil structure stabilization (Foster 1994), and AM fungi comprise the major portion of the soil microbiome (Hayman, 1978). AM fungi along with their extra-radical hyphae and plant root hairs increase the soil -root contact area (Geelhoed *et al.,* 1997b). Thus, they provide the host plants with nutrient uptake such as mainly phosphorus (P) (Bucher, 2007), then calcium (Ca) (Azcón and Barea, 1992), iron (Fe) (Treeby, 1992), manganese (Mn) (Kothari *et al.,* 1991), zinc (Zn) (Cavagnaro, 2008) and nitrogen (N) (Näsholm *et al.,* 2009). AM fungi also increase plant nutrient acquisition in less fertile soils (Brundrett, 2009). It is also assumed that they are able to substitute fertilizer use (Galvez *et al.,* 2001), thereby providing sustainable agriculture.

Apart from providing nutritional benefits to plants, AM fungi also have many other capabilities like, 1. They provide enhanced plant tolerance to biotic stress (pathogens and herbivores) and abiotic stress (salinity, drought and metal toxicity) (Bennet and Bever, 2007); 2. Improvement in nutrient cycling, energy flow, and plant establishment in disturbed ecosystems (Tiwari and Sati, 2008); 3. AM fungi with their extensive mycelial network, interconnect a number of unrelated individual plant species, thus causing an impact on the function and biodiversity of the entire ecosystem (Bonfante and Genre, 2010); 4. This fungal hyphae also produces a proteinaceous, hydrophobic, sticky substance into the soil known as 'glomalin', which helps to improve soil stability, binding, and water retention, thereby helping to reduce soil erosion (Bedini *et al*., 2009); 5. AM fungi also reduce metal toxicity to plants (Fan and Liu, 2011). These fungi thus help to restore land and vegetation; and 6. To humans, they provide antioxidants or nutrient components through agricultural produce. This symbiosis also stimulates the synthesis of secondary metabolites that are beneficial to human health through their antioxidant activity (Seeram, 2008).

In the dunes, AM fungal association helps sustain dune vegetation. The fungi also help in sand binding through their extensive colonization with the dune vegetation (Puppi *et al*., 1986). The hyphae of this fungus bind with the sand particles and form sand aggregates that are able to resist strong winds and storms, thus remaining intact despite the death of the roots and hyphae (Koske and Polson, 1984). Sand dunes encourage a highly diversified community of AM fungi mainly because of their low soil mineral content such as Phosphorus (P) (Koske, 1988). The extra-radical hyphae of the AM fungi, which are thinner and more extensive than the root hairs (Novero *et al*., 2008), are able to provide the host plant with P in soils with reduced P content as compared to the plant roots (Koske and Polson, 1984). The sand dune vegetation that holds the presence of AM fungal association is said to have an increase in the longevity of the feeder roots and improvement in soil texture through the increased aggregation of the soil particles (Nasim, 2005).

Potential of AM fungi in *in vitro* culture techniques

Examining AM species diversity is crucial as they play a role of positive fungi in terrestrial ecosystems. In order to use AM fungi as biofertilizers, researchers have found a way i.e. development of monoxenic cultures of AM fungi *in vitro* using transformed roots as a symbiotic partner (Fortin *et al.,* 2002). The AM fungal inoculum production via *in vitro* techniques is preferred as compared to the traditional pot culture methods. *In vitro* production provides many viable, contamination free, pure spores in a single Petri plate with nutrient medium. The sterile conditions and the regular monitoring of the cultures make this technique more suitable and desirable for producing a quality AM fungal inoculum. This also helps to study the morphological, physiological and taxonomic characters of AM fungi (Kokkoris and Hart,

2019). Some of the AM fungi genera brought into successful monoxenic cultivation include *Glomus, Gigaspora, Scutellospora,* and *Acualospora* (de Souza and Declerck, 2003).

Thus, pure AM fungal spores can be obtained through the *in vitro* technique which could be further used in research and industry. Keeping these benefits in mind the following objectives have been devised.

Objectives:

1. To identify and isolate spores of AM fungi.
2. To maintain trap cultures of the selected AM fungal species.
3. To prepare Root Organ Cultures (ROC) of the selected AM fungal species.
4. To study colonization of the selected AM fungal spores in ROC.

CHAPTER 02: REVIEW OF LITERATURE

AM fungi are obligate biotrophs that form symbiotic associations with plant roots to grow and complete their life cycle (Parniske, 2008). They are seen in almost all ecosystems (Brundrett, 2009). Frank (1885) was the first to identify an association between plant roots and mycorrhizal fungi (Frank and Trappe, 2005). The term mycorrhiza is coined from the Greek words' mycos' meaning fungus and 'rhiza' meaning roots (Wang and Qiu, 2006). The initial observations of AM fungal symbiosis were done by Trappe and Berch (1985) and Rayner (1926).

The name for this symbiosis has changed through years. At times the symbiosis was called 'phytomycetous endo-mycorrhiza', this was named so, to distinguish the endo-mycorrhiza formed between the members of Orchidaceae or Ericaceae and higher fungi. However, the name 'phycomycete' does not carry any significance (Koide and Mosse, 2004). The difference between ecto- and endo-mycorrhiza was found by Frank (1887). When specialized structures such as arbuscles and vesicles were observed within the roots of the symbiosis, it was named as 'vesicular-arbuscular mycorrhiza' (Gallaud, 1905). The currently accepted term for this symbiosis i.e.' arbuscular mycorrhiza' was coined when it was found that not all species of this fungus form vesicles (Koide and Mosse, 2004).

Diversity and distribution of AM fungi

AM fungi are reported in the roots of most angiosperms and pteridophytes, some gymnosperms and gametophytes of some lower plants such as mosses and lycopods (Sith and Read, 1997). In seed plants, the earliest symbiosis evidence was seen in silicified roots of Triassic cycad *Antarcticycas schopfii* (Phipps and Taylor, 1996). The first fossil evidence of the same was observed in stems of an early vascular land plant *Aglaophyton major* dating 400 million years ago from the Rhynie chert (Remy *et al.,* 1994). In *Aglaophyton* were also found *Scutellospora-*

and *Acualospora*- like spores (Dotzler *et al.,* 2009). Evidence of a 450-million-year-old fossils of mycorrhizal fungal-like structures in early land plants from the Rhynie chert in Scotland show that this symbiosis is ancestral and that it has evolved from water to land (Selosse and Le Tacon, 1998).

The single phylogenetic origin of AM fungi and its evolution from 353-462 million years ago was proved through a molecular clock analysis of ribosomal DNA sequence data from the present day Glomales (Simon *et al*., 1993). All the genes required for the symbiosis of AMF with plant roots were isolated from all major lineages of plants and it was found that these genes were present in the common ancestors of land plants and their functions got conserved during the invasion of land by plants (Wang *et al.,* 2010). There is another, where in an ancient form called *Geosiphon pyriformis* that forms a different type of AM fungal symbiosis as it produces specialized bladders that harbor symbiotic cyanobacteria called *Nostoc punctiforme* (Schüßler *et al.,*1996). Molecular phylogenetic analysis has shown that *Geosiphon* is a representative of the Glomeromycota (Schüßler *et al*., 2001).

Phylogenetic relationships

AM fungi form a monophyletic group in the kingdom Glomeromycota (Schüßler *et al*., 2001). Around 288 species that are taxonomically described are added in this group (Öpik and Davison, 2016). The taxonomic concepts have been reassessed which has revealed the polyphyly of some genera by using nuclear encoded rDNA (recombinant DNA) phylogenies (Redecker and Raab, 2006). From the rDNA phylogenies it has been revealed that the genus *Glomus* is several times polyphyletic (Schwarzott *et al*., 2001). The *Glomus* species is found in six different lineages within Glomeromycota. The rDNA phylogenies have revealed that the genus *Paraglomus* is the most primitive in the Glomeromycota lineage. Through the rDNA

phylogenies the clades, *Pacispora* and *Diversispora* have been removed out (http://tolweb.org/Glomeromycota). According to the rDNA phylogenies, the species, *Glomus mosseae* and *Glomus claroideum* are from the same monophyletic group though they are distantly related (Schwarzott *et al*., 2001). The formation of 'sporiferous saccule' in *Acualosporaceae* (*Acualospora* and *Entrophospora*) is now considered to be present in *Archaeospora.* Molecular data has shown that the presence of a 'bulbous suspensor' in *Gigasporaceae (Scutellospora* and *Gigaspora)* is a distinguishing character (http://tolweb.org/Glomeromycota). Gigasporaceae and Acaulosporaceae representatives form a clade in most rDNA phylogenies, which is in conflict with previous investigations based on cladistic analysis of morphological features that placed Glomeraceae and Acaulosporaceae together (Morton and Benny, 1990).

The rDNA phylogenies state that Glomeromycota is a monophyletic group (James *et al*., 2006). These were initially placed in the Zygomycota group however, their symbiotic nature, absence of zygospore and rDNA phylogenies indicate that they form a monophyletic group that is different from the Zygomycota lineage (http://tolweb.org/Glomeromycota). Based on this data the phylum Glomeromycota was developed by Schüßler *et al.* (2001). These authors also corrected the name from 'Glomales' to 'Glomerales'.

Classification of AM fungi

In order to classify AM fungi, the spores from the soil are isolated. Routine soil extractions are followed by wet sieving and decanting method given by Gerdemann and Nicolson, 1963. Earlier many attempts have been made to classify AM fungi. The system of classification with Latin names was decided upon by Nicolson and Gerdmann. A more descriptive classification was given by Mosse and Bowen wherein they classified based on the spore wall structure,

colour and cytoplasmic characteristics (Mosse and Bowen, 1968). Nicolson and Gerdemann (1968) divided the fungi into two groups, *Endogone* one forming extra-radical azygospores/zygospores arising from the tip of a swollen hyphal suspensor but producing no intra-radical vesicles, and the other forming extra-radical chlamydospores and intra-radical vesicles. In order to establish the relationship of AM fungi with other fungi, molecular studies were done by Schüßler *et al.* (2001). The group of AM fungi was elevated to the level of phylum Glomeromycota, which was shown to be distinct from other fungal groups.

Initially identification of strategies by taxonomists were based on anatomy and morphology characters. Later on, methods based on serology (Aldwell and Hall, 1987), isozyme variation through gel electrophoresis (Hepper, 1987) and fatty acid variation (Bentivenga and Morton, 1994) were introduced. Currently DNA-based methods are considered to be more reliable for genealogical relationships among organisms (Koide and Mosse, 2004). For AM fungal identification, the target regions of DNA are on ribosomal genes which show variations that are sufficient to distinguish between AM fungal species or isolates (Krüger *et al*., 2012).

All this has led to the modem era of molecular identification of AM fungal species (Redecker *et al.,* 2013). Next Generation Sequencing (NGS) tools represent a further step forward for biodiversity surveys of all organisms (Shokralla *et al*., 2012), including AM fungi. Over the last few years, the number of NGS based AM fungal biodiversity studies has increased, while the spectrum of the target environments has broadened (Öpik *et al.,* 2013). Furthermore, new sets of primer pair for the specific amplification of AM fungal DNA sequences, capable of providing higher accuracy and a broad coverage of the whole phylum Glomeromycota have been developed (Krüger *et al*., 2009). Nowadays, AM fungal assemblages are no longer studied only in plant roots, but also in the bulk rhizosphere soil (*Davison et al*., 2012). The main result obtained from the application of NGS to the study of AM biodiversity has been the discovery

of an unpredictable diversity within the phylum Glomeromycota (Öpik *et al*., 2013). However, this series of novel molecular tools has introduced a new issue i.e. the continuously increasing number of unidentified AM fungal DNA sequences from environmental samples with no correspondence whatsoever to sequences of known species (Öpik *et al*., 2010). This has naturally made scientists aware of the fact that the number of AM fungal species could be larger than expected. However, it is not reliable to have new species described on just the basis of short DNA sequences obtained by means of NGS tools. Instead, for each new suggested taxon, a series of steps needs to be followed to characterize the morphotype, the functional traits, and the ecological role offered when present in combination with other organisms in a given environment. Therefore, NGS tools cannot be considered as complete replacements of the traditional methods of identification and description of new species (Berruti *et al*., 2014). Routine identification of arbuscular mycorrhizal fungi will probably continue to be based primarily on morphological characters and thus an increased acceptance of the combined approach between anatomy and DNA will be important. The ability to properly name the fungi, avoid duplication of names and relate the species to one another also depends heavily on international culture collection centre's such as the International Culture Collection of Arbuscular and Vesicular-arbuscular Mycorrhizal Fungi (INVAM), and the International Bank for the Glomeromycota (BEG/IBG) (Koide and Mosse, 2004).

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

AM fungi in coastal sand dunes

Sand dunes serve as natural buffers, protecting the landward side from storm tides, waves and wind action. Stabilization of large, mobile dunes by the vegetation cover has been recognized as an effective means to decelerate the inland movement of sand (Woodhouse, 1982). The dune vegetation traps and holds windblown sand grains on the fore dunes. It contains many native plant species and is valued as a habitat of its own natural biodiversity. Loss of dune vegetation can trigger dune erosion wherein the exposed, dry sand particles are flown by high-velocity winds resulting in shifting of large volumes of sand, sometimes resulting in formation of large depressions in the dunes. (http://www.ozcoasts.gov.au/indicators/beach_dune.jsp).

AM fungi are widespread in coastal sand dune systems (Stürmer and Bellei, 1994). Coastal sand dunes favour the occurrence of AM fungi mainly because of low P content (Ranwell, 1972). Mycorrhizal diversity in sand dunes results in an increase in longevity of feeder roots and improvement in soil texture through increased aggregation of soil particles (Nasim, 2005). The AM fungal colonization of plant roots greatly increases the uptake of P, Nitrogen (N), Calcium (C), Potassium (K) and Zinc (Z) (Gupta *et al*., 2000). AM fungi provide plants with P that enables AM plants to grow better than non-mycorrhizal plants when P is limiting. An increase in yield or biomass of AM plants is often observed as compared to non-mycorrhizal plants (Mosse, 1972). Dune vegetation benefits greatly by AM fungal association through improved establishment, greater biomass accumulation, faster colonization of bare areas, improved water relations, large increase in relative growth rate, leaf area, total biomass and increased seed output (Corkidi and Rincon, 1997b). Increased nutrient supply, salinity tolerance, reduced abiotic stresses and formation of wind-resistant soil aggregates are also some of the major benefits derived by the dune vegetation through AM fungal association (Gemma and Koske, 1989).

Jehne and Thompson (1981) reported considerable number of hyphal connections of fungal mycelium in the top 20 cm of mobile sand in Cooloola (Queensland), Australia. These fungi bind loose sand grains into larger aggregates through secretion of hydrophobic 'sticky' glycoproteinaceous substance known as 'glomalin' which results in improved soil stability, binding, and water retention and hence limits sand dune loss or erosion (Bedini *et al.,* 2009). Forster and Nicolson (1981) reported that 1.5 % of the aggregate sand grains reach a diameter of 2 mm in Scotland dunes.

About 65 AM fungal species have been reported from sand dune habitats around the globe, representing 28 % of the total AM species (Stürmer *et al*., 2010), including 32 new species (Blaszkowski and Czemiawska, 2011). Studies on AM fungal associations in sand dune plants in Australia, USA, India, and Europe indicate that dominant dune plants and pioneer grasses are normally associated with AM fungi. The most common AM fungal genera in coastal sand dune systems worldwide are *Acaulospora, Gigaspora, Glomus* and *Scutellospora* (Maun, 2009).

Cultivation of AM fungi *in vitro*

The conventional method used to study the life cycle of AM fungi in situ is to associate them with root organ culture (ROC) (Fortin *et al.,* 2002). This technique has greatly influenced our understanding on various aspects of AM symbiosis by allowing non-destructive observations throughout the fungal life cycle. Although the host plant is replaced by Ri T-DNA transformed roots, the fungus is able to colonize and sporulate. The development of spores, morphologically and structurally similar to those produced in pot cultures, and the ability of the *in vitro* produced propagules to retain their viability to colonize and initiate new mycorrhizal symbiosis indicates

that the fungus is able to complete its life cycle. ROC technique has proved to be successful for cultivation and mass inoculum production of AM fungi (Rodrigues and Rodrigues, 2013).

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

Two media frequently used to culture AM fungi on ROC are the minimal (M) medium (Becard and Fortin, 1988) and the modified Strullu Romand (MSR) medium (Declerck *et al*., 1998). Both these media contain micro- and macro-nutrients as well as vitamins and sucrose (Cranenbrouck *et al*., 2005). The successful development of fungal isolates into sustainable culture has been achieved using minimal M medium (Becard and Fortin, 1988). Manipulation of medium composition and pH to suit new fungal isolates could lead to a better understanding of factors affecting the complex biology underlying the symbiosis (D'Souza *et al.,* 2013).

ROC was first developed by White and coworkers (Butcher, 1980) who used excised roots on synthetic media supplemented with vitamins and sucrose. ROC was first performed successfully by Mosse and Hepper (1975) using an *in vitro* system based on a dual culture of spores and excised roots of *Trifolium* (clover) species. Mugnier and Mosse (1987) obtained similar results using *Daucus carota* L. (carrot) roots genetically transformed by *Agrobacterium rhizogenes* Conn.

Many species and strains of AM fungi have been cultured in the ROC system. However, only a few species are fast growers and colonizers, able to produce thousands of *in vitro* propagules in a few months and thus have potential in large scale production. In most cases, two types of fungal inoculum viz., extra-radical spores or propagules from the intra-radical phase (mycorrhizal root fragments and isolated vesicles) of the fungal ontogeny can be used to initiate monoxenic cultures. Cultures of AM fungal species that do not produce vesicles (*Scutellospora*

and *Gigaspora* species) are systematically produced using spores, which are usually large and germinate vigorously. Plenchette and Strullu, (2003) demonstrated the ability of entrapped, disinfected mycorrhizal root fragments to form new mycorrhizae, even after storage for 1 month at 4°C. Intra-radical vesicles separated from roots and encapsulated were also shown to retain their inoculum potential and hence represent another practical source of inoculum.

For all AM fungal propagules, appropriate selection and efficiency of sterilization process are keys to the success of axenic or monoxenic AM fungal cultures. Isolated spores are often surface sterilized using the two-step procedure of Becard and Fortin (1988). Continuous culture is obtained by transferring mycorrhizal roots to fresh medium either with or without mycelium (Fortin *et al.,* 2002). Following this transfer, the pre-existing root-fungus association continues to proliferate. While using older mycorrhizal roots, it is preferable to transfer them to a Petri plate containing an actively growing root (Declerck *et al*., 1998).

The most evident advantage shared by all *in vitro* cultivation systems is the absence of undesirable microorganisms due to controlled conditions, rendering greater suitability for large-scale production of high-quality inoculum. Contamination by other microorganisms may occur either at the establishment of the cultivation process or at later stages of culture. Therefore, it may be useful to control the cultures visually, by standard plate-counting techniques and by molecular techniques. As a drawback, the diversity in terms of genera of AM fungi that have been grown *in vitro* is lower than under pot cultivation systems. Once successfully initiated, the cultures may be maintained for periods exceeding 6 to 12 months without intervention (Doner and Becard, 1991). Monoxenic cultures provide access to abundant and high-quality fungal material suitable for taxonomic and evolutionary studies (Fortin *et al*., 2002). In terms of biodiversity, monoxenic cultures provide a tool for basic

comparative analyses of root populations and strain potential, long-term propagation capabilities, and fungal adaptation to environment.

CHAPTER 03: METHODOLOGY

Study sites

Coastal sand dunes from beaches of Goa were randomly selected for the study. From the study sites, random plant species were selected for sand sample collection. For rhizosphere soil sample collection, pure cultures maintained in the Goa University Mycorrhizal Culture Collection (GUMCC), Botany discipline, SBSB, Goa University were used.

Sample collection

Small portions of sand were extracted from different root zones of random plants by digging 10-15cm deep and collected in zip lock bags. Root samples were also brought. The bags of samples were thoroughly mixed to form a composite sample. The roots were separated from adhering soil, washed and used for estimation of AM colonization. The composite sample was divided into two parts (3:1) one for AM spore isolation, enumeration and identification and the other as inoculum to prepare trap cultures, respectively. For the rhizosphere soil sample, a portion of the soil close to the host root zone from the selected pure cultures was taken.

Taxonomic identification of spores

For identification, the spores were mounted on glass slides in PVLG and examined under a bright-field Olympus BX41 research microscope. Spore morphology, wall characteristics, dimensions and other relevant data were observed for the identification of the AM spores. The spore characteristics were compared with the descriptions given by Rodrigues and Muthukumar (2009), Blaszkowski (2012) and the International Collection of Vesicular Arbuscular Mycorrhizal Fungi (invam.wvu.edu). Names and epithets of AM fungal species

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

Extraction of AM fungal spores

Extraction of AM fungal spores was carried out by Wet Sieving and Decanting Technique (Gerdemann and Nicolson, 1963)

- 20g of soil sample was weighed in a beaker and a 100ml of water was poured in it.
- The soil suspension was stirred using a glass rod and then the larger particles were allowed to settle for 10- 15 seconds.
- The aliquot was decanted through sieves arranged in descending order (250-37 pm).
- The procedure was repeated a minimum of four times for each sample.
- The residues from each sieve were then washed into separate beakers.
- The sievate was filtered separately through Whatman No. 1 or A grade filter paper.
- The filter paper was then placed in Petri plate and care was taken to ensure that it remained moist.
- The filter paper was examined for presence of spores and sporocarps under Olympus stereo microscope SZ2-ILST (10 x 4.5 zoom).

Preparation of trap cultures

Propagation of AM fungi was carried out by a trap culture method modified from Morton *et al.* (1993)

- Sand was dried till 80% of its moisture was lost.
- The dried sand was sterilized in hot air oven at 90°C for 3hrs for three consecutive days.

- 15 cm plastic pots were washed, dried and wiped with cotton dipped in absolute alcohol and the holes of the pots at the bottom were covered with cotton balls.
- The pots were filled with the sterilized sand by half their quantity.
- Spores of the selected AM fungal species were collected in small Petri plates on wet pieces of paper.
- These pieces of paper containing spores were sprayed with water into the pots containing sand.
- More sand was put into the pots to fill it to three-fourth its quantity.
- Coleus, *Plectrantus scutellarioides* (L.) R.Br. were used as the host plant for the AMF colonization as well as ragi (*Elusine coracana*) (Poaceae) seeds.

Monoxenic culture establishment:

Preparation of ROC plates

- ROC plates were prepared using modified White's medium given by (Becard and Fortin, 1988).
- The host plant roots used for this cultivation were of *Daucus carota* L.
- The Petri plates inoculated with Ri T-DNA roots were incubated in an inverted position in dark conditions under room temperature for further root growth.

AM fungal spore disinfection process

- Spores were isolated from the sievate of wet sieving and decanting onto a small Petri plate containing wet pieces of paper to ensure sufficient moisture conditions with the help of a needle and stereomicroscope.

- The isolated spores were then disinfected through a sterilization technique of Becard and Fortin (1988) using a laminar air flow (LAF).
- In the LAF four small Petri plates were prepared containing sterile distilled water half their quantity. To the first Petri plate a required amount of 4% sodium hypochlorite (NaClO) solution was added and in the fourth Petri plate a pinch of 0.02% streptomycin powder was added.
- With the help of a forceps the pieces of paper containing isolated spores were dipped in a second small Petri plate containing sterile distilled water.
- Under a stereomicroscope, the spores that sunk at the bottom of the Petri plate were isolated using a micropipette and put into a third small Petri plate containing NaClO solution and the contents were swirled continuously for 5min.
- Next the spores were transferred into the next two Petri plates which contained only water for removal of any traces of the first solution by swirling it continuously for a min and lastly the spores were transferred into the streptomycin solution and swirled for about two minutes.

Germination of disinfected spores

- For germination, the disinfected spores were plated on Modified Strullu-Romand (MSR) minus sucrose medium modified by Declerck *et al.,* (1998) solidified with Gellan Gum clarigel (medium without sucrose) and on plain water agar and incubated in the dark for 27^0C.
- The plates were placed in an inverted position.
- The NaClO solution was standardized in order to obtain maximum germination.

Transfer of germinated spores onto root plates

- The germinated spores were transferred onto Petri plates containing good healthy root growth.
- The germinated portion of the spore was marked and cut out.
- Similar portion was cut out on the ROC plate close to a root zone.
- The germinated spore along with its intact germ tube was transferred onto the ROC plate.
- The plates were sealed, labelled and incubated in an inverted position in dark conditions for further colonization.

AM fungal root colonization

Trypan Blue Staining for AM root colonization (Phillips and Hayman, 1970)

- Roots were cut in 1cm pieces and washed with water.
- The root pieces were cleared in 10% KOH at 90^0C for 1hour in a hot air oven.
- After pouring off the KOH solution, the root pieces were rinsed with several changes of tap water to remove traces of KOH.
- 5N HCl was added in the test tubes and the root pieces were soaked for 3-4 minutes in it.
- After pouring off the HCL solution, the root pieces were stained in 0.05% Trypan blue solution and kept overnight.
- The root segments were mounted in PVLG and examined under a compound microscope for AM colonization.
-

- Slides showing clear AM fungal colonization were sealed with DPX for permanent mounting.

Histochemical Staining for Polyphosphate granules using Toluidine Blue O (TBO) (Kumble and Kornberg, 1996).

- The root pieces were washed in tap water and cleared in 2% KOH by heating at 90^0C for 30-40 minutes in a hot air oven.
- The root pieces were then rinsed with several changes of tap water to remove traces of KOH.
- The root pieces were then acidified in 1N HCl for 2-3min.
- Later the root pieces were stained with Toluidine blue O for 20 minutes.
- The stained root pieces were observed under light microscope for Poly-P granules which stain purplish- pink.

CHAPTER 04: RESULTS

Identification of AM fungal spores

The isolated spore species was identified to be *Gigaspora albida* based upon the following characteristics: -

Whole spores: Spores are cream with pale green tint, globose to subglobose in shape and with a size of 200- 280 µm **(Plate 1.d)**

Subcellular structure of spores: Spore wall has three adherent layers (L1, L2 and L3), with the first two adherent and equal thickness in juvenile spores, L2 thickens as the spore wall differentiates. L3 differentiates as a prelude to germ tube formation.

L1- Outer permanent rigid layer with smooth surface, hyaline to pale yellow (0-0-10-0), 2.0-3.2 m thick.

L2- A semi-plastic layer of yellow (0-10-60-0) to brownish yellow (0-10-100-0) sublayers (or laminae) that increase in number with differentiation; varying considerably in thickness in mature spores; 14-26 m thick (15-25 µm in the same spore). Sublayers pale brown (0-10-60-0) in PVLG.

L3- Germinal layer concolorous with the L2 layer and adherent. Numerous "warts" or "papillae" on inner surface of this layer concentrated in regions where germ tubes form (usually in close proximity to the suspensor cell); warts 1.6-4 µm high in germinating spores **(Plate 1. e).**

Subtending hypha

Width of sporogenous cell: 32-45 µm **(Plate 1.f).**

Sporogenous cell wall: Two hyaline layers (L1 and L2- continuous with the first two layers of the spore wall). L2 Brownish yellow 2.5-4.2 µm thick near the spore and then thinning to 1.2-1.4 µm beyond the sporogenous cell.

Hence the above characterized AM fungal species is classified further as follows: -

Kingdom- Fungi

Phylum- Glomeromycota

Class- Glomeromycetes

Order- Diversisporales

Family- Gigasporaceae

Genus- *Gigaspora*

Species- *albida*

Trap cultures

Trap cultures of the isolated AM fungal spores were prepared using Coleus cuttings and Ragi seeds as the host plants and maintained. After 45 days the root samples of the host plant were taken to check for AM fungal colonization. Trypan blue staining of the root samples showed the presence of AM fungal colonization in the host roots. The root cortical cells showed the presence of AM fungal hyphae and hyphal coils were also observed **(Plate 2. a & b)**. Presence of *Arum*- type arbuscles was also observed **(Plate 2.d)**. The AM fungal hyphae also showed the presence of Poly-P granules when stained with Toluidine Blue O stain **(Plate 2.e).**

Modified White's Media for ROC production

Modified White's media using 15g of sucrose was found to be optimal for the multiplication of Ri T-DNA roots. New root growth commenced within 5 days upon plating **(Plate 3. a & b).]**

Standardization of NaClO volume for spore disinfection

Different NaClO volume were tried for the spore disinfection process. It was observed that 4%vlume of NaClO was found to be suitable for sterilization of spores. This resulted in maximum germination of the spores in MSR medium and plain agar water without sucrose.

Spore germination

Single germ tube formed in vicinity of warty protruberances on inner surface of L3 of the spore wall. The hyphal growth protruded into septate branching on further growth followed by its coiling **(Plate 4. c & d).** Auxiliary cell formation was seen after 18 days. Initially cells in aggregates of 4-10, subglobose to ovoid to clavate, borne on tightly coiled hyaline hyphae, thin-walled, hyaline to pale cream; each cell with narrow projections **(Plate 4. e & f)**. For, the spores recovered from pot cultures, the germination commenced after 10 days on MSR media and after 4 days on plain agar water. Whereas spores obtained from dunes, the germination was observed after 7 days on MSR medium and after 3 days on plain agar water **(Plate 4. a & b).** It was observed that the spore germination was faster on plain agar water compared to MSR medium.

AM fungal *in vitro* colonization

After 2 to 3 days, the germinated spores were transferred on ROC plates **(Plate 3.c)**. After 3-4 days upon plating, the germinated spores showed further growth and colonized the Ri T-DNA roots **(Plate 5. a & b)**. The Ri T-DNA roots were observed for AM fungal colonization using Trypan blue staining technique after 4 weeks. The Ri T-DNA roots showed presence of hyphal colonization **(Plate 5. c & d).**

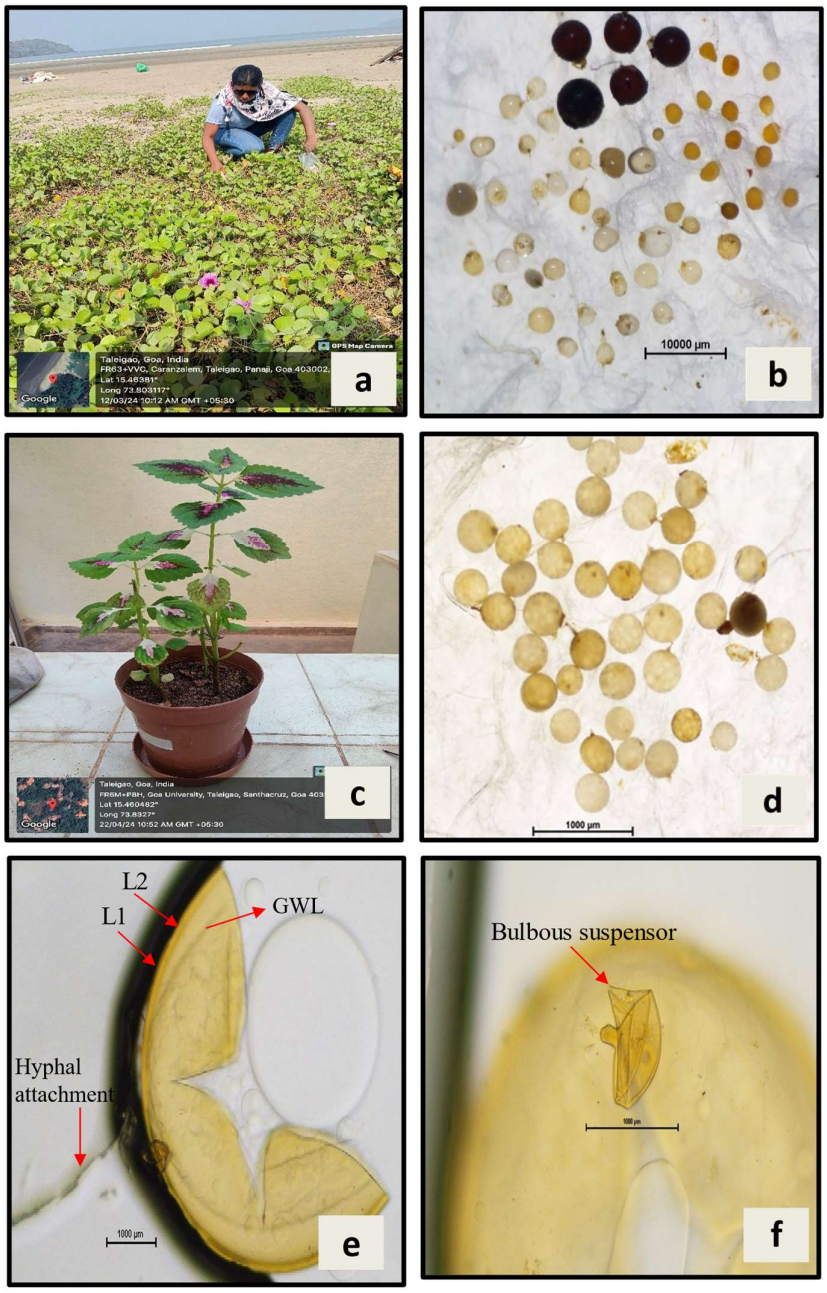

Plate 1: AM fungal spores extraction: a. Dune rhizosphere soil sample collection; **b.** Spores isolated from dune sample; **c.** Trap culture; **d.** Isolated spores from Goa University Arbuscular Mycorrhizal Fungus Culture Collection (GUAMFCC); **e.** *Gigaspora albida* broken spore showing hyphal attachment, spore wall layers (L1 & L2) and germinal wall layer; **f.** Bulbous suspensor in *G. albida.*

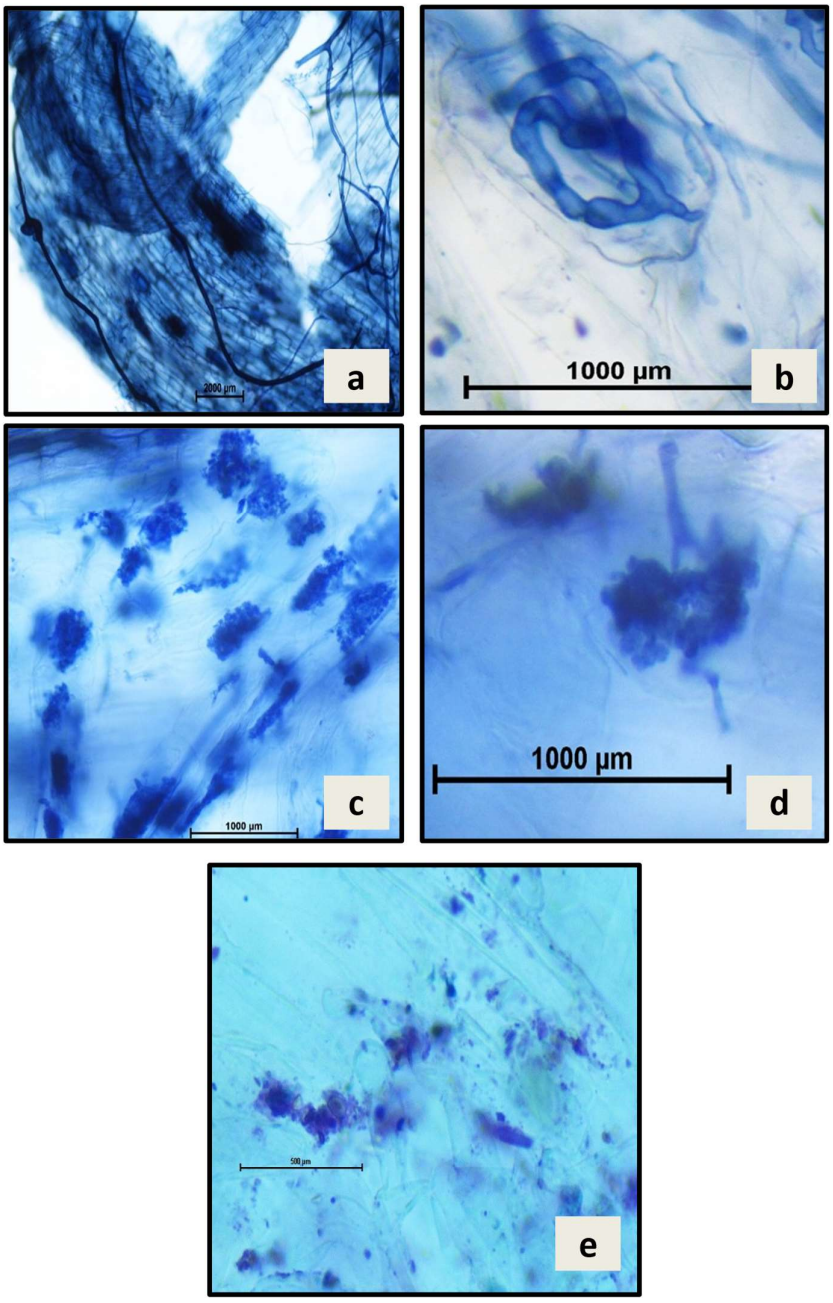

Plate 2: AM fungal root colonization in trap cultures: a. Hyphal colonization in roots; **b.** Hyphal coils; **c.** Arbuscles; **d.** *Arum*-type arbuscles; **e.** Polyphosphate granules.

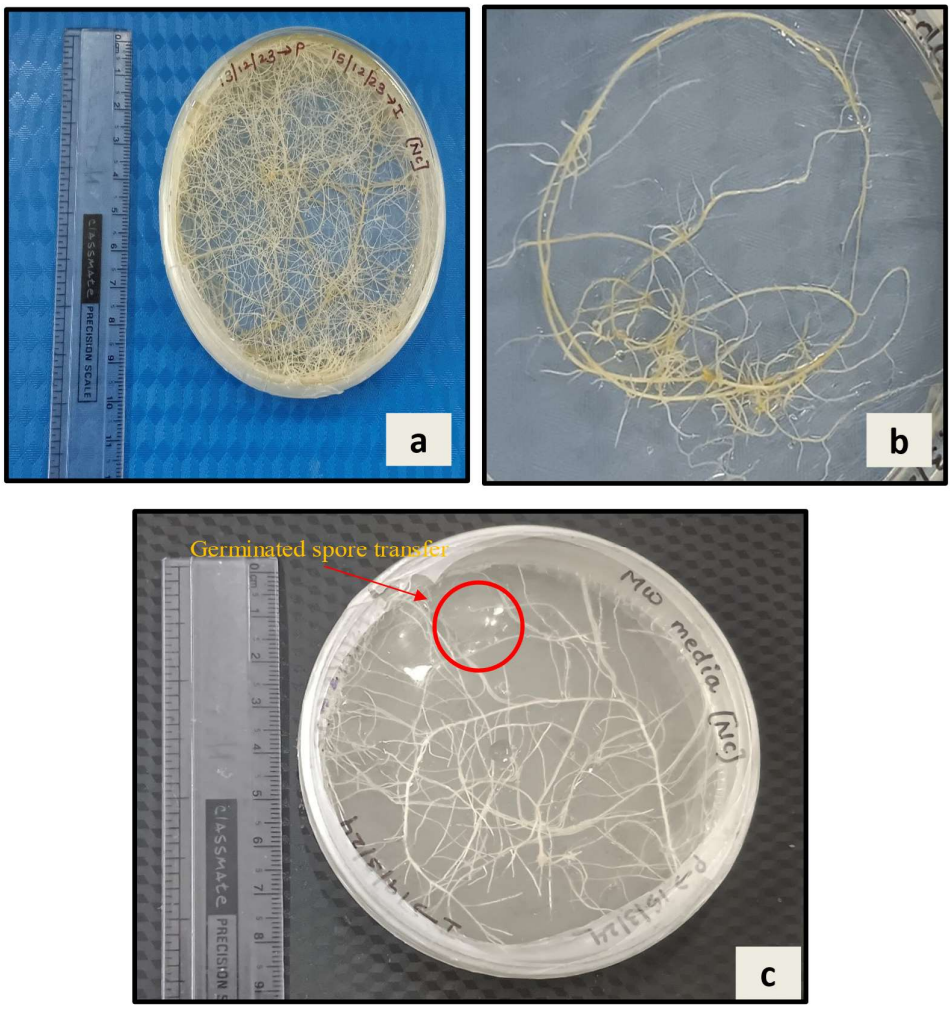

Plate 3: Establishment of Ri T-DNA cultures: a & b. Cultures of transformed roots; **c.** Transfer of germinated spore onto Ri T-DNA cultures.

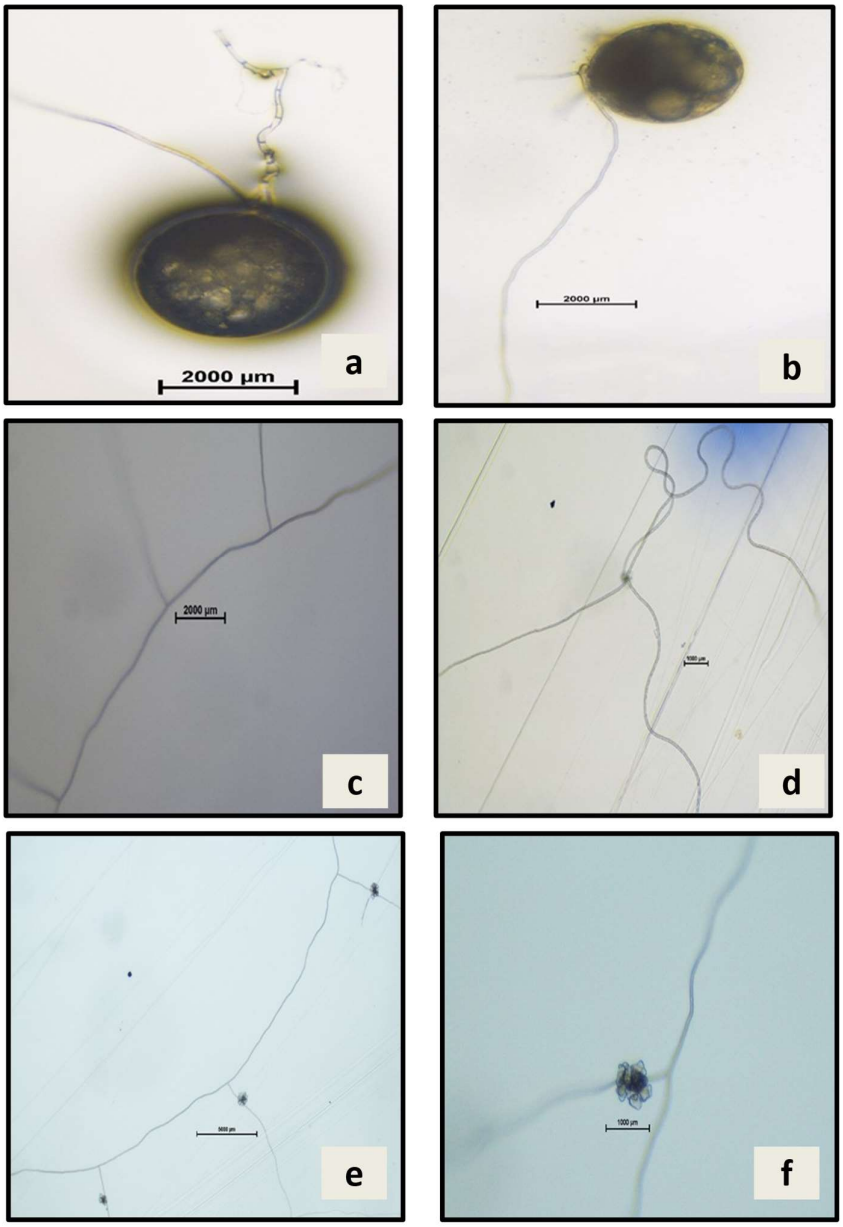

Plate 4: Germination of *G. albida* spore: a. Spore germination on MSR media; **b.** Spore germination on agar media; **c.** Hyphal branching; **d.** Hyphal coiling; **e. & f.** Auxiliary cell.

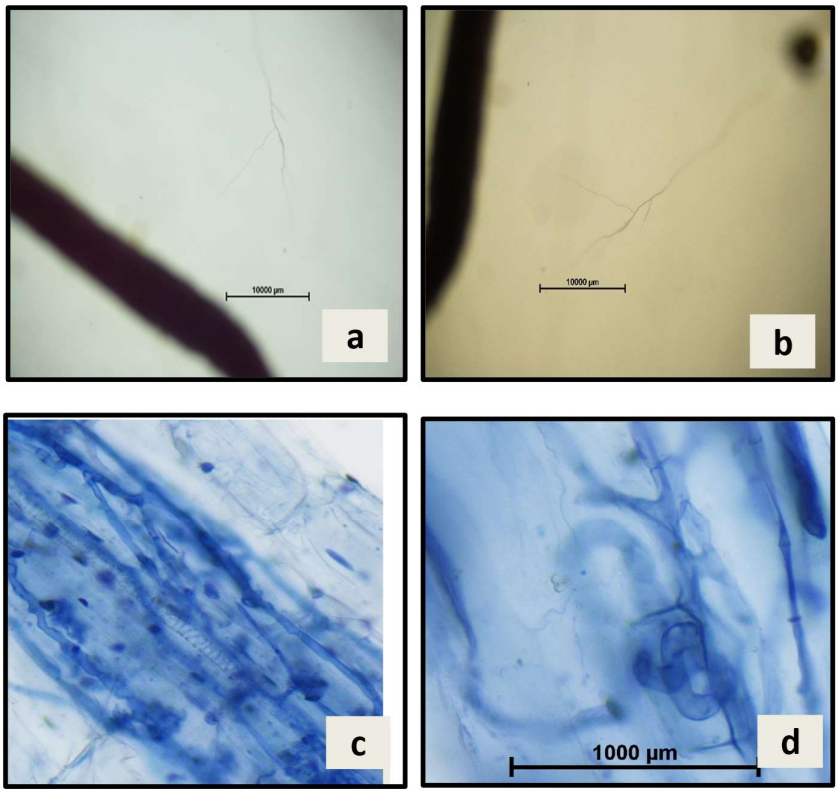

Plate 5: *In vitro* root colonization: a & b. *G. albida* hyphae entering Ri T-DNA roots; **c.** colonization in Ri T-DNA roots; **d.** Hyphal coiling.

CHAPTER 05: DISCUSSION AND CONCLUSION

Discussion:

AM fungi are considered to play a crucial role in plant community establishment and sand dune stabilization (Puppi *et al*., 1986). AM fungal bio-inoculants thus can be introduced and used for revegetation and agriculture.

An attempt was made to culture spores of *Gigaspora albida* in *in vitro* conditions with an objective to obtain sporulation by devising a standard protocol. This would facilitate the multiplication and commercial inoculum production. Successful colonization of the isolated species was observed in *in vitro*. AM fungal species such as *Scutellospora* and *Gigaspora* belonging to the Gigasporaceae family have shown successful *in vitro* spore germination and colonization (Declerck and Dalpé, 2001).

In this study, the selected AM fungal species *G. albida* spores were extracted from the dune rhizosphere soils and pure cultures, and multiplied. The taxonomy of the isolated AM fungal species was studied. Studies have shown that AM spores are the most effective propagules for germination (Klironomos and Hart, 2002). The isolated spores of AM fungi were used to prepare trap cultures using the plant *Plectranthus scutellarioides* (L.) R.Br. (Coleus) as host. Roots from the trap cultures were examined for AM fungal colonization using Trypan blue staining and for presence of Polyphosphate granules using Toluidine blue O staining.

Cultures of Ri T-DNA were established using *Daucus carota* L. (carrot) roots on White's media under *in vitro* conditions. White's media has shown successful *in vitro* cultivation of AM fungi in several species (Becard and Fortin, 1988). Using the standardized protocol, the isolated AM fungal spores were disinfected and inoculated on MSR medium and plain agar water (without sugar) for germination. This was done to maximize germination of AM spores. Germination of the spore emerged from the germinal layer by germ tube, protruding through the spore wall.

The germinated spores were transferred onto Ri T-DNA cultures. The *in vitro* growth was observed up to auxiliary cell formation in monoxenic culture systems. No sporulation has occurred till date. It is reported that Gigasporaceae members take a longer time to sporulate (Ijdo *et al*., 2011). These cultures are maintained for further examinations for sporulation to occur.

Conclusion:

Through this study successful germination and colonization of AM fungal species *G. albida* was obtained *in vitro.* With these results it is possible to cultivate AM fungal spores *in vitro* in Ri T-DNA roots. This could be used further for the production of bio-fertilizers to enhance agriculture, revegetation of degraded lands, dune establishment and maintain plant community balance.

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Solution I: Macro- Elements for 1L (100X), use	WM Medium
Nano-Pure water	
Chemicals	
MgSO4-7H2O	73.1 g
(Magnesium Sulfate)	
KNO3 (Potassium Nitrate)	8 g
KCl (Potassium Chloride)	6.5 g
(Potassium Phosphate)	
NaH2PO4-H2O (Sodium Phosphate)	1.9 g
Na2SO4 (Sodium Phosphate)	19.9 g (or 9.6 g NaCl)

Solution II of Calcium Nitrate Tetrahydrate for 1L (100X)

Ca(NO3)2-4H2O (Calcium Nitrate Tetrahydrate): 28.8g/L

Solution III of Potassium Iodide for 250 mL (1000X)

KI (Potassium Iodide): 0.1875 g/250mL

Solution IV

Micro-Elements for 250 mL (1000X)	**WM Media**
Chemicals	
MnSO4-H2O (Manganese Sulfate)	1.165 g (or 1.5 MnCl2-4H2O)
ZnSO4-7H2O (Zink Sulfate)	0.6625 g
H3BO3 (Boric Acid)	0.375 g
CuSO4-5H2O (Copper (II) Sulfate Pentahydrate)	weight and dilute 0.65 g in 50mL water and take 2.5mL of this solution to the mix
Na2MoO4-2H2O (Sodium Molybdate Dihydrate)	Weight and dilute 0.12 g in 100mL water and take 0.5mL of this solution to the mix. [Or 0.11 g of NH4(Mo7O2)4-2H2O]

Solution V of NaFe EDTA 500mL (250X)

NaFe (Sodum Iron) EDTA: 0.8g/500 mL

Solution VI of Vitamins (1000X)

Use Gamborg's Vitamin Solution 1000X.

Procedure:

Preparation of 1L medium of WM Media

1. Put 700 ml NanoPure water in a 1L medium bottle.

2. Add 30 g of sucrose

3. Add g 4 g of Phytagel (Sigma Ref: P8169), with agitation using

magnetic stirrer.

4. Add 10 mL of Solutions I and II each.

5. Add 5 mL of Solutions V.

6. Add 1 mL of Solutions III and IV each.

7. Mix with magnetic stirrer and complete to 1 L with water.

8. Adjust pH to 5.5, autoclave.

Preparation of Polyvinyl alcohol lacto- glycerol (PVLG)

Requirements

- Polyvinyl alcohol - 16.6g
- Lactic acid - 100ml
- Distilled water - 100ml
- Glycerin - 10ml

Preparation: Dissolve 16.6g polyvinyl alcohol in 100ml distilled water by heating at 90ºC. After cooling add 100ml lactic acid and 100ml glycerin. The polyvinyl alcohol should have the following properties: 99-100% hydrolyzed and viscosity of 24- 32 centipoise.

Preparation of Trypan blue stain

Requirements

- Trypan blue - 0.05g

- Lactic acid - 50ml
- Glycerin - 10ml
- Water - 40ml

Preparation: 0.05g of trypan blue powder is dissolved in 40ml of distilled water and then 50ml of lactic acid and 10ml of glycerin is added.

Preparation of Toluidine blue O

Requirements

- Toluidine blue O - 1g
- Distilled water - 100ml
- 1N HCl

Preparation: 1g of toluidine blue O is dissolved in 100ml of distilled water and the pH is adjusted to 1 by adding 1N HCl.