Mycorrhiza (2002) 12:163–167
DOI 10.1007/s00572-002-0174-9

ORIGINAL PAPER

David D. Douds Jr

Increased spore production by *Glomus intraradices* in the split-plate monoxenic culture system by repeated harvest, gel replacement, and resupply of glucose to the mycorrhiza

Received: 20 November 2001 / Accepted: 27 March 2002 / Published online: 18 May 2002

Abstract Monoxenic culture of *Glomus intraradices* Schenck and Smith with Ri T-DNA transformed roots in two-compartment Petri dishes is a very useful technique for physiological studies and the production of clean fungal tissues. Experiments were conducted to increase the efficiency of this method for the production of arbuscular mycorrhizal fungus spores. Approximately 20,000 spores could be harvested every 2 months from the distal (fungus only) compartment of a 9-cm-diameter divided Petri dish. The method requires replacement of the gelled media in the distal compartment and resupply of 200 mg glucose to the proximal (root) compartment coincident with harvest of spores. These modifications resulted in an approximate threefold increase in spore production per unit time over the standard split-plate culture technique.

Keywords AM fungi · Monoxenic culture · Inoculum production

Introduction

Monoxenic culture of arbuscular mycorrhizal (AM) fungi and Ri T-DNA transformed roots has advanced the study of many aspects of the AM symbiosis, including development of the mycorrhiza (Bécard and Fortin 1988; Chabot et al. 1992), carbon metabolism (Pfeffer et al. 1999; Fontaine et al. 2001; Lammers et al. 2001), nutrient uptake (Bago et al. 1996; Joner et al. 2000; Maldonado-Mendoza et al. 2001), morphology of the extraradical phase (Bago et al. 1998), signaling/recognition (Bécard and Piché 1989; Nagahashi and Douds 2000), and interactions with plant pathogens (St-Arnaud et al. 1995). The method produces contaminant-free tissues for the study of gene expression by the fungus (Lammers et al. 2001; Maldonado-Mendoza et al. 2001; Ubalijoro et al. 2001).

Many of these studies will provide useful information on genetic/physiological/morphological regulation, lack of which has hindered the development of axenic culture of AM fungi for inoculum production. Utilization of the monoxenic culture method itself for inoculum production was advanced by the split-plate method of St-Arnaud et al. (1996). The AM fungus *Glomus intraradices* 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 profusely on the distal side, free of roots and their accumulated waste products. These spores are infective when used as inocula in calcined clay or soil mix (Vimard et al. 1999).

Experiments were conducted to increase the efficiency of the split-plate culture technique for production of spores of *G. intraradices*. Sequential removal and replacement of media in the distal side of the plate, along with replenishment of the carbon source for the mycorrhiza, allowed for repeated harvests of inoculum from a single Petri dish culture.

Materials and methods

Split-plate cultures

Split-plate cultures of *Glomus intraradices* Schenck and Smith (DAOM 181602) and Ri T-DNA transformed roots of carrot (*Daucus carota* L.), DC1 clone (Bécard and Fortin 1988) were initiated as described by St-Arnaud et al. (1996) with slight modifications. Cores of medium (1.5 cm diameter) containing colonized roots, hyphae, and spores were removed from a pre-existing monoxenic culture of *G. intraradices* and carrot roots and placed into an empty well of the same diameter on one side of a split Petri plate (9 cm diameter). This was filled to the top of the divider with M media (Bécard and Fortin 1988) gelled with 0.4% (w/v) phytagel (Sigma) and containing 10 g l^{-1} sucrose. A fresh root explant then was transferred to grow along side the plug of inoculum. The opposite, distal, root-free side of the plate was filled to the top of the barrier with the same media but lacking sucrose.

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D.D. Douds Jr (✉)
USDA-ARS ERRC, 600 E. Mermaid Lane,
Wyndmoor, PA 19038, USA
e-mail: ddouds@arserrc.gov
Fax: +1-215-2336581

Table 1 Production of spores of *Glomus intraradices* in the distal compartment of split-plate monoxenic cultures with Ri T-DNA transformed roots of carrot as host. Medium in the distal compartment was replaced, with or without addition of 100 mg glucose to the proximal, root compartment, at 2-month intervals beginning at 3 months. Each number is the mean of 5 observations. Numbers in the same column followed by the same letter are not significantly different (α=0.05). Root fresh weight and colonization were measured at 9 months. As plates in treatments A and C were not disturbed, sporulation was not measured until termination of the experiment

Medium treatment			Spore production				Root fresh wt. (g)	Colonization (% Root length)
	Replaced	Glucose	3 months	5 months	7 months	9 months		
A	No	No	–	–	–	21991 a	0.850 b	36.0 b
B	Yes	No	20461 a	6800 a	27 b	21 c	0.878 b	38.2 b
C	No	Yes	–	–	–	25819 a	1.483 a	40.9 ab
D	Yes	Yes	19388 a	15838 a	5971 a	11245 b	1.677 a	47.8 a

Hyphae typically crossed the barrier into the distal compartment after 4–6 weeks and sporulated profusely until approximately 3 months. Roots crossing over into the distal side were removed regularly.

Experimental manipulations

Medium from the distal compartment was removed from experimental plates after 3 months of culture and at 2-month intervals thereafter. A variable amount of glucose, applied dropwise as a filter-sterilized solution, was added to the root compartment at the same time as the gel in the distal compartment was replaced with M media without carbon source. Glucose was added instead of sucrose because earlier results showed it to be readily taken up from the apoplast and metabolized by intraradical structures of AM fungi (Shachar-Hill et al. 1995; Pfeffer et al. 1999). Sucrose was used to initiate new cultures as a matter of routine procedure.

A unique experiment was conducted to attempt to separate the components of the gel removal treatment, which included removal of the extraradical phase, removal of any accumulated auto-inhibitors that may restrict continued sporulation, and replenishment of mineral nutrients. Four cores of media (see below) only were removed from the distal compartment of 3-month-old cultures. Fresh, concentrated liquid M media then was added to the empty wells to yield 100% replenishment of the mineral nutrients originally contributed by the M media (Bécard and Fortin 1988). Water was added to controls and the experiment was terminated after 2 months further growth (see below).

Collection of data

Spores in the distal compartment were counted in a subsample of four 9-mm-diameter cores of media (8% of the total volume). The cores were blended at high speed in 10 mM sodium citrate (pH 6.0) (Doner and Bécard 1991), caught on a 45-µm sieve, and transferred to a Petri dish for enumeration at ×20 magnification under a dissecting microscope.

Roots were recovered from the proximal side at the end of the experiments by solubilizing the media in 10 mM sodium citrate (pH 6.0) with gentle shaking. Roots were stained (Phillips and Hayman 1970) and assayed for percentage root length colonized via the gridline intersect method (Newman 1966).

Fourth generation spores, i.e. those produced during the replacement cycle running from 7 to 9 months, were tested for infectivity. Two hundred spores were placed on a filter paper disk midway down the soil column of three replicate conical plastic pots (SC-10 super cell, 160 cm^3, Stuewe and Sons, Corvallis, Ore., USA). The potting medium used was a mixture of field soil, sand, vermiculite, and calcined clay (0.75:1.0:1.0:0.75 by volume). Three bahiagrass (*Paspalum notatum* Flugge) seedlings were transplanted into each cone and grown in the greenhouse. Entire root systems were stained and assayed for colonization after 4 weeks of growth.

Data analysis

Data were analyzed using analysis of variance or paired *t* tests. Values for which ANOVA found significant treatment effects were characterized further using Tukey's method of multiple comparisons (α=0.05). Where appropriate, 95% confidence intervals were calculated to conclude that a mean was significantly greater than zero.

Results

Fungal regrowth after gel replacement, no addition of glucose

Medium was removed from the distal side of half of a 3-month-old cohort of split-plate cultures and replaced with fresh M media minus carbon source without adding glucose to the proximal, mycorrhizal root-containing side (n=6). Spore enumeration after 2 months further growth showed a small, but significant production of new spores by the plates in which the gel was replaced (mean=1,575 spores, 95% CI=364<μ<2786). In addition, the number of spores in the control plates, left undisturbed for the 5 months, was equivalent to that present in the treatment plates at 3 months, 29,427 versus 29,125, respectively, indicating no significant sporulation in these split-plate cultures beyond 3 months of growth under normal circumstances.

Gel replacement with addition of glucose

Another cohort was used in an experiment with two factors: gel removal and replacement on the distal half (with or without) and addition of glucose to the root side (0 or 100 mg) (Table 1). This experiment verified the conclusion above that there is no significant sporulation in undisturbed plates beyond 3 months of culture (treatment A at 9 months versus treatments B and D at 3 months). There was, however, greater sporulation in the replacement without glucose addition treatment than detailed above, indicating cohort-to-cohort variability in vigor. Furthermore, addition of 100 mg glucose to the root side of undisturbed plates at 2-month intervals did not result in a significant increase in sporulation (approximately

Table 2 Effect of the re-supply of organic nutrients other than glucose to the root compartment at the time of replacement of the gel in the distal compartment upon resulting sporulation in the distal compartment. Each number is the mean of 5 observations. Numbers in the same column followed by the same letter are not significantly different, α=0.05. Glucose addition = 100 mg

Medium treatment			Spore production			
Replaced	Glucose	Organics	3 months	5 months	7 months	9 months
Yes	Yes	No	26583 a	8605 a	2756 a	8117 a
Yes	Yes	Yes	27328 a	7788 a	2680 a	8344 a

4,000 spores, treatment C minus treatment A, Table 1). Removing the gel from the distal side in combination with adding 100 mg glucose to the root side resulted in the production of 33,000 spores over the three successive cycles (treatment D, 5 months + 7 months + 9 months). Addition of 100 mg glucose produced an increase in root growth and, when combined with gel replacement, also an increase in percentage root length colonized.

A further experiment was conducted to test the effect of the re-supply of other organic nutrients present in M medium (glycine, thiamine HCl, pyridoxine HCl, nicotinic acid, and myo inositol) to the root side upon sporulation in the cyclically replaced media in the distal compartment. No stimulation over that caused by 100 mg glucose was seen (Table 2).

Effect of increasing levels of glucose

Another cohort of plates was used to determine whether sporulation could be increased further with the re-supply of greater amounts of glucose (Table 3). Increasing the glucose addition from 100 to 200 mg increased spore production more than twofold in each cycle. Root growth also was enhanced, but not colonization. Another cohort was used to test the application of 400 mg glucose. There was no significant difference in spore production in the 3- to 5-month cycle for 400 mg glucose (29,054 spores) versus 200 mg glucose (32,200 spores) (n=6).

Mineral nutrient replenishment without gel removal

Re-supply of mineral nutrients in the distal compartment, without disturbance of the extraradical mycelium, significantly increased sporulation over cultures not receiving supplemental minerals (Table 4). There was no effect on root biomass or colonization.

Infectivity of inocula

Infectivity of spores produced during the 7- to 9-month gel replacement cycle of two cohorts was tested using *P. notatum* as host plant. Percentage root length colonized by *G. intraradices* averaged 20% to 26% after 4 weeks of growth.

Discussion

The split-plate monoxenic culture of transformed carrot roots and *G. intraradices* was developed by St-Arnaud et al. (1996) to study interactions between AM fungus

Table 3 Effect of the re-supply of an increasing amount of glucose (mg) to the root compartment at the time of gel removal and replacement in the distal compartment upon the sporulation of *G. intraradices* in the distal compartment. Each number is the mean of 5 observations. Numbers in the same column followed by the same letter are not significantly different (α=0.05). Root weight and colonization were measured after 9 months

Medium treatment		Spore production				Root fresh wt. (g)	Colonization (% Root length)
Replaced	Glucose	3 months	5 months	7 months	9 months		
Yes	0	20016 a	2025 c	0 c	63 c	0.828 c	19.7 b
Yes	100	16457 a	9550 b	6497 b	5113 b	1.744 b	28.7 a
Yes	200	22116 a	22510 a	20428 a	11617 a	2.803 a	33.7 a

Table 4 The effect of re-supply of mineral nutrients to the distal compartment, without the confounding effect of gel replacement, upon spore production of *G. intraradices* in the distal compartment of the split-plate monoxenic culture system. Each number is the mean of 6 observations. Numbers in the same column followed by the same letter are not significantly different (α=0.05; paired *t* test). Root weight and colonization were measured after 5 months of growth. The distal compartment received either approximately 1 ml of concentrated mineral salts to completely replenish the minerals found in 33 ml of M medium or 1 ml distilled water for controls. All plates received 100 mg glucose in the root compartment after 3 months of growth

Medium treatment			Spore population		Root fresh wt. (g)	Colonization (% Root length)
Replaced	Minerals	Glucose	3 months	5 months		
No	No	Yes	37804 a	65465 b	1.0723 a	42.2 a
No	Yes	Yes	39171 a	75448 a	0.9804 a	38.8 a

hyphae and root pathogens. The discovery that the fungus proliferates greatly in the distal compartment created a significant tool for AM fungus research. The work presented here is a modification to increase the efficiency of the method as a tool for inoculum production.

Published methods of in vitro production of spores of *G. intraradices* vary widely in their efficiency. Chabot et al. (1992) described the establishment of dual cultures using surface-sterilized spores as starter inoculum, but only 700 new spores were produced in 4 months of culture per Petri dish. Diop et al. (1994) used sheared roots as starter inoculum and produced 893 spores per plate in 3 months. The airlift bioreactor studied by Jolicoeur et al. (1999) produced 12,400 spores per liter (approximately 375 spores in the equivalent volume of a split-plate compartment). This places in perspective the efficiency of the method of St-Arnaud et al. (1996), which produced an average of 15,000 spores per distal side of a 9-cm-diameter split Petri dish (30 cm^3) 3–4 months after the fungus crossed the divider, 4–5 months total elapsed time.

The optimal procedure developed here requires replacing the media in the distal compartment every 2 months beginning at 3 months and concomitantly replenishing the carbon source in the proximal compartment with 200 mg glucose. This produced nearly 65,000 spores in 7 months (Table 3), i.e., almost a threefold increase in spore production per unit time relative to the previous split-plate technique. This method takes advantage of several observations.

First, *G. intraradices* in a newly initiated split-plate culture would typically cross the barrier in 4–6 weeks (St-Arnaud et al. 1996; also observed here). This lag is necessary for the fungus to spread throughout the proximal compartment and cross the barrier at random. This lag does not happen after the first harvest of spores and replenishment of media in the procedure described here. Vigorous regrowth by the fungus into the distal compartment typically occurred in less than 1 week.

Secondly, this method takes advantage of metabolic studies showing that glucose is directly taken up by intraradical fungal structures and that they are highly competitive with the root in this uptake (Shachar-Hill et al. 1995; Solaiman and Saito 1997). Initially, colonization of the root is slight, so the conversion of C into fungal biomass and the ability of the fungus to compete for C are low. This is remedied with the sequential harvests/replacements of the media. Colonization increases and, based on our earlier work with *Allium porrum* roots colonized by *Glomus etunicatum*, the fungus would be highly competitive for the freshly added glucose (Shachar-Hill et al. 1995). Uptake of ^{13}C-labelled glucose in the root compartment and its appearance in metabolites of the spores and hyphae of the distal compartment has been demonstrated (Pfeffer et al. 1999; Douds et al. 2000; Bago et al. 2000).

A third factor contributing to the success of this method is the periodic replenishment of inorganic nutrients in the distal side. Recent research has demonstrated that *G. intraradices* is capable of removing all the P provided by M medium (35 µM) in the distal compartment in a matter of days (Maldonado-Mendoza et al. 2001), though the contribution of P contamination of the phytagel to the gelled M medium used here (128 µM) must also be considered (Doner and Douds 1995). Therefore, it is possible the medium was depleted of P, and perhaps other essential nutrients, after the initial 3 months of growth. The fresh nutrient addition, made available to the mycorrhiza in the proximal half by the fungal regrowth into the distal compartment, may have contributed to the renewed sporulation. This hypothesis is supported by the data of Table 4, in which re-supply of mineral nutrients to the distal compartment increased sporulation over that caused by re-supply of carbon source alone. These data also indicate that removal/disturbance of the extraradical phase alone is not the cause of increased sporulation after gel replacement. Further, the large number of spores produced by the cohort of Table 4 indicates that *G. intraradices* does not produce auto-inhibitors that restrict sporulation beyond approximately 20,000 spores per compartment.

Given the obligate symbiotic nature of AM fungi and our inability to culture them axenically, monoxenic culture of roots and AM fungi is the surest way to produce these symbionts without contaminating microorganisms. Sequential harvests of *G. intraradices* from the distal compartment of split-plate cultures, accompanied by replacement of the media on that side and addition of 200 mg glucose to the proximal side, significantly increased the utility of this in vitro method for inoculum production. Further, the nearly synchronous recolonization of the distal compartment by the fungus after gel replacement increases the utility of the split-plate method for physiological and nutrient uptake studies.

Acknowledgements The author acknowledges initial receipt of Ri T-DNA transformed carrot root clone DC1 from G. Bécard, receipt of the initial monoxenic culture of *G. intraradices* from J. A. Fortin, technical assistance from V. Spier, G. Nagahashi for subculturing roots, and Y. Shachar-Hill for helpful discussions.

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