In vitro culture of Arbuscular Mycorrhizal Fungal spore using root organ culture technique.

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PREFACE

It is with great pleasure that I present my thesis on "*In vitro* culture of AM fungi *Funneliformis geosporum* using Ri T-DNA transformed roots". As we know, AM fungi are essential for the plant growth and for nutrient uptake, especially phosphorous from the soil. The unique features of AM fungi are the formation of vesicles and arbuscules in the plant root. These beneficial microorganisms can be utilized for enhancing agriculture, ecological restoration, research, and soil structure. Hence, this topic came about through my interest and curiosity in AM fungi.

The aim of my thesis is to explore the morphological characteristics and colonization pattern of AM fungi in transformed roots, shedding light on their symbiotic relationships with plants and their ecological significance. Through meticulous experimentations, standardization and analysis, this study seeks to deepen our understanding of AM fungi biology and their roles in sustainable agriculture and environmental remediation. Each section of this thesis builds upon the previous one to provide an overall examination of the topic, from theoretical foundations to experimental methodology and results. I hope this work serves as a valuable contribution to the field of mycorrhizal research in future.

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ABBREVATION USED

Entity	Abbreviation
Arbuscular mycorrhiza	AM
Modified Stullu-Romand	MSR
Polyvinyl alcohol- lactic acid-glycerol	PVLG
Root organ culture	ROC
White's Media	WM

ABSTRACT

Arbuscular Mycorrhizal fungi form symbiotic association with plant roots which helps in plant growth and accumulation of nutrients. *In vitro* culture of AM fungi in transformed root culture allows better observations and long-term experimentations on the life cycle of AM fungi as well as offers production of high quality and large-scale inoculum production.

This study presents *in vitro* cultivation of AM fungi species *Funneliformis geosporum* using root organ culture technique on Ri T-DNA transformed carrot (*Daucus carota* L.) roots. The WM media was standardized for the sub culture of Ri T-DNA transformed roots. The concentration and sterilization time were also standardized for disinfecting the spores. The disinfected spores were germinated on MSR media without sucrose, which was then transferred to Petri plate containing Ri T-DNA transformed roots. The successful *in vitro* germination of *F. geosporum* and colonization in Ri T-DNA roots was achieved.

CHAPTER 1: INTRODUCTION

Mycorrhiza is an intimate and mutually beneficial association between a non-pathogenic fungus and living root cells, which absorbed nutrients from the soil primarily epidermal and cortical cells. The fungus is released to the host cells which absorbed nutrients from the soil and in turn the fungus receives its organic nutrients from the host plant. The mycorrhizae assist plants to mainly absorb phosphates from the soil.

Mycorrhizas are commonly divided into ectomycorrhiza and endomycorrhiza. Endomycorrhiza mycelium is made up of aseptate hyphae which penetrate the root and form arbuscules and vesicles. In ectomycorrhiza, the hypha of fungi does not penetrate individual cells with in the root. A thick fungal sheath develops around the terminal lateral branches of roots and is connected to an intercellular network of hyphae known as the “Hartig net” in the root cortex.

1.1 Arbuscular Mycorrhizal (AM) Fungi and AM development

AM fungi (Arbuscular Mycorrhizal) are ubiquitous soil fungi belonging to phylum Glomeromycota that form symbiotic association with plant roots. AM fungi help in increasing plant growth and accumulation of nutrients mainly P, N, Ca, Fe, Mn, Zn, Cu and S through greater soil exploration by mycorrhizal hyphae. They also help plant to increased resistance against biotic and abiotic stress such as drought, salt and heavy metal.

They are obligate symbionts which form association with living plant root to complete its life cycle. AM fungi penetrates into plant through cell walls of the root and develops intra-radical

structures i.e., hyphae, arbuscules, vesicles in the cortical cells of the host root and extra-radical structures i.e., hyphae and spores in soil.

1.2 Arbuscules and vesicles

Arbuscules are repeated dichotomously branched haustoria like structures which grow intra-cellularly and are involved in interchange of nutrients between host plant and fungus. Vesicles are thin or thick-walled oval structures produced intra-cellularly and store materials like lipids, polyphosphate and other minerals.

The importance of the AM fungi to agricultural and forest plant species reside it help in plant growth and nutrition. In tropical forests incidence of mycorrhizae profoundly influence soil fertility and thus the growth and development of plants (Bagyaraj, 1989). Mycorrhizal fungal hyphae extend into the soil, penetrating into nutrient depletion zone and increased the effectiveness of immobile elements by as much as sixty times. Direct benefits are usually related to the enhancement of phosphate uptake by the plant. However, in some soils zinc, copper and ammonium are also important (Stribley, 1987). Indirect benefits may include increased soil aggregation or stabilization of soil associated with hyphae formed in the soil.

For the identification of AM fungi morphological characters play an important role in establishing the taxonomic identity and relationships of AM species such as sporocarp (size, shape, peridium), spore (colour, shape, size, content), spore wall (colour, dimension, number, ornamentation), subtending hypha (shape, width), mycorrhizal anatomy (hyphal characters, intraradical spores), spore germination (direct, indirect).

For isolation of AM fungal spores, the wet sieving and decanting method is mostly used. (Gerdemann and Nicolson 1963).

1.3 Mass production of AM fungi

The three major systems used in the mass production of AM are substrate-based production system, substrate free production system and *in vitro* production system.

In substrate-based production system the plants and their associated symbionts are cultivated in soil-based substrate which allows the initial production of AM fungal inocula. This inoculum is propagated for the mass multiplications by using a single species or an identified AM fungal species in clay or soil under controlled condition (Gaur and Adholeya, 2002). This system helps in preservation of mass production of AM fungi. Also, supply of nutrient can be easily monitor properly but there are chances of contamination.

In Substrate free production system, the nutrient solution is aerated using an aeration pump to avoid the roots suffering from oxygen deprivation. The pump is periodically switched on to minimize the flow of nutrient solution which can create air bubbles which damage the expansion of radical hyphae. The disadvantage of this system is spore production rates can be affected due to lack or less carrier substrate and contamination.

In-vitro production system is the most widely used for mass production of AM fungi. Mosse and Hepper established the first *in vitro* culture of AM fungi in year 1975 by using the dual culture of *Glomus mosseae* spores with excised roots of clover. These *in vitro* cultures consist of genetically modified roots that grow without a shoot and grown in sterile condition forming symbiosis. In 1988, the root organ system was introduced by Becard and Fortin using T-DNA transformed root of *Daucus carota* which more closely resemble a “normal” root system were colonized more readily than 9-day-old roots.

St-Arnaud *et al.,* (1996) used spilt-plate method which increase the production of propagules and facilitate the access of AM fungi. Tiwari and Adholeya (2003) used root organs culture in small containers, by which large-scale production of AM fungi was obtained. Also, Jolicoeur *et al.,* (1999), performed large scale cultivation of AM fungi in an airlift bioreactor and in a mist bioreactor with perlite as the substrate (1998) or in a bioreactor containing solid medium (Fortin *et al.,* 1996). When medium was partially replaced from the distal compartment and glucose was provided to the proximal compartment, the AM fungus continued sporulation by using split-plate method (Douds 2002). Declerck *et al.* (2009) developed the *in vitro* system in which the pre-inoculated plants produced individually introduced AM fungal inoculum in sterile growth tube in a closed system running with nutrient solution.

The most important advantage of *in vitro* cultivation system is that, it is more suitable for large-scale production of high-quality of AM fungal inoculum because of the absence of undesirable micro-organisms. It required regular monitoring and regulating cultures. 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). 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 (Strullu and Romand, 1986, modified by Declerck *et al.,* 1998).

MSR medium lacking sucrose promoted higher germination rates in *Rhizoglomus irregularis* (D'Souza *et al.,* 2013). Stunted BAS, together with typical *Paris*-type root colonization, characterize *F. caledonium* isolates (Karandashov *et al.,* 2000).

1.4 AIM AND OBJECTIVE

1. Preparation of Trap culture and it's multiplication.

2. To study the spore morphology of the AM species.

3. To study the colonization pattern and sporulation of the respective AM species.

4. To study the *in vitro* culture of the AM species.

CHAPTER 2: LITERATURE REVIEW

Pawlowski *et al.* (1999) established monoxenic cultures of *Glomus etunicatum* in association with excised Ri T-DNA transformed carrot roots. Their study revealed that the modified White's medium was optimal for the host root growth and for *G. etunicatum* spore germination and mycorrhiza formation.

Karandashov *et al.* (2000) observed sporulation after 1-3 weeks of spore germination in *F. caledonium* grown in dual culture with transformed roots of *D. carota* in M medium.

Gadkar and Adholeya (2000) successfully established *in vitro* culture in G. *margarita* using Ri T-DNA transformed carrot roots. They reported that sporulation observed in the dual culture was a temporal phenomenon.

Tiwari and Adholeya (2002) developed an *in vitro* system using Ri T-DNA transformed carrot roots as the host in AM fungi belonging to *Glomus intraradices* and *Gigaspora margarita*. Both the species showed extensive hyphal proliferation and sporulation.

De Souza and Declerck (2003) reported mycelium development, architecture, and spore production in Scutellospora reticulata from single spore isolates grown with Ri T-DNA transformed carrot root organ culture in the monoxenic system.

Diop *et al.* (2003) described *in vitro* methods to cultivate root organs and to select and purify AM fungal inoculum.

Adholeya *et al.* (2005) developed an *in vitro* system using Ri T-DNA transformed roots and the AM fungus *Gigaspora margarita*. They studied the initial events of mycorrhiza formation. They reported that sucrose, sodium, and P were critical components of the medium to establish the dual culture. They recorded abundant viable and aseptic spores and suggested that the system is especially appropriate for studying the triggering of the fungal biotrophy towards the root.

Kandula *et al.* (2006) reported the cultivation of *Scutellospora calospora* spores on MSR medium using Ri T-DNA transformed *Daucus carota* L. roots. They recorded the morphological characteristics of extra-radical mycelium, intra-radical mycelium, branched absorbing structures, and auxiliary cell formation. They recorded the formation of auxiliary cells of root contact in 3 to 4 days that continued for up to 2 months. They observed that the spores produced on the MSR medium were full of oil globules.

Bidondo *et al.* (2012) successfully developed in *in vitro* culture of *G. decipiens* using transformed roots of *D. carota* on an M medium. They reported sporulation after five months of inoculation in dual culture.

Costa *et al.* (2013) successfully developed in *in vitro* culture of *Gigaspora decipiens* and *Glomus clarus* in the transformed carrot roots. They studied the effect of temperature and pH on the sporulation of both the AM species using an M *medium.* They observed that the spore production increased at 22°C and decreased at 28°C and 32°C. Also, in *G. decipiens,* a reduction in sporulation occurred at pH 6.5, whereas in *G. clarum,* sporulation was maximum at pH 4.0.

Srinivasan *et al.* (2014) conducted comparative studies on colonization potential under *in vitro* and *in vivo* conditions in *Glomus intraradices*. Their study revealed that root colonization potential of *in vitro* produced spores was higher compared to the root segment inoculum.

Rodrigues and Rodrigues (2015), using spores, successfully developed *in vitro* culture of *Funneliformis mosseae* using transformed roots of *Linum usitatissimum L.* on a modified Strullu-Romand medium. Their study reported germ tube growth through the spore wall, arbuscules, hyphal coils, and extra-radical branched absorbing structures (BAS).

Aryal (2017) developed a protocol for *in vitro* propagation of *Glomus mosseae* spores with excised roots of *T. pretense.*

Rodrigues and Rodrigues (2018) worked on maximizing the shelf life of monoxenically produced carrier-based bio-inoculum of two AM fungal species, viz., *Rhizoglomus intraradices* and *Funneliformis mosseae*. The *in vitro* inocula produced in the organic carrier formulation and stored at 25°C remained viable for 6 months. Also, 100 % germination was recorded in both AM species when cultured back to *in vitro* conditions, indicating high viability and efficiency of the carrier formulation in maintaining vigour of *in vitro* produced propagules.

Ellatif *et al.* (2019) studied the effect of phenolic compounds on the production of *Gigaspora gigantea* propagules and root hair growth of transformed tomato seedlings (pH 5.7 and 6.5). Catechin anhydrous showed a significant increase in AM root colonization, while Tannic acid inhibited the growth of hairs and AM colonization. The pH of 5.7 of solid media was found to be suitable.

CHAPTER 3:MATERIALS AND METHODS

3.1 Preparation of trap cultures

Trap cultures of AM fungi were prepared using the modified method of Morton *et al*. (1993). The rhizosphere soil was mixed with sterilized sand in a 1:1 ratio. The mixture was then transferred to 15 cm plastic pots, pre-washed and wiped with absolute alcohol. *Plectranthus scutellarioides* (L.) (Coleus) cutting was used as the host plant. The pots were maintained for 90 days in the greenhouse to establish AM symbiosis and continue the AM life cycle. After 90 days, the pots were left to dry for 1 to 2 weeks, after which the shoot portion was cut off at the soil interface. The pot contents were then transferred in *zip-loc* plastic bags, labelled and stored at 4°C. The spore was then extracted for taxonomic identification and to set up monospecific or pure cultures.

3.2 Preparation of monospecific (single species) cultures

Monospecific cultures of AM species isolated from the trap cultures were prepared following Gilmore (1968). The AM spores from the trap culture were isolated by wet sieving and decanting method (Gerdemann and Nicolson, 1963). After repeated washing, the spores of each single species were isolated and stored in a Petri plate at 4°C. The isolated spores were examined under an Olympus stereo microscope. Before inoculation, any soil particles, hyphal fragments, or other spores were removed. The spores of a similar type were washed with distilled water. Plastic pots were filled with sterilized 1:1 sand soil mix, in which the isolated spores (single species) were placed along with the filter paper at a depth of 2-3 cm, covered with additional soil and planted with *Plectranthus scutellarioides* cuttings in the pots. The pots were maintained in the polyhouse and watered twice a week. After 90 days of growth period, watering was stopped to allow the pot contents to dry. The pot contents were then harvested for analysis of spores.

3.3 Culture media for cultivation of AM propagules and transformed roots

WM media was used to culture transformed roots (Bécard and Fortin, 1988). An MSR media without sucrose was used to cultivate AM propagules (Declerck *et al*.,1998).

3.4 Preparation and standardize the protocol for *In vitro* culture technique for AM fungal species

3.4.1. Isolation of AM fungal spores

Spores were isolated using the wet sieving and decanting method of Gerdemann and Nicolson (1963).

1. 100 g rhizosphere soil was suspended in 1 litre tap water. The mixture was stirred for 10-15 seconds, and the coarse particles were allowed to settle for 3-4 minutes.
2. The supernatant was decanted through a series of sieves arranged in descending order of mesh size (250 µm - 37 µm).

• Steps 1 and 2 were repeated twice to ensure maximum spore recovery from the soil sample.

3. Sievates from each sieve were collected separately in beakers.
4. The supernatant from each beaker was then separately filtered through the Whatman No.1 filter paper.
5. The filter papers are placed in the Petri plate. Care was taken to ensure that they remained moist.
6. The contents of the filter papers were examined for spores and sporocarps under the stereomicroscope.
7. Intact, fresh and healthy spores were isolated and used for inoculum preparation.

3.4.2. AM fungal propagule disinfection process

The isolated spores were rinsed twice with sterilized distilled water. After this, they were disinfected using sodium hypochlorite and streptomycin at 0.02 %. The concentration and

sterilization time varied from species to species and depended on the type of propagules used (spores or colonized root fragments).

Protocol for disinfection of spores

- The stereomicroscope along with other requirements, were kept in laminar air flow chamber, sterilization was done using UV light for 30 min.
- The working bench of the laminar airflow and hands were sterilized using alcohol.
- 5 ml of sterilized distilled water was added to small sterilized glass Petri plates.
- The isolated AM spore was transferred to a small sterilized Petri plate using the sterilized forceps and micropipette. A solution of sodium hypochlorite was added to this.
- The spores were disinfected with sodium hypochlorite for 2-5 min with continuous swirling.
- Then, disinfected spores are twice rinsed with sterilized distilled water for 5-10 minutes with constant swirling.
- The spores were then treated with an antibiotic (streptomycin) for 1-2 minutes.

3.4.3. Germination of disinfected propagules

For the disinfection process, the modified method of Bécard and Fortin (1988) was followed.

- The spore was inoculated on Modified Strullu-Romand (MSR) medium (pH 5.5) without sucrose.
- The inoculated Petri plate was then sealed with parafilm.
- Various details about AM species, date of inoculation and concentration of sodium hypochlorite were noted.

- The Petri plate was incubated in the dark at 27°C in an inverted position.
- After every second day, the inoculated Petri plate was checked for spore germination.

3.4.4. Establishment of *in vitro* culture

● The stereomicroscope was kept in a laminar airflow chamber and sterilized using a UV light for 30 min.

● Hands were washed using alcohol.

● Using a sterilized needle, a clarigel plug along with the germinated AM propagule was placed in the vicinity of actively growing Ri - DNA transformed roots.

● The Petri plate was then incubated in the dark in an inverted position at 27°C.

● The transferred roots were procured from Prof. B.F. Rodrigues, Discipline of Botany, SBSB, Goa University.

3.4.5. Processing of root segments for AM fungal colonization

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

Standard protocol for assessment of AM fungal colonization

■ Roots were gently washed with tap water to remove the attached soil or organic particles and cut them into 1 cm segments.

■ The cleaned root bits were treated in 10 % KOH by heating at 90°C for 2-5 minutes in the oven. The KOH solution is known to clear the host cytoplasm and nuclei and readily allows the stain to penetrate.

■ After cooling, the roots were thoroughly rinsed in water to remove the traces of KOH.

- The roots were then acidified with 5 N HCl for 2-5 minutes. The solution was then poured off.
- This was followed by overnight staining the root bits with 0.05 % Trypan blue stain
- The stained root segments were then temporarily mounted on the glass slide using polyvinyl alcohol lacto glycerol (PVLG).
- The stained root segments were then observed under a light microscope.
- The presence or absence of intra-radical and extra-radical non-septate hyphae, arbuscules, vesicles, hyphal coils and auxiliary cells in the root's segments were recorded.

3.4.6. Histochemical Staining for Lipids

The localization of lipid bodies in the roots of colonized host plants by AM fungi was carried out using the Sudan Black staining method (McGee-Russell and Smale, 1963). The acidified root segments were stained with Sudan Black for 5 minutes. Then, these root segments were mounted in PVLG and observed using a light microscope.

CHAPTER 4: RESULTS AND CONCLUSION

4.1 Results

4.1.1. Preparation of Pure cultures

The pure AM culture of *Funneliformis geosporum* was obtained from the Goa University Arbuscular Mycorrhizal Fungus Culture Collection (GUAMFCC). This monospecific culture was multiplied using *Plectranthus scutellarioides* as the host plant and maintained in the glasshouse **(Plate 1)**. The multiplied cultures were subsequently used for the further studies.

4.1.2. Standardization of WM media for ROC and sub-culturing the transformed roots

For subculturing the Ri T-DNA roots, modified WM medium was employed. It was observed that upon plating, the active growth was observed in the sub-cultured Ri T-DNA roots within 10 days.

4.1.3. Colonization pattern in *F. geosporum* using Trypan blue staining

Typical AM structures *viz.*, hyphae and vesicles were recorded in the host plant inoculated with AM fungi. These observations indicate that the AM fungi were successful in colonizing the Coleus roots **(Plate 2)**. Extensive hyphal network was observed which elongated through the inter-cellular spaces of the root cortex. The young, sub globose vesicles and mature intra-radical spores was observed.

4.1.4. Spore morphology of *F. geosporum*

Slides were prepared using PVLG and observed under Nikon Eclipse E200 digital camera microscope. The characteristics of *Funneliformis geosporum* (Nicolson & Gerdemann) Walker

& Schüßler viz., wall layers, septum, inner wall of subtending hypha, subtending hypha were recorded.

4.1.5. *In vitro* cultivation of AM fungi

For sterilization of AM spores, 4% concentration of NaClO for five minutes followed by sterilization in 0.02% Streptomycin sulphate for two minutes was most suitable. *In vitro* spore germination of *F. geosporum* are presented in **Plate 4**. Spore germination was observed in 7 to 10 days after inoculating on MSR media without sucrose.

The germinated spores were transferred to ROC plate and kept in dark at 27°C for incubation. The hyphal growth and root colonization was observed in Ri T-DNA roots using Trypan blue staining method. The hyphal proliferation in the Ri T-DNA roots was observed but there was no sporulation observed.

Fig. 1: Trap cultures of AM

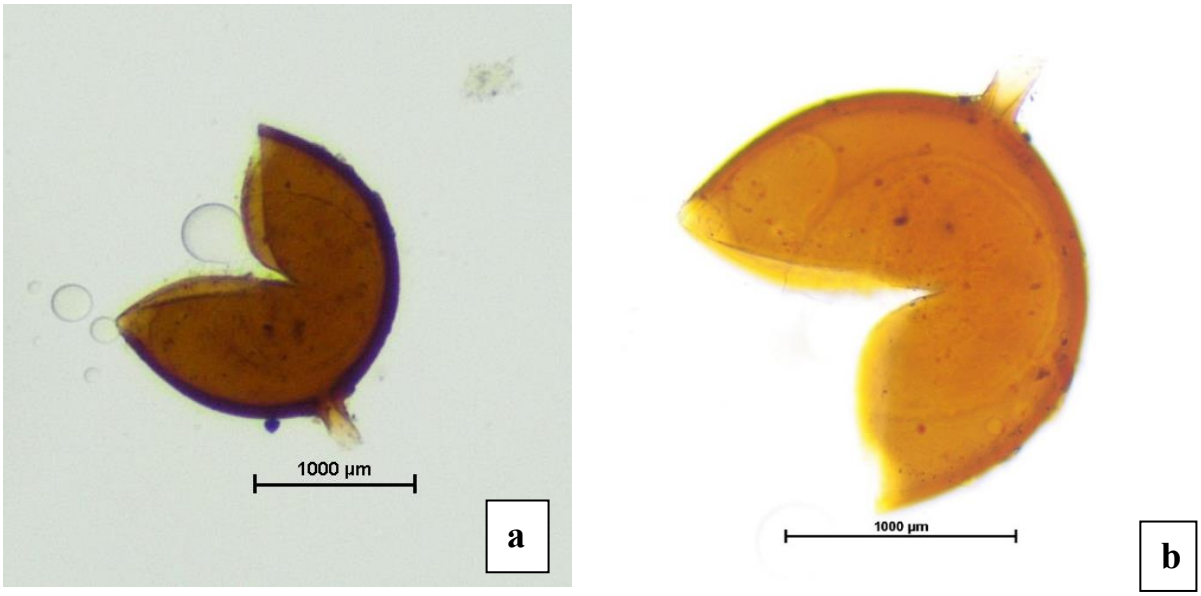

Fig 2 a & b: *Funneliformis geosporum* spore.

Plate 1: Trap cultures and spore morphology of AM fungal species undertaken for the study.

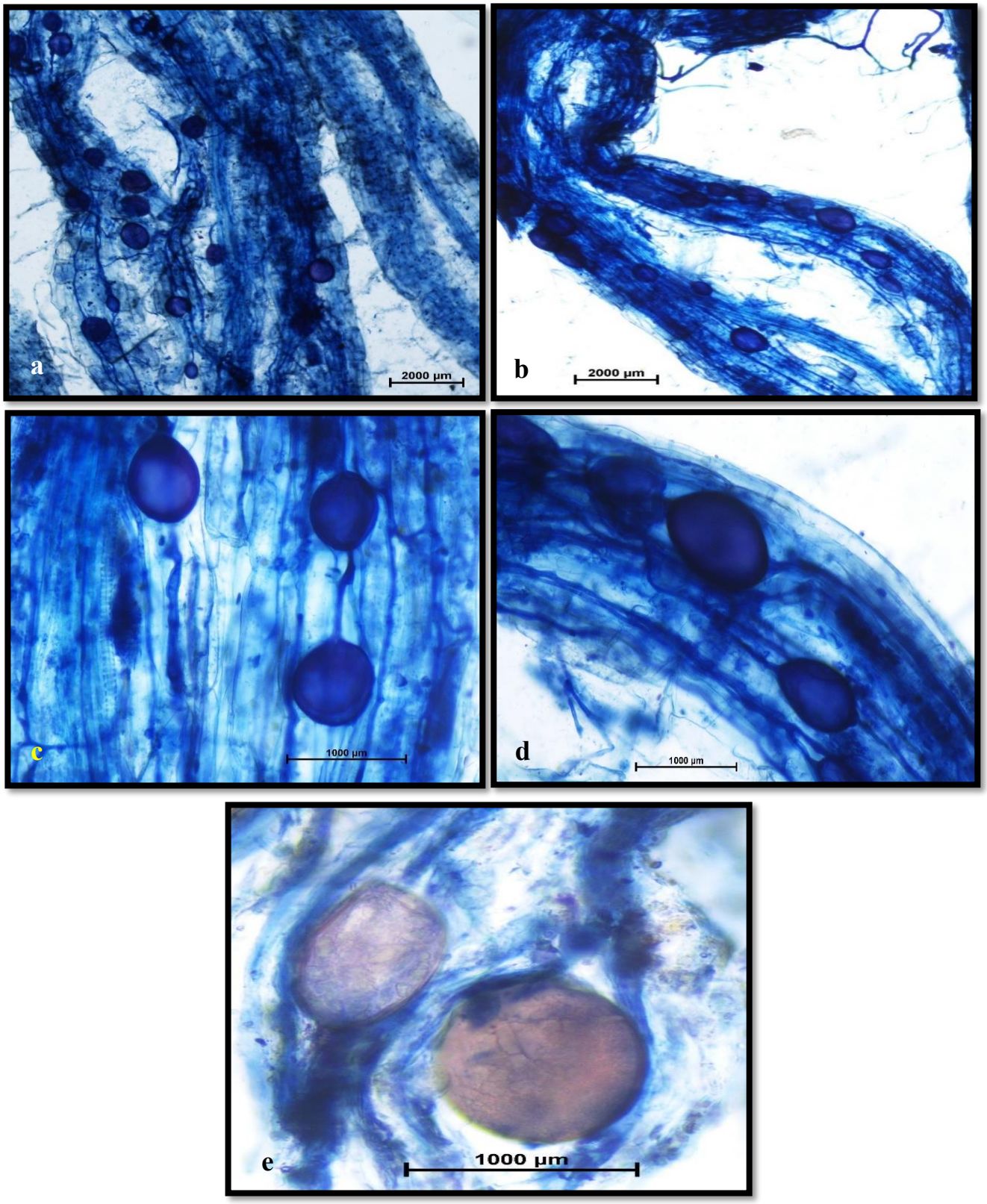

Plate 2: Root colonization in *Funneliformis geosporum* - **a**: Vesicles and hyphal colonization (10x); **b**: Hyphae (H) and vesicle (V); **c**: Young vesicles and hyphae (40x); **d**: Wall differentiation in mature vesicle; : Mature intra-radical spores.

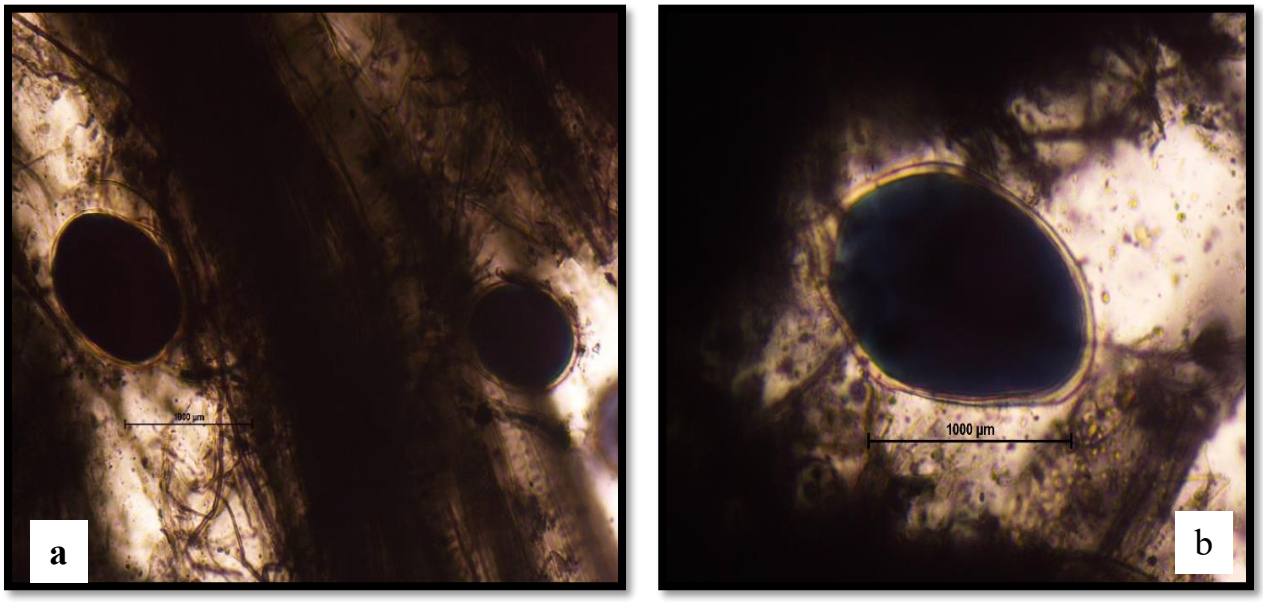

Fig. 1a & b: Sudan black staining for lipid in vesicles.

Fig. 2 : Ri T-DNA transformed roots growing on WM media

Plate 3: Sudan black staining for lipid in vesicles and Ri T-DNA transformed roots growing on WM media.

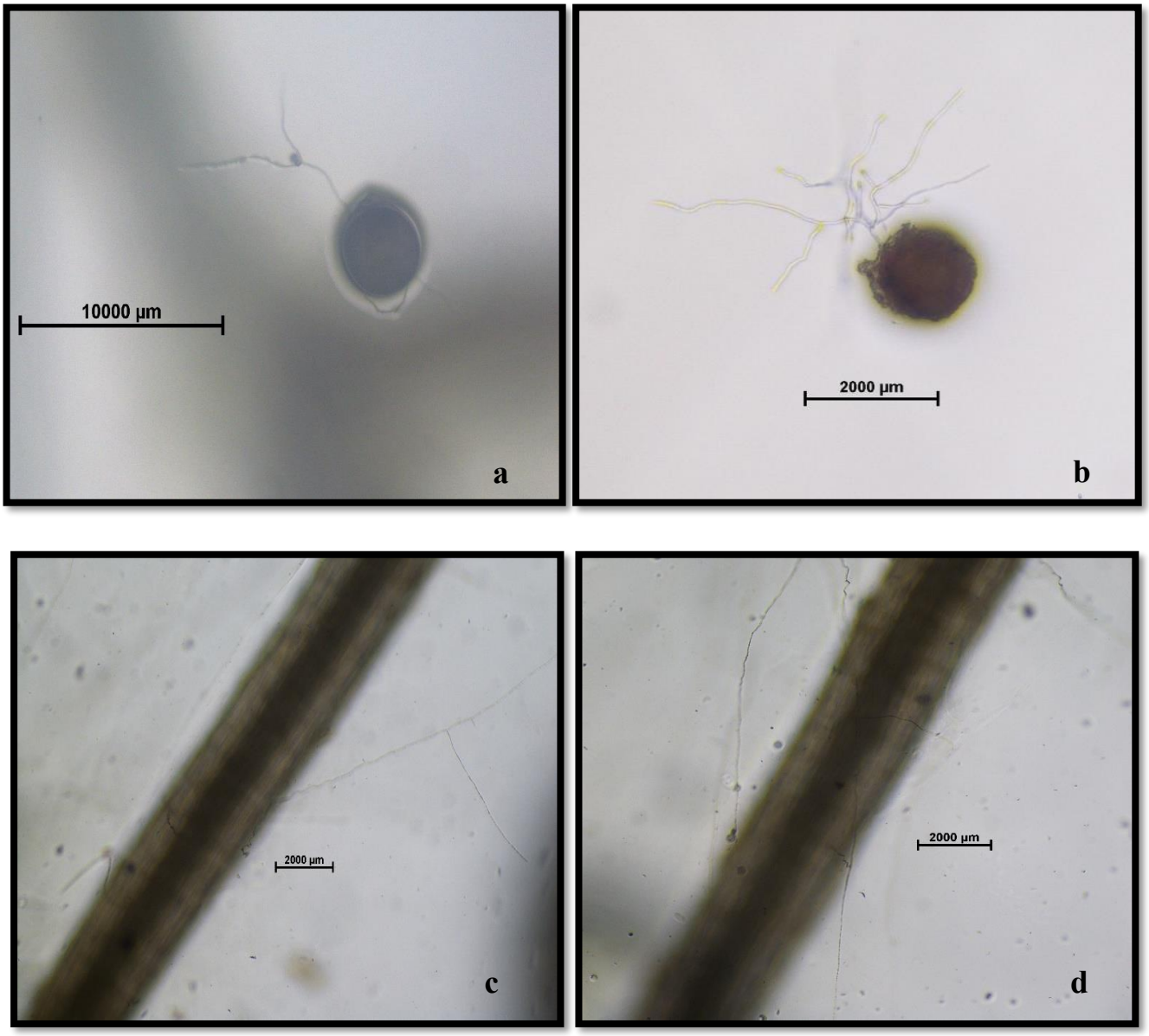

Plate 4: ***In vitro*** **spore germination in** ***Funneliformis geosporum*** **- a**. Germinated spore (4x); **b.** Profuse lateral branching in germ tube (10x)**; c& d**: Initial contact between transformed roots and the hypha.

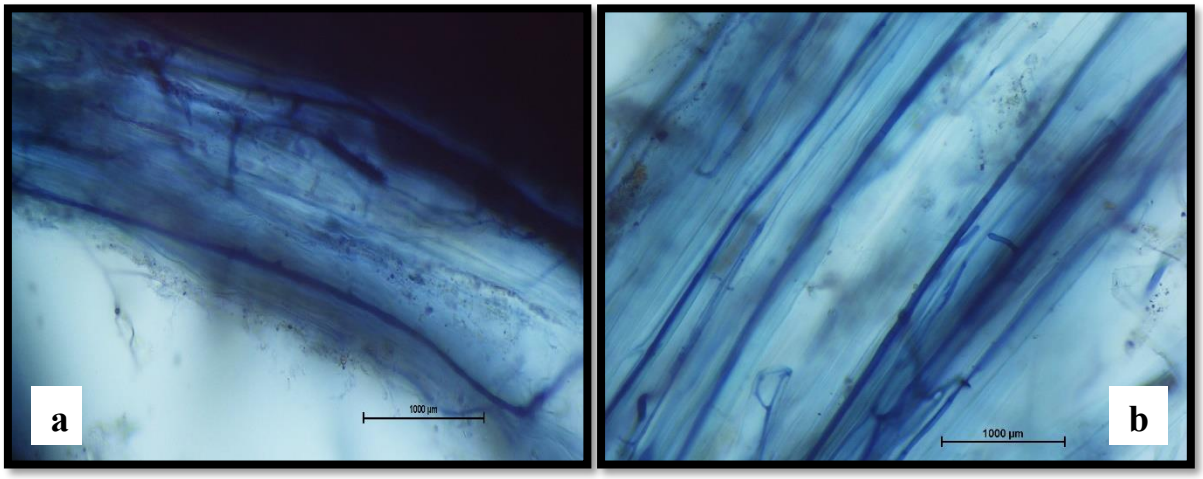

Plate 5: *In vitro* root colonization – **a & b**: Hyphal colonization in Ri T-DNA transformed roots.

4.2 Discussion

In vitro cultivation is a highly effective method for the study of AM association and its life cycle. The small growing research on monoxenic culture reported on successful establishments of variety of AM fungal species (Fortin *et al*., 2002). In this study we report the *in vitro* germination of *F. geosporum* and successful colonization in Ri T-DNA roots.

In the present study, *in vitro* germination was through the spore wall. The germ tube emerged through the spore wall followed by branching. The MSR (minus sucrose0 medium was used for germination, as it ensures fast spore germination in *in vitro* conditions (D'Souza *et al*., 2013). According to Declerck *et al.* (2000), the germ tube grows as a straight developing hypha that branch into filaments with progressively smaller diameters to explore the medium. The hyphal proliferation in the Ri T-DNA roots was observed. However, no sporulation was observed. This absence of sporulation may be due to more time required to sporulate under the *in vitro* culture condition as reported by Kandula *et al.* (2006).

4.3 Conclusion

Monoxenic cultivation of AM fungi on root culture allows detailed observations and long-term experimentations on the life cycle of AM fungi as well as offers production of high quality and large-scale inoculum production. Our results depict the successful *in vitro* germination of *F. geosporum* and colonization in Ri T-DNA roots.

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APPENDIX I:

WM MEDIA COMPOSITION AND CONCENTRATION

Solution I: Macro- Elements for 1L (100X), use Nano-Pure water **Chemicals**	**WM Medium**
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