MONOXENIC CULTURE OF THE ARBUSCULAR MYCORRHIZAL FUNGUS *SCUTELLOSPORA CALOSPORA* AND Ri-TDNA TRANSFORMED CARROT ROOTS

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ABSTRACT

This paper presents the first report of cultivation of *Scutellospora calospora* with Ri-TDNA transformed carrot (*Daucus carota. L*) roots. Carrot root transformation was done using *Agrobacterium rhizogenes*. Spore germination tests were conducted on 6% water agar and minimal media to assess the time taken for germination. Morphological characteristics of extraradical mycelium, intraradical mycelium, branched absorbing structures and auxiliary cell formation were recorded. Auxiliary cell formation started within 3-5 days of root contact and continued until 2 months. There was an extensive, thick, brown extraradical mycelial development following root infection, but spore formation was low (four spores) and was observed only after 8 months. Spores were full of oil globules and were produced on MSR medium but not M medium.
Keywords: hairy roots, auxiliary cells, *Scutellospora calospora, Agrobacterium rhizogenes*.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) colonise approximately 80% of terrestrial plants. The beneficial effects of AMF on the growth and health of plants have been recognised for some time, although their obligate biotrophic nature has limited *in vitro* culture and large-scale production, reducing their potential for use as inocula in agricultural and horticultural practices (Plenchette et al. 1996). Research on AMF has increased exponentially since the 1970s and molecular biology has provided novel tools for growing AMF under *in vitro* conditions, such as root organ culture. Root organ culture was first successfully performed by Mosse & Hepper (1975) using a system based on dual culture of *Glomus mosseae* spores with excised roots of clover. Due to their obligate symbiotic nature, less than 5% of AMF were successfully cultivated using the dual culture approach.

The monoxenic method involved growing sterile AMF spores on dual culture plates with transformed carrot (*Daucus carota. L*) roots. Carrot roots were transformed using a wild type strain of *Agrobacterium rhizogenes* containing a plasmid that induces a hairy root phenotype. These adventitious roots were then cultured *in vitro* on medium devoid of plant hormones, where they grew very rapidly, with a characteristic, highly branched and non-geotropic pattern. The combination of transformed carrot roots and sterile AMF spores can be used to produce "dual *in vitro* cultures" that provide an efficient method of producing abundant spores (typically over 5000) and mycelia in a 9 cm Petri plate (Becard & Fortin 1988).

Root organ culture has obvious advantages over traditional systems, permitting production of contaminant-free propagules. So far, 25 AMF species have been successfully cultivated in monoxenic culture (Fortin et al. 2002). However, most data generated under monoxenic culture conditions have been obtained with *Glomus* and *Gigaspora* species, while *in situ* observations on *in vitro*-produced cultures of *Scutellospora* species

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have been seldom reported. Successful root organ culture of *S. reticulata* by de Souza & Declerck (2003) has shown that it produces mycelium of two architectural patterns, one related to root colonisation and the other to resource exploitation.

Long-term sequential time-lapse relationships between extra radical mycelial growth and spore production in Gigasporaceae have not been studied previously. The present study describes the cultivation of *S. calospora* with Ri-TDNA transformed carrot roots for the first time in monoxenic culture. The lifecycle of *S. calospora* under *in vitro* culture is characterised and the results describe morphological characteristics, including extraradical mycelium, intraradical mycelium, branched absorbing structures (BAS) and auxiliary cell (AC) formation.

MATERIALS AND METHODS

Inoculum preparation, root transformation and spore germination tests

Scutellospora calospora spores were recovered from white clover (*Trifolum repens*) pot cultures (Lincoln University nursery) by wet sieving and sucrose density centrifugation (Furlan et al. 1980). Spores were surface sterilised using a modified method of Becard & Fortin (1988). Briefly, spores were washed in a 0.05% Tween solution, soaked in 2% (w/v) chloramine T solution for 10 min, and rinsed thrice in sterile distilled water. A second treatment with chloramine T followed by rinsing with water was performed in the same manner. Spores were stored in a sterile solution containing 200 mg/litre streptomycin and 100 mg/litre gentamycin until use. Spores were rinsed in sterile distilled water just before use.

Carrot roots were transformed using *Agrobacterium rhizogenes* strain A4T. Clonal cultures were maintained in pottles on modified Whites medium (Becard & Fortin 1988), modified by replacing sucrose with 3% glucose and agar with 0.4% Phytagel (Sigma).

Ten replicates of water agar (6%) and five replicates of minimal (M) medium (Becard & Fortin 1988) with the modifications described above, were poured into 2.5 cm deep, standard (9 cm) diameter Petri plates and allowed to set. Each plate had 10 sterile spores distributed evenly and the plates were incubated at 27°C in the dark. Plates were observed once a week until germination, which took approximately 40 days.

Establishment of dual culture

Dual culture plates were prepared by pouring M medium into each of two compartments in a split Petri plate. Compartment A had 20 ml of M medium with 1% glucose and compartment B contained 10 ml of M medium without glucose. An agar “bridge” consisting of M medium without glucose was raised along the partition on the side of compartment B to allow the mycelium to traverse the two compartments. Plates were also poured with modified Strullu-Romand (MSR) medium (Declerck et al. 1996) with the same changes described above.

Dual culture was initiated by uniformly placing five non-germinated spores and five 2 cm long, actively growing root tip pieces with branched primordial roots, along the circumference of compartment A of the M and MSR media plates. The plates were inverted and incubated in the dark at 27°C. Plates were observed once a week until spore germination and then every 2 days until the spores made contact with the root.

Following germination, a plug of gel medium containing a germinated spore and root piece was transferred into compartment A of a fresh Petri plate containing the same MSR or M medium from which a similar plug had previously been removed using a 9 mm cork borer. Five replicates of each medium were produced. Cultures were maintained for 8 months at 27°C in the dark. The cultures were examined weekly and the roots were trimmed, as needed, in order to prevent their growth into compartment B**.**

Data collection and harvest

A 10×10 mm grid line was marked on the bottom of the Petri plate. Total hyphal lengths and the number of auxiliary cells for each replicate were assessed as follows. Vertical and horizontal lines were observed under an Olympus (SZX-ILLB2-200) stereo-microscope at magnifications of 10× to 90× and using a compound microscope (Olympus), and the presence of roots and hyphae recorded at each point where they intersected a gridline.

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No distinction was made between runner hyphae or lower order hyphae. At the end of the 8 month period, auxiliary cells were counted individually in each cell formed by the grid and totalled for each plate. One plate was destructively harvested for staining.

Staining of roots

Phytagel was liquefied using 10 mM sodium citrate (pH 6.0) using gentle shaking. Roots were cleared by autoclaving for 15 min at 121°C in 10% KOH (w/v), then washed gently with water and rinsed in 10% HCl before being finally rinsed twice in water. They were stained in chlorazol black E consisting of equal volumes of 80% lactic acid, glycerine, and distilled water with 0.1 % (w/v) chlorazol black E (Sigma). The samples were stained overnight and colonisation events were recorded using a compound microscope.

RESULTS

Spore germination tests

Spore germination occurred between 44 and 60 days after inoculation irrespective of the medium used. After 60 days, the germination rate was 16.6%. The germ tubes emerged and once they reached the length of the spore, began to show negative geotropism by growing upwards in the inverted plates (Fig. 1a).

Primary colonisation and the process of infection

On the M/MSR media, 75% of the germinated spores initiated contact with roots. Of these, only 50% of the transferred spore/root cultures established good colonisation *in vitro*. Multiple entry points were observed following infection via appressorium on the root surface (Figs 1b & 1c). Intraradical colonisation consisted of intercellular hyphae and coil-like structures that occasionally had small projections (Fig. 1d). Following infection, there was an extensive proliferation of long light orange/brown runner hyphae (Fig. 1e) or extraradical mycelium. However, infection was patchy or localised, even though 70% of the root length was colonised. These pigmented hyphae extended in different directions for distances of up to 10 mm. Dichotomously branched short ramifications or branched absorbing structures (BAS) (Fig. 1f) developed on the runner hyphae. BAS were fine thin-walled, hyaline hyphal networks close to the root zone and became septate (Fig. 1g) at maturity.

Within 10 days after germination, auxiliary cells began to be produced on short ramifications, occurring simultaneously on one or both sides of first order branches of runner hyphae (Figs 1e, 1d & 1h). The auxiliary cells ranged in colour from pale yellow to brown and had an ornamented surface with spherical features. The mean auxiliary cell number on MSR medium was 77, with a range between 12 and 129. After 8 months, some of the auxiliary cells looked crumpled and at this stage three freshly formed spores were observed in one plate and a single spore in a second plate. Spores were full of oil globules (Fig. 1i) and in both cases were produced on MSR medium. In the M medium, only three auxiliary cells were observed in a single plate and spores were not seen in any plate.

DISCUSSION

This study describes the successful establishment of dual culture between *S. calospora* and transformed carrot hairy roots. Following infection, extensive proliferation of extraradical mycelium with a number of auxiliary cells was observed. Although spores were not produced in abundance, a standard method for dual culture of *S. calospora* and completion of the life cycle has been established. Solidification with Phytagel improved visual observation by reducing the opacity, allowing non-destructive photographs to be taken of the developmental stages. MSR was the only medium in which the fungus completed its life cycle.

Spore germination tests on water agar resulted in 16.6% germination. Four plates, two each with fungi and bacteria, were contaminated. Interestingly, these plates showed enhanced spore germination compared with non-contaminated plates. Hildebrandt et al. (2002) noted the presence of slime forming bacteria, identified as *Paenibacillus validus*,

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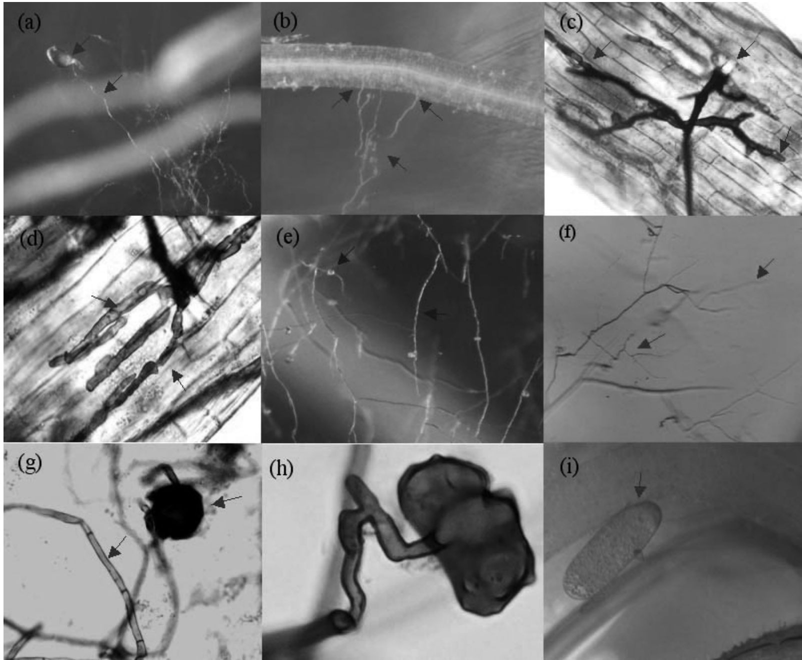

FIGURE 1: (a) Germinating spore and germ tube (40×). (b) Multiple entry points and auxiliary cells (90×). (c) Appressoria and infection pegs after staining with chlorazol black (400×). (d) Internal spread of the coiled and localised mycelium (600×). (e) Well established extraradical mycelium and auxiliary cells (90×). (f) Branched absorbing structures (BAS) that are finely branched and extending deep into the media (90×). (g) BAS becoming septate at later stages of their establishment and an auxiliary cell (400×). (h) Stained single auxiliary cell enlarged (1000×). (i) Newly formed spore near the carrot hairy root (90×) seen in intact plate. Arrows denote structures.

on the surface of sterilised spores of *Glomus intraradices*. These bacteria stimulated the growth of *G. intraradices* up to the spore formation stage in the absence of any plant tissue. In the present study with *G. geosporum* dual culture (data not shown), spores were constantly associated with some bacteria and showed successful establishment of dual culture with the carrot hairy roots, even though they did not reach spore formation stage.

Spore germination took approximately 44 to 60 days in *S. calospora*. In contrast, it occurred within 3 to 25 days in *S. reticulata* (de Souza & Declerck 2003). In *Acaulospora rehmii,* the first spore germination was observed after 15 days (Dalpe & Declerck 2002) and continued for 4-5 weeks thereafter. In *G. mosseae*, pH played an important role and the highest germination rate (60-70%) was noted in the MES amended medium with a pH range of 7.30-7.68 over a period of approximately 14 to 20 days (Douds 1997). In the present experiment a decrease in the pH level was observed in both media after autoclaving and the pH was adjusted to 5.5 after autoclaving.

Thick, looping hyphae were often detected following colonisation of the outer cortex by *Gigaspora* spp. and *Scutellospora* spp. by Dodd et al. (2000). In the present study

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following infection via appresoria on the root surface, intraradical hyphae with small projections were observed in the cortical region below the infected zone. There was an extensive proliferation of extraradical mycelium, but infections were patchy or localised. *Scutellospora calospora* produced far more extraradical mycelium from small-localised infection units and BAS developed on the runner hyphae. Mosse & Hepper (1975) reported such branching structures in *G. mosseae* monoxenic cultures, which were comparable to arbuscles and can develop outside the root. It has been shown that BAS actively take up phosphorous, nitrogen and several other nutrients (Bago et al. 1996), which are then transferred to the host plant, thus assisting the mineral nutrition of the host.

Species of *Scutellospora* and *Gigaspora* do not form vesicles but instead form auxiliary cells on extraradical mycelium (Dodd et al. 2000). The number of these structures in the present experiment was 77 per plate (on MSR medium only), on average, but this was not enough to support substantial spore production. de Souza & Declerk (2003) observed over 600 to 700 auxiliary cells for an average of 56 spores per plate. They also suggested that because these are produced in such large numbers, they are probably carbon storage structures to use as energy sources for spore initiation and development and/or mycelial production and repair. Jabaji-Hare (1998) observed high amounts of lipids within the auxiliary cells of *Gigaspora* species, supporting the storage function of these structures. Although there was no attempt at a detailed study into the auxiliary cells in the present paper, some crumpled auxiliary cells at the end of the 8-month period were noticed.

A low rate of *in vitro* spore formation was observed in *S. calospora* in the present study. Declerck et al. (2004) estimated that one spore needs resources from at least 19 auxiliary cells. Spores were probably produced in low numbers because they have a long vegetative phase of 2-3 months before sporulation and a lengthy process of spore expansion and development. Microscopic observation of mycelium by de Souza & Declerk (2003) revealed that hyphal damage caused by root growth might have negatively affected spore formation. They found that some immature spores located far from the disrupted hyphae were arrested in their juvenile stage, probably due to the damage caused to the mycelial network. In the present experiment, extensive trimming of the roots because of the need to maintain the plates over 8 months might have damaged the mycelium and thereby resulted in no spore formation in some plates.

In summary, dual culture of a previously unculturable AMF has been achieved. Although spore formation was very low, completion of the life cycle was observed. The present research increases the number of successful monoxenic cultures of *Scutellospora* species to two. The use of monoxenic cultivation of *S. calospora* provides a unique tool to help in the understanding of critical colonisation events and may lead to procedures that enable increased spore formation.

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