TIME-COURSE SPORULATION OF INDIGENOUS ARBUSCULAR MYCORRHIZAL FUNGI FROM ARID ZONES OF SENEGAL

DYNAMIQUE DE SPORULATION DE CHAMPIGNONS MYCORHIZIENS ARBUSCULAIRES INDIGENES DU SENEGAL

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Abstract:
Three indigenous arbuscular mycorrhizal fungi from arid zones of Senegal monoxenically cultivated with excised carrot roots showed different patterns of mycelial growth and sporulation. Among the arbuscular mycorrhizal fungi, *Glomus aggregatum* developed the most profuse mycelium and *Glomus mosseae* had the lowest sporulation rate after 3 months cultivation. Intensive sporulation occurred in the branched absorbing structures of arbuscular mycorrhizal fungi. A sigmoidal curve (lag, exponential, plateau) characterized the dynamics of spore production. Monoxenic culture of the intraradical forms of *Glomus sp* and isolated carrot roots appeared suitable to follow the entire life cycle of arbuscular mycorrhizal fungi and to establish *in vitro* collections.

Key-words:
Glomus aggregatum, *Glomus fasciculatum*, *Glomus mosseae*, *in vitro* sporulation, Ri T-DNA transformed root,
Running title: *In vitro* growth and sporulation of AM fungi

Résumé:
La culture *in vitro* de trois champignons mycorhiziens arbusculaires originaires de zones arides du Sénégal en présence de racines isolées de carotte a montré différentes caractéristiques de croissance mycélienne et de sporulation. *Glomus aggregatum* a le développement mycélien le plus important et *Glomus mosseae* sporule le moins. Chez les champignons étudiés, une intense sporulation se produit dans les structures arborescentes des champignons. Une courbe sigmoïde (latence, croissance, plateau) symbolise la dynamique de sporulation. La culture *in vitro* de la forme intraracinaire des *Glomus* et de racines isolées de carotte permet de suivre le cycle de développement des champignons et d'établir des collections.

Mots clés:
Glomus aggregatum, *Glomus fasciculatum*, *Glomus mosseae*, Racines transformées de carotte, Sporulation *in vitro*

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1. Introduction

Arbuscular mycorrhizal (AM) fungi are obligate biotrophic organisms associated with a wide range of vascular plants (Nicolson [1]). Their marked effects are often upon physiological aspects of host partner particularly under stress conditions (Augé[2]). Because of their biotrophic status, it is difficult to follow the developpement of AM extraradical hyphae in soil based systems. The non-destructive monoxenic system using excised roots has proven to be a powerful tool to understand the biology of the mutualistic fungi (Bécard *et al.,* [3]; Bécard *et al.,* [4]; St-Arnaud *et al.,* [5]) and to obtain mass free contaminant spores (Declerk *et al.,* [6]; Diop *et al.,* [7]; Diop *et al.,* [8]). Many physiological, genetical, and practical studies can be achieved using the mycorrhizal root-organ culture system and the only limit to its use is the user's imagination (Fortin *et al.,* [9]). The newly obtained vegetative propagules, had have a high level of viability, able to initiate well established AM mycorrhizas in both *in vivo* and *in vitro* conditions (Diop *et al.,* [8]; Plenchette *et al.,* [10]; Vimard *et al.,* [11]). Recently, three mathematic models were adapted to follow the sporulation dynamics of *Glomus* strains cultivated monoxenically (Declerck *et al.,* [12]). The tested models accurately simulated the time-course sporulation of AM fungi and their use can open new avenues for evaluating inoculum potential.

Three AM structures can be used in *in vitro* systems (germinating spores, extraradical and intraradical hyphae) to initiate new infections. Resting spores are the preferred starter inoculum material (or mother inoculum) used to follow the early events of arbuscular-mycorrhizas formation and the long term outcome of sporulation (Bécard *et al.,* [3]; Chabot *et al.,* [13]; Diop *et al.,* [7]; Hepper [14]). Extraradical AM fungal mycelia are very sensitive to mechanical damage during surface sterilization and are impracticable in monoxenic cultures. Intraradical forms (mainly vesicles) of AM fungi are also rarely used to initiate monoxenically or axenically cultures, despite their resistance and their importance in the completion of the life cycle of AM fungi (Diop *et al.,* [15]; Strullu *et al.,* [16]).

The aims of this study were first, to test the ability of three AM fungi originated from sahelian zones to growth monoxenically using isolated vesicles as starter inoculum, and finally to assess the inoculum potentials of indigenous AM fungi in dual culture with Ri-TDNA excised carrot roots by following their time-course sporulation and the hyphae morphological features.

2. Materials And Methods

2.1. Fungal inoculum

Three AM fungi: *Glomus aggregatum* Schenck and Smith emend. Koske (DAOM 227128), *Glomus fasciculatum* [(Thaxter) Gerd. and Trappe emend. Walker and Koske (DAOM 227127)] and *Glomus mosseae* (Nicol. And Gerd.) Gerd. and Trappe (DAOM 227131) isolated from the semi-arid zones of Senegal were used. Voucher specimens of *Glomus sp* were deposited at the Biosystematic Research Center, Ottawa, Canada and their morphological features are described (Dalpé *et al.,* [17] ; Diop [18]). They were routinely propagated in a sterile coarse soil of beach (2.21 ppm avalaible P) in the presence of *Zea mays* seedlings under growth chamber conditions. After four months cultivation, roots of *Zea mays* were removed and washed with water. Heavily infected AM roots were non-destructively selected prior to surface disinfection. Two steps of surface sterilization were achieved by successive baths in 96% ethanol (10 sec.), 6% calcium hypochlorite (1 min.), 2% chloramine T plus two drops of Tween 20 (10 min.), followed by rinsing for 10 min in an antibiotic solution containing 200 mg l^{-1} sptreptomycin sulfate and 100 mg l^{-1} gentamycin sulfate. A second sterilization step was achieved 48 h. after

the first one and as before. The disinfected roots were then gently shredded to extract vesicles (Diop [18]). The intraradical forms were stored on sterile water agar (0.8%) in the dark at 4 °C until use.

2.2. Root organ cultures

Excised roots of carrot (*Daucus carota L.*) genetically modified by the plasmid Ri-TDNA of *Agrobacterium rhizogenes* served as plant partner (Bécard and Fortin [3]). They were routinely cultivated in inverted Petri dishes (Ø 90 mm) containing Strullu and Romand (SR) (Strullu *et al.*, [19]) medium at 28 °C in the dark.

2.3. Monoxenic cultures and arbuscular mycorrhizal development

Pregermination of each AM fungil insolute was achieved by placing isolated vesicles on water agar medium (0.8%) in the dark at 28 °C. Following germination, single vesicle of each AM fungus was cultivated in association with Ri T-DNA carrot roots in Petri dishes containing 40 ml each of the modified Strullu Romand (MSR) medium (Diop [18]). The MSR medium contained in mg l^{-1} distilled water: $MgSO_4.7H_2O$ 739, KNO_3 76, KH_2PO_4 4.1, $Ca(NO_3)_2$. $4H_2O$ 359, KCl 65, NaFeEDTA 8, $MnSO_4.4H_2O$ 2.45, $ZnSO_4.7H_2O$ 0.28, H_3BO_3 1.86, $CuSO_4.5H_2O$ 0.22, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.035, thiamine 1, pyridoxine 0.9, nicotinic acid 1, cyanocobalamin 0.4, calcium pantothenate 0.9, biotin 0.9 x 10^{-3}, sucrose 10 000, Bacto-agar 8000. The pH of the medium was adjusted to 5.5 and autoclaved.

The experimental unit consisted of a Petri dish (diameter 90 mm) containing 40 ml MSR medium and a dual culture of one vesicle of each AM fungal isolate with a 7 cm length of apex carrot roots. Each treatment was replicated eight times. Sporulation and extradical hyphal spread of each AM fungus was monitored at weekly intervals using a binocular (100x).

3. Results

3.1. Morphological features of AM fungi

Different patterns of development of indigenous AM fungi were observed from the growth of initial germ tubes to the formation of mycelium network and spore production. Vesicles of AM fungi germinated and colonized their host partners within three days after dual cultures. Generally 3 to 5 germinating hyphae arised from vesicles and after first contacts with roots. Each fungus followed a distinct growth pattern. Among the AM fungi, *Glomus aggregatum* showed the most profusely growing mycelium and *Glomus mosseae* had the lowest sporulation rate.

3.1.1. Glomus aggregatum

Vesicles of *Glomus aggregatum* readily germinated and gave rise to an extensive mycelium network development one month after dual culture with excised carrot roots (Fig. 1a). Germinating straight running hyphae randomly grew and completely covered surface of Petri plates. However, *Glomus aggregatum* developed few branched absorbing structures which appeared after six weeks of culture. Mos of spores (30-80 µm Ø) were formed along thin secondary hyphae. Branched absorbing structures bore few newly produced spores in a average of 25 by branched absorbing structures. Clusters of hyphae were sometimes visible to the extremity of senescent part of transformed carrot roots.

3.1.2. Glomus fasciculatum

The runner fungal hyphae (4-6 µm) bore numerous branched absorbing structures and about thirty of these fungal structures are constituted at the end of two weeks of culture (Fig. 1b). The production of the spores preferentially occurred in the branched absorbing structures of *Glomus fasciculatum* with a density of more than 45 spores by branched absorbing structures what up to near 8 700 spore formation in a Petri dish after 3 months of culture.

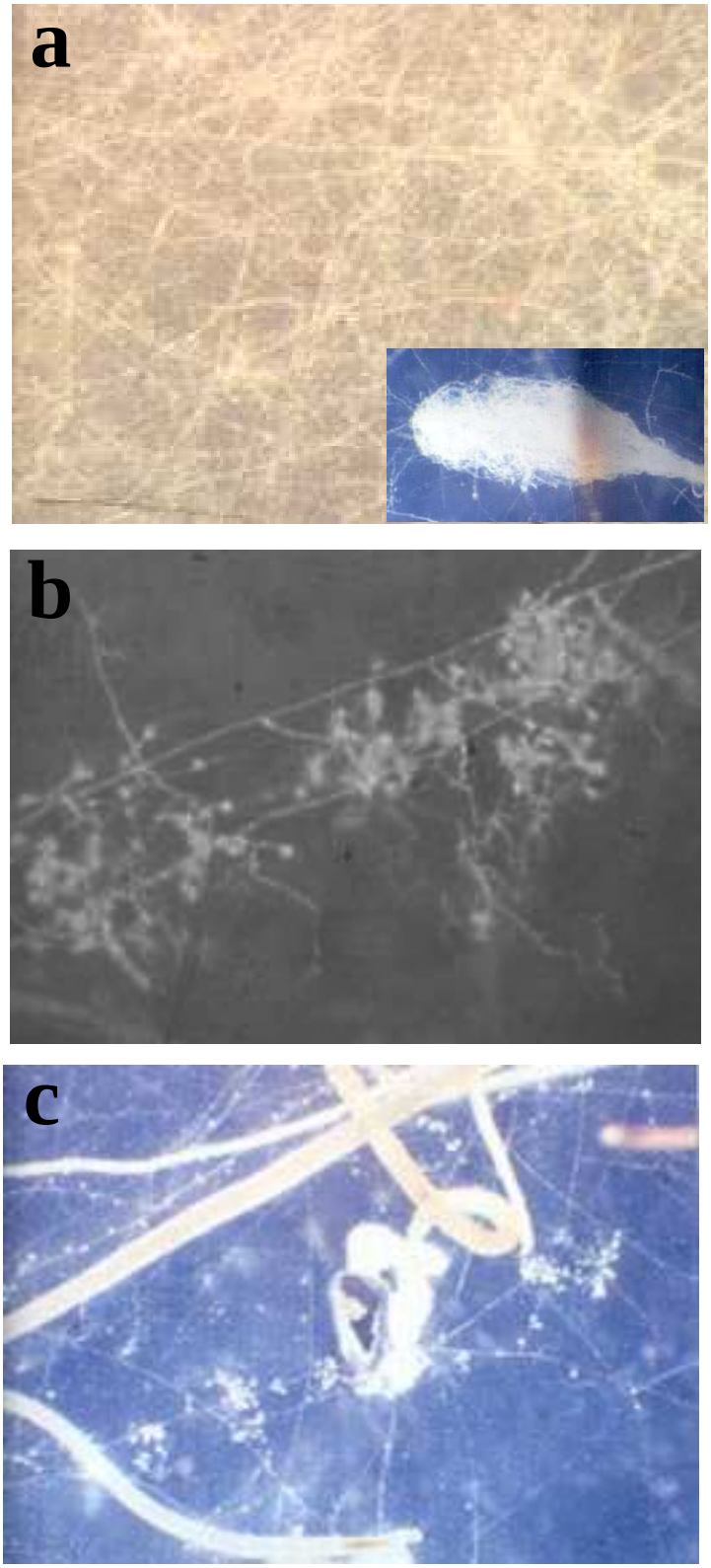

Figure 1: Morphological features of indigenous AM fungi monoxenically cultivated in Ri T-DNA carrot roots after 3 months cultivation

a. Extensive mycelium growth of *Glomus aggregatum* and a detail of condensed hyphae sometimes found near senescent roots; bar = 80 µm
b. Runner hyphae of *Glomus fasciculatum* with numerous spores in branched absorbing structures; bar = 200 µm.
c. Isolated and cluster of spores of *Glomus mosseae*; bar = 1,5 mm.

Numerous vesicles in Ri-TDNA carrot roots were also observed.

3.1.3. Glomus mosseae

The *in vitro* dual culture of an isolated vesicle of *Glomus mosseae* in presence of a transformed carrot root produced an important fungal biomass. The germinating hyphae of the AM fungus first depended to the host partner after 7 days of growth before developing thinner secondary hyphae and moving randomly in any directions. After one month of culture, a dense mycelium covered the whole solidified medium and the number of average spores produced passed more than 3200 spores in some Petri plates. The spores either appeared scattered or grouped along the runner hyphae or in the branched absorbing structures (Fig. 1c). Clusters of 30 to 50 spores are produced regularly in the branched absorbing structures of the AM fungus. Under the binocular, appresoria and internal vesicles were easily observable whereas the arbuscules were detected only after coloration of the roots.

3.2. Sporulation

Spores of indigenous AM fungi were found uniform, globular and showed dense lipidic contents on maturity. A sigmoidal curve symbolised the patterns of sporulation of AM fungi (Fig. 2). The lag period of sporulation was correlated with an extensive mycelial growth. This period was long with *Glomus mosseae* and lasted 5 weeks. A period of more active sporulation followed this latence period and was linked to numerous infection units formation and senescence of root partners. The intense period of spore production lasted about 13 weeks for *Glomus fasciculatum* and 10 weeks for *Glomus aggregatum* and *Glomus fasciculatum*. The rates of sporulation by a week were 580, 670 and 320 respectively for *Glomus aggregatum*, *Glomus fasciculatum* and *Glomus mosseae*. Later, a plateau phase of sporulation occurred and coincided with a high maturation process of spores. After 3 months of culture, *Glomus fasciculatum* produced the highest number of spores (8731), followed by *Glomus aggregatum* (around 5817) while *Glomus mosseae* formed the lowest number of spores (around 3207).

4. Discussion

In the present investigation, morphological features of indigenous AM fungi were characterized by numerous branched absorbing structures at regular distances along the running hyphae and profuse development of mycelium. These branched absorbing structures were the preferential zones of fungal sporulation and enhanced the fungus-substrate contact surface as described by Bago *et al.,* [20]. Therefore, it was advanced that branched absorbing structures could be uptake sites for extraradical mycelium and nutrient supplies to spores developing. *Glomus fasciculatum* produced the most branched absorbing structures during the period of growth of partners and sporulated more indicating intensive physiological exchanges in monoxenic culture. Karandashov *et al.,* [21], found that formation of branched absorbing structures of *Glomus caledonium* coincided with a senescence of host partner and mycelium growth. Our findings showed that indigenous AM fungi readily developed branched absorbing structures after colonization of roots as mentioned with *Glomus intraradices* Bago *et al.,* [22]1998. However, the formation of branched absorbing structures occurred within growing roots as well as ageing roots and their importance seemed not to be linked to mycelial density.

Strong correlations were found between spores and vesicles production (Declerck *et al.,* [6]) and between spores and AM infection units (Diop *et al.,* [7]). Our results with monoxenic cultures of indigenous *Glomus* species on transformed carrot roots agree with these findings. Spore production has been proven to be a

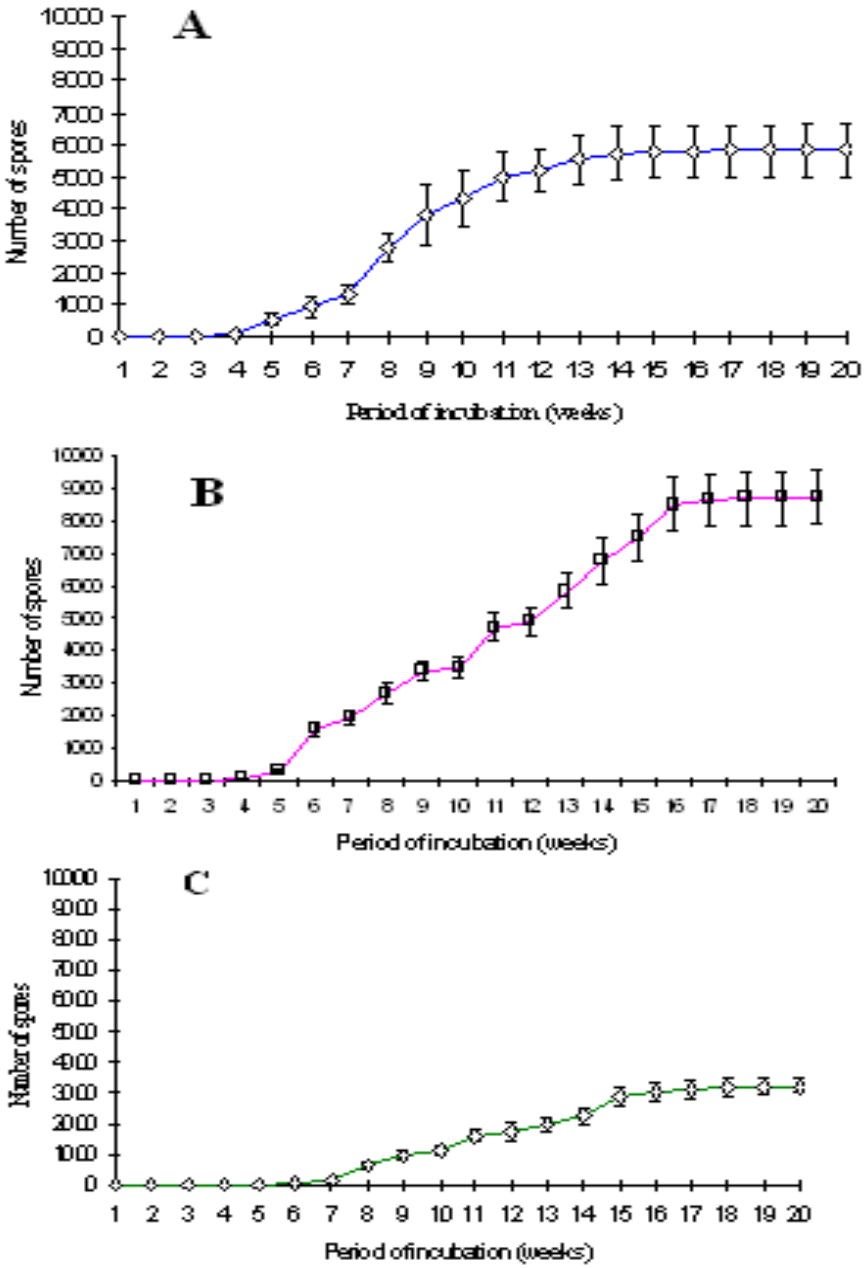

Figure 2 : Dynamic of sporulation of *Glomus aggregatum* (A), *Glomus fasciculatum* (B), *Glomus mosseae* (C). Vertical bars indicate standard deviations

good indicator of inoculum potential of AM fungi (Declerck *et al.*, [6]; Diop *et al.*, [7]). The relationship between branched absorbing structures and sporulation observed within AM fungi suggest that it could be interesting to take into account these fungal structures in mathematic models (Declerck *et al.*, [12]) to estimate the total inoculum potential of monoxenic cultures. Several authors have described the sigmoidal sporulation pattern (lag, exponential and plateau) of AM fungi (Bago *et al.*, [22]; Declerck *et al.*, [6]; Griffin [23]; Menge [24]). Our results confirmed this classic dynamic even though other fungi like *Gigaspora margarita* (Diop *et al.*, [7], 1992) and *Glomus caledonium* (Karandashov *et al.*,

[21]) did not follow this pattern. In our study, each AM fungus showed a specific period of sporulation and mycelial growth which could be controlled by intrinsic factors of the fungal isolate. Otherwise, among AM fungi, only *Glomus aggregatum* showed a regular condensation of hyphae near the necrotic zones of roots suggesting a high saprophytic phase of this fungus.

In conclusion, considering the high inoculum potential of AM intraradical forms (1cm of heavily mycorrhizal roots sometimes were able to contain more than 1500 vesicles), the approach of monoxenic cultures using vesicles as starter inoculum are promising to maintain AM fungal diversity and to better understand the life cycle of these imperfect fungi used in the present investigations. Further research is also needed to highlight the role of vesicles in regulation of AM fungal sporulation. The *in vivo* performance of inoculum potential of different generations of indigenous AM fungi produced *in vitro* is now undertaken under adverse conditions.

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