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Full Length Research Paper

Development of an *in vitro* culture system adapted to banana mycorrhization

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The beneficial impact of arbuscular mycorrhizal (AM) fungi on banana nutrition and resistance against abiotic and biotic stresses is well documented. However, most studies were conducted under greenhouse or field conditions and none reported the life cycle of the AM fungi on banana roots. It is obvious that any system associating both organisms under strict controlled *in vitro* culture conditions may help to comprehend the role of AM fungi in banana physiology. Here we developed an *in vitro* culture system associating autotrophic micropropagated banana plants with an AM fungus (*Glomus intraradices*). Intraradical root colonization, with production of arbuscules and vesicules, as well as extraradical development with production of new spores was observed. This study opens the door to investigate the role of AM fungi in banana physiology in particular for the control (through e.g. the elicitation of defence genes in bananas) of major banana root pathogens under strict *in vitro* culture conditions.

Key words: Arbuscular mycorrhizal fungi, ***Musa acuminata***, ***in vitro*** conditions, sporulation.

INTRODUCTION

Bananas and plantains are among the most important crops in the world representing a major staple food for millions of people in developing countries. They contribute to food security by producing fruit year-around and provide incomes to rural populations (Roux et al., 2008).

Pests and diseases are major constraints that limit banana and plantain production. They reduce yield and cause economic damage. Integrated management practices combining, among others, pesticide applications, utilization of resistant cultivars and production of micropropagated plantlets are used nowadays to prevent and reduce the detrimental impact of biotic stresses (Raut and Ranade, 2004).

In recent years, biological control agents (BCA) have been considered as new alternatives for pest and diseases control. Arbuscular mycorrhizal (AM) fungi have been widely recognized as BCA's (Azcón-Aguilar and Barea, 1996; Johansson et al., 2004; Barea et al., 2005). These soil microorganisms form symbiotic associations with nearly 80% of plant families and have been used to control plant diseases provoked by soil pathogens, thus improving plant resistance (Smith and Read, 2008). They were also shown to play a key role in plant nutrition and growth, in resistance to drought and in soil structure (Smith and Read, 2008). Several studies have reported their beneficial effects on nutrients uptake and growth of banana plants (Declerck et al., 1995; Yano-Melo et al., 1999; Declerck et al., 2002b) as well as control of nematodes (Elsen et al., 2003a; Elsen et al., 2008) and fungal root pathogens (Declerck et al., 2002a). However, most studies were conducted in greenhouse or field con-

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Abbreviations: AM, Arbuscular mycorrhizal; **BCA,** biological control agent; **MSR,** modified Strullu Romand.

ditions and none reported the life cycle of the AM fungi on banana roots. It is obvious that any system associating both organisms (that is, the banana and the AM fungus) under strict controlled ***in vitro*** culture conditions may help to comprehend the role of AM fungi in e.g. controlling banana root diseases.

Recently, Elsen et al. (2003b) demonstrated under strict ***in vitro*** culture conditions the protective effect of AM fungi against ***Pratylenchus coffeae,*** an important banana nematode pathogen. However, this study was conducted with excised transformed roots of carrot. In such system, the absence of photosynthetic tissues may disturb the hormonal balance and exogenous sucrose is used as the carbon source to feed the root and indirectly the AM fungus (Fortin et al., 2002). Therefore, the development of an ***in vitro*** culture system associating autotrophic banana plants with AM fungi is highly desirable to investtigate more deeply and make continuous and non-destructive observations of the effects of AM fungi on various root pathogens and diseases.

Recently, two culture systems were developed for the association of AM fungi with autotrophic plants under ***in vitro*** culture conditions (Voets et al. 2005; Dupré de Boulois et al. 2006). The system developed by Voets et al. (2005) allowed the successful association of micropropagated potato plantlets to an AM fungi. In this system, the roots and AM fungi developed under strict ***in vitro*** culture conditions, while the shoot developed in open-air conditions. In the system developed by Dupré de Boulois et al*.* (2006) both ***Medicago truncatula*** plantlets and the AM fungi developed under strict ***in vitro*** culture conditions.

In the present study, we adapted the culture systems developed by Voets et al*.* (2005) and Dupré de Boulois et al*.* (2006) for the ***in vitro*** mycorrhization of micropropagated banana plantlets. Spores production, hyphal length and intraradical root colonization were assessed.

MATERIALS AND METHODS

Biological materials

Tissue-cultured banana (***Musa acuminata***) plants, cultivar Grande Naine (AAA genome, Cavendish group) were provided by the International Musa Germplasm collection at the INIBAP Transit Centre (ITC) at K.U.Leuven, Belgium. The plant material was proliferated, regenerated and rooted on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, 10 mg/l ascorbic acid and 2 g/l phytagel. The pH was adjusted between 6.12 and 6.15 before sterilisation (Banerjee and De Langhe, 1985).

Banana plantlets were proliferated in test tubes (Banerjee and De Langhe, 1985) by adding 10^{-3} M indole-3-acetic acid (IAA) and 10^{-2} M 6-benzilaminopurine (BAP), two plant growth regulators, to the MS medium. Individual aseptic shoots (± 1 cm height) were inserted in each test tube. Plantlets were proliferated every 4 - 6 weeks. For regeneration and rooting, no plant growth regulators were added, but 0.5 g/l of active charcoal. Four aseptic shoots (± 3 cm height) were placed in sterile culture boxes (145 x 100 x 85 mm) to allow horizontal root growth. Plantlets were grown in a culture room at 27/25°C (day/night) 70% relative humidity under 12 days light cycle.

A strain of ***Glomus intraradices*** Schenck and Smith (MUCL 41833) associated to Ri T-DNA transformed carrot (***Daucus carota*** L.) roots on the modified Strullu Romand (MSR) medium (Declerck et al., 1998) was purchased from GINCO (http://www.mbla.ucl.ac.be/ginco-bel). A piece of MSR medium containing roots and spores was provided in a 90 mm diameter Petri plate. The material was proliferated thereafter by associating two weeks old transformed carrot roots with spores on Petri plates (90 mm diameter) filled with 40 ml MSR medium (Cranenbrouck et al., 2005). The Petri plates were incubated at 27°C in the dark in an inverted position. After 5 months of culture, each Petri plate contained several thousand spores.

The culture system

Petri plates of 145 mm diameter were used. A hole of 24 mm diameter was made on the edge of each Petri plate lid with a heated cork borer. The Petri plates were then cautiously enclosed in plastic bags and sterilized at 25 KGy by gamma irradiation at STERIGENICS (Fleurus, Belgium).

The sterilized Petri plates were filled with 150 ml MSR medium lacking vitamins and sucrose, buffered with 10 mM MES (pH 6) and adjusted to pH 6 before sterilization. Spores from a five-month-old culture of ***G. intraradices*** were extracted from the MSR medium by solubilization of the gellan gel (Doner and Bécard, 1991). Spores were subsequently maintained in sterile (121°C for 15 min) deionized water before inoculation. In each Petri plate, approximately 150 spores were placed on the surface of the MSR medium for pregermination. The Petri plates were then sealed and incubated in the dark at 27°C in a horizontal position.

Four days after incubation, nearly 85% of the spores have germinated. Twenty days old rooted banana plantlets were then transferred into the Petri plates*.* Each plantlet was positioned vertically in the Petri plates, with the roots on the surface of the MSR medium and the shoot in upright position. The Petri plates were then closed with the perforated lids, allowing the shoot to extend beyond the hole (Figures 1 and 2). To prevent microbial contamination, the hole was cautiously plastered with sterile Sealer BREATHseal^{TM} (Greiner Bio-one). The Petri plates were then sealed with Parafilm (Pechiney, Menasha, WI, USA), covered with a black plastic bag and incubated in a growth chamber set at 27/25°C (day/night) with 70% relative humidity under 12 day light cycle. MSR medium (100 ml) without vitamins and sucrose was added in the Petri plates at week 2, 4, 7 and 9. Each culture system was considered as an experimental unit and seven replicates were considered.

Experimental design

Non-destructive microscopic observations were made 6 and 10 weeks after association of the banana plants with the AM fungus. The total number of newly produced spores and the extraradical hyphal length were estimated. A grid of lines was marked on the bottom of each Petri plate to form 1 cm squares. The presence of hyphae was noted at each point where they intersected a line. The total extraradical hyphae length was calculated with the formula of Newman (1966). The number of spores was estimated in each square formed by the grid of lines and summed over the entire Petri plate (Declerck et al., 2001).

Banana plantlets were harvest at week 11. The leaves were counted and pseudostem height was measured from the top of the

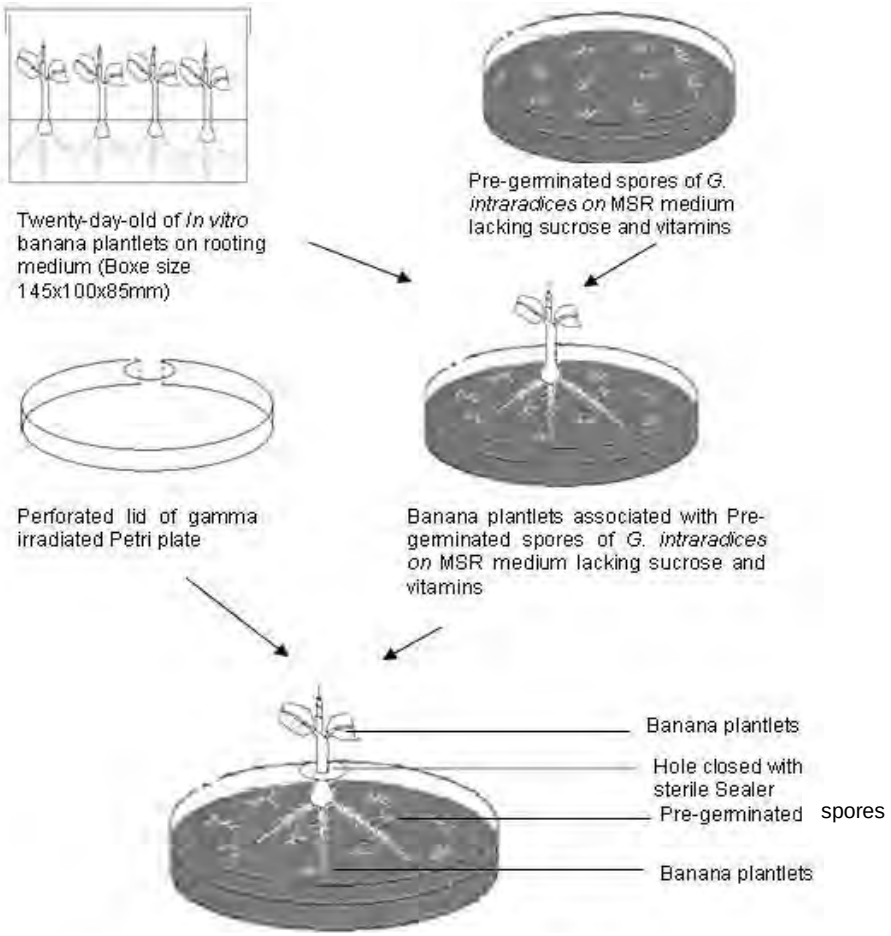

Figure 1. Cultivation system for the ***in vitro*** mycorrhization of banana plantlets.

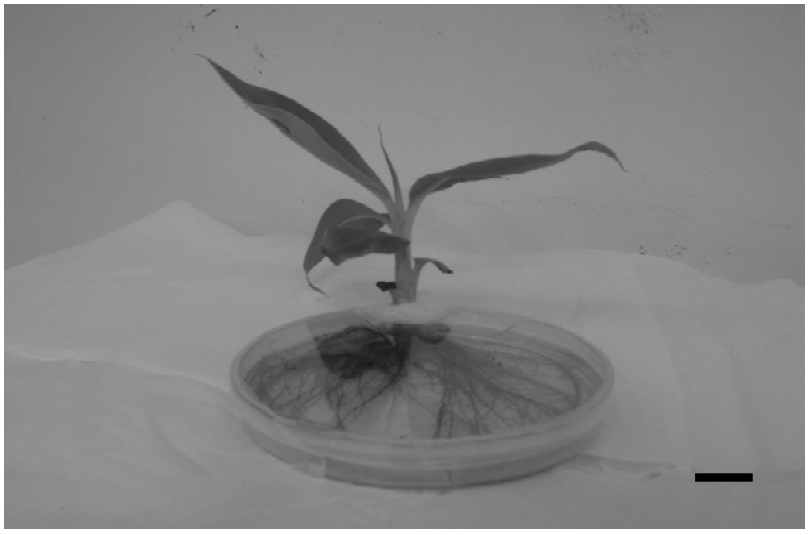

Figure 2. Banana plantlets (Grande Naine) associated with pre-germinated spores of ***G. intraradices***. Bar = 2 cm.

rhizome to the crossing point of the two unfolded leaves. The roots were subsequently removed from the Petri plates with forceps to assess root colonisation. The roots were soaked in 10% KOH at ambient temperature. Three cycles of one hour were conducted with renewed solution of KOH. Roots were then soaked in 10% KOH overnight at ambient temperature. They were washed several times with deionized water and bleached in a fresh prepared alkaline H_2O_2 for 30 min (Koske and Gemma, 1989). The roots were subsequently stained at ambient temperature for 45 min with a solution of blue ink (Parker^P Quink®) diluted in 1% HCl at 1:50 proportions (Vierheilig et al., 1998a). Root colonization was assessed by the method of McGonigle et al. (1990) to determine the number of arbuscules, vesicles, hyphae and the total percentage of colonization.

RESULTS

Banana plantlets did not suffer from transplantation into the MSR medium lacking sucrose and vitamins and buffered with 10 mM MES (pH 6). No wilted leaves were observed. The first leaf was unfurled 10 days after transfer of the banana plantlets into the Petri plates. The next unfurled leaves were longer and larger than the former ones. Primary roots emerged from the corm and developed on the surface and into the culture medium. On each primary root, secondary order roots were formed, as well as tertiary roots. Two types of primary roots were observed. The feeder roots, mostly thin with a high density of secondary roots, and the pioneer roots, larger, with a low density of secondary roots. Clumps of secondary roots, almost as large as the primary roots developed behind desiccated or damaged roots tips of primary roots. A dense cover of large root hairs was observed on some primary roots. All secondary and tertiary roots were covered by root hairs. Newly produced roots were creamy white and became brown and dark with ageing. At the end (11 weeks) of the experiment the banana plantlets had in average 6 leaves and a height of 8.67 ± 0.22 cm.

The first contact points between hyphae and roots were observed eighteen days after banana plantlets were transferred in the Petri plates containing pre-germinated spores of ***G. intraradices***. At that time, hyphae started to spread profusely on the surface of the medium. Numerous runner hyphae grew in straight lines and branched to produce second and higher order hyphae which extended the fungal colony on the surface of the Petri plate. Some of the lower branched hyphae were frequently undulated and tangled. Numerous branching absorbing structures (BAS) were observed along runner and lower order hyphae as well as anastomoses. Some runner hyphae differentiated and divided profusely forming fan-like or horsetail-like structures. The first newly produced spores were observed at week 4 following association. Spore production increased over time. An average of 195 ± 41 spores was formed at week 6 with a hyphal length of 403 ± 87 cm. At week 10, the number of spores increased three fold and reached 651 ± 92 with a mean value of hyphae length of 616 ± 70 cm. Spores were produced in terminal as well as intercalary position. They were isolated or produced in clusters, sometimes associated to BAS. Spore size ranged between 20 and 130 µm with an average of 73 ± 2.4 µm.

Colonization, estimated at week 11, was restricted to a few roots, mostly of secondary and tertiary order. The total root colonization was low and averaged 6.2 ± 1.2% at week 11. Arbuscules and vesicles were regularly observed (Figure 3). The respective proportion of hyphae, vesicles and arbuscules was 0.2 ± 0.1%, 1 ± 0.4% and 4.8 ± 0.8%, respectively.

DISCUSSION

This study reported for the first time the successful ***in vitro*** mycorrhization of banana plantlets. The AM fungus was able to colonize the root system and to complete its life cycle. Intraradical root colonization, with production of arbuscules and vesicules, as well as extraradical development with production of new spores was observed.

The culture system used for the ***in vitro*** mycorrhization of banana plantlets consisted of a Petri plate with an opening allowing the banana shoot to develop in open air conditions, while the roots developed under strict ***in vitro*** conditions in association with the AM fungus. To avoid any unconsidered risk of contamination, the opening made on the edge of the lid of the Petri plate was plastered with sterile Sealer BREATHseal^{TM} (Greiner Bio-one). Voets et al. (2005) used this system for the ***in vitro*** mycorrhization of micropropagated potato plantlets. However, plant size and pseudostem width of banana plantlets imposed slight modifications to the system. Larger Petri plates, position and size of the opening differed from the system of Voets et al. (2005).

Banana plantlets were associated with the AM fungus on the MSR medium used for the root organ culture of transformed carrot roots (Cranenbrouck et al., 2005). However, the medium was devoid of sucrose and vitamins. This medium was earlier used for the ***in vitro*** mycorrhization of potato (Voets et al., 2005) and ***M. truncatula*** plantlets (Dupré de Boulois et al., 2006). This medium, deprived of vitamins and sucrose, supported the growth of photosynthetic-active banana plantlets.

In the MSR medium, banana roots differentiated into primary roots (that is, feeder and pioneer roots), secondary and tertiary roots, and clumps of secondary roots. The architecture of the root system was comparable to the results of Banerjee and de Langhe (1985) and Buah et al. (1998). Feeder and pioneer roots of banana plants were described for the first time under hydroponics conditions by Swennen (1986). The development of clumps of secondary roots behind desiccated or damaged root tips of primary roots was previously observed

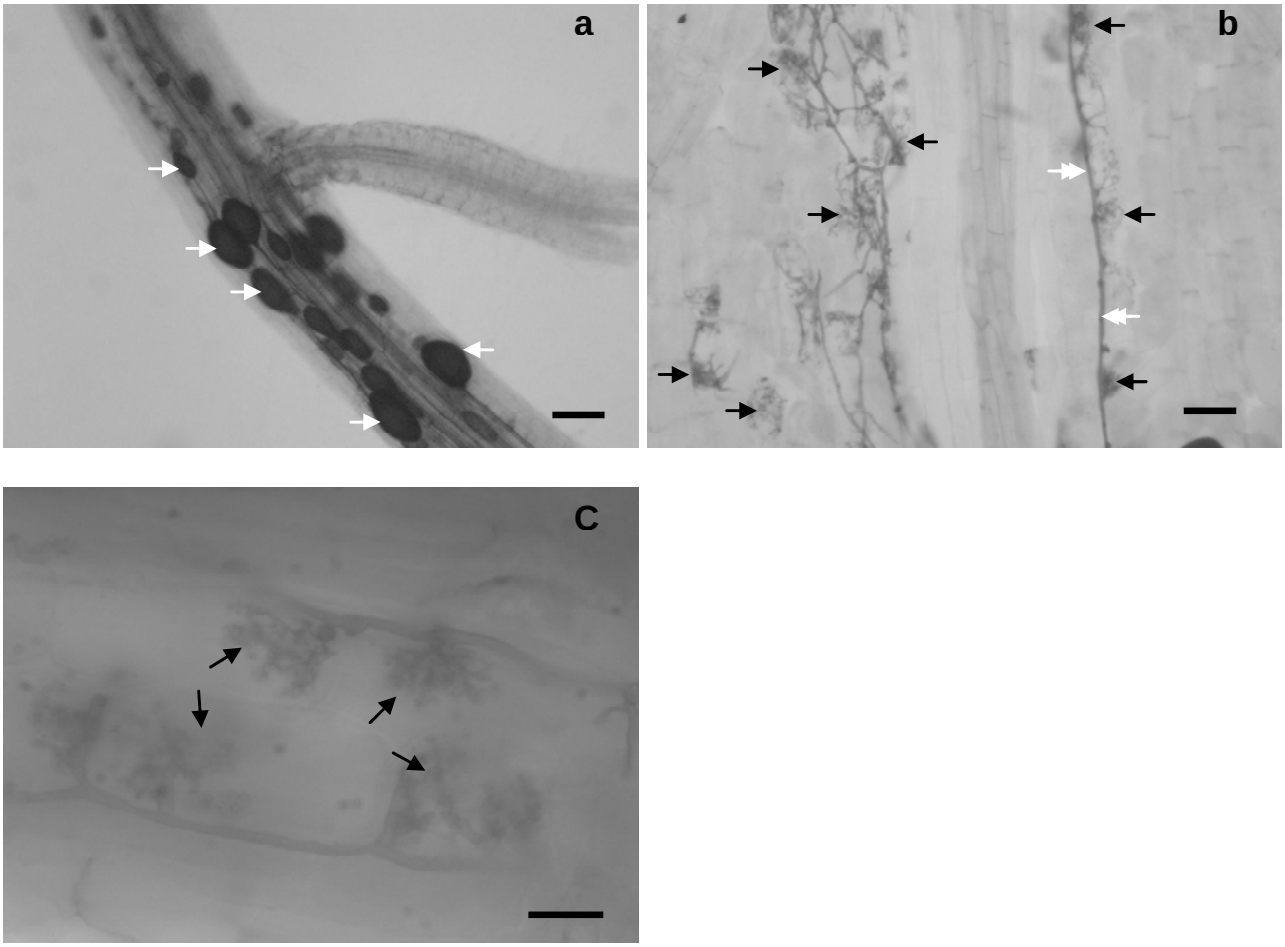

Figure 3. Intraradical structures of ***G. intraradices*** in roots of ***Musa*** cv. G. Naine after 11 weeks of associations under ***in vitro*** conditions. a.) Vesicles (white arrows), bar = 70 µm. b.) Intraradidal hyphae (white double arrows) and rows of arbuscules (black arrows), bar = 30 µm. c.) Arbuscules (black arrows), bar = 10 µm.

ved in hydroponics (Swennen, 1986) and in pot culture (Kobenan et al., 1997). Kobenan et al. (1997) called these roots “witche’s-broom” and Draye (2002) called them “replacement roots”. Roots produced in our experiment turned black after a couple of days and stained the culture medium. This blackening was most probably caused by oxidation of the phenolics compounds release by the roots regularly reported for ***in vitro*** culture of banana plantlets (Banerjee and de Langhe, 1985; Titov et al., 2006). Blackening of roots could be reduced by adding activated charcoal, antioxidants such as ascorbic acid or citric acid in the culture medium (Banerjee and de Langhe, 1985).

In presence of the banana roots, ***G. intraradices*** developed runner hyphae that branched into secondary and higher order hyphae. BAS, horsetail-like structures and fan-like structures were frequently observed. Horsetail-like structures have been proposed as preferential site for spore production and appeared to be the extremity of the fungal colony (Bago et al., 1998). Fan-like structure formation was reported to be induced by root exudates (Giovannetti et al., 1993).

Spore size ranged between 20 and 130 µm with an average of 73 ± 2.4 µm. External mycelium architecture and spore size were similar to the results of early studies (Bago et al., 1998; Bago et al., 2004).

In our experiment, at week 10, the number of spores exceeded 600 and the length of extraradical mycelium was above 600 cm. These values were low as compared to the results obtained in past research with the same AM fungal strain (MUCL 41833) associated ***in vitro*** to transformed roots of carrot (Declerck et al., 2001; Elsen et al., 2003b). Results obtained with another AM fungal strains (MUCL 43194) associated ***in vitro*** to potato plantlets (Voets et al., 2005) were also higher than our results. Identically the proportion of total root length colonized by the AM fungus, the density of arbuscular and vesicles were low as compared to the results of previous studies

conducted ***in vitro*** (Elsen et al., 2003b; Dupré de Boulois et al., 2006). These low values may be related to various factors among which the AM fungi-host plant species association (Bever et al., 1996; Voets et al., 2005; Ahulu et al., 2007) and the phenolic compounds released by the banana roots in the MSR medium (Buee et al., 2000; Requena et al., 2007). Although a number of studies reported the beneficial effects of phenolics compounds on the development of AM fungi (Siqueira et al., 1991; Vierheilig et al., 1998b), other studies reported an inhibitory effect (Chabot et al., 1992; Piotrowski et al., 2008). Fries et al. (1997) and Piotrowski et al. (2008) observed decreased hyphal elongation and root colonization in presence of increased concentrations of phenollic compounds. Banana plants are known to release large quantities of phenolic compounds (Titov et al., 2006). Many experiments have demonstrated the role of root phenolic compounds in banana resistance against ***Radopholus similis*** (Valette et al., 1998, Wuyts et al., 2007) and ***Fusarium oxysporum*** var. ***cubense*** (de Ascensao and Dubery, 2003). The large quantity of phenolic compounds released in our experiment, causing blackening of the roots and staining of the culture medium, extraradical hyphae and spores, may have inhibited hyphal elongation, spore production and root colonization as earlier postulated by Fries et al. (1997) and Piotrowski et al. (2008).

Conclusion

In this study, micropropagated banana plantlets were successfully associated for the first time to an AM fungus under ***in vitro*** conditions. The AM fungus was able to colonize the banana roots, producing arbuscules and vesicles. Extraradical hyphae extended from the mycorrhizal root system and developed in the growth medium, producing spores, BAS, fan-like and horsetail-like structures. The system developed in the present study opens the door to investigate the role of AM fungi in the control (through e.g. the elicitation of defence genes in bananas) of major banana root pathogens under strict ***in vitro*** controlled conditions.

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