Mycorrhiza (2000) 10 : 23–28

ORIGINAL PAPER

Vladimir Karandashov · Inna Kuzovkina
Heidi-Jayne Hawkins · Eckhard George

Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots

Accepted: 26 January 2000

Abstract The arbuscular mycorrhizal fungus *Glomus caledonium* was established in a dual culture with Ri T-DNA-transformed carrot roots. A modification of the minimal M medium buffered at pH 6.50 with 10 mM MES and solidified with 0.4% unpurified gellan gum allowed spore germination and formation of the symbiosis, together with the development of an extensive extramatrical mycelium and sporulation. Spore production increased with culture generation and most spores were viable. These spores colonized carrot roots and completed the fungal life cycle. In many cultures, sporulation was accompanied by the formation of arbuscule-like structures on short and thickened lateral branches of main hyphae. Root colonization was of the *Paris*-type with hyphae spreading intracellularly. Most colonized root cells contained coils of thickened hyphae, sometimes surrounded by fine hyphae, but no typical arbuscules were observed.

Key words Arbuscule-like structures (ALS) · Branched absorbing structures (BAS) · Dual culture · *Glomus caledonium* · Sporulation · Transformed carrot roots

V. Karandashov (✉) · I. Kuzovkina
K. A. Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences, 35, Botanicheskaya Street, 127276 Moscow, Russia

H.-J. Hawkins · E. George
Institute of Plant Nutrition (330), Hohenheim University, D-70593 Stuttgart, Germany

Present address:
V. Karandashov, Institute of Plant Nutrition (330), Hohenheim University, D-70593 Stuttgart, Germany
Fax: +49-711-4593295
e-mail: volodkar@ippras.ru

Introduction

Monoxenic culture on plant roots is a valuable tool for the study of arbuscular mycorrhizal (AM) fungi. This technique avoids effects of other rhizosphere micro-organisms on, for example, root growth or mycorrhiza formation. Mosse and Hepper (1975) were the first to use successfully a simple in vitro system to investigate the development of the AM fungus *Glomus mosseae* on excised clover roots. Mugnier and Mosse (1987) improved this technique by using Ri T-DNA-transformed roots as the plant partner. These roots exhibit phytohormone-based autotrophic growth and have low nutrient requirements, which allows root growth on minimal media also suitable for AM fungi.

Monoxenic cultures of AM fungi on either transformed or non-transformed roots have been used recently to study factors involved in the establishment of arbuscular mycorrhiza (Bécard and Piché 1989, 1990; Balaji et al. 1995) and to investigate functional aspects of the symbiosis (St-Arnaud et al. 1995; Bago et al. 1996; Villegas et al. 1996). However, only a few fungal species have been used in these experiments, for example *Gigaspora margarita* (Bécard and Fortin 1988) and *Glomus intraradices* (Chabot et al. 1992). Using another approach, monoxenic cultures of *Glomus versiforme* (Diop et al. 1994), *Glomus fasciculatum* and *Glomus macrocarpum* (Declerck et al. 1998) were established by using sterilized mycorrhizal root fragments or intraradical vesicles as fungal inoculum. These species do not reflect the great morphological, ecological and physiological diversity of AM fungi as a whole. Thus, the contribution of in vitro techniques to our general knowledge of arbuscular mycorrhizas is limited.

Successful introduction of fungal isolates into a sustainable culture has been achieved using minimal M medium (Bécard and Fortin 1988). While this medium has been widely used for the study of AM fungi in vitro, it appears unsuitable for culturing some AM fungal

species (Nuutila et al. 1995; Douds 1997). Manipulation of medium composition and pH to suit new fungal isolates could lead to a better understanding of factors affecting the complex biology underlying the symbiosis.

The aim of the present study was to investigate factors required for growing an isolate of the AM fungus *Glomus caledonium*, in an easily reproducible monoxenic culture on transformed carrot roots by making further modifications to the M medium. The experiment was also conducted to assess the potential of the spores formed under monoxenic conditions as a source of contamination-free inoculum.

Materials and methods

Culture medium

The minimal M medium (Bécard and Fortin 1988) was modified as described below and used for both routine maintenance of carrot roots and establishment of the dual culture. The composition of the medium in mg l^{-1} of double-distilled water was: $MgSO_4 \cdot 7H_2O$, 731; KNO_3, 80; KCl, 65; $Ca(NO_3)_2 \cdot 4H_2O$, 288; NaFeEDTA, 8; $MnSO_4 \cdot 4H_2O$, 0.04; $CuSO_4 \cdot 5H_2O$, 0.025; H_3BO_3, 0.45; $ZnSO_4 \cdot 7H_2O$, 0.22; KI, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.025; thiamine, 0.1; pyridoxine, 0.1; nicotinic acid, 0.5; myo-inositol, 50; sucrose, 10000. The micronutrients of the M medium were replaced by those of the minimal medium used by Mugnier and Mosse (1987), which contains Mn and Zn at much lower concentrations. These two elements are known to inhibit germination of *Glomus caledonium* spores (Hepper 1979). The medium was solidified with 0.4% gellan gum (Gel-Gro, ICN). Because of the high P contamination of gellan gum (Doner and Douds 1995), KH_2PO_4 was omitted from the medium. The organic buffer MES was added (10 mM) to stabilize the medium pH, which was adjusted to 6.5 with 1 M KOH before sterilization at 121 °C for 15 min. This buffer was shown to improve hyphal growth from germinating spores of *Glomus caledonium* (Carr 1991) and *Glomus mosseae* (Douds 1997).

Root culture

A culture of Ri T-DNA-transformed carrot roots was kindly provided by Prof. G. Bécard (Laboratoire de Mycologie Végétale, Université Paul Sabatier, Toulouse). The roots were routinely cultured on the modified M medium in inverted Petri dishes at 26 °C in the dark.

Fungal material

The AM fungus *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerdemann (isolate RIS 42, BEG 15), originally provided by Dr. I. Jakobsen (Risø National Laboratory, Roskilde), was grown in pot cultures with onion (*Allium cepa* L. cv. Stuttgarter Riesen) under glasshouse conditions at Hohenheim (Stuttgart, Germany), using a nutrient-poor Luvisol. Spores were isolated by wet sieving (Gerdemann and Nicolson 1963) and density gradient centrifugation (Ohms 1957). Collected spores were surface sterilized twice for 10 min with a 2% solution of Chloramine-T containing a drop of Tween 80, thoroughly rinsed with several changes of sterile tap water and soaked in a solution of antibiotics (streptomycin, 200 mg l^{-1}, and gentamycin, 100 mg l^{-1}). Prepared spores were be stored in this solution at 4 °C for up to 1 year. The sterilization procedure was repeated before using the spores as inoculum (Mertz et al. 1979).

Dual culture

Lengths of carrot root (3–5 cm) with several lateral branches were placed singly in 9-cm Petri dishes containing 40 ml of the modified M medium. Mycorrhizal formation was achieved by placing a single, ungerminated spore of *Glomus caledonium* 5 mm from a lateral root. Only spores which appeared to be viable (without visible damage and containing lipid droplets) were used as inoculum. The dishes were inverted and incubated at 26 °C in the dark.

Mycorrhiza development

Extraradical hyphal spread and sporulation were monitored non-destructively, using either a binocular- or an inverted microscope. At the end of the culture period, roots were extracted from the medium after solubilizating with 10 mM sodium citrate (Doner and Bécard 1991), cleared with 10% KOH at 121 °C for 15 min, and stained with trypan blue as described by Koske and Gemma (1989). Percentage root colonization was quantified using the gridline intersection method (Giovannetti and Mosse 1980). Viability of newly formed spores was determined by reinoculating carrot roots under the same culture conditions.

Medium pH

In order to follow changes in the medium pH caused by the sterilization procedure or by root exudates, carrot roots were grown on modified M medium amended with the pH indicator bromocresol purple (Merck) (0.001%).

Results

Growth of carrot roots

Autoclaving did not alter the pH of the modified M medium buffered at 6.5. However, the beginning of active root growth was associated with a local decrease in pH from 6.50 to 5.5–5.8 around the roots. Further root development was followed by extension of the zone of acidified pH and, in most cultures, the pH decreased to 5.5–5.8 after about 1 month. Transformed carrot roots grew vigorously on the modified M medium. Although most roots penetrated the solid medium, some grew on the surface and into the air space. Roots were initially white, turning yellow-brown when growth slowed at the end of the incubation period.

Spore germination

The procedure used for spore sterilization was efficient. Less than 5% of spores appeared contaminated and Petri dishes containing those were discarded. In the presence of carrot roots, most spores germinated within 1 week. However, only actively growing roots supported prolonged hyphal growth. Germinating spores produced 1–4 germ tubes which grew out from the subtending hypha. When the germ tubes reache a length of several maternal spore diameters (0.5–2 mm), they developed lateral hyphae which

grew backwards or perpendicular to the germ tube. Colorless secondary spores with a diameter of approximately 20 μm were formed at this time. Both branches produced by germ tubes and secondary spores were seen within 1–2 days of spore germination.

Dual culture

By 1–3 weeks after germination, germ tubes and lateral hyphae had formed a network approximately 2–3 cm in diameter around the maternal spore. Many shorter lateral hyphae appeared at this time among carrot roots. Some grew towards roots, bifurcated or trifurcated and formed appressoria on the root surface. Shortly after the first contact between root and fungus, the pattern of fungal development changed drastically with many so-called runner hyphae (Friese and Allen 1991) developing in all directions. These runner hyphae branched to form many higher-order laterals, which developed at 45° and produced numerous oil-filled secondary spores. Within several days, the external mycelium had explored the whole volume of the medium. Main hyphae seldom grew on the medium surface and did not colonize carrot roots growing in the air. Growing hyphae exhibited typical bi-directional protoplasmic streaming as judged from the active movement of optically dense particles observed under the microscope.

After 5–7 weeks growth, four of the seven dual cultures had produced much larger (180–300 μm) spores. All spores were produced within a period of 2–3 days. The number of spores varied markedly between Petri dishes: 11, 19, 24 and 34 (only apparently viable spores being counted). Spores appeared singly both on or in the vicinity of carrot roots or at some distance from them. Subtending hyphae were short lateral branches of main hyphae. Spores were initially white and the contents were homogenous but they became yellow when mature and the contents merged into larger lipid droplets. Not all spores remained viable over the experimental period, with some showing loss of internal contents or coalescence of lipid droplets.

Hyphal density increased greatly during the second and the third month after establishment of the culture, with numerous anastomoses observed. In several cultures, main hyphae bore arbuscule-like structures up to 1 mm in diameter (Fig. 1). These were formed by proliferation of thick, short hyphae, which often developed into spore-like enlargements or spore clusters (Figs. 2, 3). The arbuscule-like structures often appeared in clusters consisting of hundreds of individual structures in an area of 2–3 cm^2. They developed for a period of more than 1 year. At least part of the external mycelium was still alive after 4 months of culture, even though carrot root growth had ceased.

The external mycelium also gave rise to distinct units of secondary mycorrhizal colonization. These were easily observable in the optically clear, young lateral roots. The secondary colonization was initiated by short branches of main hyphae. These short hyphae reached the same diameter as the main hyphae and very intensive protoplasm exchange between the infection unit and extraradical mycelium was observed throughout the experimental period. Staining of root samples showed colonization levels of about 5%. Almost all colonized cortical cells were penetrated by thick hyphae, which spread mostly intracellularly, becoming smaller in diameter between or at the interface of cells (Figs. 4–6). These hyphae formed lateral branches or coils, sometimes surrounded by fine hyphae. Typical arbuscules were not observed and vesicles were rare. Intracellular hyphae usually occupied the entire cell volume and frequently grew into two or more neighboring cells (Fig. 6).

Viability of spores

All newly formed spores of *Glomus caledonium* germinated after being transferred to fresh medium. Spores taken from actively growing cultures (soon after spore maturation) germinated within 12–24 h. Spores isolated from older cultures, e.g., after 4 months, required up to 1 week to germinate. Further fungal development was similar to that observed for spores isolated from pot cultures: production of resting spores took place at the same time as in the initial monoxenic culture. Second-generation cultures produced a mean of 15 spores (range 2–43), third generation cultures a mean of 54 (range 14–91). Further maintenance of the fungus over six generations resulted in an increase in mean spore number, especially when cultured on agarose-solidified medium (data not shown).

Discussion

Minimal M medium has a relatively low pH (5.55) and was originally designed for the establishment of an in vitro culture of *Gigaspora margarita* on transformed carrot roots (Bécard and Fortin 1988). This medium also proved appropriate for obtaining monoxenic cultures of *Glomus intraradices* (Chabot et al. 1992) and *Glomus versiforme* (Diop et al. 1994). On this medium, these fungi produce viable resting spores and so complete their life cycle. However, *Glomus fistulosum* (Nuutila et al. 1995) and *Glomus mosseae* (Douds 1997) grown on the M medium colonized transformed roots but failed to sporulate.

Since the first attempts to culture AM fungi, several studies have concentrated on the effects of medium pH on the establishment of the symbiosis. Spores of *Glomus mosseae* only germinated when the pH of M medium was buffered above pH 7 (Douds

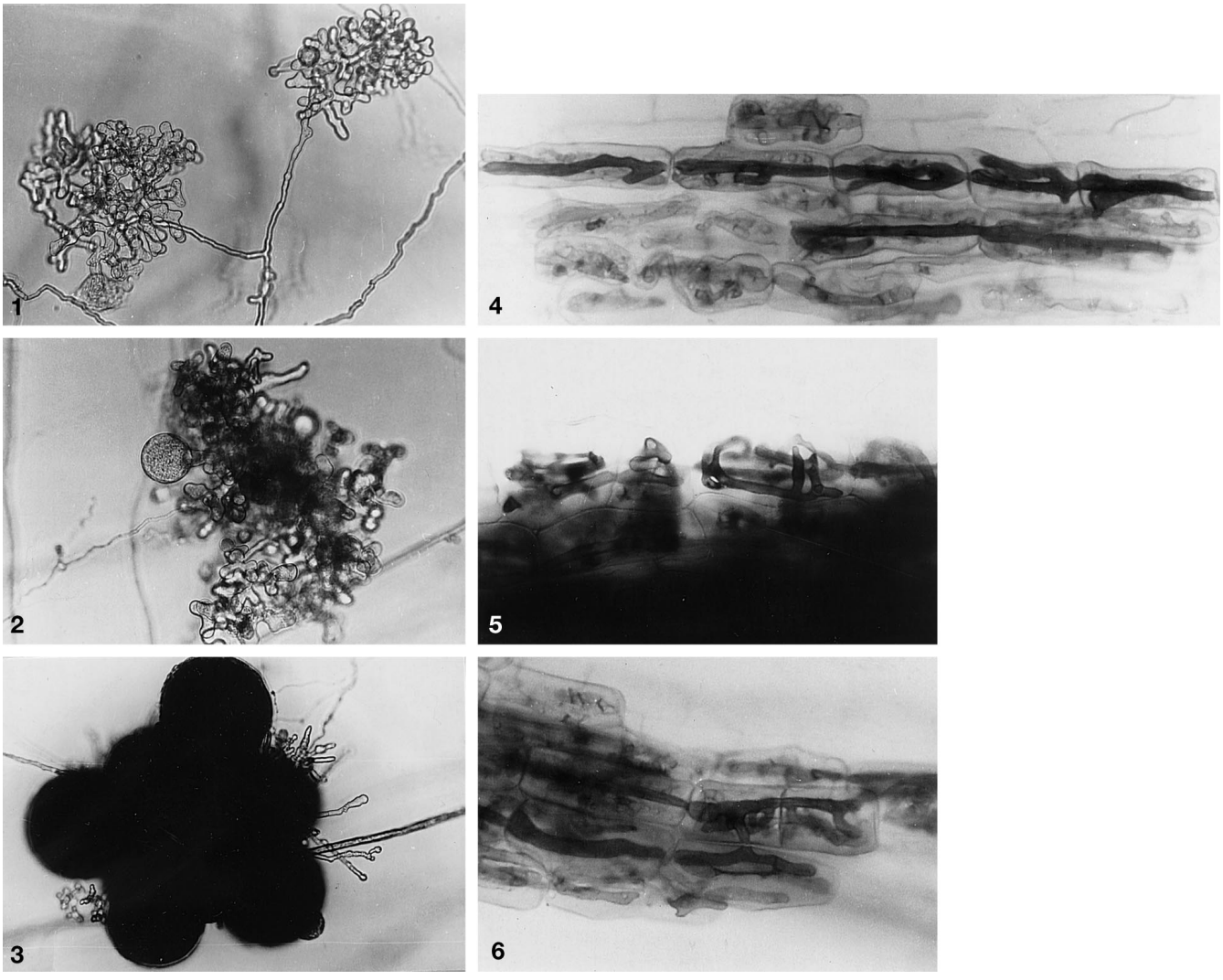

Fig. 1 Arbuscule-like branches formed on the external mycelium of *Glomus caledonium*; *bar* 50 μm

Fig. 2 Spore formation on arbuscule-like branches; *bar* 50 μm

Fig. 3 A spore cluster developing from an arbuscule-like structure; *bar* 100 μm

Fig. 4 Intramatrical phase of *Glomus caledonium* in carrot roots; *bar* 25 μm

Fig. 5 Intracellular coiled hyphae of *Glomus caledonium*; *bar* 25 μm

Fig. 6 An intracellular hypha of *Glomus caledonium* penetrating three neighboring carrot cells; *bar* 25 μm

1997). In other studies, development of *Glomus mosseae* (Mosse and Hepper 1975; Mugnier and Mosse 1987) and *Glomus intraradices* (Mosse 1988) was inhibited at low medium pH and hyphae only grew after the pH was increased. To increase medium pH, either pH is adjusted before or after autoclaving (Mugnier and Mosse 1987) or the medium is amended with organic buffers (Mosse 1988; Carr 1991; Douds 1997). The second method is preferable since minimal media normally have low buffering capacities and pH may change during sterilization or due to root nutrition and/or exudation during the incubation period.

Preliminary tests showed that germination of *Glomus caledonium* spores in the presence of growing carrot roots was not affected by pH. However, germ tube growth ceased within a few days of germination when the pH was adjusted to 5.55 (results not shown). Addition of 10 mM MES stabilized medium pH at 6.5 during sterilization and maintained it at this level during the initial part of the experiment. This allowed the fungus to colonize the roots. A decrease in medium pH to 5.5–5.8 during incubation of the dual cultures did not affect the formation or function of secondary colonization units. This suggests that early developmental events, such as spore germination, germ tube growth, host recognition and initial root contact determine the success or failure of the symbiosis and are the stages most dependent on environmental factors. Subsequent access to root carbohydrates may allow the fungus to grow under a wider range of environmental conditions.

Fragments of mycorrhizal roots and isolated vesicles have also been used successfully as fungal inoculum for the establishment of monoxenic cultures of several isolates of *Glomus macrocarpum*, *Glomus fasciculatum*, *Glomus intraradices* and *Glomus versiforme* (Diop et al. 1994; Declerck et al. 1996, 1998). In preliminary tests in our laboratory, sterilization (Diop et al. 1994) of freshly collected roots produced up to 30% contamination-free root fragments. However, intraradical structures of *Glomus intraradices* germinated and formed many hyphae, whereas those of *Glomus caledonium* did not (data not shown). Burggraaf and Beringer (1989) also observed negligible hyphal growth from intraradical vesicles of *Glomus caledonium*. Furthermore, the sterilization procedure was not satisfactory when applied to roots after several months storage in dried soil (data not shown; Mosse 1988). Therefore, for the present study, fungal resting spores appeared the most suitable source of inoculum, because of simple and efficient procedures of spore extraction and sterilization and because of high inoculum potential.

The first sporulation of *Glomus caledonium* in monoxenic culture was obtained by Hepper (1981) using entire clover plants. In her experiment, spores were produced after 4 months dual culture. However, in our case, up to 363 spores were collected after 3 months (data not shown). The number of spores recovered from the dual culture was similar to that reported for fungi forming larger resting spores, *Gigaspora margarita* (150 spores, ⦰ 300 μm, Diop et al. 1992) and *Glomus macrocarpum* (250 spores, ⦰ 160–180 μm, Declerck et al. 1998). Successive subcultures resulted in increased spore production, perhaps due to adaptation of the fungus to specific conditions of in vitro culture.

In the present study, sporulation did not follow the classic three-phase development (lag, exponential, and plateau) common for most biological populations, including AM fungi (Declerck et al. 1996; Bago et al. 1998a) and all spores were formed within several days. This may be due to the time scale of the observations or it may be a natural feature of the fungal isolate or an effect of certain culture conditions on the fungus. To investigate this further, a time-course pot experiment to determine sporulation rates should be performed using intact plants. Concurrently, in vitro experiments involving alterations of the medium composition, for example P concentration, should also be carried out.

The spores produced in the present study retained high inoculum potential over successive cultures and could be used to form mycorrhiza in new plant roots. No specific tests were performed to study the capacity of the spores to colonize plant roots in vivo. However, it was shown previously that both spores and mycorrhizal root fragments obtained in monoxenic culture can colonize roots of *Acacia albida* (Diop et al. 1994) and leek (Declerck et al. 1998) grown in a glasshouse.

Apparently, most of the infection units did not produce arbuscules. It was reported that arbuscules produced in older roots of monoxenic cultures of *Glomus mosseae* (Mosse and Hepper 1975) and *Gigaspora margarita* (Miller-Wideman and Watrud 1984) "remained vestigial and looked stumpy with few fine branches. In such infections the main feature was massive, much swollen hyphae growing longitudinally within the intercellular spaces" (Mosse and Hepper 1975). In our case, intraradical hyphal spread was almost exclusively intracellular, forming coils of thickened hyphae inside the cells. The intensive bi-directional protoplasmic exchange and the similar diameter of main hyphae and hyphae connecting infection units to the main hyphae suggests that the infection unit is important for exchange of material, even in the absence of arbuscules. The appearance of "arbuscule free" colonization sites was not due to aging of the dual cultures. Thus, under the present culture conditions, *Glomus caledonium* formed a *Paris*-type mycorrhiza (Smith and Smith 1997) on transformed carrot roots. The fact that under the same culture conditions *Glomus intraradices* formed typical *Arum*-mycorrhiza (data not shown) supports the suggestion of Smith and Smith (1997) that carrot has an unclear status with respect to the formation of *Arum*- or *Paris*-types of mycorrhiza.

Development of extraradical mycelium under sterile conditions is usually accompanied by the production of so-called arbuscule-like structures (ALS) (Mosse and Hepper 1975; Bécard and Fortin 1988; Chabot et al. 1992; Bago et al. 1998a) or branched absorbing structures (BAS) (Bago et al. 1998b). These ephemeral structures are comprised of finely branched hyphae. The suggestion has been advanced that the appearance of these structures is a specific response to a more or less intimate interaction with a root (Bécard and Fortin 1988), or a localized response to an unknown stimulus within the medium (Mosse and Hepper 1975). Recently, Bago et al. (1998a, b) suggested that ALS/BAS play an important role as preferential sites for nutrient uptake by the extramatrical mycelium of AM fungi and increase nutrient supplies to spores developing on individual ALS/BAS. Besides typical ALS/BAS, fungal mycelium in the present study also produced long-lived hyphal structures more closely resembling intracellular arbuscules. Their development coincided with a decrease in active root and mycelium growth. We suggest that the development of dual cultures causes some changes in the nutrient medium, such as a decrease in medium pH to the level corresponding to the apoplastic pH (5.5, according to Kurkdjian and Guern 1989) or an accumulation of certain root metabolites. This may trigger the extraradical fungal mycelium to form structures more typical of intraradical fungal mycelium (arbuscules). However, the fact that these structures were observed only in a few dual cultures up to now poses the question of whether they are produced only under

sterile conditions or are also formed in soil. It is also not known whether these hyphal structures occur with other fungal species. Factors controlling the formation of these structures and their possible functions require further study.

Acknowledgements This study was initiated by the late Prof. Horst Marschner. We thank Dr. Iver Jakobsen for supply of the fungal material and Prof. Guillaume Bécard for very helpful advice and supply of the plant material. This research was financed by the Volkswagen-Stiftung, Hannover, Germany.

References

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoaxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Azcón-Aguilar C, Goulet A, Piché Y (1998b) Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular-mycorrhizal fungi. New Phytol 139:375–388

Balaji B, Poulin MJ, Vierheilig H, Piché Y (1995) Responses of an arbuscular mycorrhizal fungus, *Gigaspora margarita*, to exudates and volatiles from the Ri T-DNA-transformed roots of nonmycorrhizal and mycorrhizal mutants of *Pisum sativum* L. Sparkle. Exp Mycol 19:275–283

Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Piché Y (1990) Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA-transformed roots. Can J Bot 68:1260–1264

Burggraaf AJP, Beringer JE (1989) Absence of nuclear DNA synthesis in vesicular-arbuscular mycorrhizal fungi during in vitro development. New Phytol 111:25–33

Carr GR (1991) Use of zwitterionic hydrogen ion buffers in media for growth tests of *Glomus caledonium*. Soil Biol Biochem 23:205–206

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Declerck S, Strullu DG, Plenchette C (1996) In vitro mass-production of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Diop TA, Bécard G, Piché Y (1992) Long-term in vitro culture of an endomycorrhizal fungus, *Gigaspora margarita*, on Ri T-DNA-transformed roots of carrot. Symbiosis 12:249–259

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared-root inocula of vesicular-arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Doner LW, Bécard G (1991) Solubilization of gellan gels by chelation of cations. Biotechnol Tech 5:25–28

Doner LW, Douds DD (1995) Purification of commercial gellan to monovalent cation salts results in acute modification of solution and gel-forming properties. Carbohydr Res 273:225–233

Douds DD (1997) A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA transformed carrot roots. Mycorrhiza 7:57–61

Friese CF, Allen MF (1991) The spread of VA mycorrhizal hyphae in the soil: inoculum types and external hyphae architecture. Mycologia 83:409–418

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal *Endogone* species extracted by wet sieving and decanting. Trans Br Mycol Soc 46:235–244

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular infection in roots. New Phytol 84:489–500

Hepper C (1979) Germination and growth of *Glomus caledonius* spores: the effects of inhibitors and nutrients. Soil Biol Biochem 11:269–277

Hepper C (1981) Techniques for studying the infection of plants by vesicular-arbuscular mycorrhizal fungi under axenic conditions. New Phytol 88:641–647

Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 94:486–488

Kurkdjian N, Guern J (1989) Intracellular pH: measurement and importance in cell activity. Annu Rev Plant Physiol Plant Mol Biol 40:271–303

Mertz SM, Heithaus III JJ, Bush RL (1979) Mass production of axenic spores of the endomycorrhizal fungus *Gigaspora margarita*. Trans Br Mycol Soc 72:167–169

Miller-Wideman MA, Watrud LS (1984) Sporulation of *Gigaspora margarita* on root cultures of tomato. Can J Microbiol 30:642–646

Mosse B (1988) Some studies relating to "independent" growth of vesicular-arbuscular endophytes. Can J Bot 66:2533–2540

Mosse B, Hepper C (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infection in transformed root-inducing roots grown axenically. Phytopathology 77:1045–1050

Nuutila AM, Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria* × *Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Reports 14:505–509

Ohms EE (1957) A flotation method for collecting spores of a phycomycetous mycorrhizal parasite from soil. Phytopathology 47:751–752

Smith FA, Smith SE (1997) Structural diversity in (vesicular)-arbuscular mycorrhizal symbioses. New Phytol 137:373–388

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f. sp. *chrysanthemi* in an in vitro dual culture system with the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effects of N source on pH and nutrient exchange of extramatrical mycelium in a mycorrhizal Ri T-DNA-transformed root system. Mycorrhiza 6:247–251