World Applied Sciences Journal 17 (1): 20-28, 2012
ISSN 1818-4952

4532y00Biotechnological Aspects for VAM Aseptic Mass Production

[1]Sawsan A. Abd-Ellatif, [2]R.A. Abdel Rahman,
[3]M.B.H. Mazen, [3]A.E. El-Enany and [3]Nivien Allam

[1]Department of Bioprocess Development, City for Scientific Research and Technological Applications, Borg El-Arab, Alexandria, Egypt
[2]Department of Pharmaceutical Bioproducts Research, City for Scientific Research and Technological Applications, Borg El-Arab, Alexandria, Egypt
[3]Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt

Abstract: Arbuscular mycorrhizae (AM) are symbiotic associations, formed between plants and soil fungi that play an essential role in plant growth. Interest in AM fungi propagation for agriculture is increasing due to their role in the promotion of plant health, in soil nutrition improvement and soil aggregate stability. The present work shows the successful aseptic mass production of biologically active spores of vesicular arbuscular mycorrhizae (VAM) from a consortium of spores in an axenic transformed dicotyledonous root organ culture. The root organ culture system supported extensive root colonization with the formation of many arbuscular and vesicles. The fungus both within root segments and as spores produced were viable and able to complete its life cycle in vitro and maintained for several successful periods each reach for 3-4 months. The mycorrhizal root segments, however, exhibited higher inoculum potential due to the numerous vesicles and extensive intraradical mycelium.

Key words: Monoxenic culture · Arbuscular mycorrhizae (AM) · Root organ culture · Vesicles · Arbuscular

INTRODUCTION

Firstly, the word technology here means that the technology involves the extraction of potential viable propagules from soils, surface sterilization and optimization of growth conditions for germination under aseptic conditions. This is followed by the association of the propagules with a suitable excised host root for propagule production and recovery. Mass-produced propagules are then formulated in an utilizable form and stored before application to the target plant. This sums up the long journey from the soil system to laboratory propagation and subsequent application to land or potted plants, allowing practical exploitation of their potential. The major biological characteristic of AM fungi is their obligate biotrophic nature. This means that each of their life cycle steps requires the association with a living plant. This limit large-scale production of mycorrhizal inoculums to satisfy the farmer needs which force us to make another strategy for more aseptic production of AM propagules. The use of excised roots as host partner in AM symbiosis was first proposed by Mosse and Hepper [1]. Clonal roots of some 15 plants have been established and this list has been enlarged during the last decades. Transformation of roots by the soil-borne microorganism *Agrobacterium rhizogenes* has provided a new way to obtain mass production of roots in a very short time. Genetically modified carrot (*Daucus carrota* L.) roots by *A. rhizogenes* show profuse roots two to four weeks later. Transformed roots have a quick, vigorous and homogenous growth in relative poor substrates without supplementation of hormonal substances. The negative geotropism of transformed roots facilitates contacts with hyphae of AM fungi [2-4] indicated they can survive for a long time without subculture and [5] observed less contamination in culture of transformed carrot roots due to their negative geotropism. For all AM propagules, proper selection and efficiency of sterilization process are keys of the success of axenic or monoxenic AM fungal cultures. Monoxenic cultivation has several advantages over conventional pot cultivation systems regarding inoculum production. This technique offers pure, sterile,

Corresponding Author: Sawsan A. Abd-Ellatif, Department of Bioprocess Development, City for Scientific Research and Technological Applications, Borg El-Arab, Alexandria, Egypt

bulk, contaminant-free propagules, hitherto not practicable using conventional modes of pot culture, aeroponic or hydroponic techniques. In addition, this technique has an edge over other conventional modes of mass production, whereby a several-fold increase in spore/propagule production is achieved in less time and space. The main object of our work is the production of large scale pathogen-free VAM inoculums. All types of AM propagules (isolated spores or vesicles, mycelia, sheared mycorrhizal roots) are virtually able to initiate AM symbiosis. Chlamydospores of *Glomus sp.* [1, 3] and non-sporocarpic azygospores of *Gigaspora margarita* [6] are obviously the preferred inoculum starter even though dormancy and strain mutation may occur under greenhouse conditions. The preference is due to availability of facility to recover, sterilize and to germinate these propagules. Developing countries will benefit more VAM symbiosis using the monoxenic system, which is cheaper than greenhouse culture [7].

MATERIALS AND METHODS

Tomato Hairy Root Culture: Seeds of tomato (*Lycopersicon esculentum* L.) were surface sterilized in 70% (v/v) ethanol for 30s, then in 50% (v/v) commercial Clorox solution containing two drops of Tween 20 for 10-15 min, finally rinsed five times with sterile distilled water. Seeds were germinated on MS medium [8] supplemented with 3% sucrose. Seedlings 1-1.5 month old were used as source of explants for transformation.

Bacterial Strains and Culture Conditions: *Agrobacterium rhizogenes* strains A4 and R1000 were used for transformation. Bacteria from glycerol stock culture were cultured on LB solid medium [peptone (10g/L), yeast extract (5g/L), NaCl (10g/L); pH 7.0] contains 50mg/L (w/v) kanamycin for screening and selecting the desired colony. Bacterial cultures were prepared by incubation of one colony in 20 ml LB medium at 28°C for 12-16 h on rotary shaker (130 rpm) [2]. The bacterial cells were harvested by centrifugation at 4200 rpm, 4 °C for 15 min and the pellet was resuspended in 20 ml MS liquid medium and used for infection of tomato explants.

Transformation and Establishment of Hairy Root Cultures: Leaves of 1-1.5 month old tomato seedlings were cut into 1cm^2 pieces and immersed in 20ml bacterial suspension for 30 min. The explants were blotted with sterile filter paper and placed in hormone free MS solid plates. Plates incubated at room temperature for 3 days in the dark. After 3 days leaf explants were transferred onto modified MS medium to satisfy both of growth of plant material and production of VAM fungi, the modification was in supplementation with 500 mg/L carbencillin, 10g/L sucrose, low p content, the ratio of NH_4SO_4: KNO_3 must be 5:1, also we maintain N content less than 0.2mM, 8g/L agar. Cultures incubated at 25°C in complete darkness. Bacteria-free explants that had produced hairy roots were subcultured onto fresh MS plates containing 400 mg/L carbencillin. Hairy roots were excised from explants when they were 4-5cm in length and transferred to new MS plates, then they were transferred into liquid MS medium. Hairy root cultures were maintained on a rotary shaker (80-90 rpm) in complete darkness.

Establishment of AMF Symbiosis with Tomato Root: spores extracted from our pot culture of AM fungi from the twelve-week-old root-based culture under aseptic conditions using a sterile scalpel, several successive sterilization with chloramin T, ethyl alcohol and finally with mixture of antibiotics were used to inoculate tomato hairy root on modified MS media [2]. These were allowed to grow for 12 weeks at ±28°C in the dark. Visual observations were recorded under desiccating microscope of high power magnification.

Initiation of Dual Culture System: Ten spores of AM fungi were placed in the center of the Petri dish containing the fungal medium solidified with gellan gum and were incubated at 28°C. for five days as described earlier [9]. Then a 10 cm-root segment, which was pre-grown in the fungal growth medium for one week, was placed near the emerging germ tube of the spore. The proximal end of the root tip is oriented towards the tip of germ tube. The inoculated roots were incubated in the dark at 28°C. The Petri dish was observed periodically for the growth of external hyphae and sporogenesis.

Host Root Cultivation: From a Petri plate filled with the modified MS medium, remove a disc of agar with a cork borer. This disc should be identical in size to the disc of gel supporting the germinated spore. The diameter of the disc should be large enough to include the spore and germinating hyphae (9-mm diameter is usually enough). Transfer the disc of gel supporting the germinated spore of into the hole of the removed disc. Place the root carefully in the near vicinity of the propagule (spore, sporocarp or mycorrhized root fragment) into the Petri plate, so that the growing hyphae

are perpendicular to a secondary root. Seal the Petri plates with plastic wrap and incubate in an inverted position at 27°C in the dark.

Continuous Culture

Association Establishment: After a few days a new spores are produced very quickly thereafter. The mycelium grows extensively, rapidly invading the complete volume of the Petri plate. The sporulation capacity of the fungal strain and the development of mycelium depend on the strain. After 3 months, a culture of spores can produce more than 10,000 spores.

Root-Organ Cultures as a Tool for AM Fungal Systematic: The taxonomy of AM fungi is based almost entirely on spore morphology, with description, identification and classification to the species level being difficult. Isolation of AM fungi from pot cultures often produces spores lacking subtending hyphae and (or) with damaged spore wall layers (especially the outer evanescent layer). As a consequence, poor quality spore reference material has generated incomplete and sometimes unusable species descriptions. Moreover, the absence of living cultures of type specimens has dramatically reduced studies of spore ontogenesis. However, the root-organ culture system has renewed interest in AM fungal taxonomy. The contaminant-free cultures give constant access to clean fungal propagules, which can be observed and harvested at any stage during fungal development [9-12]. This material is much more appropriate for morphological, ultrastructural, physiological, biochemical and molecular studies than pot-cultured fungi. The in vitro grown AM fungi constitute a reliable material for species characterization and description [13] and for evolutionary and interspecific studies [14].

RESULTS

***In vitro* Propagation of Arbuscular Mycorrhizal Fungi:** Our objective was to explored natural genetic transformation of tomato (*Lycopersicon esculentum* L.) by the ubiquitous soil bacterium *Agrobacterium rhizogenes* produces a condition known as hairy roots, which is biochemically and genetically stable. They are able to grow in a hormone-free medium. Also, use these Ri T-DNA transformed roots in the study of the AM symbiosis (sporulation of AM fungi *in vitro*).

Tomato Seeds Cultivation: Seeds of tomato after surface sterilized were germinated on MS medium supplemented with 3% sucrose. Seedlings 1-1.5 month old were used as source of explants for transformation (Fig. 1).

Transformation: 6 days after inoculation with *A. rhizogenes*, transformed roots appeared on the inoculated sites (Fig. 2). These roots (4-6 cm long) were cut off and transferred to a sterile hormone-free MS medium and cultivated as described in Materials and Methods. Hairy roots were maintained by regular subcultures in the dark at 4-wk intervals. A typical tomato hairy root culture is shown in Fig. 2.

Here, axenic culture through hairy root organ culture of tomato, produced by transforming tomato plant of Ri plasmid (Ri, root inducing) of *Agrobacterium rhizogenes,* can be easily propagated and maintained provide a method for the *in vitro* mass production of AM, mainly spore, which have more advantages over traditional method in contaminant free cultures throughout life cycle (Fig. 3).

On Ri T-DNA Transformed Roots: From one week after, transformed roots developed many lateral roots growing both on the surface and penerating into the media. On the third day after the inoculation, hyphal extension was observed from the spores. Extension of the germ tube from the spores also started simultaneously. The system showed extensive hyphal proliferation. Spores applied in this experiment was a consortium of spores suggested that multiple colonization, i.e. use of a consortium, rather than a single spore effective under different environmental conditions, was probably more beneficial to the plant, because a single endophyte may not be able to withstand certain environmental changes. Dual axenic cultures of sheared-root inocula of AM and tomato roots showed significant sporulation and the production of extensive biomass were associated with excised tomato roots. As many as $(10)^2$-$(10)^3$ axenic mature spores were recovered in Petri dishes after three months incubation in the dark.

Developmental Stages of AM Fungal Growth

Spore Germination: It occurred within 25 d and the first germination was observed 15 d after incubation on the MS medium (Fig. 4). After 1 month, the germination rate was 50%. Germ tubes often crossed right over the roots and continued to elongate without altering their direction or branching. In some

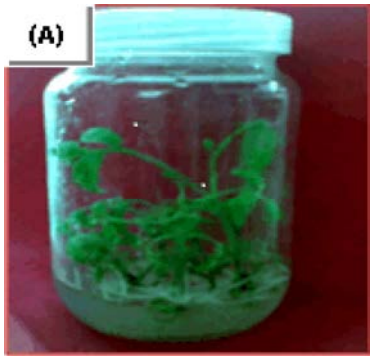

Fig. 1: (A) Tomato plants after 4 weeks of culture incubated in a growth room under 16 hrs light, 8 hrs dark at 25°C

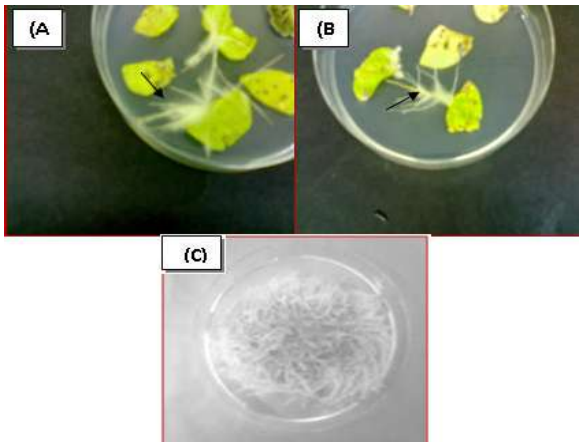

Fig. 2: (A-B) Induction of hairy roots from leaf explants of *Lycopersicon esculentum* L. (C) Tomato hairy root. Arrows showed the adventitious roots produced at the site of infection.

Fig. 3: Axenic culture of dual culture of transformed tomato root inoculation with fungal consortium of AM species. Note, the actively growing transformed roots soon spread over and covered the culture plat. The roots were initially white and became yellowish-brown when their growth ceased.

Fig. 4: Spore germination on MS medium after two weeks.

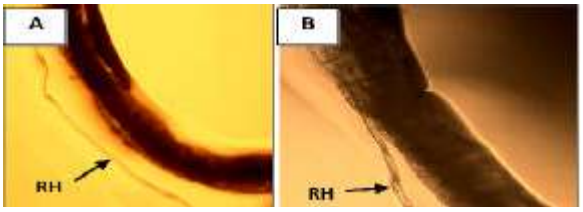

Fig. 5: (A-B): Show running hyphea (RH) on MS medium.

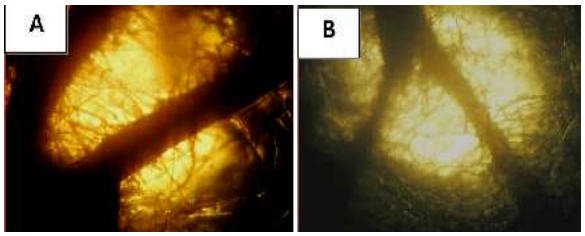

Fig. 6(A-B): Intensive hyphen interactions, each one constitute one propagule so thousands of propagules used as inoculums, was obtained from monoexinic culture.

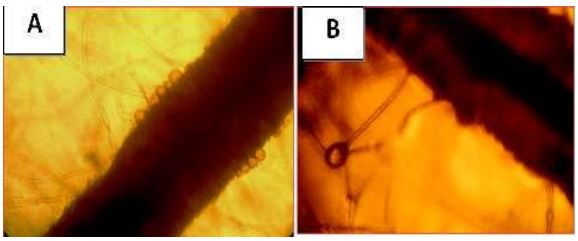

Fig. 7(A-B): Invasion of spores to tomato roots on MS medium.

cases, however, the hyphae did branch once or twice either before or after crossing the root of a mycotrophic species.

Pre-symbiotic growth of AM is characterized by formation of so-called running hyphae (RH). After some weeks with additional host partner, growth of germinated AM propagules increased continuously. The progress on

extraradical fungal development may be easily, frequently and non-destructively observed with a microscope (Fig. 5).

The attachment of germ tubes to the root surface, followed by highly branched hyphal growth, results in a fan-like structure. Rapid growth of extraradical fungal growth (Fig. 6) and formation of small vegetative spores (Fig. 7A) were seen directly under a microscope. Secondary infections on growing roots were formed by the newly formed spores (Fig. 7B) and the AM fungal hyphae spread all over the entire Petri dish in high density, followed by spores' formation within three weeks after the initiation of the dual culture system (Fig. 8).

At the end of the three month period, spores both mature and under-developed was observed and the number of spores ranged from at least 1,000 to 2,000 per Petri plate. Spore sizes were comparable to those of typical culturing plate.

Continuous Propagation of Dual Am-root Culture by Subculturing: Four-to five-month-old dual cultures of AM in fungal growth medium of the present work were successfully used as the starting material for subculture. The entire gel was cut into sixteen pie-shaped pieces; each slice was transferred to a Petri dish containing the fungal growth medium. The roots and AM fungi started to grow further immediately; the fungal hyphae grew all over the gel along with new growth of the roots. One to two thousand spores were produced in ten weeks. The dual culture could be continuously propagated to produce large numbers of spores.

Multiple Germinations: From three week after inoculation, spores in this work reaching up to 40% successive germinations for single spores of *in vitro* conditions. Spore germination does not require external factors other than humidity and temperature to germinate. Germination rates reached 80-100% at the end of the experiments. The most striking feature of the system is that the dual cultures can be subcultured, generation after generation and produce abundant aseptic spores. The spores are important inocula for the horticulture, field and
specialty crop plants to form beneficial AM symbiosis.

Monoxenic Cultures Establishment, Sporulation and Subculture: Seventy percent of the pregerminated spores colonized transformed tomato roots and produced spores. For 30% of non-infective spores, failure was attributed to damage to the germination tube after transfer to a Petri plate or failure of the germ tube to contact actively growing roots. The extramatrical mycelium development was extensive and numerous infection units were formed in active growing roots. The first daughter spores were formed approximately 3 months after colonization and sporulation was observed thereafter for 3-5 months. The spores were globose (Fig. 9A-D) in all the cultures, In Petri plates with extensive colonization, spores were distributed over the entire plate, mostly deep in the growth media, close to the roots, but also in the external cells layers of older roots (data not shown) and on the plate lid. These germinated spores were able to re-associate with Ri T-DNA transformed roots on the MS medium and produced new spores.

Extraradical Mycelium Architecture and Development: The extraradical mycelium of spores was characterized by thick, orange-brown hyphae similar to runner hyphae (RH) forming the main branching network of the culture. Thinner lower-order hyphae branched from this network and formed auxiliary cells, sporogenic hyphae and spores, hyphal swellings (HS) and branched absorbing structures-like (BAS-L) (Fig. 10).

BAS were formed as ramifications of primary, secondary or higher-order hyphae and also as ramifications of sporogenous hyphae (Fig. 10). These structures were formed by numerous thin, generally straight but sometimes contorted, hyphae extending radially in the medium. These structures were transient and their cytoplasm content retracted after septation. This process occurred 1-2 wk after the BAS-L formation (Fig. 10). During cytoplasm contraction cytoplasm frequently was arrested inside small, thin single-wall hyphal swellings (HS), which typically developed at the hyphal extremity of the BAS-L (Fig. 10). These HS were hyaline, globose to ovoid. These structures resembled extraradical vesicles or immature spores because they frequently contained dense cytoplasmic material typically found in fully expanded spores and. The number of these HS varied from just a few to several hundred for a single BAS-L in a single culture. Extraradical mycelium may also form coils, whose frequency of occurrence depends on their location in a root and the generic affiliation of the arbuscular fungal species. Generally, coils more abundantly occur at entry points (Fig.11). Intraradical hyphae of Glomus spp. are infrequently coiled in the other regions of a mycorrhizal root. In contrast, coils produced by species of the other genera of arbuscular fungi usually are abundant and evenly distributed along mycorrhizal roots.

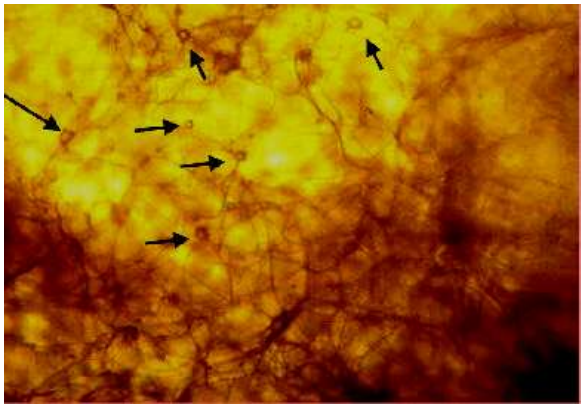

Fig. 8: Arrows show the newly formed spores Fig. 9(A-D): Spore proliferations and newly formed spores arise showing many new immature spores.Fig. 10: Arrow show Branched absorbing structures (BAS) with swollen tips of growing hyphea (HS) in Ri T-DNA transformed tomato root. A swollen tip resembles in form an immature spore.Fig. 11: Arrows points to coiled hyphea arise with mature spore.

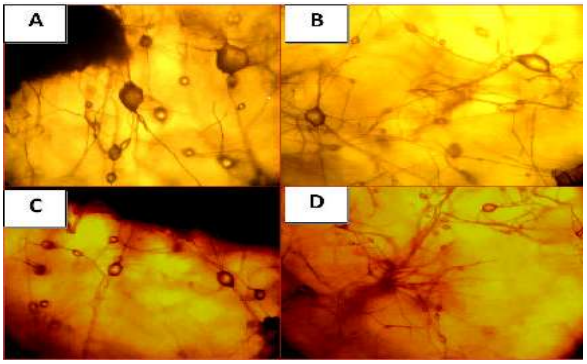

Fig. 9(A-D): Spore proliferations and newly formed spores arise showing many new immature spores.

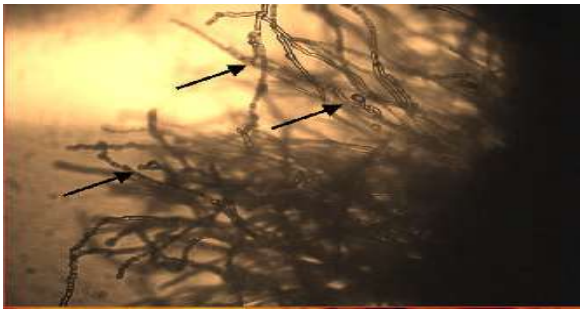

Fig. 10: Arrow show Branched absorbing structures (BAS) with swollen tips of growing hyphea (HS) in Ri T-DNA transformed tomato root. A swollen tip resembles in form an immature spore.

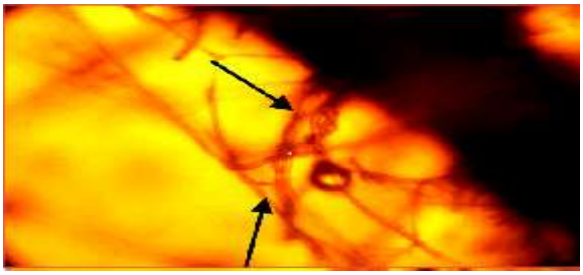

Fig. 11: Arrows points to coiled hyphea arise with mature spore

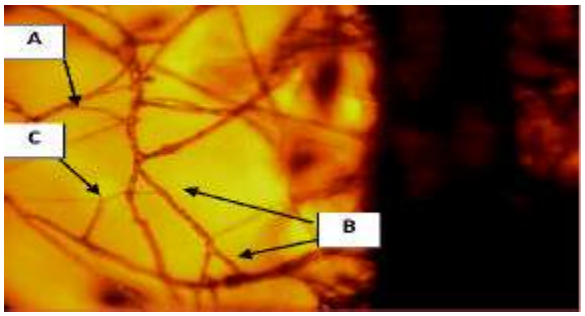

Fig. 12: Intesractions among hyphae growing in monoxenic root organ culture. A: Anastomosis on secondary hyphae. B: Branched hyphae without anastomosis formation. C: Wound healing of hyphae. Note that parts of the hyphae where the color is lighter are dead parts separated by septa.

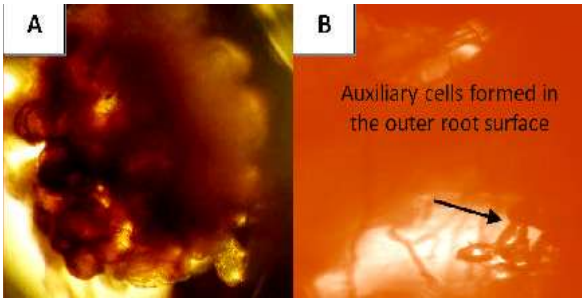

Fig. 13 (A-B): Show auxiliary cells.

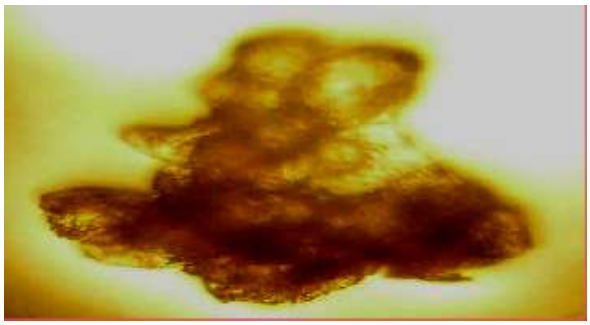

Fig. 14: Collapsed auxiliary cells after 8 months.

The number of hyphal branches always was higher. This pattern facilitated the radial spreading of the hyphae and was associated with primary root colonization. It was formed during the nonsymbiotic (after spore germination) and symbiotic (after root colonization) phases. The other pattern was characterized by RH that grew straight or were only slightly curved, expanding for several centimeters and branching in a radial pattern with irregular distances between branches. These RH mostly were formed by extraradical hyphae during root colonization. They exhibited intense cytoplasm flow in contrast with lower-order hyphae, were active throughout the 5 months of observations and seldom formed septa.

Anastomoses Formation: In spite of the many points of hyphal contact within the hyphae network, less than 1% anastomosis was observed. We observed anastomoses only in the branched hyphae (Fig.12A-B). No anastomoses were observed among the RH. On the other hand, a mechanism to repair hyphae damage was detected (Fig. 12C). Hyphal bridges were formed when flow of cytoplasm was obstructed or to reconnect broken hyphae (Fig. 12C)

Auxiliary Cells: Auxiliary cells were formed by short ramifications occurring at one or simultaneously at both sides of hyphae (Fig. 13A). Each ramification generated one to several branches that swell at the extremity to form an independent cell that was smooth-surfaced to angular to knobbed, subglobose, ovoid to cleavage and with an inflated thin-walled ‘‘cell’’, orangish-brown under transmitted light (Fig. 13A). The subtending hyphea, at the base of the ‘‘cell’’, had no occlusion or septa. The number of cells in each auxiliary cell ranged from two to more than twenty and the total number of auxiliary cells ranged from 100-200 per Petri plate. The auxiliary cells were formed mainly on first-order branches from a RH, where one or more groups of auxiliary cells could be found. They also formed abundantly on the root surface (Fig. 13B) and sporadically in the external cortical cells of roots.

At full expansion, young auxiliary cells contained dense cytoplasmic inclusions resembling the lipid droplets found in healthy looking AMF spores. After 8 months many auxiliary cells were empty or collapsed, leaving a scar at their origin (Fig. 14).

DISCUSSION

This *in vitro* system also allowed to observe the structural development of the extraradical phase of spores, which comprises an organized radial network of runner hyphae from which lower order branches (at a 45° angle) develop at regular intervals (between 25 and 300 mm). Some of these ramifications developed into new runner hyphae and others bore arbuscule-like structures (ALS) and spores [15]. Ultrastructural investigations revealed that ALS (renamed branched absorbing structures or BAS [16] are very similar to intraradical arbuscules and that, like arbuscules, they are sites of intense metabolic activity. Arbuscules and BAS are also similar in terms of their gross morphology (thinner diameter with increased dichotomous branching). The extent to which these structures are functionally comparable remains to be elucidated. However, prolific branching of the fungus to form BAS results in an important increase in surface area and so produces extraradical Mycelium.

A vigorous extraradical hyphal development and subsequent differentiation of VLS, BAS and spores [17]. The structural development of the mycelial phase has been described exhaustively by Bago *et al.* [15], from a single isolate of *G. intraradices* and has since been used as the reference model to which further descriptions are compared. The basic structure of the mycelium is made of large, straight-growing thick-walled hyphae named runner hyphae (RH), due to their capacity to extend rapidly, to colonize the substrates and to establish root contact. Microscopically, runner hyphae are similar to pre-symbiotic hyphae. Microscopic cellular and subcellular observations allow detection of protoplasmic streaming, nuclei migration and organelle morphology [18, 19]. Hyphae are either single-walled, as with *G. versiforme* [20], or double-walled as found through ultrastructure works on *G. fasciculatum*. Both walls showed almost equal thicknesses and separated easily at hyphal break. This morphology recalls the hyphae within *in vivo* propagated hyphae [21], where the inside hyphal wall was attributed to self-invasion by a hyphal outgrowth following wounding or cutting. RH and branched hyphae (BH) abundance determines the mycelium architecture. In spite of a common general organization, finer details of mycelium architecture may vary considerably between *Glomus* species as well as their isolates [17]. Experienced microscopists can often differentiate fungal colonies under the binocular by observing branching and sporulation patterns. With some isolates, where the mycelium expansion is restricted to the vicinity of roots, sub-culturing usually failed.

The contact between the root and fungal hyphae may take one to several weeks to become established [22]. Repetitive investigations under in vivo growth conditions

revealed that, once the root-fungi contact is established, the fungal morphology changes drastically, with a reorientation of hyphal apical growth giving rise to either a direct entry point or to an intensive hyphal branching called "fan-like structure" [20,23,24]. The biological function of auxiliary cells remains speculative [25]. Jabaji-Hare [26] observed high amounts of lipids within the auxiliary cells of a *Gigaspora* species, supporting the storage function of these structures. Roles in transitory storage [27] and reproduction [28] have been hypothesized, or they might represent vestiges of relict reproductive structures [29]. The root organ culture system supported extensive root colonization with the formation of many arbuscular and vesicles. The fungus both within root segments and as spores produced was viable and able to complete its life cycle in vitro.

The mycorrhizal root segments, however, exhibited higher inoculum potential due to the numerous vesicles and extensive intraradical mycelium. The present work shows the successful mass production of biologically active spores of vesicular arbuscular mycorrhizae (VAM) in an axenic transformed dicotyledonous root organ culture. As demonstrated here, the extramatrical mycelium play a major role in the *S. reticulata* life cycle and survival because spores are the main propagule in this family [30- 32]. The observed properties of spore production also might dictate the ecology of this species to a large extent in the natural environment as well as in agricultural soils. However, further experiments are necessary to prove that monoxenic culture can be used to predict AM species behavior in field soils. The use of monoxenic culture should help us unravel some of the basic and applied aspects of the biology and ecology of the AMF

REFERENCES0

1. Mosse, B. and C.M. Hepper, 1975. Vesicular-arbuscular infections in root-organs cultures. Physiol. Plant Pathol., 5: 215-223.
2. Bécard, G. and J.A. Fortin, 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol., 108: 211-218.
3. Mugnier, J. and B. Mosse, 1987. Vesicular-arbuscular infections in Ri T-DNA transformed roots grown axenically. Phytopathol., 77: 1045-1050.
4. Tepfer, D., 1989. Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology and evolution. *In* Plant-microbe interactions. Vol. 3. Edited by T. Kosuge and E.W. Nester.
5. Diop, T.A., 1990. Méthodes axéniques de production d'inocula endomycorhiziens à vésicules et à arbuscules: étude avec le *Gigaspora margarita*. MSc Thesis, University of Laval, Quebec.
6. Strullu, D.G. and C. Romand, 1986. Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques C.R. Acad. Sci. Ser. III Sci. Vie, 303: 245-250.
7. Diop, T.A., C. Plenchette and D.G. Strullu, 1994. *In vitro* culture of sheared mycorrhizal roots. Symbiosis, 17: 217-227.
8. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiolo. Plant. 15: 473-497.
9. Becard, G. and Y. Piche, 1992. Establishment of vesicular arbuscular mycorrhiza in root organ culture: review and proposed methodology. In: Norris JR, Read D, Varma AK (eds) Methods in microbiology: techniques for mycorrhizal research. Academic Press, New York, pp: 550-568.
10. Chabot, B., G. Becard and Y. Piche, 1992. Life cycle of Glomus intraradices in root organ culture. Mycologia, 84: 315-321.
11. Strullu, D.G., T. Diop and C. Plenchette, 1997. Realsation de collection sin vitro de Glomus intraradices (Schenck et Smith) et Glomus versiforme (Karsten et Berch) et Proposition d, un cycle de developpement. C R Acad Sci. Paris. 320: 41-47.
12. Dalpe, Y., 2004. The *in vitro* monoxenic culture of arbuscular m ycorrhizal fungi: a majortool for taxonomical studies. In: Fri, as Herngndez jz JT, Ferrera Cerrato R, Olalde V V Portugal (eds) Advances en conocimiento de la biologia de las Micorriza. Universidad de Guanajuato, Mexico.
13. Declerck, S., D. D'or, S. Cranenbrouck and E. Le Boulenge, 2001: Modelling the spoulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. Mycorrhiza, 11: 225-230.
14. Declerck, S., D. D'or, S. Cranenbrouck, Y. Dalpe, A. Gramougin-Ferjani, J. Fontaine and M. Sancholle, 2000. *Glomus proliferum sp*. nov: a description based on morphological, biochemical, molecular and monoxenic cultivation data. Mycologia, 92: 1178-1187.
15. Bago, B., C. Azcón-Aguilar and Y. Piché, 1998a. Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic culture conditions. Mycologia, 90: 52-62.

16. Bago, B., C. Azcón-Aguillar, A. Goulet and Y. Piché, 1998b. Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol., 139: 375-388.
17. Declerck, S., D.G. Strullu and C. Plenchette, 1998. Monoxenic culture of the intraradical forms of Glomus sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia, 90: 579-585.
18. Mosse, B., 1988. Some studies relating to "independent" growth of vesicular arbuscular endophytes Can. J. Bot., 66: 2533-2540.
19. Bago, B., W. Zipfel, R. M. Williams, J. Jun, R. Arreola, P.J. Lammers, P. E. Pfeffer and Y. Shachar-Hill, 2001. Translocation and utilization of fungal storage lipid in the Arbuscular mycorrhizal symbiosis. Plant Physiol., 128: 108-124.
20. Garriock, M.L., R.L. Peterson and C.A. Ackerley, 1989. Early stages in colonization of *Allium porrum* (leek) roots by the vesicular-arbuscular mycorrhizal fungus Glomus versiforme. New Phytol., 112: 85-92
21. Lim, L.L., B.A. Fineran and A.L.J. Cole, 1983. Ultrastructure of intrahyphal hyphae of *Glomus fasciculatum* (Thaxter) Gerdemann and Trappe in roots of white clover (*Trifolium repens* L.). New Phytol., 95: 231-239.
22. De Souza, F.A. and R.L.L. Berbara, 1999. Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. Mycologia, 91: 343-350.
23. Friese, C.F. and M.F. Allen, 1991. The spread of VA mycorrhizal fungal hyphae in the soil: inoculums types and external hyphal architecture. Mycologia, 83: 409-418.
24. Giovannetti, M., C. Sbrana, L. Avio, A.S. Citernesi and C. Logi, 1993. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol., 125: 587-593.
25. Bonfante, P. and V. Bianciotto, 1995. Presymbiotic versus symbiotic phase in arbuscular endomycorrhizal fungi: morphology and cytology. In: Varma A, Hock B, eds. Mycorrhiza: structure, function, molecular biology and biotechnology. Springer-Verlag, pp: 229-247.
26. Jabaji-Hare, S., 1988. Lipid and fatty-acid profiles of some vesicular-arbuscular mycorrhizal fungi: contribution to taxonomy. Mycologia, 80: 622-629.
27. Pearson, J.N. and P. Schweiger, 1993. *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders associated with subterranean clover: dynamics of colonization, sporulation and soluble carbohydrates. New Phytol., 124: 215-219.
28. Pons, F. and V. Gianinazzi-Pearson, 1985. Observations on extrametrical vesicles of *Gigaspora margarita in vitro*. Trans Br Mycol. Soc., 84: 168-170.
29. Morton, J.B. and G.L. Benny 1990. Revised classification of arbuscular mycorrhizal fungi (*Zygomycetes*): a new order, *Glomales*, two new suborders, *Glomineae* and *Gigasporineae* and two new families, *Acaulosporaceae* and *Gigasporaceae*, with an emendation of *Glomaceae*. Mycotaxon, 37: 471-491.
30. Brundrett, M.C., L.K. Abbott and D.A. Jasper, 1999a. Glomalean mycorrhizal fungi from tropical Australia. I. Comparison of the effectiveness and specificity of different isolation procedure. Mycorrhiza, 8: 305-314.
31. Brundrett, M.C., D.A. Jasper and N. Ashwath, 1999b. Glomalean mycorrhizal fungi from tropical Australia. II. The effect of nutrient levels and host species on the isolation of fungi. Mycorrhiza, 8: 315-321.
32. Klironomos, J.N. and M.M. Hart, 2002. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza, 12: 181-184.