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Establishment of monoxenic culture between the arbuscular mycorrhizal fungus *Glomus sinuosum* and Ri T-DNA-transformed carrot roots

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Abstract

We report for the first time the establishment of an arbuscular mycorrhizal association between *Glomus sinuosum* (= *Sclerocystis sinuosa*) and transformed Ri T-DNA carrot (*Daucus carota* L.) roots in monoxenic culture. The *G. sinuosum* sporocarps survived not as single spores, but as sporocarps in the environment. The mode of germination of *G. sinuosum* was by extension of hyphae around the sporocarp. Numerous vegetative spores, arbuscules and vesicles were produced after the roots were infected by the hyphae. New mature sporocarps started to form after four months in the culture system. Forty-seven sporocarps were produced on average in each culture dish after six months, and these newly produced sporocarps were capable of germination in the growth medium.

Introduction

Numerous studies have demonstrated the beneficial role of arbuscular mycorrhizal (AM) fungi in improving plant growth by increasing resistance to drought and disease or by enhancing the efficiency of nutrient absorption (Smith and Read, 1997). Although axenic cultures of these fungi cannot be established, it is possible to grow AM fungi in sterile conditions in root-organ culture (Fortin et al., 2002).

An increasing number of AM fungal species have been cultured monoxenically as described in a number of papers reporting the production of root-organ cultures. These include *Acaulospora rehmanii* (Dalpé and Declerck, 2002), *Gigaspora rosea* (Bago et al., 1998), *Gigaspora margarita* (Diop et al., 1992; Miller-Wideman and Watrud, 1984), *Gigaspora gigantea* (Gadkar et al., 1997), *Glomus etunicatum* (Schreiner and Koide, 1993), *Glomus intraradices* (Chabot et

al., 1992; St-Arnaud et al., 1996), *Glomus versiforme* (Declerck et al., 1996; Diop et al., 1994), *Glomus caledonium* (Karandashov et al., 2000), *Glomus fasciculatum* and *Glomus macrocarpum* (Declerck et al., 1998), *Glomus proliferum* (Declerck et al., 2000), *Glomus deserticola* (Mathur and Vyas, 1995) and *Glomus fistulosum* (Gryndler et al., 1998). *Glomus sinuosa* (Gerdemann and Bakshi) Almeida and Schenck, formerly named *Sclerocystis sinuosa* (Gerdemann and Bakshi), produces complex sporocarps that have recently been proposed as an advanced character of some *Glomus* species (Redecker et al., 2000). Gerdemann and Bakshi (1976), Wang et al. (1992) and Wu (1993a,b) provided limited information on some of the morphological characteristics, including the size, colour and shape of the sporocarps and the arrangement of spores inside the sporocarps. Successful monoxenic culture of *G. sinuosum* would certainly help increase our knowledge of this AM fungus and would represent a powerful tool for taxonomic investigations. The present study reports for the first time the

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cultivation of *G. sinuosum* on Ri T-DNA transformed carrot roots with sporocarps and describes some of the morphological characteristics of the cultures.

Materials and methods

Fungal inoculum

The AM fungus used was *Glomus sinuosum* (Gerde-mann and Bakshi) Almeida and Schenck, originally recovered from soil collected in Guiling, Guangxi province, China by Wang et al. (1992) and maintained in pot culture on red clover (*Trifolium pratense* L.) at the Institute of Soils and Fertilizers, Chinese Academy of Agricultural Sciences, Beijing. The fungus was propagated in pot culture by inoculation with single sporocarps in black PVC tubes. Sporocarps were extracted by wet sieving and surface sterilized by treatment with 2% Chloramine T plus two drops of Tween 20 for 15 min, then soaking in an antibiotic solution containing 200 mg L⁻¹ streptomycin sulphate and 100 mg L⁻¹ gentamycin sulphate for 15 min and rinsing 3–5 times with sterile water. The sporocarps were then placed in one half of Petri dishes (10 sporocarps per dish) containing 0.8% Bacto-Agar medium. The dishes were incubated at 27 °C in a constant temperature box to promote germination. *Streptomyces orientalis* (an unspecified strain purchased from the Institute of Microbiology, Chinese Academy of Sciences) was added to the medium in the other half of each Petri dish to speed up sporocarp germination (Mugnier and Mosse, 1987). The helper bacterium remained physically separate from the sporocarps throughout the procedure. The germinated sporocarps were then transferred axenically into the experimental dishes containing M medium using a sterile 7-mm cork borer and cultured with transformed Ri T-DNA carrot roots.

Source of root organ culture

Transformed Ri T-DNA carrot roots (*Daucus carota* cv. Takii) were obtained. They were routinely cultivated on a minimal medium (M medium) by the method of Bécard and Fortin (1988).

Experimental culture

The experiments were conducted by placing a single germinating sporocarp with a single transformed root in the same Petri dish and incubating at 27 °C in the dark. Every two days the dishes were checked for

formation of a plant-fungus association. After two months some of the roots were examined for mycorrhizal colonization by clearing and staining with trypan blue using the method of Phillips and Hayman (1970). The cultures were maintained for a further four months to observe the establishment of the symbiotic association and the formation of new sporocarps. All the dishes were kept inverted during the period of culture to compensate for the negative geotropism of the transformed roots.

Results

Structure of the sporocarps

This fungus has a very characteristic pattern of sporocarp formation (Figure 1), as described by Wang et al. (1992) and corresponding closely to the description by Gerdemann and Bakshi (1976). The sporocarps are globose to subglobose and brownish-black to black, with a diameter of 260–330 µm. The fruiting body of each sporocarp is composed of chlamydospores whose lateral walls adhere to one another. The connecting hyphae are embedded in a central hyphal plexus. Chlamydospores are formed radially in a single, tightly packed layer around a central plexus of hyphae. Chlamydospores are yellow-brown to brown, obovoid to ellipsoid, and with a wall thickness of about 2.8–8.5 µm. The base is composed of sterile hyphae. Mature sporocarps have a peridium up to 13.5–25.0 µm thick which is composed of hyphae of 2–3 µm wall thickness and 6.0–7.5 µm diameter. They are visible to the naked eye.

Sporocarp germination and hyphal growth

Germination of sporocarps of *G. sinuosum* occurs by extension of hyphae around the sporocarp from different directions. Some sporocarps can produce 6–7 germ tubes (Figure 2). In contrast with the germination of *Gigaspora margarita* (Bi et al., 1999), each chlamydospore can be considered as a germinating body and this may increase the probability of contact with the host plant roots.

Infection of transformed Ri T-DNA carrot roots by G. sinuosum The roots were excised, placed in a Petri dish and stained. Entry points were readily observed (Figure 5), together with vesicles (Figure 6) and arbuscules in the root cells (Figure 7). This is a new

Figures 1–6. Sporocarp of *Glomus sinuosum*; bar 50 μm . (2) Germination of *G. sinuosum* sporocarp; bar 200 μm . (3) Formation of vegetative spores of *G. sinuosum*; bar 60 μm . (4) Root infection by *G. sinuosum* hyphae and numerous vegetative spores; bar 120 μm . (5) *G. sinuosum* hyphae infecting a root (arrow); bar 100 μm . (6) A vesicle of *G. sinuosum* (arrow); bar 40 μm .

demonstration of the use of these sporocarps for the establishment of monoxenic culture.

Formation of vegetative spores of G. sinuosum

After two months it was observed that the hyphae had grown extensively with much branching. The direction of branching was almost perpendicular to the

thick-walled hyphae. We observed numerous vegetative spores of *G. sinuosum* that were produced in the medium (Figure 3). They were globose to subglobose, and were 24 μm in diameter on average (range 15–30 μm). We observed numerous inclusions resembling oil droplets in the vegetative spores under the microscope. As the spores matured they changed from a light colour to a dark colour. Hyphae encircled

Figures 7–10. Arbuscules of *G. sinuosum* (arrow); bar 100 μm . (8) Formation of new sporocarps showing no peridium (arrow); bar 100 μm . (9) Formation of a mature sporocarp; bar 135 μm . (10) Germination of a new sporocarp of *G. sinuosum*; bar 100 μm .

the roots, formed many vegetative spores (Figure 4) and grew very prolifically. Hyphae started to infect the fine roots in the medium, thus producing an extensive hyphal network and forming a mycorrhizal association.

Formation of new sporocarps

After culture of the symbiosis for four months, we found that the symbiosis produced numerous vegetative spores. After culture for six months, about 47 mature sporocarps were produced on average in each Petri dish. When young sporocarps formed, the peridium was virtually absent (Figure 8), with an average diameter of only 128 μm (range 67–155 μm). As the sporocarps matured, their size increased and the diameter of the peridium reached 272 μm on average with a range of 207–363 μm (Figure 9). The mature sporocarps had the same appearance as the mother sporocarps.

Germination of the newly matured sporocarps

In order to check the germination ability of the newly formed sporocarps, we transferred some of them axenically onto 0.8% Bacto-Agar medium. After culture for one week, new hyphae had extended from the sporocarps (Figure 10) and the hyphae were still capable of colonizing the roots.

Discussion

Monoxenic culture technology is a very powerful tool for the experimental establishment of arbuscular mycorrhizal associations. A small but growing number of papers have reported the successful establishment of AM associations using a number of AM fungal species (Fortin et al., 2002). Our experiment has added *G. sinuosum* to the list of fungi by using monoxenic culture with transformed Ri T-DNA carrot roots.

Different AM fungal species have different modes of germination. The *G. sinuosum* sporocarp is com-

posed of many chlamydospores. In theory, each chlamydospore within a sporocarp has the potential to germinate, and in contrast with the spores of other species, for example *Gigaspora margarita* and *Glomus intraradices* (Bi et al., 1999, 2000), their spore germination consists of the extension of only one germ tube with subsequent branching, a germination pattern that may increase the probability of root infection. Hyphal branching is prolific and *G. sinuosum* may therefore be an excellent choice of fungal species to select for studies on the extramatrical hyphae and the hyphosphere.

Mycorrhizal association was established after the formation of arbuscular and vesicular structures and hyphal coiling in the root cells. Taken together, all of these characteristics indicated the successful establishment of a mycorrhizal association. Numerous vegetative spores were then produced. These were about 24 μm in diameter, with somewhat variable shape. These spores may possess similar levels of survival and propagation potential to those of mature spores (Dalpé and Declerck, 2002). The production of large numbers of mature sporocarps in monoxenic culture may provide a good supply of experimental material for genetic and physiological studies on the mycorrhizal association and suggests that *G. sinuosum* can complete its life cycle in the monoxenic culture system *in vitro*. Vesicles were also observed in the monoxenic cultures and more detailed investigation is required on the vesicles of *G. sinuosum*.

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