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A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA-transformed carrot roots

Abstract A stepwise procedure was investigated to determine the optimal conditions for the establishment of *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe in dual in vitro culture with Ri T-DNA-transformed roots of *Daucus carota* L. *Glomus mosseae* spores germinated best in 10 mM Tris or MES-buffered medium at pH values just above neutral. Growth of hyphae from germinated spores was much greater in the presence of Tris than MES, eg. 8 mm versus 4 mm per spore for Tris and MES, respectively, at pH 7.2. Roots exhibited a broad pH optimum for growth of 6.0–7.0 in both MES and Tris, but did not grow well above pH 7.5. In addition, purified gelling agent, gellan gum, was utilized to lower the P concentration of media. With these factors combined, mycorrhizas were successfully established in 14% of dual cultures.

Key words Dual culture · Mycorrhizal fungi
Transformed roots · *Daucus carota*

Introduction

In vitro dual culture of Ri T-DNA-transformed roots of Carrot (*Daucus carota* L.) and vesicular-arbuscular mycorrhizal (VAM) fungi is a powerful tool for the study of these symbionts (Mugnier and Mosse 1987; Bécard and Fortin 1988; Bécard and Piché 1992). Among the characteristics of VAM fungi described using in vitro methods, with or without roots, are: the stimulation of germination and hyphal growth by CO_2 (Bécard and Piche 1989), regulation of hyphal growth and branching by root exudates (Nagahashi et al. 1996b), the reactions to compounds from host and nonhost roots (Bécard and Piché 1990; Schreiner and Koide 1993), uptake, transfer, and metabolic fate of ^{13}C-labeled metabolites (Pfeffer et al. 1996), and response of VAM fungi to cell wall-associated phenolics (Douds et al. 1996) and flavonoids (Bécard et al. 1992; Chabot et al. 1992b; Morandi et al. 1992). Other potential uses for this system are the production of pure, concentrated inoculum and sterile fungal tissue for genetic and physiological studies.

To date, few VAM fungus species are reported to have been successfully established in dual, in vitro culture. Among these are *Gigaspora margarita* (Bécard and Fortin 1988), *Gigaspora gigantea* (Douds and Bécard 1993), *Glomus etunicatum* (Schreiner and Koide 1993), and *Glomus intraradices* (Chabot et al. 1992a; St. Arnaud et al. 1996). Mugnier and Mosse (1987) reported successful establishment of *Glomus mosseae* on Ri T-DNA-transformed roots of *Convolvulus sepium*. Their method required a two-compartment system. Roots grew in nutrient medium in one compartment and crossed into a water agar plus peat medium in another compartment, where they were inoculated with pregerminated spores of *Glomus mosseae*. No secondary infections were found when peat was excluded from the water agar. The presence of peat, however, would make observation of fungus-root interactions in situ difficult.

Different VAM fungi have unique characteristics making them particularly suited for individual lines of research. For example, *Gigaspora margarita* was quite useful in initial studies of the response of VAM fungi to flavonoids, notably quercitin (Bécard et al. 1992; Chabot et al. 1992a). However, *Gigaspora gigantea* does not respond to quercitin (Douds et al. 1996). In addition, *Gigaspora gigantea* responds to exudates of Carrot in a more quantifiable manner than does *Gigaspora margarita*, making it a better subject for the study of signaling between root and fungus (Nagahashi et al. 1996a). Further, hyphae of *Glomus intraradices* prolif-

erate and sporulate profusely after crossing a barrier in vitro into root-free medium (St. Arnaud et al. 1996). *Gigaspora gigantea* hyphae do not proliferate under similiar circumstances (Douds, unpublished results). Given that the long-term goal of all such research is the establishment of VAM fungi in axenic culture, it is essential to establish many individual species in dual culture to obtain an optimal subject for investigation of particular aspects of the symbiosis.

Successful establishment of a VAM fungus species in dual in vitro culture may require manipulation of the conditions proposed by Bécard and Piché (1992) to suit the new fungus. Even so, establishment of in vitro dual cultures with transformed roots "is extremely difficult" (Redecker et al. 1995). A buffer system and pH may need to be found which is conducive to both germination and hyphal growth of the fungus and growth of the root partner. Media P concentrations may require adjustment because VAM fungi differ in their "P tolerance" (Sylvia and Schenck 1983). Experiments were conducted with one of the most ubiquitous and often studied of the VAM fungi, *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, to demonstrate a methodical approach to dual cultures in a single compartment system.

Materials and methods

Fungal germination and growth

Spores of *Glomus mosseae* (INVAM 156) were isolated from pot culture with *Paspalum notatum* Flugge via wet sieving and centrifugation (Gerdemann and Nicolson 1963; Jenkins 1964). Spores were surface sterilized using chloramine T (2% w/v), streptomycin sulfate (0.02% w/v) and gentamicin sulfate (0.01% w/v) (Bécard and Fortin 1988), and stored at 4 °C on Petri plates of "M" medium (Bécard and Fortin 1988) with 0.4% (w/v) gellan gum ("Phytagel," Sigma) as gelling agent. Spores were used anytime within the first 4 months of storage.

Preliminary experimentation showed no germination of *Glomus mosseae* spores at 32 °C in 2% CO_2 when inserted into routine dual culture M medium (unbuffered pH 5.5). Germination trials were then conducted using 10 mM MES [2-(N-morpholino) ethansulfonic acid] and 10 mM Tris [tris (hydroxymethyl) aminomethane] (Trizma acid and base, Sigma) buffers at pH values in the range 6.0–7.5. Three replicate 9-cm-square plastic Petri plates (experimental units) each with 20–25 spores inserted into the gel, were prepared for each pH and incubated vertically at 32 °C in 2% CO_2 for 25 days. Total hyphae length of individual germinated spores was measured microscopically using an occular micrometer.

Root growth

Growth of Ri T-DNA-transformed carrot roots was measured in the presence of buffers at the pH values utilized for spore germination and growth. Roots were typically subcultured on unbuffered M medium (Bécard and Fortin 1988) with 0.2% (w/v) gellan gum to facilitate transfer of roots, and transferred into 9-cm-square Petri plates of experimental media with 0.4% (w/v) gellan gum and incubated horizontally at 24 °C. Total root length was measured using the gridline intersect method (Newman 1966) after 23 days growth. Root fresh weight was measured after solubilizing the gels in 10 mM Na citrate, pH 6.0 (Doner and Bécard 1991).

Dual culture

Once optimal buffer and pH combinations were found for spore germination and initial hyphal growth, and it was determined that the roots grew sufficiently well at those buffer and pH combinations at 24 °C, the two organisms were grown together in 9-cm-square Petri plates. Roots were subcultured for these experiments in plates of M medium with 0.2% (w/v) gellan gum and 10 mM Tris buffer pH 7.2 and 7.5. Two pregerminated *Glomus mosseae* spores were transferred to each plate with a Carrot root and incubated horizontally for 3–6 months at 24 °C. Germinated *Glomus mosseae* spores exhibited no geotropism, so it was unnecessary to incubate vertically as for *Gigaspora margarita* (Bécard and Fortin 1988).

The P content of the medium was adjusted in later experiments. Commercially available gellan gum contains approximately 0.1% P by weight (Doner and Douds 1995), due to precipitation from *Sphingomonas elodea* cultures along with gellan gum. This would supply approximately 0.2 mg P per 50 ml of 0.4% gellan in a 9-cm Petri dish, compared with 0.055 mg P contained in that volume of M medium. Experiments were then conducted to compare mycorrhiza formation using unpurified gellan (M medium) to that using purified gellan (Doner and Douds 1995) (M medium with and without P). Cultures were initiated with the optimal buffer and pH combinations and incubated at 24 °C for 3–6 months.

All plates were monitored routinely for colonization (e.g. proliferation of hyphae). Gels were solubilized with 10 mM Na citrate (Doner and Bécard 1991) following the incubation period and roots were cleared and stained with trypan blue (Phillips and Hayman 1970) to detect colonization by *Glomus mosseae*.

Results

Spore germination and hyphal growth

Initial attempts to germinate spores of *Glomus mosseae* in M medium at the unbuffered pH of 5.5 failed. In addition, incubation in M medium with 10 mM MES buffer at pH 5.5 resulted in no germination after 24 days. Incubation in MES pH 5.8 resulted in only 6.7 ± 1.3% germination after 24 days. Therefore, buffered media spanning pH 7 were examined.

Glomus mosseae spores exhibited the highest germination in MES above pH 6.7 and at 7.3 and 7.6 in Tris (Fig. 1). Maximal germination after 25 days was approximately 80% with Tris or MES. Hyphal growth, however, was much greater for spores in the presence of Tris than MES (Fig. 2).

A second experiment was conducted to focus on Tris buffer in the range pH 7.0–8.0. The optimal pH range for spore germination, measured after 24 days, was 7.06–7.38 and hyphal growth showed an optimum at pH 7.68 (Fig. 3). Thereafter, routine germination of *Glomus mosseae* spores conducted with 10 mM Tris, pH 7.1–7.3 resulted in 60–70% germination in 14–20 days.

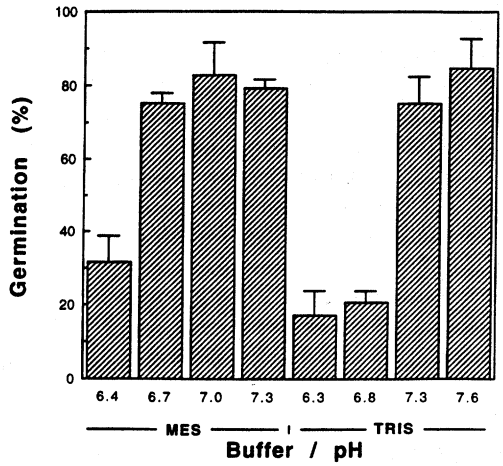

Fig. 1 Germination of spores of *Glomus mosseae* after 25 days incubation at 32 °C and 2% CO_2. Means of three replicates of 20 spores each ± SEM

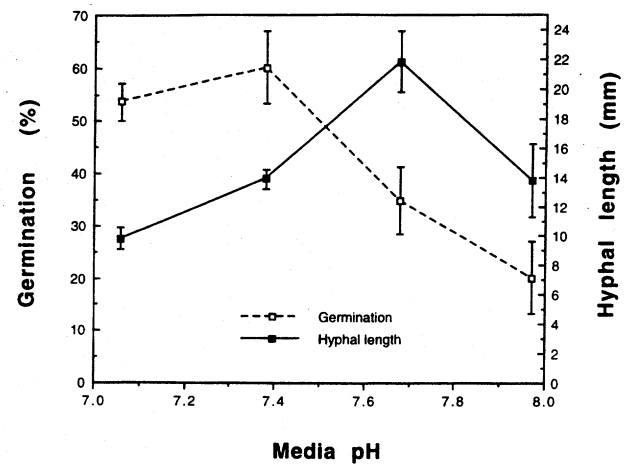

Fig. 3 Germination and length of hyphae from germinated spores of *Glomus mosseae* after 25 days incubation in 10 mM Tris buffer at the indicated pH. Means of three replicate plates, each with 25 spores, ± SEM

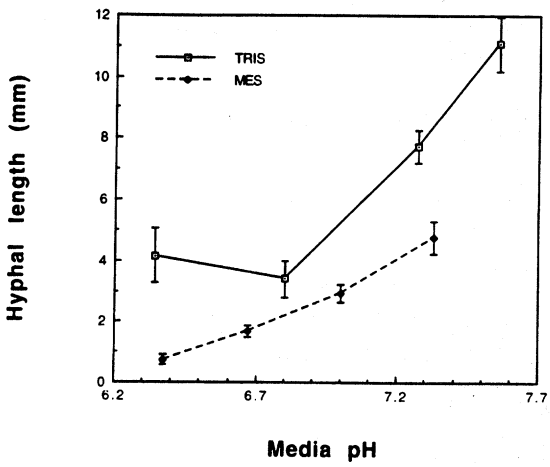

Fig. 2 Length of hyphae from individual germinated spores of *Glomus mosseae* after 25 days incubation in 10 mM MES or Tris buffer at the indicated pH. Data presented indicate the mean hyphal length of germinated spores per plate, three replicate plates per treatment ± SEM

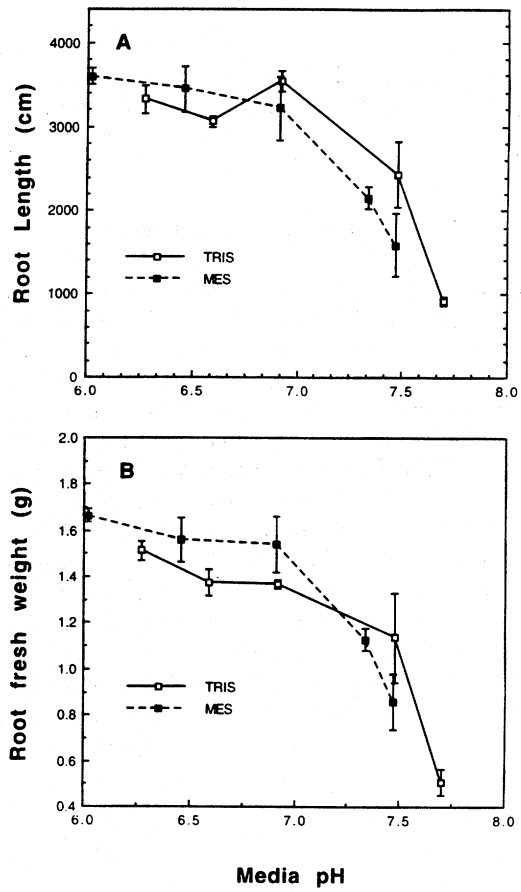

Fig. 4 Length (**A**) and fresh weight (**B**) of Ri T-DNA-transformed roots of Carrot grown for 23 days in the presence of 10 mM Tris or MES buffer at the indicated pH. Means of three replicates ± SEM

Root growth

Roots appeared to grow equally well with MES or Tris (Fig. 4) for the 23 days of the root growth experiment. Roots exhibited similar length and biomass accumulation through pH 7.0, above which growth was inhibited. A second experiment, examining more closely the buffer and pH range best for spore germination and growth, showed no difference in root growth for 10 mM Tris at pH 7.2 or 7.5. Root growth rate was linear over the 27 days of this experiment ($y = 0.168(x)-0.736$; $r = 0.997$; y = root length in meters, x = days of growth).

Growth of roots in the presence of variable amounts of P was tested in unbuffered gels (pH 5.5–6.0) using purified gellan with or without the M level of P

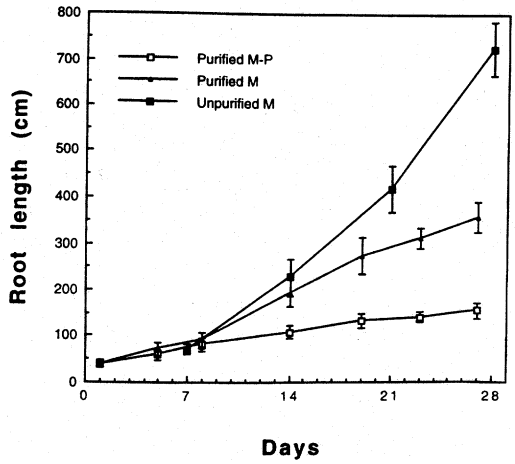

Fig. 5 Length of Ri T-DNA-transformed roots of Carrot grown in unbuffered media in the presence of purified or unpurified gellan gum as gelling agent. Means of five replicates ± SEM. The phosphorus contents (mg/plate) were 0.016, 0.071, and 0.271 for purified gellan M-P, purified gellan M, and unpurified gellan M media, respectively

(35 mM) and unpurified gellan with the M level of P. As expected, roots grew better with successive increments of P (Fig. 5).

Dual culture of *Glomus mosseae* and Carrot

Initial attempts to produce mycorrhizae in M media with unpurified gellan, 10 mM MES or Tris, were unsuccessful. Several experiments were conducted utilizing purified gellan to more tightly regulate P levels. Each Petri plate with 50 ml of medium contained 0.064–0.09 mg P when M media minus P was made with 4 g l^{-1} purified gellan. Plates contained 0.119–0.145 mg P when M media with P was made with purified gellan. Plates contained 0.272–0.295 mg P when made with unpurified gellan and M media with P. There was evidence of P dependency of mycorrhiza formation. A 14% success rate was achieved in separate experiments for plates with purified gellan with M medium (with and without P). Even though successful cultures grew 17–24 weeks before termination, and hyphae had spread throughout the Petri plates, no new spores were produced.

Discussion

Germination and hyphal growth observed here agree with published values for *Glomus mosseae* spores. Meier and Charvat (1992) observed 80% germination within 14 days and Hardie (1985) observed maximal germination only after 35–40 days of incubation. Hyphal lengths of over 10 mm per germinated spore have been reported under the best experimental conditions (Azcon 1987; Douds and Schenck 1991).

Mugnier and Mosse (1987) noted a dependence of mycorrhizal development upon N concentration of the nutrient medium in the root compartment of their in vitro system. Nitrogen concentrations less than 0.2 mM led to the development of mycorrhizae. Though these results are not directly applicable to the system utilized here, the NO_3^- concentration of M medium (3.2 mM) is another factor that could be manipulated to establish dual cultures of VAM fungi and carrot roots.

Even with the manipulation of buffer, pH, and P levels in an attempt to increase the opportunity for successful formation of mycorrhizae between carrot and *Glomus mosseae* in vitro, few cultures were successful. Further, no successful cultures sporulated. Over 90% of *Gigaspora margarita* spore production in vitro occurred after 6 months (Diop et al. 1992), indicating that *Glomus mosseae* may have sporulated after the 16–23 weeks the cultures were allowed to grow in these experiments. Given the low rate of establishment of cultures, future subculture of *Glomus mosseae* could be done from successful cultures, as with routine propagation of *Glomus intraradices* in vitro dual cultures (St. Arnaud et al. 1996).

Acknowledgements The author thanks G. Bécard for transformed roots and shared expertise.

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