In vitro culturing of Arbuscular Mycorrhizal (AM) fungi using Ri T-DNA roots

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"*In vitro* culturing of Arbuscular Mycorrhizal (AM) fungi using Ri T-DNA roots"** is based on the results of investigations carried out by me in the Botany discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Prof. Bernard F. Rodrigues and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities/College will be not be responsible for the correctness of observations/experimental or other findings given the dissertation.

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This is to certify that the dissertation report **"*In vitro* culturing of Arbuscular Mycorrhizal (AM) fungi using Ri T-DNA roots"** is a bonafide work carried out by **Ms. Ashwini Ramdas Ghadi** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master's** in the Discipline Botany at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The cultivation of arbuscular mycorrhizal (AM) fungi have long been of interest due to their crucial roles in plant nutrition, growth promotion, and environmental sustainability. This dissertation delves into the *in vitro* study of two prominent AM fungal species, *Scutellospora scutata* and *Gigaspora albida*, utilizing Ri T-DNA transformed carrot roots as a model system.

Born out of a desire to unravel the intricacies of AM fungal biology and explore their potential applications in agriculture and ecosystem management, this study aims to shed light on cultivation, colonization, and sporulation dynamics. Through meticulous experimentation and methodological precision, insights into growth patterns, morphological characteristics, and ecological significance are uncovered. This preface sets the stage for a comprehensive exploration of AM fungal biology, offering a glimpse into the meticulous process of scientific inquiry and the pursuit of knowledge in symbiotic plant-fungal interactions.

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Ms. Ashwini Ghadi

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ABBREVIATIONS USED

Entity	Abbreviation
Branched absorbing structures	BAS
Dibutylphthalate Polystyrene Xylene	DPX
Gigaspora albida	*Gi. albida*
Hydrochloric	HCl
The International Collection of Vesicular Arbuscular Mycorrhizal Fungi	INVAM
Potassium hydroxide	KOH
Modified Strullu and Romand	MSR
Sodium hypochlorite	NaClO
Nitrogen	N
Phosphorus	P
Root Organ Culture	ROC
Root system architecture	RSA
Polyvinyl-Lacto-Glycerol	PVLG
Scutellospora scutata	*Sc. scutata*
Minus sucrose	-Sucrose
Toluidine Blue O	TBO
Whites media	WM

ABSTRACT

The study was initiated to investigate the *in vitro* cultivation of two arbuscular mycorrhizal (AM) fungi species, *Scutellospora scutata* and *Gigaspora albida*, utilizing Ri T-DNA transformed carrot roots. The methodology involved pre-germinating AM fungal spores on MSR (Modified Strullu-Romand) media without sucrose and allowing them to colonize Ri T-DNA roots grown on WM (Whites Media) for further development and sporulation. Various morphological characteristics of extra-radical and intra-radical mycelium, branched absorbing structures (BAS), and auxiliary cell formation were assessed in the AM symbiosis with the transformed carrot roots.

The study found that the optimal range for surface sterilization of AM spores using 4% Sodium hypochlorite (NaClO) was between 45µl to 55µl, resulting in minimal contamination while maintaining spore viability. Additionally, successful germination of *Gi. albida* and *Sc. scutata* was observed on MSR plates within 5 to 10 days in the absence of sucrose. After 40 days of inoculation on sub-cultured Ri T-DNA root plates, knobby-type auxiliary cells were observed on *Sc. scutata,* while no echinate type of auxiliary cells were observed in *Gi. albida.* Furthermore, hyphal and arbuscular colonization was seen in both species after 60 days, indicating successful establishment within the root system, But no sporulation was observed in both the species.

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1: What is Mycorrhiza and Arbuscular Mycorrhiza

The term “mycorrhiza” coined by A.B. Frank, 1885, means fungus-roots (Habte and Osorio, 2001). It is the mutualistic association between fungus (myco) and roots (rhiza) of the plants. It increases surface area for absorption of water and minerals where plant roots are unable to reach.

In the realm of mycorrhizal associations, the phylum Glomeromycota takes precedence, although other fungal groups are also capable of forming these associations (Rodrigues and Rodrigues, 2015). Within these symbiotic relationships, various structures emerge, including the mycelium, arbuscules, vesicles, auxiliary cells, and spores (Nicolson, 1967).

AM fungi are characterized by the presence of arbuscules, tree-like branching hyphal structures, which are observed in the cortical cells of the host plant, arbuscules develop within the root cells of the host, while extraradical mycelia extend outside the roots to enhance the area for water and nutrient uptake (Smith & Read, 2008). Additionally, certain genera/species of AM fungi produce vesicles that serve as nutrient reservoirs and propagules. These anatomical features facilitate a close association between the mycorrhizal fungi residing within the root cells of the host plant (Goltapeh et al., 2008). Consequently, root samples must undergo cellular clearance, and the endophytic fungi should be stained prior to microscopic examination to accurately interpret and comprehend the colonization process and functionality of AM fungal symbiosis (Smith & Read, 2008).

When fungal hyphae make contact with a host plant's root surface, they undergo specialization to create appressoria, enabling root penetration. Inside the root cortex, hyphal branches pierce cortical cell walls and form arbuscules, pivotal for nutrient exchange. Meanwhile, the fungus expands its mycelium network in the soil surrounding the root, scavenging nutrients and seeking new colonization sites (Bonfante-Fasolo, 1984; Harrison, 1999).

AM fungal propagules exist in various forms, including spores, living hyphae, isolated vesicles, mycorrhizal root segments, or colonized soil (Diop et al., 1994).

1.1.2: Traditional and conventional method of AM culturing

Traditionally, root segments and spores isolated from open-pot culture have served as the primary source of AM inoculum for research purposes. However, this type of inoculum is spatially demanding and susceptible to contamination, even with meticulous phytosanitary care (Ames and Linderman, 1978). The production of propagules under aseptic conditions presents one of the most promising approaches to obtain high-quality pathogen-free inoculum required for research purposes.

The conventional method employed to study the life cycle of AM fungi is through the association of these fungi with root organ culture (ROC) (Fortin et al*.,* 2002). The establishment of ROC *in situ* has significantly enhanced the understanding of various aspects of the AM symbiosis, allowing non-destructive throughout the fungal life cycle and its applicability to research and inoculum production.

The cultivation of AM fungi in association with Ri T-DNA transformed roots has

opened new possibilities for studying the extra-radical mycelium of AM (Fortin et al., 2002).

1.1.3: Significance of AM fungi in maintaining soil health and plant productivity

AM fungi play a crucial role in ecosystem functioning, particularly in soil health and plant productivity. These fungi form symbiotic associations with the roots of the majority of terrestrial plants, facilitating nutrient uptake and enhancing plant resilience to environmental stresses.

1. Nutrient acquisition: AM fungi significantly improve the uptake of essential nutrients, particularly phosphorus, which is often limited in soils. This symbiotic relationship allows plants to access phosphorus more efficiently, even in low-phosphorus environments. Research by Smith and Read (1997) extensively documents the role of AM fungi in phosphorus acquisition.
2. Improved plant growth and health: By enhancing nutrient uptake, AM fungi contribute to improved plant growth, vigor, and overall health. The symbiosis with AM fungi has been shown to increase plant biomass, root development, and resistance to various biotic and abiotic stresses (Dodd et al., 2002).
3. Disease suppression: AM fungi play a crucial role in suppressing soil-borne diseases caused by fungal pathogens and nematodes. They reduce disease incidence in plants and inhibit the growth of pathogenic microorganisms in the soil (Azcon-Aguilar and Barea, 1996).
4. Soil structure and stability: The extraradical hyphae of AM fungi produce a

stable glycoprotein called glomalin, which binds soil particles together, improving soil aggregation and stability. This enhances soil structure, water retention, and nutrient cycling (Miller and Jastrow, 2000).

5. Ecosystem restoration: AM fungi contribute to the restoration of disturbed ecosystems by accelerating the natural process of plant community development. Research conducted by Dodd et al. (2002) demonstrates the rapid invasion of AM fungi into newly created land platforms, facilitating revegetation efforts in nutrient-poor substrates.
6. Reduced chemical input: Utilization of AM fungi in agriculture and horticulture has the potential to reduce the reliance on chemical fertilizers and pesticides. Studies have shown that AM fungi can enhance nutrient acquisition in crops, leading to decreased chemical inputs while maintaining or even increasing yields (Giovannetti, 2001).
7. Promotion of sustainable agriculture: AM fungi offer opportunities for promoting sustainable agricultural practices by improving soil fertility, reducing soil erosion, and enhancing crop resilience to environmental stresses. This aligns with the goals of sustainable agriculture, focusing on long-term productivity and environmental stewardship (Atkinson et al., 2002).
8. Horticultural and agricultural applications: In horticulture, AM fungi inoculation has been successfully utilized to improve plant establishment, growth, and productivity, particularly in micropropagated plants. This approach reduces acclimatization losses and enhances plant development, ultimately

leading to increased productivity (Lovato et al., 1996).

1.1.4: Ri T-DNA transformation and its revolutionary impact

One method for large-scale production of AM inoculum involves utilizing root organ culture (ROC), where the growth and production of AM fungi occur on transformed plant roots induced by Ri T-DNA of *Agrobacterium rhizogenes*. This technique, known as ROC, has been successfully employed in commercial-scale AM production systems (Adholeya et al., 2005). ROC-based systems entail isolating potential viable propagules from monosporal cultures, surface sterilization, and optimizing growth conditions for spore germination under aseptic conditions. The resulting mass-produced mycorrhizal propagules can be formulated and stored for extended periods, meeting industrial requirements. Comparisons between ROC-based methods and conventional techniques highlight the superiority of *in vitro* methods in terms of product quality, purity, and ease of production (Verma and Adholeya, 1996; Douds et al., 2000)

1.2 Hypothesis

By monoxenic culture, so far, 25 species of AM fungal species have been successfully cultivated (Forti et al*.,* 2002). However, most data generated under monoxenic culture conditions have been obtained with species of *Glomus* and *Gigaspora*, while *in situ* observations on *in vitro*-produced cultures of *Scutellospora* species have been rarely reported. Successful ROC of *S. reticulata* by de Souza and Declerck (2003) have shown that it produces mycelium of two architectural patterns, one related to root colonization

and other to resource exploitation.

It's been well recognized that *scutellospora* and *Gigaspora* are known to form auxiliary cells.

1.3 Scope

The Scope of the present study lies in its potential to unravel the secrets of the intimate symbiosis between AM fungi and host plants. By employing Ri T-DNA transformed roots, researchers can gain unprecedented access to the intricate life cycles of AM fungi under controlled laboratory conditions. This approach offers a unique window into the world of these soil organisms and their crucial roles in enhancing plant health, productivity, and ecological resilience.

Table 1.1: Some distinguishing features of *Sc. scutata* and *Gi. albida.*

Features	***Sc. scutata***	***Gi. albida***
Germination	**Indirect germination** takes place by the development of **"germination shield"** before the emergence of **germ tube**. (Sequeira et al., 1985)	**Direct germination** takes place when the inner wall layers protrude though a weakened area of the outer wall layer as a germ tube initially, afterwards elongating into a typical hypha. (Sequeira et al., 1985).
Auxiliary cells	The projections on the surface of the auxiliary cells are knobby and highly variable in shape and size (Morton, 1995)	The auxiliary cells are echinulate with spines that are forked dichotomously (Bentivenga and Morton, 1995)

Spore wall layers	2 (according to INVAM)	3 (according to INVAM)
Spore internal structures	Germination shield present. (according to INVAM)	Germination shield absent. (according to INVAM)

The plant root association with the AM fungi overcome the challenges like water stress (Liu et al., 2015), heavy metal resistance against aluminum and lead in *in vitro* (Gavito et al*.,* 2014), chromium immobilization (Wu et al*.,* 2016), mercury contaminated soils, copper stress (Almeida-Rodríguez et al*.,* 2015), arsenate stressed soils (Cattani et al*.,* 2015), against oxidative stress (Driai et al*.,* 2015). Hence, they play a crucial role in increasing plant health and soil fertility.

As the mysteries of AM fungi through the synergy of biotechnology and plant-fungal interactions are delved, one unlocks the new possibilities for sustainable agriculture, biodiversity conservation, and ecosystem restoration.

The first reported *in vitro* root organ culture system was first reported by White (1943). AM fungal culture development under *in vitro* root organ culture process is one of the best ways to obtain good amount of extra-radical spores in a short span of time with contamination free inoculum (Binondo et al*.,* 2012).

AM fungal cultivation techniques based on *Agrobacterium rhizogenes* transformed roots provide the development of the AM fungal inoculum *in vitro* (Schultze, 2013). After the development of monoxenic cultures of AM fungi through root organ culture, the continuous observations of fungal colonization and mycelium development as well

as the sporulation is possible.

1.4 Aims and Objectives

The present work aims to highlight cultivation systems of AM fungi, encompassing a particular type of host, culture media and types of AM fungal propagules used to initiate monoxenic cultures.

The main purpose of this study is to bring about the *in vitro* culturing of two AM species, one each of the genus *Scutellospora* and *Gigaspora* using Ri T-DNA transformed hairy roots system.

Stains such as Trypan blue, can be used to check successful colonization. It can be used to check successful AM fungal colonization, mycelium development and sporulation. Besides, various morphological characteristics of extra-radical mycelium, intra-radical mycelium, branched absorbing structures and auxiliary cell formation, in the AM symbiosis in root-inducing transferred-DNA transformed roots of carrot (*Daucus carota* L.).

To detect major storage compounds, histochemical stains such as Sudan black can be used to detect lipids and Toluidine blue O is used to detect polyphosphates.

The objectives of the present work are highlighted below:

1. Isolation of spores,
2. Preparation of trap cultures,
3. *In vitro* germination of AM spores,
4. AM fungal colonization in Ri T-DNA roots.

CHAPTER 2: LITERATURE REVIEW

Table 2.1: Authors and their publications

Authors	Inference
Mosse & Hepper, (1975)	Successfully performed the root organ culture using a system based on dual culture of *Glomus mosseae* spores with excised roots of clover.
Diop et al., (1994)	Studied sporulation and colonization potential of two AM fungi, viz., *Glomus intraradices* and *G. versiforme*, using surface-sterilized sheared-root inocula. After rigorous surface sterilization, the inocula were transferred to Petri dishes with tomato roots. Both AM species exhibited significant sporulation and hyphal biomass production within three months of incubation in the dark. Spores of *G. intraradices* were larger, with 893 mature spores recorded, while *G. versiforme* produced 2065 mature spores. Both fungi effectively colonized tomato roots, with *G. versiforme* showing slightly higher root colonization (35%) compared to *G. intraradices* (24%). Additionally, both spores and fragments of colonized roots were capable of germinating on minimal M medium without prior treatment. Successfully recolonized seedlings of *Acacia albida* under greenhouse conditions, with spores of *G. intraradices* and a mixture of mycelia and colonized roots of *G. versiforme* being the most efficient inocula. These findings highlight the potential of surface-sterilized sheared-root inocula as high-quality starter inocula for various plant species.
St-Arnaud et al.	Investigated the potential for producing aseptic spores of *Glomus intraradices*, using a two-compartment *in vitro* system. This system involved growing genetically transformed

(1996)	*Daucus carota* roots in one compartment with a complete growth medium, while allowing only the AM fungus to grow in the adjacent compartment lacking sugar. The results showed that colonization of the sugarless compartment by the fungus occurred between six and eight weeks after subculturing the mycorrhizal roots. Hyphal and spore densities were significantly higher in the sugarless compartment, with up to 34,000 spores counted per plate. This suggests the possibility of producing aseptic spores, not only for research purposes but also for large-scale inoculum production. Factors such as the absence of sucrose and physical separation from host roots were hypothesized to contribute to the enhanced hyphal and spore densities. The study sheds light on the ecological role of chemicals, such as flavonoids, in AM fungal symbiosis formation and mycelium architecture.
Filion et al., (1999)	The study highlights the influence of soluble substances released by the extraradical mycelium of *Glomus intraradices* on soil microorganisms. Although the specific chemicals involved are not identified, the study suggests that these substances could include amino acids, proteins, and other organic compounds. The composition of these substances is crucial for their interaction with soil microorganisms, leading to either stimulation or inhibition of their growth. Additionally, the study emphasizes that the pH of the growth medium is affected by the presence of the extra-radical mycelium, which in turn influences the microbial equilibrium.
Hawkins et al., (2000)	Reviewed new findings on the uptake and transport of inorganic and organic nitrogen (N) by AM fungi, focusing on the species *Glomus mosseae*. Experiments conducted in semi-hydroponic, non-sterile pot cultures demonstrated that AM fungal hyphae could

	transport N, supplied as ^{15}N-labeled glycine, to wheat plants. The amount of transported N varied depending on the N supply to the plants, with higher uptake observed under sufficient N conditions. *In vitro* studies confirmed that AM fungal hyphae could acquire N from both inorganic and organic sources. Long-term experiments with monoxenic cultures further demonstrated the ability of AM fungi to transport N from organic sources to plant roots. However, the contribution of N transported by AM fungal hyphae to plant nutrition was relatively small, suggesting that other factors may limit their effectiveness in enhancing plant N uptake. The article concludes by highlighting the potential ecological significance of AM fungi in accessing spatially unavailable N sources in soils and the need for further research to elucidate the mechanisms of N uptake and transfer by AM fungi.
de Souza & Declerck, (2003)	Their study utilized a modified Strullu-Romand (MSR) medium for culturing *Scutellospora reticulata* in a monoxenic system. The MSR medium was solidified with Gel-Groy and adjusted to a pH of 5.5 before autoclaving. Additionally, the study employed water agar (0.8%) for spore germination. The composition of the MSR medium, with its pH adjustment, appeared suitable for supporting the fungal life cycle, including mycelium development and spore production. The chemicals used in the culture medium likely influenced various aspects of fungal growth and development. For example, the pH adjustment could have affected nutrient availability and fungal metabolism, thereby influencing spore germination, mycelium growth, and spore production. Gel-Groy, used for solidification, might have provided essential nutrients or physical support for fungal growth. Moreover, the absence of

	specific nutrients such as P in the culture medium may have influenced fungal behaviour, as indicated by the sporulation dynamics. Overall, the composition of the culture medium, including the chemicals used and their concentrations, likely played a crucial role in supporting the growth and development of *S. reticulata* in the monoxenic system. Further studies could explore the specific effects of individual components of the culture medium on fungal physiology and ecology, providing insights into optimizing culture conditions for AM fungi and their applications in various soil ecosystems.
Diop, (2003)	The mini-review provides a comprehensive overview of the *in vitro* culture of AM fungi, essential for the growth and survival of vascular plants. It highlights recent advances in cultivation techniques, aiming to achieve AM fungal growth independent of a plant host. Methods for cultivating root organs, selecting and purifying fungal inocula, and achieving continuous culture without losing infectivity are described. The review emphasizes the significance of *in vitro* systems in studying both fundamental and practical aspects of AM symbiosis, complementing *in vivo* experimental approaches. It also proposes areas for further research to fully exploit the potential of AM symbiosis. Overall, the review underscores the importance of continuous advancements in *in vitro* techniques for enhancing our understanding and utilization of AM symbiosis in agriculture and environmental restoration.
Kandula et al., (2006)	Carried out successful cultivation of *Scutellospora calospora* with Ri-TDNA transformed carrot roots using *Agrobacterium rhizogenes.* Spore germination tests showed germination occurring between 44 and 60 days, with a rate of 16.6%. Following

	infection, extensive extra-radical mycelium proliferation was observed, along with auxiliary cell formation, indicating a potential C storage function. However, spore formation was low, with only four spores observed after 8 months, primarily on MSR medium. The study highlights the importance of monoxenic culture in understanding critical colonization events and suggests further research to improve spore production.
El Meskaoui et al., (2008)	Introduced a novel method for establishing AM symbiosis in micropropagated strawberry plantlets. Through a tripartite system *in vitro*, the study achieved successful root colonization by AM fungi, resulting in enhanced growth during acclimatization. In comparison to non-inoculated plantlets, the AM inoculated plantlets exhibited significant increases in shoot and root length, as well as shoot fresh weight. The symbiosis positively influenced plant behaviour by augmenting relative water content and modifying root cell composition to alleviate water stress. These findings suggest promising applications for improving the growth of micro propagated plants through AM symbiosis.
Redecker et al., (2013)	Carried out revision of the taxonomy of AM fungi to address confusion from conflicting schemes and numerous taxon names. They analyzed molecular and morphological evidence to propose a consensus classification, urging caution in proposing new names. The results provide a revised taxonomy aligned with current phylogenetic understanding, aiming to provide a stable framework for future research on these vital plant symbionts.
Rodrigues and Rodrigues, (2013)	They discussed *in vitro* cultivation of AM fungi. They discussed various concepts like system description, AM fungal species cultivated on ROC, culture media, AM fungal inoculum, continuous cultures, fungal morphological features in ROC system, fundamental and practical studies. They further discussed the advantages and

	disadvantages of the root organ culture system.
Srinivasan et al., (2014)	Successfully established monoxenic cultures of *Glomus intraradices* in conjunction with transformed carrot hairy roots, utilizing *Agrobacterium rhizogenes* for transformation and Modified Strulla and Romand (MSR) medium for culture. The research achieved significant spore production and root colonization potential, with up to 9000 spores per Petri dish after three months of incubation. This approach offers a promising method for mass-producing contaminant-free inoculum of AM fungi, which could have significant implications for enhancing crop production in agriculture and horticulture.
Rodrigues and Rodrigues, (2015)	Established monoxenic cultures of *Funneliformis mosseae* with *Linum usitatissimum* roots on modified Strullu-Romand (MSR) medium (pH 5.5). Germ tubes emerged from spores within five days, followed by hyphal contact with transformed linum roots. *Paris*-type arbuscules, hyphal coils, and extra-radical branched absorbing structures (BAS) were observed, along with terminal and intercalary secondary spores with 83% viability. *In vivo* experiments demonstrated successful root colonization and sporulation. Findings highlight the suitability of MSR medium for spore germination, root colonization, and sporulation, indicating potential for large-scale inoculum production.
Kehri et al. (2018)	Studied intricate taxonomy and systematics of AM fungi. Initially, identification relied on morphological features, leading to extensive species descriptions. However, recent molecular advancements have transformed the field, resulting in robust classifications based on morphological, ontogenic, and nucleotide sequence data (SSU, ITS, LSU, β-tubulin, and nrDNA). This evolution underscores the importance of molecular tools in refining our understanding of AM fungal diversity and relationships.

Velip and Rodrigues, (2019)	Demonstrated successful *in vitro* culturing and sporulation of *Gigaspora decipiens* using transformed roots of *Linum usitatissimum*. Additionally, it introduces a technique for simultaneous spore germination and root colonization in a single Petri plate, minimizing relocation effects and accelerating colonization. This advancement holds promise for more efficient mass multiplication of pure AM fungal cultures, facilitating their use as biofertilizers.
Nivedha et al., (2019)	The study investigates the symbiotic relationship between AM fungi and rice, particularly in modern rice production systems. Using *in vitro* conditions, the research has standardized an optimal spore load per seedling of *Rhizophagus irregularis* and evaluated its impact on Root System Architecture (RSA). Results demonstrated that different spore loads of AM fungi significantly modified RSA traits of rice seedlings compared to the control. Specifically, seedlings with 31 spores per seedling exhibited maximum root colonization of 100% by AM fungi, highlighting the potential benefits of AM fungal inoculation in rice cultivation.
Moukarzel et al., (2020	Their study addresses the challenges of visualizing AM fungal colonization in grapevine roots by evaluating various fixing, clearing, and staining techniques. The dark pigmentation of grapevine roots posed difficulties in distinguishing AM fungal structures. Through methodological evaluation, the study identified an optimized technique involving fixing the roots in 70% ethanol overnight, followed by autoclaving in 10% potassium hydroxide for 15 minutes, and staining with Trypan blue overnight at room temperature. This method significantly enhanced the clarity of AM fungal structures, allowing for improved visualization without the use of toxic fixatives. The

	study underscores the importance of methodological refinement in overcoming challenges associated with visualizing AM fungal colonization in grapevine roots.

CHAPTER 3: METHODOLOGY

3.1 Study site and Sample collection

For *in vitro* culturing of AM fungal spores, the spores were isolated from sand of Miramar beach, Goa (Plate 4.1). Shovel was inserted at an angle of 15^0 into soil upto 20-25cm at the root zone. The soil sample was divided into two parts, out of which one part was used for *in vitro* culturing and other for trap culture.

3.2 Spore isolation

Spore isolation was done by Wet sieveing and Decanting method (Gerdemann and Nicolsan, 1963). First, 100 grams of sand was added to 1000 milliliters of tap water and stirred for 10-15 seconds to ensure thorough mixing. The mixture is then left undisturbed to allow coarse particles to settle down. Sieves ranging from 250 micrometers to 37 micrometers are arranged in descending order, and the supernatant is decanted through them. This process is repeated multiple times to maximize spore recovery. The sievates collected from each sieve are transferred into different beakers. Subsequently, the sievate from each beaker is individually filtered through Whatman No. 1 filter paper. After draining excess water, the filter papers are carefully placed on Petriplates and observed under a stereomicroscope (Olympus SZ51), ensuring that the filter paper remains moist throughout the observation.

3.3 AM fungal spore numbers estimation

The methodology employed for estimating AM fungal spore numbers, (Gaur and

Adholeya, 1994) referred from “Arbuscular Mycorrhizae of Goa – A Manual of Identification Protocols by B.F. Rodrigues and T. Muthukumar, Published by GOA UNIVERSITY” book involves several sequential steps. Initially, a Whatman no. 1 filter paper is utilized, which is folded in half and then into quarters, resulting in four equal parts. Subsequently, a straight line is drawn across the paper, dividing it into two equal halves, followed by the marking of vertical lines on one half at 1mm intervals to create 10 columns. Each column is numbered, and an arrow is marked on each line to indicate the direction of counting. The paper is then folded to ensure that the marked portion receives the sievete for filtration, thereby containing the spores within the designated area. After draining excess water, the filter paper is placed on a large Petriplate and observed under a microscope. The number of spores between each numbered column is counted and recorded in an observation table. This methodology allows for the estimation of spore density, diversity, abundance, and the dominant taxon present at a particular site.

3.4 AM fungal spore mounting on glass slide

The method for morphological identification of AM fungal spores on glass slides, by Koske and Tessier in 1983 referred from “Arbuscular Mycorrhizae of Goa – A Manual of Identification Protocols by B.F. Rodrigues and T. Muthukumar, Published by GOA UNIVERSITY”, involves several sequential steps. Initially, a solution of Polyvinyl alcohol lacto-glycerol (PVLG) is used to mount intact and crushed spores onto clean glass slides. This mounting process entails applying slight pressure on the cover slip

with the blunt end of a needle to crush the spores. To ensure the slide's permanence, the cover slip is sealed with DPX.

The preparation of PVLG involves dissolving Polyvinyl alcohol in distilled water through heating, followed by the addition of lactic acid and glycerin once cooled. The Polyvinyl alcohol utilized should be 99-100% hydrolyzed and possess a viscosity ranging from 24 to 32 centipoise.

For the preparation of the spore slide, a drop of PVLG is placed on a clean, grease-free glass slide, onto which the spores were transferred using a needle. The slide is then covered with a coverslip, and slight pressure is applied to break the spores using the blunt end of a needle. Excess dried mount is removed by scraping with a razor blade and cleaning with a wet cloth. Finally, the edges of the slide are dried using a blotter and sealed with DPX.

The slide was observed and photos were taken using Nikon Eclipse E200 digital camera microscope.

3.5 Preparation of AM fungal Trap Culture

The process of creating a spore trap culture from a sand sample and initiating a monospecific (single species) culture involves several steps aimed at mass multiplying spores while maintaining the growth conditions similar to their natural habitat. To begin with, the spores are extracted using the Wet sieving and Decanting method (Gerdemann

and Nicolson, 1967). Following extraction, the spores are separated based on morphological characteristics, and only those belonging to a single morphotype are retained. These selected spores are surface sterilized using 4% sodium hypochlorite (NaClO) for approximately 3-5 minutes, followed by washing with water for several times to remove all the NaClO traces. Then the spores are treated with Streptomycin sulfate for 2 minutes.

For the initiation of the monospecific culture, plastic or clay pots with a diameter of 15cm are prepared by washing and sterilizing them with ethanol. Sterilized sand-soil mix (1:1 ratio) is filled into the pots after undergoing sterilization through heating three times at 85^0C for 8 hours, with 24-hour intervals between each heating. The washed and sterilized spores are then placed at a depth of 2-3cm in the soil, covered with additional soil, and host species cuttings or seeds (e.g., Coleus sp. or *Eleusine coracana*) are planted on top after being surface sterilized with a 0.1% mercuric chloride solution and washed with sterilized distilled water. The pots are transferred to a polyhouse and watered twice weekly for 45 days, with bi-weekly addition of Hoagland's solution (without phosphorus). After 45 days, a portion of roots is taken to assess AM colonization.

This methodology ensures the establishment of a monospecific culture under controlled conditions, although the timeframe for achieving a pure culture may vary depending on factors such as the species growth rate, initial inoculum, and environmental conditions.

3.6 Detection of AM fungal colonization

After certain period root portions are collected and stained with trypan blue to assess AM fungal colonization. Trypan blue technique (Phillips and hayman, 1970) is chosen for its ability to selectively stain fungal cell walls while leaving plant cells unstained. To prepare the trypan blue stain, 0.05g of trypan blue powder is dissolved in 40ml of distilled water, to which 10ml of glycerin and 50ml of lactic acid are added.

For the slide presentation of roots treated with trypan blue, root samples are first cut into 1cm pieces and washed thoroughly with water. They are then placed in a test tube and treated with 10% potassium hydroxide (KOH), heated at 121^0C for 10 minutes in an autoclave or at 90^0C for 1 hour in an oven. After draining off the KOH solution, the roots are washed several times with distilled water to remove any remaining traces of KOH. Subsequently, the roots are acidified by adding 5N hydrochloric acid (HCl) to the test tube and soaking them for 3-4 minutes.

Following acidification, the roots are stained with 0.05% trypan blue and left overnight. The stained roots are then mounted on clean slides using polyvinyl alcohol lacto-glycerol (PVLG) and observed under a compound microscope to visualize AM fungal colonization. Finally, to create permanent slides, the slides are sealed with DPX.

This methodology enables the detection of AM fungal colonization in root samples,

with appropriate precautions taken to ensure accurate staining and visualization of fungal structures.

3.7 Detection of major storage compounds by histochemical staining

To detect major storage compounds in AM fungi, histochemical staining techniques are utilized. Lipid bodies, essential for cell growth, are primarily detected using Sudan black staining technique (McGee-Russell and Smale, 1963). Triacylglycerols, the main neutral lipid in AM fungi, are crucial for carbon metabolism and transport.

To prepare the Sudan black stain, 100mg of Sudan black is dissolved in 10ml of 70% saturated ethyl alcohol and filtered before use. For the staining procedure, AM fungi root samples are initially washed under tap water, then treated with 2% potassium hydroxide (KOH) and heated at 90^0C for 30-45 minutes in an oven. After draining off the KOH, the roots are thoroughly washed with distilled water to remove any remaining traces of KOH. Acidification with 1N hydrochloric acid (HCl) follows for 10 minutes. Subsequently, the roots are stained with Sudan black for 1-5 minutes. The stained root segments are mounted on clean glass slides with glycerin, covered with a cover slip, and gently crushed with the blunt end of a pencil. Observation under a compound microscope reveals bluish-black staining of lipid bodies.

Polyphosphates, another major storage compound in AM fungi, are detected using toluidine blue O (TBO) staining technique of Kumble and Kornberg, 1996, which

specifically targets polyphosphate granules. To prepare the TBO stain, 1g of toluidine blue O is dissolved in 100ml of distilled water, and the pH is adjusted to 1 using 1N HCl. The staining procedure begins with washing root samples with tap water, followed by treatment with 2% KOH and heating at 90^0C for 30-45 minutes in an oven. After draining off the KOH and thorough washing with tap water, acidification with 1N HCl is performed. Roots are then stained with TBO for 20 minutes. Finally, the stained roots are mounted with glycerin on clean glass slides for observation under a compound microscope.

These histochemical staining techniques provide valuable insights into the presence and distribution of major storage compounds in AM fungi, aiding in the understanding of their physiological functions and interactions with host plants.

3.8 *In vitro* culturing of AM spores

To culture AM fungi *in vitro* on WM (White media), several key steps are involved. Initially, spores undergo sterilization, with the duration varying based on the specific species of spores. Surface sterilization is performed using 4% sodium hypochlorite (NaClO) for 3-5 minutes, with the concentration optimized through testing different concentrations ranging from 10 to 250µl to determine the optimal germination. Following NaClO treatment, spores are thoroughly washed multiple times to eliminate any residual NaClO traces. Subsequently, spores are treated with Streptomycin sulfate at a concentration of 0.02% to 0.05% for 2 minutes to further sterilize them.

Spore germination is initiated by inoculating the sterilized spores onto MSR media without sucrose. Ri T-DNA roots made from Carrot root transformation using *Agrobacterium rhizogenes* were taken from Prof. Bernard F. Rodrigues, botany discipline, SBSB, Goa University and subcultured on WM media with sucrose. The germinated spores are subsequently transferred onto WM media plates containing Ri T-DNA roots. These plates are properly sealed using paraffin tape and placed inverted in the dark to facilitate colonization.

By following these steps, *in vitro* culturing of AM fungi on WM media can be achieved, allowing for the study of their growth and colonization patterns under controlled laboratory conditions.

CHAPTER 4: RESULT AND DISCUSSION

4.1 Results

4.1.1 Pot culture

Pot culture of AM fungal spores isolated from the sand was accomplished using Coleus (*Plectranthus scutellarioides*) and Finger millet (*Eleucine coracana)* as the host plants and maintained as cultures in the poly house of Arbuscular Mycorrhizal Culture Collection at Goa University. After 45 days the cultures were checked for colonization using Tryphan blue, which showed hyphal, vesicular, and arbuscular colonization. Further major storage compounds i.e. lipids were stained using Sudan black and polyphosphates were stained using Toludine blue O.

4.1.2 WM media standardization for sub-culturing of Ri T-DNA roots

WM (Whites Media) used for sub-culturing was standardized. The Ri T-DNA showed active growth within 10-15 days of inoculation on this media. The AM spores germinated on MSR media (-Sucrose) were then inoculated on these plates.

4.1.3 Spore isolation and identification

Wet sieving and decanting method (Gerdemann and Nicolsan, 1963) was used to isolate spores. The 2 spore species *Sc. scutata* and *Gi. albida* were selected for *in vitro* culturing. The spore identification was done using INVAM & "Arbuscular Mycorrhizae of Goa – A Manual of Identification Protocols by B.F. Rodrigues and T. Muthukumar, Published by GOA UNIVERSITY."

Spore slides were done using PVLG and observed under Nikon Eclipse E200 digital camera microscope.

Table 4.1: features observed under microscope in of *Sc. Scutata* and *Gi. Albida*

Features	***Sc. scutata***	***Gi. albida***
Spore wall layers	2	3
Spore internal structures	Germination shield present.	Germination shield absent.
Bulbous suspensor	Present	Present

4.1.4 Sterilization of spores

AM spores were surface sterilised using 4% Sodium hypochlorite (NaClO) for 10-15 minutes, with varying volumes of 4% NaClO ranging from 10µl to 100µl in 10ml of water. In 10µl to 40µl, fungal and bacterial contamination was observed. Optimal sterilization and germination was observed within the range of 45µl to 55µl. Concentrations exceeding 60µl showed no contamination, yet failed to induce germination.

4.1.5 Germination

In the absence of sucrose, germination of *Gi. albida* and *Sc. scutata* was observed on MSR plates within a span of 5 to 10 days.

4.1.6 Colonization

After 40 days of inoculation of germinated spores on sub-cultured Ri T-DNA root plates, Knobby-type of auxiliary cells were observed on *Sc. scutata* and no echinate auxiliary cells were observed in *Gi. albida.*

After 60 days, hyphal and arbuscular colonization was seen in *Gi. albida* and *Sc. Scutata.*

4.2 Discussion

Trap cultures are most importantly prepared to trap as many indigenous AM fungal species as possible, live colonized roots of field plants can also be used to do so (shah 2014). In this study, trap cultures had showed presence of many AM spore species out of which *Gi. albida* and *Sc. scutata* were selected for *in vitro* culturing due to their abundance. In coastal sand dune environments, Gigasporaceae can dominate (Stürmer and Bellei, 1994; Beena et al., 2000). However, in agricultural soils cultivated with annual crops and in arid ecosystems, they are often less abundant or entirely absent (Sieverding, 1991; Helgason et al., 1998; Stutz et al., 2000; Jansa et al., 2002).

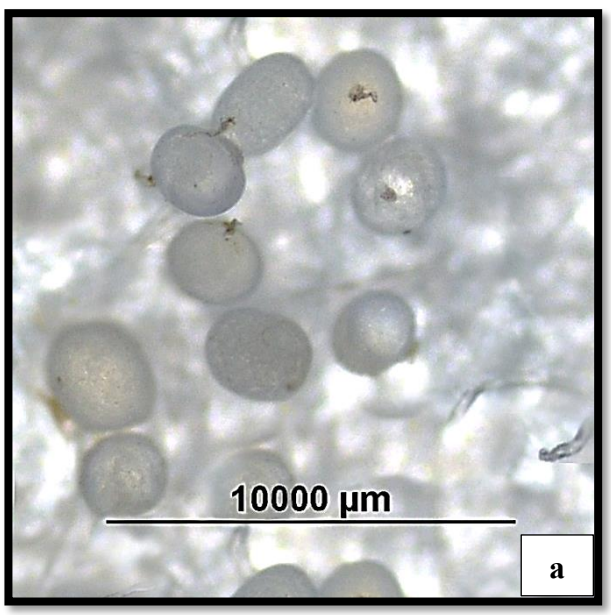
Spore germination is also affected by sterilization process, the temperature, heat, chemicals used for a particular duration also influence the spore viability (Hu, 2020). Therefore might be spore germination is also subject to seasonal and environmental variables. In this study, It was observed that of 45µl, 50µl, and 55µl of 4% NaClO solution for 15 minutes is effective for sterilizing *Sc. Scutata* and *Gi. albida* spores.

In Gigasporaceae, the germination process is linked with the spore wall organization (Walker & Sanders, 1986: Spain et. al., 1989). Multiple germ tubes were reported for Gigasporaceae (Koske 1981: Giovannetti et al., 2000). Here in this study *Sc. scutata*

species showed the germination through multiple germ tubes. The germination is indirect germination which is development of a “germination shield” prior to the emergence of a germ tube.

Members of the Gigasporaceae family have distinct intra- and extraradical mycelium morphologies, characterized by the absence of intraradical vesicles and the presence of extraradical auxiliary cells (Morton, 1995).

Knobby type auxiliary cells were reported in *Sc. scutata* but no auxiliary cells were seen in *Gi. albida.* Intra and extraradical mycelium were also detected while checking for hyphal colonization. Overall successful colonization was achieved in both the species within 60 days of inoculation, but sporulation was not seen in either of them. Sporulation may occur after some more months in both the species. It is found that *Scutellospora* species undergo an extended vegetative phase lasting 2-3 months *in vitro* before sporulation (Kandula et al., 2006), while members of the Gigasporaceae family, noted are more challenging to sub-culture and proliferate in a monoxenic environment due to their lengthy spore development process (Kandula et al., 2006). Glomeraceae family members allocate resources to reproduction (Ijdo et al., 2011), favoring *in vitro* environments, whereas Gigaspora family members prioritize somatic growth for survival. Dodd et al. (2000) observed that Gigasporaceae family members produce auxiliary cells on extra-radical hyphae instead of vesicles. These cells, identified as carbon storage structures (de Souza and Declerck, 2003), require at least 19 auxiliary cells worth of resources to generate a single spore.



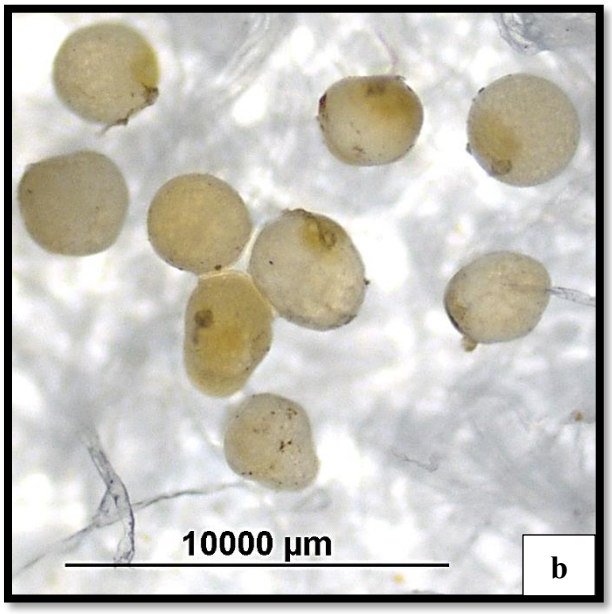


Plate 4.1: A. Study site: Miramar beach, Goa.

Spores isolated from sand dune sample; **a.** *Gi. albida*; **b.** *Sc. scutata.*

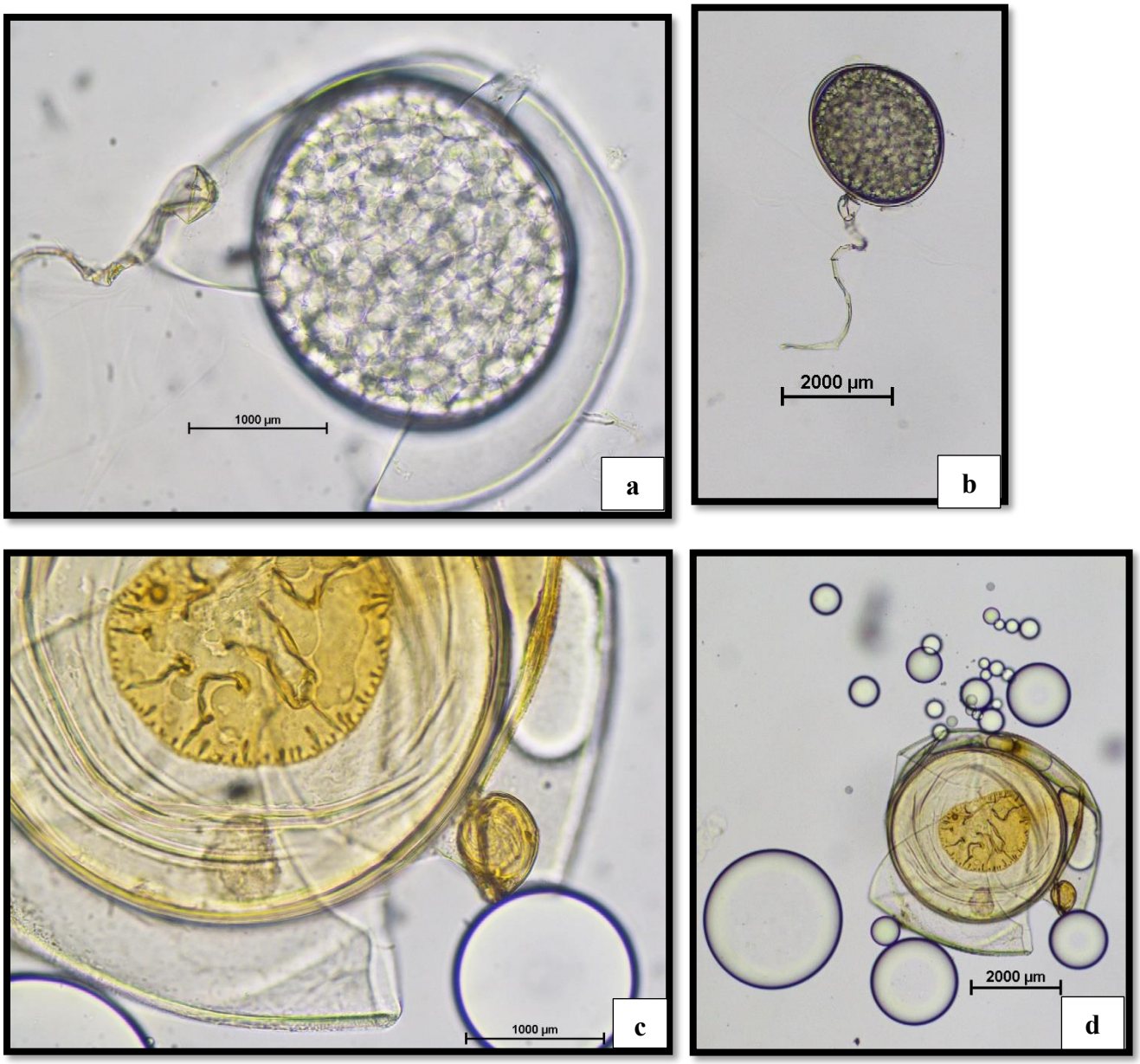


Plate 4.2: Selected spore species for culturing; a. Broken spore of *Gi. albida* (40X); **b.** Intact spore of *Gi. albida* (10X); **c.** Broken spore of *Sc. scutata* (40X); **d.** Broken spore of *Sc. scutata* (10X).

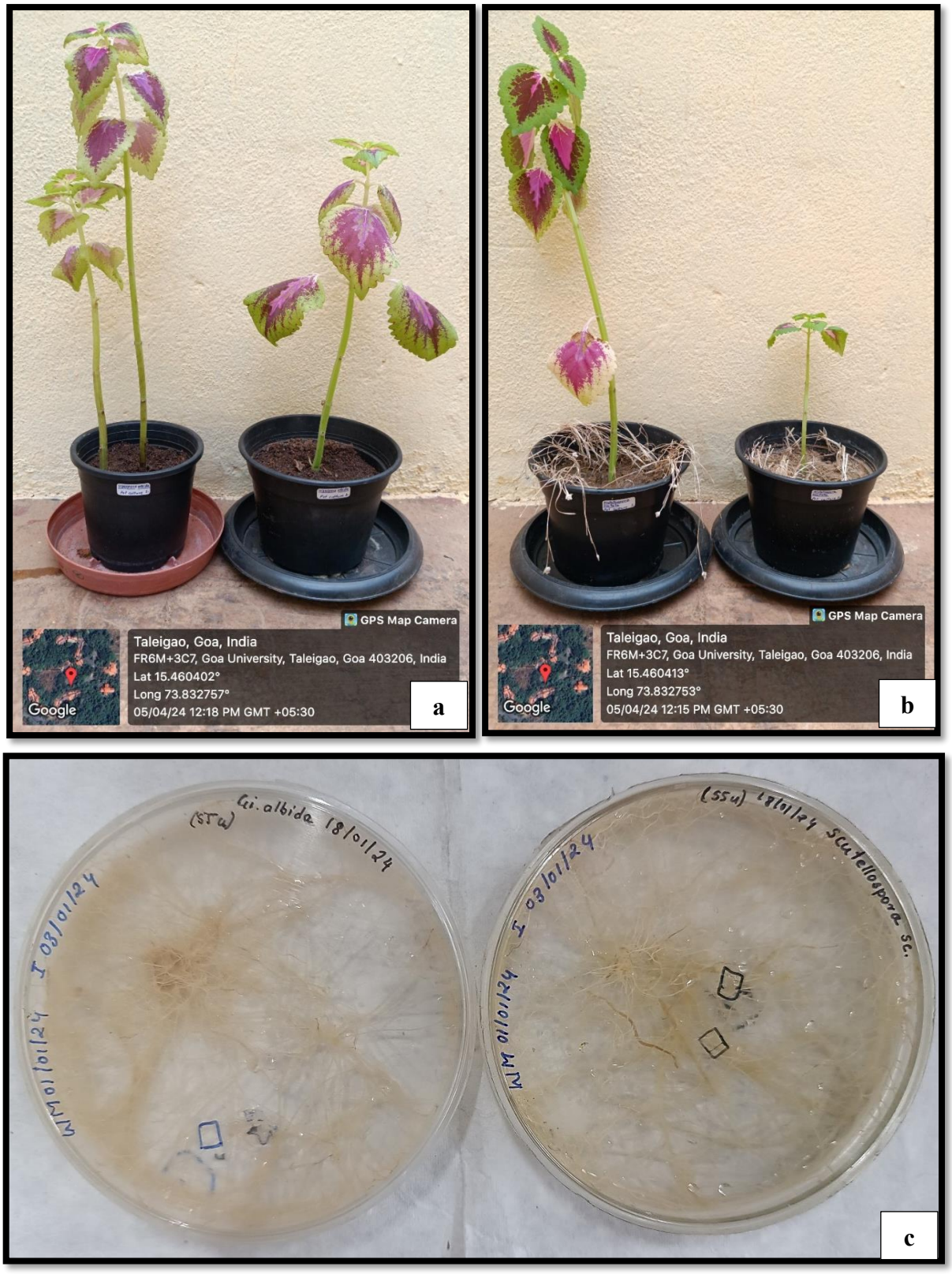


Plate 4.3: 2 methods of AM fungal culturing; a & b. Open pot trap cultures;

c. *In vitro* culture of *Gi. albida* & *Sc. scutata.*

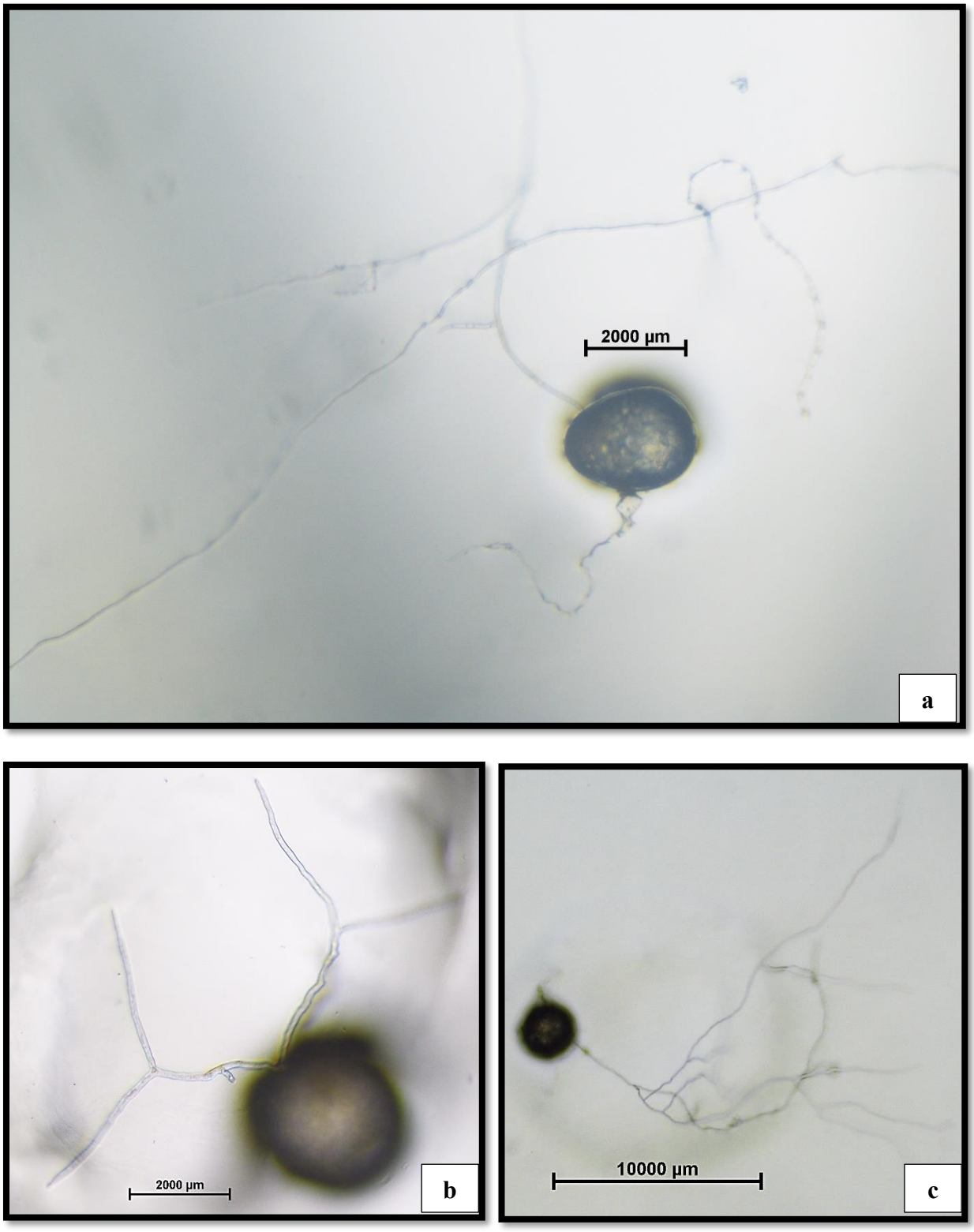


Plate 4.4: *Gi. albida* germination on MSR (-sucrose) media;

a & b. under 40X; **c.** under 4X

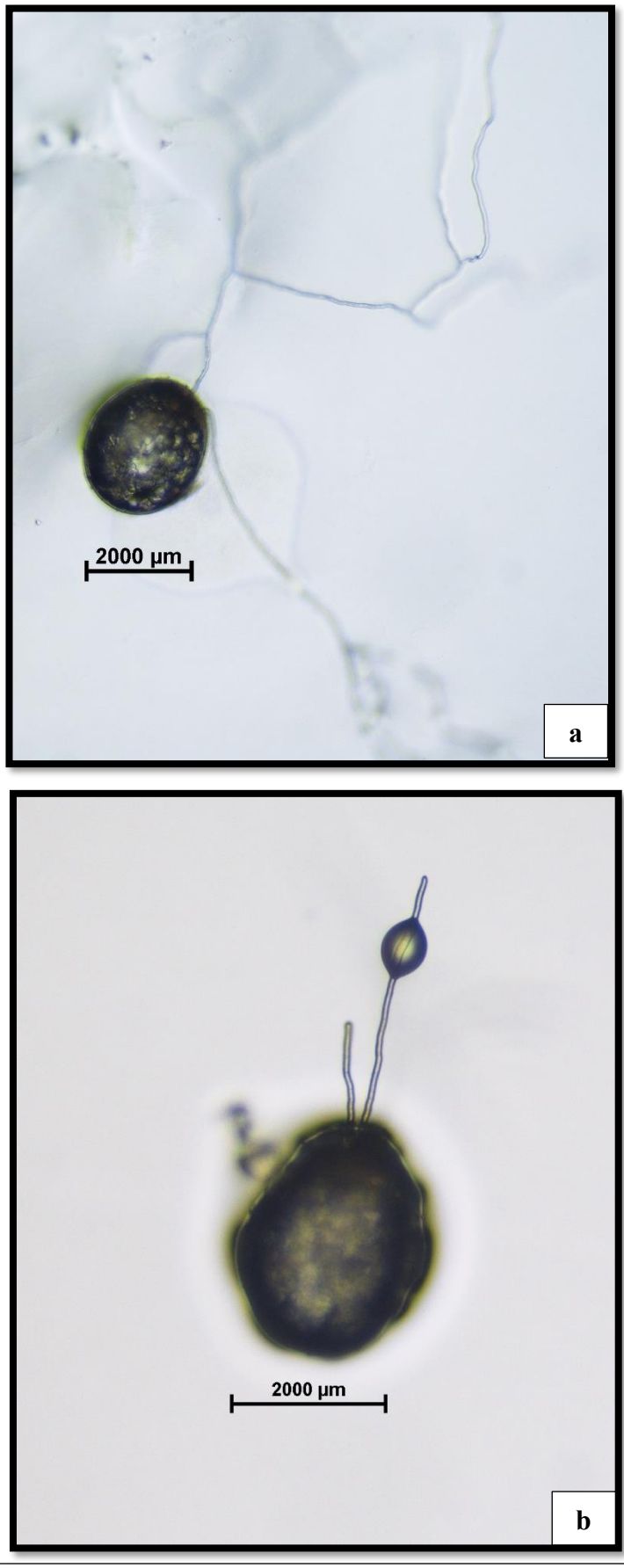


Plate 4.5: *Sc. scutata* germination on MSR (-sucrose) media;

a & b. Under 40X.

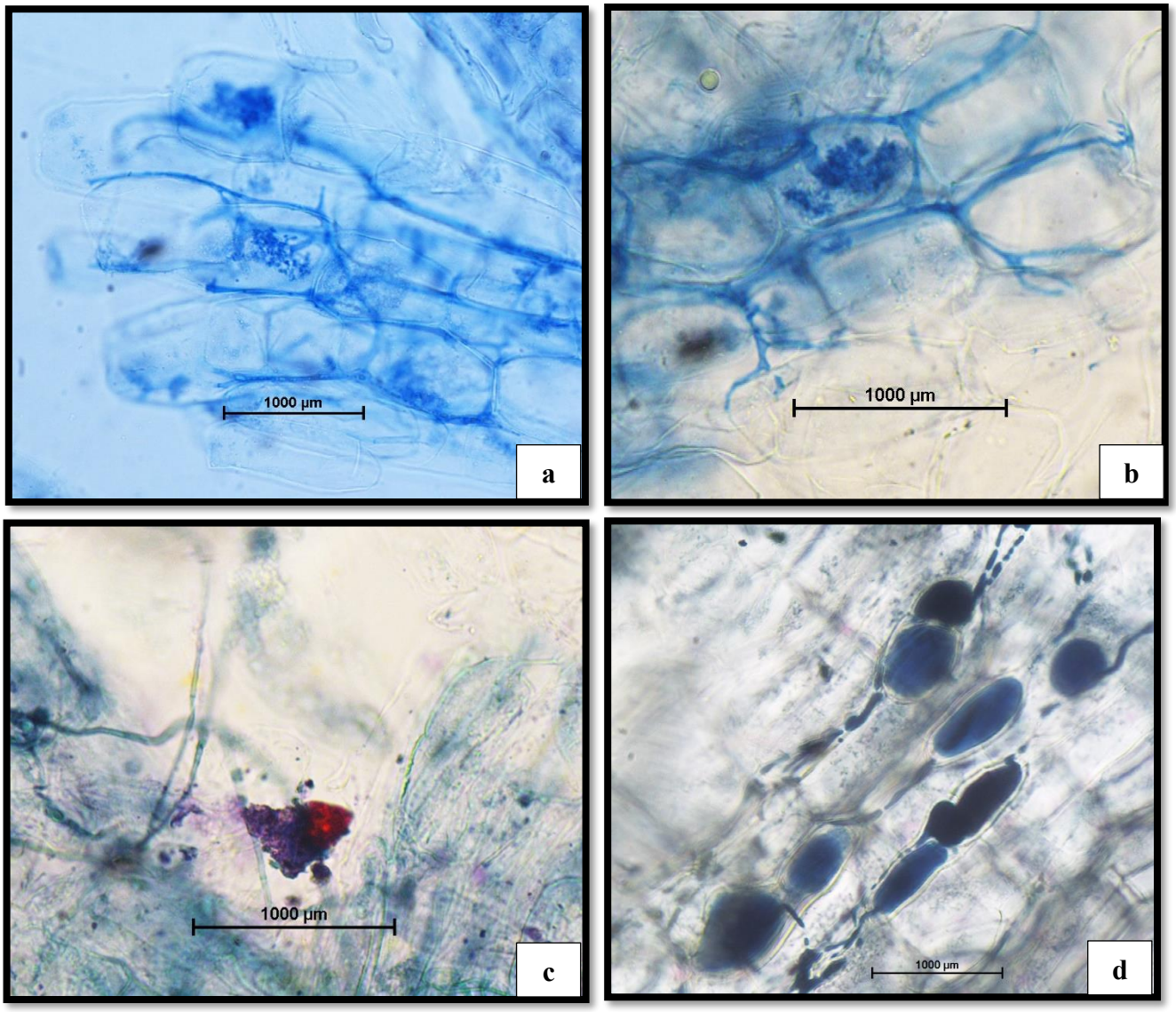


Plate 4.6: Colonization in trap cultures; a & b. Arbuscular colonization; **c.** Toludine blue O staining of polyphosphate granules; **d.** Sudan black staining in vesicles.

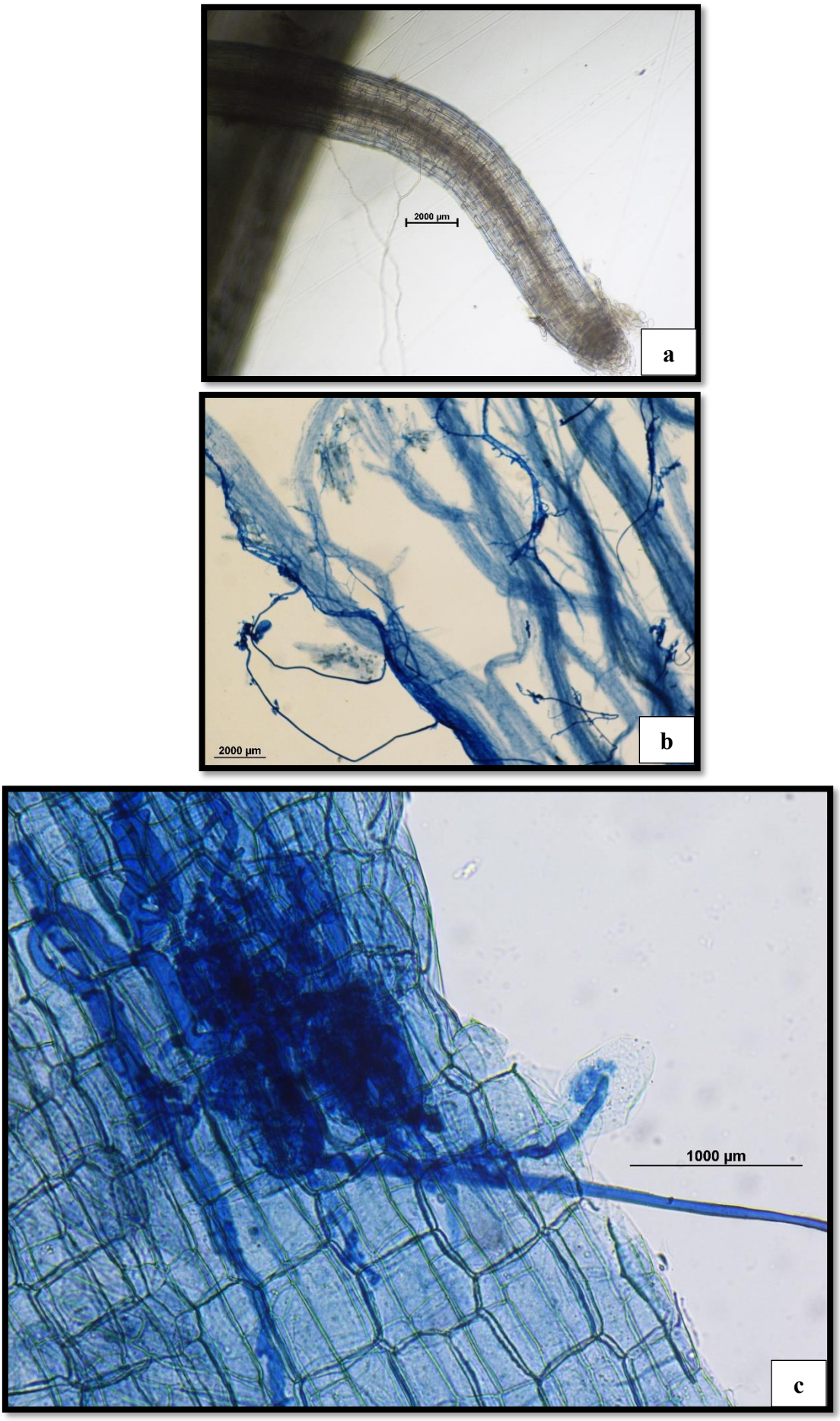


Plate 4.7: *In vitro* colonization in *Gi. albida*; a. Initiation of colonization in Ri T-DNA roots; **b.** Hyphal colonization; **c.** Hyphal and arbuscular colonization.

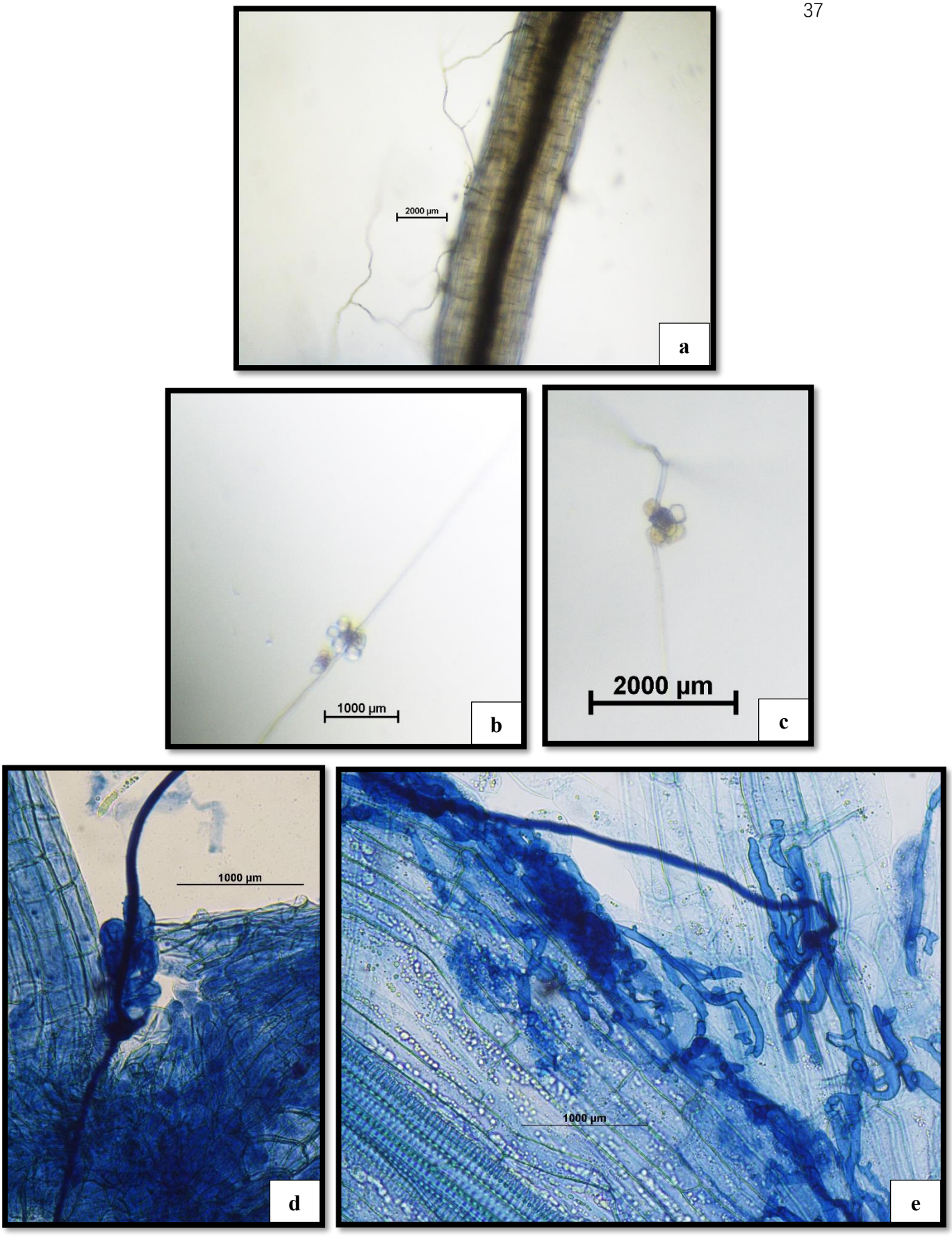


Plate 4.8: *In vitro* colonization in *Sc. scutata*; a. Initiation of colonization in Ri T-DNA roots; **b & c.** Auxiliary cells; **d.** Auxiliary cells stained with tryphan blue; **e.** Hyphal & arbuscular colonization.

CHAPTER 5: CONCLUSION

Based on the results presented above, several conclusions can be drawn regarding the cultivation and characterization of arbuscular mycorrhizal (AM) fungi:

1. **Successful Pot Culture and Spore Isolation:** The use of Coleus and Finger millet as host plants facilitated the cultivation of AM fungal spores, with colonization confirmed through microscopy. The wet sieving and decanting method helped to identify the dominant AM family found in sand dunes i.e. Gigasporaceae, out of which two species, *Sc. scutata* and *Gi. albida* were one of the abundant ones.

2. **Optimization of WM Media for Sub-Culturing:** The standardization of Whites Media (WM) for sub-culturing Ri T-DNA roots demonstrated active growth of Ri T-DNA and provided a suitable medium for the inoculation of germinated AM spores.

3. **Effective Sterilization Protocol:** Surface sterilization of spores using 4% NaClO was optimized, ensuring minimal contamination while maintaining spore viability. The optimal sterilization range was identified to be between 45µl to 55µl of 4% NaClO in 10ml of water.

4. **Germination and Colonization:** Both *Gi. albida* and *Sc. scutata* exhibited

successful germination on MSR plates, and subsequent colonization of Ri T-DNA roots was observed. Auxiliary cells were detected, indicating the development and growth of AM fungal structures.

5. **Implications for AM Fungi Cultivation:** The findings suggest that AM fungi can be successfully cultivated *in vitro*, offering insights into their growth dynamics and colonization patterns. The identified species, *Sc. scutata* and *Gi. albida*, hold potential for further research into their ecological roles and applications in agriculture and ecosystem management.

In conclusion, the results contribute to our understanding of AM fungal biology and provide valuable methods for their cultivation and characterization, laying the groundwork for future studies in this field. Further research may focus on optimizing cultivation techniques, exploring the ecological functions of specific fungal species, and assessing their potential applications in sustainable agriculture and environmental remediation.

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APPENDIX I: WM MEDIA COMPOSITION AND CONCENTRATIONS

Constituent of Stock solution I, II, III, IV, & V for WM media are as below: -

Macro elements involved in solution I		
Element name	**Chemical formula**	**Per 500ml**
Magnesium Sulfate	$MgSO_4 \cdot 7H_2O$	36.55g
Potassium nitrate	KNO_3	4g
Potassium chloride	KCl	3.25g
Sodium phosphate monobasic monohydrate	$NaH_2PO_4 \cdot H_2O$	0.95g
Sodium chloride	NaCl	4.8g

Elements involved in solution II		
Element name	**Chemical formula**	**Per 500ml**
Calcium nitrate tetrahydrate	$Ca(NO_3)_2$-$4H_2O$	14.4g

Elements involved in solution III		
Element name	**Chemical formula**	**Per 250ml**
Potassium iodide	KI	0.1875

Micro elements involved in solution IV		
Element name	**Chemical formula**	**Per 250ml**
Manganese Sulfate	$MnSO_4$-H_2O	1.165 g
Zink Sulfate	$ZnSO_4$-$7H_2O$	0.6625g
Boric Acid	H_3BO_3	0.375g

Copper (II) Sulfate Pentahydrate	CuSO4-5H2O	weight and dilute 0.65g in 50mL water and take 2.5mL of this solution to the mix

Sodium Molybdate Dihydrate	Na_2MoO_4-$2H_2O$	Weight and dilute 0.12 g in 100mL water and take 0.5mL of this solution to the mix.

Elements involved in solution V		
Element name	**Chemical formula**	**Per 125ml**
Sodium iron EDTA	NaFe EDTA	0.2g

Preparation of 500ml of WM Media with sucrose for Ri T-DNA root subculturing:

1. Put 500 ml Nano Pure water in a 500ml media bottle.
2. Add 15 g of sucrose
3. Add 5 mL of Solutions I and II each.
4. Add 2.5 mL of Solutions V.
5. Add 0.5 mL of Solutions III and IV each.
6. Mix with magnetic stirrer.
7. Adjust pH to 5.5 using pH meter.
8. Add 2 g of clerigel with agitation using magnetic stirrer.
9. Autoclave the media at 121°c at 15 psi for 30 minutes.

Preparation of WM media:

1. Put 500 ml Nano Pure water in a 500ml media bottle.
2. Add 15 g of sucrose
3. Add 5 mL of Solutions I and II each.
4. Add 2.5 mL of Solutions V.
5. Add 0.5 mL of Solutions III and IV each.

6. Mix with magnetic stirrer.
7. Adjust pH to 5.5 using pH meter.
8. Add 2 g of clerigel with agitation using magnetic stirrer.
9. Autoclave the media at 121°c at 15 psi for 30 minutes.

After autoclaving, cool the media bottle with periodic shaking. Add 1ml Gamborg solution. Using a Laminar Air Flow, pour the media into sterilized Petri plates.