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ORIGINAL PAPER

A novel in vitro cultivation system to produce and isolate soluble factors released from hyphae of arbuscular mycorrhizal fungi

Vijay Gadkar · James D. Driver · Matthias C. Rillig

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Abstract The Petri plate dual in vitro culture system for arbuscular mycorrhizal fungi (AMF) is ill-suited for the production and isolation of extraradically released soluble factors, thereby preventing the further characterization of such compounds. To overcome this technical bottleneck, we here describe a novel cultivation system using the standard AMF *Glomus intraradices*-carrot dual culture as a model. This new system is capable of producing soluble factors in copious amounts without any interference from host roots. The greatest advantages of this culture method include ease of handling and reusability of the culture vessel, thus making it a very cost effective system.

Keywords Arbuscular mycorrhizal fungi · Extramatrical · Glomalin · Hairy roots · Root organ culture · Symbiosis

Introduction

Arbuscular mycorrhizal fungi (AMF) colonize the roots of most terrestrial plant species and provide their hosts with nutritional benefits, a role centrally played by the extraradical mycelia (Smith and Read 1997). The extraradical mycelium of AMF has been shown to release soluble compounds which include factors capable of activating plant symbiotic genes (Kosuta et al. 2003; Olah et al. 2005) and proteins capable of modifying soil properties (Wright et al. 1998; Rillig 2004). Both these functions are of immense interest in the areas of plant–microbe interactions, bioremediation and soil restoration.

The conventional method to cultivate AMF in vitro is the dual root organ culture system (Bécard and Piché 1992; St Arnaud et al. 1996). This system uses solid microbiological gelling agents (e.g. Phytagel, agar, Gelgro) to cultivate the fungi, making it extremely difficult to extract fungal-derived aqueous exudates from this solid phase. Substitution of liquid media in the ''fungus only'' compartment of the split Petri plate in lieu of the solid gelling agent has been attempted (Joner et al. 2000). However, these liquid cultures are difficult to handle and maintain, and hence unreliable; as a consequence, these cultivation systems have not been widely adopted by mycorrhizal biologists. To alleviate this technical bottleneck, we here describe the novel application of a plant bioreactor which could be used for both large scale AMF cultivation and production of extramatrical soluble compounds. This system can facilitate the large-scale production and

V. Gadkar (✉) · J. D. Driver · M. C. Rillig
Division of Biological Sciences, University of Montana, 32 Campus Drive #4824, Missoula MT 59812-0004, USA
e-mail: vijay.gadkar@mso.umt.edu

retrieval of soluble compounds in sufficient quantities for subsequent biochemical characterization.

Materials and methods

Establishment of root organ culture system in the plant bioreactor

Plant bioreactors (Fisher Scientific, Pittsburgh, PA) were adapted for developing the novel AMF cultivation system. This bioreactor consists of a cylindrical chamber (150 mm × 100 mm) which contains a removable nylon sieve (5–6 μm) assembly. This mesh assembly fits tightly into the central chamber. The whole assembly can be closed using a central screw-cap lid for sterile usage (Fig. 1). The central chamber has a side-arm with a screw-cap which allows sampling of the vessel contents without dismantling the whole system. The whole assembly is made up of polypropylene and is thus repeatedly autoclavable.

The bioreactor was adapted for AMF cultivation as follows. Two separate cultivation zones were created using the central mesh assembly as the separating barrier. An inert substrate, either pre-washed expanded clay or glass beads was added in each vessel to an approximate height of 5 cm and the central mesh assembly was placed on top of it. The bottom chamber was exclusively for fungal proliferation. The whole bioreactor containing the inert beads was sterilized by autoclaving for 30 min at 121°C prior to adding growth medium. After cooling, the bottom section was filled with 40 ml of sterile M-medium minus sucrose and vitamins (Bécard and Piché 1992) through the side arm. The clay beads rapidly soaked up the liquid and the excess liquid formed a layer at the bottom of the chamber. A previously established Petri dish (90 mm) of actively growing *Glomus intraradices* (DAOM 181602) in association with carrot hairy roots (clone DC2; dual culture a kind gift from Dr. David Douds, USDA-ERRC) was axenically placed on the nylon mesh by opening the central

Fig. 1 (**A**) Layout of the cultivation system used for isolating AMF released extramatrically factors. Close up view of the cultivation system showing the (**B**) carrot hairy roots in association with the AMF *G. intraradices*. The cotton rolls soaked in glucose are also visible. (**C**) the bottom ''fungus only'' zone containing the sterile expanded clay and liquid M-medium

screw cap lid. To accelerate the proliferation of *G. intraradices* mycelia into the bottom chamber, two pre-sterilized cotton dental rolls, Parotisroll (Coltène/Whaledent GmbH, Langenau, Germany), each soaked in 4 ml sterile glucose solution (50 mg/ml stock solution) (Douds 2002) were carefully placed in each individual bioreactor. No leakage of glucose into the bottom chamber was possible because glucose could only be accessed by the roots in the top compartment through the cotton rolls. The bioreactors were incubated in the dark at 30°C (±2°C) for development of the dual culture. Each individual bioreactor was analyzed for fungal proliferation under a dissecting microscope.

Harvest of bioreactor and culture analysis

Forty days post inoculation (d.p.i.) the dual culture growth in each bioreactor was terminated. The liquid culture from the bottom chamber from each individual bioreactor was drained into sterile containers via the side arm. The central membrane assembly was removed, and AM fungal hyphal length present in the hyphal compartment was determined with an aqueous floatation extraction method following Rillig and Steinberg (2002). After the pH of the solution was measured, the exudate was subject to the following analysis.

Secreted glomalin concentration

Total protein levels of control and experimental samples were estimated by the Bradford assay. Glomalin concentration was measured by ELISA using the monoclonal antibody, MAb32B11, as described earlier (Wright and Upadhyaya 1996). The ELISA analysis was performed using 50 µl of the exudates, fraction Briefly, the fungal exudates, collected in M-media in the fungus-only compartment, were placed in individual wells of 96-well polypropylene plates. The exudates were air dried prior to exposure to MAb32B11. Glomalin levels were estimated by comparison to glomalin extracted from soil standards following the protocol of Wright and Upadhyaya (1996).

Elemental analysis

Inductively coupled plasma emission spectrometry (ICP-ES) was performed on individual aliquots of M-medium from each bioreactor to determine changes in the mineral content of the medium. Individual 8 ml bioreactor samples were acidified with 1 ml nitric acid (1.2 M, ACS grade) and 1 ml of HCl (1.2 M, trace metal grade), immediately prior to performing the ICP-ES analysis. Samples were analyzed on the ICP-ES instrument (TJA Corp, model IRIS) using the Thermospec/CID analysis program. Elemental concentrations were determined in mg l^{-1} in each sample.

Results and discussion

The bicompartmental Petri dish cultivation system (St. Arnaud et al. 1996) provides an excellent system for studying the extramatrical phase of the AM fungus. However, it uses solid gelling agents in the ''fungus only'' hyphal compartment, making it unsuitable for isolating fungal derived aqueous factors. Attempts to isolate aqueous factors from the solid gelling agent have included either boiling or ionic liquefaction (Doner and Bécard 1991; Filion et al. 1999). The former could potentially destroy any heat labile moieties and the latter complicate downstream analytical process since this fraction also contains the depolymerized gelling agent. Attempts to substitute the solid gelling agent with liquid M-medium has been attempted (Joner et al. 2000). However, liquid dual cultures suffer from technical problems. These include the unavoidable occurrence of condensation of the liquid M-media on the lids of the Petri dish (potentially leading to the undesirable transfer of material into the root compartment), and the constant and highly laborious steps required to prune the hairy roots to prevent it from accessing and thereby potentially contaminating the liquid medium with root-derived compounds.

The present bioreactor cultivation system is based on the same basic bicompartmental design (St. Arnaud et al. 1996) but does not suffer from the above described technical problems; most

notably the constant pruning of the rapidly growing hairy roots has been eliminated. In the bioreactors the same hairy roots grew negatively geotropically, a genetic characteristic of these roots, and thus were air pruned due to lack of nutrition. Moreover, due to the design of the mesh holder, the central nylon mesh on which the dual culture is placed is not in direct contact with the expanded clay beads. Hence, there is only a negligible chance of any concentration mediated diffusion of plant factors to contaminate the fungus derived liquid medium in the bottom chamber.

The establishment of the bioreactor system for the in vitro culture of the AMF *G. intraradices* resulted in a prolific growth of the fungal mycelia in the ''fungus only'' zone, i.e. the root free bottom chamber. The fungal mycelium was visible in the root free zone anytime after 15 days post initiation of the dual culture. The spores were formed on the expanded clay beads and prolific mycelium was visible on the walls of the chamber.

The first parameter measured after the 40 day fungal proliferation was the change in pH of the M-medium in which the fungal mycelia had proliferated. The pH of the nutrient solution was found to be 7.30, both in the glucose and non-glucose supplemented bioreactors (Table 1). Using a dual compartment Petri plate system, Bago et al. (1996) also observed alkalization of the M-medium, suggesting that our bioreactor system functions similarly to the classical Petri plate system. The explanation for such alkalization by the mycelium was the active NO_3^-/H^+ symport system, which released OH^- ions into the surrounding solution (Bago et al. 1996).

The experimental setup described in this paper allowed us to monitor the utilization of Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and PO_4^{3-} by the *G. intraradices* mycelia. The elemental analysis of the M-media from the fungus-only zone revealed an active mechanism of nutrient uptake and extrusion of ions over the 40 day period of fungal proliferation. The K^+ levels decreased by 1.5-fold with respect to non-inoculated controls, and Na^+ levels increased ca. 19-fold. The level of PO_4^{3-} also markedly decreased after 40 days of fungal proliferation. Among the divalent ions analyzed, Fe^{2+} was be almost completely depleted in the culture medium; Ca^{2+} levels increased ca. 3-fold and Mg^{2+} levels decreased 2.8-fold compared to the control. This represents a clear demonstration of the suitability of this in vitro culture system for the purpose of nutrient translocation in the absence of potentially confounding factors contributed by solid growth media.

The present work further clearly demonstrates the applicability of the plant growth bioreactor for isolating soluble compounds released by *G. intraradices* mycelia during its symbiotic interaction with the carrot roots. We could obtain and quantify the AMF-produced protein, glomalin in this culture system (Table 1). Previous work in this laboratory (Rillig and Steinberg 2002) has demonstrated that glomalin is produced in bicompartmental AMF in vitro cultures. However, amounts produced were too low to permit biochemical characterization. The present culture system clearly allows a straightforward scale-up of production that should easily yield material sufficiently abundant for further biochemical characterization of this compound. (Table 2)

The bioreactor system described in the present study represents a practical tool for performing studies pertaining to AMF nutrient dynamics and for the large scale production of compounds

Table 1 pH, hyphal length and glomalin content from M-medium isolated from bioreactors with glucose and non-glucose treatment

Parameters	M-medium from ''fungus only'' compartment (n = 10)		M-medium from non-inoculated control (n = 5)
	Glucose$^+$	Glucose$^-$	
pH	7.3a	7.3a	5.20b
Hyphal length (mm g^{-1})	1556.4a	459.8b	–
Glomalin (μg ml^{-1})	0.38a	0.48a	–

Data followed by different letter within a column are significantly different ($P < 0.05$) using Student–Newman–Keuls test. Values are means of 10 and 11 replicates for Glu$^-$ and Glu$^+$ treatments, respectively

Table 2 Inductively coupled plasma (ICP) analysis of common elements from M-medium from mycorrhizal and non-mycorrhizal treatment

	Ca^{2+} mg l^{-1}		Potassium (K^+) mg l^{-1}		Magnesium (Mg^{2+}) mg l^{-1}		Sodium (Na^+) mg l^{-1}		Phosphorus (PO_4^{3-}) mg l^{-1}		Iron (Fe^{2+}) mg l^{-1}	
	Glu^+	Glu^-	Glu^+	Glu^-	Glu^+	Glu^-	Glu^+	Glu^-	Glu^+	Glu^-	Glu^+	Glu^-
Mycorrhizal treatment	155.7a	162.9a	46.1b	49.8b	26.6b	31.2c	9.6a	10.1a	0.21b	0.28b	BDL	BDL
Control non-inoculated (M-medium)	54.1b		73.3a		81.1a		0.52b		1.4a		0.97	
Average fold change	+2.9		−1.5		−2.8		+18.9		−2.8			

Glucose (Glu) are those bioreactors which received the glucose supplements. Data followed by different letter within a column are significantly different ($P < 0.05$) using Student–Newman–Keuls test. Values are means of 10 and 11 replicates for Glu^- and Glu^+ treatments, respectively. BDL refers to below detection level

produced by the mycelium under sterile conditions. The very construction of the system circumvents problems of previous culture methods. In addition to the advantages mentioned above, our design also alleviates the problem of concentration-dependent diffusion across the two chambers (the hyphal and the hyphal/root chamber). This is the case because the mesh holder is positioned in such a way that the mesh is 2 cm from the bottom of the holder, precluding any direct contact of the solid culture medium and the components of the bottom chamber (glass beads or expanded clay). This gap nevertheless permitted mycelia to traverse the mesh, pass into the lower chamber and proliferate profusely as seen in our experimental system.

Our culture system, capable of producing AM fungal derived soluble factors in copious quantities, would find a variety of applications, mostly related to better defining the spectrum of compounds released by the mycelium under sterile conditions. A detailed analysis of the liquid media from the fungus-only chamber would allow for the detection and characterization of the hypothesized Nod-like factor currently hypothesized to be produced by AMF in the course of its signaling cross-talk with the host (Olah et al. 2005). With the availability of advanced metabolomics screening platforms, novel compounds released by AMF having potential biotechnological application could be identified.

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