In vitro cultivation of Arbuscular Mycorrhizal (AM) fungal spores using root organ culture technique.

A Dissertation for

Course code and Course Title: BOT-651 Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc.in Botany

By

PRANALI PREMANAND NAIK

Seat Number: 22P0480015

ABC ID:855-370-443-967

PRN: 201802269

Under the Supervision of

PROF. BERNARD F. RODRIGUES

School of Biological Sciences and Biotechnology

Department of Botany

GOA UNIVERSITY

April 2024

GOA UNIVERSITY SBSB TALEGAO 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 technique"** is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Prof. Bernard F. Rodrigues and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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

Date: 08/04/2024

Place: Goa University

Pranali Premanand Naik

Seat no: 22P04S0015

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"*In vitro* cultivation of Arbuscular Mycorrhizal (AM) fungal spores using root organ culture technique."** is a bonafide work carried out by **Ms Pranali Premanand Naik** under my supervision in partial fulfilment of the requirements for the award of the degree of M.Sc. in the Discipline of Botany at the School of Biological Sciences and Biotechnology, Goa University.

Date: 8-4-2024

Prof. Bernard F. Rodrigues

Signature of Dean of the School

School Stamp

Date: 08/04/2024

Place: Goa University

CONTENTS

PREFACE

Arbuscular Mycorrhizal (AM) fungi are essential for plant health and ecosystem function, enhancing nutrient uptake and soil structure. However, their obligate biotrophic nature makes large-scale cultivation challenging. The root organ culture (ROC) technique has emerged as a promising approach for *in vitro* cultivation of AM fungi. This dissertation explores the potential of the ROC technique for AM fungi cultivation, including optimizing culture conditions, evaluating host plant species, examining the ROC technique's impact on morphology, physiology, and genetic diversity, and exploring the applications of *in vitro* produced AM fungal inoculum in agricultural practices. The findings aim to advance research and applications related to AM fungi, paving the way for efficient and sustainable strategies for utilizing these beneficial microorganisms in agriculture and ecological restoration.

ACKNOWLEDGEMENT

Completing this dissertation has involved many obstacles, personal development and priceless assistance from many people and the institution. I would like to acknowledge everyone who has helped me with this project.

First and foremost, I would like to express my sincere gratitude to Prof. Bernard F. Rodrigues (Dean of the School of Biological Sciences and Biotechnology), my Guide, for all of his advice, guidance and constant encouragement during this project work.

I am greatly thankful to Prof. S. Krishnan (Vice Dean of Research, SBSB), Dr. Rupali Bhandari, (Program Director, Department of Botany, SBSB) for their encouragement and providing me with the resource's facilities used to carry out the research work in the department. I am thankful to all the teaching and non-teaching stuff for their help during this time.

I wish to thank Dr. Tanvi N. Prabhu, Dr. Dhillan Velip and research scholar Mrs. Amisha Shirodkar for their constant and valuable help, guidance and encouragement I received during my work.

I would like to acknowledge my lab mates and classmates who contributed ideas and perspectives that enriched the project.

Finally, I am grateful to my parents for their patience and boosting my morale during this whole project.

Ms. Pranali Premanand Naik

PLATES

ABBREVATION USED

Entity	Abbreviation
Arbuscular mycorrhiza	AM
Modified Stullu-Romand	MSR
The International collection of Vesicular Arbuscular Mycorrhizal Fungi	INVAM
Polyvinyl alcohol- lactic acid-glycerol	PVLG
Root organ culture	ROC
Modified Whites media	MW

ABSTRACT

Arbuscular mycorrhizal (AM) fungi are essential for plant health and ecosystem function, enhancing nutrient uptake, stress tolerance, and soil structure. However, their obligate biotrophic nature presents challenges for large-scale cultivation. The root organ culture (ROC) technique has emerged as a promising approach for *in vitro* cultivation of AM fungi, using excised plant roots grown under sterile conditions. This dissertation investigates the potential of the ROC technique for AM fungi cultivation, including optimizing culture conditions, evaluating host plant species, examining the ROC technique's impact on morphology, physiology, and genetic diversity, and exploring potential applications of *in vitro* produced AM fungal inoculum in agricultural practices. The findings aim to contribute to a deeper understanding of the ROC technique and its potential for advancing research and applications related to AM fungi. By overcoming limitations associated with the obligate biotrophic nature of AM fungi, this research can lead to the development of efficient and sustainable strategies for utilizing these beneficial microorganisms in agriculture and ecological restoration.

Keywords*: in vitro, Gigaspora gigantea,* transformed roots.

CHAPTER 1: INTRODUCTION

1.1. Arbuscular mycorrhizal fungi and their significance

Mycorrhiza is a symbiotic association between fungi and plant roots that plays a significant role. In the environment, mycorrhizal fungi and plant roots frequently coexist in symbiotic relationships (Ijdo et al., 2011). Mycorrhizal associations are typically from the phylum Glomeromycota, although other fungal groups can also form these associations (Rodrigues and Rodrigues, 2015). The different structures formed in this symbiotic association are mycelium, arbuscules, vesicles, auxiliary cells, and spores (Nicolson, 1967). Different AM fungi are cultivated using different *in vitro* techniques and various media. A variety of hosts, AM fungal propagules, are used for the production of specific cultures of the spores and to study in its *in vitro* condition (Diop, 2003). Most studies involve *in vitro*, axenic, monoxenic, or organ cultures.

From an ecological perspective, most vascular plants depend on arbuscular mycorrhiza (AM) fungi for their growth and survival. More than 80% of the terrestrial plant groups and AM fungi work in symbiosis (Harrier, 2001). *In vitro* cultivation has increased significantly in the last few years. Some findings show that there are effective efforts to produce AM fungi without needing a plant host. It is done through studies and procedures of growing root organ cultures and cleaning intraradical and extraradical types of fungal inoculum. AM fungi comprise an external phase known as the extraradical mycelium phase that creates an extensive network in the soil and an interior phase inside the root known as the intraradical mycelium (Barrett et al., 2014).

All terrestrial plants have AM fungus as an integral part of their lives as they help in nutrient exchange and provide additional advantages to plants (Corradi and Bonfante, 2012). They also can

regulate carbon, nitrogen, and P cycling across above and below-ground ecosystem components (Kivlin et al., 2011).

AM fungal propagules are bulk-produced *in vitro* using transformed root cultures (TRC). These propagules are utilized in research, farming, and ecological restoration (Kokkoris and Hart, 2019). This symbiosis is essential for plant growth and survival and has important implications as it improves soil health and biodiversity. It has also been the focus of extensive research in plant-microbe interaction (Rodrigues and Rodrigues, 2015).

1.2. *In vitro* cultivation of AM fungi

Mycorrhizal fungi have been difficult to study due to their obligate biotrophic nature and their dependence on host plants for growth; it is possible to grow them in sterile culture with plant root explants or with hairy roots transformed with *Agrobacterium rhizogenes* (Plenchette et al., 1996).

In *in vitro* cultivation of AM fungi, they are grown in a sterile laboratory environment outside their natural host plant. One of the numerous benefits of this technique is its ability to examine AM fungal biology in a controlled setting. This pure and contaminant-free inoculum production is used in field experiments and isolation for further studies (Adholeya et al., 2005).

The most common method for *in vitro* AM fungi cultivation is using a monoxenic culture system, which involves growing AM fungi in association with transformed plant roots. These transformed roots are typically obtained through genetic modification using *Agrobacterium rhizogene* or other methods and are used as a substrate for AM fungal growth (Ho-Plagaro et al., 2018). Monoxenic culture systems are useful for observing AM fungal growth and development, including sporulation and spore germination, in a non-destructive manner (Srivastava et al., 2014). Other

methods include root organ culture, which involves growing with excised plant roots and using a liquid culture system, i.e., growing AM fungi in a liquid medium (Hildebrandt et al., 2002).

In vitro grown AM fungal inoculum can improve plant growth and development in nutrient-deficient soils or polluted environments (Rodrigues and Rodrigues, 2014).

1.2.1. Commonly used techniques for *in vitro* AM fungal cultivation.

Several studies have been conducted into different approaches for AM fungal *in vitro* cultivation. Hydroponics systems, aeroponics, and nutrient flow techniques have been developed for mass production in *in vitro* cultivation (Akhtar and Abdullah, 2014). The trap culture method, which includes cultivating AM fungus with a host plant in a controlled environment, is frequently used.

Other techniques that are frequently used for growth is the monoxenic culture system. The physiology and genetics of AM fungi can be studied in a controlled environment using monoxenic cultures (Bécard and Fortin, 1988; Cranenbrouck et al., 2005). AM fungi are grown in a sterile culture medium along with a single host plant species using this method.

1.2.2. Advantages and disadvantages in *in vitro* cultivation

The most evident benefit in *in vitro* culture techniques is that they are more appropriate for large-scale production of good-quality inoculum since they do not include undesirable microbes (Ijdo et al., 2011). In this technique, it is constantly necessary to monitor and manage the cultures. However, the requirement for frequent additions of growth media in *in vitro* cultures may cause cross-contamination (Akhtar and Abdullah, 2014).

1.3. Root colonization of AM fungi

Several steps are involved in the root colonization of AM fungi. When AM fungi come in contact with the host plant's root, the plant secretes exudates that stimulate the germination of AM spores. As per Bago et al. (2000), carbon storage compounds are mobilized after the fungus grows, fueling the development of coenocytic germ tubes and providing carbon residue for anabolism. Symbiotic growth is maintained for 1-2 weeks, during which germ tube development reaches several centimeters.

When the symbiosis is successfully established within the time, the AM fungi penetrate the host plant's root by secreting wall-degradation enzymes, and the hyphal tip exerts hydrostatic pressure (Zeilnger et al., 2015). Enzymes like cellulase, pectinase, and xyloglucanase are actively found in colonizing roots and external mycelium of AM fungi (Aggarwal et al., 2011).

Once the AM fungi penetrate the roots, they develop extensively between and within root exodermal and cortical cells and form intraradical structures such as the arbuscules and lipid-rich vesicles (Bago et al.,2000).

1.4. Viability of AM fungi spores

Spores can be extracted from the soil and used as inoculum. Still, such spores tend to have very low viability or may be dead or parasitized, so these soil samples can be taken up to set up a "trap culture" using a suitable host plant and to boost the number of viable spore propagules for isolation, further multiplication and also to produce pure or monospecific cultures (Rodrigues and Rodrigues, 2015).

The medium must be carefully selected for the trap culture to be effective. Rodrigues and Rodrigues (2014) stated that the host plants should resist pests and diseases in the inoculum production environment.

According to Xueguang Sun et al. (2016), the spores in the rhizosphere can be divided into four kinds based on their viability, i.e., dead spores with exhausted (empty spores), dead spores with content, viable spores that could not germinate, and spores that could germinate.

The presence of the extraradical mycelium characterizes them as branched haustoria-like structures within the cortical cells, also known as the arbuscules (Sharma et al., 2017). Its primary role is to increase the surface of the roots during nutrient transfer. After colonizing the plant roots, the fungi penetrate the surrounding soil, extending to the root-depleting zone (Mohmmadi et al., 2011).

1.5. AM fungi: Their Function in Reducing Abiotic and Biotic Stress in Plants

Drought significantly reduces plant productivity. This occurs as water constraints lead to stomatal closure, resulting in decreased CO_2 influx and photosynthetic activity. As a result, there is a decline in plant productivity and agricultural yield. However, it has been found that AM fungi can enhance plant performance under drought stress (Tang et al., 2022). AM helps mitigate water deficit and improve the plant's ability to tolerate drought by enhancing water uptake and providing direct benefits.

Temperature is a crucial stress factor impacting plant growth and productivity. AM fungi can enhance plant tolerance to temperature stress by improving water and nutrient uptake, photosynthesis, and protecting against oxidative damage (Jajoo and Mathur, 2021).

AM fungi naturally occur in saline environments and are known to enhance the growth of plants in saline conditions (Porcel et al., 2012).

AM fungi found in mining and polluted sites are adapted to heavy metal contamination. Over 80% of plants on mining sites are colonized by AM fungi, which shows a diverse range of these species (Karimi et al., 2011). Studies indicate that AM fungi positively affect plant survival, growth, nutrition, soil quality, and re-establishment.

Aims and objective

The main aim of this study is to highlight AM fungal cultivation methods, which include specific host type, culture media and AM fungal propagule used to begin monoxenic culture.

The primary goal is to study the Ri T-DNA transformed hairy roots system to facilitate the *in vitro* culturing of Gigaspora gigantea species.

The objective of this project is;

- Isolation of AM spores
- Preparation of trap culture
- *In vitro* germination of AM spores
- *In vitro* colonization of AM spore in Ri T-DNA transformed roots.

CHAPTER 2: REVIEW OF LITERATURE

- Bécard and Fortin (1998) explored the early stages of mycorrhizal development using Ri T-DNA altered roots and *Gigaspora margarita*. The medium required essential nutrients like sodium, phosphorus, and sucrose. The colonization process was monitored within five days, and aseptic, viable spores were obtained in large quantities. This method is suitable for studying fungal biotrophy in the root direction.
- The study monitored the *in vitro* spore formation of the arbuscular mycorrhizal fungus *Glomus versifome* using a mycorrhizal root-segment inoculum with carrot roots. Three stages of sporulation were observed: lag phase, intense phase, and plateau phase. After five months, 9500 spores were generated per Petriplate. The root-organ culture system facilitated vast colonization, leading to the formation of arbuscules and vesicles. Mycorrhizal root segments showed greater inoculum potential. (Declerck et al., 1996)
- Spores of *Glomus intraradices* were cultivated in a bi-compartment system using genetically modified *Daucus carota* (carrot) roots. Mycorrhizal roots developed in the proximal compartment, which had full growth media, and in the distal compartment, which had a sucrose-free medium. The mycelium colonized the distal compartment, resulting in larger hyphal and spore concentrations. This allows for the production of aseptic spores for large-scale inoculum manufacturing and research objectives (St-Arnaud et al., 1996)
- This study reveals three stages of the spread of arbuscular mycorrhizal fungi, *Glomus intraradices*, grown monoxenically with tomato roots. These stages include runner hyphae proliferation, arbuscule-like structure formation, and spore production. The medium's pH varies with the mycorrhiza's decrease in development, with a higher pH in areas with more fungal spores. The complex structure of the extraradical mycelium suggests increased

nutrient absorption by mycorrhizal roots and their role in preserving and improving soil structure. (Bago et al., 1998)

- Hairy roots can grow in minimal media, but they persist in MS medium. A new spore surface sterilization technique is presented, making it easier to use. Surface sterilization *G. margarita* spores are used to inoculate altered roots on M medium, promoting spore germination and development. Mycorrhizal spore penetration into the root cortex, arbuscular development, and mycelium expansion are observed in the plant's altered roots. (Khaliq and Bagyaraj, 2000)
- Karandashov et al. (2000) established AM fungal species of *Glomus caledonium* in dual culture with transformed carrot root on minimal M medium. The spores colonized the roots and completed the life cycle. Sporulation with the formation of arbuscules like structures (*Paris*-type) was observed.
- Carrot roots transformed with Ri T-DNA were used to create an *in vitro* system that successfully supported arbuscular mycorrhizal (AM) fungi from the genera *Glomus intraradices* and *Gigaspora margarita*. The co-culture model is suitable for future research on interaction and competition mechanisms in natural associations and mass production of multiple mycorrhizal fungal isolates. This is the first successful co-culture of these genera of AM fungus *in vitro* (Tiwari and Adholeya, 2002)
- Diop (2003) studied the procedure for selecting and purifying AM fungal inoculum in intraradical and extraradical forms and cultivating root organs. The use of *in vitro* technique can be further explored, and the potential of AM symbiosis can be studied using axenic and monoxenic cultures of AM fungi to restore and maintain an ongoing culture while maintaining their infection.

- Rodrigues and Rodrigues (2013) provide an update on the growing range of AM fungal species using ROC and the advancements in producing AM fungal inoculums. They discuss the techniques for cultivating root organ culture, culture media, selected host roots, propagule selection and sterilization, and continuous cultures for maintaining inoculum colonization. They also discuss the development of AM fungal cultures, their applications, and their drawbacks.
- This study aimed to establish monoxenic cultures of *Gigaspora decipiens* and *Glomus clarum* in transformed roots and to understand their symbiosis. The cultures were established from spores germinated on agar and water and transferred to Petri plates containing transformed carrot roots. The effects of temperature and pH on spore production of *Gi. decipiens* occurred at a pH of 6.5, while *G. clarum* had a pH of 4.0. (Costa et al., 2013)
- Rodrigues and Rodrigues (2015) demonstrated the potential of the AM fungus *Funneliformis mosseae* in co-cultivation with transformed linum roots. The spores emerged through the spore wall in 88% of cases after five days, and the procedure showed potential for mass production of AM inoculum and potential application in various applications. The results suggest the potential of this method in promoting AM inoculum production.
- Silvani et al. (2019) developed a new *in vitro* technique to study AM symbiosis in a natural environment. This method involves developing AM fungal species of *Rhizophagus intraradices* and *Rhizophagus aggregatus* in an artificial medium with sterilized soil. This method produces an extensive extra-radical mycelia network and viable spores, similar to pot cultures.

- Kokkoris and Hart (2019) discussed the possible effects of TRC propagation on the traits of AM fungi and how this may impact their utility.
- A successful effort was made to cultivate and sporulate the AM fungal species *Gigaspora decipiens* (Hall and Abbott) *in vitro* using transformed *Linum usitatissimum* L. (Flax) roots. It explained a method that eliminates the need to distribute a previously germinated AM spore among the T-DNA roots to achieve spore germination and *in vitro* root colonization on the same Petriplate. (Velip and Rodrigues, 2019)

CHAPTER 3: METHODOLOGY

3.1. Sample collection and isolation of spores

Soil sample was collected from the dunes of Miramar Beach, Goa, to isolate the AM fungal spores. The sample was collected from rhizosphere soil close to the roots, from 0-25 cm depth. AM fungal spores were isolated using the Wet Sieving and Decanting method (Gerdemann and Nicolson, 1963). In 20g of fresh or refrigerated soil sample, tap water was added and stirred with the help of a glass rod for 10-15 seconds. The sediments were allowed to settle. The supernatant was poured through sieves positioned from 250 to 53µm in descending order. This process was carried out for a minimum of four times. From each sieve, the residue was cleaned and placed in a different beaker. Using Whatman No. 1 filter paper, the aliquot was filtered out individually carefully the filter paper was placed on a Petri plate, and care was taken to keep it wet. The filter paper was observed under a stereo microscope (Olympus SZ51) for the presence of spores and sporocarps.

3.2. Taxonomic identification of the spores

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

AM fungi were propagated by the trap culture method. Pots to be used for this method were cleaned with diluted Dettol and wiped with 70% ethanol to sterilize the pots. Soil sterilization was done by

keeping the soil in hot air at over 100^0C for three days. AM fungal spores were isolated by wet sieving and decanting method (Gerdemann and Nicolson, 1963) from the rhizosphere soil sample and introduced in sterilized pots with sterilized soil. Coleus (*Plectranthus scutellariodies*) cuttings or Ragi (*Eleusine coracana*) were used as the host plant. These pots were maintained in the Polyhouse for colonization of the roots.

3.4. Culture media for cultivation of transformed roots and AM fungal propagules

Culture media used for the cultivation of transformed roots was MW media (Bécard and Fortin, 1988). An MSR medium (Declerck et al*.,* 1988) and agar water without sucrose were used to cultivate AM propagules.

3.5. Disinfection process of AM propagules.

The isolated spores were cleaned twice with sterilized distilled water before being disinfected. After that, the spores were placed in a 4% sodium hypochlorite solution for surface sterilization. The concertation and time depended on the spore size. Then the spores were rinsed with sterile distilled water twice and treated with streptomycin for 1-2 minutes.

3.5.1. Germination of the Disinfected Spores

The spores, after disinfection, were placed in the MSR medium/medium containing only agar and water. These plates were then kept for incubation in the dark at 27^0C by sealing the plates with parafilm or cling wrap.

3.6. Growing of the Ri T-DNA roots.

Ri T-DNA roots of Carrot (*Dracus carota)* were provided by Prof. B. F. Rodrigues, Botany discipline, SBSB, Goa University. WM media was used for sub-culturing. This plate contained sucrose with clerigel, and the pH was maintained at pH 5.5. Gamborg vitamin solution was added

for the active growth of the Ri T-DNA transformed roots. The plates were kept inverted and incubated at 27^0C in the dark.

3.7. Trypan Blue staining for root colonization (Phillips and Hayman, 1970)

The roots were washed under running tap water to remove dirt or organic particles attached and cut into small segments. These roots were placed in a test tube with 10% KOH at 90^0C for 1 hour in a hot air oven, as KOH clears the cytoplasm and nuclei for stain penetration. The KOH was poured out, and the roots were rinsed with tap water to remove the traces of KOH. 5N HCl was added, and the roots were soaked in it for 3-4 minutes and then poured off. In this, 0.05% Trypan blue stain was added and kept overnight. The root segments were mounted on a clean slide with PVLG and observed under a compound microscope (Nikon Eclipse E200) for AM colonization.

CHAPTER 4: RESULT AND DISCUSSION

4.1. Results

4.1.1. Spore isolation and taxonomic identification

The spores isolated using the wet sieving and decanting method, were selected based on their abundance in the soil sample. Taxonomic identification of the spores was done by using the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (invam.ku.edu), Rodrigues and Muthukumar (2009) and Blaszwski (2012). Based on this study, *Gigaspora gigantea* was selected for further study (Plate 4.1).

4.1.2. Modification of White media (MW) media for ROC and sub-culturing the transformed roots

Modified WM media was used for growing the Ri T-DNA roots. After ten days of inoculation on the MW medium, the Ri T-DNA roots showed active growth (Plate 4.2). Later, the germinated spores of *Gi. gigantea* were placed in the medium containing Ri T-DNA roots.

4.1.3. Surface sterilization, germination, and colonization of spore

Spores sterilized using 4% sodium hypochlorite (NaOCl) were then rinsed in sterile distilled water. The germination of the spores was observed after 12 days of inoculation in the MSR media (-sucrose), while the spores germination after seven days on the agar media (Plate 4.4). The germinated spores were transferred to a ROC plate containing transformed roots and kept inverted for incubation in dark at 27^{o}C. Colonization was observed after one month of spore inoculation in the transformed roots also auxiliary cells were observed (Plate 4.4)

4.1.4 Preparation of trap cultures.

Mass multiplication of AM fungal spores isolated from the soil was accomplished using Coleus (*P. scutellarioides*) as the host plant. The AM culture was maintained as culture in the polyhouse,

Arbuscular Mycorrhizal culture collection at Goa University. After 45 days, the roots were used to check AM fungal colonization, which showed arbuscule (Plate 4.2 & 4.3).

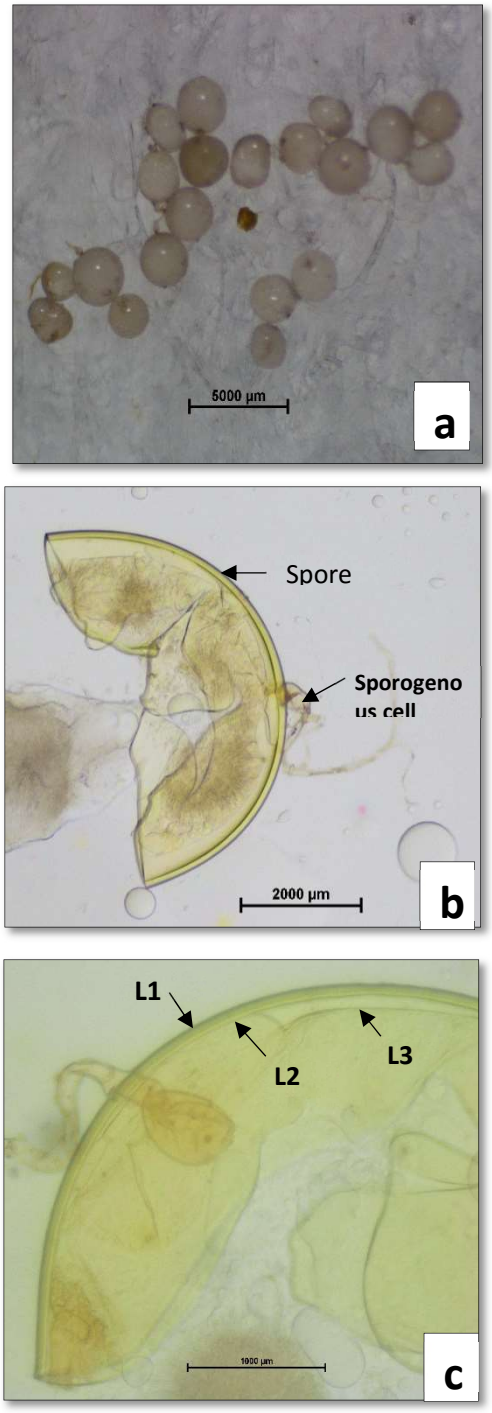

Plate 4.1: AM spore isolation: a. Isolated spores of *Gi. gigantea* from sand dunes; **b.** Broken spore of *Gi. gigentia* showing spore wall and sporogenous cell; **C.** Broken spore showing wall layer (L1, L2 & L3)

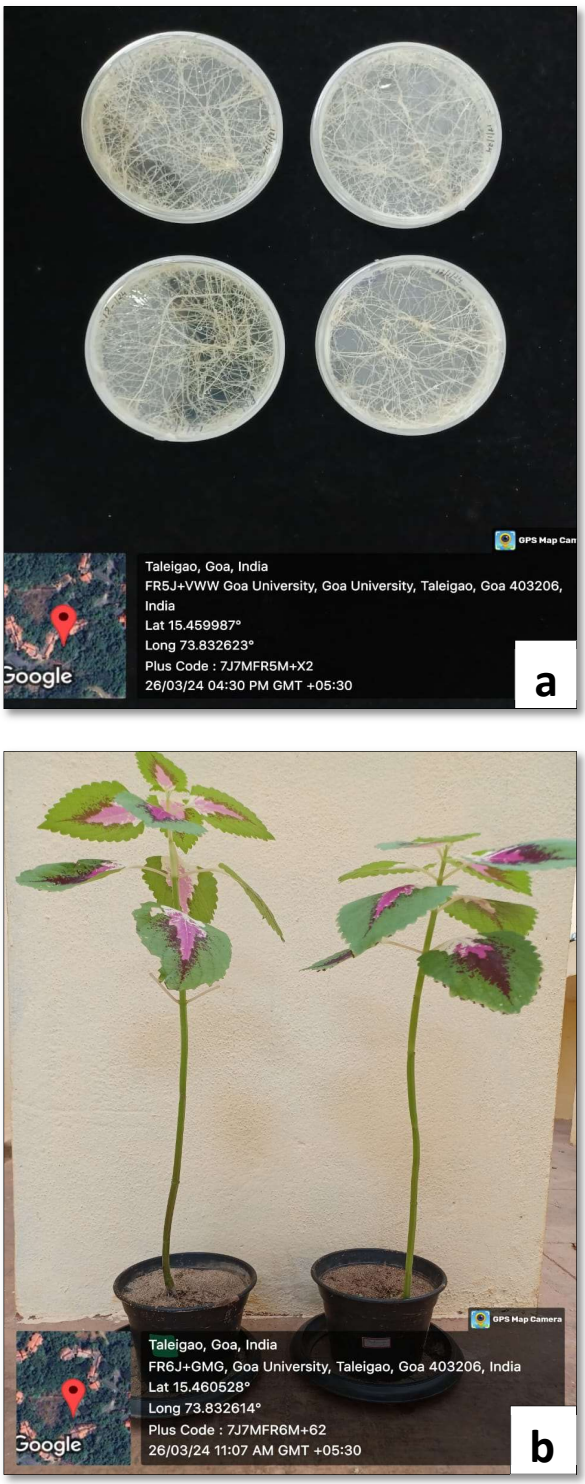

Plate 4.2: Establishment of Ri T-DNA cultures and Trap culture: **a.** Culture of transformed Ri T-DNA roots: **b.** Trap culture.

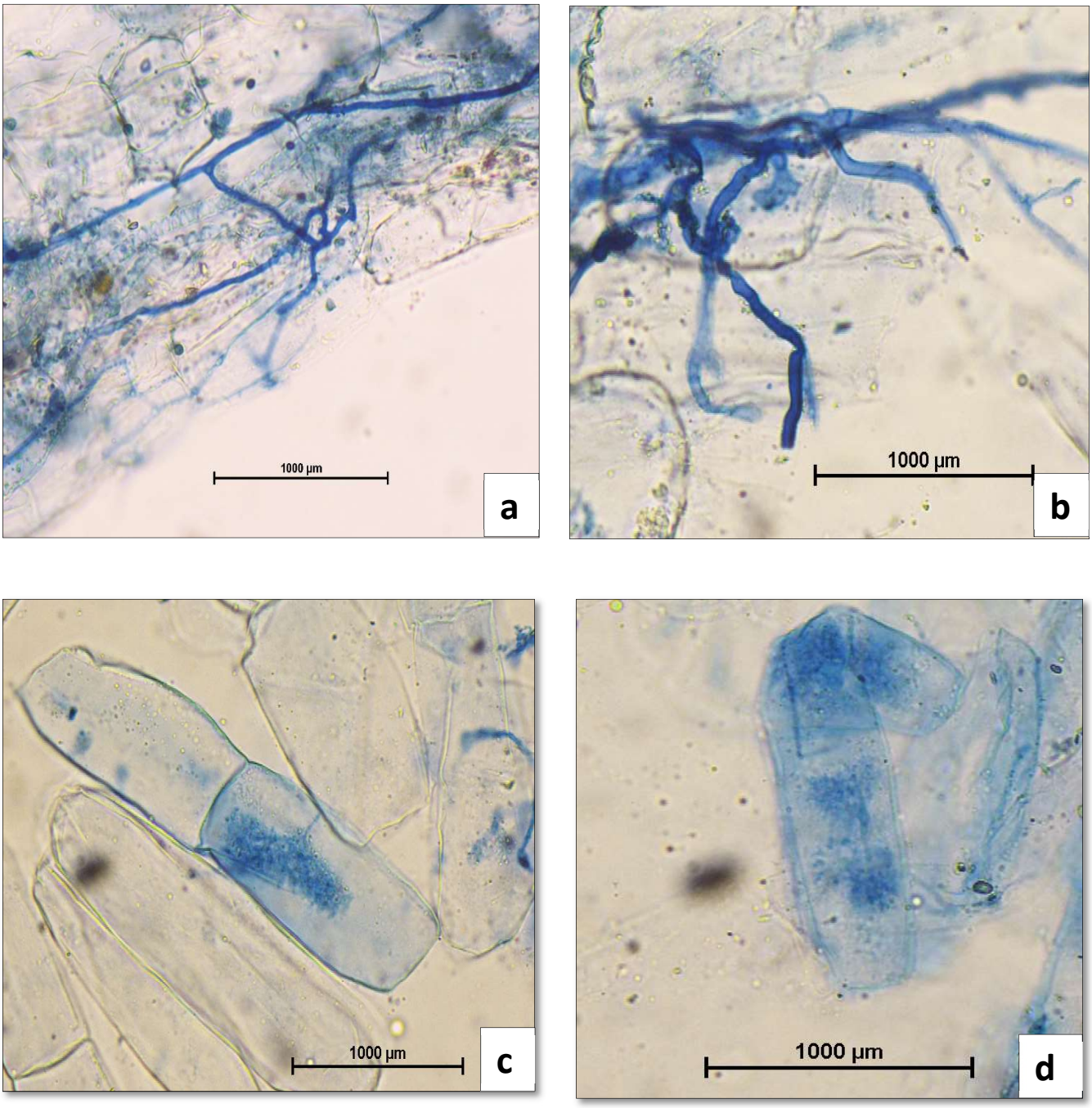

Plate 4.3: AM fungal root colonization in trap culture: a. Hyphal colonization; **b.** Hyphal coiling **c & d.** Formation of arbuscules.

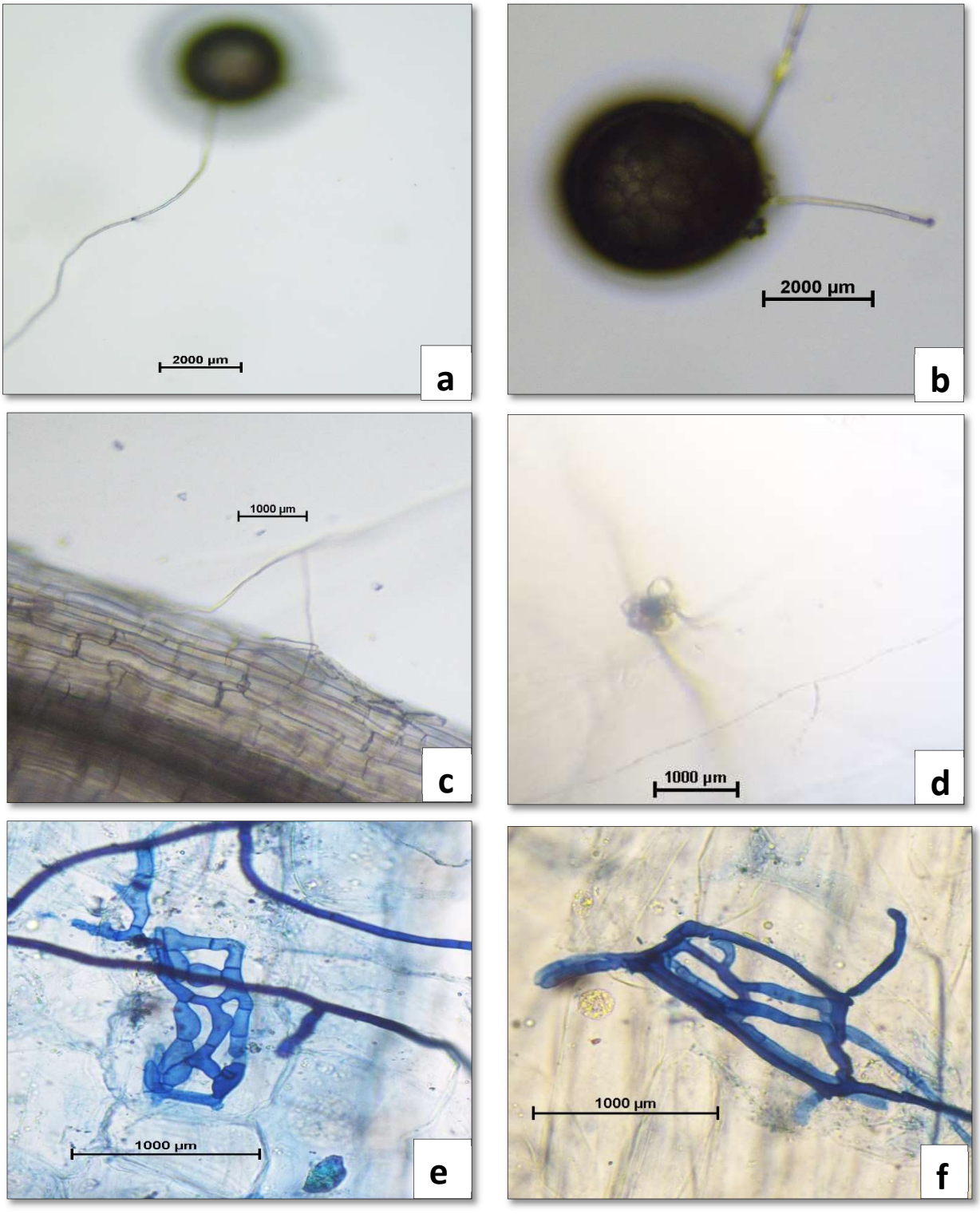

Plate 4.4: Germination of *Gigaspora gigantea* spore and *in vitro* root colonization in Ri T-DNA roots: a & b. Spore germination; **c.** hyphae entering Ri T-DNA roots; **d.** Auxiliary cell formation. **e & f.** Hyphal coiling

4.2. Discussion

In vitro cultivation is a highly effective method for the experimental establishment and investigation of AM association and its life cycle. A small but increasing quantity of research on monoxenic culture reports that AM relationships have been successfully established utilizing a variety of AM fungi (Fortin et al., 2002). In this study, we report *in vitro* germination of *Gigaspora gigantea*. However, sporulation was not observed.

In the present study, *in vitro* germination occurred via the emergence of a germ tube through the spore wall. Emergence of single or multiple germ tubes were recorded. This was followed by hyphal proliferation and branching. According to Giovannetti et al. (2010), the germ tubes of the *Gigaspora, Scutellospora*, and *Acaulospora* species emerge straight through the spore wall. The capacity of fungal spores to germinate repeatedly by generating consecutive germ tubes is known as multiple germination (Koske, 1981b). Multiple germination is an extra survival strategy used by the spores to boost the chances of making successful contact and colonizing the host root (Costa et al., 2013).

MSR media minus sucrose was used for germination, ensuring 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 branches into filaments with progressively smaller diameters to explore the medium.

Germination is usually followed by sporulation. However, in the present study, even after several attempts no sporulation was recorded. This could be due to their adapting time to the *in vitro* culture condition and a long vegetative phase as reported by Kandula et al. (2006). Members of the Gigasporaceae family are said to be more challenging to sub-culture and proliferate in a monoxenic environment (Dalpe et al., 2005). According to Ijdo et al. (2011), members of the

Glomeraceae family use their resources in reproduction, making them more suited to *in vitro* environments. In contrast, members of the Gigasporaceae family invest their energy and resources in somatic growth, which allows them to grow and survive. Members of the Gigasporaceae family generate auxiliary cells on extra-radical hyphae rather than vesicles (Dodd et al., 2000). At least 19 auxiliary cells worth of resources are required to generate a single spore (Declerck et al., 2004).

CHAPTER 5: CONCLUSION

The *Gigaspora gigantea* from the sand dunes of Miramar can be successfully isolated onto MW media under specific lab conditions. The spore germination was observed after 14 days thus indicating its viability. The germinated spores successfully colonized in the Ri T-DNA transformed roots of carrot. Monoxenic growth of AM fungus on root culture provides a high quality for large-scale inoculum generation. It also enables thorough observations and long-term experiments on the life cycle of AM fungi. However, sporulation was not observed, but it can be used in the production of spores that are free from undesirable microbes.

REFERENCES

- Abdul Khaliq, A. K., & Bagyaraj, D. J. (2000). Colonization of arbuscular-mycorrhizal fungi on Ri T-DNA transformed roots in synthetic medium. https://doi.org/10.5555/20013004180
- Adholeya A, Tiwari P, Singh R (2005) Large-scale production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. Declerck S, Strullu DG, Fortin JA (Eds.) In: *In vitro* culture of mycorrhizas. pp: 315-338. Heidelberg: Springer.
- Aggarwal A, Kadian N, Tanwar A, Yadav A, Gupta KK (2011) Role of arbuscular mycorrhizal fungi (AMF) in global sustainable development. Journal of Applied and Natural Science 3(2):340–351 https://doi.org/10.31018/jans.v3i2.211
- Akhtar, M. S., & Abdullah, S. N. A. (2014). Mass production techniques of arbuscular mycorrhizal fungi: major advantages and disadvantages: a review. Biosci Biotechnol Res Asia, 11, 1199-1204. doi: http://dx.doi.org/10.13005/bbra/1506
- Barrett G, Campbell CD, Hodge A. (2014). The direct response of the external mycelium of arbuscular mycorrhizal fungi to temperature and the implications for nutrient transfer. Soil Biol. Biochem. 78:109–17 https://doi.org/10.1016/j.soilbio.2014.07.025
- Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108: 211-218. https://doi.org/10.1111/j.1469-8137.1988.tb03698.x
- Bago, B., Pfeffer, P. E., & Shachar-Hill, Y. (2000). Carbon metabolism and transport in arbuscular mycorrhizas. *Plant physiology*, *124*(3), 949-958.https://doi.org/10.1104/pp.124.3.949

- Blaszkowski, J. (2012). Glomeromycota. W. Szafer Institute of Botany, Polish Academy of Sciences.
- Costa FA, Haddad LSM, Kasuya MCM, Oton WC, Costa MD, Borges AC (2013) *In vitro* culture of *Gigaspora decipiens* and *Glomus clarum* in transformed roots of carrot: the influence of temperature and Ph. Acta Sci., Agron. 35: 315-323.
- Corradi N, Bonfante P. (2012) The Arbuscular mycorrhizal symbiosis: origin and evolution of a beneficial plant infection. PLoS Pathog 2012; 8:8–10 https://doi.org/10.1371/journal.ppat.1002600
- Cranenbrouck S, Voets L, Bivort C, Renard L, Strullu DG, Declerck S (2005) Methodologies for *in vitro* cultivation of arbuscular mycorrhizal fungi with root organs. Declerck S, Strullu DG, Fortin JA (Eds.) In: *In vitro* culture of mycorrhizas. pp: 341-375. Springer-Verlag, Heidelberg https://doi.org/10.1007/3-540-27331-X_18
- Diop, T.A., (2003). *In vitro* culture of arbuscular mycorrhizal fungi: advances and future prospects. Afr. J. Biotechnol. 2, 692-697. https://doi.org/10.5897/AJB2003.000-1128
- de Souza, F. A., & Declerck, S. (2003). Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. *Mycologia*, *95*(6), 1004-1012.https://doi.org/10.1080/15572536.2004.11833016
- Declerck, S., Risede, J. M., & Delvaux, B. (2002). Greenhouse response of micropropagated bananas inoculated with *in vitro* monoxenically produced arbuscular mycorrhizal fungi. *Scientia Horticulturae*, *93*(3-4), 301-309. https://doi.org/10.1016/S0304-4238(01)00347-8

- 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. https://doi.org/10.1139/b01-139
- Giovannetti, M., Avio, L., & Sbrana, C. (2010). Fungal spore germination and pre-symbiotic mycelial growth–physiological and genetic aspects. *Arbuscular mycorrhizas: physiology and function*, 3-32. https://doi.org/10.1007/978-90-481-9489-6_1
- Harrier, L. A. (2001). The arbuscular mychorrizal symbiosis: a molecular review of the fungal dimension. J. Exp. Bot. 52, 469-478. https://doi.org/10.1093/jexbot/52.suppl_1.469
- Hildebrandt, U., Janetta, K., and Bothe, H. (2002). Towards growth of arbuscular mycorrhizal fungi independent of plant host. Appl. Environ. Microbiol. 68, 1919-1924. https://doi.org/10.1128/AEM.68.4.1919-1924.2002
- Ho-Plagaro T, Huertas R, Tamayo-Navarrete MI, Ocampo JA, Garcıa-GarridoJM. (2018). An improved method for *Agrobacterium rhizogenes*-mediated transformation of tomato suitable for the study of arbuscular mycorrhizal simbiosis. Plant Methods14: 34. https://doi.org/10.1186/s13007-018-0304-9
- https://invam.ku.edu/
- Ijdo M, Cranenbrouck S, Declerck S. (2011). Methods for large-scale production of AM fungi: past, present, and future. Mycorrhiza, 21:1–16. https://doi.org/10.1007/s00572-010-0337-z
- 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. https://doi.org/10.1007/s005720050283

- Kivlin, S.N.; Hawkes, C.V.; Treseder, K.K. (2011**)** Global diversity and distribution of arbuscular mycorrhizal fungi. *Soil Biol. Biochem*, *43*, 2294–2303. https://doi.org/10.1016/j.soilbio.2011.07.012
- Kokkoris V, Hart M (2019). *In vitro* propagation of arbuscular mycorrhizal fungi may drive fungal evolution. Front Microbiol, 10:2420. https://doi.org/10.3389/fmicb.2019.02420
- Koske RE (1981b) Multiple germination by spores of *Gigaspora gigantea*. Trans. Br. Mycol. Soc. 76: 328-330. https://doi.org/10.5555/19811963095
- Mohammadi, K., Khalesro, S., Sohrabi, Y., & Heidari, G. (2011). A review: beneficial effects of the mycorrhizal fungi for plant growth. *J. Appl. Environ. Biol. Sci*, *1*(9), 310-319.
- Nicolson TH (1967) Vesicular-arbuscular mycorrhiza—a universal plant symbiosis. Science Progress, Oxford 55:561–581 https://www.jstor.org/stable/43419694
- Plenchette C, Declerck S, Diop TA, Strullu DG (1996) Infectivity of monoxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot root. Appl. Microbiol. Biotechnol. 46: 545-548. https://doi.org/10.1007/s002530050858
- Rodrigues KM, Rodrigues BF (2014) Arbuscular mycorrhizal (AM) fungi and plant health. SIES college, Sion, Mumbai. In: Gosavi M (ed) Fungi in biotechnology. SIES College, Sion, Mumbai, pp 8–24
- Rodrigues BF and Muthukumar T (2009) Arbuscular mycorrhizae of Goa- a manual of identification protocols, pp: 109-135. Goa University, Goa.
- Rodrigues, K.M., and B.F. Rodrigues. (2015). Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum*: germination,

colonization, and sporulation studies. Mycology 6(1): 42–49. https://doi.org/10.1080/21501203.2015.1024777

- Schüßler A. and Walker C (2010). The Glomeromycota: a species list with new families and genera. Schüßler A, Walker C (Eds.) In: Libraries at the Royal Botanic Garden Edinburgh, The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University.
- Sharma, S., Sharma, S., Aggarwal, A., Sharma, V., Singh, M. J., & Kaushik, S. (2017). Mass multiplication of arbuscular mycorrhizal fungi. *Mycorrhizal Fungi*, 154-173.
- 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. https://doi.org/10.1007/s13199-019-00637-z
- Srinivasan, M., Kumar, K., Kumutha, K., & Marimuthu, P. (2014). Establishing monoxenic culture of arbuscular mycorrhizal fungus *Glomus intraradices* through root organ culture. Journal of Applied and Natural Science,6(1),290–293. https://doi.org/10.31018/jans.v6i1.417
- Sun, X., Hu, W., Tang, M., & Chen, H. (2016). Characterizing and handling different kinds of AM fungal spores in the rhizosphere. *World Journal of Microbiology and Biotechnology*, *32*, 1-8. https://doi.org/10.1007/s11274-016-2053-0
- Tang, H., Hassan, M. U., Feng, L., Nawaz, M., Shah, A. N., Qari, S. H., ... & Miao, J. (2022). The critical role of arbuscular mycorrhizal fungi to improve drought tolerance and nitrogen use efficiency in crops. *Frontiers in Plant Science*, *13*, 919166. https://doi.org/10.3389/fpls.2022.919166

- Tiwari, P., & Adholeya, A. (2002). *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. *FEMS microbiology letters*, *206*(1), 39-43. https://doi.org/10.1111/j.1574-6968.2002.tb10983.x
- Zeilinger, S., Gupta, V. K., Dahms, T. E., Silva, R. N., Singh, H. B., Upadhyay, R. S., et al. (2015). Friends or foes? Emerging insights from fungal interactions with plants. FEMS Microbiol. Rev. 40, 182-207. https://doi.org/10.1093/femsre/fuv045

APPENDIX I: MW MEDIA COMPOSITION AND CONCENTARTAION

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

Solution II 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 250mL (1000X)	**MW media**
Chemicals	
MnSO4-H2O (Manganese Sulfate)	1.165 g (or 1.5 MnCl2-4H2O)
ZnSO4-7H2O (zinc 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 2.5 mL of this solution to the mix
Na2MoO4-2H2O (Sodium Molybdate Dihydrate)	Weight and dilute 0.12 g in 100mL water and take 0.5 mL of this solution to the mix [or 0.11 g of NH4(Mo7O2)4-2H2O]

Solution V of NaFe EDTA 500mL (250X)

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

Solution VI of Vitamins (1000X)

Use Gamborg's Vitamins Solution 1000X