**Advances and Current Perspectives on Root Organ Culture of Arbuscular Mycorrhizal Fungi**

**ABSTRACT**

**Arbuscular mycorrhizal fungi (AMF) play an important role in enhancing plant growth through nutrient uptake, restoring soil quality, and increasing plant resistance from various biotic and abiotic stresses, making them vital for sustainable agriculture and ecosystem management. The development of AMF-root organ cultures (ROC) is the most promising technique for large-scale production and wider application for sustainable agriculture. The AMF-ROC system facilitates high-quality contaminant-free bulk inoculum in less time and limited space. Despite this, the system has also many constrained such as the system is not universal for all AMF species, variability in sporulation rate, need for cost-effective scaling. Recent developments in bioreactor-based production and liquid culture-based cultivation enhanced hope for large-scale production. However, further research is required for protocol optimization, high spore production, and reducing the cost of production. This review highlights the current status, challenges, and future perspective of AMF-ROC development and their scaling for mass production as well as emphasizing their wider application for sustainable agriculture.**

***Keywords: AMF, ROC, Mass production, Inoculum, Sustainable agriculture, Symbiotic Fungi***

**INTRODUCTION**

Arbuscular mycorrhizal fungi (AMF) are essential soil fungi that form symbiotic relationships with approximately 80 % of land plants. Fungi are an important symbiotic partner and help plants in their growth improvement, plant health, and soil quality maintenance (Brundret & Tedersoo, 2018). These fungi are obligate biotrophic in nature and form the world's most abundant mutualistic association. AMFs are important for sustainable agriculture as involve in the bi-directional movement of nutrients, helping both the plants and the soil which further reduces the need for various chemical fertilizers. The AMF and the roots of plants work together to exchange nutrients, mainly phosphorus from the host to meet the requirement of carbohydrates and fatty acids, (Bravo et al., 2017; Luginbuehl et al., 2017; Rich et al., 2017). Researchers have created novel in vitro systems called root organ cultures (ROCs) to investigate the advantages of AMF. The process involves growing AMF in ROCs under controlled conditions, which is a non-invasive method for studying extraradical fungal development and interactions with the roots of the host. *Rhizobium rhizogenes* is responsible for producing the Ri T-DNA plasmid, which serves as a key mechanism for creating "hairy roots" that can grow indefinitely and have similar physiological and morphological features to normal roots (Oliveira et al., 2024; Asmelash et al., 2016). The introduction of this change enables the propagation of sterile AMF inoculum for agricultural purposes in controlled conditions.

According to Kokkoris and Hart (1999), while choosing plant species for ROC systems, carrot (*Daucus carota*) roots are particularly preferred for their robust growth, and ability to support high spore production levels when inoculated with AMF species (Bécard and Fortin, 1988; Rosikiewicz et al., 2017). Other than carrot, plant species, such as *Chichorium intybus*, *Glycine max*, *Linum usitatissimum*, *Lycopersicon esculentum*, *Medicago truncatula*, and *Solanum tuberosum*, have also been explored for AMF cultivation (Goh et al., 2022), however, there remains a substantial lack in experimentation and research that provide a comprehensive framework to compare the performance of these root systems in germplasm studies. Hence, it is essential to standardize methodologies and characterize the potential of hairy roots across various plant species in order to make advancements in the field.

In the last three decades, researchers have revealed that AMF-ROCs have the ability to produce high-quality and large-scale AMF inoculum under sterile conditions within a comparatively short time frame is necessary for sustainable agriculture practices. Additionally, monoxenic culture techniques which involve the cultivation of AMF in association with a single host root system, are another eco-friendly alternative to chemical fertilizers. Despite several benefits, in order to fully realize the potential of AMF-ROCs, it is necessary to address issues and challenges such as optimizing growth conditions, preserving propagules intact, and decreasing production costs (Basiru et al., 2020).

*In vitro* cultivation of AMF involves several considerations ranging from the choice of gelling agents, initial biomass from starter cultures, and aging periods, to incubation conditions. Advancements have been made in the field of biotechnology and microbial ecology that have provided new understandings of the various mechanisms underlying AMF-host interactions, paving the way for the refinement of AMF-ROC systems (Genre et al., 2020). For further advancement of the field, these systems can be integrated with bioinformatics and high-through put screening techniques which can offer exciting opportunities for the identification and selection of AMF strains optimized for field applications.

Scaling up ROC systems for commercial use still has some gaps, despite so many innovations made so far. Although more than 300 species of AMF have been reported and only a few species are limited for mass production under ROC. Therefore, careful surveillance is required to provide constant AMF propagation considering various factors such as diversified nutrient composition, temperature, pH, and light conditions for a wide range of AMF. Furthermore, even though carrot roots remain the typical choice for ROCs, it is worth exploring alternative, and cost-effective host root systems that could help reduce the cost of AMF cultivation. Targeted research and innovation are critical for the wider adoption of AMF in modern agriculture to address these challenges.

The current status of the ROC of Arbuscular mycorrhizae fungi is discussed in this review. The potential advantages, drawbacks, and progress in this domain are also discussed here. Additionally, the analysis scrutinizes the obstacles faced in AMF production on a large scale and ultimately seeks to foster more research and innovation in this area. A comprehensive examination of the concerns related to the widespread implementation of AMF is essential for achieving sustainable global food security and environmental health.

**Arbuscular Mycorrhizal Fungi (AMF) and Root Organ Culture (ROC)**

As highlighted by Smith and Read (2010), the symbiotic relationship between fungi called AMF and plants' roots enhances their productivity, diversity, and ability to resist a wide range of biotic and abiotic stresses. AMF has received significant attention in sustainable agricultural, horticultural, and environmental restoration efforts due to its various benefits (van Der Heijden et al. 2015). Additionally, the use of AMF-based products has been on the rise in recent times, with benefits that span from gardening to enhancing specific environments like golf greens.

Despite their advantages, AM fungi are obligate biotrophs which means that they need to live in host roots in order to complete their lifecycle, which comes as a challenge for its large-scale production, especially when developing a cost-effective and contaminant-free inoculum. Several methods of cultivation have been created to address these issues, with Root Organ Culture (ROC) being a significant approach.

The development of AMF cultivation was initiated by Mosse's (1959) work, which laid the foundation for *in vitro* studies of AM fungi. Later in 1962, Mosse established the first successful connection between a plant and an *Endogone* species. A breakthrough was achieved by Mosse and Hepper (1975), who cultured AM fungi using (*Lycopersicum esculentum Mill*.) and red clover (*Trifolium pratense L*.) roots on gelled media.

The development of the ROC system gained momentum in the 1980s with contributions from Mugnier and Mosse (1987) and Bécard and Fortin (1988) where they Ri T-DNA transformed carrot roots as host. The innovation of the split-plate method by St-Arnaud et al. (1996) made a new innovation, allowing the separation of root and fungal compartments using a bi-compartmental ROC system which led to improved production of propagule and spore harvest. Later in context to the study, Douds (2002) found that sporulation continued in the system after partial medium replacement in the distal compartment and glucose supplementation in the proximal compartment, allowing for repeated harvests from the same culture.

Later, ROC systems have undergone significant changes in order to enhance their scalability and efficiency. Early efforts by Tiwari and Adholeya (2003) utilized small containers to grow root organs culture of AM fungi, optimizing the system for mass production (Adholeya et al., 2005). Other than these, innovations like airlift bioreactors, perlite mist bioreactors, and the use of solid support systems like gelled media have also been made for large-scale cultivation of AM fungi. Wang in the year 2004 patented a hydroponic culture system that employed liquid media and exfoliation methods, further branching out the techniques available for AMF cultivation. Later, in 2006, Gadkar and others presented a unique system where AM fungi were propagated using ROC in recombinant clay balls, and significant improvements in spore yield were revealed and allowed for repeated harvests from the same culture.

ROC system was first used to investigate the in vitro production of *Glomus intraradices* spores (Douds, 2002; St-Arnaud et al., 1996), followed by extensive experimentation with plant systems (Voets et al., 2009). While carrot (*Daucus carota L*.) roots are the primary cause of ROC, chicory (*Chichorium intybus L*.) and barrel medicago (*Medicago truncatula Gaertn*.) medic roots have also been used to cultivate AM fungi (Fontaine et al., 2004). Other hosts, such as banana (Koffi et al., 2009) and grapevine, are also compatible with AM fungal associations, even though they have been found to be less effective for large-scale spore production.

Different media compositions have been used in ROC systems, using M-medium by Bécard and Fortin (1988) and modified Strullu Romand (MSR) medium by Strullu and Romand (1986) which was later modified by Declerck et al., in 1998 and the most commonly used. These media provide essential nutrients and vitamins, required for the optimal growth of both roots as well as fungi, and the medium is further stabilized by the addition of gelling agents like Phyta Gel and Gel Gro.

In bioreactor-based ROC systems, liquid M-medium is aerated to promote fungal growth. Gadkar et al. (2006) devised specialized culture setups, such as using glucose-soaked cotton rolls in a separate compartment, to enhance spore production along with the maintenance of proper aeration and medium composition which is critical for successful cultivation (Berruti et al, 2016). Additionally, Voets et al. (2009) also reported that plantlets are precolonized by an AM fungus, which results in increased sporulation when the plantlet is placed in a Petri plate with a reconstituted culture medium.

Many AM fungal species and strains have been successfully cultivated in vitro using the highly efficient ROC system. Over 100 strains are currently *in vitro*, with GINCO being the most significant among such collections, which contain at least 20 species and 30 strains. The *Glomeraceae* and *Gigasporaceae* families have only a few species, including *Glomus intraradices*, which have demonstrated rapid propagule production, making them suitable for large-scale applications. Re-classification into the *Glomeromycota* phylum has been achieved through purely theoretical studies of its evolutionary history. Molecular analyses have led to the relocation of strains that were previously classified as *Glomus intraradices* to their current designation in the genus *Glomus irregulare* (Schwarzott et al, 2001; Stockinger a. n. 2009). The improvements highlight the significance of molecular techniques in improving AM fungal taxonomy.

Despite the advancements made by ROC systems, AMF cultivation still faces obstacles. The major challenge is to balance cost-effective production on a large scale with the preservation of spore viability and quality. In addition, further research is needed to optimize the system for different AM fungal species and host roots. Improvements in bioreactor design, medium composition, and host root selection could be further advanced by understanding host-fungal compatibility and spore development through the use of molecular tools. The applicability of ROC to diverse AM fungal species can also be expanded by exploring novel host plants and alternative media formulations.

**Current Techniques in Root Organ Culture of AMF**

The global mycorrhizal inoculant market has experienced exceptional growth during the past twenty years because of increased field crop and horticultural sector need for AMF. The fungi enable land plants to improve their nutrient performance by enhancing phosphorus uptake and building symbioses with almost 80% of all terrestrial vegetation. Large-scale AMF cultivation presents difficulties because these fungi need host plants for existence and development although they demonstrate specific advantages. Research into overcoming these difficulties led scientists to investigate root organ culture (ROC) as a technique that enables controlled production of sterile AMF inoculum on a mass scale. Research into AMF production and biology has established ROC as a fundamental technology driven by technological advancements and a greater understanding of fungal biology.

The fundamental principle behind ROC depends on its ability to grow AMF in sterile settings through the maintenance of host plant roots in media without contaminants. Experiment methods within ROC have experienced substantial progress which has let researchers deeply investigate the complex biology of AMF-host relationships. ROC achievement of improved spore production and colonization rates became possible by optimizing culture conditions including nutrient formulations together with co-culture systems. The developments strengthen research on AMF genetic variety and functional mechanics so researchers can enhance sustainable agricultural applications.

Jolicoeur et al. (1999) conducted important initial ROC research by studying the submerged culture conditions of *Daucus carota* hairy roots with AMF. Hairy roots produced by *Agrobacterium* (*Rhizobium)* rhizogenes) strains helped researchers show how inoculum-to-medium ratios heavily influenced fungal and root development. Due to root growth suppression in dense cultures, researchers studied Petri dish cultures to uncover optimal spore production conditions during their AMF production scale-up research. The research revealed a methodology to optimize AMF yield production through adaptive culture system development which subsequently established foundational principles for bioreactor applications. ROC has advanced substantially through bioreactor technology which delivers scalable systems to propagate AMF. The research by Jolicoeur et al. demonstrated that airlift bioreactors outperformed petri dishes in spore output yet served as a foundation for generating subsequent-generation bioprocess technologies. Researchers seek to enhance spore production and expand system capabilities through improvements in bio-reactor design technology and nutrient supply methods. The research noted that bioreactor-based AMF production requires continued innovation efforts to enhance the optimization of growth and sporulation conditions.

Among the pioneers of the methodology, Declerck and his colleagues based on 2001's work embarked on ROC methodologies with the help of AMF using a monoxenic culture system for their inoculum production. They found that nutrient agar enriched with sucrose, vitamins, and essential nutrients was the best medium to take the role of sporulation as the number of *Glomus* strains—*G. intraradices*, *G. proliferum*, and *G. caledonium* emerged. The implementation of mathematical models, like Schnute and Gompertz, became a solution to predicting sporulation dynamics and optimizing culture conditions. These types of models indicated the phases of multicellular interactions associated with sporulation and pointed out the differences between the strains of the same genus. This study provided a mathematical procedure for an ROC system to make it robust and highly effective by which it can be easily applied on a larger scale.

Molecular tools integrated into ROC have advanced the science by providing accurate methods for studying both AMF-host relationships and plant genetics. The successful host plant root transformation stands confirmed through the implementation of polymerase chain reaction (PCR) according to Puri and Adholeya (2013). Through collaborations between potato tubers transformed with *Agrobacterium rhizogenes* strain Ri1600 and co-culture of *Glomus intraradices* (CMCCROC7) using *Solanum tuberosum* var. 'Pukhraj', scientists produced hairy roots as a novel approach. The dual culture system produced high spore yields at excellent viability levels through this development thus demonstrating ROC's potential for mycorrhizal biofertilizer production. The investigation demonstrated how potato-based ROC systems are accelerating sustainable agricultural development through their ability to create efficient breeding systems that promote AMF multiplication at industrial scales.

The development of three-dimensional culture systems alongside bioreactor technology now enables ROC to advance toward commercial usage. Modern cultural control systems that offer temporal and spatial manipulation help both accelerate AMF propagation rates and study their symbiotic processes. Real-time monitoring combined with automation technology has improved production processes through reduced labor costs and improved inoculum quality consistency while minimizing resource expenses.

Root organ culture (ROC) functions as a critical laboratory method to cultivate arbuscular mycorrhizal fungi (AMF) while advancing our scientific knowledge of fungal life cycles and vineyard sustainability in agriculture. The paper by Srinivasan et al. (2014) detailed how carrot hairy roots evolved into single-species cultures of *Glomus* (*Rhizophagus*) *intraradices*. Transformed hairy roots of carrot roots using *Agrobacterium rhizogenes* methods were then co-cultured with *Rh. intraradices* fungi spores on Modified Strulla and Romand (MSR) co-culture medics. The experimental medium developed for AM spore growth and germination successfully supported extensive hyphal development and produced 8,500 to 9,000 spores per petri dish under dark growth conditions for three months. Monoxenic culture systems showed exceptional capacity for producing contaminant-free propagules which reinforces their role in AM fungal inoculum generation as well as detailed biological research. By emphasizing the importance of these systems as efficient devices for the mass production of AM fungal inoculum, this research enables thorough investigations into their biology.

Radha et al. (2015) expanded the groundwork with *Agrobacterium rhizogenes* MTCC culture 532 to generate *G. intraradices* spores within carrots through Ri-TDNA propagation methods. Their research evaluated different parameters which included experimental conditions for pH values along with sucrose content, inoculum origins, and spore cultivation strategies. The study optimized dual culture methods when researchers discovered that agar plugs that contained at least fifty viable spores matched with mycorrhized root apices produced the best spore multiplication rates. Spore multiplication led to 9,772 ± 770 spores under conditions of complete darkness with 27°C incubation over 16 weeks at pH 5.5 on enriched MSR with 1.0% sucrose. This study demonstrated ROC as a dependable system for cultivating AMF in vitro while offering an efficiency standard to advance AMF growth methods.

Scientists studying *G. intraradices* lifecycle achieved important findings through their work on carrot hairy roots as transformation hosts according to research by Rezaee Danesh et al. (2016). Following the removal of wheat rhizosphere samples, scientists combined fungal spores and root samples which grew between days 3 to 5 after surface sterilization. Radial growth of hyphal germinations developed 2.5-millimeter diameter networks during a 2-10-day interval and generated true spores following 25 days of initial contact. Each growth plate delivered between 1000 and 2500 spores during the twelve weeks of development as the spores transformed from whitish hyaline bodies to brownish-yellow coloration. The research showed AMF growth patterns which demonstrates its capability to improve soil qualities and agricultural output through structured mycorrhizal treatments.

The ROC methodology serves purposes beyond AMF laboratory cultivation and supports various studies from morphology to taxonomy and biochemistry to ecology according to Aryal (2017). The research presented an advanced experimental approach that incorporated well-known procedures from AMF investigations to create consistent results at a laboratory scale. Standardized cultivation methods increase both the accessibility and reliability of Arbuscular Mycorrhizal fungi growth which enables significant developments in mycorrhizal research together with new applications.

Raj et al. (2017) investigated technological developments in Aseptic AMF inoculum production through their research on large-scale methods. Through root organ culture researchers assessed carrot roots along with tomato, potato, sweet potato, and soybean roots for their potential in mass production. The modification of ROC protocols through split plate techniques produced substantial improvements in extraradical spore counts. After 90 days of dark period incubation in sugar-free MW medium *Rhizophagus irregularis* spore production reached 99,204 ± 1,438.10 propagules per container. The M medium containing 1% sugar produced 16,236 ± 1,186.70 intraradical spores and 39,458 ± 1,098.00 extraradical spores. The integration of *R. irregularis* into transformed callus root clumps provided an ongoing system for both propagation and sustainable inoculum supply. By using a sugar-free medium the researchers achieved both reduced contamination and improved stability to ensure proper inoculum quality for all stages of the process from harvest through packaging. transportation and storage. The laboratory-scale capability along with the dependable nature of this approach makes it an essential tool for agricultural use, ecological applications, and commercial purposes as it improves sustainability standards through enhanced productivity and decreased contamination.

The effectiveness of AMF, Aqueous neem (*Azadirachta indica*) leaf extract, and synthetic NPK fertilizer in mitigating damage caused by *Cylas puncticollis* in sweet potato (*Ipomoea batatas*) cultivation was investigated in a field study conducted by Issa et al. (2017). The research was conducted in Cameroon over the 2013 and 2014 cropping seasons using two agro-ecological regions (Garoua and Ngaoundere) as the focus. By applying AMF treatment, stem length was increased by 58% in local sweet potato varieties and 64% in improved TIB1 sweet potatoes through quantitative analysis. Stem length improved by 59% and 66% in the presence of AMF and neem extract, respectively, as compared to controls. Across both agro-ecological zones, there was observable statistical evidence of varying degrees of tuber damage caused by *C. puncticollis* through AMF and AFM-neem treatments (p 0.05). Despite being more pest resistant, the local sweet potato variety had lower productivity (1265 t/ha) compared to the TIB1 variety with greater success (15.82 T/HA), according to yield analysis. However, it was still considered to be superior. AMF-neem combination was found to be the most successful in reducing pest-induced damage severity and infestation rates. In contrast, the use of NPK fertilizer resulted in increased vegetative growth and a greater vulnerability to *C. puncticollis* infestation by insects. The study underscores that AMF and neem extract are sustainable alternatives to synthetic fertilizers and insecticides due to environmental and health concerns related to chemical pesticide use. The ability of neem to selectively eliminate pests and become biodegradable makes it a practical integrated pest control method. Based on these results, AMF and neem extract are ecologically sound options for increasing sweet potato yield and pest resistance in northern Cameroon.

Scientists have transformed arbuscular mycorrhizal fungi (AMF) research through recent enhancements in root organ culture (ROC) methods which allow their controlled *in vitro* cultivation. The research community depends on ROC technology as an essential instrument for both producing AMF inoculum and studying fungal symbiotic behavior along with sustaining agricultural sustainability. Researchers have documented multiple advancements in root organ culture methods to optimize ROC systems and understand their developing applications.

Abd Ellatif et al. (2019) evaluated how culture media composition combined with environmental factors affects AMF growth patterns. The research studied eight phenolic compounds affecting *Gigaspora gigantea* growth in tomato root organ cultures showing different stimulatory and inhibitory effects based on compound concentration and solution conditions. Studies confirmed that at pH 6.5 the flavonoid catechin improved mycorrhizal root colonization in addition to enhancing arbuscular formation but tannic acid at pH 5.7 proved inhibitory. More effective spore development occurred with solid culture environments compared to liquid culture environments in these specific experiments. In vitro, cultures produce aseptic spores to demonstrate how ROC systems drive sustainable agriculture by enhancing both land fertility and plant output.

A new method introduced by Perera García et al. (2022) used transformed chicory roots to culture *Rhizophagus irregularis* (Cuban strain INCAM 11) in a modified Strullu and Romand (MSR) medium. Their research established an exact timeline which showed AMF development starting with hyphal interaction with roots through spore formation into mycelium growth. Experiments using the *in vitro* system successfully cultured many AMF species by reaching highly significant spore production exceeding 2000 spores per plate within five months. The investigation uncovered *Paenibacillus* species together with microbial partnerships as vital elements for both AMF survival and multiplication. The coexistence of these microorganisms reveals opportunities to build useful microbial consortia which enhance agricultural inoculum function as well as efficiency. The research establishes regional AMF species collections as a fundamental biological resource for future studies addressing indigenous fungal adaptation in local ecological settings.

Goh et al. (2022) achieved a methodological breakthrough by creating RocTest which serves as a solid evaluation system to determine the behavior of AMF during testing with various root plants. Such a tool measured both fungal reproduction counts and sporulation and monitored extraradical fungal structural development in various combinations of host plants partnered with AMF. Promising combinations for spore production and fungal development emerged from testing these root organ culture species *Daucus carota*, *Medicago truncatula*, and *Nicotiana benthamiana* alongside the fungal species *Rhizophagus clarus*, *Rhizophagus irregularis*, and *Glomus sp*. through the RocTest framework. Standardized AMF cultivation techniques based on this framework's clear graphical outputs allow researchers to dependably track research development for their germplasm collections and sustainable agricultural work. The findings demonstrate that matching suitable host-fungal strains is crucial to achieving the highest possible fungal propagation and spore yield.

Large-scale AMF production through ROC shows overwhelming economic significance due to its global application potential. Distinguished ROC techniques enable affordable inoculum production which matches both farmer expenditures and market value. The release of superior-quality AMF propagules enhances nutrient acquisition along with plant development in addition to enhancing soil structural integrity and stress resistance. These advantages realize the sustainable farming objectives while aligning with ecological management objectives. Furthermore, the development of refined protocols guarantees product quality improvement alongside reduced contamination risk and better pH maintenance which results in predictable performance of ROC-based systems across different applications.

One of the most exciting frontiers in the integration of microbial interaction to ROC systems is being widely practiced. The results of different research revealed that AMF co-culturing with beneficial microorganisms, e.g. *Paenibacillus* species, increases fungal development as well as inoculum efficiency. These interconnections are a mutually supportive accelerator of AMF reproduction hence, microbes that can be specifically designed for certain crops and locations could be grown. Just looking at the microbial dynamics in ROC systems not only are very helpful for the research of AMF symbioses from ecological and functional aspects but also is key to the investigation of the missing elements in these relationships.

ROC technologies being used give the possibility of AMF biology and their practical applications to be more intensively understood. This work of art in terms of the ability to play with environmental factors at the best possible level lets scientists find the answer to the questions of how AMF and the host organism interact when the spore can germinate and the hyphal development can happen. Studies similar to these are for the production of biofertilizers, biocontrol agents, and sustainable soil management techniques. Not only that, the potentiality of enhancing these services to incorporate ROC-derived AMF inoculum into agroecosystems turns into a new approach for mitigating soil degradation, nutrient depletion, and climate change-related problems.

In conclusion, the current methods used for root organ culture of AMF have achieved significant progress regarding their cultivation together with their ecological and agricultural applicability. Researchers achieved large-scale production capabilities of AMF through the optimization of culture media conditions, host-AMF pairing techniques and sustainable environmental conditions. Advanced methodological frameworks and the study of microbial interactions accelerate our knowledge of AMF biology while broadening its applications. Current advances in ROC methods will define agricultural sustainability by maintaining healthy soils and boosting yield outputs while supporting long-term ecological health.

**Table 1.** Summarizing all the relevant previous studies has been provided below

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Study** | **Technique** | **Gelling agent/** | **Media** | **Inoculum (Starter Culture)** | **Genus** | **Host** | **Incubation conditions** | **Duration** | **Spore, Propagule Yield** | **Biomass** | **Colonization** |
| **Substrate** |
| Wood et al. (198) | Plant ROC of herbaceous plants | Porous substrate (vermiculite & vermiculite-peat) and a nutrient solution | N/A | 10-20 surface-sterilized pregerminated Gigaspora margarita spores | *Rhizophagus, Gigaspora* | *Trifolium incarnatum, Arachis hypogaea* | 25°C-28°C | 4-8 weeks | 2766 sp. L-1 | N/A | Up to 32% |
| Fortin et al. (199) | Petri dish culture, Bioreactor | Agar (1%w/v), vermiculite | Modified White medium, MS medium, M-medium (pH 5.2 to 5.5) | 9.8 g FW 3.7g FW | *Rhizophagus, Gigaspora, Scutellospora, Sclerocystis* | *Daucus carota, Trifolium pratense,* etc | 24°C | 9 weeks | 48,300 sp. L-1 120,000 sp. L-1 | N/A | N/A |
| St-Arnaud et al. (1996) | Classical ROC | 0.5% w/v gellan gum | Modified M-medium | N/A | N/A | *Daucus carota* | 27°C | 4 months (16 weeks) | 500,000 sp. L-1 | N/A | N/A |
| Jolicoeur et al. (1999) | Airlift bioreactor, Petri dish | N/A | Low salt M-medium | 0.6 g DW g L-1 | *Rhizophagus irregularis* | *Daucus carota* | N/A | 10-13 weeks | 20,000 sp. L-1 30,000 sp. L-1 | 0.13 g DW L-1 | 25%-75% |
| Declerck et al. (2001) | ROC in solid medium | Gel Gro (0.4% w/v) | MSR medium | 5 mm mycorrhizal root piece or spore | *Rhizophagus* | *Daucus carota* | N/A | 12 weeks | 280,000 sp. L-1 | N/A | N/A |
| Wang (2004) | N/A | N/A | N/A | 1 g FW (0.065 g DW) | *Rhizophagus irregularis* | N/A | 26°C | 2 months (8 weeks) | 48300 sp. L-1 | 20.7±0.57 | N/A |
| Puri & Adholeya (2013) |  |  | M-medium |  | *Rhizophagus irregularis* | *Solanum tuberosum* var ‘Pukhraj’ |  | 12 weeks | 60,250 spores/jar | ~400,000 sp. L-1 | >50% |
| Srinivasan et al. (2014) | ROC in solid medium | Gallon gum (0.3% w/v) | MSR medium (pH 5.5 before autoclave) | 10-15 sterilized spores | *Rhizophagus irregularis* | *Daucus carota* | 27°C | 3 months | 300,000 sp. L-1 | N/A | N/A |
| Raj et al. (2017) | ROC with solid medium | 0.4% CleriGel (Phytagel in India) | Sugar free MW-media | N/A | *Rhizophagus irregularis* | *Daucus carota* | *26±2ºC* | 90 days | 210,770 sp. L-1; 992,040 ppg L-1 | N/A | 75% |
| M-media fortified with 1% sugar | 556,940 sp. L-1 |
| Sugar free MSR media | 196,390sp. L-1 |
| Radha et al. (2015) | Dual Culture Technique | Agar | Modified Strullu and Romand (MSR) + 1% sucrose | Agar plug with viable spores and mycorrhized root apexes | *Glomus intraradices* | *Carrot (Daucus carota)* | 27°C, dark, pH 5.5 | 16 weeks | 9772±770 spores | Not reported | Mycorrhized root apexes |
| Schuessler (2016) | Root organ liquid (ROL)-based ROC | Any group of polysaccharides like gellan gum, agar-agar, etc (0.1% or 0.05%w/v) | M-medium or MSR medium | 0.1-0.3 FW of AMF colonized root material | *Acaulospora, Rhizophagus, Scutellospora, Funneliformis* | Chicory, clover, bindweed, etc | 27°C | 42-84 days | 106 ppg L-1, 300,000 sp. L-1 | 80-120 mg FW L-1 | >53% |
| Rezaee Danesh et al. (2016) | Root Organ Culture (ROC) | N/A | N/A | Mycorrhizal spores and roots | Glomus intraradices | Carrot (*Daucus carota*) | 25°C, dark | 12 weeks | 1000-2500 spores | Not reported | Not reported |
| Raj et al. (2017) | Root Organ Culture (ROC), Split Plate Method | Sugar-free medium | MW medium, M medium, MSR medium | Transformed callus root clumps with Rhizophagus irregularis | *Rhizophagus irregularis* | Carrot (*Daucus carota*) | 24-27°C, dark | 90 days | 99204±1438.10 propagules | High | >75% |
| Abd Ellatif et al. (2019) | Root Organ Culture (ROC) | Solid and liquid media | MSR medium with phenolic compounds | Spores | *Gigaspora gigantea* | Tomato | 25°C, dark, pH 5.7/6.5 | N/A | Not reported | Moderate | Superior in solid media |
| Perera García et al. (2022) | Root Organ Culture (ROC) | N/A | Modified Strullu and Romand (MSR) | Rhizophagus irregularis propagules | *Rhizophagus irregularis* | Chicory (*Chichorium intybus*) | 25°C, dark | 5 months | 2000 spores per plate | Not reported | High |
| Goh et al. (2022) | RocTest | N/A | N/A | N/A | *Rhizophagus clarus, R. irregularis, Glomus* sp. | Carrot, *Medicago truncatula, Nicotiana benthamiana* | N/A | N/A | Modeled performance | Not reported | Modeled performance |

**Potentials and Limitations of Root Organ Culture for AMF**

By utilizing mycorrhizal root-organ cultures, researchers have greatly enhanced their understanding of AMF symbiosis by creating an environment conducive to research on fungal biology and interactions with host roots. Nonetheless, this in vitro system presents both significant benefits and drawbacks that require careful examination.

During the entire life cycle of AMF, root-organ culture systems have been proven to be highly effective in catalyzing the development of common fungal structures like appressoria, arbuscules, vesicles, extraradical mycelium, and spore. Fortin et al. (2002) found that the structures formed in these environments are very similar to those created when plants were grown in pot cultures, suggesting that mechanisms for fungal colonization in nature mirror those observed in natural conditions. This fidelity provides a well-established foundation for fungal biology under controlled circumstances. The complete life cycle has been made possible by in vitro systems, enabling the successful cultivation and preservation of many AMF species and isolates. They are now indispensable for taxonomic studies and long-term conservation. By optimizing media composition and growth conditions, researchers have the potential to culture even more complex species within the Glomales order, addressing a significant gap in AMF research.

The *in vitro* method (Figure 1) is a potential tool for investigating the genetic and physiological factors that contribute to the obligate biotrophic status of AMF. In this controlled environment, researchers are able to make variables dependent, and fungal material is analyzed at different stages of interaction. PCR-based subtractive hybridization and other techniques are particularly useful in identifying fungal genes expressed in plants and mycorrhizal roots, as noted by St-Arnaud et al. (1996). This approach is highly efficient. Also, root-organ cultures are useful for the production of research and commercial AM inocula. Bioreactor-based production methods, as advocated by Jolicoeur et al. (1999) and Jolicioeux and Perrier (2001), have enabled the expansion of inoculum production. Although significant improvements in efficiency and cost are still necessary, these systems hold the potential to be highly effective for large-scale, aseptic inoculum production.

The controlled conditions of root-organ cultures make them a valuable tool (Figure 2) for studying interactions between AMF and soil organisms. Several investigations have focused on polysaccharide decomposition, nitrogen fixation, nitrate reductase activity, ammonification, and phosphate solubilization which makes it crucial to understand the roles of AMF in soil health and nutrient cycling. Moreover, the use of in vitro systems has been conducted to determine the sublethal effects of pesticides on AMF. Root-organ cultures were used by Wan et al. (1998) and Wanch and Rahe (1998) to study the effects of pesticides like benomyl, glyphosate, dimethoate, or azadirachtin on Glomales species in vitro. The research provides an opportunity to utilize root-organ cultures as standard testing methods for pesticide regulation, which may help mitigate the risk of environmental contamination by AM-toxic chemicals.

Despite its benefits, the root-organ culture system is not entirely free of limitations. The deficiency of photosynthetic tissues is one of the main limitations, as it disrupts *in vivo* natural source-sink relationships and hormonal balance. By substituting photosynthates with sucrose in the culture medium, these systems produce an artificial sugar solution at the root-fungus interface. Fortin et al. (2002) in their study argued that this unique condition could change the biochemical dynamics of the plant-fungal interaction. Carbohydrates in natural systems travel through the epidermis and then enter the cortex and vascular system. The symbiotic benefits of AMF may be shortened by the in vitro accumulation of sugars at the root-fungus interface, which creates an unnatural biochemical environment. This constraint makes it difficult to extend in vitro research to field environments.

One more significant limitation is the tendency for species bias in present-day in vitro settings. Some AMF species or isolates, such as *Rhizophagus intraradices*, are more likely to be successful under these systems than others. This bias limits the use of root-organ cultures for studying full AMF diversity. St-Arnaud et al. (1996) highlighted the importance of optimizing media and growth conditions for a wider range of species in their research. Furthermore, evidence suggests that mycorrhizal roots secrete compounds that inhibit mycelial growth and spore production in vitro. They found inhibitory effects that highlight the challenge of reproducing natural interactions in a laboratory setting and call for further methodological refinement.

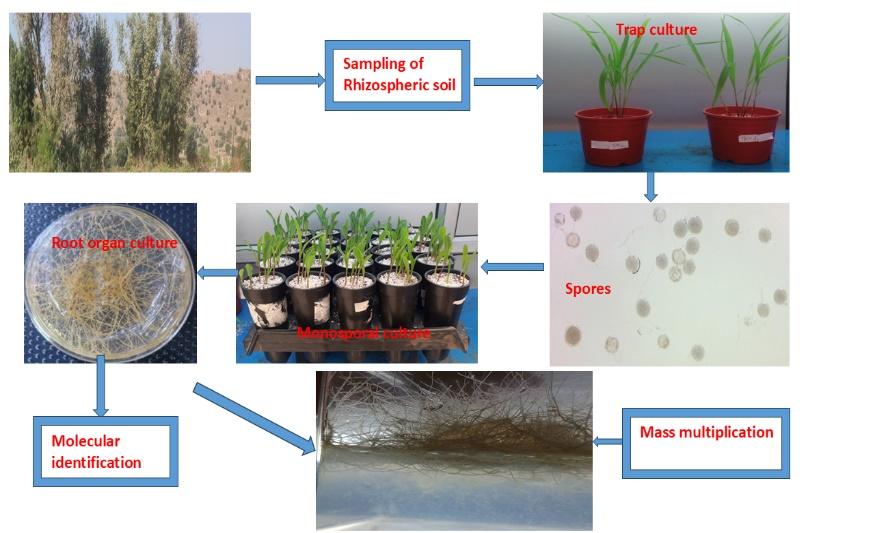
The value of root-organ cultures in creating AMF inoculum is significant, but scaling up production to meet commercial demands remains a major challenge. Optimization of current methods is necessary to improve productivity and cost-effectiveness. The recommendations of Moutoglis and Béland (2001) are a starting point, but more research is necessary to develop adaptable solutions. Moreover, root-organ cultures offer a controlled environment for studying AMF, but they do not replicate the complexity of natural ecosystems. Soil heterogeneity, microbial diversity, and dynamic environmental conditions are difficult to simulate in vitro. The necessity of conducting field-based research in addition to in vitro studies is highlighted by this limitation.

The development of AMF through root-organ cultures has proven to be a significant improvement in understanding fungal biology, symbiosis, and ecological interactions. They have great prospects in terms of genetics, biology, and practical applications, such as in vitro fertilizers and toxicity testing. However, the limitations of this method including its artificiality, bias towards species, and difficulties in increasing production require further methodological developments. The resolution of these difficulties will enable researchers to enhance their understanding regarding the potential of root-organ cultures for promoting sustainable agriculture, ecosystem management, and environmental conservation.

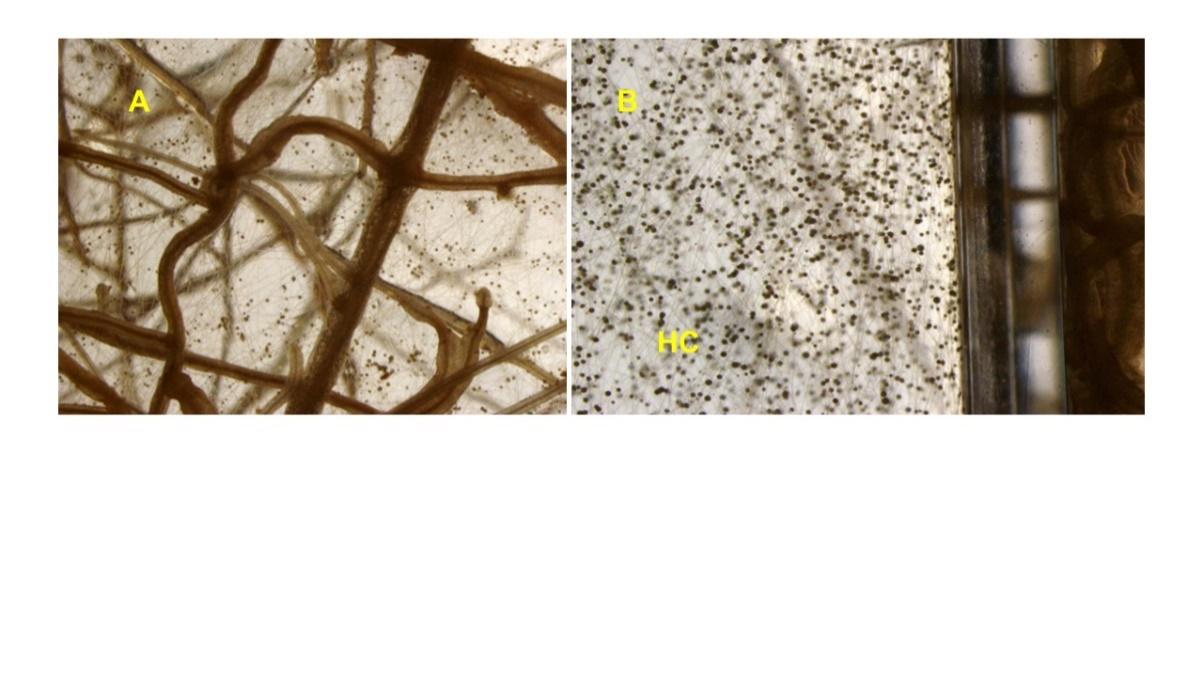
**Future Perspectives and Research Directions**

AMF-ROC systems present important chances to tackle agricultural and environmental challenges across the globe. Optimal growth media, gelling agents, and incubation conditions are being enhanced to optimize spore production, propagule viability, or scalability. These enhancements are noteworthy. Even though the use of advancements in Molecular biology like gene editing and transcriptomic methods is not very common, they can help understand the genetic mechanisms of AMF-host interactions and help identify appropriate strains for specific environments, such as agriculture or food production. Further, bioinformatics and high-throughput screening methods can also be utilized to locate and produce AMF strains more quickly and efficiently.

ROC systems could be made more accessible and economically viable by exploring alternative, less expensive host root lines beyond transformed carrot roots. The integration of ROC systems with bioreactor technology could enable the transfer of research from laboratory work to practical field testing, resulting in a paradigm shift towards large-scale AMF production. By avoiding biochemical inhibitors and optimizing nutrient cycling, research may promote the growth of more complex AMF species. Furthermore, ROC systems can restore ecosystems by employing high-quality AMF inoculum to rejuvenate damaged soils. The implementation of sustainable agriculture and commercial production of AMF inoculum could decrease reliance on agrochemicals, enhance crop resilience, and contribute to global food security.



**Figure 1.** Representation of Overall Process Followed for Development of AMF-ROC and Mass Production



**Figure 2.** AMF -ROC culture of *Rhizophagus irregularis* growing with Ri-transformed roots of *Daucus carota* (A. Growing in monocompartmental petri plate) and B. Growing in bi-compartmental petri plate and hyphal compartment (HC) showing very high sporulation without host roots.

**CONCLUSION**

ROC systems are a novel approach which have been instrumental in understanding AMF biology and symbiosis, despite their shortcomings, such as the lack of photosynthetic tissues in host roots. Controlled environments can be utilized to study and spread AMF through root organ culture (ROC) systems. AMF inoculum can be produced without any contamination by conducting highly effective molecular and ecological experiments. ROC systems can be used to create backup sources that can adapt to agricultural and environmental challenges, such as soil restoration and sustainable crop production. Despite the challenges posed by cost, scale, and strain-specific optimization, AMF propagation has progressed due to advancements in biotechnology and molecular techniques. In order for ROC systems to become the cornerstone of sustainable agriculture and ecosystem management, their worldwide applications must be realized through continuous innovation and collaboration across disciplines.

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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