In vitro Cultivation of Arbuscular Mycorrhizal (AM) Fungal Spores using Root Organ Culture Techniques

A Dissertation for

BOT -651: Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc. in Botany

By

AUSTINA BARBOSA

Seat Number: 22P0480002

ABC ID – 224-237-485-595

PRN: 201608718

Under the Supervision of

DR. BERNARD F. RODRIGUES

School of Biological Sciences and Biotechnology
Botany Discipline

GOA UNIVERSITY

April 2024

GOA UNIVERSITY
SBSB
TALEIGAO
GOA
BOTANY DISCIPLINE

Seal of the School

Examined by:

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, ***"In vitro* Cultivation of Arbuscular Mycorrhizal (AM) Fungal Spores 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 in the dissertation.

I hereby authorize the University authorities to upload the dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Austina Barbosa
22PO480002

Date: 08 April 2024
Place: Goa University

COMPLETION CERTIFICATE

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

Dr. Bernard F. Rodrigues

Date: 08 April 2024

Dean of the School
Date: 08 April 2024
Place: Goa University

GOA UNIVERSITY
SBSB
TALEIGAO
GOA
BOTANY DISCIPLINE

School/Dept Stamp

CONTENTS

PREFACE

AM fungi play a crucial role in enhancing the plant health and soil fertility, making them essential component of sustainable agricultural practices. Despite their importance, the efficient cultivation of AM fungi *in vitro* remains a challenge, which this research aims to address through innovative application of root organ technique. The present study sparks the potential benefits of AM fungi in improving crop yields and soil structure. The motivation for this study stems from the need to overcome existing limitations in AM fungal cultivation methods and contribute to the broader understanding of their symbiotic relationships with plants. The methods employed in this study focus on cultivating AM fungal spores in association with Ri T-DNA transformed roots, offering a controlled environment to explore their growth and interactions.

AKNOWLEDGEMENT

I would like to express my deepest gratitude to all those who have supported me during the completion of this discipline specific dissertation submitted in partial fulfilment of Master's degree.

I am deeply grateful to my Guide Professor Bernard F. Rodrigues (Dean of the School of Biological Sciences & Biotechnology), for their continuous guidance, encouragement, and insightful feedback throughout this research journey. Their expertise and mentorship have been valuable to me.

I would like to thank Professor S. Krishnan (Vice- Dean of Research, SBSB), Dr. Rupali Bhandari (Program Director, Botany Discipline, SBSB) for their assistance in carrying out this dissertation research. I would also like to thank Dr. Siddhi K. Jalmi and Dr. Aditi Naik for their motivation and support.

I am immensely thankful to Dr. Dhillan M. Velip, Assistant Prof. Tanvi Prabhu, Research scholar Mrs Amisha Shirodkar, GUFCC culture assistant Ms Lenora Vaz, for their constructive feedback, guidance and valuable advice throughout my research. Your insights have greatly improved the quality of this work.

A special thank you to Goa University Mycorrhizal laboratory, Botany Discipline, School of Biological Sciences & Biotechnology, where I conducted my research. The facilities and resources provided have been essential to the successful completion of this dissertation.

I would also like to thank the non-teaching staff of Botany Discipline for their constant support and cooperation.

I extend my gratitude to my colleagues and peers, for their support during my studies. Your discussions and encouragement have greatly influenced my work.

I am immensely thankful to my family and friends for their persistant support and encouragement throughout this journey. Your belief in me has been my strength.

I offer my sincere thanks to the almighty. The journey has not been without its challenges, but I have emerged with a greater understanding of complexities of AM fungi and their interactions with plants.

LIST OF TABLES

LIST OF PLATES

Plate 4: Micrographs of *in vitro* germination:

a. *Claroideoglomus etunicatum* spore germination;

b. *Cetraspora pellucida* spore germination on plain agar water;

c. Hyphal branching;

d. Auxiliary cells and Septate hyphae;

e, f. Hyphal proliferation and entry in the Ri T- DNA carrot roots.

Plate 5: *In vitro root* colonization:

a, b. *Cetraspora pellucida* hyphal colonization in roots;

c. Hyphal swelling;

d. Hyphal coils.

ABREVIATIONS USED

ENTITY	ABBREVIATION
Arbuscular Mycorrhizal	AM
Root Organ Culture	ROC
Phosphorus	P
Modified Strullu Medium	MSR
White's medium	WM
Minimal medium	M
Murashige and Skoog	MS
Monoxenic culture	MC
Goa University Arbuscular Mycorrhizal Culture Collection	GUAMCC
The International collection of Vesicular Arbuscular Mycorrhizal Fungi	INVAM
Polyvinyl Lacto Glycerol	PVLG
British Standard Sieve	BSS
Sodium Hypochlorite	NaClO
Potassium Hydroxide	KOH
Polyvinyl Lacto Glycerol	PVLG

ABSTRACT

Ri T- DNA transformed roots serves as an important tool in the studies of *in vitro* cultivation of Arbuscular Mycorrhizal (AM) Fungi. This tool is widely used to study and investigate the *in vitro* root colonization by several species of AM Fungi. The aim of this study was to establish *in vitro* cultures of *Claroideoglomus etunicatum* and *Cetraspora pellucida* AM Fungal spores using Ri T- DNA transformed carrot (*Daucus carota*) root organ cultures (ROC). Sterilized AMF propagules were co- cultured with hairy transformed carrot roots on Whites Media (WM). AMF Spore germination was observed after surface disinfection with 4% NaClO and 0.02% streptomycin. The first contact between AMF hyphae and roots occurred several days after germination. The colonization was confirmed by staining a part of ROC.

CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

Many chemical products are widely used in the horticulture and agricultural industries to increase yields. As a result, the soil's biological potential is lost. There is also a significant impact on the soil's quality. Using composts, bio-stimulators, bio-fertilizers, and microbially enhanced bio-pesticides is an alternate strategy for achieving higher yield. About 80% of plants trade advantages with obligatory biotropes called Arbuscular Mycorrhizal (AM) fungi (Berruti et al., 2016). They are known as bio-fertilizers because they have the ability to supply nutrients, water, and provide protection to the host plants from various diseases. The AM fungi in return receive photosynthetic products.

1.1 COASTAL SAND DUNES

The coastal sand dunes can be described as mounds and narrow strips of sand with distinct boundaries. They are determined by the sea and are the landward limits of sand transport. These are natural barriers against the wave action of the sea. They protect the shoreline and the natural habitats or the developed areas towards the inland. Plants in dunes and in general have specific rhizospheric microflora. This microflora depends on the influx of mineral nutrients to roots and the abundance, density, composition, and diversity of plant- derived exudates. Interactions with microbes like AM fungi in the roots appear crucial in obtaining in-organic nutrients or growth-influencing substances (Nayak et al., 2019).

1.2 ARBUSCULAR MYCORRHIZAL FUNGI

AM fungi are soil-dwelling obligate symbiotic fungi. The AM fungi mycelium regarded as the key elements of the rhizosphere, serves to enhance the absorptive surface area of the host plant roots. The external mycelium attains as much as 3% of root weight (Jakobsen and Rosendahl, 1990). Mycorrhizal fungi improve soil structure by growing external hyphae into the soil, forming a skeletal framework that keeps soil particles together (Nwoko, 2014).

The external hyphae created are suitable for the production of micro-aggregates. The micro-aggregates interwoven by external hyphae and roots eventually generates macro-aggregates and contributes to the plant's carbon (C) resources in the soil (Miller and Jastrow, 2000). Soil aggregates, affected by soil C, are vital for forming organic compounds required to bind soil particles (Otgonsuren and Lee, 2010). Therefore, AM fungi improves the overall microbial activity in the root zone (Nwoko, 2014).

1.2.1 ORIGIN AND TAXONOMY OF AM FUNGI

AM fungi are regarded as having the most prevalent symbiotic relationships on Earth. Franciszek Kamienski, a Polish mycologist, identified the symbiotic relationship between a fungus and roots in *Monotropa hypopitys* (1881). The word "mycorrhiza" was later coined for the association by Frank (1885). Oehl et al., (2011) proposed a classification where phylum *Glomeromycota* was divided into three classes: Glomeromycetes, Archaesporomycetes, and Paraglomeromycetes with five orders, 14 families and 29 genera. Further, Goto et al., (2012) proposed a new classification formed based on both morphological and molecular studies introducing a new classification formed based on both morphological and molecular studies introducing a new family Intraornatosporaceae with two new genera *Intraornatospora* and *Paradentiscutata*. Recently, (Redecker et al., 2013) proposed a new classification and rejected the splitting of the phylum *Glomeromycota* by Oehl et al., (2011) into three classes.

1.2.2 AM STURCTURES

Arbuscular mycorrhizae consists of intra- and extra- radical structures. The intra-radical structures are arbuscules, vesicles, and intra-radical hyphae. The extra- radical structures are extra-radical hyphae, spores and auxiliary cells.

a. Arbuscules

Arbuscules are the sites of exchange for phosphorus (P), C, water, and other nutrients. They differ in morphology, depending on the generic affiliation of the AM fungal species (Morton, 2002). The *Paris* and *Arum* are the two types of arbuscules formed.

b. Vesicles

Globose or ovoid, thin walled vesicles are the storage organs filled with lipids and glycolipids (Mosse, 1981). Vesicles may be inter- or intra-cellular and may be found in both inner and outer layers of the cortical parenchyma. Vesicles are generally ellipsoidal in *Glomus* spp., whereas members of the family Gigasporaceae never produce them.

c. Auxiliary cells

Auxiliary cells are swollen structures produced terminally by extra-radical hyphae. The cells are either spiny and smooth or knobby. They are formed only by members of the family Gigasporaceae and Pacisporaceae.

d. Spores

Spores are multinucleate single cells mainly produced blastically at the tip of sporogenous hyphae continuous with mycorrhizal extra-radical hyphae. They are made up of spore wall layers with many lipid bodies within them. The spore walls consist of chitin and other compounds. These structures aid in the survival and germination of spores playing a crucial role in the life cycle AM fungi. Spore colour, shape, and size vary significantly depending on developmental stage and environmental factors. Spore colour vary between hyaline to white, yellow, red, brown, and black, with all intermediate tints.

1.2.3 Viable propagules

AM fungi has a range of viable propagules. These include the spores, hyphal fragments, and hyphae within senesced or living roots, also called mycorrhizal root fragments. The spores are the commonly used propagule. Spores can be collected from the field or pot cultures by wet sieving and decanting method of Gerdemann and Nicolson (1963). These AM fungal spores can be isolated individually from the sievates under a stereo microscope using a needle or a fine tweezer.

1.2.4 SIGNIFICANCE

The significant benefits of plants from the AM fungal relationships are the improvement of uptakes in water and inorganic nutrients, especially P, nitrogen (N), potassium (K), iron (Fe), and manganese (Mn) (Anders and Koide, 1994). The AM fungal hyphae are more efficient than root hairs in terms of nutrient/water acquisition since they are narrower, longer, and more versatile in their direction of growth (Goltapeh et al., 2008). AM fungi produce pools of organic C, such as glomalin, which is known to increase the soil microbial biomass (Rillig et al., 2001). Additional benefits include increased tolerance to environmental stresses such as nutrient deficiency, diseases, drought, and salinity (Smith and Read, 2008; Gupta and Kumar, 2000).

1.3 ROOT COLONIZATION

Hyphal growth is frequently orientated along epidermal grooves, and appressoria are formed over periclinal and anticlinal wall junctions between adjacent epidermal cells (Garriock et al., 1989). The formation of appressoria is the most significant sign of fungal recognition of a potential host plant (Staples and Macko, 1980). Appressoria is the bulbous formation produced by AM fungi that is well attached to the host cuticle cells. After the differentiation of appressoria, AM fungi usually colonize host roots by forming inter-cellular and intra-cellular

hyphae and intra-cellular arbuscules (Giovannetti and Lioi, 1990). The absence of appressoria and arbuscules in these interactions between AM fungi and plant tissues suggests the lack of any recognition event leading to the establishment of a functional symbiosis (Giovannetti and Sbrana, 1998).

1.4 *IN VITRO* CULTIVATION

In vitro culture is an efficient and reliable artificial system. It is a suitable method for investigating basic and practical aspects of AM symbiosis and complements experimental approaches. *In vitro* conditions involve the replacement of the plant host by the ROC aseptically. As a result, the symbiotic benefit to the plant is affected by the absence of photosynthetic tissues, a normal hormonal balance, and physiological source-sink relationships. Sucrose is added to the culture medium to compensate for the absence of photosynthates. Therefore, the root-fungus interface is bathed in a sugar solution, which does not occur *in vivo*. In this case, carbohydrates reach the cortex and the vascular system *via* the epidermis. The presence of sugars at this interface may modify the biochemistry of the root-fungal interaction. This might explain why arbuscules and vesicles are often scarce in Ri T-DNA transformed carrot roots despite abundant intra-cortical mycelium (Kumar and Yadav, 2019).

1.4.1 GERMINATION OF SPORES

Spores must be surface sterilized before being used as *in vitro* inoculum (Bécard and Piché, 1992). This step is critical because success depends on the elimination of all contaminants. However, in some cases spores may carry bacteria between wall layers, making disinfection difficult or even impossible (Walley and Germida, 1996). If spores are not to be used immediately, they should be stored at 4^0C, either in distilled water or on water agar.

Hydro-metabolism activation is a primary pre-requisite for the germination of AM propagules like spores and root fragments (Dalpe et al., 2005). The time required for spore germination of different genera may range from 2 to 90 days (D'Souza et al., 2013). Attempts to assess the requirements for spore germination and germ tube formation on artificial media have yielded mixed results due to variations in methodology, fungal species, and culture conditions. The reserve materials from propagules are used to grow AM spores, and after germinating, they develop germ tubes in the pre-symbiotic phase. Important factors responsible for initiating germination and germ-tube growth are pH, temperature, moisture, mineral and organic nutrients (Clark, 1997), substrate (Maia and Yano-Melo, 2001), and flavanols (Bécard and Piché, 1992).

Germ tube length was significantly increased in MSR (Modified Strullu Medium) without sucrose (D'Souza et al., 2013). Sucrose is one of the nutrients exchanged during the symbiotic phase (Smith and Read, 2008). A higher sucrose concentration produces an inhibitory effect on hyphal growth, resulting in decreased hyphal length. Siqueira et al., (1982) observed that germ tube growth was favoured in low concentrations of sucrose (4g/L), while concentrations above 4g/L reduced germ tube growth.

1.4.2 ARTIFICIAL MEDIA TYPES

A modified White's medium (MW) and a minimal medium (M), both described by Becard and Fortin (1988), are used for the routine maintenance of root culture (Chabot et al., 1992). The M medium (Bécard and Fortin, 1988) was initially developed for Tomato ROC (Butcher, 1980). The macro-element composition of White's medium is considerably lower than that of MS (Murashige and Skoog) and B5 media, commonly used for *in vitro* plant cultures (Bécard and

Piché, 1992). However, this dilute medium is adequate for root growth. The composition of the M medium is even poorer and was developed following a bioassay that compared the effects of different element concentrations on mycorrhiza formation (Fortin et al., 2002).

The MSR medium is used *in vitro* to optimize the growth of the intra-radical phase of the fungus. The macro element composition of MSR is similar to that of the M medium. Differences between the two media occur in oligo element and vitamin concentrations: MSR medium lacks iodide, myoinositol, and glycine, and M medium lacks pantothenate, biotin, and cyanocobalamine. These various components are perhaps not essential since their absence in either medium has no apparent adverse effect on the AM symbiosis. Both media are adjusted to pH 5.5 before autoclaving and are solidified with Clerigel. Almost 30 AM fungal isolates from the families of Acaulosporaceae, Gigasporaceae, and Glomaceae have been successfully grown on these media (Fortin et al., 2002).

1.4.3 ROOT ORGAN CULTURE (ROC)

ROC were first developed by White and co-workers (White 1943; Butcher and Street 1964; Butcher 1980). Excised roots were used on synthetic Mineral media supplemented with vitamins and a carbohydrate source. Pioneering work by Mosse and Hepper (1975) used root cultures obtained from *Lycopersicum esculentum* (Tomato) and *Trifolium pratense* (Red Clover) to establish *in vitro* mycorrhiza with *Glomus mosseae.* It was demonstrated for the first time that spores of an AM fungus could be successfully used to colonize excised roots growing on a mineral based medium. Later, Strullu and Romand (1986, 1987) showed that it was also possible to re-establish mycorrhiza on excised roots of *Fragaria ×Ananassa Duchesne* (Strawberry), *Allium cepa* (Onion), and Tomato, using the intraradical phase (i.e., vesicles or entire mycorrhizal root pieces) of several species of *Glomus* as inoculum (Fortin et al., *2002)*.

1.4.4 *IN VITRO* ROOT COLONIZATION

Mycorrhizal ROC has elucidated many aspects of the intimate symbiotic plant-fungal association. Although Ri T-DNA transformed roots replace the host plant, the fungus can colonize and sporulate. The development of spores is morphologically and structurally similar to those produced in pot cultures. The ability of the *in vitro* produced propagules to retain their viability to colonize and initiate new mycorrhizal symbiosis indicates that the fungus can complete its life cycle. Thus, the success achieved by the culture of AM species using Ri T-DNA transformed roots demonstrates that this technique can be exploited for large-scale inoculum production (Rodrigues and Rodrigues, 2013).

Mugnier and Mosse (1987) achieved the first culture of hairy roots colonized by an AM fungus. They successfully colonized *Convolvulus sepium* hairy roots using spores of *G. mosseae* (Hepper and Mosse, 1975). Bécard and Fortin (1988) showed that 13-day-old *D. carota* hairy roots, which more closely resemble a "normal" root system (i.e. vigorous taproot, long elongation zone, and a pyramidal pattern of lateral root development), were colonized more readily than 9-day-old roots. ROC has apparent advantages over traditional systems, permitting significant production of contaminant-free propagules (Declerck et al., 1996). Bécard and Fortin (1988) used carrot hairy roots colonized by *Glomus intraradices* to obtain the first *in vitro* sporulation of an AM fungus.

1.5 MONOXENIC CULTURES

Monoxenic culture (MC) consists of an explant of Ri T-DNA transformed carrot root associated with AM fungal propagules on a synthetic nutrient-agar media. MC has been successful as a cultivation system for more than 25 AM species (Fortin et al., 2002) and is proving useful for studies of the fungal symbiont (Harrison et al 1999).

1.6 AIMS AND OBJECTIVES

The following are the aims and objectives of the present work:

1. To identify and isolate spores of AM fungi.
2. To prepare Trap cultures of AM fungal spores.
3. To study spore morphology of *Claroideoglomus etunicatum* and *Cetraspora pellucida* AM fungal spores.
4. To prepare *in vitro* ROC for AM fungal spores.
5. To study colonization in transformed roots and *in vitro* sporulation.

CHAPTER 2:

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

2.1 GLOBAL SCENARIO

- Diop et al., (1992) study showed that the ROC system is a promising method to produce clean AM mycorrhizal inoculum, since with only three spores used as inoculum, more than 450 spores of *G. margarita* were produced on colonized roots, on average, in 40 ml of medium. The new spores (450/dish) were of the expected colour, shape, and size, germinating (95%) on fresh medium.

- Declerck et al., (1996) found that the dual-culture of mycorrhizal root segments containing *G. versiforme* with transformed carrot roots on the MSR medium permits mass production of pathogen free AM fungal inoculum. The study also stated that this system allowed extensive internal root colonization and spore production in a small space and over a short time.

- Douds (1997) in his studies revealed that *Glomus mosseae* spores germinated best in 10 mM Tris or MES-buffered medium at pH values just above neutral. Growth of hyphae from germinated spores was much greater in the presence of Tris than MES at pH 7.2. Roots exhibited a broad pH optimum for growth of 6.0-7.0 in both MES and Tris, but did not grow well above pH 7.5. In addition, a purified gelling agent, gellan gum, was utilized to lower the P concentration of media. With these factors combined, mycorrhizas were successfully established in 14% of dual cultures.

- An *in vitro* system using Ri T-DNA transformed roots and *Gigaspora margarita* was developed to study the initial events of mycorrhiza formation. Sucrose, sodium, and P were found to be critical components of the medium used to establish the dual culture. The study stated that the system is especially appropriate for studying and triggering fungal biotrophy towards the root (Becard and Fortin, 1988).

- Vimard et al., (1999) investigated the colonization of *Allium porrum* (leeks) from monoxenic *in vitro* produced *Glomus intraradices* spores. Monoxenically-produced spores as AM fungal inoculum in a soil mix growth medium yielded an AM colonization level similar to a root-segment inoculum 16 weeks after inoculation, with a much lower level of fungal contamination. This inoculum was more suitable for large scale production and biochemical and molecular investigations of the AM symbiosis.

- Pawlowska et al., (1999) reported the establishment of monoxenic cultures of *Glomus etunicatum* in association with excised Ri T-DNA transformed carrot roots. Modified White's medium buffered with 10 mM MES (pH 6) or MOPSO (pH 6±5) was found to be most optimal for host root growth as well as for *G. etunicatum* spore germination and mycorrhiza formation. Sporulation was asynchronous and continued until root senescence. Although *G. etunicatum* spores formed *in vitro* exhibited general morphological and anatomical similarity to soil-borne inoculum, they were significantly smaller and had thicker spore walls than their soil-borne counterparts.

- Bever and Morton (1999) found substantial variation among single-spore cultures established from a single population of the arbuscular mycorrhizal fungus *Scutellospora pellucida.*

- The studies done by Karandashov et al., (2000) revealed that *Glomus caledonium* formed a Paris-type mycorrhiza (Smith and Smith, 1997) on transformed carrot roots. The fact that under the same culture conditions *Glomus intraradices* formed typical *Arum*-mycorrhiza, supported the suggestion of Smith and Smith (1997) that carrot has an unclear status concerning the formation of *Arum*- or *Paris*-type of mycorrhiza.

- Klironomos and Hart (2001) suggest that there is a higher success in culturing *Gigaspora* and *Scutellospora* species using spores as inoculum, while *Glomus* and *Acaulospora* species can be cultured using any combination of inoculum like spores, hyphae, and roots.

- Fortin and Becard (2002) studies on endomycorrhizal symbiosis describe how transformed roots stimulate the production of highly branched structures absent on hyphae growing saprophytically. The production of these structures was likely to be necessary for nutrient uptake and formation of the symbiosis.

- Voets et al., (2009) revealed that the mycelium donor plant (*Medicago truncatula) in vitro* culture system allowed fast, extensive, and homogenous colonization of roots at the seedling stage. It was also found that the newly colonized seedlings could reproduce the fungal life cycle, producing thousands of spores within four weeks.

- *Ron* et al., (2014) showed that *Agrobacterium rhizogenes* (or *Rhizobium rhizogenes*) was able to transform plant genomes and induce the production of hairy roots. *A. rhizogenes* was used in tomato to assess gene expression rapidly, and the function was described.

- Kokkoris and Hart (2019) discussed the potential consequences of Transformed Root Culture (TRC) propagation on AM fungal traits and how it would affect their functionality.

- Novais et al., (2020) assessed whether nitrogen-fixing rhizobia can be transferred on the host legume *Glycine max* by extra radical mycelium (ERM) produced by *Glomus formosanum* isolate colonized the grass *Urochloa decumbens*. The findings reveal that, besides its main activity in nutrient transfer, ERM produced by AMF may facilitate bacterial translocation and the simultaneous associations of plants with beneficial fungi and bacteria, representing an essential functional structure to establish symbiotic relationships.

2.2 NATIONAL SCENARIO

- Srinivasan et al., (2014) study indicated that dual culturally produced *G. intraradices* spore inoculum in a soil and sand mixture growth medium showed higher level of root colonization in comparison to open pot culture-produced inoculum after 60 days of incubation period. This *in vitro* produced inoculum is highly suitable for commercial production of the AM in a shorter time.

- Kumar and Yadav (2018) Studies suggest that mycorrhizal fungi enhance plant growth and development, stimulate plant resistance, reduce the absorption of heavy metals, and promote plant sustainability under metal stress; they can also be used as biotic elicitors for secondary metabolite production in plants.

2.3 LOCAL SCENARIO

- A germ tube emergence from the germ tube initial of the germination shield was recorded in a spore of *S. verrucosa* isolated from *Naregamia alata* (Meliaceae). The germ tube was present in the inner germinal walls of the spore and penetrated the outer wall. The germ tube emergence from the germination shield can be used as a marker to determine the systematic position of *Scutellospora* (Radhika et al., 2009).

- Radhika and Rodrigues (2012) reviewed various developments in the *in vitro* culturing of AM fungi. It was shown that *in vitro* culture system offers versatility and potential application in many fields of AM research.

- D'Souza and Rodrigues (2013) surveyed seventeen mangrove species belonging to eight families at seven riverine and fringe habitats in Goa, West India, for AM fungal diversity. Maximum root colonization was recorded in *Excoecaria agallocha* (77%) and minimum colonization in *Avicennia marina* (6%). *Paris*-type colonization was predominant while auxiliary cells was recorded in roots of *Acanthus ilicifolius, Ceriops tagal* and *Sonneretia alba*.

- Rodrigues and Rodrigues (2013) studies revealed that the continued development of high quality and low-cost methods for producing AM fungal spores under *in vitro* systems lead to new and advanced methods of large-scale inoculum production of AM fungi. Intra-radical propagules served as a source of high-quality inoculum. The encapsulation of AM fungi produced monoxenically in alginate beads offered an opportunity to diversify inoculation process.

- During an AM fungal spore survey on a primary coastal sand-dune system in Goa on the west coast of India, *entrophosporoid* spores tightly covered with a dense hyphal mantle were recovered. Morphological differences separating *S. felinovii* and *S. baltica* reside in the structure and phenotypic features of spore wall 1. In single-species cultures with *S. scutellarioides* as host plant, *S. felinovii* formed mycorrhiza with arbuscules and intra- and extra-radical hyphae. In the field, *S. felinovii,* the novel fungi, was likely associated with roots of *Z. matrella* (Willis et al., 2016).

- The study by Rodrigues and Rodrigues (2017) assessed the performance of the organic carrier formulation consisting of vermiculite as the main component along with cattle manure, wood powder and wood ash in different proportions and its ability to retain inoculum potential of the *in vitro* produced AM fungal propagules of *Rhizoglomus intraradices* and *Funneliformis mosseae*. The *in vitro* produced propagules of both AM species were viable and effectively colonized the roots of *Eleusine coracana*. Such an attempt indicates a strong possibility for enhancing plant growth and productivity.

- Rodrigues and Rodrigues (2018) showed how to maximize the shelf life of the monoxenically produced carrier-based AM fungal bio-inocula of *Rhizoglomus intraradices* and *Funneliformis mosseae.* The *in vitro* produced inocula stored at 25^0C, remained viable for six months in the organic carrier formulation. 100% germination was recorded when the spores of both AM species were cultured back to *in vitro* conditions, indicating high viability and efficiency of the carrier formulation in maintaining the vigor of *in vitro* produced propagules.

- Velip and Rodrigues (2019) studies exhibit a successful attempt towards *in vitro* culturing and sporulation of *Gigaspora decipiens* in transformed roots of *Linum usitatissimum* (Flax). The present study describes a technique wherein spore germination and *in vitro* root colonization can be brought about in the same Petri plate rather than transferring a prior germinated AM spore among the T-DNA roots. This technique minimizes the effect of relocation of germinating spores, thereby hastening root colonization.

CHAPTER 3:

MATERIALS AND METHODS

CHAPTER 3: MATERIALS AND METHODS

3.1 SOIL SAMPLES

Pure cultures maintained in the Goa University Mycorrhizal Culture Collection (GUMCC), Botany discipline, SBSB, Goa University were used for rhizosphere soil sample collection. 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.

3.2 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 discrioption given by Rodrigues and Muthukumar (2009), Blaszkowski (2012) and the International Collection Of Vesicular Arbuscular Mycorrhizal Fungi (invam.wvu.edu). Names and epithets of AM fungal species were followed according to the recommendation of Schüßler and Walker (2010) and Redecker et al., (2013).

3.3 ISOLATION OF SPORES

AM fungal spore isolation was carried out using the wet sieving and decanting method (Gerdemann and Nicolson, 1963). 20g of rhizosphere soil sample was placed in a beaker, and tap water was added. The soil suspension was stirred continuously using a glass rod, and the sediment was allowed to settle for one minute. After settling down the soil particles, the upper layer of soil suspension is passed through different sieves 250BSS, 150BSS, 75BSS and 56BSS arranged in descending order. The procedure was repeated twice for each sample. The residues from each sieve were then washed into separate beakers. The aliquot was filtered separately

through Whatman No. 1 filter paper. The filter paper was then placed on a Petri plate to ensure that it remained moist. The filter paper was examined for the presence of spores and sporocarps under Olympus stereomicroscope SZ2-ILST (10 x 4.5 zoom).

3.5 PREPARATION OF TRAP CULTURES USING AM FUNGAL SPORES

AM fungal spores were isolated from 20g of rhizosphere soil from the monoxenic pot cultures in Goa University Arbuscular Mycorrhizal Fungus Culture Collection (GUAMFCC). Pot cultures were prepared using sterilized sand and AM inoculum. Cuttings of *Plectranthus scutellarioides* (Coleus) were used as host plant. The plant pots prepared were kept in Goa University Polyhouse and were watered as and when required for 30- 45 days.

3.6 *IN VITRO* ROOT ORGAN CULTURE

Transformed carrot (*Daucus carota*) roots were procured from the Mycorrhiza laboratory, Goa University. Routine maintenance of the roots was made using the sterilized Petri plates containing WM medium kept in an inverted position at room temperature in dark. The composition of the media is given in mg/L of distilled water except for sucrose. The WM media contained of six solutions: the solution one included the macro elements namely $MgSO_4$-$7H_2O$, KNO_3, KCL,$NaH_2PO_4.H_20$, and NaCl. The solution II was $Ca(NO_3)^2.4H_2O$. The solution III consists of KI. The solution IV included the microelements like the $MnSO_4.H_2O$/ $MNCl_2.4H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $CuSO_4.5H_2O$, Na_2Mo_4. $2H_2O$. The solution V included NaFeEDTA. The solution VI was Gamborg's vitamin solution 1000X. Sucrose serves as the carbohydrate source. The pH was adjusted to 5.5 by adding 1N HCl/NaOH before sterilization in autoclave for 15- 30 mins.

3.7 ROOT ORGAN SUBCULTURE

After 13- 20 days of incubation, the Petri plates containing numerous fine root apexes that were straight, white, and turgescent were selected for further sub-culturing on WM media. Under aseptic conditions, the apexes of about 3cm were cut and removed from the gel medium with the help of sterilized forceps and scalpel. Two apexes per Petri plate in a "head to tail" position were placed to favour their growth in opposite direction. The tip of the apexes were placed in such a manner to ensure they were placed slightly into the medium to favour further root growth in the medium and not on its surface. The Petri plates were then sealed and incubated in an inverted position at 27^0C in the dark.

3.8 SPORE STERILIZATION

A solution containing sodium hypochlorite (NaClO) was widely used to disinfect the isolated AM fungal spores. Although five minutes in 4% NaClO solution gave satisfactory results, concentration and treatment duration was altered depending on contaminant levels and spore sensitivity. Ideally, spores were gently agitated during sterilization. Spores were subsequently double rinsed in sterilized distilled water and finally in a streptomycin sulfate (0.02% w/v) antibiotic solution for 10-15 minutes (Bécard and Fortin, 1988). To reduce the risk of contamination by bacteria or fungi that were not eliminated during the sterilization process, the spore number was kept limited in each Petri dish (Fortin and Becard, 2001). The Sterilized AM fungal spores were placed in Petri dish containing plain agar water media for its further germination. These Petri plates were incubated in inverted position at room temperature and were kept in dark.

3.9 ASSESSMENT OF MYCORRHIZAL COLONIZATION:

Two coleus plants were randomly harvested from each pot on 15, 30, 45 and 60 days after inoculation. The roots were cut to one cm length and were used for estimating AM colonization by root clearing and staining technique (Phillips and Hayman 1970).

3.9.1 Tryphan blue (Phillips and Hayman, 1970)

The root pieces were boiled in 10% KOH solution to soften the tissues. After repeated washing with water, they were neutralized with 2% hydrochloric acid for about 5 to 10 minutes. The cleared root segments were stained in 0.05 % Typhan blue, and the root bits were kept in the stain for 48 hours. The roots were examined for AM colonization (Srinivasan et al., 2014).

3.9.2 Histochemical staining using Sudan Black stain (Mc Gee-Russell and Smale, 1963)

The root pieces were boiled in 10% KOH solution at 90^0C for 30-45 minutes in a well-ventilated exhaust hood to soften the tissues. After repeated washing with tap water to remove the traces of KOH, they were acidified with 1N hydrochloric acid for about 5 to 10 minutes. The cleared root segments were stained in Sudan Black, and the root bits were kept in the stain for 1-5mins. The roots were examined for lipid bodies which are stained bluish- black.

3.9.3 Histochemical staining using TBO (Toluidine Blue O) by Kumble and Kornberg, 1996.

The root pieces were boiled in 10% KOH solution at 90°C for 30-45 minutes in a well-ventilated exhaust hood to soften the tissues. After repeated washing with tap water to remove the traces of KOH, they were acidified with 1N hydrochloric acid for about 5 to 10 minutes. The cleared root segments were stained in Toluidine blue O for 20 minutes. The roots were examined for poly- P granules which were stained purplish- pink.

CHAPTER 4:

RESULTS, DISSCUSSION AND CONCLUSION

4.1 RESULTS

4.1.1 TRAP CULTURE

Trap culture of AM fungal spores isolated from the dunes were maintained as cultures in the poly-house of GUAMFCC. After 45 days the cultures were checked for colonization using Tryphan blue (Phillips and Hayman, 1970). The presence of hyphal and vesicular colonization was observed **(Plate 2a, b)**.

The presence of intra-radical spores and polyphosphate granules **(Plate 2c, d)** were also observed by staining the root fragments using Sudan black (McGee-Russell and Smale, 1963) and Toludine blue O (Kumble and Kornberg, 1996).

4.1.2 AM FUNGAL SPORE MORPHOLOGY AND IDENTIFICATION

With the help of 'The international Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM)' and the manual of 'Glomeromycota by Janusz Blaszkowski', the spore morphology of the isolated AM fungal spores was studied and identified.

Table 4.1. Classification of the AM fungal spores:

	Claroideoglomus etunicatum W.N. Becker & Gerd.	*Cetraspora pellucida* (T.H. Nicolson & N.C. Schenck) C. Walker & F. E. Sanders
KINGDOM	Fungi	Fungi
PHYLUM	Glomeromycota	Glomeromycota
CLASS	Glomeromycetes	Glomeromycetes
ORDER	Diversisporales	Diversisporales
FAMILY	Claroideoglomeraceae	Gigasporaceae
GENUS	*Claroideoglomus*	*Cetraspora*
SPECIES	*etunicatum*	*pellucida*

a. *Claroideoglomus etunicatum*

Based on the following characteristics the AM fungi isolated from GUAMFCC culture pots was identified and confirmed to be *Claroideoglomus etunicatum* **(Plate 1e)**.

The spores were found singly in the soil. The colour of the AM spore ranges from orange to reddish brown. The shape was globose to subglobose. The spore wall consisted of two layers, an outer mucilagenous layer and a second layer consisting of thin adherent sublayers. The spore contents were separated from attached hypha by a thin curved septum.

b. *Cetraspora pellucida*

Based on the following characteristics the AM fungal species isolated from the dunes was confirmed to be *Cetraspora pellucida* **(Plate 1f)**.

Cetraspora pellucida previously assigned as *Scutellospora pellucida* (Oehl et al., 2008). The spores were found singly in the soil, and formed terminally on a bulbous subtending hypha, hyaline to yolk yellow, globose to sub-globose. Subcellular structures of the spores consists of a spore wall and two inner walls. Spore wall composed of two layers. Layer one is smooth and hyaline while layer two is also hyaline to yolk yellow.

4.1.2 STANDARDIZATION OF WHITES MEDIA (WM) AND MAINTAINING THE ROC CULTURES

Modified MW with 15g sucrose and with pH 6.6 was found suitable for growing the Ri T DNA carrot roots. The root growth occurred 4-5 days after inoculation and was found to be suitable for further sub-culturing. The ROC Petri plates were kept inverted in the dark at room temperatures. The roots were sub-cultured after every 20- 25 days **(Plate 3b).**

4.1.3 SPORE STERILIZATION

The isolated spores were surface sterilized by treating with 4% NaClO for 5 minutes. This concentration was most suitable for the optimum germination of the spores **(Plate 3a).** Any increase in treatment time, affected the germination. After treatment with NaClO, the spores were washed twice with distilled water (DW). Further, the spores were rinsed with 0.02% streptomycin. The spores were then placed in the plain agar water, kept inverted in the dark at room temperature for germination.

4.1.4 SPORE GERMINATION

Claroideoglomus etunicatum spores recorded germination within four days of plating **(Plate 4a)**. Pawlowska et al., (1999) observed *in vitro* spore germination in *C. etunicatum* 4 to10 days after plating. The germination always occurred through the subtending hypha.

Cetraspora pellucida spores recorded germination after 29 days of plating **(Plate 4b)**. Spores of both the AM fungal species showed better germination on plain agar water compared to other media. Hyphal branching and septate hypha was prominently observed **(Plate 4c, d).**

4.1.5 SPORE COLONIZATION

The germinated spores were transferred on the ROC Petri plates and were kept undisturbed in dark conditions at room temperatures **(Plate 3c)**. The germ tubes showed branching and moving towards the root **(Plate 4e)**. Knobby type of auxiliary cells and septate hyphal growth were observed **(Plate 4d).**

Root colonization by *Cetraspora pellucida* occurred after 12 days after transferring the germinated spores in the Petri plate containing actively growing Ri T-DNA carrot roots. This was confirmed by Trypan blue staining method (Phillips and Hayman, 1970) **(Plate 5)**.

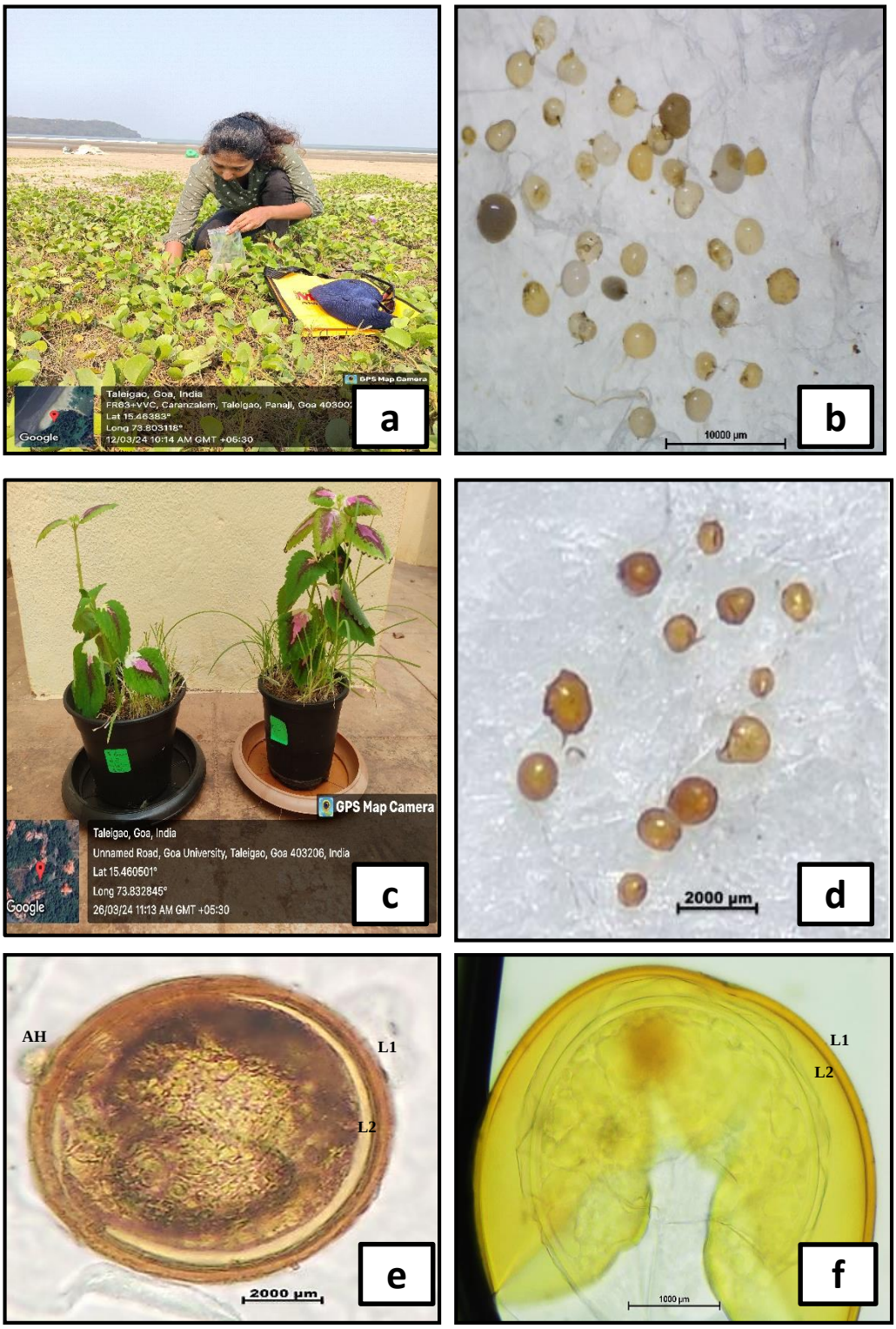

Plate 1: AM fungal spore isolation, trap cultures and identified AM spores: **a.** Collection of dune rhizosphere soil samples; **b.** AM fungal Spores isolated from dune rhizosphere soil samples; **c.** Trap cultures; **d.** AM fungal spores isolated from Goa University Arbuscular Mycorrhizal Fungus Culture Collection (GUAMFCC); **e.** *Claroideoglomus etunicatum* spore; **f**. *Cetraspora pellucida* spore.

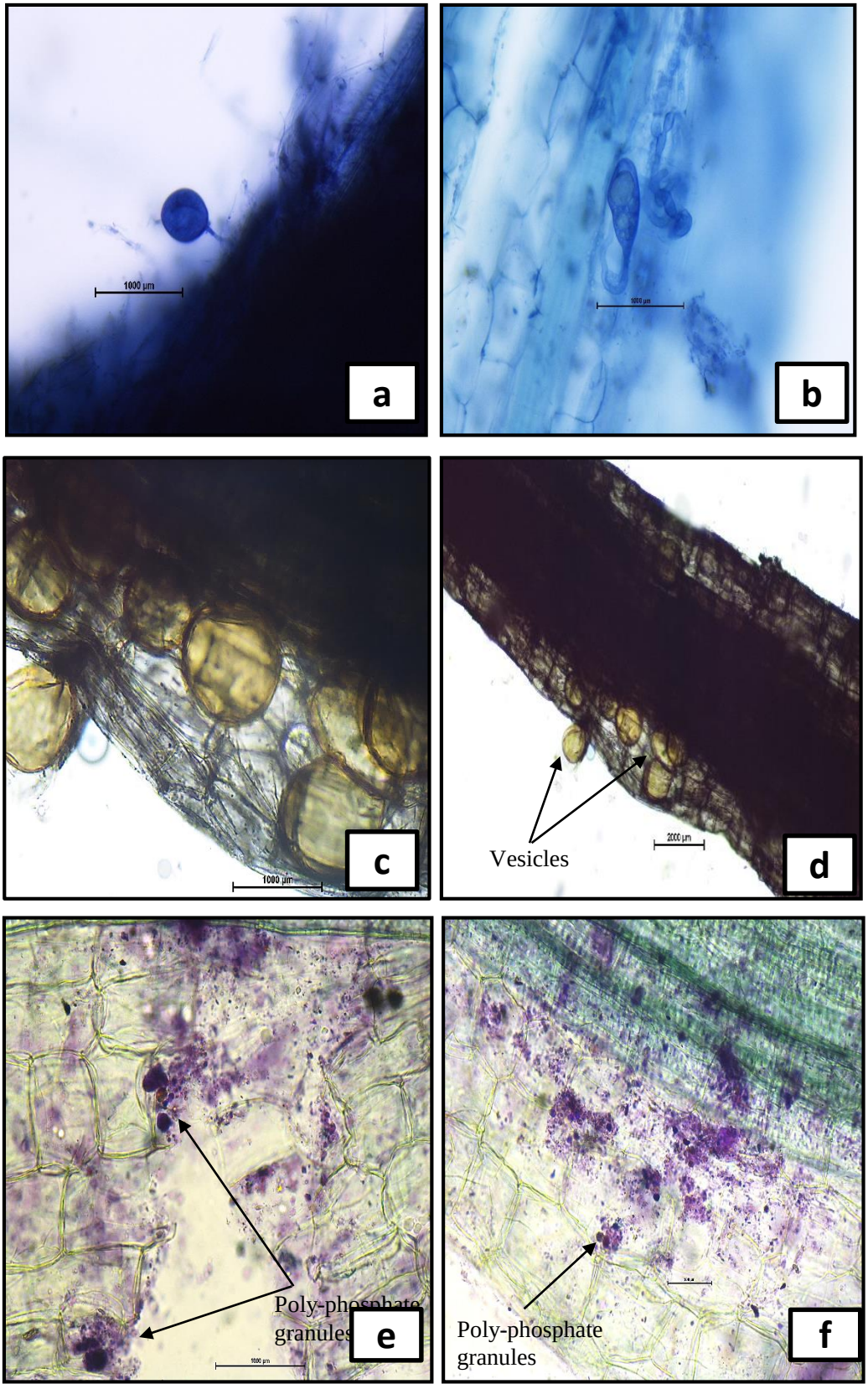

Plate 2: AM fungal root colonization in *Plectranthus scutellarioides and Eleucine coracana*: a & b. Vesicles; **c, d.** Intra-radical spores; **e, f**. Poly-phosphate granules.

Plate 3: AM fungal spore germination and establishment of dual cultures a: AM fungal spores on plain agar water; **b:** Culture of Ri T-DNA carrot roots on WM medium; **c:** Transferring germinated AM fungal spore in Petri plate containing Ri T-DNA carrot roots.

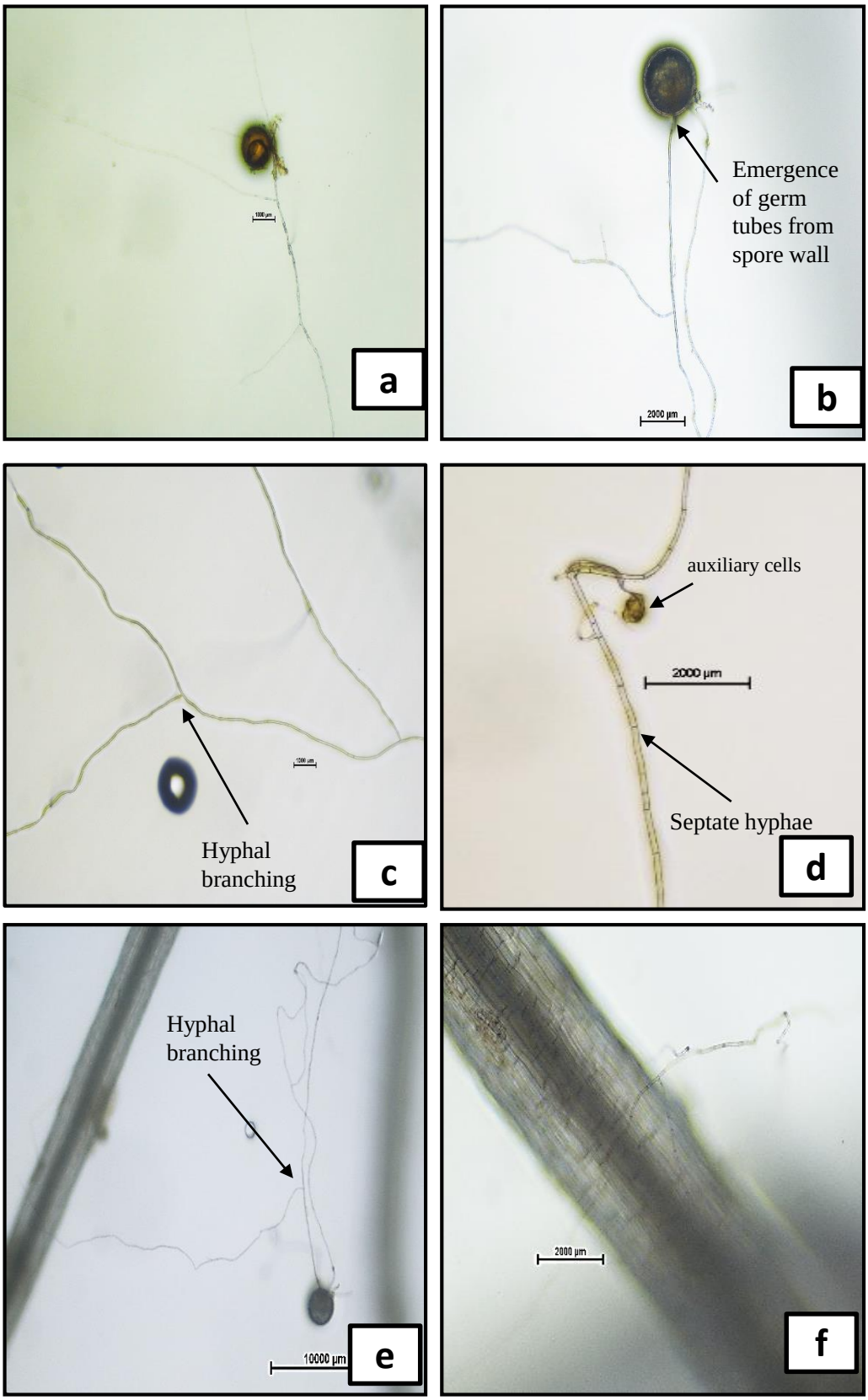

Plate 4: Micrographs of *in vitro* germination: a. *Claroideoglomus etunicatum* spore germination; **b.** *Cetraspora pellucida* spore *g*ermination on plain agar water; **c.** Hyphal branching; **d.** Auxiliary cells and Septate hyphae; **e & f.** Hyphal proliferation and entry in the Ri T-DNA carrot roots.

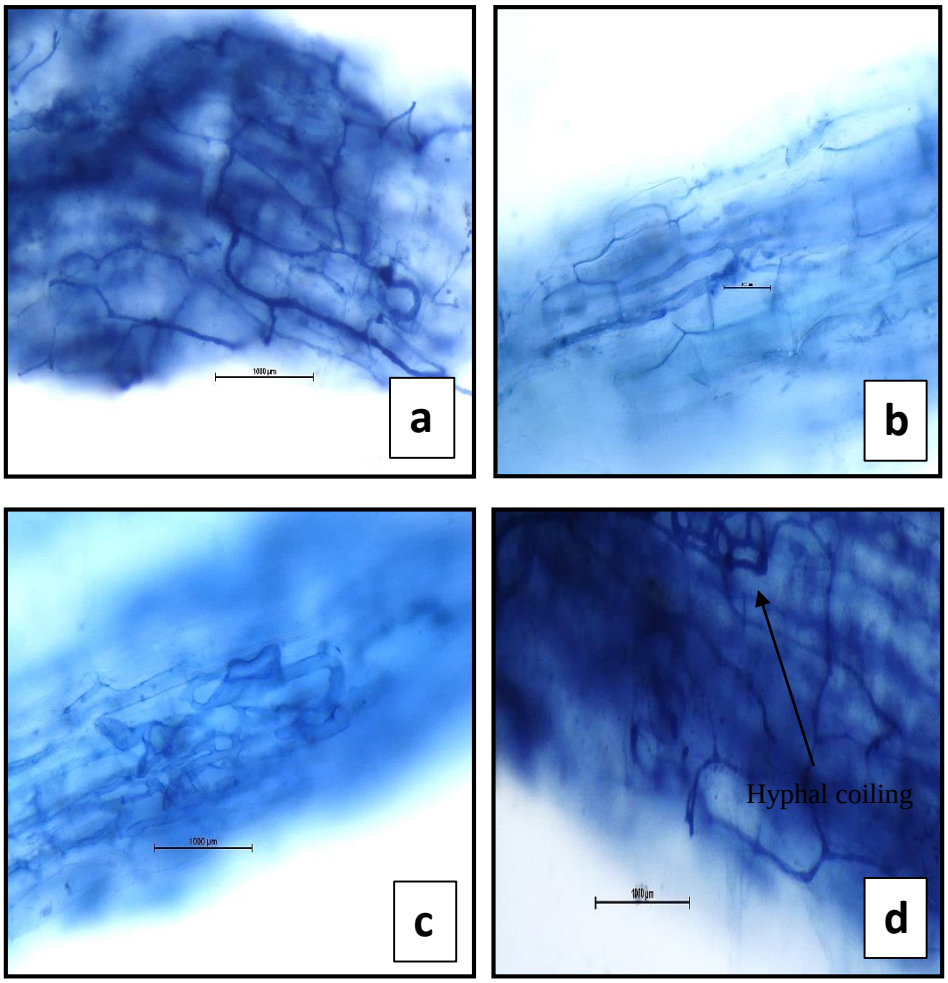

Plate 5: *In vitro* root colonization: a, b. *Cetraspora pellucida* hyphal colonization in roots; **c. .** Hyphal swelling; **d.** Hyphal coils.

4.2 DISSCUSION

In vitro cultivation of AM fungi has the capacity to provide high quality, contaminant-free AMF inoculums for research into potential sources of bio-fertilizers (Kumar and Yadav, 2019). It is well known that mycorrhiza are obligate symbiosis fungi, and therefore the excised root required as host partner of AM symbiosis was first suggested by Mosse and Hepper (1975). Gerdemann and Nicolson (1963) describe isolation of healthy AM spores from pot culture using the wet sieving method. Fresh, healthy mycorrhizal spores can be isolated from approximately 100 g of rhizospheric soil from trap culture. Monoxenic culture of AM fungi provides substantial quantity of contamination-free fungal material that is suitable for taxonomic and evolutionary studies (Fortin et al., 2002).

In the present study, the host plants like *Coleus* and Finger millet used for maintaining the trap cultures helped in studying the colonization pattern *in vivo* conditions. The colonization in the prepared Trap cultures was studied by staining techniques and the same staining techniques were used for studying colonization *in vitro* conditions. Studies by Kumar and Yadav (2019) have shown that *in vitro* culture is an efficient and reliable artificial system which is also a suitable method for investigating basic, practical aspects of AM symbiosis and complements experimental approaches.

The minimal (M) medium (Bécard and Fortin 1988) and the modified Strullu Romand (MSR) medium (Strullu and Romand 1986, modified by Declerck et al 1998) are the two media which are frequently used to culture AM fungi on ROC. While this medium has been widely used for the study of AM fungi, it appears unsuitable for the culture of several other AM fungal species (Douds 1997). In the present study, Ri T-DNA roots were actively growing in

the modified WM. It was suitable for further sub-culture on fresh WM and thus the media was standardized.

Some AM fungal spores are collected from 300BSS, while larger spores such as *Gigaspora margarita* can be obtained on 60 BSS fractions (Kumar and Yadav, 2019). However, the identified AM fungal species, *Cetraspora pellucida* and *Claroideoglomus etunicatum*, were isolated from the sievates collected from 250BSS and 75BSS respectively.

The concentration of 4% NaClO for surface sterilizing the AM fungal spores was standardized. This ensured minimal contamination and spore viability. The AM fungal spores were found germinating actively on plain agar. *Cetraspora pellucida* colonized the Ri T-DNA roots after several days of its germination and Plating into the ROC Petri plates. However, no sporulation was observed yet after six weeks of colonization.

According to De Souza and Declerck (2003) *Scutellospora reticulalta* AM fungal spore germination occurred within 25 days, and the first germination was observed three days after incubation on the water-agar medium. After one month, the germination rate was 59%. The germination tube exhibited negative geotropism after 2-3 days. While Schreiner and Koide (1993) reported the formation of hyphal networks close to the roots, and appressoria on the root surface for *Claroideoglomus etunicatum* spores germinated near Ri T-DNA-transformed carrot roots.

4.3 CONCLUSION

The results of this study has provided data on AM fungal spore germination and colonization *in vitro.* From the present study, it can be concluded that the identified AM fungal spores can be cultured *in vitro* conditions and can be used for mass production of the inoculum. It can be further used to prepare carrier inoculum and used as bio-inoculant in agriculture, horticulture and forestry.

CHAPTER 5:

REFERENCES

CHAPTER 5: REFERENCES

Bécard, G., & Fortin, J. A. (1988). Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New phytologist*, 108(2), 211-218.

Bever, J. D., & Morton, J. (1999). Heritable variation and mechanisms of inheritance of spore shape within a population of *Scutellospora pellucida*, an arbuscular mycorrhizal fungus. *American journal of botany*, *86*(9), 1209-1216.

Bi YinLi, B. Y., Li XiaoLin, L. X., Wang HongGang, W. H., & Christie, P. (2004). Establishment of monoxenic culture between the arbuscular mycorrhizal fungus *Glomus sinuosum* and Ri T-DNA-transformed carrot roots.

Bianciotto, V., Victorino, I., Scariot, V., & Berruti, A. (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: Current role and potential for the horticulture industry. In III International Symposium on Woody Ornamentals of the Temperate Zone 1191 (pp. 207-216).

Bukhari, M. J., & Rodrigues, B. F. (2008). Effect of Arbuscular Mycorrhizal Fungi on growth on *Artocarpus heterophyllus* and *Syzygium cumini* seedlings in iron ore mine rejects.

Calvet, C., Camprubi, A., Pérez-Hernández, A., & Lovato, P. E. (2013). Plant growth stimulation and root colonization potential of *in vivo* versus *in vitro* arbuscular mycorrhizal inocula. *HortScience*, *48*(7), 897-901.

Chabot, S., Bécard, G., & Piché, Y. (1992). Life cycle of *Glomus intraradix* in root organ culture. Mycologia, 84(3), 315-321.

Cranenbrouck, S., Voets, L., Bivort, C., Renard, L., Strullu, D. G., & Declerck, S. (2005). Methodologies for *in vitro* cultivation of arbuscular mycorrhizal fungi with root organs. *In vitro culture of mycorrhizas*, 341-375.

Dalpé, Y., & Declerck, S. (2002). Development of *Acaulospora rehmii* spore and hyphal swellings under root-organ culture. Mycologia, 94(5), 850-855.

Declerck, S., Strullu, D. G., & Plenchette, C. (1996). *In vitro* mass-production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycological Research, 100(10), 1237-1242.

Declerck, S., Strullu, D. G., & Plenchette, C. (1998). Monoxenic culture of the intraradical forms of *Glomus sp.* isolated from a tropical ecosystem: a proposed methodology for germplasm collection. *Mycologia*, *90*(4), 579-585.

De Novais, C. B., Sbrana, C., da Conceição Jesus, E., Rouws, L. F. M., Giovannetti, M., Avio, L., & de Faria, S. M. (2020). Mycorrhizal networks facilitate the colonization of legume roots by a symbiotic nitrogen-fixing bacterium. *Mycorrhiza*, 30(2), 389-396.

Diop, T. A. (2003). *In vitro* culture of arbuscular mycorrhizal fungi: advances and future prospects. *African Journal of Biotechnology*, *2*(12), 692-697.

Diop, T. A., Bécard, G., & Piché, Y. (1992). Long-term *in vitro* culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA transformed roots of carrot. Symbiosis.

D'Souza, J., Rodrigues, K. M., & Rodrigues, B. F. (2013). Modified Strullu and Romand (MSR) medium devoid of sucrose promotes higher *in vitro* germination in *Rhizophagus irregularis.*

Danesh, Y. R., Kariman, K., Keskin, N., & Najafi, S. (2022). Characterization of arbuscular mycorrhizal fungal communities associated with vineyards in north western Iran. *Turkish Journal of Agriculture and Forestry,* 46(3), 271-279.

Derkowska, E., Paszt, L. S., Harbuzov, A., & Sumorok, B. (2015). Root growth, mycorrhizal frequency and soil microorganisms in strawberry as affected by bio-preparations. *Advances in Microbiology*, 5(1), 65-73.

Douds Jr, D. D. (1997). A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA-transformed carrot roots. *Mycorrhiza*, 7(2), 57-61.

Fortin, J. A., Bécard, G., Declerck, S., Dalpé, Y., St-Arnaud, M., Coughlan, A. P., & Piché, Y. (2002). Arbuscular mycorrhiza on root-organ cultures. *Canadian journal of Botany*, 80(1), 1-20.

Garcia, S. S. P., Suarez, K. F., José, E., Ortega, P., Perez, Y. M., Perez, R. P., & Rodríguez, Y. *In Vitro* Propagation of a Cuban Arbuscular Mycorrhizal Fungal Strain.

Garriock, M. L., Peterson, R. L., & Ackerley, C. A. (1989). Early stages in colonization of *Allium porrwn* (leek) roots by the vesicular—arbuscular mycorrhizal fungus, *Glomus versiforme. New phytologist*, 112(1), 85-92.

Giovannetti, M., & Sbrana, C. (1998). Meeting a non-host: the behaviour of AM fungi. *Mycorrhiza*, 8(3), 123-130.

Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A., & Giovannetti, M. (2020). Arbuscular mycorrhizal fungi and associated microbiota as plant bio-stimulants: research strategies for the selection of the best performing inocula. *Agronomy*, *10*(1), 106.

Goto, B. T., Silva, G. A., Assis, D., Silva, D. K., Souza, R. G., Ferreira, A. C., ... & Oehl, F. (2012). *Intraornatosporaceae* (Gigasporales), a new family with two new genera and two new species. *Mycotaxon*, 119(1), 117-132.

Jakobsen, I., & Rosendahl, L. (1990). Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytologist*, 115(1), 77-83.

Karandashov, V., Kuzovkina, I., Hawkins, H. J., & George, E. (2000). Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. *Mycorrhiza,* 10, 23-28.

Klironomos, J. N., & Hart, M. M. (2002). Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza*, 12, 181-184.

Kokkoris, V., & Hart, M. (2019). *In vitro* propagation of arbuscular mycorrhizal fungi may drive fungal evolution. *Frontiers in Microbiology*, 10, 484727.

Kokkoris, V., Miles, T., & Hart, M. M. (2019). The role of *in vitro* cultivation on asymbiotic trait variation in a single species of arbuscular mycorrhizal fungus. *Fungal biology,* 123(4), 307-317.

Kumar, S., & Yadav, S. (2018). *In vitro* cultivation of AMF using root organ culture: factory of bio-fertilizers and secondary metabolites production. *Microbial Cell Factories*, 95-108.

Labour, K., Jolicoeur, M., & St-Arnaud, M. (2003). Arbuscular mycorrhizal responsiveness of *in vitro* tomato root lines is not related to growth and nutrient uptake rates. *Canadian journal of botany*, *81*(7), 645-656.

Miller, R. M., & Jastrow, J. D. (2000). Mycorrhizal fungi influence soil structure. Arbuscular mycorrhizas: physiology and function, 3-18.

Mosse, B. (1962). The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. *Microbiology*, 27(3), 509-520.

Mosse, B., & Hepper, C. (1975). Vesicular-arbuscular mycorrhizal infections in root organ cultures. *Physiological plant pathology*, 5(3), 215-223.

Muthukumar, T., Radhika, K. P., Vaingankar, J., D'Souza, J., Dessai, S., & Rodrigues, B. F. (2009). Taxonomy of AM fungi-an update.

Nayak, S., Behera, S., Dash, P.K. (2019) Potential of microbial diversity of coastal sand dunes: need for exploration in Odisha coast of India. *The scientific world Journal*, 2019.

Nwoko, C. O. (2014). Effect of arbuscular mycorrhizal (AM) fungi on the physiological performance of *Phaseolus vulgaris* grown under crude oil contaminated soil. J. *Geosci. Environ. Prot*, 2, 9-14.

Oehl, F., Silva, G. A. D., Goto, B. T., & Sieverding, E. (2011). *Glomeromycota*: three new genera and glomoid species reorganized. *Mycotaxon,* 116(1), 75-120.

Oehl, F., de Souza, F. A., & Sieverding, E. (2008). Revision of *Scutellospora* and description of five new genera and three new families in the arbuscular mycorrhiza-forming Glomeromycetes. *Mycotaxon*, 106(1), 311-360.

Olsson, P. A., Thingstrup, I., Jakobsen, I., & Bååth, E. (1999). Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. *Soil Biology and biochemistry*, 31(13), 1879-1887.

Otgonsuren, B., & Lee, M. J. (2010). A native arbuscular mycorrhizal fungus, *Acaulospora scrobiculata* stimulated growth of Mongolian crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.). *Mongolian Journal of Biological Sciences*, 8(2), 33-41.

Pawlowska, T. E., Douds, D. D., & Charvat, I. (1999). *In vitro* propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. *Mycological Research*, 103(12), 1549-1556.

Radhika, K. P., Willis, A., & Rodrigues, B. F. (2009). Emergence of germ tube from germination shield in *Scutellospora verrucosa* Walker & Sanders. *Journal of Mycology and Plant Pathology*, 39(2), 317.

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S. L., Morton, J. B., & Walker, C. (2013). An evidence-based consensus for the classification of arbuscular mycorrhizal fungi *(Glomeromycota*). *Mycorrhiza,* 23, 515-531.

Riker, A. J., Banfield, W. M., Wright, W. H., Keitt, G. W., & Sagen, H. E. (1930). Studies on infectious hairy root of nursery apple trees. J. Agric. Res. 41: 507–540.

Rillig, M. C., Wright, S. F., Nichols, K. A., Schmidt, W. F., & Torn, M. S. (2001). Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. Plant and Soil, 233, 167-177.

Rodrigues, B. F., & Jaiswal, V. (2008). Effect of disturbance on Arbuscular Mycorrhizal (AM) fungal population in coastal sand dune vegetation of Goa, India. Mycol Plant Pathol, 155-168.

Rodrigues, K. M., & Rodrigues, B. F. (2013). *In vitro* cultivation of arbuscular mycorrhizal (AM) fungi. J Mycol Pl Pathol 43(2): 155-168.

Rodrigues, K. M., & Rodrigues, B. F. (2017). Development of carrier based *in vitro* produced arbuscular mycorrhizal (AM) fungal inocula for organic agriculture. *Annals of Advanced Agricultural Sciences*, 26-37.

Rodrigues, K. M., & Rodrigues, B. F. (2018). Maximizing the shelf life of monoxenically produced carrier-based am fungal bio-inocula for maintaining its long-term viability. *Journal of Phytology*, 43-48.

Ron, M., Kajala, K., Pauluzzi, G., Wang, D., Reynoso, M. A., Zumstein, K.,& Brady, S. M. (2014). Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. *Plant physiology,* 166(2), 455-469.

Rosikiewicz, P., Bonvin, J., & Sanders, I. R. (2017). Cost-efficient production of *in vitro Rhizophagus irregularis*. *Mycorrhiza*, *27*, 477-486.

Saito, M. (1995). Enzyme activities of the internal hyphae and germinated spores of an arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. *New Phytologist*, *129*(3), 425-431.

Silvani, V. A., Statello, M., Scorza, M. V., Pergola, M., Colombo, R. P., & Godeas, A. M. (2019). A novel *in vitro* methodology to cultivate arbuscular mycorrhizal fungi combining soil and synthetic media. *Symbiosis*, *79*, 163-170.

Siqueira, J. O., Hubbell, D. H., & Schenck, N. C. (1982). Spore germination and germ tube growth of a vesicular-arbuscular mycorrhizal fungus *in vitro*. Mycologia, 74(6), 952-959.

Srinivasan, M., Kumar, K., Kumutha, K., and Marimuthu, P. (2014). Comparison of Colonization Potential of AM Fungus *Glomus intraradices* Cultured under *In Vitro* and *In Vivo* Condition. *Trends in Biosciences* 7(3): 437-440.

Staples, R. C., & Macko, V. (1980). Formation of infection structures as a recognition response in fungi. *Experimental Mycology*, 4(1), 1.

Tepfer, D. (1989). Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology, and evolution.

Velip, D. M., & Rodrigues, B. F. (2019). *In vitro* cultivation of *Gigaspora decipiens* using transformed roots of *Linum usitatissimum*. Kavaka, 53, 96-99.

Voets, L., de la Providencia, I. E., Fernandez, K., IJdo, M., Cranenbrouck, S., & Declerck, S. (2009). Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. *Mycorrhiza,* 19, 347-356.

Vimard, B., St-Arnaud, M., Furlan, V., & Fortin, J. A. (1999). Colonization potential of *in vitro*-produced arbuscular mycorrhizal fungus spores compared with a root-segment inoculum from open pot culture. *Mycorrhiza,* 8, 335-338.

Willis, A., Błaszkowski, J., Prabhu, T., Chwat, G., Góralska, A., Sashidhar, B., & Adholeya, A. (2016). *Sacculospora felinovii*, a novel arbuscular mycorrhizal fungal species (*Glomeromycota)* from dunes on the west coast of India. *Mycological Progress*, 15, 791-798.

APPENDIX I:

WM MEDIA COMPOSITION AND CONCENTRATION

Solution I: Macro- Elements for 1L (100X), use Nano-Pure water	**WM Medium**
Chemicals	
MgSO4-7H2O (Magnesium Sulfate)	73.1 g
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