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Long-Term *in vitro* Culture of an Endomycorrhizal Fungus, *Gigaspora margarita*, on Ri T-DNA Transformed Roots of Carrot

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Abstract

The vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita* was inoculated on Ri T-DNA transformed roots of carrot. Both organisms were grown for a long period (one year) and various features of the symbiosis were studied quantitatively and correlated to evaluate the *in vitro* system. Root biomass, intramatrical mycelium and production of spores were quantified. The infection units (penetration points with typical arbuscules) simultaneously increased in number and length over the culture period, and mostly when root growth declined. Sporulation of *Gigaspora margarita* took place after establishment of extensive hyphal biomass (1mo) and was still actively occurring after one year in culture. The new spores (450/dish) were of the expected color, shape and size, and germinated (95%) on fresh medium without pretreatment.

Keywords: *Gigaspora margarita*, Ri T-DNA transformed root, *in vitro* sporulation

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1. Introduction

The potential benefit of utilizing vesicular-arbuscular (VA) mycorrhizal fungi for plant propagation in horticulture, agriculture and forestry depends on the availability of high quality and low cost VA mycorrhizal inoculum (Plenchette, 1991). Different methods are still under investigation for producing such an inoculum including hydro- and aeroponic cultural techniques (Thompson, 1986; Hung and Sylvia, 1988). Until the axenic cultivation of endomycorrhizal fungi is achieved, the use of root organ culture (Mosse and Hepper, 1975; Mugnier and Mosse, 1987a) offers an alternative for producing inoculum free of pathogens. Recently, Bécard and Fortin (1988) developed a simple and reproducible system to cultivate, through its complete life cycle, the VA mycorrhizal fungus *Gigaspora margarita* Becker and Hall. They used carrot roots, transformed by the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes*, as plant host. Our objective was to examine the performance of the *in vitro* system during one year of culture to evaluate its potential for inoculum production.

2. Materials and Methods

Fungal symbiont

The VA mycorrhizal fungus used in this study was *Gigaspora margarita* Becker and Hall (DAOM 194757, deposited at the Biosystematic Research Center, Ottawa, Canada). Spores of this fungus were produced in association with leek (*Allium porrum* L.) after 6 months in green house culture. They were then extracted by wet sieving (Gerdemann and Nicolson, 1963), purified by density gradient centrifugation (Furlan et al., 1980) and finally surface sterilized according to the procedure described by Bécard and Fortin (1988). Spores germinated at 27°C on M medium (Bécard and Fortin, 1988) after being slightly (2 mm) inserted into the gel. The dishes were incubated vertically so the emerging germ tubes (third day), which have a negative geotropic mode of growth (Watrud et al., 1978), grew upward into the solid medium. Germinating spores then (fourth day) were transferred axenically into the experimental dishes by using a 15 mm cork borer.

Root organ culture

Roots of carrot (*Daucus carota* L.) transformed by the Ri T-DNA of *Agrobacterium rhizogenes* served as plant partner (Bécard and Fortin, 1988). They were routinely cultivated on a minimal M medium (Bécard and Fortin,

1988) except that Bacto Agar was replaced by 0.4% (w/v) gellan gum (Gel-Gro, ICN Biochemicals, Cleveland, OH, 44128, USA). This is a clear gelling agent which allows better root growth and facilitates microscopic observations.

Experimental culture

The experiments were conducted in square Petri dishes (9×9 cm) incubated vertically at 27°C in the dark. Each dish contained 40 ml of M medium solidified with 0.4% Gel-Gro. Two sterile cotton dental rolls (Healthco, DDL Montréal, Canada) were placed at the lower-edge of each Petri dish to absorb condensation. A single transformed root of carrot (7 cm long) with a vigorous elongation zone and some lateral roots was introduced along with three germinated spores on the upper part of the dish (Fig. 1). Dishes without spores served as control units.

Experimental set-up for assessing mycorrhizal formation in vitro

Several biometric measures of the dual culture were made after 2, 4, 6 and 12 months: fresh weight of inoculated and uninoculated roots, number and length of mycorrhizal infection units, degree of root colonization, and number of new spores.

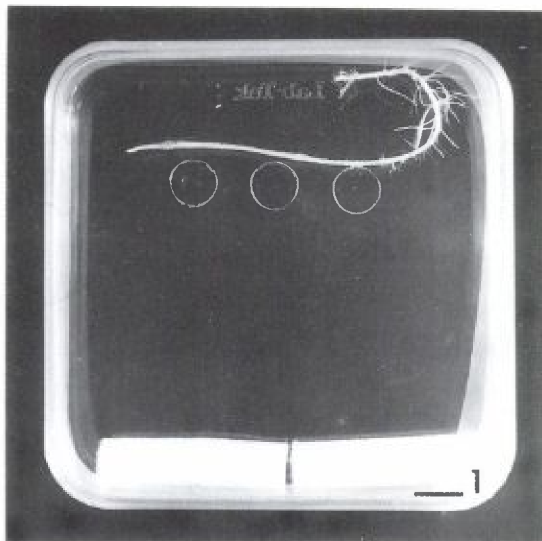

Figure 1. Experimental unit: dual culture of a Ri T-DNA transformed root of carrot with 3 pregerminated spores of *Gigaspora margarita*. The dots indicate the initial position of fungal symbiont. The two sterile cotton rolls in the bottom absorb the condensed water. Bar = 1 cm.

The number and length of mycorrhizal infection units were examined after clearing (10% KOH at 90°C for 60 min), rinsing the roots with deionised water and staining with 0.1% (w/v) chlorazol black E at 90°C for 60 min (Brundrett et al., 1984). Stained roots were delicately mounted on slides and viewed under a microscope at 100×.

The percentage of root length colonized by *G. margarita* has not been established by using an estimation method like the gridline intersect method but by direct calculation. The length of infected root was exhaustively measured under a microscope and the total root length was calculated once (23 m). This latter was considered constant enough to be used for all replicates to calculate colonization percentage at the 4th, 6th and 12th month.

The ability of spores produced *in vitro* to germinate on fresh M medium was used as an indication of their viability. Sixty-eight spores were chosen randomly and transferred axenically from a one-year-old culture, directly into the experimental dishes. Four dishes were used as replicates, each with 13 to 19 spores, and were incubated in an inverted position at 32°C in a CO_2 (2%) incubator for 10 days.

Statistical analysis

Each Petri dish was an experimental unit. Three to ten dishes were assayed per sample. All tests were analysed by ANOVA with the general linear model procedure and Waller-Duncan test (P 0.05) (Anderson and Mclean, 1974). Data for root colonization and the number of mature spores were transformed to logarithms and square roots, respectively. Correlations between sporulation and colonization variables were calculated.

3. Results

Fresh weight of mycorrhizal and nonmycorrhizal transformed roots

Fresh weight of inoculated and uninoculated transformed carrot roots were not significantly different during the 12 months of culture (Fig. 2). The fresh root weights rapidly increased during the first 2 months and reached a peak at month 4. Then, for the last 8 months, no noticeable root tissue was synthesized and the observed progressive decrease of fresh weight can be attributed to loss of water and root aging. Fresh root weight was highly and negatively correlated with most of the other sequences of mycorrhizal formation (Table 1).

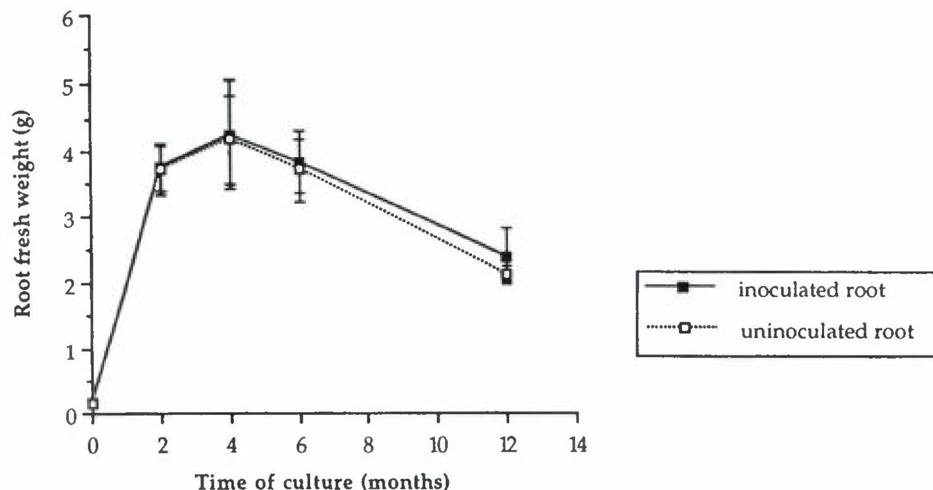

Figure 2. Fresh weight of transformed roots of carrot in inoculated plates and uninoculated plates during 12 months of culture. Vertical bars indicate standard deviations.

Table 1. Linear correlation coefficients between the different measured variables after 12 months of culture

	Spores	Length of infection unit	Number of infection unit	% of Colonization	Weight
Spores	1.000	0.899	0.921	0.956	–0.579
Length of infection unit		1.000	0.949	0.969	–0.759
Number of infection unit			1.000	0.977	–0.766
% of colonization				1.000	0.807
Weight					1.000

Pearson correlation coefficients are significant at r = 0.005

Mycorrhizal infection units

Infection units were characterized by the formation of many penetration points and typical arbuscules (Fig. 3). The number and length of infection units were directly correlated [r=0.95, Table 1)] at one year with a mean rate of production of 1.3 infection units and 13.7 μm per day, respectively. During the last 8 months of culture, however, the number of infection units increased more rapidly than length, by a factor of 5.3 and 2.7, respectively (Fig. 4). These two variables were as expected, positively correlated (> 96%) to root colonization.

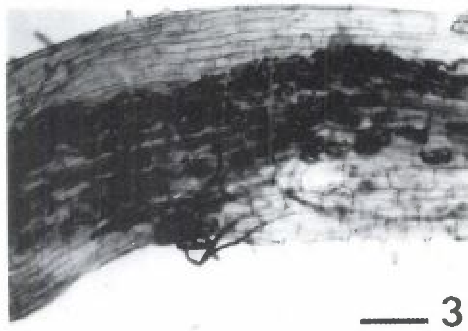

Figure 3. Typical infection unit: a root segment highly colonized by fungal hyphae with one or several penetration points (arrows). Note the presence of numerous arbuscules in the cortical region of the root. Bar = 7.0 μm.

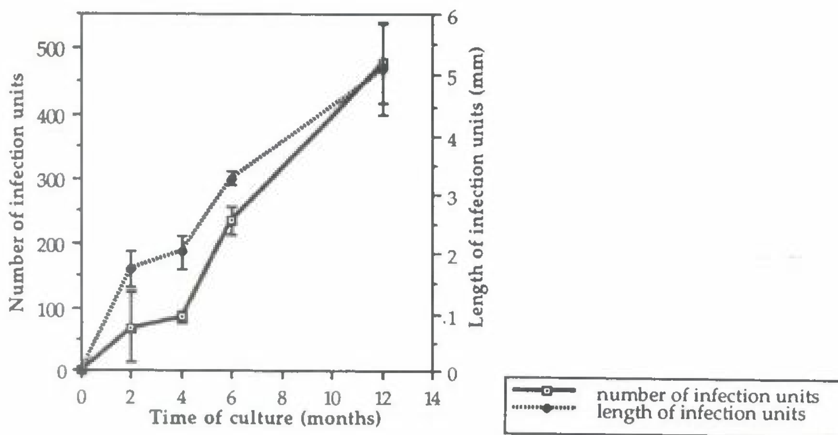

Figure 4. Root colonization during 12 months of culture expressed by the average number and length of infection units. Vertical lines indicate standard deviations.

Percentage of root colonization

The percentages of root length colonization were 0.8, 3.3, and 10.6% respectively after 4, 6, and 12 months of culture. This represents an average length of 2.43 m of roots highly colonized and about 500 infection units (Fig. 4) at the end of the culture.

Production and viability of spores produced in vitro

Newly-matured spores were uniform, globular, and white with an average diameter of 300 μm and did not show a zone of preferential formation within

Figure 5. *In vitro* production of spores of *Gigaspora margarita*. Bar = 1.5 mm.

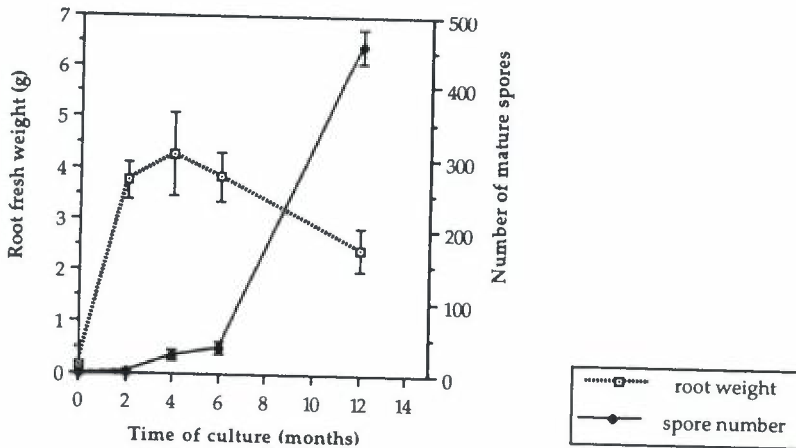

Figure 6. Spores and root biomass produced in dual culture for 12 months. Vertical lines indicate standard deviations.

dense mycelium in Petri dishes (Fig. 5). *In vitro* sporulation of *G. margarita* was slow during the first 4 months of observation, as root weight rapidly increased (Fig. 6). More active sporulation started in the 6th month of culture with no apparent slowing in production after 12 months, leading to an average of 449 spores per dish. The number of spores was negatively correlated to root weight (Fig. 6), but positively correlated to root colonization (96%).

An average of 94.8% (coefficient of variation, C.V. = 7.6%) of the spores produced *in vitro* germinated within 10 d of removal and placement on fresh plates. Forty percent of the spores germinated within 2 days. All germinating

spores exhibited a single, vigorous growing germ tube producing branches and auxiliary cells.

4. Discussion

Dual culture of the vesicular-arbuscular mycorrhizal fungus *G. margarita* and transformed carrot roots produced an extensive root and fungal biomass with typical infections and numerous spores. These results confirm that root organ cultures allow VA mycorrhizal fungi to complete their life cycles (Bécard and Piché, 1992).

Inoculated and uninoculated transformed roots of carrot exhibited similar growth. No advantage for root growth was associated with the presence of the VA mycorrhizal fungus. This result is not surprising since the *in vitro* system used was a closed system, where all nutrients are freely and equally available (until they become progressively limiting) for both colonized and uncolonized roots. There is no formation of a depletion zone in such hydroponic systems.

More surprising and difficult to interpret is that root infection (as expressed by the number of infection units) occurred mostly during the last 8 months of culture, i.e. with non growing roots. More than 80% of the infection units were produced during this period of root aging. One explanation is that the early stages of root colonization had to overcome some defense mechanisms, and later the fungus took advantage of the reduced metabolic activity of roots. We do not have sufficient knowledge about the susceptibility of the non growing roots to mycorrhizal infection in a natural system to conclude that this *in vitro* development is atypical. Intraradical spreading of colonization (as expressed by length of infection units) seemed to occur within growing roots as well as aging roots. We hypothesize that growth of the mycosymbiont followed a biotrophic mode along with, progressively, some necrotrophy. This latter growth ability can be related to the prolonged viability of VA mycorrhizal hyphae in senescent and dead roots reported by Hall (1976) and Tommerup and Abbott (1981).

The VA mycorrhizal fungus *G. margarita* produced numerous mature spores in one year of dual culture with transformed roots. An average number of 150 times the initial number of mother spores has been obtained. The first daughter spores were produced after 2 months. The mean production of 3 new spores of *Gigaspora margarita* after 2 months of dual culture agreed with results of Miller-Wideman and Watrud (1984) who used tomato roots as the plant partner. However, they used 10 germinated spores of *G. margarita* as inoculum and many of the daughter spores aborted. Spore production is correlated with root aging and strongly correlated with mycorrhizal infection. It is not clear

whether sporulation was a survival reaction caused by stress conditions when roots became senescent or whether sporulation was strictly dependent on root colonization. In the first hypothesis, the fungus would utilize material already existing in the extramatrical phase while in the second hypothesis the fungus would directly convert biotrophic material through the intraradical phase into material for sporulation. Interestingly, the number of spores and infections units produced at the end of the culture were approximately the same with a high correlation coefficient (92%).

VAM fungus spores can appear healthy and not be viable (Miller-Wideman and Watrud, 1984; Mugnier and Mosse, 1987b). Spores produced under our culture conditions germinated readily without the presence of roots or any previous treatments. The ability of their germ tubes to form hyphae and produce auxiliary cells was identical to that of spores produced in the greenhouse. Their infectivity *in vitro* on transformed roots and *in vivo*, on plants grown in pot culture, has been verified several times. The mechanism which prevents them from germinating *in situ*, even after several months at 27°C, and why germination is triggered after a simple transfer into another Petri dish, are two interesting and fundamental questions.

This study shows that the root organ culture system is a promising method to produce clean VA mycorrhizal inoculum since, with only 3 spores used as inoculum, more than 2 m of colonized roots and 450 spores of *G. margarita* were produced, in average, in 40 ml of medium. This represents a yield of 11.25 spores per cm^3 of medium. In a 4-month greenhouse experiment with seedlings of *Paspalum notatum*, Douds and Schenk (1990) obtained, with the best nutrient regime, 5.5 to 6.5 spores per cm^3 of soil. They used 30 to 40 mother spores as inoculum and two isolates of *G. margarita* comparable with our isolate in terms of spore size (Douds, personal communication). The comparison for spore production would be very much in favor of the *in vitro* system if the culture (12 months) could be significantly reduced. We hypothesize that this could be achieved by utilizing a poorer culture medium in order to shorten the period of active root growth. The most active root colonization along with sporulation would be triggered earlier, leading to a final greater percent root colonization. The size of a Petri dish is certainly suitable for research purposes and also for collection of fungal isolates, but efforts must now be made to scale up the system for quantitative production of VA mycorrhizal inoculum.

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