Biology, ecology and evolution of the family Gigasporaceae, arbuscular mycorrhizal fungi (Glomeromycota)

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BIOLOGY, ECOLOGY AND EVOLUTION OF THE FAMILY *GIGASPORACEAE*, ARBUSCULAR MYCORRHIZAL FUNGI (GLOMEROMYCOTA)

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Cover: Front picture coexistence between *Scutellospora reticulata* and *Glomus intraradices* in dixenic culture with Ri-T DNA transformed carrot roots, back cover DGGE patterns of two strains of *Gi. gigantea* isolated from the same site.

BIOLOGY, ECOLOGY AND EVOLUTION OF THE FAMILY *GIGASPORACEAE*, ARBUSCULAR MYCORRHIZAL FUNGI (GLOMEROMYCOTA)

Proefschrift

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door

Francisco Adriano de Souza

Geboren in 1964 te Santos, São Paulo State, Brazilië

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To my beloved parents “Maurício & Dolores”,
In memory

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Chapter 1

General Introduction

GENERAL INTRODUCTION

"Plants don't have roots, they have mycorrhizas (J.L. Harley)". This strong statement was made to warn plant biologists and ecologists of the fact that under natural conditions 80% of land plants are associated with soil fungi in a mutualistic symbiosis, termed mycorrhizas (fungus-root, from the Greek: myco [fungus] and rhiza [roots]). Recently, van der Heijden and Sanders (2002) stated, "mycorrhizal associations need to be considered in order to understand the ecology and evolution of plants, plant communities and ecosystems". This statement is based on experimental research showing the effects of mycorrhizas on the outcome of plant competition and succession (van der Heijden et al 1998), and on the fact that the evolution of land plants was dependent upon the presence of mycorrhizas (Cairney 2000; Brundrett 2002).

In the course of evolution six different types of mycorrhizal evolved (Arbuscular-, Arbutoid-, Ericoid-, Ecto-, Monotropoid-, and Orchid-mycorrhiza). This thesis focused on Arbuscular Mycorrhizal, in particular because their vital role in sustainable agriculture.

The arbuscular mycorrhizal (AM) is the most ancient and widespread type of mycorrhiza. The origin of Arbuscular Mycorrhizal Fungi (AMF), based on molecular clock dating, is estimated to have occurred around 600 million years ago (MYA) (Redecker et al. 2000). Moreover, fossil evidence demonstrated the presence of arbuscular mycorrhizal-like fungi in the first bryophyte-like land plants in the early Devonian, approximately 400 MYA (Remy *et al*. 1994; Taylor *et al.* 1995), supporting the hypothesis that AMF were instrumental in the colonization of land by ancient plants (Pyrozynski & Malloch 1975; Simon et al 1993; Remy *et al*. 1994). Nowadays, AMF occur in the majority of the extant plant families and terrestrial ecosystems (Smith & Read, 1997).

In the AM symbiosis, the plant supplies the fungus with energy for growth and maintenance via photosynthetic products and the fungus provides the host plant with an array of services. The benefits that plants can derive from the AM symbiosis range from slightly negative to highly beneficial (Sieverding 1991; Guo et al. 1994; van der Heijden 2002) and the direction and magnitude of the effect depends on the plant-fungus partner(s) (Bever 2002) and environmental characteristics. The major fungal services, or at least the most evident so far, are carried out by the fungal extraradical mycelium (ERM). These services, consists of uptake, assimilation and translocation of nutrients to the plant roots from locations beyond the rhizospheric zone (Jakobsen et al. 1992; Johansen et al. 1993; Ezawa et al. 2002). Therefore, the mycorrhizal fungi usually increase the fitness of the symbiotic plant. Moreover, other relevant services are, increased resistance to root pathogens and water uptake capacity. In addition, the ERM of AMF contributes largely to soil aggregation.

The economic significance of the AMF to sustainable agriculture (Jeffries et al. 2003), land reclamation (Jasper 1994; de Souza & Silva 1996) and efficient use of non-renewable resources such as phosphate (Jakobsen 2000) has been widely accepted. Many plant species cannot survive in low fertility soils without these fungi. Among them, are important cash (example coffee, citrus) and food security crops (example cassava, sweet-potato) and native species of trees in tropical ecosystems. To facilitate the efficient use of AMF in agricultural systems and to study their significance in natural ecosystems, it is necessary to be able to assess the diversity of AMF and to known their life history strategies. These were the major objectives of the study described in this thesis.

The Arbuscular Mycorrhizal Fungi.
AMF are obligatorily biotrophic, which is a characteristic that is to be expected in highly compatible symbioses. Therefore, they need to be associated with a compatible host plant to complete their life cycle. These fungi are putative asexuals (Rosendahl & Taylor 1997). However, there has been one report of sexual structures in *Gigaspora decipiens* (Tommerup & Sivasithamparam 1990), but this result has remained unconfirmed until now. AMF form large spores ranging from 22 to 1050 μm in diameter (Schenck & Perez 1990). The spores were thought to be heterokaryotic as revealed by *in situ* hybridization assays (Trovelot et al. 1999; Kuhn et al. 2001). Recently, PCR typing of individual nuclei of *Glomus etunicatum* and *Glomus intraradices* demonstrated that AMF might be homokaryotic (Pawlowska & Taylor 2004), however, new research on this issue suggest that at least another strain of *Glomus etunicatum* is indeed heterokaryotic (Hijri & Sanders 2005). Stains with homo and heterokaryotic states might indicate that parasexual cycle is operating in AMF (see chapter 7). Nevertheless, considerable interspecific variation has been reported in the rRNA genes obtained from individual spores (for a review see Sanders 2002). AMF spores typically contain thousand's of nuclei (Cooke et al. 1987; Bécard & Pfeffer 1993). Recently, Hijri & Sanders (2004) characterized the genome size, complexity and ploidy of *Glomus intraradices*. They found *G. intraradices* to be haploid, with a small genome size (~ 16.54Mb), in the lower limit of eukaryotes. It contained of 88.36% single copy DNA, 1.59% repetitive DNA, and 10.05% fold-back DNA. It is interesting to notice that other AMF were reported to have much larger genomes than *G. intraradices*. For instance, the genome sizes of 12 AMF species ranged from ~ 127.4 Mb in *Scutellospora pellucida* to 1058.4Mb in *Scutellospora gregaria* (Hosny et al 1998; Bianciotto & Bonfante 1992). These contrasting genome sizes probably reflect differences in ploidy and or amount of repetitive sequences among these species. Despite the recent advances in AMF genomic characterization, the source and significance of the high intraspecific genetic variation in AMF is still largely unknown.

Classification of Arbuscular Mycorrhizal Fungi.

AMF comprise a monophyletic group of soil fungi, recently reclassified from the polyphyletic phylum *Zygomycota* to a new proposed phylum *Glomeromycota* (Schüβler et al. 2001). This phylum was proposed after analysis of a large data set of 18S rRNA gene sequences of all known groups of fungi.

To date, more than 190 AM species have been described (http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylogeny.html). They are classified in four orders encompassing six families and eight genera (Table 1). *Glomus* is the largest genus containing 54.8% of all described species. However, this genus is not monophyletic and has subsequently been sub-divided into three separate groups (Schwarzott et al, 2001). In addition to *Glomus,* AMF species fall into the following recognized genera: *Scutellospora,* 17.0%; *Acaulospora,* 16.5%; *Gigaspora,* 3.6%; *Pacispora*, 3.6%; *Entrophospora,* 2.5%; *Archaeospora,* 1.5%; *Paraglomus,* 0.5% and *Diversispora*; of the described species.

Table 1. Orders, families and genera in *Glomeromycota* and species distribution per genus

Order	Family	Genus	Number of described species[2]
Diversisporales	*Diversisporaceae*	*Diversispora*	3
	Gigasporaceae	*Gigaspora*	7
		Scutellospora	32
	Pacisporaceae	*Pacispora*[3]	7
	Acaulosporaceae	*Acaulospora*	33
		Entrophospora	5
Glomerales	*Glomeraceae*	*Glomus*[1]	104
Archaeosporales	*Archaeosporaceae*	*Archaeospora*	3
Paraglomerales	*Paraglomeraceae*	*Paraglomus*	2
Total 4	7	9	196

([1]) The genus *Glomus* is polyphyletic and has been divided in *Glomus* groups A, B and C (Schwarzott et al. 2001). *Glomus* group C belong now to the genus *Diversispora*, order *Diversisporales*.
([2]) The total number of species includes synonymous species.
([3]) Recently the family *Pasisporaceae* and the genus *Pacispora* were proposed to accommodate *Glomus*-like species as well as new species that share germination and internal walls features as well as molecular support that link them to *Scutellospora* and *Acaulosporaceae* species (Oehl & Sieverding 2004; Walker et al 2004; Walker & Schüβler 2004). *Pasispora* was described within *Glomeraceae* (Oehl & Sieverding 2004), and reclassified to the order *Diversisporales* based on cytological, molecular and morphological evidence (Walker & Schüβler 2004).

AMF LIFE CYCLE AND MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF THE AMF MYCELIUM

The life cycle of an AMF can be divided in three main steps:

A. Establishment of the symbiosis. This involves propagule activation, host search, appressorium formation, root penetration and arbuscule formation.
B. Vegetative growing phase. This involves intra and extraradical mycelium growth, and an overall increase of fungal biomass, formation of mycelial structures and expansion of the AMF colonization within and between plants.
C. Reproductive phase. This involves the formation of reproductive structures. Resting spores are the major type of propagule.

AMF life history strategies seem to differ highly between the AMF families. For instance, *Glomeraceae* and *Acaulosporaceae* species tend to be more prolific than *Archaeosporaceae* and *Gigasporaceae* species, in both controlled and natural conditions (Sieverding 1991, Morton et al. 1993; de Souza et al. 1999; Bever et al 2001; Franke et al. 2001; Franke-Snyder et al. 2001; Lovelock et al. 2003). *Acaulosporaceae*, *Glomeraceae* and *Pacisporaceae* also possess intraradical vesicles that are effective propagules, which the other families do not have. *Paraglomeraceae* seem to behave like a ruderal species, because it is found preferentially in disturbed ecosystem (Sieverding 1991, Spain & Miranda 1997). Little is known about the ecology of *Pacispora* and *Diversospora*. AMF life cycles and specially life history strategies are rather difficult to study mainly due to problems associated with the biotrophic nature of AMF, which imposes the necessity to grow them together with roots under controlled conditions, and the challenges of identifying and quantifying AMF species under natural conditions. Recently, Pringle & Taylor (2002) reviewed the importance of fitness in filamentous fungi, including AMF. They said: “without data on fitness, hypotheses about the adaptive significance of phenotypes or basic mechanisms of evolution, for example natural selection, remain speculative”. In addition, they highlighted the importance of defining species and species boundaries in fungi to properly assess their fitness under natural conditions.

Below, I provide a brief description of the AMF mycelium and its main structures, with special attention to the extraradical mycelium, as it is one of the central subjects of this thesis. For further information on basic aspects of AMF the reader is referred to Smith & Read (1997).

The AMF mycelium is dimorphic and essentially non-septated or coenocytic (Mosse 1981). The non-septate hyphae allow a fast cytoplasmic flow in a bi-directional way, not only carrying resources from source to sink regions of the fungal colony and/or symbiotic root, but also transporting fungal organelles, such as mitochondria and nuclei (Bago et al. 1998b; Bago et al. 1999).

The major mycelial structures are characterized as follows:

Arbuscules: The arbuscule is the defining specialized morphological structure hypothetically shared by all arbuscular mycorrhizal fungal species (Morton & Benny 1980). Arbuscules are haustoria-like structures that are formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface between fungal tissue and the plant plasma membrane. This interface is thought to be the major site for nutrient and carbon exchanges between both partners, and it is considered to be the key structure for establishment of a functional symbiosis (Smith & Read 1997; Harrison, 1999). The arbuscules are usually short-lived (1 to 3 weeks), and are preferentially found in young thin roots during early stages of root colonization (Mosse 1981; Smith & Read 1997; Harrison 1999). However, long-lived arbuscules have also been reported in woodland plants (Brundrett & Kendrick 1990). The arbuscule formation is genetically controlled by the host plant, and the numbers of arbuscules formed is dependent on plant species, availability of nutrients, as well as on the fungal partner (Harrison 1999).

Vesicles: Vesicles are lipid-filled sack-like structures formed within roots. Their functions are primarily as storage organs, but they can also function as propagative structures (Biermann & Linderman 1983; Declerck et al. 1998). Vesicles increase in numbers with the progress of root colonization. Vesicles are formed only by members of the families *Acaulosporaceae, Glomeraceae,* and *Pacisporaceae*.

Auxiliary cells (AC): Auxiliary cells are formed by short ramifications occurring at one or simultaneously at both sides of extraradical hyphae. Each ramification generates several branches that swell, and form clusters, which are composed of 2 to more than 20 balloon-like structures, of about 12-39 µm in diameter. AC are metabolically active structures, rich in nuclei, organelles and lipids (Jabaji-Hare et al. 1986; Jabaji-Hare 1988; Bonfante & Bianciotto 1995). However, little is known about the biological function of AC. There is no evidence that AC are functional substitutes for vesicles (Bonfante & Bianciotto 1995), and available evidence speaks against an infective capacity for AC (Biermann & Linderman 1983). It has been suggested that AC are reminiscent of relict

reproductive spores (Morton & Benny 1990). AC has only been found in *Gigasporaceae*. They have a spine or smooth surface in *Gigaspora* and *Scutellospora,* respectively.

Extraradical mycelium (ERM): The extraradical mycelium is of key importance for the fungus and to the function of the symbioses. The majority of spores are formed in the external mycelium. The ERM is also responsible for spreading the root's colonization within and between plants, generating an underground link between plants in a community (Smith & Read 1997).

The great majority of the research carried out on ERM has been performed using *Glomus* species, and the results obtained with *Glomus* have in some extent been generalized to other AMF groups. However, such generalization is often not warranted. For instance, *Glomus* is reported to form anastomosis in the ERM (Gerdermann 1955; Giovannetti et al. 1999; Giovannetti et al. 2001; Giovannetti et al. 2003) while the *Gigasporaceae* has not (Giovannetti et al. 1999). *Gigasporaceae* are no doubt distinct from *Glomus* in numerous respects regarding ERM distribution, organization and function, but these issues have yet to be studied in detail. Hart & Reader (2002) studied the mycelium development and biomass of a total of 21 species from the families *Acaulosporaceae*, *Glomeraceae* and *Gigasporaceae*. They found evidence for distinct AMF intra- and extraradical mycelium development strategies which could be related to taxonomic differences between the families, and the results were independent of the host plant used. For instance, *Glomeraceae* colonized roots earlier than *Acaulosporaceae* and *Gigasporaceae*, and produced the most extensive root colonization. On the other hand, *Glomeraceae* showed low soil colonization measured by ERM development, while *Gigasporaceae* showed the highest ERM development (2 to 4 times longer than the other two families). *Acaulosporaceae* showed low root colonization and ERM development. These results indicate that *Gigasporaceae* exhibit a different root-soil colonization strategy than *Acaulosporaceae* and *Glomeraceae*.

An alternative way to study the life cycle of AMF *in situ* is to associate them with Root-Organ Culture (ROC) (Bécard & Fortin 1988; Fortin et al. 2002). The advantages of using ROC as a model system is that both symbionts (AMF and host root) are able to grow in a transparent, defined medium, allowing nondestructive *in vivo* observations throughout the fungal life cycle. ROC can be obtained by direct excision of root organs of suitable plants (ex. Tomato) or by transformation using virulent *Agrobacterium rhizogenes* harboring the Ri plasmid. The resulting "hairy roots" can be indefinitely cloned on growth-hormone-free culture medium. The cultivation of AMF in association with the Ri T-DNA transformed roots has opened new possibilities to study the extraradical mycelium of AMF (Bago et al 1998ab; de Souza & Berbara 1999; Fortin et al 2002). However, to date only a few AMF species of the genera *Glomus* and *Gigaspora* have been cultivated *in vitro* with ROC. Recently,

Acaulospora rehnii (Dalpé & Declerck 2002) and *S. reticulata* (this thesis) were also successfully cultivated in ROC conditions.

Archaeosporaceae and *Paraglomeraceae* are considered to be the oldest lineages of glomeromycotan (Morton & Redecker 2001), and almost nothing is known about their ERM.

CHALLENGERS AND QUESTIONS ABOUT AMF ECOLOGY

Despite the relevance of AM symbioses to the functioning of terrestrial ecosystems, the study of the AMF ecology is still in its infancy (Hart & Klironomos 2002). Recently, it has become clear that AMF families exhibit remarkable differences that may be indicative of disparate ecological behavior. Although significant progress has been made in understanding AMF ecology, a number of fundamental questions and paradoxes still remain unresolved. For instance, despite the very close association of AMF with their plant partner, they are commonly thought to be rather non-specific with respect to the plants with which they can be associated. However, this view is changing as molecular techniques applied directly to field conditions have shown specificity among AMF species (for a review see Sanders 2003), and the extent and consequences of this specificity is as yet mostly unknown. Another paradox lies in the relatively low number of described AMF species. Highly evolved symbiosis are typically highly specific, yet despite the description of approximately 4 million plant species, three-fourths of which are mycorrhizal, fewer than 200 AMF species have been described. Does this point to a remarkable generality of infection or a lack of description of ecologically relevant units in AMF?

All AMF colonize essentially the same niche within plant roots, as exemplified by van Tuin et al (1998), who detected 3-4 AMF species from 3 different genera within single 1 cm root pieces in a microcosm experiment. The selective forces that allow and shape such complex coexistence can only be speculated at present. Knowledge about AMF life history strategy is fundamental to our understanding of how different fungi coexist, how they explore the available resources, and how they perpetuate themselves (Pringle & Bever 2002; Hart & Klironomous 2002).

Although, little is known about life history strategies in AMF, the scarce knowledge available suggests the existence of differences at the family level. For instance, *Acaulospora colossica* and *Gigaspora gigantea* maintained different and contrasting seasonalities by colonizing and sporulating more frequently when associated with cool and warm season plants, respectively. Other examples are apparent in the development of the ERM as discussed in the previous section (Hart & Reader 2002). Moreover, AM fungal species distribution in an ecosystem can be highly heterogeneous and spatially

aggregated at a fine scale. Contrasting seasonal and spatial niches may therefore facilitate the maintenance of diversity within an AMF community (Pringle & Bever 2002). However, the above mentioned mechanism does not explain the simultaneous occurrence of a diverse AMF community in single plant species or individuals, as revealed by molecular based techniques (van Tuinen et al. 1998; Helgason et al. 1998; Kowalchuk et al. 2002; Husband et al. 2002ab; Opik et al. 2003;Vandenkoornhuyse et al. 2003).

SPECIES ASSESSMENT

Species are fundamental units in ecology and evolution, and they are defined in AMF on the basis of spore morphology. The main characteristics used for species identification are: mode of spore formation (family to genus level), size, color and wall components, germination features and subtending hyphae characteristics or absence thereof. Thus, the traditional procedure to assess AMF diversity has been based on spore identification and quantification. Identification of species can be done directly, after spore extraction from (soil) samples, or after enrichment of the fungal spore populations via trap or pot culture techniques. The latter approach makes use of a mycorrhizal plant to enrich the spore population and improve spore quality for taxonomic identification. Besides, it facilitates further isolation of the fungi. However, such enrichment procedures can bias for those species that propagate well under the given conditions (de Souza & Guerra 1998; de Souza 2000). Also, the morphological identification of AMF spores is difficult and time-consuming and depends largely on the investigator's level of expertise. Furthermore, the identification procedure can be biased or inaccurate due to phenotypic variation of the spores. Moreover, spores are not present in all stages of the fungal life cycle nor are they produced in similar numbers by all species.

Recently, the use of DNA based techniques has facilitated the detection and identification of microorganisms in all stages of their life cycles. Application of DNA-based techniques to characterize AMF has led to important advances in our understanding of their phylogeny, ecology and genomic organization (Schüβler et al 2001; Clapp et al 2002; Hijri & Sanders 2004). The nuclear ribosomal (nrRNA) have been the most commonly used marker for characterization of AMF in the lab as well as in natural assemblages (Clapp et al 2002). Eukaryotes typically possess nuclear ribosomal RNA (nrRNA) genes with a large number of nearly identical tandemly repeated arrays, encoding the small sub unit (SSU nrRNA or 18S), the 5.8S, and the large sub unit (LSU nrRNA or 25S) nrRNA genes, with two internal transcribed spacers ITS1 and ITS2. Although AMF show typical eukaryote nrRNA gene organization, it has been demonstrated that AMF nrDNA sequence shows a much higher

variation than what would be expected between gene copies within a single organism (spore) (for a review see Sanders 2003). AMF possess higher intragenomic polymorphism in the nrDNA copies than most eukaryotes, i.e. the nrDNA copies are not always nearly identical within a species. Furthermore, the number of tandem repeats arrays may be unevenly distributed between the nuclei within a single spore (Trovelot et al. 1999). However, few studies have assessed the implications of such polymorphism with respect to the phylogeny, evolution and ecology of these organisms.

THE FAMILY GIGASPORACEAE, ECOLOGY AND APPLICATIONS

The main focus of this thesis is to explore the diversity of an AMF family, the *Gigasporaceae*. *Gigasporaceae* is one of the most diverse and economically important AMF families. An example of the economical value of *Gigasporaceae* can be seem by the importance of *Gigaspora margarita* isolates in enhancer coffee plants in Brazil and India (Lopes et al. 1983; Colozzi et al. 1994; Bhattacharya & Bagyaraj 2002). However, almost nothing is known about the ecology of these fungi in natural conditions. Such knowledge would improve the chances of successful and durable inoculation responses. *Gigasporaceae* is one of the youngest AMF lineages. Fossil records of *Gigasporaceae*-like spores are from approximately 240MYA (Phipps & Taylor 1996), and molecular clock estimates place the split between *Gigasporaceae* and *Acaulosporaceae* at approximately the same period (243-252 MYA, Simon et al 1993). The genus *Scutellospora* is much more diverse than *Gigaspora* in terms of spore morphology, genetic diversity (nrRNA genes), number of described species (Table 1) and occurrence in natural and man-made ecosystems. *Gigasporaceae* species occur in natural ecosystems across the globe together with other AMF, but usually at lower abundance in high diverse plant ecosystems [Tropical Forest, Central America (Lovelock et al. 2003); various ecosystems, Brazil (Siqueira et al 1989); Temperate, Europe (Helgason et al. 1998); Tropical Forest, Southwest China (Zhao et al. 2001; Zhao et al. 2003); Western Ghats of Goa, India (Khade & Rodrigues 2003). In sand-dune ecosystems, *Gigasporaceae* can sometimes be the dominant species (USA, Bergen & Koske 1984) (USA, Gemma et al. 1989; Brazil, Stürmer & Bellei 1994; India, Beena et al 2000) , whereas in agricultural soils cultivated with annual crops, they tend to be less abundant or even absent (Sieverding 1991; An et al. 1993; Hendrix et al. 1995; Helgason et al. 1998; Daniell et al. 2001; Jansa et al. 2002;Vandenkoornhuyse et al. 2002; Oehl et al. 2003; Johnson et al. 2004). For instance, Sieverding (1991) made a careful and extensive evaluation of various agricultural practices

such as tillage, crop rotation, fertilization and pesticides on community structure and propagule densities of AMF in tropical soils. In that study, the dominant species were usually from the *Glomeraceae* or *Acaulosporaceae*. An adequate explanation for these patterns has yet to be found.

AIM, RESEARCH QUESTIONS AND OUTLINE OF THIS THESIS

RESEARCH AIMS

The overall goal of this study was to gain further insight into the biology, ecology and evolution of *Gigasporaceae*, a family of Arbuscular Mycorrhizal Fungi (AMF). This research aim is based on the assumption that the ecology of AMF cannot be fully understood without comprehending AMF life history strategies and species diversity. To better address the research focus, two major aims have been defined.

The first aim was to study the life history strategy of *Gigasporaceae*. In particular, the research focused on the process of spore production. While it is well known that spores are the main, if not the sole, type of propagule in *Gigasporaceae*, the functional relationships involved in their formation has been unclear until now. The intention was to explore the role of morphostructures involved in the spore formation such as the extraradical mycelium and auxiliary cells.

The second aim was to assess the genetic variability of *Gigasporaceae* through nuclear ribosome (nrDNA) inter and intragenomic polymorphism, to use this variability as a marker to characterize and discriminate *Gigasporaceae* species and to gain insights into evolutionary and phylogenetic relationships in this family. Although heterogeneity in *Gigasporaceae* has been previously reported, this study, for the first time, systematically describes this heterogeneity and analyses this phenomenon with respect to *Gigasporaceae* phylogeny and evolution.

RESEARCH QUESTIONS

On the basis of the research aims presented above, several research questions were formulated:

1. What are the roles of Auxiliary Cells in the life cycle of *Gigasporaceae*?

2. Are members of the *Gigasporaceae* K-strategists in relation to other common AMF species?
3. Can nrDNA intragenomic polymorphism be used to differentiate species of AMF?
4. Do different geographic isolates of an Arbuscular Mycorrhizal Fungi morphospecies differ in their nrDNA copies? If yes, how can species boundaries be defined?
5. What are the phylogenetic and evolutionary implications of nrDNA variation in Arbuscular Mycorrhizal Fungi?

The first two questions are related to biological aspects, and they are addressed in Chapters 2, 3 and 4 of this thesis. The last three questions relate to genetic and evolutionary issues, and they are addressed in Chapters 5 through 7.

OUTLINE OF THIS THESIS

Chapters 2, 3 and 4 focus on the biological aspects of *Gigasporaceae*. Their aims are to evaluate the contribution of mycelial morphostructures to spore production and to study the life history strategy in *Gigasporaceae*. This research was carried out in collaboration with the group of S. Declerck (UCL, Louvain-La-Neuve, Belgium). *S. reticulata* was used as a model species to study the role of the extraradical mycelium on spore formation. The fungal development was studied in root-organ culture (ROC) conditions, and each experimental unit was composed of a Petri-plate containing nutrient medium and a monoxenic culture of Ri T-DNA transformed carrot root explants inoculated with single germinated spores of *S. reticulata*. The major advantage of this system is that it allows for detailed, nondestructive observation of the entire fungal development over time, thereby offering the possibility to study the life cycle of such biotrophic obligatory organisms.

In **Chapter 2,** extraradical mycelium development and architecture and spore production were studied, using *S. reticulata* ROC. Culture establishment, anastomosis occurrence and auxiliary cell development were also examined. Among the results obtained, a novel possible biological function of auxiliary cells in the life cycle of *Gigasporaceae* was observed. This brought us to advance the hypothesis that auxiliary cells play a role in carbon storage, providing energy reserves for spore and/or mycelial production and repair.

To test this hypothesis, the development of the extraradical mycelium, auxiliary cells and spore production of *S. reticulata* were quantified using ROC conditions (**Chapter 3**). Four variables (root and extraradical hyphae lengths, spore and auxiliary cells numbers) were monitored over time and data modeled using a modified Gompertz model and statistically analyzed.

In **Chapter 4,** a comparison of life history strategy (LHS) of *Gigasporaceae* and *Glomus* species was made. I re-examine data generated in the two previous chapters in comparison to other published data generated using the root organ culture to address LHS issue.

For **Chapters 5** through **7**, our research focus changed from fungal biology to the genetics of the AMF. Recent application of DNA-based techniques to characterize AMF has led to important advances in our understanding of the phylogeny and ecology of this symbiotic obligatory group of fungi. At the same time, it has revealed the complexity of AMF genomic organization. For example, nrRNA genes in AMF exhibit an unexpectedly high intragenomic polymorphism. Unfortunately, little progress has been made in the interpretation of this heterogeneity. To gain more insights into nrDNA polymorphism, we characterized this feature for several *Gigasporaceae* species.

One study targeted *Scutellospora* species, with the aim to compare species characterization by spore morphology with that predicted by nrDNA (**Chapter 5**). Furthermore, we sought to characterize our model species *S. reticulata* further. We studied the ontogenesis of *S. reticulata* spores and compared it with eight previously studied *Scutellospora* species. Nuclear ribosomal DNA was also extracted, PCR amplified, cloned and sequenced, and the data were used to assess the phylogenetic position of *S. reticulata*. In addition, PCR-DGGE was used to differentiate *Scutellospora* species by their sequence variation in their nrRNA genes (**Chapter 5**). PCR-DGGE and sequence analysis revealed intragenomic 18S nrDNA polymorphisms in four out of six *Scutellospora* species. These results also indicated that 18S nrDNA intragenomic polymorphism could be used as a marker to differentiate some closely related *Scutellospora* species.

The molecular characterization performed with *Scutellospora* was then extended to the sister genus *Gigaspora* (**Chapter 6**). I focused this part of my study on *Gigaspora* species for two main reasons. First, intragenomic polymorphism had previously been reported in several *Gigaspora* species. Second, I had access to a large and representative collection covering all known species and many geographically distinct isolates. Furthermore, species diversity in *Gigaspora* is much lower than that of his sister genus *Scutellospora.* This apparently low diversity could be the result of a low speciation or of a lack in distinctive morphological characters used in taxonomy, which could limit the discrimination between genetically (genotypes) and ecological (ecotypes) distinct organisms. PCR-DGGE was used to explore inter and intragenomic variation of 18S nrRNA genes and to test the usefulness of this gene as a marker to discriminate between *Gigaspora* species and *Gigasporaceae* populations from environmental samples. To achieve the latter goals, *Gigasporaceae*-specific primers were used in a nested PCR approach with PCR-DGGE.

Results obtained in Chapters 5 and 6 suggested the occurrence of nrDNA trans-species polymorphism. Such polymorphism is commonly found in hybrid species, but such an assumption would require

detailed analysis to reveal the evolutionary path and phylogenetic history of the species in question. To evaluate the phylogenetic and evolutionary implications of the inter- and intragenomic polymorphism found in *Gigaspora,* a detailed analysis of nrDNA variability was performed (Chapter 7). Eight species out of the 48 accessions tested previously were selected, representing one isolate of each species and one putative new *Gigaspora* species. We cloned fragments of approximately 2300bp, covering the 18S, 5.8S and the ITS1 and ITS2 regions, using DNA extracted from single spores. Clones containing different haplotypes were selected using PCR-DGGE targeting the V9 region of the 18S nrDNA and sequenced. Phylogenetic, recombination and test of rate constancy of nucleotide substitution analyses were performed to gain insight into the evolutionary trajectories driving nrDNA evolution in the genus.

Chapter 8 presents a general summary and overall discussion of the most important results, and evaluates them in relation to the research aims and questions and the current state of knowledge within this field of study.

Chapter 2

Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots

de Souza FA, Declerck S. 2003.

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ABSTRACT

Mycelium development and architecture and spore production were studied in *Scutellospora reticulata* from single-spore isolates grown with Ri T-DNA transformed carrot root-organ culture in monoxenic system. Culture establishment, anastomosis occurrence and auxiliary cell development also were examined. Seventy percent of the pre-germinated disinfected spores colonized the transformed carrot roots. After 8 mo, the average spore production was 56 (24–130) per 30 cm^3 of medium. Of the spores produced, 75% germinated and produced new generations in monoxenic culture. The mycelium network was formed by thick light-brown hyphae, which exhibit two major architecture patterns related to either root colonization or resource exploitation, and lower-order hyphae, bearing auxiliary cells, branched absorbing structures (BAS), hyphal swellings (HS) and forming anastomoses. BAS were formed abundantly in extramatrical mycelium and frequently had HS resembling vesicles, a feature not previously reported in the *Gigasporaceae*, to the best of our knowledge. Few anastomosis were observed within the mycelium and most often corresponded to a healing mechanism that form hypha bridges to reconnect broken hyphae or overcoming obstructed areas within a hypha. Numerous auxiliary cells were produced during culture development and their role was inferred.

INTRODUCTION

Arbuscular Mycorrhizal (AM) fungi form a monophyletic group of obligate plant symbiotic fungi belonging to phylum *Glomeromycota* (Schüβler et al 2001). Among the nine AM fungi genera, *Scutellospora* contains approximately 17% of the described species (see chapter 1). This genus was erected following dichotomy of the genus *Gigaspora sensu* Gedermann & Trappe (Walker and Sanders 1986). Despite the high diversity and occurrence of viable spores of *Scutellospora* species in natural ecosystems, some species are difficult to isolate and maintain in culture collections. For example, *Scutellospora crenulata* Herrera-Peraza; Cuenca & Walker is reluctant to grow in pot cultures (Herrera-Peraza et al 2001) while *S. projecturata* Kramadibrata & Walker and *S. spinosissima* Walker & Cuenca can be grown in mixed-species pot cultures but not as single species (Kramadibrata et al 2000, Walker et al 1998).

Monoxenic culture (MC) consists of an explant of Ri T-DNA transformed carrot root associated with AM fungal propagules on a synthetic nutrient-agar media. MC has been successful as a cultivation system for more than 25 AM species (see Fortin et al 2002 for a review) and is proving useful for studies of the fungal symbiont (Harrison et al 1999). However, most data generated under

MC conditions were obtained with *Glomus* and *Gigaspora* species while *in situ* observations on in vitro-produced cultures of *Scutellospora* species were seldom reported. Our main objectives were: (i) to establish and describe the longterm culture of *Scutellospora reticulata* (Koske, Miller& Walker) Walker & Sanders under MC in association with Ri T-DNA transformed carrot roots; and (ii) to describe the fungal development, i.e., spore germination, root colonization, extraradical mycelium development and architecture, and spore production.

MATERIAL AND METHODS

Fungal isolate.

Scutellospora reticulata accession EMBRAPA CNPAB11 was provided by the germplasm collection of Embrapa Agrobiologia, Seropédica, RJ, Brazil (de Souza 2000). The strain was isolated from eroded soil in Brazil (see Santos et al 2000 for site characteristics) and cultured from a single spore on *Brachiaria decumbens* Stapf & Prain. The plants were grown in plastic pots containing 1L of a mixture of clay soil and sand (1:1 v/v), pH 4.8–5.2, amended with 5.5 g/Kg of rock phosphate and fertilized intermittently with 1/10 strength nutrient solution (Hoagland and Arnon 1950) without P. After 8 mo of growth, pot contents were dried in situ. Roots were removed, chopped and homogenized again with the remaining substrate. The pot culture presented an average of 2 spores per cm^3 having 343 µm diam (mean 203–476 µm). The pot culture was stored at room temperature (under tropical conditions) until required. After 2 yr, spores were extracted by wet sieving and centrifugation, selected under a stereomicroscope as described by de Souza and Berbara (1999). After cleaning, the spores were re-examined under a stereomicroscope, and those appearing clean and in good condition were transferred to a blood-collect glass tube with a micropipette.

Establishment of monoxenic cultures.

Spores were surface sterilized at room temperature by a procedure adapted from Bécard and Piché (1992). After surface sterilization, spores were transferred individually with a micropipette to Petri plates containing water agar 0.8% (Agar bacteriological No. 1, OXOID Ltd., Hampshire, U.K.) pH 6.0 and incubated in an inverted position in the dark at 27 ^oC until germination.

Two to 3 d after germination, a block of agar containing a single germinated spore was transferred to another Petri plate close to a transformed carrot (*Daucus carota* L.) root approximately 70 mm long (Dalpé and Declerck 2002). The modified Strullu-Romand (MSR) medium (see Declerck et al 1998, modified from Strullu and Romand 1986) was solidified with 4 g/L Gel-Gro^{TM} (ICN

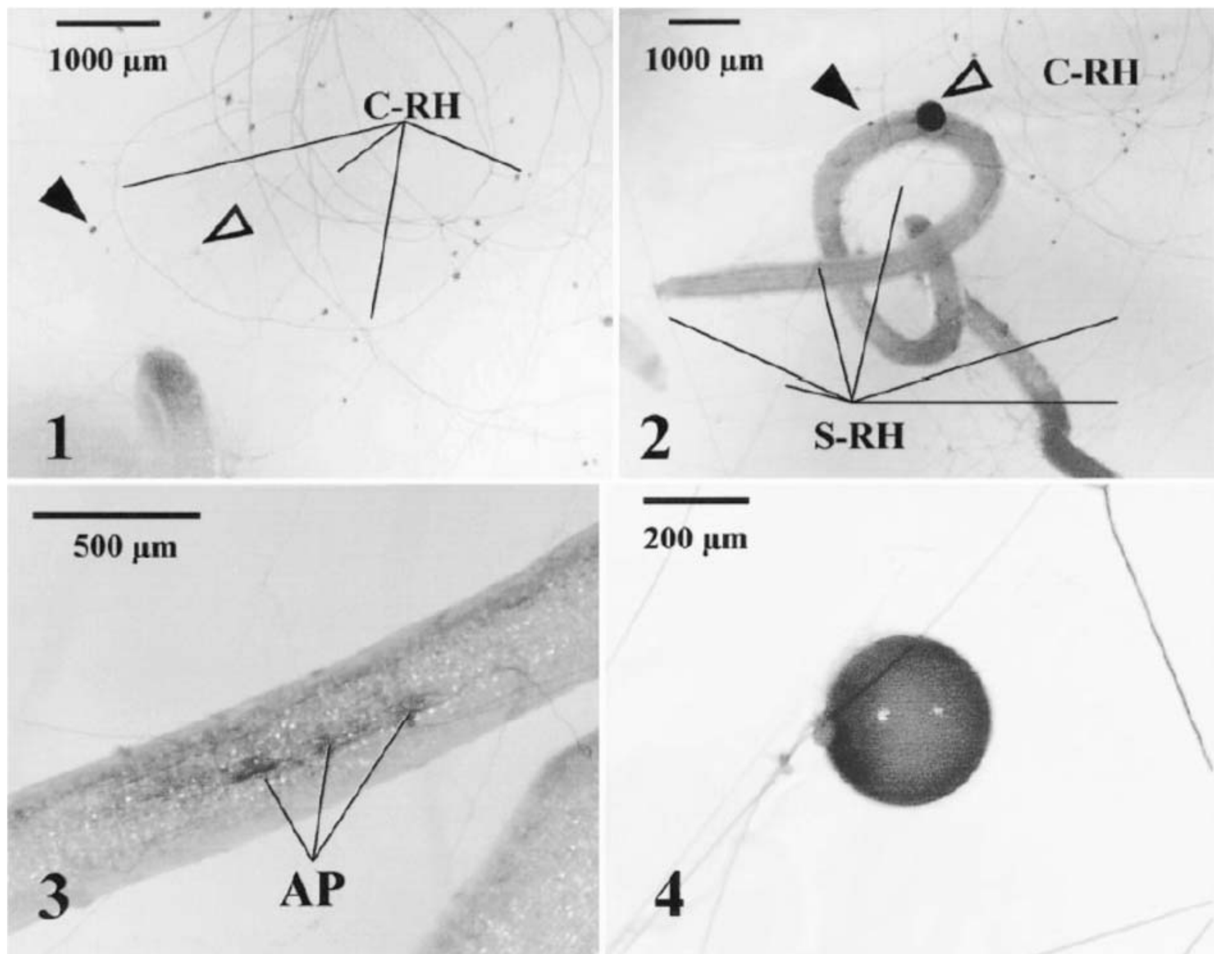
Biomedicals Inc, Irvine, California, U.S.A.) and used as growing medium. Each experimental unit consisted of a successful single spore-carrot root culture in a Petri plate (90 mm diam) containing 30 mL MSR medium. Cultures were incubated in an inverted position in the dark at 27 ^{o}C for up to 8 mo.

Data collection and harvest.
The germination rate of surface-sterilized spores was assessed for three sets of 100 spores each during a 4 wk period. Ten Petri plates each containing 10 spores composed a set. The development of fungal extraradical mycelia and the differentiation of hyphal swelling tips, auxiliary cells and spores were observed for up to 8 mo in 10 plates arranged randomly. Spore dimensions were assessed using 12 randomly chosen, mature spores in each experimental unit. Mature and juvenile spores were differentiated by color, septa formation in the subtending hyphae and by the absence of cytoplasm activity in the sporogenous bulbous subtending hyphae. Germination of monoxenic propagated spores was assessed at the end of the experiments. After 8 mo, spores were removed from the growth medium with forceps, germinated as described above and re-associated with transformed carrot roots on the MSR medium to test their ability to form new colonies and spores. The occurrence of anastomoses was evaluated in 100 intersection points per experimental unit. The intersections were observed in primary as well as secondary and lower-order hyphae. Observations were made through a dissecting microscope and an inverted compound microscope. Digital pictures were captured with a Sony DXC-950 P Power HAD 3-CCD digital camera, and the spore and mycelia measurements were taken with Leica Qwin V 1.0 software (Leica Imaging Systems Ltd., Cambridge, U.K.).

RESULTS

Spore germination.
Spore germination occurred within 25d, and the first germination was observed 3 d after incubation on the water-agar medium. After 1 mo, the germination rate was 59%. The germination tube exhibited negative geotropism and covered an average volume of 1.1 cm^3 (range 0.3–1.9; N = 20) after 2–3 d.

Monoxenic cultures establishment, sporulation and subculture.
Seventy percent of the pregerminated spores colonized transformed carrot roots and produced spores. For 30% of noninfective 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.



FIGS. 1–4. Mycelium architecture, infection units and mature spore of *Scutellospora reticulata* growing in Ri T-DNA transformed carrot root. 1. General view of the mycelium architecture showing circular-runner hyphae (C-RH) and secondary hyphae bearing numerous auxiliary cells (bold arrowhead). Hollow arrowhead shows sporogenous hypha bearing an immature spore. 2. Same culture 9 d later, with the same auxiliary cells and spore (arrows). Observe the colonization and spreading of straight-runner hyphae (S-RH) in the root zone originating in the circular-runner hyphae area far from the root. 3. Infection units showing appressorium (AP) formation on the root surface and darker areas on the root cells below the infection points. 4. *S. reticulata* mature spore.

The extramatrical mycelium development was extensive (FIGS. 1, 2) and numerous infection units were formed in active growing roots (FIG. 3). The first daughter spores were formed approximately 10–12 wk after colonization (FIGS. 1, 2) and sporulation was observed thereafter for 5–8 mo. After 8 mo, the average spore production per Petri plate was 56 (24–130) per 30 cm^3 of MSR medium. The spores were globose (FIG. 4) in all the cultures, with an average diam of 376 µm (280–500 µm coefficient of variation of 10.67%, N = 120). 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. The spores produced in the

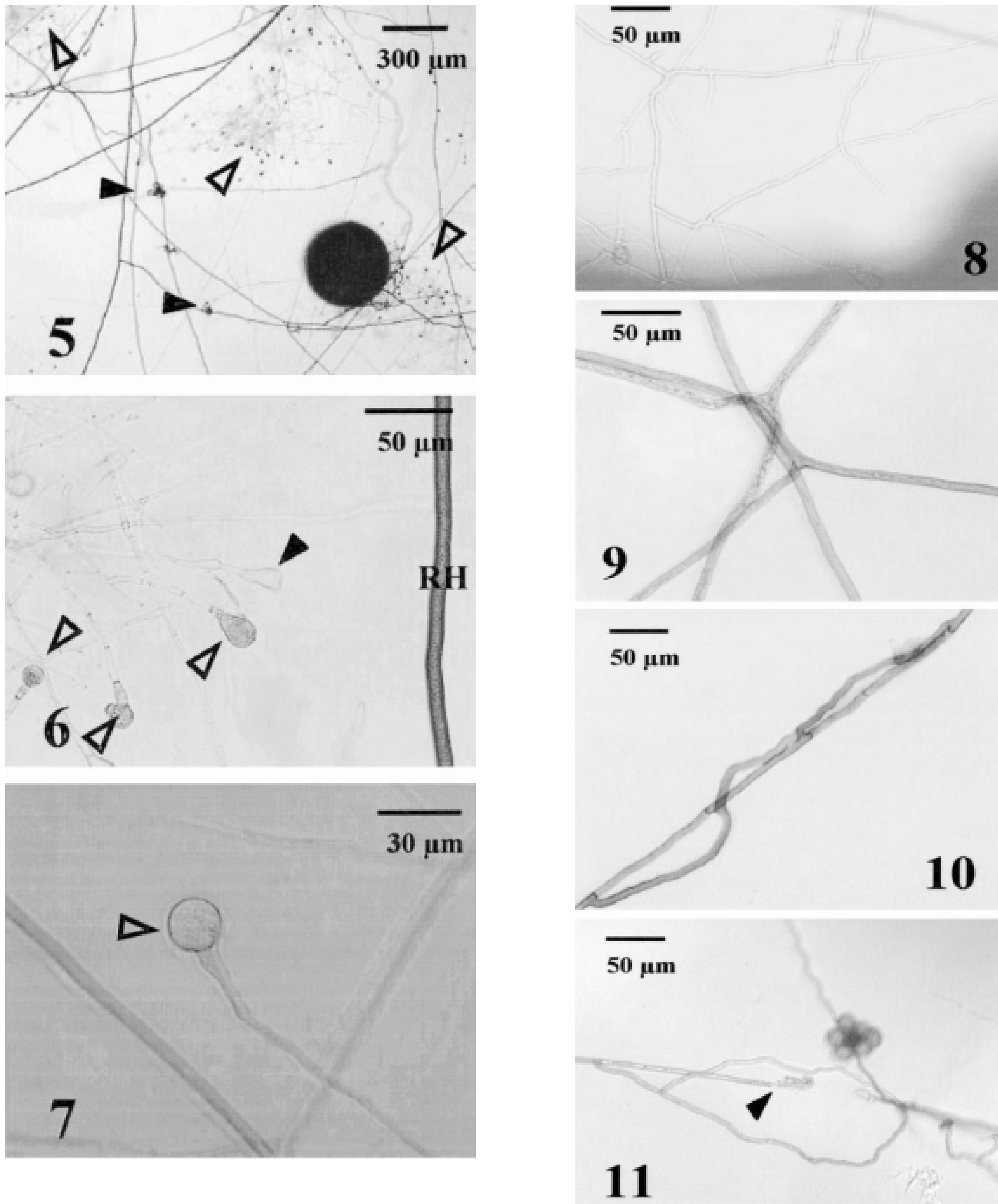
MC germinated within 2–3 d, averaged 76% germination in water and 75% in sugar-free MSR medium after 2 wk incubation at 27 ^{o}C. These germinated spores were able to re-associate with Ri T-DNA transformed carrot roots on the MSR medium and produced new spores (data not shown).

Extraradical mycelium architecture and development.

The extraradical mycelium of *S. reticulata* was characterized by thick, orange-brown hyphae (5.7–17.6 μm) similar to runner hyphae (RH) described by Friese and Allen (1991) and Bago et al (1998a) (FIGS. 1, 2) forming the main branching network of the culture. Thinner lower-order hyphae branched from this network and formed auxiliary cells, sporogenic hyphae and spores (FIGS. 1, 2), hyphal swellings (HS) and branched absorbing structures-like (BAS-L) (Bago et al 1998b). The RH presented two architectural patterns occurring concomitantly in the same colony (FIGS. 1, 2). In one pattern, RH formed open circles or spirals. The number of hyphal branches always was higher on the convex side of the partial circle or spiral (FIG. 1). 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 (FIG. 2), 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 8 mo of observations and seldom formed septa.

Branched absorbing structures and hyphal swellings.

Structures resembling BAS-L reported by Bago et al (1998b) were formed as ramifications of primary, secondary or higher-order hyphae and also as ramifications of sporogenous hyphae (FIG. 5–7). These structures were formed by numerous thin (< 1–5.3 μm), generally straight but sometimes contorted, hyphae extending radially in the medium in a volume frequently larger than 2 mm^3 (FIG. 5). These structures were transient and their cytoplasm content retracted after septation. This process occurred 1–2 wk after the BAS-L formation (FIG. 6). 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. 6). These HS were hyaline, globose to ovoid with dimensions of 13–18 μm by 26–36 μm. These structures resembled extraradical vesicles or immature spores because they frequently contained dense cytoplasmic material typically found in fully expanded spores and auxiliary cells (FIG. 7). The number of these HS varied from just a few to several hundred for a single BAS-L. In a single culture, HS could be 20 times as abundant as differentiated *S. reticulata* spores.



FIGS. 5–7. Branched absorbing structures (BAS) with swollen tips of *Scutellospora reticulata* growing in Ri T-DNA transformed carrot root. 5. Distribution of BAS (hollow-arrowhead) close or not to a spore, dark dots are swelled tips containing cytoplasm. 6. Magnification of an old BAS, showing hyphae septation and the presence (hollow-arrowhead) or absence (filled-arrowhead) of cytoplasm in the swolled tips. Compare the thickness of those hyphae with the runner hyphae (RH). 7. A swollen tip that resembles in form an immature *Glomus*-like spore (arrowhead). Note the appearance of the cytoplasm inside the structure.

FIGS. 8–11. Interactions among hyphae of *Scutellospora reticulata* growing in monoxenic root organ culture. 8. Anastomoses on secondary hyphae. 9. Contact point of three hyphae without anastomosis formation. 10. Wound healing of a hypha. Note that parts of the hypha where the color is lighter are dead parts separated by septa. 11. Bridge hypha formed to reconnect a broken hypha, with extruded cytoplasm as indicted by the arrow.

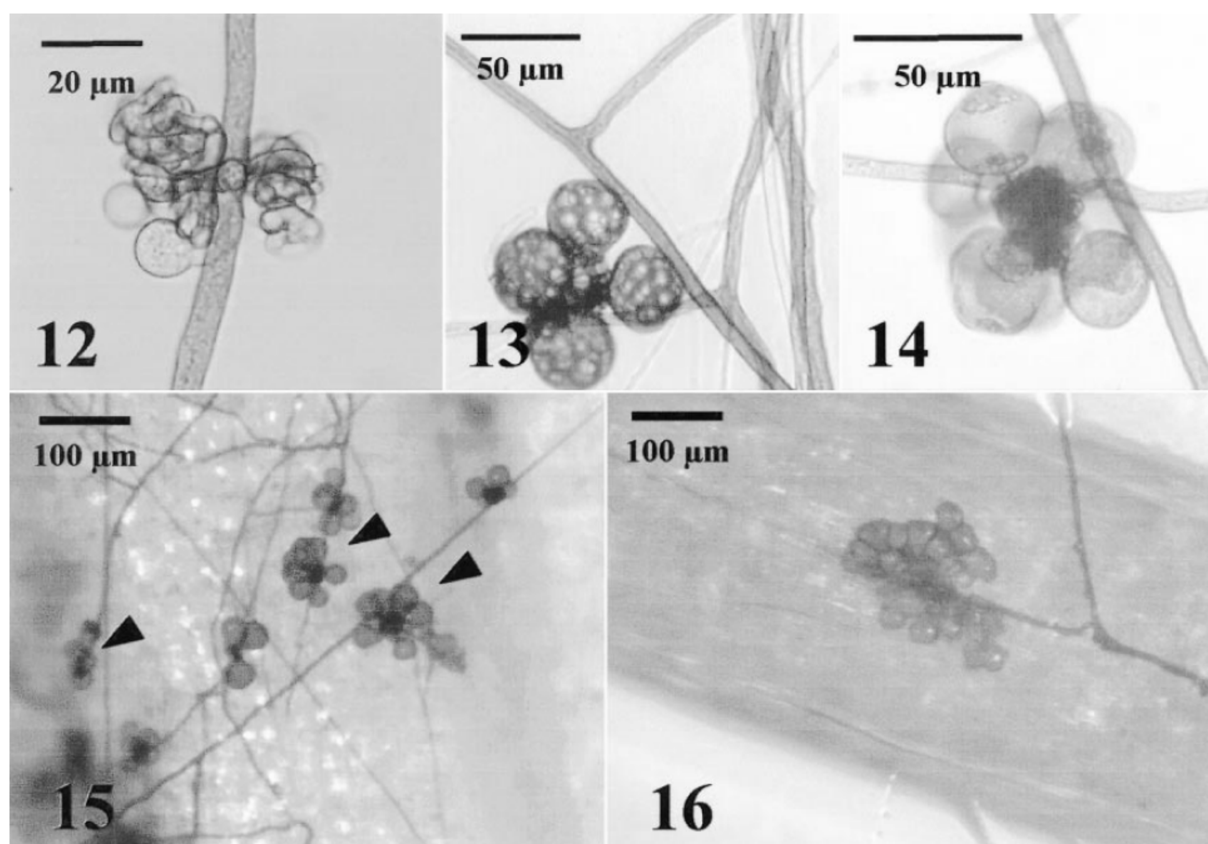
Anastomoses formation.
In spite of the many points of hyphal contact within the hyphae network, less than 1% anastomosis were observed. We observed anastomoses only in the secondary hyphae forming BAS-L (FIG. 8). No anastomoses were observed among the RH (FIG. 9). On the other hand, a mechanism to repair hyphae damage was detected (FIGS. 10, 11). Hyphal bridges were formed when flow of cytoplasm was obstructed (FIG. 10) or to reconnect broken hyphae (FIG. 11).

Auxiliary cells.
Auxiliary cells were formed by short ramifications occurring at one or simultaneously at both sides of a hypha (FIG. 12). 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 clavate, and 29.8 (12–16) μm high by 31.1 (19–39) μm wide with an inflated thin-walled (< 1 μm thick) ‘‘cell’’, orangish-brown under transmitted light (FIGS. 12). The subtending hypha, 4.2 μm (3–6) diam at the base of the ‘‘cell’’, had no occlusion or septa (FIG. 12). The number of cells in each auxiliary cell ranged from two to more than twenty (FIGS.12–16), and the total number of auxiliary cells ranged from 600–700 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 (FIGS. 1, 2). They also formed abundantly on the root surface (FIG. 15) and sporadically in the external cortical cells of roots (FIG. 16). At full expansion, young auxiliary cells contained dense cytoplasmic inclusions (FIG. 13) resembling the lipid droplets found in healthy looking AMF spores. After 8 mo many auxiliary cells were empty (FIG. 14) or collapsed, leaving a scar at their origin.

DISCUSSION

In contrast to the high diversity of *Scutellospora* species in nature, a relative low number of species are maintained in living culture collections (see the collections at BEG-France, http://www.ukc.ac.uk/bio/beg/; INVAM-USA, http://invam.caf.wvu.edu.; MAFF Gene Bank-Japan, http://www.gene.affrc.go.jp/micro/index.html and TARI-Taiwan http://www.tari.gov.tw/ ACT/ACT.htm) and these are in the form of pot cultures, which often have problems with viability and contamination (Walker and Vestberg 1994). For example, Morton (1995) reported contamination of a putative *Microdochium* species in a culture of *S. gregaria* INVAM CL756 and relates this contamination to the low production of spores, while Hijri et al (2002) reported the contamination by ascomycetes in apparently healthy spores of *S. castanea* BEG1. The use of *S. castanea* spores

contaminated with ascomycetes caused problems for interpreting DNA analysis (Schussler 1999). The MC of *S. reticulata* provides a new perspective in the cultivation of this genus and extends the range of AM fungi genera maintained on excised roots (Fortin et al 2002). This system allows nondestructive observations of extraradical mycelium (Bago et al 1998a) and spore developmental sequence (de Souza and Berbara 1999, Dalpe´ and Declerck 2002).



FIGS. 12–16. Auxiliary cell formation in *Scutellospora reticulata* growing in monoxenic root organ culture. 12. Bilateral hyphae branching from a secondary hypha, showing swollen hyphal tip forming immature auxiliary cells (AC). 13. Mature auxiliary cells with smooth surface and filled with lipid droplets. 14. Old auxiliary cells from 8 mo old culture with depleted lipid droplets. 15. Auxiliary cells in the vicinity and on a root surface. Note the differences in number of the cell units. 16. Auxiliary cells formed in the outer root cortical cells, present a difference in shape from the auxiliary cells shown in FIG. 13–15.

Culture and subculture of S. reticulata*.*

The spore germination rate was high for both 2 yr dried pot culture (59%) and ROC-produced (75%) spores, demonstrating the long-term survival potential of this accession and the viability of the MC produced spores. The composition of the MSR medium with pH adjusted to 5.5 before autoclaving (Declerck et al 1998) appeared adequate for this isolate, since the fungal life cycle was properly

completed. In addition, a high number of successful single spore MC (70%) was obtained and could be increased by manipulation of the Petri plate, such as vertical incubation (Diop et al 1992) or by a better placement of the germinated spore and the root organ culture explant. Spore production within this system varied between 24–130 per Petri plate with 30 mL medium, which is comparable to the production in single-spore open-pot culture (mean two spores per mL dry soil). The sporulation dynamics of *S. reticulata* was similar to that of *Gigaspora margarita* Becker & Hall (CNPAB 1 and 16), *Gi. albida* Schenck & Smith (INVAM 927 and BR607A, de Souza unpublished results) and *Gi. rosea* Nicolson & Schenck DAOM194757 (Diop et al 1992), which formerly was identified as *Gi. margarita* (Bago et al 1998c) or in competition with *Glomus intraradices* Schenck & Smith (Tiwari and Adholeya 2001). Compared with *G. intraradices* Schenck & Smith, *G. versiforme* (Karsten) Berch, *G.proliferum* Dalpe´ & Declerck (Declerck et al 2000, 2001), which start to produce spores in less than 1 mo in MC, these *Gigasporaceae* species exhibit a long vegetative phase before sporulation (2–3 mo). The sporulation dynamics of *S. reticulata* and *Gi. margarita*, as compared with *G. intraradices*, *G. versiforme* and *G. proliferum* under similar growing conditions, are consistent with ‘‘K’’-like strategists for these three *Gigasporaceae* species, as compared to these three *Glomus* species, which behave like ‘‘r’’ strategists.

Mycelium architecture and development.

MC technique let us study the development of the external mycelium and its structures for lengthy periods and detect structures and patterns of mycelium growth previously reported. Bago et al (1998a) divided the extraradical spreading of the *Glomus intraradices* in monoxenic system into three stages: (i) proliferation of runner hyphae acting as conducting channels, which divide dichotomously and extend the fungal colony radially; (ii) development of arbuscule-like structures, which are formed at regular intervals along the runner hyphae and which might play a preferential role in nutrient uptake; and (iii) formation of spores in zones already colonized by runner hyphae and arbuscule-like structures. Those three stages also occur in *S. reticulate*. However, the pattern of mycelium branching is distinct. The *Glomus* mycelium branches more profusely and with lower angles than *S. reticulata*. In addition, *S. reticulata* forms the circular or spiraled mycelium that has not been reported to occur in *Glomus*. The RH observed here are nearly identical to what Friese and Allen (1991) defined as runner hyphae. However, we observed that these hyphae also play a role in the allocation of resources within the fungal network. This was supported by the dense bidirectional cytoplasmic/protoplasmic streaming observed from roots to the surrounding environment, i.e., the various structures of lower-order hyphae and from lower-order hyphae to roots. In that sense, RH works as a ‘‘circulatory system’’. Microscopic observations revealed that hyphal damage, caused by root growth, might have negatively

affected spore formation in the MC. Some immature spores, located far from the disrupted hyphae, were arrested in their juvenile stage, probably due to the damage caused to the mycelia network. Similar damage and arrested spore development also might be expected in disturbed soils such as in tilled agricultural fields. This negative selection might help explain the low abundance of some *Gigasporaceae* species in arable fields (Daniell et al 2001, Helgason et al 1998, Jansa et al 2002), because spores are the main propagules for *Gigasporaceae* (Brundrett et al 1999 a, b, Klironomos and Hart 2002). Other factors observed in MC that might negatively affect fitness in agricultural soils include the long vegetative period before sporulation, the lengthy process of spore expansion and development.

Branching absorbing structures-like.

BSA-L reported here, resemble those described by Bago et al (1998b) for *G. intraradices* but were less ramified and frequently terminated in swollen tips, the HS. Bago et al (1998b) proposed that most BAS were involved in nutrient uptake capabilities and those appearing at the spore's subtending hyphae were implicated in spore formation. BAS-L longevity was ephemeral, and cytoplasm often was arrested inside the HS after septation of the subtending hyphae. For *S. reticulata*, these structures were localized near sites of sporulation, perhaps indicating a function related to sporulation, as demonstrated for the spore-BAS structures in *G. intraradices* (Bago et al 1998b). In some cases, i.e., *G. clarum* Nicolson & Schenck (de Souza and Berbara 1999), *G. etunicatum* Bercker & Gerdemann (Pawlowska et al 1999) and *G. proliferum* (Declerck et al 2000), HS were juvenile spores that differentiated into mature spores after successful culture establishment. In *A. rehmii* Sieverding & Toro, HS did not develop further, but they might help provide energy storage used to support further sporulation (Dalpé and Declerck 2002), thereby serving a similar role as suggested for auxiliary cells (see below).

Anastomoses.

Data on anastomoses formation mostly were reported for *Glomus* species (Giovanneti et al 1999, 2001, 2003). Anastomoses were observed to occur between hyphae originating from the same spore and from different spores of the same isolate (Giovannetti et al 1999), while no anastomoses were detected between hyphae belonging to different isolates (Giovannetti et al 2003). Concerning *Scutellospora* species, i.e., *S. castanea* Walker, neither interspecific nor intergeneric hyphal fusions were observed with germlings (Giovannetti et al 1999). In our experiment, we recorded anastomoses in intact arbuscular mycorrhizal networks of *S. reticulata* but in a low frequency as compared with the *Glomus* species studied (Giovanneti et al 1999, 2001, 2003). Bago et al (1999) also reported the

existence of healing mechanisms in *Glomus intraradices* and *Gigaspora rosea* that restrict the damage induced by ageing or lytic events. These authors suggested that such mechanism could improve hyphal survival under adverse conditions. We also hypothesize that the healing mechanism reported in *S. reticulata* might decrease damage caused in the mycelium network that can negatively affect spore formation.

Auxiliary cells.

The biological function of auxiliary cells remains speculative (Bonfante and Bianciotto 1995). Jabaji-Hare (1988) observed high amounts of lipids within the auxiliary cells of a *Gigaspora* species, supporting the storage function of these structures. Roles in transitory storage (Pearson and Schweiger 1993) and reproduction (Pons and Gianinazzi-Pearson 1985) have been hypothesized, or they might represent vestiges of relict reproductive structures (Morton and Benny 1990). Our observations suggest a possible role in carbon storage, for use as energy for spore initiation and development and/or mycelial production and repair. Specific details supporting this supposition were (i) the abundance of auxiliary cells (approximately 600–700 per Petri plate, data not shown) for an average of 56 spores produced, (ii) the apparent changes in lipid content during the transition from young to older auxiliary cells and (iii) the high lipid content within these cells. The transitory role of auxiliary cells in spore production also was supported by previous work conducted with *S. calospora* (Nicol. & Gerd.) Walker & Sanders (Pearson and Schweiger 1994). These authors said that carbon stored in the auxiliary cells could be used during spore formation.

Final comments.

Although artificial, MC supports the life cycle of AMF, thereby generating viable propagules (spores). The major advantage of this system is that it allows for detailed, nondestructive observation of the entire fungal development over time. This is particularly useful in the study of extraradical mycelium development and function. 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 (Brundrett et al 1999a, b, Klironomos and Hart 2002). 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 MC can be used to predict AM species behavior in field soils. The use of MC should help us unravel some of the basic and applied aspects of the biology and ecology of the AMF symbiosis.

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Chapter 3

Development of extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells

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ABSTRACT

The development of the extraradical mycelium and auxiliary cells and spore production of *Scutellospora reticulata* in association with Ri T-DNA transformed carrot roots was followed under root-organ culture conditions. Extraradical mycelium development followed classical lag-exponential-plateau phases, with an additional late decline phase in number of auxiliary cells. Spore production started in parallel with a critical extraradical mycelium biomass produced, continued long after root growth ceased and during the late decline in auxiliary cells number. Isolated auxiliary cells were shown to exhibit hyphal re-growth, but not root colonization, either in situ or in vitro. These results showed that root and extraradical mycelium development were intimately associated in a sequence where both grew together during active root growth, followed during root aging by a period in which only the fungus developed. Spore production appeared dependent on a critical extraradical mycelium biomass and on the re-allocation of resources from both the intraradical mycelium and the auxiliary cells via the hyphal network.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi can colonize roots from three sources of inoculum: spores, mycorrhizal root fragments, and extraradical hyphae (Smith & Read 1997). However, not all AM fungi are equally able to colonize roots with these propagules. Clear differences exist between AM families (genera), which may reflect differences in life-history strategies and ecology (Brundrett, Abbott & Jasper 1999). Knowledge of these differences is also essential for culture propagation designed for research purposes and practical applications. Glomeraceae and Acaulosporaceae species can colonize roots from all sources of propagules, while in Gigasporaceae (*Gigaspora* and *Scutellospora*), spores are the best identified source of inoculum (Brundrett et al. 1999, Klironomos & Hart 2002) and are consequently of central importance for species survival.

Spore production is influenced by a large range of factors, including season (Gemma & Koske 1988, Clapp et al. 1995, Bever et al. 2001), nutrient level (Douds & Schenck 1990, Bever et al. 2001), plant and fungal species (Hendrix, Guo & An 1995, Bever et al. 1996, Bever 2002), physiological changes in the host plant (Sylvia & Schenck 1983), interaction with predators (Bakhtiar et al. 2001) or beneficial (Paula, Reis & Dobereiner 1991) soil organisms. Factors strictly related to the development of the intraradical phase of the fungus have also been addressed. Gazey, Abbott & Robson (1992)

demonstrated that sporulation of *Acaulospora laevis* and *Acaulospora* sp. started after root colonization has reached a critical level. This finding was confirmed with *Gigaspora margarita* and *A. longula* (Douds & Schenck 1991). Pearson & Schweiger (1993) observed that sporulation of *Scutellospora calospora* occurred after the decline in root colonization and increased from then onwards.

Components of the extraradical mycelium of the AM fungus may also impact spore production and, consequently, species survival. Members of the Gigasporaceae are characterized by the formation of unique structures named auxiliary cells, while in the families Glomeraceae and Acaulosporaceae these structures are absent. The function of auxiliary cells as transitory storage (Pearson & Schweiger 1993) or reproductive structures (Pons & Gianinazzi-Pearson 1985) are still speculative. Pearson & Schweiger (1993) and de Souza & Declerck (2003) suggested a possible role of the auxiliary cells in carbon storage, for use as energy for spore initiation and development and/or mycelial production and repair. However, no quantitative analysis was performed to ascertain this hypothesis. Despite the importance of the extraradical mycelium in spore production, the relationships between both components have been poorly investigated, primarily due to the inherent below ground nature of AM fungi. Most studies involved destructive extractions of mycelium from successive collection of soil samples (Gazey et al. 1992, Jakobsen, Abbott & Robson 1992, Boddington et al. 1999). A few non-destructive studies have been conducted, with soil-based root observation chambers (Friese & Allen 1991) and two-dimensional sandwich systems (Giovannetti et al. 2001), providing information on growth dynamics of hyphae spread from colonized roots into the environment, anastomosis formation and nuclear exchanges. Despite these major observations, in situ soil systems may present intrinsic limitations, among which are the difficult non-destructive, direct, repeated and long-term 3-dimensional visualisation of extraradical mycelium, and the unambiguous distinction between AM fungal hyphae, in particular the fine hyphal branches, and those of soil saprophytes and root pathogens. In the last decade, the emergence of the root-organ culture (ROC) system (Fortin et al. 2002) has improved our understanding of extraradical hyphae, notably with respect to its carbon and nutrient uptake capabilities (Bago, Shachar-Hill & Pfeffer 2000, Joner, Ravnskov & Jakobsen 2000) and for its developmental dynamics (Bago, Azcon-Aguilar & Piche´ 1998). Sporulation dynamics (Declerck, Strullu & Plenchette 1996, Declerck et al. 2001) and extraradical mycelium growth dynamics (Bago et al. 1998), of some *Glomus* species, have further been studied by using this ROC system. In these studies, the extraradical mycelium growth and spore production were shown to follow a three-phase development: likely a lag-phase, an exponential growth phase and a plateau phase, as evidenced in most other biological populations (Menge 1984). However, these results were mostly restricted to a few *Glomus* species and virtually nothing is known about other families such as

Gigasporaceae. In addition, no study considered the long-term sequential time-lapse relations between extraradical mycelium growth and spore production in Gigasporaceae. Recently the successful ROC of *Scutellospora reticulata* was reported (de Souza & Declerck 2003). The species was shown to produce profuse mycelium characterized by thick hyphae that exhibit two major architecture patterns related to root colonization or resource exploitation, and lower order hyphae bearing high amounts of auxiliary cells, hyphal swellings at the tip of branched structures, and infective spores (de Souza & Declerck 2003).

The aim of this study was to quantify the dynamics of extraradical mycelium growth, spore and auxiliary cells production under ROC, and to test the hypothesis that spore production in S. reticulata may be dependent on a critical extraradical mycelium biomass and on the re-allocation of resources from roots and auxiliary cells to developing spores via the hyphae network.

MATERIAL AND METHODS

Biological material

Spores of *Scutellospora reticulata* (CNPAB 011) and *Agrobacterium rhizogenes* transformed carrot roots (*Daucus carota*) clone DC1 were obtained from EMBRAPA-Agrobiologia (Brazil, http://www.cnpab.embrapa.br) and from GINCO (http://www.mbla.ucl.ac.be/ginco-bel) respectively. The AM fungus was propagated on Terra Green (Agsorb 8/16 LVM-GA; Chicago) in pot culture with leek (*Allium porrum* cv. 'Bleu de Solaise') plants, under greenhouse conditions (24/20 ^{0}C day/night, with natural light). Plants were fertilized at regular intervals with Long Ashton solution (Hewitt 1966) and water was applied every 2–3 d. The transformed carrot roots were routinely maintained and multiplied on the MSR medium (Declerck, Strullu & Plenchette 1998) modified from Strullu & Romand (1986) in an inverted position at 27 ^{0}C in the dark.

Establishment of root-organ cultures

Spores were surface-sterilized following a procedure adapted from Bécard & Piché (1992) except that all steps were performed with room temperature solutions. Disinfected spores were subsequently incubated on the MSR medium in the dark at 27 ^{0}C. Following germination, a plug of gel medium supporting a germinated spore was transferred with a 9 mm cork borer into another Petri plate containing the MSR medium in which a similar plug had been previously removed. An actively growing transformed carrot root (70 mm long) was then placed in the near vicinity of the spore. Due to the negative geotropism of the transformed carrot roots and germ tubes of the spores, the Petri plates

were incubated in an inverted position at 27 ^{0}C in the dark. Each replicate thus consisted of a 90 mm Petri plate containing 40 mL of the MSR medium with one germinated spore and a 70 mm long Ri T-DNA transformed carrot root. Five replicates were used for progressive monitoring.

Data collection and harvest

A 10 x 10 mm grid of lines was marked on the bottom of each Petri plate. The total root and extraradical hyphal lengths and the number of spores and auxiliary cells for each replicate were assessed as follows. Vertical and horizontal lines were observed under an Olympus SZ-40 stereo microscope at 10 to 40x magnification, and the presence of root and hyphae recorded at each point where they intersected a line. No distinction was made between runner hyphae and lower order hyphae or between primary root and sub-order roots. The total numbers of intersects between root or hyphae and gridlines were then included in the formula of Newman (1966) used to relate the total length of roots (or hyphae) in a given area to the number of times they intersect a number of straight lines placed within this area (see Giovannetti & Mosse 1980). Full-size dark brown colored developed spores and auxiliary cells were counted individually in each cell formed by the grid (Declerck et al. 2001) and summed over the entire Petri plate. Details about S. reticulata spore development and ontogenesis are provided by de Souza & Declerck (2003).

Root and extraradical hyphal length, spore and auxiliary cell number were counted at regular intervals on each experimental unit for 36 wk, i.e. until growth of fungus ceased (week 0 = time of spore-root association).

At the end of the experiment, the gellan gel was liquefied (Doner & Bécard 1991). Roots were removed and internal root colonization quantified after staining with Trypan blue (Phillips & Hayman 1970). Fifty randomly selected root pieces were mounted on microscope slides in groups of ten and examined using an Olympus BH2-RFCA compound bright-field light microscope at 125x magnification. The frequency of mycorrhizal colonization (%*F*) was then calculated as the percentage of root pieces containing either hyphae or arbuscules.

Cytoplasmic streaming, as an indicator of living hyphae and vitality of colony was estimated on the ROC at 1, 3 and 8 months, on twenty randomly chosen runner hyphae per Petri plate under a Olympus BX50 inverted microscope at 300 to 600x magnification. For each runner hyphae, a group of cytoplasmic granules was aligned to a precise micrometer position and the time needed to reach another micrometer position was estimated with a stop watch. The results were then calculated as $\mu m\ s^{-1}$.

Hyphal re-growth and root colonization from auxiliary cells

Auxiliary cells also were removed with forceps and mycelium gently discarded. Groups of ten separated auxiliary cells were placed along transformed carrot roots on the MSR to test their ability to initiate the fungal life cycle (i.e. germination and colonization). Ten replicates were considered. The same test was conducted on pre-germinated leek plants grown in multi-pots (Somapo-Sopirec, Diemeringen, France) containing 15 g sterilized (121 ^{0}C for 15 min) Terra-green, under greenhouse conditions (24/20 ^{0}C day/night, with natural light). Plants were fertilized once a week with Long Ashton solution (Hewitt 1966) and watered every 2–3 d. The auxiliary cells were placed in the planting hole prior to planting the leek plants.

Statistical analysis

The time-course for the four variables (root and extraradical hyphae lengths, spore and auxiliary cells numbers) was modeled using a modified Gompertz model (Zwietering et al. 1990), recently shown to be appropriate for sporulation kinetics of AM fungi in vitro (Declerck et al. 2001):

$$E[Y_t] = A\exp\left(-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right)$$

where $E[Y_t]$ is the expected growth for variable Y at time t, λ is the lag phase, μ_m is the maximum growth rate, and A, the maximum total growth. The model was fitted to the data by non-linear regression using a Nelder–Mead simplex optimisation algorithm (Nelder & Mead 1965), in order to minimize the residual sum of squares (RSS), which is the sum of the squared differences between the predicted and measured values. The mathematical software MATLAB Version 5.3 (1999) was used for the computations.

For each of the five replicates, the value of the three parameters, i.e. λ, μ_m and A, was recorded for each variable. A set of 3 x 4 parameters per Petri plate is thus obtained, describing the dynamics of the development of the root-fungi system. To allow biological interpretation of data, the mean ($\bar{X}$) and standard deviation (SD) of each parameter were computed over the 5 replicates. In the results section, the variability between replicates is expressed as $\bar{X}$ ±SD. Three types of statistical analyses were performed: (1) two-by-two comparisons of the lag parameter λ were computed using a classical *t*-test for paired data sets in order to establish the chronology of the in vitro development of the extraradical mycelium of *S. reticulata*; (2) the Pearson correlation coefficients between the four variables were computed for each parameter in order to analyze the links between root and extraradical hyphae growth, and between the production of auxiliary cells and spores – a classic t-test with n -2 df

was used to determine whether the estimated correlation coefficient was significantly different from 0 (i.e. to test the independence); and (3) the assumption that the root stopped its growth before sporulation started was verified. To achieve this last point, the cessation of growth, which is not a parameter of the Gompertz model, was estimated arbitrarily, as the point on the time axis where the instantaneous growth rate (slope) was less than 10% of the first time after the inflection point. The assumption was verified using a t-test for paired data sets with $H_0 : m_D = 0$ *vs* $H_1 : m_D < 0$, with m_D being the mean of the differences between the end of the root growth and the lag parameter for the spore number.

The data for cytoplasm streaming in hyphae were subjected to an analysis of variance and treatment means were separated by Newman–Keuls test.

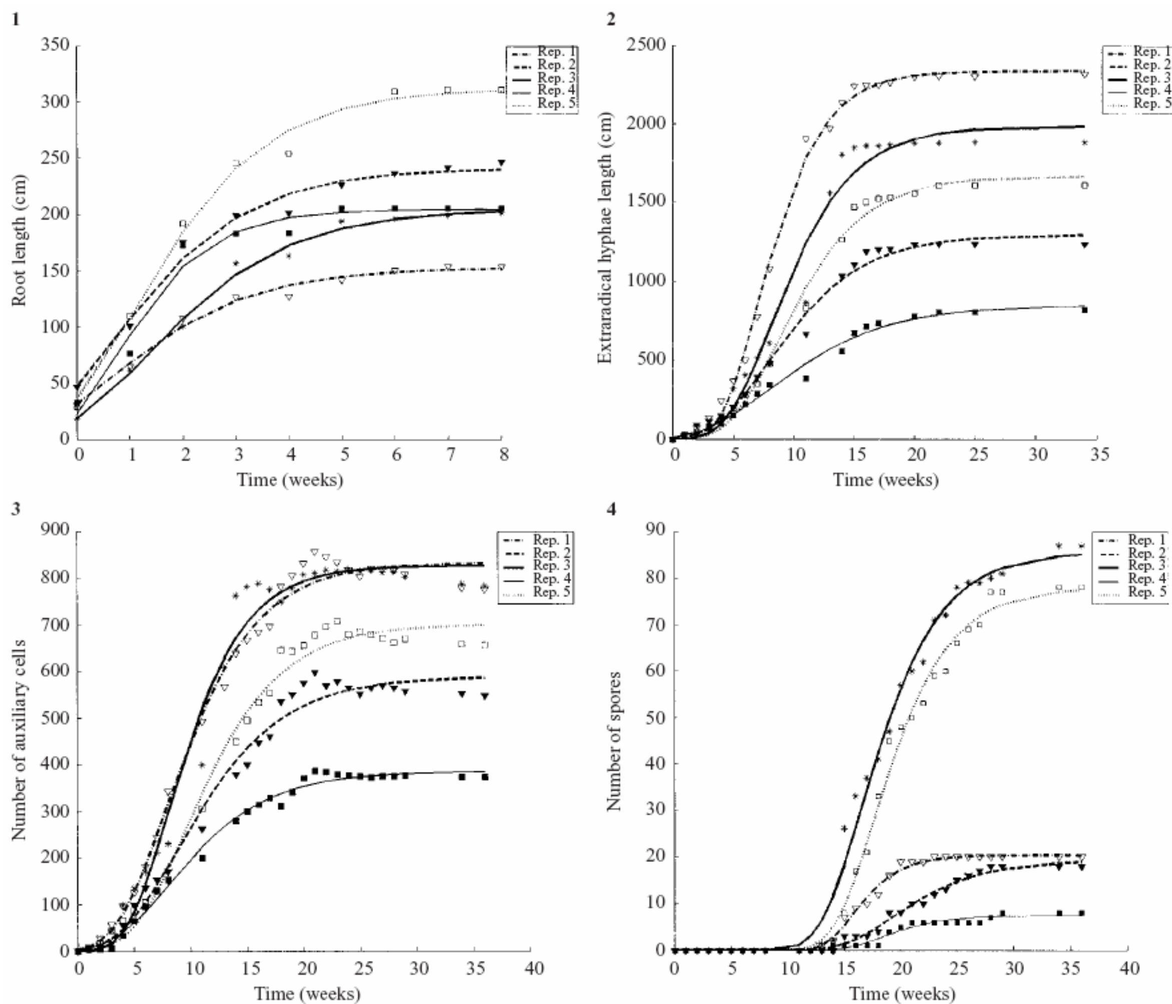
RESULTS

Adequacy of the Gompertz model

The Gompertz model was fitted to the four variables i.e. root and extraradical hyphae lengths, and spore and auxiliary cells numbers. The model fitted the data quite well (Figs 1–5). The coefficient of determination (R^2) estimated for each replicate was high and varied between 0.9658 and 0.9968 (data not shown).

Root growth and intraradical colonization

No lag phase was observed in root growth (Fig. 1). Root length increased linearly up to 6 wk after association with a spore, with a maximum growth rate (μ_m) of 61.58 ± 18.41 cm wk^{-1}. It reached a plateau after 7 wk (Fig. 1) and did not increase any further until the end of the experiment (week 36). At this time, root length varied in the different replicates from 153 to 311 cm and the mean maximum total growth (*A*) was 223.54 ± 58.89 cm. Many lateral roots, initially white and becoming brown-yellow when growth ceased, were observed within the medium and on the surface. At harvest, typical intraradical structures were observed, i.e. arbuscules and hyphae. Arbuscules consisted of many thin branches developing from a swollen trunk. They were locally abundant, occupying nearly all cells of the root cortex in colonized segments. Hyphae, often with knobs or projections, usually formed coils near entry points on the root epidermal cell walls. The frequency (%*F*) of root colonization by *Scutellospora reticulata* at harvest was 34.8 ± 12.7.



Figs 1–4. Time-course of parameters associated with transformed carrot root-organ culture of *Scutellospora reticulata* on the modified Strullu-Romand medium. Each curve illustrates the evolution of the parameter in one replicate. All parameters were fitted with the Gompertz model Fig. 1 Transformed carrot root growth (cm), Fig. 2 Extraradical hyphae length (cm). Fig. 3 Auxiliary cell number and, Fig 4 Spore number. Rep 1 to Rep 5 are the replicates number.

Development of extraradical hyphae and auxiliary cells

The first germinating spores were observed 7–10 d after incubation on the MSR medium. The germinating spores transferred to the vicinity of a transformed carrot root started to proliferate actively. The first auxiliary cells were observed even before any contact with the host root. The first appressoria were observed within 5–10 d of co-cultivation. A lag phase (λ) period of approx. 4 wk (3.8 ± 0.9 wk) was observed in the development of extraradical hyphae. An average of approximately 36.5 cm hyphae was produced per week during this period (Fig. 2). With increasing number of appressoria, considerable growth of hyphae was noted. Hyphae rapidly covered the Petri plate forming a dense hyphal network composed of hyphae entering and exiting the roots. This exponential growth phase

lasted approx. 12 wk during which the total extraradical hyphae length increased about 10 fold. The maximum growth rate (μ_m) was 160 ± 87 cm wk^{-1}. At the end of this period (from week 16 until week 20), extraradical hyphae growth slowed-down, reaching a plateau (Fig. 2). During this period, the extraradical hyphae length increased 17.4 cm wk^{-1}. No growth was observed after week 20. At this time, the maximum total growth (*A*) reached 1631 ± 580 cm and was highly correlated with %*F* (r = 0.83, *P*-value for the independence test = 0.011).

Cytoplasmic streaming was observed at 1, 3 and 8 months with nearly all the runner hyphae exhibiting active bi-directional movements. Rate varied from 3.6 ± 1.8 μ_m s^{-1}, 1.5 ± 1.1 μ_m s^{-1}, 2.3 ± 1.0 μ_m s^{-1} for the 1, 3 and 8 months old cultures, but did not differ significantly.

The number of auxiliary cells in the Petri plates followed approximately the same dynamic as the extraradical hyphae. A lag phase (λ) of 4 wk (3.8 ± 0.7 wk) was followed by an exponential increase during the following 12 wk, with a maximum growth rate (μ_m) of 55 ± 20.5 auxiliary cells produced per week, and ended in a plateau phase, with a maximum number of auxiliary cells (*A*) of 668 ± 187 (Fig. 3). This coordinated development of auxiliary cells and hyphae was evidenced by the highly positive correlations observed between the number of auxiliary cells and the length of extraradical hyphae for the three parameters of the Gompertz model i.e. the lag time (λ) (r = 0.88, *P* value= 0.011), the maximum growth rate (μ_m) (r = 0.83, *P*-value = 0.011), and the maximum total growth (*A*) (r = 0.97, *P*-value < 0.001). The number of auxiliary cells was further highly correlated with %*F* (r = 0.85, *P*-value = 0.009). Furthermore, the lag parameters (λ) for extraradical hyphal length and auxiliary cell number were not significantly different (*P*-value = 0.435), indicating that the dynamics for these two variables not only have the same shape, but were really simultaneous. Significant negative correlations were established between root growth and both number of auxiliary cells (r = - 0.69, *P*-value = 0.033) and extraradical hyphal length (r = - 0.81, *P*-value = 0.013) during the exponential phase of growth of extraradical hyphae and number of auxiliary cells. Following the plateau phase, a decline in the number of auxiliary cells was observed from week 21 until the end of the experiment. In some auxiliary cells, cytoplasm that had previously accumulated flowed backward into the hyphae. As a consequence, these empty cells frequently loosed their turgor and shrinked, leaving small knobby structures on the hyphae. In old Petri plates, the number of auxiliary cells that shrinked was higher than the number of new auxiliary cells produced, resulting in a decline in number of auxiliary cells enumerated. The decrease in the number of auxiliary cells was about 7%, i.e. an average of 46 auxiliary cells per Petri plate, between weeks 21 and 36. In addition, the remaining auxiliary cells looked empty or with few visible lipid content. Life span of auxiliary cells, estimated between full-size development and shrinkage, exceeded 6 months.

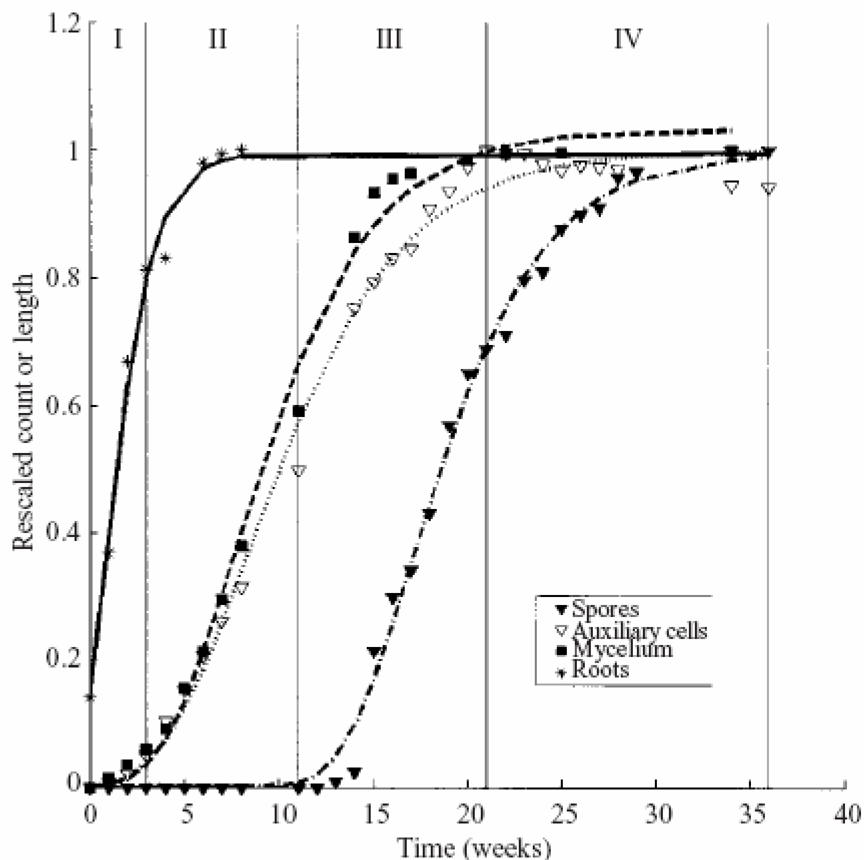


Fig. 5. Rescaled time-course of the extraradical phase of *Scutellospora reticulata* associated with transformed carrot root on the modified Strullu-Romand medium, fitted with the Gompertz model. Each fungal structural component i.e. hyphae, auxiliary cells and spores followed a classical lag – exponential – plateau phase, with an additional late decline phase for the auxiliary cells, whereas the development of the culture as a whole followed a four-stages sequence with (I) a radial hyphae extension and first appresoria formed, (II) a rapid increase of colony with concomitant development of auxiliary cells, (III) the production of spores once sufficient mycelium is produced and (IV) it's continuation after hyphal growth ceased, maximum number of auxiliary cells is reached and during decrease of auxiliary cells number.

Spore production

As with extraradical hyphal growth, spore production followed a three phase dynamic, but delayed in time (λ significantly longer, P – value ≤ 0.001; Fig. 4). The lag phase lasted approx. 14 wk (14 ± 1 wk) with the first spores produced 12 wk after co-culture. The number of spores increased exponentially from week 14 until week 28, with a maximum growth rate (mm) of 4.3 ± 3.4 spores produced wk^{-1}. The spore production entered the plateau phase at week 29, with a maximum spore number (A) of 42 ± 37 at week 34, which was highly correlated with $\%F$ ($r = 0.78$, P – value = 0.018). Sporulation started five weeks after root growth ceased (P - value = 0.0188) and intense spore production, corresponded approximately to the late exponential and early plateau phase of the

extraradical hyphal growth. At mid-phase of exponential spore production (week 21), more than 80% of auxiliary cells had already been produced. Sporulation was still occurring 7 wk after growth of extraradical hyphae reached a plateau and during the phase of decrease in number of auxiliary cells: 30% of the final spore numbers were produced during this period. Spore production was positively correlated with auxiliary cell production during the phases of exponential growth (r = 0.74, P-value = 0.023) and plateau (r = 0.59, P-value = 0.059) and was highly negatively correlated to extraradical hyphal length during the lag time (r = -0.78, P-value = 0.017). No significant correlation was observed between spore number and auxiliary cell number during the lag phase (r = -0.43, P-value = 0.125) nor between spore number and extraradical hyphal length during the exponential phase (r = 0.39, P-value = 0.149) and plateau (r = 0.42, P-value = 0.127).

Hyphal re-growth and root colonization from auxiliary cells

Auxiliary cells exhibited hyphal re-growth within 2 d after transfer to the vicinity of the carrot root (Fig. 6). Multiple hyphae (1–4), emerged from the auxiliary cell subtending hyphae, extended radially, to 3–4 μ_m, and grew straight into the medium, whereas auxiliary cells became gradually empty. Hyphal ramification appeared only occasionally and no new auxiliary cells were formed either before or after contact with the root. After one week, growth of hyphae ceased, and even where they contacted a root, no colonization was observed or was there subsequent abundant growth within the medium. The same result was obtained under pot culture conditions. After two months in contact with leek plants no root colonization was observed. Time–course sequential development of the extraradical mycelium in root-organ culture Colonization of the synthetic medium by *Scutellospora reticulata* was a dynamic process. The development of the extraradical mycelium of the fungal colony followed a four-stage sequence in which each structural component developed in a classical lag – exponential – plateau phase, with an additional late decline phase in number of auxiliary cells (Fig. 5). After germination of the spore, a few long hyphae, frequently curved, extended into the medium and branched at regular intervals in secondary and lower order hyphae bearing auxiliary cells. Once the first appresoria were established, the hyphae started to spread into the medium (Stage I). A phase of rapid radial increase of colony followed, with the concomitant development of numerous auxiliary cells (Stage II). Sporulation began once a sufficient extraradical fungal biomass, measured as hyphal length and auxiliary cells number, was attained (Stage III), and continued after hyphal development had ceased, maximum number of auxiliary cells was reached and decrease in number of auxiliary cells was observed (Stage IV). Root growth was observed during stage I and early stage II, entered the plateau phase at the middle of stage II and remained unchanged during stages III and IV. At this late

moment, root internal colonization highly correlated with each structure of the extraradical mycelium i.e. spores and auxiliary cells numbers and with the extraradical hyphae length.

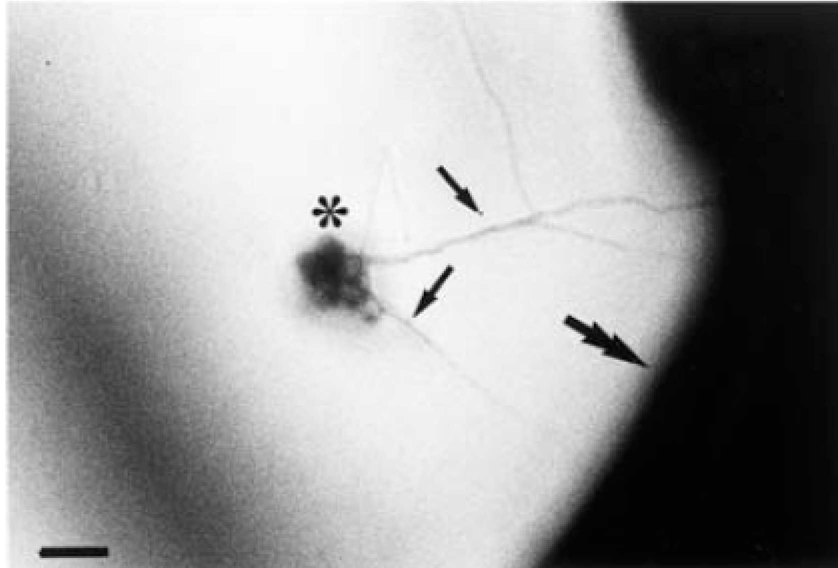


Fig. 6. Hyphal re-growth (arrow) from auxiliary cells (*) in the vicinity of a transformed carrot root (double arrow) under root-organ culture conditions. Bar=80 μ_m.

DISCUSSION

Extraradical mycelium development versus root growth

Extensive growth of extraradical mycelium began once the root was penetrated by the invading fungal hyphae, i.e. during the phase of exponential root growth, and continued during the root plateau phase growth. More than 70% of the extraradical hyphae length and auxiliary cells number were produced during this non-growing root phase, while the first spores were produced 5 wk after root growth ceased. The initiation and/or continuous spore production of AM fungi after root-growth ceased has been reported in other studies conducted in ROC (Diop, Bécard & Piché 1992, Declerck et al. 2001), although no relationship between both processes were evidenced. The bi-directional cytoplasmic flow in hyphae observed in our experiment with young (one-month old) active growing roots and in cultures with non-growing roots (3 and 8 months old) suggested that intraradical fungal structures, at least part of them, were viable in old non-growing roots and that the mycelium was as vital as ever. The speed of cytoplasmic streaming did not differ between the youngest and oldest cultures observed suggesting a metabolically active status of the fungus in the ROC, whatever the age of the root (in the

range of 1–8 months). Even though our experimental approach did not focus on the development of the intraradical phase of the fungus, it highlights the ability of the fungus to grow and translocate elements in the absence of a growing root.

Sporulation versus extraradical mycelium development

The AM fungus started sporulation in parallel with a critical extraradical fungal biomass, i.e. extraradical hyphae and auxiliary cells, produced. The critical extraradical biomass was reached with 1360 ± 625 cm extraradical mycelium length and 501 ± 196 auxiliary cells, recorded at 14 wk (the end of the lag phase). In addition, the sporulation peak occurred when both extraradical mycelium and auxiliary cells reached the plateau phase. It is noteworthy to observe that species producing large spores in ROC such as *Glomus caledonium* (Karandashov et al. 2000, Declerck et al. 2001), *Acaulospora rehmii* (Dalpé & Declerck 2002), *Gigaspora margarita* (Diop et al. 1992, Tiwari & Adholeya 2002) necessitate a longer period for spore initiation than species with small spores such as *G. intraradices* (Declerck et al. 2001), *G. versiforme* (Declerck et al. 1996), or *G. proliferum* (Declerck et al. 2000). This could presumably be related to the high amount of resources needed for the full-size development and maturation of large spores, therefore requiring a higher critical extraradical fungal biomass than small spores. This was earlier hypothesized by Diop et al. (1992) with a *Gigaspora* species cultured on Ri T-DNA transformed carrot roots. These authors demonstrated that sporulation took place after establishment of an extensive mycelium biomass (approx. 1 mo) and before root growth stopped (approx. 4 mo). From the intense cytoplasmic streaming observed in young and old cultures, it seems realistic that the fungus could mobilize resources stored in the mycelial structures for sporulation. The fungus converts sugars taken up in the host root into lipids, which are then stored or exported to the extraradical mycelium where they are stored or metabolised (Pfeffer et al. 1999). During root ageing and concomitant decline in resources availability within roots, spore production may be aided by the material stored in the mycelial structures. Auxiliary cells in Gigasporaceae (Pearson & Schweiger 1993, 1994) and material-rich hyphal swellings in *Acaulospora rehmii* (Dalpé & Declerck 2002) and *Glomus* species with large-size spores such as *G. caledonium* and *G. monosporum* (Dalpé & Declerck 2002) were attributed a potential transitory carbon storage function possibly supporting sporulation. Although speculative, the abundance of auxiliary cells (approx. 700 per Petri plate), the high positive correlation between auxiliary cells number and spore production during the phases of exponential culture development and plateau, and the late decline in auxiliary cells number paralleled to the continuous increase in spore production enumerated in our experiment, coupled to the richness in lipids (Jabaji-Hare 1988), may possibly be interpreted as a transitory structure of colony development insuring energy storage to support further sporulation.

Considering one spore and one compartment of an auxiliary cell perfect spheres of radius 0.188 and 0.031 μ_m respectively (for details in spore dimensions, see de Souza & Declerck 2003) and considering an average of 12 (range 2–22) compartments per auxiliary cells and a conversion rate of 1:1 auxiliary cell:spore, it would be necessary to use the resources stored in 19 auxiliary cells to form one spore. Thus, using that rough calculation the resources stored in the auxiliary cells would allow the formation of approximately 36 spores per Petri plate, which is close to the average 42 spores enumerated in our experiment. In parallel to this hypothesis, one may also suggest that species which sporulate gradually during a long period (an average of 26 wk before reaching the plateau in the case of *S. reticulata*) may deplete the resources of hyphae within roots at a slower rate than species which sporulate excessively in a short period such as *G. intraradices* and *G. proliferum* (6–15 wk before reaching the plateau) (Declerck et al. 2001), an hypothesis earlier suggested by Abbott & Robson (1981). Auxiliary cells colonization potential Auxiliary cells were formed within the mycelium, irrespective of root colonization; at the root surface; and, for some, in the first layers of root cells (de Souza & Declerck 2003). They are produced by the non intraradical vesicle producing family Gigasporaceae, whereas they are absent in the Glomeraceae and Acaulosporaceae, which do produce intra-root vesicles, and also absent in the Paraglomaceae and Archaeosporaceae which produce neither auxiliary cells nor intra-root vesicles. However, there is no indication that auxiliary cells are substitutes to the internal vesicles (Morton & Benny 1990). Even though they exhibit hyphal re-growth, as shown for *Gigaspora margarita* (Pons & Gianinazzi-Pearson 1985) and *Scutellospora reticulata* in the present study, no root colonization, either in vivo or in vitro, was observed in our experiment. Therefore, they appeared functionally, at least for fungal colony initiation, different from the internal vesicles, which are able to germinate, re-colonize roots, and complete the fungal life cycle (Strullu & Romand 1987, Declerck et al. 1998). However, further experimentations using long pieces of hyphae bearing numerous auxiliary cells, i.e. not isolated auxiliary cell, like we used here, should be tested for possible synergistic ability for root colonization.

CONCLUSIONS

This study has provided clear indication on the sequential development of the extraradical mycelium of *Scutellospora reticulata*. It suggested that root growth and extraradical mycelium development are intimately associated in a sequence were both grew concomitantly during active root growth, followed during root aging by a period in which only the fungus continues to develop. The constant cytoplasmic streaming observed in hyphae of cultures with old non-growing roots demonstrated the vitality, at least

partly, of the mycelial network and its capability to translocate elements. The continuous spore production is, therefore, dependent on a critical extraradical mycelium biomass and on the re-allocation of resources from both the intraradical mycelium and the auxiliary cells via the hyphal network.

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