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

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Chapter 4

Life History Strategies in Gigasporaceae: Insight from Monoxenic Culture

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INTRODUCTION

During the past years there has been an increased interest in the role of arbuscular mycorrhizal (AM) fungal biodiversity for the functioning of terrestrial ecosystems and in the application of AM fungal technology for agricultural and land rehabilitation schemes. However, one major bottleneck in AM research is the lack of knowledge on ecology, and in particular on life history strategies (LHS) among the different AM fungal families (Hart et al. 2001; Hart and Klironomos 2002).

The LHS of an organism is a product of its evolutionary past, and is expressed in the fungal life cycle, i.e. patterns of growth, differentiation, storage and, especially, reproduction (Begon et al. 1996). Different LHS patterns seem to have been developed by AM fungi (Pringle and Bever 2002; Hart et al, 2001) and knowledge about these patterns is fundamental to our understanding of AM fungi mode of coexistence, optimal performance, soil competition, survival and perennity. Most important, using the LHS patterns is possible to predict the environment that the organism performs best.

One important characteristic of AM fungi is their capacity of coexistence. For instance, why might several AM fungi infect a single host plant? Host specificity in AM fungi is considered weak (Sanders, 2003). All AM fungi colonize essentially the same niche within plant roots, where, in theory, they form arbuscules, which is a key structure for the functionality of the symbiosis (Harrison 1999). For example, van Tuinen et al (1998) detected 3-4 AM fungi species from 3 different genera within 1 cm root pieces in a microcosm experiment. Furthermore, under field conditions, molecular techniques have revealed rather complex AM fungi coexistence in single plant species or individuals (Helgason et al. 1998; Kowalchuk et al. 2002; Husband et al. 2002; Vandenkoornhuyse et al. 2003). The selective forces that allow and shape such complex coexistence remain mostly at the speculation status.

Patterns of life history strategies are considered to obey to a set of rules related to organism size, rates of growth and development, and mainly reproduction. The cultivation system of AM fungi in monoxenic is explored here as a tool to study these issues. The chapter focused on the family *Gigasporaceae* with useful comparisons to *Acaulosporaceae* and *Glomeraceae* life history patterns.

THE FAMILY *GIGASPORACEAE* AND ITS OCCURRENCE

The family *Gigasporaceae* comprises the two genera *Gigaspora* and *Scutellospora*, with approximately 8 and 33 described species, respectively. *Scutellospora* is more diverse than *Gigaspora* in terms of described species, but also of spore morphological characteristics (Morton 1995), and

occurrence in natural ecosystems. Members of this family possess unique intra- and extraradical mycelium morphologies characterised by the absence of intraradical vesicle and the differentiation of extraradical auxiliary cells (AC).

Gigasporaceae occur in terrestrial ecosystems usually at low spore densities and high species richness in diverse and or stable plant ecosystems (Siqueira et al. 1989; Lovelock et al. 2003; Zhao et al. 2003). In coastal sand-dune ecosystems, *Gigasporaceae* can be dominant (Stürmer and Bellei 1994; Beena et al. 2000 and references cited therein), while in agricultural soils cultivated with annual crops and arid ecosystems, they tend to be less abundant or even absent (Sieverding 1991; Helgason et al. 1998; Stutz et al. 2000; Jansa et al. 2002). An adequate explanation for these patterns has yet to be found. Evidences obtained from monoxenic AM fungi cultures were used to clarify these patterns (see section 4).

LIFE CYCLE

The AM fungi life cycle can be divided in three main steps: (1) Pre-symbiotic phase and establishment of the symbiosis. It involves propagule activation, host search, appressorium formation, root penetration and arbuscule formation; (2) Vegetative growing phase; (3) Reproductive phase. The steps 2 and 3 occur almost concomitantly, because in general AM fungi show an iteroparous reproductive phase. Although simple, there are evidences that different AM fungi are using different strategies to accomplish each of these steps.

Pre symbiotic phase.

Propagules.

The production of infective propagules insures the survival of the AM fungi in times of adverse conditions. Therefore, they are the most important elements of the fungal life cycle. In AM fungi three types of propagules are generally considered: (1) spores, (2) intraradical mycelium within colonised roots and (3) extraradical mycelium. Spores are the most effective propagules for *Gigasporaceae* isolate, while *Acaulosporaceae* and *Glomeraceae* have been demonstrated to induce new colonisation using all three sources of inoculum (Biermann and Linderman 1983; Brundrett et al. 1999; Klironomos and Hart 2002). However, it has been suggested that colonised roots of *S. calospora* and *S. heterogama* are able to trigger plant root colonisation *in vivo* (Tommerup and Abbott 1981; Braunberger et al. 1996; Klironomos and Hart 2002). Intraradical sporulation has been observed from field and pot-culture *Gigaspora* and *Scutellospora* species (Schenck and Perez 1990; INVAM

http://invam.caf.wvu.edu; Dalpé, unpublished). One isolate of *Gi. margarita* was reported to produce 10-15% of the total number of spores intraradically under monoxenic cultures (Gadkar and Adholeya 2000). Thus, it might be possible that intraradical spores were the cause of the infective capacity of colonised roots of some *Gigasporaceae*. The infective capacity of the extraradical mycelium of *Gigasporaceae* has only been demonstrated *in vivo* with *S. calospora* isolates from Australia (Tommerup and Abbott 1981), while in some other cases, colonisation failed (Biermann and Liderman 1983; Klironomos and Hart 2002; Declerck et al. 2004). Declerck et al. (2004) reported, under monoxenic culture conditions, the hyphal re-growth from individual AC of *S. reticulata* and they suggested that using long pieces of intact mycelium harbouring several AC might possibly induce colonisation. The apparent discrepancy in these results might be explained by differences in the integrity of the mycelium used to perform these experiments and the amount of resource available in the mycelial structures. For instance, de Souza and Declerck (2003) observed that, in monoxenic culture, young AC contained lipid drops, while older ones appeared empty.

A comparison of spore diameter of species in the families *Gigasporaceae, Acaulosporaceae* and *Glomeraceae* (average diameter 314, 158 and 127 µm respectively), shows that *Gigasporaceae* species produce, in general, large spores (Data from Schenck and Perez (1990), *Glomus* species from the former *Sclerocystis* genus were not included). Common traits related with the spore quality are germination rates, survival dormancy, and size. Large spore must contain more resources to support multiple germinations, mycelial growth and to sustain the metabolism while searching for a host.

Spore germination, dormancy and life span

The germination process in *Gigasporaceae* is linked with the spore wall organisation. (Walker and Sanders 1986; Spain et al. 1989) Multiple germinations were reported for *Gigaspora* species (Koske 1981a; Giovannetti et al. 2000), reaching up to forty successive germinations for single spores of *Gi. margarita* under *in vitro* conditions (P. Jargeat, Pers. Comm.). If the germination tube (GT) does not meet a root, then the cytoplasm may retract (Beilby and Kidby, 1980).

Spore germination does not require external factors other than humidity and temperature to germinate. Germination rates reached a 80-100% level for *Gigaspora* isolates (Koske 1981b; Bécard and Piché 1989; Diop et al. 1992; Romero and Siqueira 1996; Maia and Yano-Melo, 2001) compared to a 60% with *Scutellospora* ones (de Souza unpublished). In the latter case, spores might enter into dormancy a phenomenon frequently associated with a higher survival capacity. *Gi. gigantea* and *Gi. margarita* spores obtained in monoxenic cultures exhibit a dormancy that does not exist when the spores are produced on whole plants (Séjalon-Delmas unpublished). With *Gigaspora* strains, a 3 weeks cold treatment may relieve the dormancy (Jargeat and Séjalon-Delmas, unpublished). The life

span of *Gigaspora* spores has been estimated to be up to 5 months under natural growing conditions (Lee and Koske, 1994; Pringle and Bever 2002). A broad comparison of germination and survival capacity of different *Gigasporaceae* species is still lacking, and it would be interesting to study these traits using a phylogenetic framework, based on morphological and molecular data.

Symbiotic Vegetative and Reproductive growing phases

In relation to LHS, two interconnected characteristics revealed by studies of *Gigasporaceae* species under monoxenic culture are highlighted here: the colonisation pattern and development and maintenance of arbuscules. In addition, hyphal healing mechanism and anastomosis were also discussed.

Colonisation pattern

Gigasporaceae seems to be slower root colonisers than *Glomeraceae* and *Acaulosporaceae* species (Brundrett et al. 1999; Santos et al. 2000, Tiwari and Adholeya 2002). Hart and Reader (2002) compared the colonisation strategy of 21 isolates from the families *Acaulosporaceae* (4), *Gigasporaceae* (5) and *Glomeraceae* (12) using 4 different host plants under pot culture conditions. They reported that *Glomeraceae* isolates colonise roots before *Acaulosporaceae* and *Gigasporaceae*, and the results were independent of the host plant used.

Under monoxenic culture, *Gigasporaceae* is able to contact and colonise a root explants within 3 to 10 days, after coming in the vicinity of an active root. However, the exponential extraradical mycelium growth was only observed to begin 3-5 weeks after colonisation, with *S. reticulata* (Declerck et al. 2004). An interesting characteristic of *Gigasporaceae* behavior is that they increase the overall colonisation (number of infection points) and extraradical mycelial growth exponentially when the root activity has decreased or ceased (Diop et al. 1992; Declerck et al. 2004).

Maintenance of Arbuscules

Arbuscules are considered short-lived (1 to 3 weeks) fungal structures found preferentially in young thin roots during early stages of root colonisation (Smith and Read 1997; Harrison 1999). However, arbuscules differentiated by some *Gigasporaceae* isolates were found, surprisingly, to occur in hairy root cultures that had ceased growth for several months as observed for *Gi. rosea* and *S. reticulata* (Diop et al. 1992; Declerck et al. 2004). The formation of arbuscules is controlled by the host plant's genetic machinery, and the number of differentiated arbuscules has been found tributary of plant identity, of the availability of nutrients, and of the fungal partner (Smith and Read 1997; Harrison 1999). Are arbuscules controlled in a different way in *Gigasporaceae* monoxenic cultures and in pot

culture? Morton provides information regarding this question via the INVAM website (http://invam.caf.wvu.edu). In *Gigasporaceae*, arbuscules are "…in pot cultures, still abundant long after plants (and roots) have ceased growth", and the persistence of the total arbuscular network in mycorrhizal roots of pot cultures is longer for species of the family *Gigasporaceae* than those of *Glomeraceae*.

ANASTOMOSIS AND HYPHAL HEALING MECHANISM (HHM)

Anastomosis is a process of hyphal fusion between compatible fungi resulting in the formation of mycelial networks and allowing exchange of genetic material. Tommerup (1988), described anastomosis in *G. monosporum* and *A. laevis*. This author demonstrated the absence of anastomosis between different species and recorded anastomosis events only between isolates of the same species. These results were confirmed on different *Glomus* strains (Giovannetti et al. 2003). Regarding *Gigasporaceae,* no anastomosis could be found on germinating spores of *Gi. rosea* and *S. castanea* (Giovannetti et al. 1999). Later, anastomosis was observed in *S. reticulata* growing under monoxenic culture, but it was restricted to branches of the same hypha and only observed in thin hyphae linked with branch absorbent structures and never between runner hyphae (de Souza and Decleck 2003). Recently, the significance of anastomosis formation and HHM for functionality and integrity of the AM fungal mycelium network was studied by de la Providencia et al. (2005). They compared anastomoses and HHM in four *Glomeraceae* (*Glomus intraradices* MUCL43194 and MUCL43204, *G. proliferum* MUCL41827 and *G. hoi* MUCL45686) and three *Gigasporaceae* (*Gi. margarita* BEG34, *Gi. rosea* BEG9 and *Scutellospora reticulata* CNPAB11) strains cultivated monoxenically. Despite of the higher hyphal density of *Gigasporaceae* (92.23 cm cm^{-3}) in relation to *Glomeraceae* (55.25 cm cm^{-3}) strains studied. Anastomoses formation in *Glomus* strains was seven times higher per hyphal length than in *Gigasporaceae*. Besides, anastomosis in *Gigasporaceae* more often concerned hyphal bridges within the same hyphae, which probably are related with the HHM. While any of the *Glomeraceae* strains studied were able to do anastomosis in the same hyphae. These results demonstrate clear differences in these families regarding the maintenance of their mycelium.

The hyphal healing mechanism (HHM) has been reported in AM fungi since Gerdermann (1955). More recently, Kang-Hyeon et al. (1994) reported wound healing in *Gi. margarita, S. verrucosa* and *S. heterogama* grown on agar media. Artificial wounding (with a razor blade, Fig 5.1) performed on 4-5 days old GT of *Gi. rosea* resulted in dead section of hyphae. The injured section darkened and separates from the living hyphae by a septum, within 15 minutes. After 4 hours, two branches grows from both sides of the section, just behind the septa, one branch becomes dominant, in

all cases it is the branch emerging from the hypha linked to the spore. Branches are attracted to each other and a fusion tip to tip is observed, around 7 hours after wounding. Average attraction distance may be calculated with the formula: ∑distance/ number of tip to tip contact, established for anastomoses recording. In *Gi. rosea* this average attraction distance is of 396 µm and 512 µm for *Gigaspora gigantea.* These values are much higher than for other fungi like *Rhizoctonia oryzae* (150 µm) (Kurshed et al, 1993). When the distance between the two parts of hyphae to repair is too long, several branches may be formed. But in normal case, 100% of severed germ tubes are repaired. Some failure in the repair mechanism may occur, when, for example, the wounding is too close to the apex, one lateral branch of the hypha becomes simply dominant, replacing the injured apex. When the wounding occurs too close to the spore, there is no repair attempt; and a new germ tube is formed.

The characterisation of the mode of action and efficiency of the HHM can give clues about organisms LHS, because K-strategists and or stress resistant organisms are expected to evolve better defence and repair mechanisms than r-strategists (Pianka 1970). he study of de la Providencia (2005) found evidences for highly distinctive mechanism of repair damage between the families *Gigasporaceae* and *Glomeraceae*. In the *Glomus* strains the injured hypha form several branches that could fuse to reconnect the hyphal network or to colonize a root. In the *Gigasporaceae* strains, the healing mechanism operates exclusively to repair the damaged hyphae and presented 100% efficiency at short distance injuries. In *Glomus* species this mechanism could increase the capacity of the fungus to colonize the roots due to the proliferation of new hyphae at the apex of the cut hyphae but could also reconnect the affected area by networking several hyphae in relative small vicinity. In *Gigaspora* and *Scutellospora*, the healing process is presented as the most probably means of maintaining the viability of the hyphae in adverse conditions (de Souza & Declerck, 2003), being a mechanism for hyphae to survive (Bago *et al*., 1999).

GENETIC DIVERSITY AND PHENOTYPIC VARIATION

The genetic diversity and phenotypic variation in AM fungi needs to be understood within a phylogenetic context to be linked with life-history traits. In addition, the species concept in AM fungi must be refined to address ecological and evolutionary questions. In this sense, the cultivation of AM fungi in monoxenic culture offers an excellent basis to undertake these studies. Koch et al. (2004) published the first article exploring the potential of monoxenic culture to study quantitative genetic traits in a population of AM fungi obtained from the field. To relate phenotypic traits with life history they measured hyphae growth rate and spore production between different isolates. The maintenance

of isolated individuals from a population in a constant environment (monoxenic culture) for several generations, allowed them to direct link phenotypic variation with variation in quantitative genetic traits. In addition, they took advantage of the monoxenic system to produce fungal cultures free from alien DNA, in quantity and quality necessary for applying genomic fingerprint techniques such as Amplified Fragment Length Polymorphism (AFLP^{TM}) (Koch et al. 2004). Using this approach would be possible to track differences in LHS of close related species and understand constrains related with speciation in this fungi.

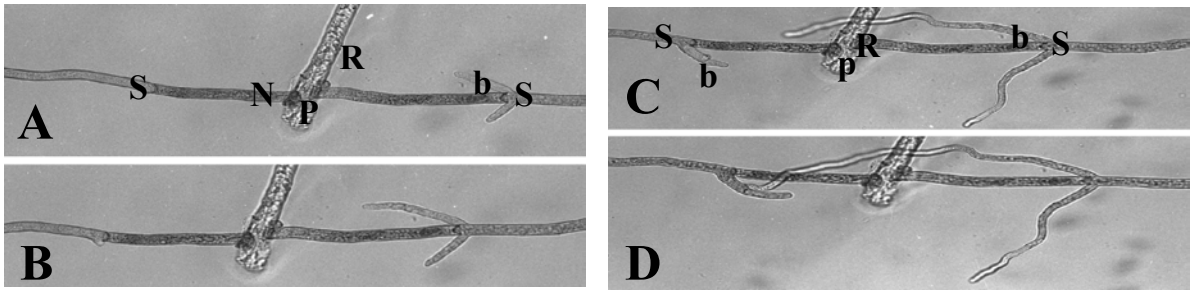

Fig. 1. Hyphal healing mechanisms in germinating hyphae of *Gigaspora gigantea* and *G. rosea*. A. Few minutes after wounding, necrosis appears at both hyphal ends. Some cell materials form a plug, which obstructed the wounded hypha preventing cytoplasmic leakage. 15 minutes later, a septum forms to isolate the hyphal necrotic ends; B. Emergence, after 4h, of two lateral branches from one live section. Note the difference of growth of the two branches; C: Growth of the two lateral branches towards a new single branch emerging at the opposite side of the injured hyphae. D. Both ends are reconnected (16 h). Letters inside the figures are showing: b. lateral branches; N. necrotic part of the hypha; P. plug; R. scrape of the razor-blade; and S. septum. From N. Sejalon-Delmas unpublished.

Vegetative compatibility test (VCT)

In terms of species concept VCT can be used to determine species boundaries in fungi. In *Gigasporaceae,* spontaneous pairing between germinating spores has never been observed. For this reason, Sejalon-Delmas developed a simple and efficient VCT method, based on wound-healing mechanism. The method consists in germinating two spores side by side. After germination, the apices of the GT of the two spores are cut off. The spore A remains in the plate and its cut GT apex is removed, while the spore B is removed and its GT apex remains in the plate. When the two spores are compatible, lateral branches develop from each part forming a connection bridge. When somatic crossing experiments involved daughter spores from a monosporal monoxenic culture, 80% of fusions were obtained. This result is slightly lower than the percent of repair observed for HHM. The 20% of failure in the crossings may be due to a traumatic cutting. Somatic crossings were never observed between spores from different species and seldom between spores originating from different

monoxenic cultures (Sejalon-Delmas unpublished). These results strongly suggest that hyphal fusion in *Glomeromycota* is genetically controlled. It is interesting to notice that in *Glomus* like in *Gigaspora* genera, no tropism occur between hyphae of different spores, incompatibility response was represented by protoplasm retraction and septum formation in the approaching hypha, prior to any physical contact (Sejalon-Delmas unpublished).

LIFE HISTORY STRATEGY (LHS) OF GIGASPORACEAE, AS REVEALED USING MONOXENIC CULTURES

The few available data for comparative analyses of *Gigasporaceae* and *Glomeraceae* LHS were obtained from experiments focused on growth kinetics and development characteristics such as the timing of the first daughter spore produced, the rate of sporulation, and the duration of the reproductive phase. These characteristics differed between *S. reticulata* and the following *Glomus* species: *G. caledonium, G. intraradices* and *G. proliferum* (Fig. 2 A). *G. proliferum* and *G. intraradices* formed their first daughter spores after one week in culture, and *G. caledonium* after two weeks, while *S. reticulata* produced its first daughter spore only after 12 weeks of continuous culturing. *G. caledonium* and *G. intraradices* reached the stationary phase after 15 weeks and *G. proliferum* after 17 weeks. In contrast, *S. reticulata* continued to produce spores until week 33, i.e. more than eight months after starting the culture (Fig. 2 A).

Life histories patterns are often related to variation in reproductive activity, or reproductive effort that measures the amount of available resources allocated to reproduction over time (Begon et al. 1996). However reproductive effort is difficult to measure. One simple way to have an idea about the reproductive effort is by calculating the Malthusian fitness (MF). The MF, in this case, compared the instantaneous change of spore production overtime in relation to the starting inoculum (Fig 2 B). For a discussion about application of MF to filamentous fungi see Pringle and Taylor (2003). The evolution of the MF in *S. reticulata* was clearly different from the *Glomus* species (Fig. 2 B). The same trend was observed in other species of these two families cultured under monoxenic ROC (Fig. 3). *Gigasporaceae* species studied in monoxenic culture have shown a short overlap of sporulation with the active growing phase of the roots, while *Glomus* species sporulate concomitantly with the root growth (Fig. 3). These observations suggest that, for these species cultured under monoxenic

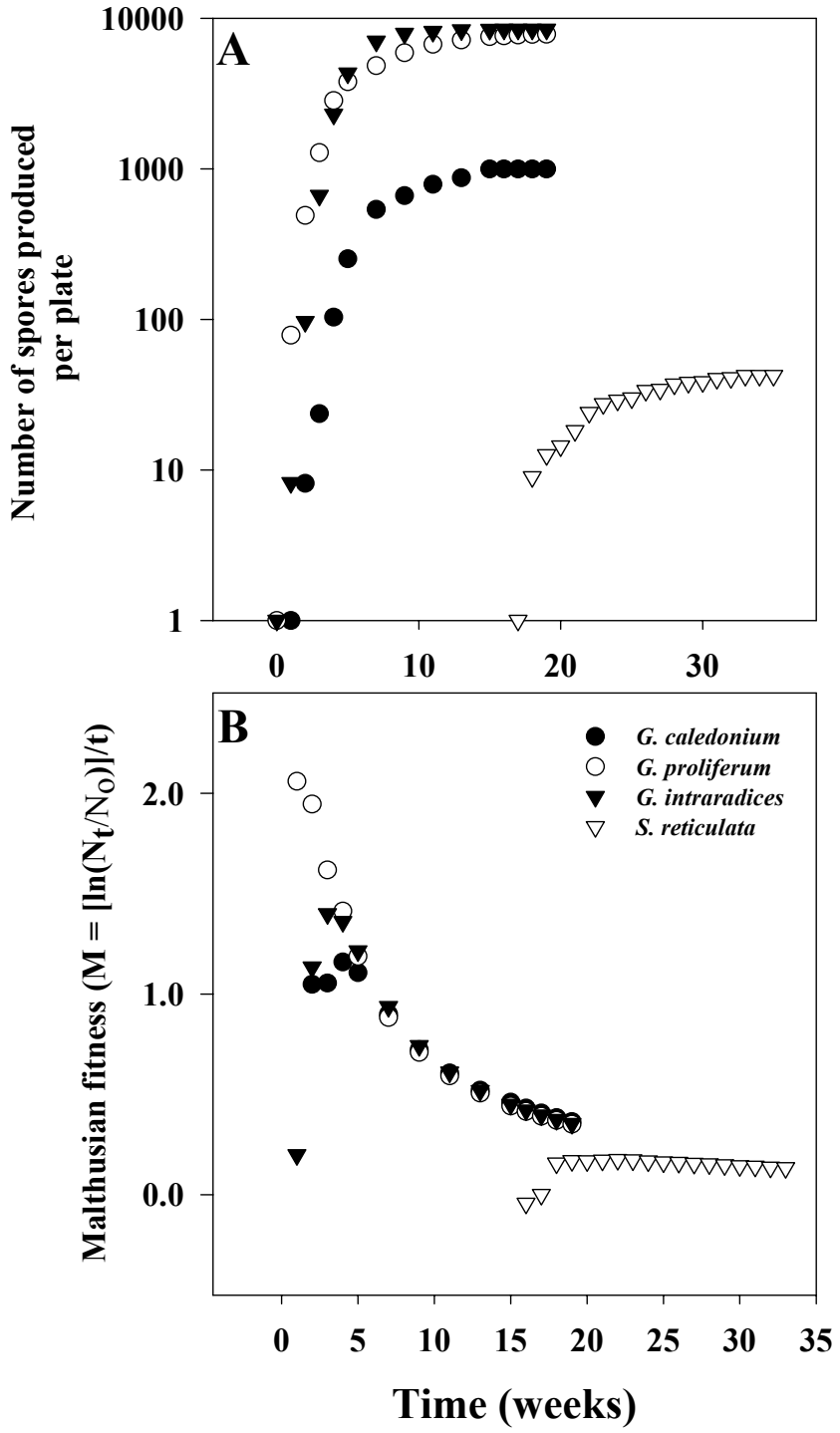

Fig. 2 A. Sporulation dynamics of three *Glomus* spp. and *Scutellospora reticulata* under ROC conditions; B. Weekly evolution of the Malthusian Fitness for the sporulation dynamics showed at A; N_o was 1, 10, 10, and 1 for *G. caledonium*, *G. proliferum*, *G. intraradices* and *S. reticulata*, respectively. Modified from Declerck et al. 2001 and 2004.

culture, *Gigasporaceae* and *Glomeraceae* concentrate their reproductive efforts at different times. Where *Gigasporaceae* favor the somatic growth and *Glomeraceae* the reproduction.

The reproductive phase in *Gigasporaceae* seems to be linked with a critical extraradical mycelium biomass. For *S. reticulata* the first daughter spores were produced after 12 weeks, when a biomass of 1360 ± 625 cm of extraradical mycelium length and 501± 96 AC was reached (Declerck et al. 2004). *Gi. margarita* and *Gi. rosea* produced the first daughter spores after 8 to 10 weeks of culturing, however, the reproductive phase can be extended over one year suggesting a long mycelium life span (Diop et al. 1992; Gadkar and Adholeya, 2000).

An interesting point, comes from the fact that the majority of fungal biomass, including spores, obtained from *Gigasporaceae* isolates was generated after the root had ceased growth. At that time, one part of the resources in the medium was already consumed by the root culture (Diop et al. 1992), indicating a capacity of *Gigasporaceae* to live and reproduce with a small portion of the resources available (Fig. 3). This scenario is similar to the conditions expected for competitive species (Grime 1979), referring the K-strategist concept (McArthur and Wilson 1967; Pianka 1970).

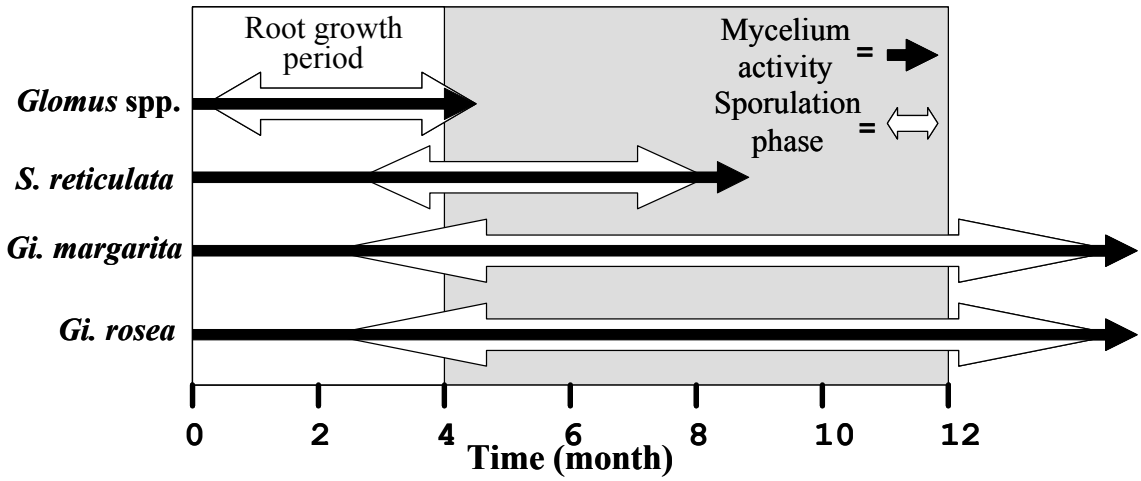

Fig. 3. Schematic representation of mycelium development and activity (black arrow) and sporulation (double head arrow) periods of various *Glomus* and three *Gigasporaceae* species under ROC conditions. *Glomus* and *Gigasporaceae* species represented were *G. caledonium*[b]; *G. clarum*[d], *G. etunicatum*[g], *G. fasciculatum*[a], *G. intraradices*[b], *G. macrocarpum*[a], *G. proliferum*[b], *G. vesiforme*[a], *Gi. margarita*[f]; *Gi. rosea*[e]; *S. reticulata*[c], respectively. (a) Declerck et al. (1998); (b) Declerck et al. (2000); (c) Declerck et al. (2004). (d) de Souza unpublished; (e) Diop et al. 1992 (f) Gadkar and Adholeya (2000); (g) Pawlowska et al. (1999).

The comparative sporulation rates of *Gigasporaceae* and *Glomus* species revealed a clear negative correlation between the size and the number of differentiated spores (Fig. 4), which is a typical tradeoff (Begon et al. 1996). One consequence of a higher allocation of resource for a small

progeny is that the offspring will have a higher quality or vigor and consequently higher chances of survival than species that produce low energy cost propagules.

Coexistence and competition experiments under dixenic culture

The direct assessment of coexistence and competition of AM fungi under dixenic culture can be exemplified by the completion of *Gi. margarita* and *G. intraradices* life cycles when co-cultured on a excised root culture (Tiwari and Adholeya 2002). The sporulation patterns observed in that dixenic culture were similar to the patterns reported for monoxenic cultures, i.e. *G. intraradices* started and ceased to form spores earlier than *Gigaspora*. The assessment of fungal competition can be carried out by comparing, for example, the sporulation of two AM fungi growing in monoxenic and dixenic culture. Such system can also be adjusted to assess the effect of predators on different AM fungal species, for instance collembolans. The possibility of studying competition under monoxenic culture might facilitates the implementation, execution and quantification of experiments by allowing a precise control of the resources used, easy maintenance, and direct quantification overtime.

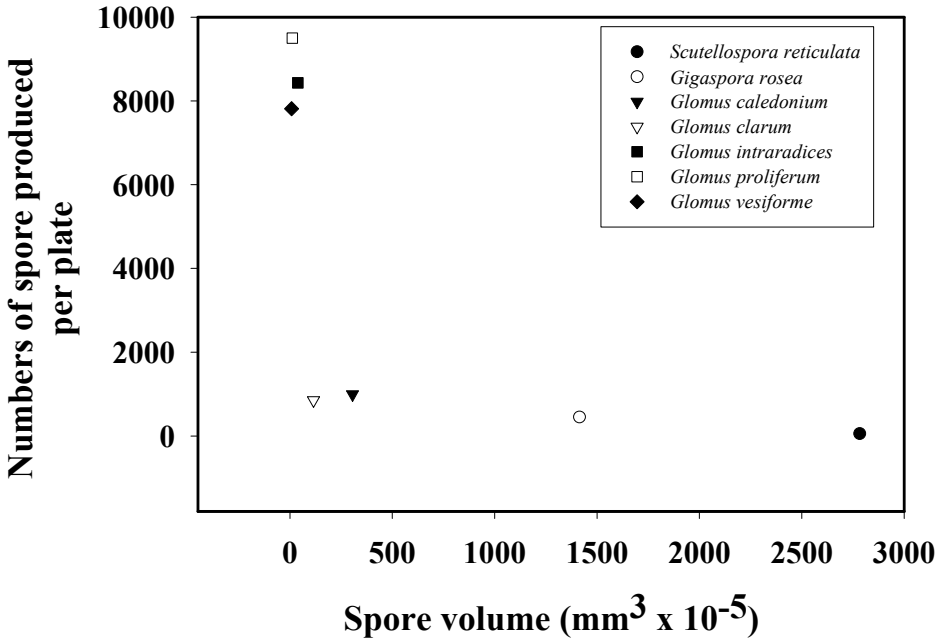

Fig. 4. Spore production in relation to spore volume of 5 *Glomus* and two *Gigasporaceae* under monoxenic culture conditions (data from de Souza & Berbara unpublished, Declerck et al. 2001, 2004; Diop et al. 1992).

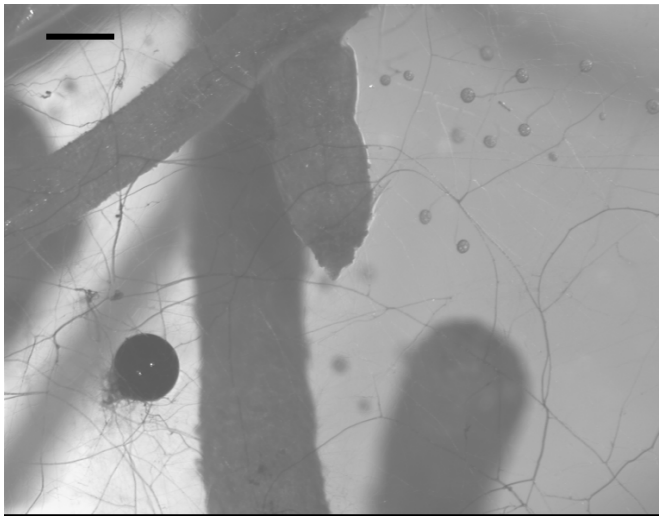

Fig. 5 Coexistence of *Scutellospora reticulata* CNPAB11 and *Glomus intraradices* MUCL 43194 under ROC. Note the difference in size between the *Scutellospora* (large dark spore) and *Glomus* (small grey spores). The majority of the mycelium showed is from *S. reticulata*. Bar = 400 μm. (de Souza unpublished).

Ecological implications of the Gigasporaceae life history strategy

The *Gigasporaceae* isolates studied in monoxenic ROC exhibited several traits (investment in somatic growth rather than in reproduction, development of large spore size and few offspring) suggesting that they are adapted to live in stable ecosystems, where inter and intra species competition is high for resources, and somatic growth is favoured above reproduction. The fast sporulation of *Glomus* isolates differentiating single spores in the soil followed the opposite trend, i.e. they seemed to be adapted for growth in disturbed ecosystems that are rich in available resources, which favour reproduction over somatic growth. It is important to remember the polyphyletic origin of the genus *Glomus* (Schwarzott et al. 2001), which implies that different subgroups have different evolutionary histories and potentially different LHS. For instance, Brundrett et al. (1999) reported that sporocarp-forming *Glomus* species needed much longer cultivation periods under pot culture conditions to produce spores than *Glomus* species that formed single spores in the soil, and this time was even longer than that observed for species of *Gigasporaceae* and *Acaulosporaceae*.

Life history traits reported for *Gigasporaceae* may imply that those species would be negatively selected in agricultural fields cultivated with annual crops and submitted to frequent plowing that disrupts the mycorrhizal mycelium (Jasper et al. 1989; Fairchild and Miller 1990). On the other hand, fast sporulating *Glomus* isolates would show the opposite trend i.e. positive selection. Another

important advantage of *Glomus* in adapting to agricultural soils is their ability to survive and propagate well using intraradical vesicles, which are formed earlier than spores (see chapter 4).

The root growth and AM fungi colonisation consist in a dynamic process where new and old colonisation stages exist in a single root system. Consequently, the coexistence of *Gigasporaceae* and *Glomus* isolates in one root should be logically facilitated by different LHS that allow fungi to explore different phases of their host's life cycle. The coexistence between *Gigasporaceae* and *Glomus* species can be directly observed under monoxenic culture (Fig. 5). Within the context of this hypothesis regarding coexistence mechanism, *Glomus* isolates first colonised an active growing root, differentiate arbuscules that subsequently disappear with root ageing; meanwhile the colonisation evolved forming vesicles. Later *Gigasporaceae* colonise the same root fragment differentiating new arbuscules and expanding colonisation to other roots and soil. The microcosms experiment of van Tuinen et al. (1998), seem to support this hypothesis. In their experiment, two *Gigasporacaeae* (*Gi. rosea* and *S. castanea*) were usually found only co-colonising a root fragment together with a *Glomus* isolate. They suggested a mechanism of synergism between the different fungi for colonisation. Interestingly, all four species are able to grow and sporulate when cultivated as single species.

CONCLUSIONS

A tremendous amount of knowledge remains to be acquired through the practice of monoxenic and dixenic cultures of AM fungi. Several of the avenues already explored using monoxenic cultures can be used as stepping-stones for future investigations. Whatever discipline is concerned, ecology, genetic, physiology, the cultivation of AM fungi associated with transformed roots appears to be useful for investigations for all of them. For example, the advances in genetics of AM fungi will sooner or later be involved in mutant comparisons easily foreseen the advantageously of using monoxenic cultures. So as understand the functional significance of AM fungi genetic variation as well as the cost and benefits of key phenotypic traits. The evaluation of fungicides, the test of soil and plant pollutants, the synergy with biocontrol agents, and the behaviour of fungi predators are all practical research avenues. The framework of research constructed using monoxenic culture allows close follow up and precise measurement of growth dynamic parameters. However, the relevance of collected data to the complexity of the natural community has to be strictly verified using data from various sources, from a diversity of fungal strains and cultivation methods in order to confirm the LHS patterns observed using monoxenic cultures. The cultivation of AM fungi in association with

transformed roots offers a standard way to compare different AM fungi, in monoxenic or dixenic cultures. In addition, this approach allows for detailed observation and long-term experimentations.

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Chapter 5

Morphological, ontogenetic and molecular characterization of *Scutellospora reticulata*, Glomeromycota

de Souza FA, Declerck S, Smit E, Kowalchuk GA. 2005.

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ABSTRACT

The Arbuscular Mycorrhizal (AM) fungus *Scutellospora reticulata* accession CNPAB11 was characterized using morphological, ontogenetic and molecular approaches. Spore ontogenesis was studied using Ri T-DNA transformed carrot roots and observations were compared with those published for eight other, pot-cultured *Scutellospora* species. The sporogenesis of *S. reticulata* exhibited an unreported pattern of outer spore wall differentiation. In addition, Denaturing Gradient Gel Electrophoresis (DGGE), targeting the V9 region of the small subunit nuclear ribosomal DNA (SSU nrDNA), was used to differentiate *S. reticulata* from 16 other *Scutellospora* species and results were confirmed by sequencing analysis. Phylogenetic analyzes, using nearly full length SSU nrDNA sequences, grouped *S. reticulata* in a cluster together with *S. cerradensis* and *S. heterogama*, species that share similar spore wall organization and also possess ornamented external walls. PCR-DGGE and sequence analysis revealed intragenomic SSU nrDNA polymorphisms in four out of six *Scutellospora* species tested, and demonstrated that SSU nrDNA intragenomic polymorphism could be used as a marker to differentiate several closely related *Scutellospora* species.

INTRODUCTION

Species identification and phylogeny of arbuscular mycorrhizal (AM) fungi have traditionally been based on analysis of morphological characteristics of spores and fungal mycelium (Morton & Benny 1990). However, the recent application of molecular phylogenetic analysis, based on small subunit nuclear ribosomal DNA (SSU nrDNA) sequences, has resulted in profound changes in AM fungi classification, with proposal of a separate phylum (*Glomeromycetes*), containing new orders, families and genera (Schüβler, Schwarzott & Walker 2001; Walker & Schüβler 2004).

Nevertheless, an integrated analysis combining developmental patterns during colony growth and spore ontogenesis with molecular phylogenetic analysis has never been performed with AM fungi. The comparative studies of development patterns during a morphostructure ontogenesis are a powerful way to establish homology between morphological character states and achieve a natural classification (Givnish & Sytsma 1997). Such an approach has been successfully used for cladistic analyses of *Scutellospora* species (Franke & Morton 1994; Morton 1995). However, these studies involved soil-based systems that require destructive sampling of the fungal material. One-way forward to overcome this problem is to use the monoxenic culture system to grow AM fungi (see Fortin et al 2002). The

monoxenic culture of AM fungi allows for cultivation, real time observation and precise sampling of fungal material throughout the life cycle. This technique has already been used successfully to study spore ontogeny (de Souza & Berbara 1999) and species characterization using a polyphasic approach (Declerck et al 2000).

Scutellospora belong to the family *Gigasporaceae*, order *Diversisporales*. This genus represents 17% of the known AM fungi species (de Souza, 2000). Recently, a *Scutellospora* species was successful cultured in monoxenic system with transformed carrot roots (de Souza & Declerck 2003). The in vitro culture of *S. reticulata* CNPAB11 was successfully applied to investigate the extramatrical mycelium development, i.e. hyphal morphology and branching (de Souza & Declerck 2003), the dynamics of spore production, and the function of auxiliary cells (Declerck et al. 2004).

The objective of the present study was to provide a thorough characterization of *S. reticulata* and perform an integrated analysis combining developmental patterns during spore ontogenesis with molecular phylogenetic analysis. In addition, the application of PCR-Denaturing Gradient Gel Electrophoresis (DGGE) was tested as a rapid identification tool to discriminate *Scutellospora* species.

MATERIAL AND METHODS

Fungal material

The *Scutellospora* species used in this study are listed in Table 1. The accessions used were obtained from culture collections, except for *S. coralloidea*, which was collected from a trap culture (for site characteristics see de Souza et al. 2004). Spores were extracted from soil using a wet sieving technique and prepared for molecular analyses according to de Souza et al. (2004). From the isolates obtained, only *S. reticulata* was established in monoxenic culture, while the other strains were used for molecular analysis.

Establishment of monoxenic culture of S. reticulata CNPAB11

S. reticulata CNPAB11 was established under monoxenic culture as described by de Souza and Declerck (2003). Briefly, surface-sterilized spores were germinated in water-agar at pH 6.0. After germination, single spores were used to inoculate explants of Ri T-DNA transformed carrot roots. Ten cultures were used as replicates (experimental units) for assessing spore development and size. Each experimental unit consisted of a transformed carrot root organ explants inoculated with a single *S. reticulata* spore in a Petri-plate containing 30mL of the Modified Strullu-Romand (MSR; Declerck et

al. 1998, following Strullu & Romand, 1986) medium, and incubated in inverted position at 27°C for up to eight months.

Sporogenesis in monoxenic culture conditions and data collection

In order to compare the data obtained with monoxenic cultures with the data generated using pot culture conditions, all development stages used here followed the definitions and procedures established by Franke & Morton (1994). To assess subcellular differentiation during spore ontogeny, spores were sampled at different developmental stages, which were differentiated by changes in spore size and color, ranging from white opaque to dark brown (de Souza & Declerck 2003). Juvenile and mature spores were differentiated by color, septa formation in the subtending hypha and by absence of cytoplasmic activity in the sporogenous subtending hypha. Spore dimensions were assessed using 12 randomly chosen, mature spores in each experimental unit. Sampled spores were mounted on microscope slides with Polyvinyl-lactic acid-glycerol medium (PVLG) (Omar et al 1979) and PVLG plus Melzer's reagent (5:1 v:v). Observations were made under a dissecting microscope and under bright field through inverted and common compound microscopes.

Table 1: Species, code, contributor, origin and germplasm collection of the *Scutellospora* spores or isolates used in this study.

Species	Code	Contributor	Origin	Germplasm Bank[a]
S. calospora	BEG32	V. Gianinazi-Pearson	UK	BEG
S. castanea	BEG1	V. Gianinazi-Pearson	France	BEG
S. cerradensis	MAFF520056	M. Saito	Japan	MAFF
S. coralloidea	Trap culture	F. A. de Souza	Brazil	-
S. gregaria	CNPAB7	F. A. de Souza	USA	CNPAB
S. heterogama	CNPAB2	F. A. de Souza	Brazil	CNPAB
S. heterogama	UFLA	J.O. Siqueira	Brazil	UFLA
S. reticulata	CNPAB11	F. A. de Souza	Brazil	CNPAB

[a] BEG = European Bank of *Glomeromycota*, Dijon, France; CNPAB = Empresa Brasileira de Pesquisa Agropecuaria - Embrapa Agrobiologia, Rio de Janeiro, Brazil; MAFF = Ministry of Agriculture, Forest and Fisheries, Ibaraki, Japan; UFLA = Universidade Federal de Lavras, Minas Gerais, Brazil;

Intraradical structure assessments

Colonized roots were harvested from eight-month-old cultures, cleared with 2.5% KOH overnight at room temperature, washed with tap water and soaked in 1% HCl solution for one hour. Roots were then stained with 0.5% Quink Parker blue ink for 20 minutes at 60°C (C. Walker personal

communication). After staining, the roots were rinsed with tap water and preserved in 50% Glycerol solution with 1% HCL and stored at room temperature until required.

DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) analysis of Scutellospora isolates

DNA was extracted from individual spores, of each of the fungi listed in Table 1, according to procedures described previously (de Souza et al. 2004). PCR-DGGE was used to provide rapid fingerprint identification of *S. reticulata* CNPAB11, which was compared to 6 other *Scutellospora* species. The DGGE analysis was performed according to de Souza et al. (2004), which developed a PCR-DGGE system to assess the diversity of *Gigasporaceae* species, targeting the V9 region of the SSU nrDNA using a nested PCR approach. Briefly, in the first PCR round, a set of specific *Gigasporaceae* primers (FM6 and GIGA5.8R) was used. The resulting PCR product was diluted 1:1000 and used as template for a second reaction using the primer NS7 (White et al 1990), with a GC-clamp extension in its 5' end, in combination with the fungal specific reverse F1Ra primer (de Souza et al 2004). Spore-to-spore variation within accessions was analyzed using PCR-DGGE, five separate single-spore DNA isolations for each fungus were compared.

To predict PCR-DGGE separation of sequences deposited in the GenBank® in relation to sequences obtained from *S. reticulata* and *S. gregaria* here, *Scutellospora* sequences that contain the fragment used for PCR-DGGE analysis, were aligned and compared to relate the sequence data with the migration of selected strains observed under DGGE. The PCR-DGGE was also used to study the intraspecific SSU nrDNA polymorphism of the species tested.

Cloning and sequencing

For cloning and sequencing purposes, only the DNA extracted from spores of *S. reticulata* and *S. gregaria* were used. The genomic DNA obtained from individual spores was amplified (for details see de Souza et al. 2004) with the forward primer NS1 in combination with the reverse ITS4 (White et al 1990). After amplification the PCR product was purified and cloned into the pGEM-T easy vector, with *Escherichia coli* strain JM109 used for transformation, according to procedures given by the manufacturer (Promega Benelux, Leiden, The Netherlands). The clones obtained were cultured and, after plasmid extraction used as template for PCR-DGGE and for sequencing. Sequencing reactions were performed for both DNA strands of each clone using the Perkin Elmer Biosystems Big Dye Terminator Sequence Reaction kit (Perkin Elmer, Foster City, Calif.) and the reactions were analyzed on a Perkin Elmer 3700 capillary sequencer (RIVM; Bilthoven, The Netherlands). The primers used

for sequencing were NS1, NS2, NS6, NS7, ITS1, ITS4 (White et al. 1990), NS31 (Simon et al 1992), AM1 (Helgason et al 1998) and F1Ra (de Souza et al 2004).

Selecting polymorphic ribotypes

The PCR-DGGE targeting the V9 SSU nrDNA of *S. reticulata* revealed the occurrence of intraspecific polymorphism between the nrDNA copies. In order to analyze clones for different variants of the ribosomal copies (ribotypes) occurring in *S. reticulata* CNPAB11 and *S. gregaria* CNPAB7, plasmids containing inserts obtained from those strains were used as templates for PCR-DGGE analysis, as described above. To help the selection of different ribotypes, PCR products obtained from original isolates were used as reference to select clones that matched to each of the ribotypes detected in each isolate examined. We analyzed 46 clones of each strain. Plasmids containing the desired inserts were purified using Quiaquick purification columns, and sequenced as described above.

Phylogenetic analysis

Sequences were aligned with those obtained from GenBank® (Benson et al 2003) using Clustal-X (Thompson et al 1997), and the alignment was improved afterwards by visual inspection. Phylogenetic trees were constructed using distance, parsimony and maximum-likelihood (ML) methods. The substitution model was chosen after comparison of 56 different models using the program ModelTest (Pousada & Crandall 1998) version 3.5. The phylogenetic analyses were performed using PAUP* version 4.0 Beta 10 (Swofford 2003).

Nucleotide sequence accession and alignment numbers

The sequences and alignment generated in this study were deposited in EMBL-EBI nucleotide sequence database (http://www.ebi.ac.uk) under the accession numbers AJ871270 to AJ871275 and the alignment number ALIGN_000832

RESULTS

Spore ontogeny

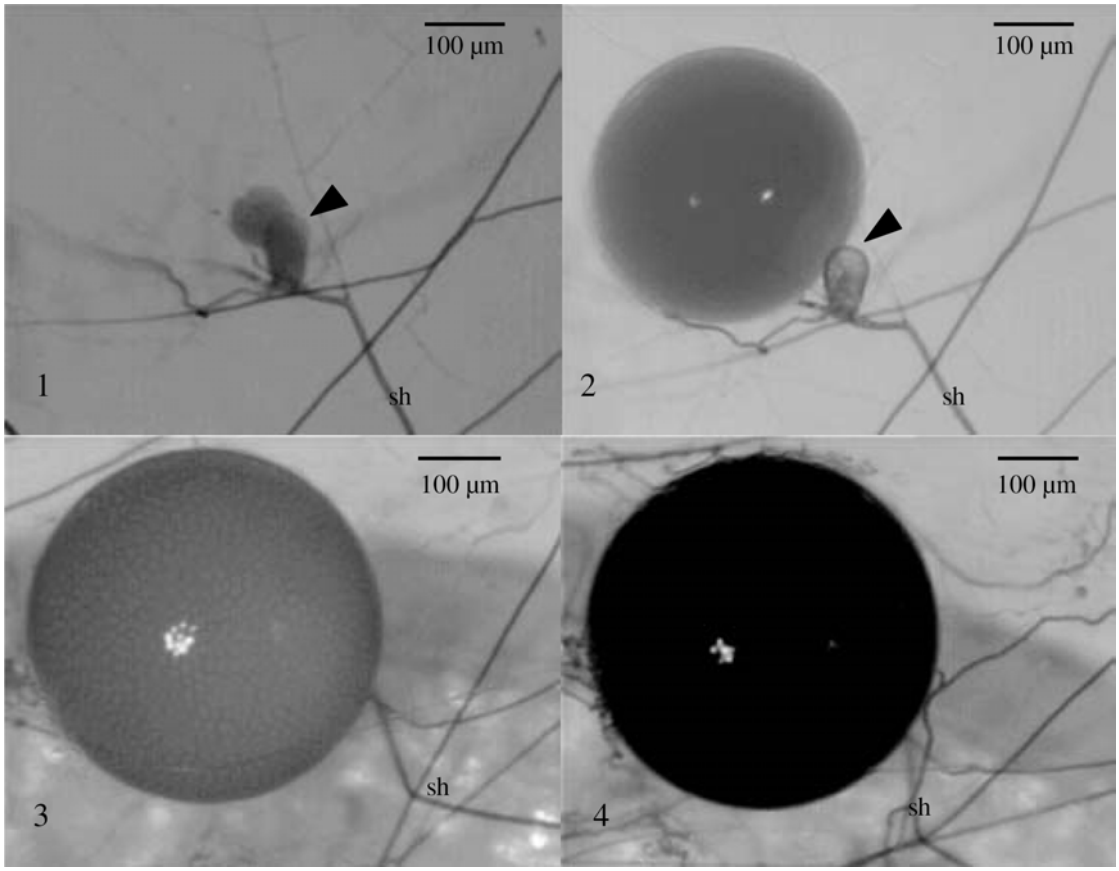
Spore formation in *S. reticulata* takes 6 to 10 days to be completed, and the spore development exhibits changes in size, spore ornamentation, and color (Figs. 1 – 4). Mature spores were globose, with an average diameter of 379µm (280-500; CV= 10.67%, n=120). The main characteristics of the spore morphology and ornamentation were in accordance with the original description of this species (Koske et al 1983). The spore ontogenesis of *S. reticulata* consisted of 6 discrete stages defined by synthesis of specific wall layers (see Fig. 5A, murographic representation). During stage 1, two layers were synthesized (Fig. 6). The external layer was hyaline (0.5 - 0.8 µm thick) and the internal layer was pale yellow (1-2 µm thick) and turned rust-red in Melzer's reagent. At that stage, some spores burst and released their contents into the medium. The second stage began after the spores reached their full expansion in size. The second wall layer increased in thickness (6 - 20 µm thick in PVLG, Fig. 7), with differentiation of external (reticulate Figs. 8) and internal ornamentations (spines, Figs. 9). In both stages, the spores were white to pale white in color (Figs. 1- 2). The third stage was characterized by a change in color from pale white to greenish yellow (Fig. 3), and later to dark red-brown (Fig. 4). At that stage the typical ornamentation of the *S. reticulata* outer wall layer could be seen (Figs. 9-11). In the sub cellular structure, a laminar layer up to 2 - 3 µm in thickness and consisting of very thin adherent sublayers (laminar wall as defined by Walker, 1993), was synthesized (Fig. 9). In the stages 4 and 5 two bi-layered inner wall layers (IW) were synthesized (Fig. 12). These layers were difficult to observe in the accession CNPAB11 used in this study, as they do not detach easily from the spore wall. The IW1 was hyaline (1 to 1.8 µm thick in PVLG), and the IW2 was light yellow (1 µm thick in PVLG). No reaction in Melzer's reagent was observed in these layers. In stage 5, the spores reached their mature dark red-brown color. Stage 6 was characterized by the synthesis of the yellowish-brown germination shield (GS) between IW1 and IW2 (Fig. 13).

Intraradical mycelium structures

S. reticulata CNPAB11 formed typical *Gigasporaceae* structures in excised carrot roots, characterized by course mycelium and arbuscules, and the absence of intraradical vesicles. The arbuscules exhibited profuse hyphal coiling and were found in eight-month-old roots.

PCR-DGGE analysis

Four out of the seven *Scutellospora* species tested using PCR-DGGE produced more than one band for the V9 region of the SSU nrDNA (Fig. 14), demonstrating the occurrence of intraspecific polymorphism in those species, and no spore-to-spore variation was found. Also, no difference was found between the DGGE profiles of the two isolates of *S. heterogama* tested (Fig. 14).



Figures. 1-4: Changes in size and color during the development of one spore of *Scutellospora reticulata*. As the spore develops spore color changes from white opaque (immature) to dark brown (mature). The subtending hypha (sh) moved due to the growth of a root. Bold arrowheads show the sporogenous cell.

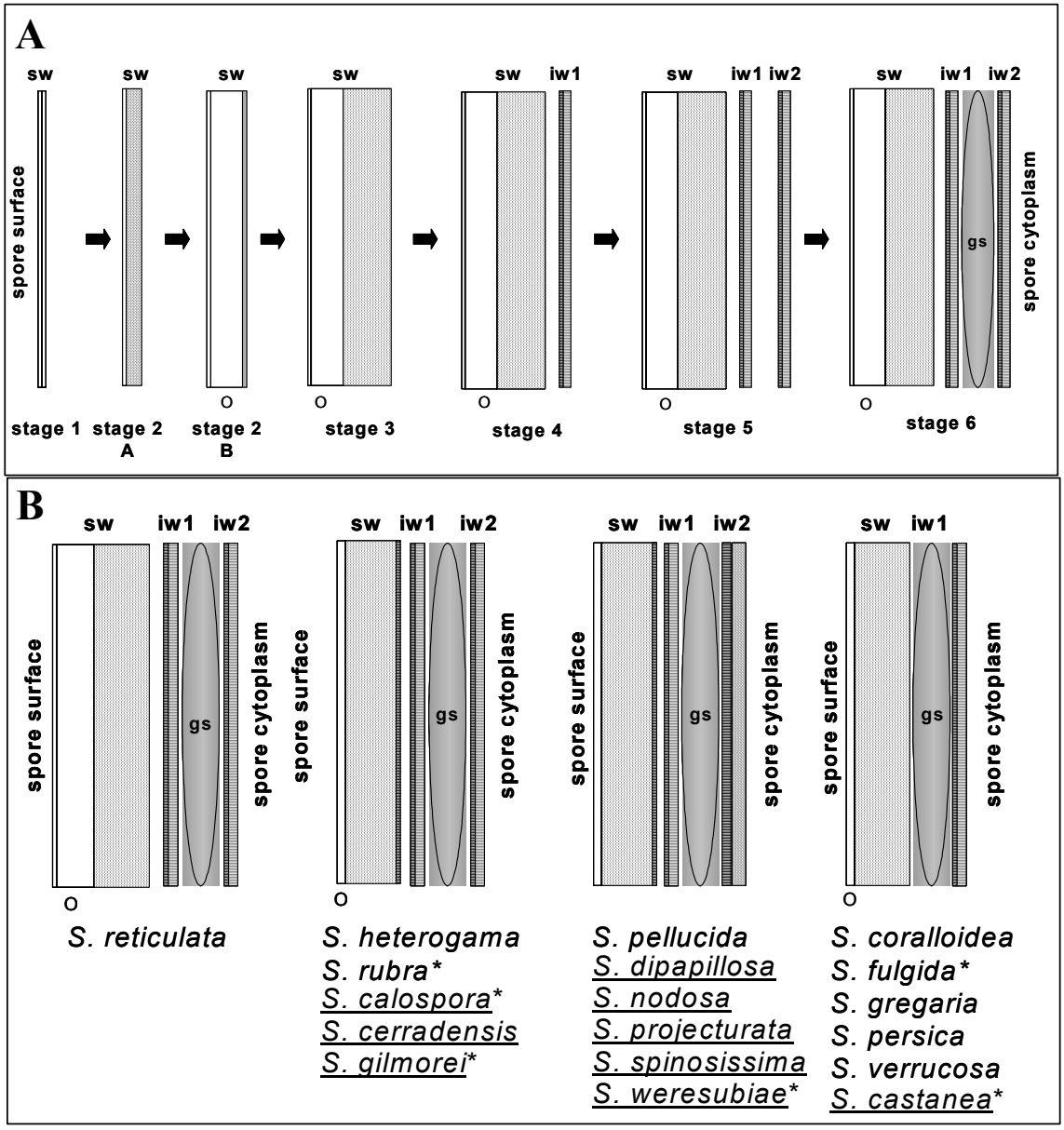
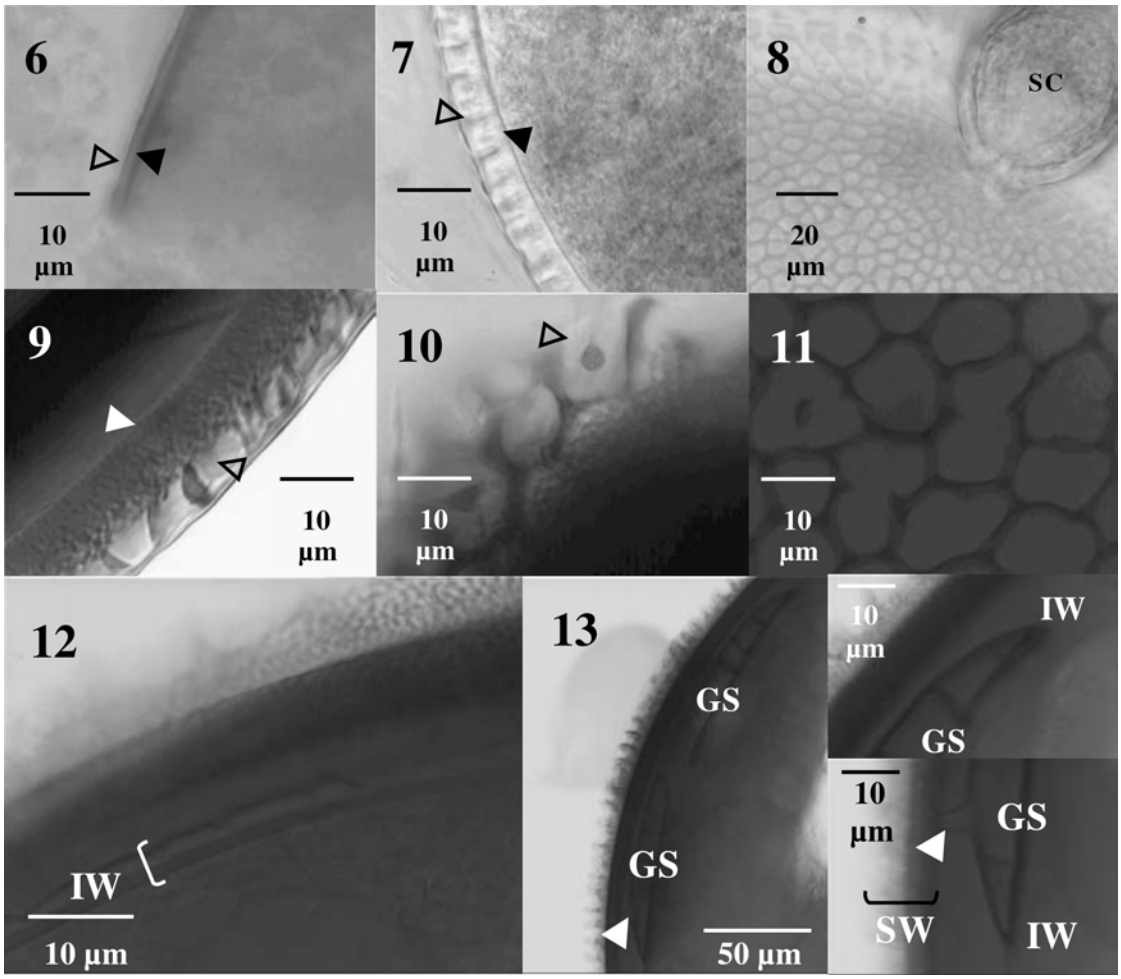


Figure 5. Murographic representation of spore wall layers. Panel A. *S. reticulata* CNPAB11 spore ontogenesis. Six discrete stages of differentiation were observed. The first three stages comprised the Spore Wall (SW) layers and the last three the Inner Wall (IW) layers and germination shield (GS) differentiation. Stage 1 is characterized by spore expansion, stage 2A and 2B by differentiation of the second SW layer (wave lines) into a double ornamented layer (O), followed by differentiation of a laminar layer (vertical dashes) in stage 3. The spore color changes from pale white to dark brown during the transition between stages 2 and 3. Stage 4 is characterized by formation of the first bilayered IW layer (horizontal lines). In stage 5 the second bilayered IW layer is formed and in stage 6 the GS is differentiated. Panel B. Groups of *Scutellospora* species based on comparative developmental sequences. (*) Asterisk designates species possessing a smooth outer layer. Underlined species were grouped based on their description. Modified from Franke & Morton (1994), Morton (1995) and Sturmer & Morton (1999) and INVAM.



Figures. 6-13: Spore wall differentiation of *S. reticulata* CNPAB11. Fig. 6. Spore wall with two thin layers at stage one. First layer is hyaline 0.5 µm thick (open arrowhead), the second one is pale yellow and 2.0 µm thick (bold arrowhead). Fig. 7. Lateral view of layer two of the spore wall with an external reticulum (open arrowhead) and internal spines (bold arrowhead). Fig. 8. Reticular ornamentation on the surface spore wall layer of a pale cream-colored juvenile spore at stage 2. Fig. 9. Lateral view of mature spore wall showing space between outer and double ornamented layers (open arrowhead) and transition between the spine and laminate layers (bold arrowhead). Fig. 10. Lateral view of reticular ornamentation of the surface spore wall layer of dark-brown-colored mature spores at stage 6 and spore wall layer 1 sealing the reticulum (open arrowhead). Fig. 11.Upper view of the reticular ornamentation of a mature spore where the layer 1 is present obstructing the view of the internal spines. 12. Two bi-layered flexible inner walls (IW) layers. 13. Lateral view of the germination shield (gs), bigger panel. Lower panel in the upper corner, magnification of the left upper side of the bigger panel, separation of the two inner layers to form the GS. Small panel in the lower corner, spore wall (SW), inner wall (IW) layers and the tip of the germination tube (arrowhead).

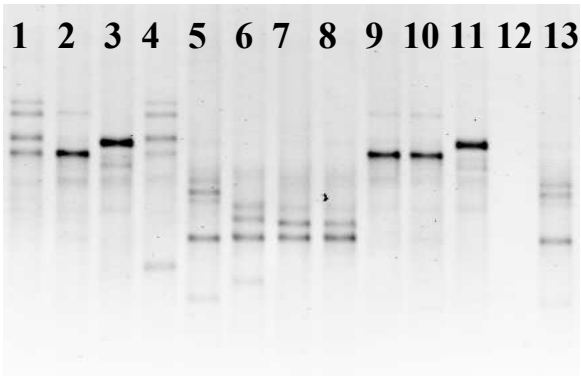


Figure 14. PCR-DGGE analysis of the V9 region of the SSU nrDNA sequences amplified from *Scutellospora* species and run for 17 hours at 95 volts. Lane designations were as follow, 1. *S. calospora* BEG32; 2. *S. gregaria* CNPAB7; 3. *S. castanea* BEG1; 4. *S. calospora* BEG32; 5. *S. reticulata* CNPAB11; 6. *S. cerradensis* MAFF520056; 7. *S. heterogama* CNPAB2; 8. *S. heterogama* UFLA; 9. *S. gregaria* CNPAB7; 10. *S. coralloidea* trap culture; 11. *S. castanea* BEG1; 12. Blank; 13. *S. reticulata* CNPAB11.

The different species tested could be separated into two major groups based on their DGGE profile. The first group was composed of species with bands located relatively high in the gel (*S. calospora*, *S. castanea*, *S. coralloidea* and *S. gregaria*), while the second group had lower bands (*S. cerradensis*, *S. heterogama*, *S. reticulata*, see Fig. 14). In the first group, *S. gregaria* and *S. coralloidea* bands had the same migratory behavior and could not be discriminated from each other, but they could be separated from the two other species in that group (*S. calospora* and *S. castanea*). In the second group, all the species analyzed contained intraspecific polymorphism (i.e. multiple bands) and the lower band of each species displayed the same migratory behavior (position) in the gel (Fig. 14). These species could be discriminated from each other on the basis of the PCR-DGGE profiles.

Sequence comparison (Table 2) and phylogenetic analysis (Fig. 15) confirmed the sequence similarity within the two DGGE migration groups. The first group was composed of sequences containing a higher AT content than the sequences from the species of the second group. In addition, sequences from SSU nrDNA V9 region from *S. aurigloba*, *S. nodosa*, *S. projecturata,* not analyzed directly by DGGE, were found to contain an intermediate AT/GC content compared to the other two groups. Despite having a more intermediate AT content in its V9 region, the sequences of *S. calospora* migrated in the region typical of high AT species.

Representative clones for each of the three *S. reticulata* DGGE bands were subjected to sequence analysis: 8 (upper position), 9 and 18-2 (middle position), and 10 (lower position), see Fig. 14 and Table 2. Clones corresponding to the three *S. reticulata* bands were recorded in a ratio of 11:15:20 (upper: middle: lower; n = 46) as determined by DGGE screening. The two clones of *S. gregaria* sequenced were identical within the region analyzed by DGGE (Table 2), and were in agreement with the predicted and observed melting behavior (Table 2, Fig. 14).

Table 2. 23 DNA sequences from 15 different *Scutellospora* species, showing 43 variable positions in the SSU nrDNA V9 region and AT/GC ratio of those positions.

	Position in the Alignment[(b)]	
	11111111111111111111111111111111111111111	
	44455555555566666666666667777777777777777	AT/GC
	49901234678911157888999990000011111122223355	
Species/clone code[(a)]/GeneBank accession number	86820623349612469279245780456924589134916 57	richness
S. castanea **AF038590**	AAATATAATCAAATCTACACAGAATGACCCGGTCTATTGATAA	30/13
S. fulgida **AJ306435**	AAATATAATCAAATCTACACAGAATGACCTGGTCTATTGGTAA	30/13
S. weresubiae **AJ306444**	GATTATAATCAGATCTACATAGAATGATCTGGTCTGTTGATAA	30/13
S. gregaria **F31; F37**	AAATATAATCAGATCTACACATCATGACCCGGTCTATTGATAA	29/14
S. nodosa **AJ306437**	AATTACAATTAGATCTATGCAGAATAGCTACGCCCGTTGATAA	28/15
S. gilmorei **AJ276094**	AAATATAATCAGACTTACACATCATGACCCGGTCTATTGATGA	28/15
S. spinosissima **AJ306436**	AAATATAATAAGACTTACACGTCGTGACCCGGTCTATTGATAA	28/15
S. calospora **AJ306444**	AATTATAATTAGACTTATACAGAACGGCTACGCCCGTTGGTAA	27/16
S. calospora **AJ306443**	AATTATAGTTAGACTTTTACAGAACGGCTACGCCCGTTGGTAA	26/17
S. calospora **AJ306445**	AATTGTAATTAGACTTATACAGAACGGCTACGCCCGTTGGTAA	26/17
S. pellucida **Z14012**	AAATATAATCAGACTTACACATCGTGACCCGGTCCATTGGTAA	26/17
S. auriglobа **AJ276092**	AATTATAATTAAGTCTACACAGAACAGCCACGCCCGTTGGTAA	26/17
S. projecturata **AJ242729**	AGTTATAATTGAATCTACACATAACGGCCACGCCCATTGGTAC	25/18
S. heterogama **AJ306434**	AATTATAACCAGACTAACACAGCGTGACCCGGTCTACTAGTAA	25/18
S. reticulata **8**	AATTATAACCAGACTTACACAGCGTGACCAGGTCTACTGGTAA	25/18
S. auriglobа **AJ276093**	AATTATAATTAAGTCTACACAGAATGGCCCCGCCCGTCGGTAA	24/19
S. dipapillosa **Z14013**	AATCATAACCAGACTTACACAGCGTTACCCGTTCTTCTGGCAA	24/19
S. cerradensis **AB041344**	AATTATGACCAGACTTACACAGCGTGACCCGGTTTACTGGTAA	24/19
S. cerradensis **AB041345*; S. reticulata*** **18-2; 9**	AATTATAACCAGACTTACACAGCGTGACCCGGTCTACTGGTAA	24/19
S. reticulata **10**	AATTATAACCAGACTTACACAGCGTGACCCGGTCCACTGGTAA	23/20

(a) Different clones in the same line are identical in DNA sequence for the fragment analyzed by PCR-DGGE.

(b) The 5' and the 3' end of the primers NS7 and F1Ra are located at positions 1420 and 1747 respectively, according to the alignment provided at EMBL-EBI.

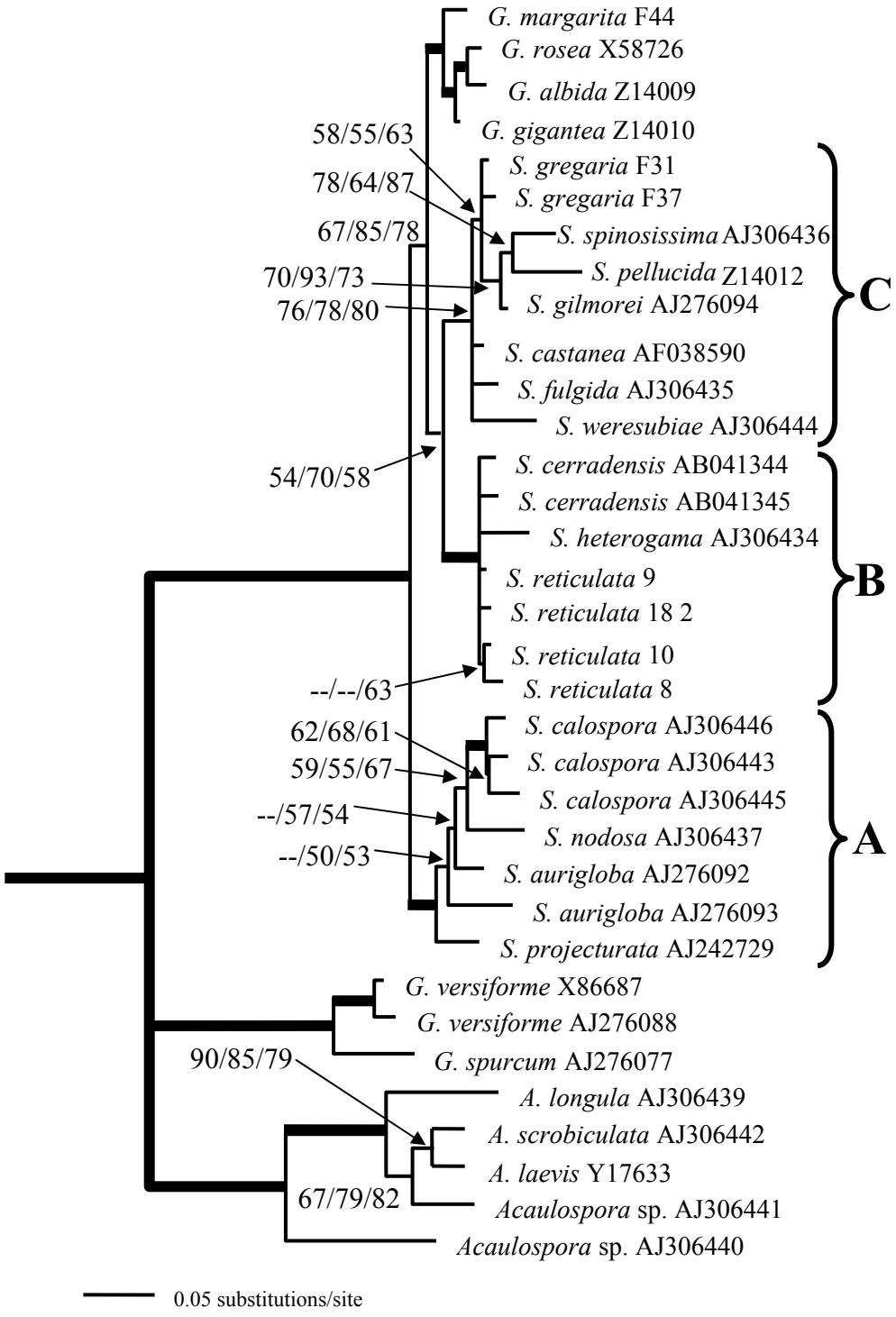


Figure 15. Maximum likelihood SSU nrDNA phylogenetic tree focusing on the family *Gigasporaceae*. *Scutellospora* sequences were clustered in three clades denominated A, B and C. The tree was constructed using Maximum Parsimony, Minimum Evolution (ME) and Maximum Likelihood (ML) methods. The bootstrap supports (1000 repetitions) for each of these methods are shown, respectively; thicker lines represent clades with support higher than 80% for all three methods, and clades supported by bootstrap values lower than 50% were reduced to polytomies. Sequences from *Glomus* group A

(Schwarzott et al. 2001) were used as out group (*Glomus mosseae* AJ306438 and *G. clarum* AJ276084).

Molecular Phylogeny

Prior to phylogenetic analysis the *Scutellospora* origin of the sequences was confirmed by BLAST search (Altschul et al 1990). The substitution model that best fit the data, after removing the constant and the gapped sites, was HKY +G (Hasegawa et al 1985; Pousada & Crandall 1998), and the parameter were as follows: proportion of invariable sites - I = 0; gamma distribution shape - G = 9.5134; number of substitutions type - NST = 2; transition/transversion ratio = 2.9585 and base frequencies - A=0.2727; C=0.2009; G=0.1971; T=0.3211. Using nearly full-length SSU rDNA sequences, *Scutellospora* sequences were grouped in three separated clades, denominated A, B and C in Figure 15. The tree topology was consistent using distance, parsimony and maximum likelihood methods. Clade A was composed of *S. aurigloba*, *S. calospora*, *S. nodosa* and *S. projecturata*. The three sequence types of *S. reticulata* clustered in clade B, together with sequences of *S. cerradensis* and *S. heterogama*. Sequences of *S. dipapillosa* also cluster in that clade, however, the sequence available is only partial, and therefore it was not used in the phylogenetic analysis. *S. gregaria* sequences clustered in clade C together with *S. castanea*, *S. fulgida*, *S. gilmorei, S. pellucida, S. spinosissima* and *S. weresubiae.*

DISCUSSION

The monoxenic culture system proved ideal for the study of spore development of *S. reticulata*, as it facilitated sampling of spores at different development stages and did not stimulate microbial activity that can cause alterations in the outer wall layer. Recently, mycelial characteristics and the role of auxiliary cells on spore production of this isolate were also studied successfully using monoxenic culture system (de Souza & Declerck 2003; Declerck et al 2004).

Spore ontogeny

In *S. reticulata*, the first spore wall layer is a thin evanescent layer, adherent to the second layer, not reported in the original description of the species (Koske et al 1983). However, it breaks easily during spore manipulation and may be decomposed by microbial activity in soil-based systems, probably explaining why it has never been reported before. Layers similar to the one we observed actually may

occur in two highly ornamented *Scutellospora* species from La Gran Sabana, Venezuela: *S. spinosissima* (Walker et al 1998) and *S. crenulata* (Herrera-Peraza et al 2001).

The development pattern of the outer ornamented layer in *S. reticulata* has never been reported before. We found that this layer first expands and later differentiates into the double ornamented layer characteristic of that species. The discrimination of spore wall (SW) layers during ontogeny is not easy and in some species they are more evident than in others. For example, Spain & Miranda (1996) found an additional inner wall layer in the SW of *S. cerradensis.* Later, a similar layer was found also in *S. heterogama, S. pellucida* and *S. rubra* (INVAM – http:\\invam.caf.wvu.edu). Such an additional layer was not observed for *S. reticulata*. This, of course, does not demonstrate the absence of that layer in *S. reticulata*. It may simply be too thin to be differentiated using light microscope.

Comparison between S. reticulata *sporogenesis with eight other* Scutellospora *species*

We compared the sporogenesis of *S. reticulata* with other *Scutellospora* species studied using the same criteria (*S. coralloidea*, *S. fulgida*, *S. heterogama*, *S. gregaria*, *S. pellucida, S. persica*, *S. rubra*, *S. verrucosa*; Franke & Morton, 1994; Morton, 1995; Stürmer & Morton 1999). Based on Morton et al. (1995) species-level definition is limited mainly to characters of the SW, and the IW layers define higher taxonomic groups. The latter appear to be linked with germination events. Using these criteria was possible to cluster these species into three groups (Fig. 5B). The first group is composed of *S. reticulata*, *S. heterogama*, *S. rubra*, which all have two bilayered IW groups and the GS positioned between them (Franke & Morton 1994; Sturmer & Morton 1999). *S. calospora, S. cerradensis* and *S. gilmorei* (Spain & Miranda, 1996, INVAM website) not studied using developmental patterns also belong to this group. The second group contains *S. pellucida,* and is discerned by characteristics of the second layer of the IW2, which expand under light pressure in mounting medium (Franke & Morton, 1994). Species related to *S. pellucida* are *S. dipapillosa, S. erythropa*, *S. nodosa* and *S. weresubiae* (Blaszkowski, 1991; Franke & Morton, 1994), also possibly *S. projecturata* and *S. spinosissima*. The third is composed of *S. fulgida*, *S. verrucosa*, *S. persica*, *S. coralloidea* and *S. gregaria,* which are species with a single bilayered IW group and the GS located between the SW and the IW (Fig 5; Morton 1995). The last two species in this group (*S. coralloidea* and *S. gregaria*) are most closely related based on cladistic analyzes, and *S. castanea* is also known to belong to this group (Morton 1995). *S. aurigloba*, was used in the molecular analysis, but due to poor information on spore wall components it was not included in any of developmental groups.

Phylogenetic analysis based on SSU nrDNA

The molecular phylogeny based on SSU nrDNA has been proved to discriminate from genus and sub-genus level to above (Redecker et al. 2000; Schwarzott et al. 2001; Schüβler et al. 2001; Walker & Schüβler 2004) but it fails to discriminate between species, because nrDNA is too conserved, a fact confirmed by our analysis. In *Scutellospora* the SSU nrDNA sequence produced three shallow clades supported by strong bootstrap values. These clades have poor resolution to discriminate between species. However, they are indicative of sub-groups within *Scutellospora*. We have not tested for polyphyly in our analysis, the tree topology suggests a different ancestor for clade A in relation to the clades B and C, and the latter clades were most closely related with the sister genus *Gigaspora*. The polyphyly has to be confirmed through the analysis of more taxa and genes.

Comparing Molecular Phylogeny with morphological groupings

The three clades obtained by molecular phylogeny clustered species that are closely related based on developmental patterns of the spores, but within each clade more than one developmental group is present. For instance, species on clade B are closely related based on developmental patterns, except for *S. dipapillosa* (Fig. 5, not show in the tree, Fig. 15). Using this criterion, species on clade C would be separated in three distinct groups: 1. *S. castanea*, *S. fulgida* and *S. gregaria*; 2. *S. gilmorei*; and 3. *S. pellucida*, *S. weresubiae* and *S. spinosissima* (Fig. 5). In clade A, most of the species would cluster in the *S. pellucida* group (Fig. 5). Besides, *S. calospora* (clade A) and *S. gilmorei* (clade C) should have clustered in clade B. On one hand, these results could indicate the poor resolution of SSU nrDNA to resolve species, or the occurrence of recurrent mutation in these sequences due to absence of selection (Givnish & Sytsma 1997). On the other hand, if the molecular phylogeny is correct then the developmental analysis might cluster species with different ancestry based on convergence of the IW layers. This might be possible if constraints related to germination events take place during species radiation. In such a scenario, nrDNA data must give better resolution to discriminate sub-genus level than developmental patterns based on IW layers because nrDNA are probably under low selective pressure during speciation in asexual organisms. The comparison between molecular and morphological characters is not easy, particularly in organisms were genetics and evolutionary processes are poorly understood. Besides, both data sets are subject to homoplasy. Clearly more research is needed to integrate morphological and molecular characters in *Scutellospora* phylogeny.

PCR-DGGE analysis

PCR-DGGE targeting the V9 region of the SSU nrDNA was used to discriminate *S. reticulata* from all other *Scutellospora* species tested here. Due to the high level of sequence similarity, some closely related species (*S. gregaria* and *S. coralloidea*) were not discriminated. Also, species in clades B and C could be better discriminated from each other than species from clade A (*S. calospora*). This poor separation within clade A may be due to the GC-rich nature of the 3'end of amplicons in this clade, causing some sequence differences to fall within a domain of relatively high melting temperature, hampering discrimination (Table 2).

PCR-DGGE can only reveal changes that affect melting behavior and thus some mutations (specifically A↔T and G↔C changes) may go undetected. The same sort of problems can occur during distinction of sequences by RFLP. This technique overlooks mutations that cause no changes in the restriction sites. Also, the cloning procedure is known to affect the integrity of the cloned DNA fragments, when using mixed PCR products, as demonstrated by Speksnijder et al (2001). Here, prior to sequencing we screened cloned products for correspondence to DGGE profiles obtained from direct amplification of spores genomic DNA. This step decreases the chances of selection of clones containing PCR or cloning artifacts (Speksnijder et al 2001). Therefore we can be highly confident of the sequence data. The SSU nrDNA sequences of *S. cerradencis* deposited in the GeneBank were obtained using the same accession used here. Based on our DGGE, analysis the sequence deposited for *S. cerradensis* (accession AB041345) should have matched with *S. reticulata* clone 10 as opposed to clones 9 and 18-2 (Table 2, Fig. 14), which suggests a possible cloning or PCR error in that sequence.

The great advantage of PCR-DGGE for analysis of AM fungi is that it takes advantage of the intraspecific polymorphism of the nrDNA copies to discriminate closely related species. This was the case of *S. reticulata, S. cerradensis* and *S. heterogama*. The same approach could be used to discriminate all known species and even isolates in *Gigaspora* (de Souza et al 2004). The characterization of intraspecific nrDNA polymorphism using PCR-DGGE provides a detailed molecular fingerprint suitable for species identification. These characteristics make PCR-DGGE a good technique to control purity of AM fungal inoculum in culture collections and to select clones before sequencing, in addition to its utility in assessing the diversity of AM fungi species in environmental samples (Kowalchuk et al 2002; Opik et al 2003; de Souza et al 2004).

CONCLUSIONS

(i). The sporogenesis of *S. reticulata* revealed an novel pattern of outer spore wall differentiation; (ii). Phylogenetic analyzes grouped *S. reticulata* in a cluster together with *S. cerradensis* and *S. heterogama*, which are species that share similar spore developmental patters. (iii) *S. reticulata* could be discriminated from all 16 known *Scutellospora* species by PCR-DGGE analysis of the V9 region of the SSU nrDNA, a result confirmed by sequence analysis.

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