A novel technique for preparation of gel-entrapped fungal spores

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Introduction

Arbuscular mycorrhiza (AM) is the commonest mycorrhizal type involved in agricultural systems. Many beneficial effects have been described for AM fungi with regard to phosphate plant nutrition, plant water potential under drought stress, bioprotection against various pathogens, improvement of soil structure, etc. (Strack et al., 2003). Since (AM) symbiosis can benefit plant growth and health, there is an increasing interest in AM inoculant formulation techniques (including entrapment in natural polymer matrixes), application and management (Vassilev et al., 2005).

The use of AM fungi in plant production systems is not a common procedure. The main obstacle is the absence of appropriate methods for mass inoculum production which further determine the lack of formulations which can guarantee a standard quality. AM fungal inoculum is typically produced in scaled-up pot culture always in the presence of the host plant. Development of soil-less AM cultures which include nutrient film technique, aeroponics, has certain advantages and disadvantages which have been reviewed by Verma and Aldholeya (1996).

The use of the AM root-organ methodology and the increasing number of fungal species cultivated in vitro offer many possibilities for the production of mycorrhizal inoculum for commercial purposes including in immobilized form. Utilization of the monoxenic culture method itself for inoculum production was significantly improved by the split-plate technique of St-Arnaud et al. (1996). The AM fungus colonizes Ri T-DNA transformed carrot roots on one side of a divided Petri dish. Hyphae cross the divider after 4–6 weeks of culture and sporulate on the distal side, free of roots and their accumulated waste products. Sequential removal and replacement of gelled media in the distal side of the plate, along with re-supply of the carbon source to the root compartment, allowed for repeated harvests of inoculum from a single Petri dish culture (Douds 2002). However, to entrap AM spores, produced by the split-plate technique, it is necessary to solubilize the solid medium. In order to avoid this step and to save labour and material costs, experiments were conducted to elaborate a method for a direct entrapment of AM spores and mycelium.

Materials and Methods

Split-plate cultures and data collection

Two-compartment Petri plates (90 mm diam.) were used to established split-plate cultures of the arbuscular mycorrhizal fungus *Glomus intraradices* and Ri T-DNA transformed roots of carrot (*Daucus carota* L.) as described by St-Arnaud et al. (1996). The first compartment was filled to the top of the plate divider with M medium (Bécard and Fortin, 1988) and further solidified with 4 g l^{-1} Gel Gro^{TM} (ICN Biomedicals Inc., Irvine, CA, USA). A small (2 mm) plug of inoculum which consisted of colonized roots, hyphae, and spores, removed from a previously grown monoxenic culture of *G. intraradices*, was placed on the solid medium. Several fresh (4-5 cm long) root explants were placed to grow along side of the inoculum plug. Inoculated Petri plates were incubated in an inverted position in the dark at 27^{o} C until hyphae of the AM fungus reached the divider.

Dissolved in dH_2O and sterilized locust bean gum (LBG) and k-carrageenan were added to the distal compartment 6 weeks after the split-plate establishment at a concentration of 1.0 %. Petri plates were than placed in the incubator in normal (horizontal) position under the same conditions. Only hyphae of the external mycelium were allowed to cross the barrier (plate divider) while roots were trimmed back to prevent them entering the distal compartment containing the polysaccharide.

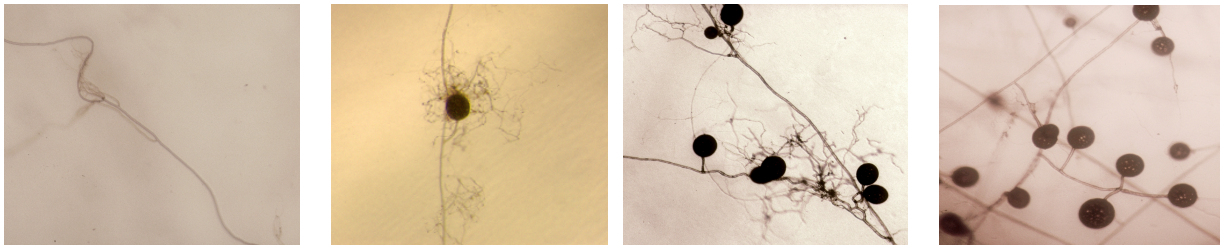
Three months after the establishment of the split plate experiments, spores in the hyphae compartment were counted as described by Declerck et al. (1996) using the 10x10 mm grid marked with a scalpel on the bottom of each plate before the culture establishment. Polysaccharide gels were then replaced with fresh LBG or k-carrageenan dissolved in distilled water as described above. At the same time, glucose (200 mg), mineral salts, or glucose + mineral salts were added dropwise to the root/AM compartment to prove the possibility of continuous production of the fungal mycelium. Petri split plates were placed again at the same conditions. Newly formed spores were enumerated after two months.

Entrapment of AM fungal mycelium and further introduction into soil-plant system

Second generation spores and mycelium from the Glu+MinSalts treatment were processed directly to form LBG-containing or k-carrageenan beads by techniques described elsewhere (Nishida et al., 1979). Infectivity potential of the mycorrhiza/gel formulation was proved using tomato as a test plant. 30 beads/tomato seedling were tested. Root length colonization was measured after 5 weeks by methods described elsewhere (Vassilev et al., 2001).

Results and Discussion

G. intraradices hyphae proliferation, formation of external mycelium network, and spore production in the distal compartment containing locust bean gum or k-carrageenan was observed non-destructively using light microscopy. Single fungal hyphae crossed the divider entering the distal part of the split-plate cultures 35-40 days after their establishment. At this early stage of mycelial formation, zigzag mode of hyphae development was observed particularly into the locust bean gum treatment (Fig. 1A). Hyphal ramifications were observed in both treatments which, in some points of the mycelial network, formed densely packed structures. Formation of *G. intraradices* spores was observed mainly in association with these hyphal structures (Fig. 1B, C) and differed from spore formation in the plate compartment containing root/*G. intraradices* (Fig.1D). Independently of the polysaccharide employed, all these mycelial formations and spores formed later on were observed within the entire gel volume: in the interior and on the surface. This mode of fungal development made difficult but not impossible the spore enumeration.



A B C D

Fig. 1. Proliferation and development of hyphae and spores of *Glomus intraradices* in media containing locust bean gum and k-carrageenan (A-C) as observed in the distal part of split-plat cultures compared to mycelium developed in the root/AM compartment

Different number of spores were found in the split-plates depending on the polymer used (Table 1). However, this difference was found to be insignificant 3 months after the culture establishment and two months after the polymer replacement without addition of medium components to the root/AM compartment. In the second stage of the experiments (after polysaccharide replacement), the number of second generation spores was significantly higher in both glucose supplemented (to the root plate side) treatments compared with the respective control treatments. The addition of mineral salts did not enhance the production of second generation AM spores. It is interesting to note that their number was equal that found after 3-month-growth in both the "alone" treatments. The highest number of AM spores was registered in both experimental series after the addition of glucose + mineral salts to the root/AM compartment. In this case, LBG-treatment provided the best conditions for spore production while the number of spores was lower in the split-plates with k-carrageenan.

Table 1. Spore production of *Glomus intraradices* in media containing locust bean gum (LBG) and k-carrageenan 3 months after the establishment of the split-plate cultures. Polysaccharides and AM mycelium were then replaced with fresh media while glucose (Glu), mineral salts (MinSalts), and glucose/minerals salts (Glu+MinSalts) were added to the root/AM compartment

Treatment	Number of spores	
	3 months	+ 2 months
LBG Alone	2392a	753d
+ Glu	-	3350b
+ MinSalts	-	2490c
+ Glu+MinSalts	-	5042a
k-Carrageenan		
Alone	1718a	521d
+ Glu	-	2115c
+ MinSalts	-	1767c
+ Glu+MinSalts	-	3638b

Each number is the mean of 6 observations;

Numbers in the same column followed by the same letter are not significantly different

Several variants of direct entrapment of the produced AM propagules could be performed:

- Direct formation of gel beads in the case of k-carrageenan-grown AM propagules;
- Formation of gel beads after mixing LBG (carrying AM propagules) with another gel;
- Formation of gel beads after mixing LBG/AM propagules) with k-carrageenan/AM propagules.

In this work we produced beads containing k-carrageenan/AM mycelium (i), LBG/AM mycelium+k-carrageenan (ii), and LBG/AM mycelium+k-carrageenan/AM mycelium (iii). The spore number per bead ranged from 1 to 7, 3 to 11, and 4 to 10, respectively. The proportion LBG:k-carrageenan was 1:2. Between 3 and 5 % of the total number of beads contained only hyphae but not spores depending of the combination. Root length colonization ranged from 21% (i) to 33% (ii) and 38 % (iii).

The described system is easy to establish and simple to handle. It does not require expensive and complicated facilities for the production of large quantities of AM inocula. Further studies may include utilization of other gelling agents with varying concentrations and other arbuscular mycorrhizal fungi. Another advantage of this method of AM inoculum formulation include the possibility of introducing other plant beneficial microorganisms such as nitrogen-fixing, phosphate-solubilizing, and biocontrol microorganisms thus preparing double inoculants with higher biofertilizing effect as shown earlier (Vassilev et al., 2001).

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