CORPUS PUBLISHERS

# Environmental Sciences and Ecology: Current Research (ESECR)

Volume 3 Issue 5, 2022

**Article Information**

Received date : May 31, 2022

Published date: June 10, 2022

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**Keywords**

*Arbuscular Mycorrhizae; In Vitro Culture;* MSR Medium

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Research Article

# *In Vitro* Propagation of A Cuban Arbuscular *Mycorrhizal Fungal* Strain

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## Abstract

In vitro root cultivation techniques based on modified root systems are often used in studies on *Arbuscular Mycorrhizal* Fungi (AMF). It is a simplified but powerful tool to investigate AMF root colonization and development of the extraradical mycelium. The aim of this study was to establish and characterize the in vitro culture of a Cuban strain of *Rhizophagus irregularis* (INCAM 11) by using transformed chicory roots. For that, superficially disinfected propagules of *R. irregularis* were co-culture with the hairy transformed chicory roots on Modified Strullu and Romand (MSR) medium during five months. Spore germination was observed 3-5 days after surface disinfection. The first contact between AMF hyphae and roots occurred 1-3 days after germination and a significant production of extensive extraradical mycelium was observed. New spore formation started within 21-25 days. After 5 months, 2000 spores could be observed per plate which were able to germinate, colonize, establish and reproduce again spores when associated to young transformed roots of chicory. The most frequent associated microorganism to the *in vitro* culture of INCAM 11 was isolated and identified as *Paenibacillus* sp.

## Introduction

*Arbuscular Mycorrhizal* (AM) fungi are soil fungi that occur worldwide forming symbiotic associations with most plant families. Their importance in natural and semi-natural ecosystems is commonly accepted and characterized by improved plant growing as well as, an increasing plant resistance against biotic and abiotic stresses [1]. In Agricultural ecosystems under less favorite growing conditions they play an important role by enhancing productivity and sustainability [2]. The plant-AMF interaction studies are difficult due to the obligate symbiont condition of the fungi [3]. AMF cannot complete their life cycle neither grow in axenic conditions without establishing a functional symbiosis with host plants [4]. However, *in vitro* root cultivation techniques, as a simplified system for the establishment of the *arbuscular mycorrhizal* symbiosis, have been widely used [5] and it has shed new light on their molecular biology, cytology, genetics, physiology, systematics and phylogeny [6].

The most obvious advantage of *in vitro* cultivation system is the absence of undesirable microorganisms, which makes it more suitable for large-scale production of high-quality inoculum and for construct *in vitro* culture collections. Several AM fungi species had been cultured in root organ cultures (ROC) systems. Based on scientific literature and culture collections, it is estimated that over 100 different strains are maintained *in vitro* [7]. However, there is evidence that many species are not able to germinate or colonize transformed roots under these conditions. Therefore, it is a challenge for many researchers to improve *in vitro* system by taking into account culture media composition, grows conditions, propagules and AM hosts. Although, in Cuba much progress is made in the cultivation and reproduction of AMF as well as in the production of AMF inoculum, there is less experience with *in vitro* culture conditions. Constructing an *in vitro* collection of Cuban AMF strains would be an important achievement not only for research on plant-AMF interaction but also for building up an indigenous collection for agriculture use. The main objective of this study was to establish and characterize an *in vitro* culture of *Rhizophagus irregularis* INCAM11 based on transformed chicory (*Cichorium intybus* L.) hairy roots.

## Material and Methods

### Biological material

The strain INCAM 11, DAOM 711363 *Rhizophagus irregularis* (Walker & Schüßler) belongs to the AMF collection of the 'Instituto Nacional de Ciencias Agrícolas, (INCA)' from Cuba. Fungal spores were obtained from pot culture by using the wet sieving and decanting [8] technique followed by sucrose centrifugation [9]. Isolation was done under a dissecting microscope (Novel) at 10 -50 X magnification. Spores clusters were stored at 4°C until their surface disinfection. The Ri-t-DNA transformed chicory (*Cichorium intybus* L.) hairy roots, supplied by Montreal University, Canada, were used to establish the *in vitro* root-organ culture.

### Spores disinfection procedure

INCAM 11 spores were surface disinfected according to a modified methodology of [10]. Among 150 and 200 healthy young spores of INCAM 11 were transferred to the filtration membrane (0.44 µm), rinsed 3 times with sterile distilled water, and treated with Chloramin T 2% (with 2 drops of Tween 20) for 10 min. Then spores were washed 3 times with sterile distillated water and treated 10 min with antibiotic solution containing Streptomycin sulphate (0.02 %) and Gentamycin sulphate (0.01 %). The solution was filter on a disinfection apparatus through a sterile millipore filter (type HA, diameter 4.0 cm, pore 0.22 µm). Afterward, the membrane supporting the disinfected spores was gently transferred into plastic Petri plates (90 mm diam.) containing 20 mL of antibiotics solution and kept in the plates for 24 hours.

## Monoxenic culture of INCAM 11 by root organ culture (ROC)

Thirty INCAM 11 surface disinfected spores associated in clusters and Ri T-DNA transformed chicory hairy roots of 2 cm length were transferred to plastic petri plates (90 mm diam.) on a Modified Strullu and Romand (MSR) medium [11]. Forty plates were set up and incubated on inverted position at 27˚C in the dark until the hyphae colonized the young roots, the mycelium dispersed profusely into the medium and new spores were produced. Plates were checked every 48 h for spore germination and hyphal growth. First contact between fungus and host roots, new spore formation and extraradical hyphal length were recorded. Evaluations were carrying out under a dissecting microscope (Novel) at 10-50X magnification. A grid of lines was marked on the bottom of each Petri plate for the extraradical hyphal length and new spore formation. Hyphal length was monitored weekly during 3 months and spore's numbers were determined at the end of the experiment, following the methodology described by [12]. The newly produced spores were sub-cultivated on fresh medium to confirm if they were able to form associations with a new *in vitro* host and complete their life cycle. Forty spores from the *in vitro* culture associated in clusters were extracted. Small pieces of medium containing spores were gently removed with a scalpel and located in the vicinity of fresh roots apex of 10 days of culture growing in new MSR medium. Plates were sealed with parafilm and incubated at 27 °C in the dark.

## Isolation and molecular identification of associated microorganism

The most frequent associated microorganism colony to the *in vitro* culture of INCAM 11 was isolated and molecularly identified. When bacterial growth around fungus were observed, individual colonies were isolated on Nutrient Agar [13] medium and incubated at 30°C for two weeks. Subsequently, the obtained isolate was identified by amplification and partial sequencing of 16S rDNA. DNA extraction: Extraction of genomic DNA was performed by alkaline lysis of fresh colonies according to [14]. The obtained DNA was quantified by spectrophotometry at 260 nm using the NanoDropTM 2000 (TermoFisher). PCR amplification and sequencing: 16S rDNA gene from isolate was PCR amplified using bacterial universal primers 27f(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') [15] and the premixed solution GoTaq® Green Master Mix (Promega Corporation, USA). PCR conditions were: an initial denaturation (95 °C, 10 min), 35 cycles of 92 °C, 1 min; 50 °C, 45 s and 72 °C, 1.5 min; followed by a final extension (72°C, 5 min). PCR was performed in an MS mini thermocycler (Major Science, USA). The PCR reaction mixture (25 µL volume) contained 1X GoTaq®Green Master Mix, 0.4 µM of each primer and 50 ng of DNA. The amplification product was verified by 1 % agarose gel electrophoresis at 80 V for 45 min. Sequencing was performed by the Sanger method in the company Macrogen®. Sequences edition and identification: Quality analyses of the sequences were performed with FinchTV1.4.0 (Geospiza, Inc). The obtained sequence was compared with the Gen Bank database using the BLASTn (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information (NCBI) [16].

## Results and Discussion

### *In vitro* propagation of INCAM 11 (R. irregularis)

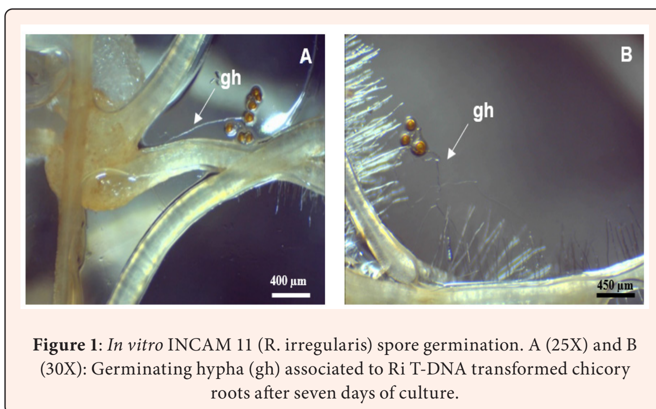


**Figure 1**: *In vitro* INCAM 11 (R. irregularis) spore germination. A (25X) and B (30X): Germinating hypha (gh) associated to Ri T-DNA transformed chicory roots after seven days of culture.

The monoxenic culture of R. *irregularis* INCAM 11 was successfully established in transformed chicory roots. Ri T-DNA transformed chicory roots show greater AM intraradical colonization and sustain higher extraradical hyphal development than non-transformed roots [17]. After 3 days of culture, spore germination and hyphal growth was observed, with simultaneous growth of germ tube towards hairy the roots (Figure 1). The first interaction between fungus mycelium and roots occurred 5-7 days after inoculation.

The germ tube produced multiply laterals branches on media surface and toward roots with extensive hyphal proliferation (Figure 2 A-B, C and D). Figure 2 (A) shows "fan-like structure" (FLS) formation after 5th day of cultivation. Hyphal length was recorded during 90 days. Some investigations under *in vitro* 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 a hyphal branching called FLS, which may lead to the formation of an appressorium [18].

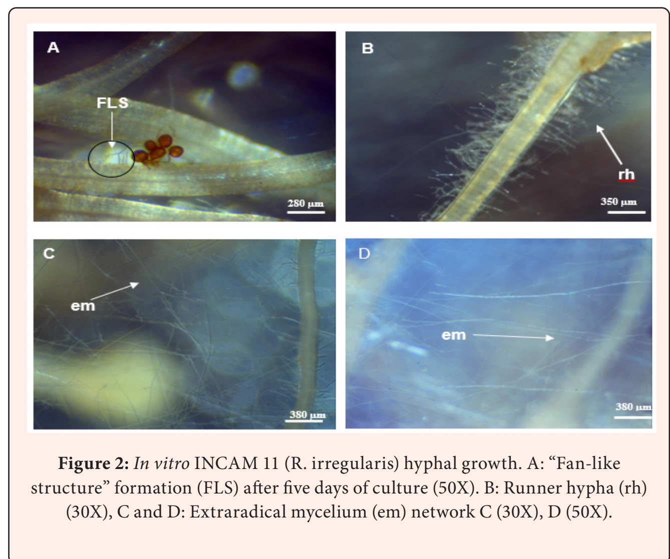


**Figure 2:** *In vitro* INCAM 11 (R. irregularis) hyphal growth. A: "Fan-like structure" formation (FLS) after five days of culture (50X). B: Runner hypha (rh) (30X), C and D: Extraradical mycelium (em) network C (30X), D (50X).

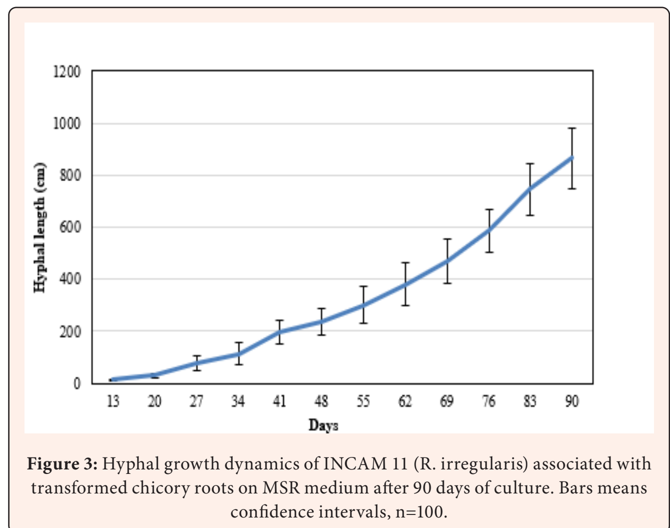


**Figure 3:** Hyphal growth dynamics of INCAM 11 (R. irregularis) associated with transformed chicory roots on MSR medium after 90 days of culture. Bars means confidence intervals, n=100.

During the experiment, when hyphae reached the root surface the pattern starts to change, their growth became fast and in a straight way toward hairy roots. Hyphae branched grew in all directions followed by hyphae proliferation intensively near the roots (Figure 2 B) and a rapidly increment of hyphal density into the medium (Figure 2 C, D) [19] reported in the *in vitro* co-culture of Glomus microcarpum with Vigna vexillata hairy roots that almost 60 % of media surface was covered with a heavy mycelial network and several FLS were observed on root surface after 12-20 days of incubation. On the other hand, [20] observed that in *in vitro* culture of Gigaspora decipiens and R. clarus produced typical structures like "branched absorbing structure" (BAS) after spore's germination. Even if the AMF species are different their behavior is similar

in root organ culture conditions. The mean hyphal length was increasing rapidly during the experiment dynamic reaching 865.6 cm length at week 13 (90 days) (Figure 3). This value is similar compared to those reported by other authors from experiments conducted on potato plantlets with a R. *intraradices* strain a mean hyphal length of 1 300 cm at week 22, seven weeks more than in this experiment.

Results from experiments developed by [21] on transformed carrot roots with two different strains of R. *intraradices* showed values of 1 831 cm and 1 442 cm in a period of 6 to 8 months. The architecture of the extraradical mycelium as well as the growth characteristics was similar to those observed in the classical monoxenic culture systems on excised root organs [11, 22] emphasized the importance of such extraradical mycelium networks to initiate rapid colonization in new hosts. The formation of new spores began within 21- 25 days after association (Figure 4 A, B). The spore's formation rate was slow during first 60 days and increased progressively after 90 days of incubation (Figure 4 C, D). The spore number was around 2 000 per petri dish after 5 months of incubation. This result is in agreement with Voets et al. (2005) [12], who observed the first newly produced spores at week 3.

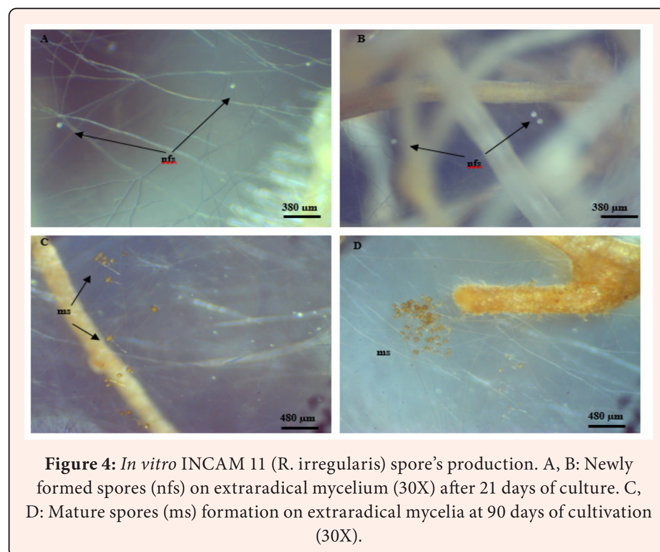


**Figure 4:** *In vitro* INCAM 11 (R. irregularis) spore's production. A, B: Newly formed spores (nfs) on extraradical mycelium (30X) after 21 days of culture. C, D: Mature spores (ms) formation on extraradical mycelia at 90 days of cultivation (30X).

First spores were smaller than mature spores and were formed almost terminally on short hyphal branches (Figure 4 A, B). These vegetative spores were hyaline whitish colored but turned to brownish yellow after several weeks and become in mature (Figure 4 C, D). After 5 months of culture matured spores with a light brown to a dark brown color were observed. In this investigation, the rate of sporulation increased after 90 days of incubation. It might be due to the decrease of sucrose and other mineral component level in the medium [4]. Similar trend was observed by [23] who showed that the rate of sporulation depended on the sucrose concentration of the media. Besides, the sporulation rate was not the same in all plates; such behavior it could be attributed to root – AMF communication in the pre-symbiotic and symbiotic phase, due it is a complex multi-step process under genetic control, regulated by an intimate molecular dialog between the host root and the obligate symbiont [24]. Such multi-step process could impact root colonization and subsequent spore production (Voets et al., 2005) [12]. The spore's number produced by INCAM 11 is around 2 000 per petri dish after 5 months of incubation, is low compared with [4, 2]. They recorded around 8 500 spores and 2 500 spores of R. *intraradices* after 3 months of culture, respectively. However, only a few AMF species are fast growers and colonizers that are able to produce many thousands *in vitro* propagules in a few months [7]. The newly produced spores were able to form associations with new hairy roots *in vitro* (Figure 5), following sub-cultivation, reproduced them self and completed their life cycle. *Rhizophagus irregularis* INCAM 11 *in vitro* spores have the ability to form new mycorrhizal associations after a subculture period indicating that this strain is capable to complete their life cycle under *in vitro* conditions. After 15 days of culture newly spore start to growth in the extraradical mycelium. They were small and transparent and after 95 days of incubation they exhibited general morphological similarity to soil borne inoculum (Figure 5 B), although they had thicker spore walls than their soil borne counterparts.

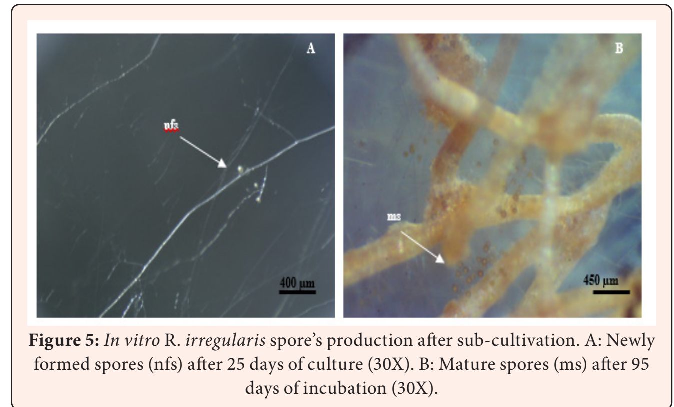


**Figure 5:** *In vitro* R. *irregularis* spore's production after sub-cultivation. A: Newly formed spores (nfs) after 25 days of culture (30X). B: Mature spores (ms) after 95 days of incubation (30X).

AM root-organ cultures facilitates the study of environmental, nutritional, and physiological effects on fungal growth and spore morphology. A range of parameters related to sporulation, hyphal architecture, and spore ontogenesis can now be compared according to the systematic approaches used [17]. The multiple characteristics (anatomical, ultrastructural, or biochemical) that can be generated using *in vitro* produced AM fungal colonies need to be classified according to their taxonomic significance, to discriminate between environmentally related and phylogenetically driven taxonomic parameters. For example, *in vitro* differentiated spores may be slightly smaller and are often less pigmented than soil-borne spores [25].

### Isolation and identification of the most frequent associated microorganism to the *in vitro* culture of INCAM 11

A microorganism colony of bacterial origin was found to be frequently associated with INCAM 11 spores (Figure 6 A and B). Bacteria colonies were round shaped, white and medium size (2-4 mm), concentric, of convex surface, with rounded, flat borders and moderate mucosity (Figure 6 A and B).

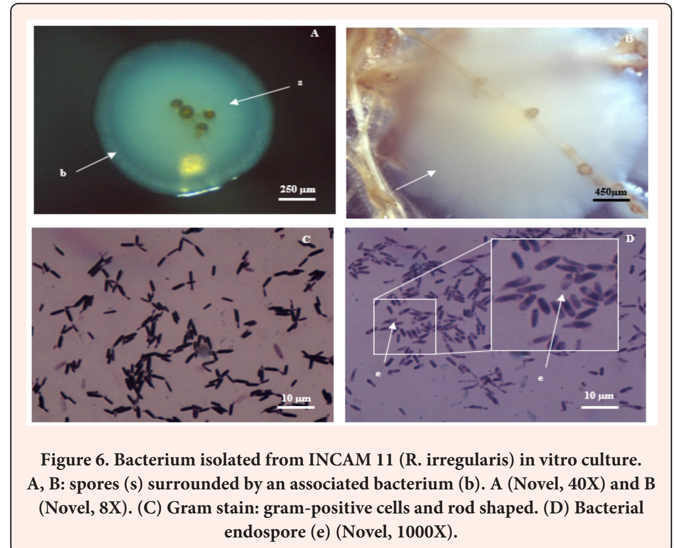


**Figure 6. Bacterium isolated from INCAM 11 (R. irregularis) in vitro culture. A, B: spores (s) surrounded by an associated bacterium (b). A (Novel, 40X) and B (Novel, 8X). (C) Gram stain: gram-positive cells and rod shaped. (D) Bacterial endospore (e) (Novel, 1000X).**

After 30 h of incubation on Agar nutrient medium some microbial growth was detected. After application of the Gram stain, predominance of bacillary and Gram positive microorganisms (Figure 6 C) as well as bacterial endospores (Figure 6 D) could be observed. According to the 16S rDNA gene sequence, the strain ABRi1 was identified as *Paenibacillus* sp (Table 1). This bacterial genus commonly grows associated with *in vitro* culture of AMF strains [26] isolated *Paenibacillus* sp. associated with external mycelium of R. *intraradices*. Accordingly [22] found that slime-forming bacteria, identified as P *validus*, frequently pop up when spores of R. *intraradices* germinated on modified M medium concluding that these bacteria were able to support fungus growth on agar medium.

**Table 1:** Bacterial strain associated to INCAM 11 *in vitro* culture identified by partial 16S rDNA sequencing.

| Isolate | Identity | Long (bp) | Access No | More Probable Strain | | |
|---|---|---|---|---|---|---|
| | | | | Name | Similarity | Access no. |
| ABRi1 | *Paenibacillus* sp. | 1447 | MW139949 | *Paenibacillus* pocheonensis Gsoil 1138T | 97.79 | NR_112565 |

In the presence of P. *validus*, hyphae branched profusely and formed coiled structures [27] identified a bacterial isolate as *Paenibacillus* sp. from the mycorrhizosphere of Sorghum bicolor inoculated with *Funneliformis mosseae*. This *Paenibacillus* strain stimulates AM fungal root colonization, spore germination, and hyphal growth of F. mosseae [27]. *Paenibacillus* species are widely distributed in the mycorrhizosphere and could have a positive effect on the development of the in vivo and *in vitro* AM symbiosis [28, 29] suggested that the most AM fungi-associated bacteria are located on the surface of spores and hyphae, although some bacteria have been found in the cytoplasm of AM fungi spores. They could stimulate spore germination by eroding spore walls (Maia et al.,1998), by producing stimulatory compounds such as $CO_2$ and other volatiles [30], or by influencing AM fungi phosphorus acquisition [31,32] showed that most of the bacteria found in association with spore walls of the AM fungi, F. geosporum and *Septoglomus constrictum* were able to degrade biopolymers such as cellulose and chitin. These authors suggested that the increase in germination of Glomus sensu lato spores could be related to the degradation of external layers of spore walls by soil bacteria. According to those authors some toxic compounds that inhibit germination of AM propagules and mycelial growth could be also reduced by these bacteria [28] reported a significant promotion in pre-symbiotic mycelium development occurred after inoculation of two *Paenibacillus* species isolated from AM propagules under *in vitro* conditions. P. *rhizosphaerae* TGX5E significantly increased the extraradical mycelium network, the rate of sporulation, and root colonization on an *in vitro* symbiotic association. These results were also observed in the rhizosphere of soybean plants grown under greenhouse conditions, when P. *rhizosphaerae* was co-inoculated with R. *intraradices*. Species of *Paenibacillus* associated with AM fungus structures in the soil, may have a promoting effect on short term pre-symbiotic mycelium development, and little impact on AM propagule germination [28-34].

## Conclusion

The *in vitro* system has proved to be a useful tool for the cultivation and conservation of a large number of species and isolates of AM fungi. The monoxenic cultures of Cuban strain R. *irregularis* INCAM 11 was successfully established on chicory transformed roots, providing spore production in a small space and over short period of time. Effects of *Paenibacillus* sp. on R. *irregularis in vitro* germination and establishment most be evaluated. Our results are the first reported in Cuba in terms of *in vitro* establishment of AM fungus and are the basis for the creation of an *in vitro* AM fungal collection.

## Acknowledgments

The authors thank the financial support of VLIR-UOS to the TEAM Project "*Arbuscular Mycorrhizal* fungi as an efficient tool to improve the agricultural production of small-scale local farmers in Cuba", developed among INCA, Cuba and Ghent University, Belgium which facilitate all laboratory materials to accomplish this research.

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