

Colonization of *Glomus microcarpum* in Ri T-DNA transformed roots of *Ipomoea batatas* (S1010) in synthetic medium

R. PRATAP CHANDRAN* and V.P. POTTY¹

Central Tuber Crops Research Institute, Thiruvananthapuram - 695 017 (India).

¹Cashew Export Promotion Council Lab and Technology Division,
Mundakkal East, Kollam - 691 001 (India).

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs and they cannot complete their life cycle in the absence of a host plant root. AM fungus, *Glomus microcarpum* was successfully co-cultivated in transgenic hairy roots of *I. batatas* (variety S1010) initiated through the mediation of *Agrobacterium rhizogenes* ATCC 15834. Murashige and Skoog (MS) medium composition was modified to support maximum hairy root growth and MS vitamins were replaced with B5 vitamins for better growth of hairy roots. Temperature, pH and light regimens were standardized for optimum hairy root growth and AM colonization. Eighty percent mycorrhizal colonization was observed after 20 days of co-cultivation in modified MS medium in petri dish. Various stages of mycorrhizal infection, pre-infection, penetration, vesicle/arbuscule formation were observed and viability of fungal structures was also studied. This technique can be used for the large scale production of monospecific culture of AM fungi.

Key words: *Agrobacterium rhizogenes*, Arbuscular mycorrhizal fungi, colonization, *Glomus microcarpum*, hairy roots, *Ipomoea batatas*, vesicles.

INTRODUCTION

The AM fungal association is geographically ubiquitous, occurring in plants from arctic to tropical regions, over a broad ecological range from aquatic to desert environments. The AM fungus plant association is a mutualistic beneficial event, in which plant supplies the fungus with carbon from its fixed photosynthates, while the fungus assists plants in its uptake of phosphate and other mineral nutrients from the soil¹. The AM fungi may produce an extensive network of extrametrical hyphae² and can significantly increase phosphorous inflow rates of plants they colonize³. The harmonious symbiotic relationship is reflected in the obligate biotrophic nature of fungi⁴. The obligate symbiotic nature of AM fungi is a crucial factor in the mass multiplication of AM spores *in vitro*. The existing

methods of AM fungal cultivation are pot culture technique, aeroponics and root organ culture. Microbial contamination is the drawback of these methods. So the best strategy is to co-cultivate AM fungi with Ri T-DNA transformed roots.

Transformed roots were induced by wounding and infecting the host plants with *A. rhizogenes*. The rhizogenicity is conferred to plant cells by a fragment of DNA (Ri T-DNA), which is transferred from the large root-inducing (Ri) plasmid, harbored by the bacterium, to the genome, where it is stably integrated and expressed. Integration of a DNA segment (T-DNA) of pRi into the host genome leads to active proliferation of adventitious roots (hairy roots) at or near the site of infection⁵ and produce growth hormones in them.

The Ri T-DNA transformed roots are used for a wide range of fundamental and applied studies. One of the most important of these has been the study of the AM symbiosis⁶ and successful colonization of *Glomus mosseae* spores in *Convolvulus sepium* hairy roots for the first time. Mosse and Hepper⁷ developed the first simple *in vitro* systems for culturing AMF and these crude systems have since been refined to current monoxenic, hydroponic and aeroponic systems. Numerous authors^{6,8,9,10} used Ri T-DNA transformed hairy roots of carrot or tomato for cultivating mycorrhizal fungi. The use of transformed carrot roots, in place of whole plants, provided a simple *in vitro* system, which allowed reproducible observation of all stages of vesicular-arbuscular mycorrhizal (VAM) development under aseptic conditions¹¹. The combination of transformed carrot roots and sterile AMF spores can be used to produce "dual *in vitro* cultures" that provide an efficient method of producing abundant (typically over 5000) spores and mycelia in a nine centimeter petri plate^{8,12}. The monoxenic method involves growing sterile AMF spores on dual culture plates with transformed carrot (*Daucus carota*) roots¹³. Potty and Chandran¹⁴ successfully co-cultivated *G. microcarpum* in Ri T-DNA transformed cassava roots.

The objective of this study was to successfully co-cultivate *G. microcarpum* in transgenic hairy roots of *I. batatas* (variety S1010) initiated through the mediation of *Agrobacterium rhizogenes* ATCC 15834 and to study the various stages of mycorrhizal infection and its viability. Most of the authors so far used carrot hairy roots because of its vigour and ease with which it can be propagated. In the present investigation, attempts were made to colonize mycorrhizal spores of *G. microcarpum* in hairy roots of *I. batatas* variety S 1010 and this is the first report. This technique can also be used for the large scale production of monospecific AM fungal inoculum.

MATERIAL AND METHODS

Sterilization and germination of seeds

The seeds of *I. batatas* (variety S1010) were surface sterilized primarily with mild detergent

solution followed by treatment with 0.1% mercuric chloride solution for 10 minutes and the seeds were washed well with distilled water. Second stage sterilization was done with 70% ethanol for 3 minutes and washed well with distilled water. The surface sterilized seeds were placed in 1% sterilized agar plates and kept at room temperature for incubation.

Hairy root induction in *I. batatas*

A. rhizogenes ATCC 15834 was used to induce hairy root infection and cultured in yeast extract medium¹⁵ which contained beef extract - 5g, yeast extract - 1g, peptone - 5g, sucrose - 5g, MgSO₄ 7H₂O - 0.49g, bacto agar - 12g, distilled water - 1000 ml and pH - 7.2. Bacterial culture was incubated at 24°C in the dark for 48 hours. *In vitro* seedlings of *I. batatas* (variety S1010) were wounded on the stem using a sterile scalpel, which was smeared with 48 h old culture of *A. rhizogenes* and incubated at 24°C for two days in dark. After two days of incubation the explants were transferred to fresh MS medium, containing cefataxime 250 mg/l to eliminate excess bacterial growth. Then the explants were transferred to fresh Murashige and Skoog¹⁶ medium without antibiotic and kept for further incubation for hairy root emergence¹⁷. Hairy roots emerged were cut approximately 2 centimeter long and were transferred to standardized modified MS medium and incubated at 24°C.

Isolation and surface sterilization of mycorrhizal spores

AM fungal spores were isolated from pot culture maintained in glass house condition in Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala State, India, by wet sieving and decanting method¹⁸. Mycorrhizal spores of *Glomus microcarpum* were surface sterilized according to Mertz *et al.*¹⁹. The sterilized spores were kept in a solution containing 2% Chloramine T and 1-2 drops of Tween 20 for 10 minutes and rinsed them with sterile distilled water and repeated treatment with Chloramine T for 10 minutes and rinsed them with sterile distilled water. The spores were transferred to sterile solution containing 200 mg/l streptomycin and 100 mg/l gentamycin and stored the solution at 4°C.

Mycorrhizal infection

Standardized modified Murashige and Skoog (MS) medium was prepared and poured in 9 cm petri plates. Fresh transformed roots approximately 3 to 4 centimeters long were placed in the medium. In order to obtain dual culture of AM fungus in transformed roots, fungal inoculum, the surface sterilized spores were placed near the transformed roots at a distance of 5 millimeter and kept for incubation at 28°C. Different stages of mycorrhizal infection such as pre-infection, penetration, vesicle/arbuscule formation and colonization were studied.

Viability of mycorrhizal fungi

Activity of succinate dehydrogenase (SDH), a mitochondrial enzyme was considered as an indicator of viability of mycorrhizal fungi²⁰. The transformed root samples were cut approximately to 1 cm and were washed with ice-cold water and kept in ice. Weighed 0.2 to 0.5 g of root samples in a test tube and added 20 ml 0.05 M Tris/ HCl pH 7.4, 50 mg/ml sorbitol, 15 units/ml cellulase (from *Aspergillus niger*) and 15 units/ml pectinase (from *A. niger*) to the root samples and kept it for 2 hours at room temperature for clearing. Clearly rinsed the roots with water on a fine sieve. Transferred the root samples to a bottle and added 20 ml of SDH staining solution [Tris/HCl, pH 7.4 (0.2 mol.l⁻¹) 2 ml, MgCl₂ (5 mmol.l⁻¹) 2 ml, Nitro-blue Tetrazonium (4 mg ml⁻¹) 5 ml, Na-succinate (2.5 mol.l⁻¹) 6 ml and H₂O 2ml] and incubated the root pieces overnight at room temperature. After incubation poured out the staining solution and thoroughly washed with

distilled water. Treated the roots with sodium hypochlorite solution (containing 3% active chlorine) for 5 minutes and washed with distilled water. Transferred the root bits to petri dish and observed the stained mycorrhizal hairy roots under the microscope.

RESULTS AND DISCUSSION

Transgenic hairy roots emerged from the wounded sites of *in vitro* plants of *I. batatas* variety S1010 (Fig. 1) on the 10th day and the hairy roots were initially white and turned slightly yellow. They were finely branched and exhibited negative geotropism. The roots were successfully grown in modified MS medium, in which the MS salt concentration was reduced to 1/5 strength. Full and 1/2 strength MS concentrations resulted in the browning of hairy root tissues. So further dilutions were made and 1/5 strength was found to be the optimum concentration for hairy root growth. MS vitamins were replaced with B5 vitamins²¹ and sucrose concentration was reduced from 30g/l to 15g/l. Cystine HCl was used as an antioxidant which prevented the browning of plant tissues. The standardized medium composition and other growth parameters are given in Table I.

The hairy roots emerged from the wounded sites were successfully transferred to standardized modified MS medium containing cefataxime 250 mg/l to contain residual *Agrobacterium* growth. Prolonged exposure of plant tissues to cefataxime leads to the death of tissues

Table 1: Standardized medium and growth conditions

Medium Composition		Temperature	pH	Light
MS salts	1/5 strength	24°C	5.7- 6	16 hours photo period
B5 vitamins	Full strength			
Myoinositol	100 mg/l			
Nicotinic acid	1.0 mg/l			
Pyridoxine HCl	1.0 mg/l			
Thiamine HCl	10 mg/l			
Sucrose	15 g/l			
Cysteine HCl	0.5 g/l			
Agar	7 g/l			
Distilled water	1000 ml			

and two to three successive transfers were made to free the hairy roots from *Agrobacterium*. The bacteria free hairy roots were used to infect mycorrhizal fungi.

The surface sterilized spores served as infective propagules and the fungal development initiated from them. Surface sterilized spores of *G. microcarpum* placed near the hairy roots germinated and infected the hairy roots. After sterilization, germination percentage of spores was 95 % and they germinated within 2 to 4 days. Five percent of the spores failed to germinate and this may be either due to the strong sterilization treatment or the spores were immature, dormant, or non viable.

The growth medium, 1/5 strength of MS medium used for supporting hairy roots did not affect the mycorrhizal (*Glomus microcarpum*) infection in hairy roots. The higher concentration of P and N in MS medium was found to be inhibitory for AM fungal colonization²². The pH 5.7 to 6 was found to be the optimum for the growth of *I. batatas* hairy roots in modified MS medium and this pH range also supported the establishment of mycorrhizal fungi in hairy roots even though a

neutral to alkaline pH was said to be optimum to *Glomus* species²³. Green *et al.*²⁴ also reported that *Glomus* spp. germinated between pH 6 and 9. These observations varied slightly with the present investigation.

Optimal temperature for spore germination seemed to vary among AM fungal species. The optimum temperature used for *I. batatas* hairy root proliferation was 24°C and this was also found optimum for the germination of *G. microcarpum* spores where as, *Glomus mosseae* and *Acaulospora leavis* germinated between 10 to 18 and 30°C with an optimum between 20 and 25°C. *Gigaspora* germinates between 10 and 30°C with an optimum between 20 and 30°C²⁵ and germination was best at 10 to 25°C for *Glomus caledonium*²⁶ and 25°C for *Glomus epigaeum*²⁷.

Different stages such as pre-penetration, penetration, vesicle/ arbuscule formation was observed (Table II). In the primary infection, fungus penetrated the roots between the epidermal cells and forms appressorium in the first cell layers. The pre-penetration stage was observed on 6th day of infection. Vesicle/arbuscules were found on the mycorrhiza infected hairy roots of *I. batatas* from

Table 2: Stages of mycorrhizal infection in hairy roots

DaysStages	2	4	6	8	10	12	14	16	18	20
Pre infection	-	-	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+
						+	+	+	+	+
								+	+	+
Primary infection (Root penetration)	-	-	-	+	+	+	+	+	+	+
Vesicles and arbuscule formation	-	-	-	-	+	+	+	+	+	+
Colonization	-	-	-	+	+	+	+	+	+	+
				+	+	+	+	+	+	+
					+	+	+	+	+	+
						+	+	+	+	+

"Each + represents 20% colonization"

the 10th day onwards (Fig. 2, 3) and this clearly indicated the successful mycorrhizal colonization in Ri T-DNA transformed *I. batatas* hairy roots. After 20 days of co-cultivation growth, the hairy roots were subjected to SDH analysis and confirmed the viability of AM fungi. The stained root bits showed a dark purple spots indicated SDH activity (Fig. 4). This enzyme was chosen because SDH activity is a marker for respiratory activity.

During the pre-infection stage, the spores of *G. microcarpum* were kept near the hairy roots of *I. batatas* and infection was observed on 6th day and 40 percent colonization was found in the hairy roots. Penetration stage was found in the eighth day of infection with 40% mycorrhizal colonization.

During the course of this study 80% mycorrhizal colonization was achieved and also found that *I. batatas* was highly susceptible for mycorrhizal colonization, where as Abdul-khaliq and Bagyaraj²⁸, 2000, colonized *Gigaspora margarita* in tomato hairy roots and reported 30% mycorrhizal colonization after 30 days. The higher mycorrhizal colonization percentage in *I. batatas* hairy roots was attributed to the higher dependency of mycorrhizal fungi towards *I. batatas* variety S 1010. The successful colonization of AM fungi *Glomus microcarpum* in transgenic hairy roots of *I. batatas* is for the first time. This variety is also resistant to root knot nematode and this fungi is also known to inhibit the growth and establishment of root knot nematode²⁹. Diop³⁰ also reported that the AM fungus



Fig. 1: Hairy roots emerging from *in vitro* plant of *I. batatas*

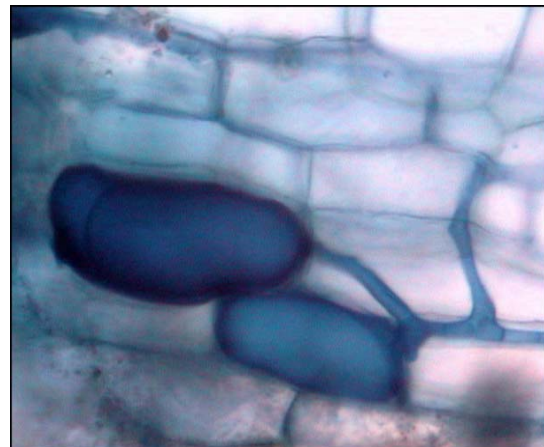


Fig. 3: Arbuscules in stained hairy roots



Fig. 2: Vesicles and fungal filaments in stained hairy roots

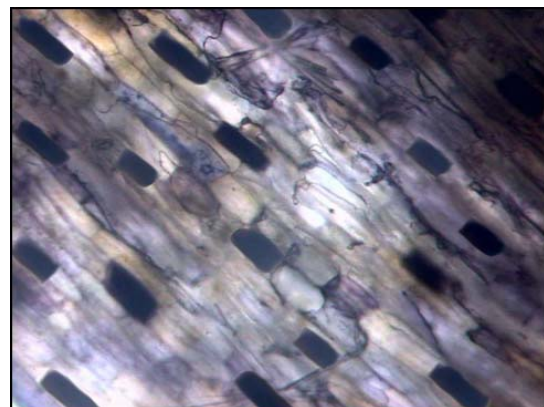


Fig. 4: SDH activity in mycorrhized hairy roots

increased protection of the root by reducing the nematode population by 50%. *I. batatas* variety S 1010 showed a higher colonization percentage more than 80% in normal roots in field conditions and prevents the entry of nematodes. This variety is also used as a trap crop for nematodes and because of this reason this variety was chosen for this study.

This mycorrhized inoculum can be recommended to field crops to reduce root knot nematode incidence. These mycorrhized hairy roots can also be grown in bioreactor using liquid medium for the large scale production of axenic mycorrhizal inoculum.

REFERENCES

- Smith S. A. and Read D., *Mycorrhizal Symbiosis*, 2nd Edn., Academic Press, Inc., San Diego (1997).
- Sylvia D. M., *Rhizosphere Dynamics*, Westview Press, Boulder, 145-167 (1990).
- Jakobsen I., Abbott L. K. and Robson A. D., *New Phytol.*, **120**: 509-516 (1992).
- Williams P. J., *Method Microbiol.*, **24**: 203-220 (1992).
- Chilton M. D., Tepfer D. A., Petit A., David C., Casse-Delbart F. and Tempe J., *Nature*, **295**: 432-434 (1982).
- Mugnier J. and Mosse B., *Phytopathology.*, **77**: 1045-1050 (1987).
- Mosse B. and Hepper C., *Physiol. Plant Pathol.*, **5**: 215-223 (1975).
- Becard G. and Fortin J. A., *New Phytol.*, **108**: 211-218 (1988).
- Declerck S., Strullu D.G. and Plenchette C., *Mycol. Res.*, **100**: 1237-1242 (1996).
- Jolicoeur M., Williams R. D., Chavarie C., Fortin J. A. and Archambault J., *Biotechnol. Bioeng.*, **63**: 224-232 (1999).
- Chabot S., Becard G. and Piche Y., *Mycologia.*, **84**: 315-321 (1992).
- Diop T. A., Becard G. and Piche Y., *Symbiosis.*, **12**: 249-259 (1992).
- St-Arnaud M., Hamel C., Vimard B., Caron M. and Fortin J. A., *Mycol. Res.*, **100**: 328-332 (1996).
- Potty V. P. and Chandran R. P., Proceedings of 5th international meeting of the Cassava Biotechnology Network, St. Louis Mo, USA (2001).
- Vervliet G., Holsters M., Teuclay H., Van Montagu M. and Schell J., *J. Gen. Virol.*, **26**: 33-48 (1974).
- Murashige T. and Skoog F., *Plant Physiol.*, **15**: 473-473 (1962).
- Ooms G., Karp A., Burell M. M., Twell D. and Roberts., *Theor. Appl. Genet.*, **70**: 440-446 (1985).
- Gerdemann J. W. and Nicolson T.H., *Trans. Br. Mycol. Soc.*, **46**: 235-244 (1963).
- Mertz Jr. S. M., Heithas III J. J. and Bush R. L., *Trans. Br. Mycol. Soc.*, **72**: 167-169 (1979).
- Vierheilig H. and Ocampo J. A., *Agricult. Ecosys. Environ.*, **29**: 439-442 (1989).
- Gamborg O. L., Miller R. A. and Ojima K., *Exp. Cel. Res.*, **50**: 155-158 (1968).
- Bressan, W. Braz. *J. Microbiol.* **33**: 33-34 (2002).
- Siqueira J. O., Hubbell D.H. and Mahmud A.W., *Plant and Soil*, **76**: 115-124 (1984).
- Green N. E., Graham S. O. and Schenck N. C., *Mycologia.*, **68**: 929-934 (1976).
- Safir G. R., VA Mycorrhizae: an ecophysiological approach, Safir GR (ed) *Ecophysiology of VA mycorrhizal plants*, CRC Press, Boca Raton, 1-3 (1986).
- Tommerup I. C., *Trans. Br. Mycol. Soc.*, **81**: 381-384 (1983).
- Graham J. H., *Mycologia.*, **74**: 831-835 (1982).
- Abdul-Khaliq and Bagyaraj D. J., *Indian J. Exp. Biol.*, **38**: 1147-1151 (2000).
- Mohandas C. and Ramakrishnan S., *J. Root Crops.*, **22**: 135-136 (1996).
- Diop T. A., *Afr. J. of Biotechnol.*, **2**: 692-697 (2003).