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Initiation of hairy roots from *Canavalia* sp. using *Agrobacterium rhizogenes* 15834 for the co-cultivation of Arbuscular mycorrhizal fungi, *Glomus microcarpum*

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Arbuscular mycorrhizal (AM) fungi have symbiotic relationship with plants and which mainly helps the plants for the uptake of phosphorus, other micro nutrients and are fundamental for soil fertility and plant nutrition. The obligate biotrophic nature of AM fungi makes it difficult to grow them in synthetic medium and this prevents large scale inoculum production. In the present investigation, we were successful in raising hairy roots from *Canavalia* sp. using *Agrobacterium rhizogenes* ATCC 15834 and successfully co-cultivated AM fungi *Glomus microcarpum* in hairy roots. The hairy root obtained was confirmed for the presence of *rol* B genes of *Agrobacterium rhizogenes*. Different stages of AM fungal colonization were also observed and 60 % of mycorrhizal colonization was observed on the 20th day of co-cultivation in petri dish. Mycorrhized *Canavalia* hairy roots were tested for its potential to use as an mycorrhizal inoculum to infect *Ipomoea batatas* roots were tested and its showed 76% colonization.

Key words: *Agrobacterium rhizogenes, Canavalia* sp., *Glomus microcarpum,* colonization, mycorrhiza, hairy roots, arbuscules, fungi

Introduction

Agrobacterium rhizogenes are the most frequently exploited gene transfer agents in a wide variety of plant species (Hooykaas, 1992). *Agrobacterium rhizogenes* is a gram negative soil bacterium was selected for its capability to induce the infected cells to form roots in a large number of plants. This is due to the transfer, the integration and the subsequent expression of a portion of bacterial DNA (T-DNA) from a little ring of bacterial DNA named Ri (root

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inducing) plasmid to that of the plant. In fact this portion of DNA contains the necessary information to change the normal cell growth programme towards the roots production (Chilton *et al*., 1982).

Hairy roots have a lot of advantage over normal roots, as it can grow much faster than normal roots and are capable of unlimited propagation in culture media and showed genetic and biochemical stability (Shanks and Morgan, 1999, Flores and Filner, 1985). Hairy roots can be cultivated in a simple medium without the addition of phytohormones and can grow from low inocula to high final biomass densities with only minimal lag phase (Toivonen, 1993).

Arbuscular mycorrhiza (AM) is a symbiotic or mutualistic association between roots of about 90% of the species of plants, including angiosperms, gymnosperms, pteridophytes and bryophytes (Williams *et al*., 1994). Fungal symbionts get shelter and food, *i.e*., reduced carbon from the plant which in turn acquires and array of benefits ranging from better uptake of phosphorus and relatively immobile micronutrients, like zinc and copper and other minerals increase in nitrogen fixing capacity of leguminous plant species, salinity and drought tolerance, maintenance of water balance, increased rate of photosynthesis to overall increase in plant growth and development (Anjana Singh 2007**)**. Mycorrhizal plants show higher tolerance to high soil temperature and various soil and root borne pathogens.

In order to overcome the biotrophic nature of AM fungi, hairy roots are being tried to grow them in petri dishes and in bioreactors. At present hairy roots from *Daucus carota*, *Solanum lycopersicum* and *Ipomoea batatas* were used as a best host plant roots for culturing and growing axenic (pure) inoculum of AM fungi. Potty & Chandran (2001) successfully grown *Glomus microcarpum* var. *microcarpum* in Ri-TDNA transformed cassava roots. Chandran & Potty (2002) proved that Hairy Root Technology (HRT) is the best alternative to grow AM fungi and it can be used for the production of monoxenic inoculum.

In this study hairy roots were initiated from *Canavalia* sp. using *Agrobacterium rhizogenes* ATCC 15834 and co-cultivated AM fungi, *Glomus microcarpum* and tested the efficiency of mycorrhized hairy roots as mycorrhizal inoculum *in Ipomoea batatas* plants. This is the first report on the colonization of AM fungi in hairy roots of *Canavalia* sp.

Materials and methods

Biological material

Seeds of *Canavalia* sp. collected from CTCRI, Thiruvananthapuram were used for hairy root induction. *Canavalia* seeds were kept in concentrated

sulphuric acid for ten minutes to dissolve seed coat. Surface sterilization of seeds were done using 0.1 percent mercuric chloride (0. 1% Hg Cl_2) for 5 - 10 minutes and washed with sterile distilled water twice and dipped in 1 percent sodium hypochlorite solution (1% $NaOcl_3$) for 5 minutes and washed well with sterile distilled water. All the pretreated and surface sterilized seeds were aseptically placed in 1% plain agar plates for germination in incubator at 25^{o}C for two weeks.

Bacterial strain and culture condition

Agrobacterium rhizogenes ATCC 15834 strain kindly donated by Dr. Usha Mukundan, Head, Department of Plant Biotechnology R.J. College, Mumbai were used for the induction of hairy roots. *Agrobacterium rhizogenes* were grown in YEB media (Vervliet *et al.,* 1974) in culture plates at 24^{0}C in incubator for 48 hours and this was used for infection and cultures were preserved in YEB slants at 4^{o}C for further studies. YEB media composition includes Beef Extract - 5g, Yeast Extract- 1g, Peptone - 5g, Sucrose - 5g, MgSO4 7H2O - 0.49g, Bacto agar - 12g, Distilled water - 1000ml and pH - 7.2.

Gene transfer technique and decontamination

Aseptically grown seedlings were used for infection. Small cuts were made on the stem by using a sterile scalpel, which was touched with 48 hour old culture of *A. rhizogenes* 15834 (Ooms *et al.*, 1985). The hairy roots obtained were frequently sub cultured in fresh media containing antibiotics cefotaxim 500 mg/l to make the hairy roots free form *A. rhizogenes*. The bacteria free hairy roots were to colonize AM fungi.

DNA extraction and detection of rol B genes from hairy roots

The T-DNA localization in plant genome serves as a reliable genetic marker to confirm transformation. Polymerase Chain Reaction (PCR) was employed to detect transformation. For DNA extraction 200 mg of transformed hairy roots of *Canavalia* sp. was collected and crushed with liquid nitrogen and further extraction steps were done using DNeasy Plant Maxi kit, Qiagen Inc. USA.

The *rol B* gene is in the T-DNA of pRi (plant root inducing) plasmid. For *rol B* gene the 5' primer sequence was TGGATCCCAAATTGCTATTCC TTCCACGA and 3' primer sequence was TTAGGCTTCTTTCTTCAGG TTTACTGCAGC. These primers will amplify a 780- base pairs (bp) fragment. Primers were supplied by Qiagen Operon, United Kingdom. The cycling conditions were denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 1

minute and extension at 72°C for 7 minutes, with samples being subjected to 30 cycles. Amplification products were separated on 2% agarose gels and detected by staining with ethidium bromide. The DNA bands were cut with a knife and eluted using Millipore amicon DA column. The eluted DNA were subjected to restriction analysis using restriction enzymes Bgl 1 and Eco RII. Bgl 1 enzyme cleaved the DNA fragment at 446 bp and Eco RII can cleave at 125 bp and 486 bp of 780 bp DNA of *rol* B gene.

Co-cultivation of AM fungi in hairy roots

Standardized modified Murashige and Skoog (MS) medium (1962) 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 of *Glomus microcarpum* was 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 for a period of twenty days.

Estimation of mycorrhizal percentage of colonization

The mycorrhized hairy roots were cut into 1 cm long and used for estimating AM colonization. The root pieces were boiled in 10% KOH solution to soften the tissues. After repeated washing with water, they were neutralized with 10 % HCl and washed again. The cleared root segments were stained in trypan blue-lactophenol. The root bits were kept in the stain for 48 hours. The roots were destained with lactophenol and examined for VAM colonization. One hundred root bits were examined for mycorrhizal infection for each treatment unless otherwise stated.

Mycorrhizal infection was expressed as percentage colonization or the length of roots becoming mycorrhizal.

$$\text{Percentage of infection} = \frac{\text{No. of root bits showing AM infection x 100}}{\text{Total number of root bits examined}}$$

Mycorrhizal infection analysis of mycorrhized hairy root inoculum

Pot culture experiment was designed to test the viability and infectivity of mycorrhized hairy root inoculum of *Canavalia* sp. grown in 9 cm petri dish. Sandy soil was sterilized with 10 % formaldehyde to kill microorganisms and

existing mycorrhizal spores. Formaldehyde treated soil was kept in plastic cups for a week. Thirty days old mycorrhized hairy roots were chopped (approximately to 1 cm in length) and 5 to 6 root bits were mixed with the soil in each cup. Fresh *Ipomoea batatas* stem bits of approximately 3 to 4 inches with 4 to 5 inter nodes were planted in each cup and watered regularly and they were kept in glass house. The data collected for a period of 17 days include root biomass, mycorrhizal colonization percentage, presence of arbuscules and vesicles. The experiment was done in triplicate and the mean values are presented.

Results and discussion

Surface sterilized *Canavalia* seeds were placed in plain agar, germinated after six days of incubation. The germinated seeds were transferred to Murashige and Skoog (MS) media (1962) and 10 day old plantlets were infected with sterile fine blade touched with 48 hour old culture of *A. rhizogenes* 15834 strain. The infected plates were kept in dark for 2 days and after incubation the infected plants were placed in MS media supplemented with cefotaxime 500 mg/l. Then the infected plants were kept in MS media with out antibiotic.

After 10 days of incubation numerous hairy roots appeared from the infected sites (Fig 1). Elongated hairy root tips were cut and placed in MS media containing antibiotic to eliminate residual Agrobacterial growth. Four successive transfers were made to free the hairy roots from *Agrobacterium rhizogenes*. In *Canavalia* sp. before the emergence of hairy roots, tumour formation was observed and from the tumourous region hairy roots emerged (Fig. 2, 3). The fresh growing regions of the roots were purely white while the older region became slightly brownish. The axenic hairy roots grew well in hormone free MS media and exhibited extensive branching (Fig. 4). The hairy roots were grown in modified MS media and various growth parameters were standardized for optimum production of hairy roots (Table 1). Full and ¾ strength of MS salts did not support the hairy root growth and caused browning of hairy root tissues. So the concentration of MS salts was reduced to half. MS vitamins were also replaced by B5 vitamins (Gamborg *et al*., 1968) for better hairy root growth. The transformed roots have a quick, vigorous and homogenous growth in relative poor substrates without the supplementation of hormonal substances**.** This is one of the major advantages of using hairy roots for culturing AM fungal inoculum.

Table 1. Growth conditions of *Canavalia* hairy roots.

Media Composition		**Temperature**	**pH**
MS media	½ strength		
B5 vitamins (instead of MS vitamins)	Full strength	25 – 26 °C	5.6
Sucrose	15 gram/liter		
Polyvinylpyrrolidone (as antioxidant)	0.3 ram/liter		
Distilled water	1000 ml		

Fig. 1. Emergence of hairy roots.

Fig. 2. Tumor formation.

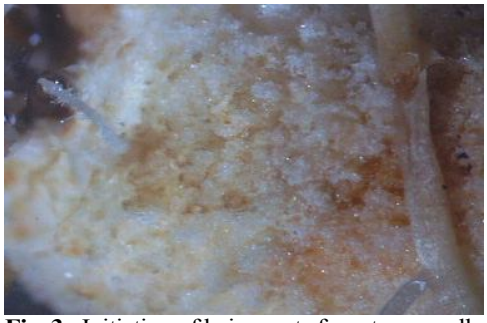

Fig. 3. Initiation of hairy roots from tumor cell.

Fig. 4. Mycorrhized hairy root of *Canavalia* sp.

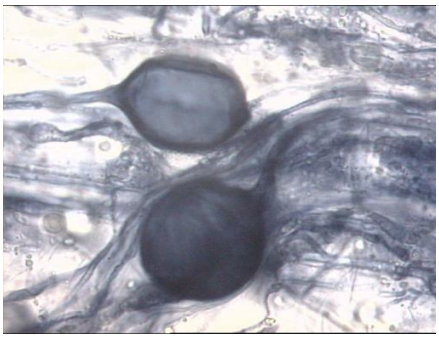

Fig. 5. Vesicles in the stained mycorrhized hairy root of *Canavalia* sp.

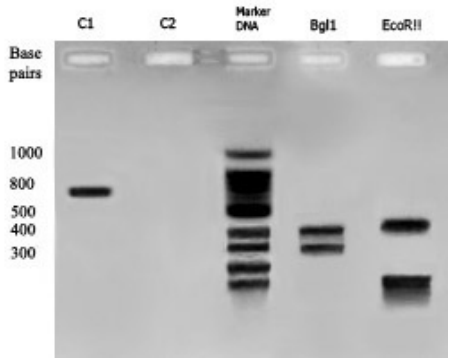

Fig. 6. PCR detection of *rol* B gene and restriction analysis of PCR product C1- DNA from transformed roots, C2 - DNA from non-transformed plant root Bgl 1 and EcoR II- restriction enzymes).

Hairy root DNA was extracted and analyzed for the presence of *rol* B gene. DNA isolated from the roots of a normal *Canavalia* seedling were kept as control. After PCR analysis the products were subjected to electrophoresis in 2% agarose gel and stained in ethidium bromide. It amplified a 780 base pair (bp) fragment and the presence of *rol* B gene in hairy root DNA was confirmed (Fig. 6). This confirmed the stable integration of T-DNA, a mobile segment of Ri plasmid which is transferred to the plant cell nucleus and stably integrated into the plant chromosome. The 780 bp PCR product eluted from the gel was further subjected to restriction analysis for confirmation by using two restriction enzymes Bgl 1 and Eco RII. These enzymes can cleave 780 bp fragments at different restriction sites. Bgl 1 enzyme cleaved the eluted PCR product at 446 bp and Eco RII cleaved at 125 bp and 486 bp of 780 bp DNA of *rol* B gene (Fig. 6). Similar analysis were also conducted by Amador *et al.,* (2002) for detecting *rol* B gene from root samples of *Pinus maximartinezii* and the results obtained from *Canavalia* sp. hairy roots were also similar. These results

showed that the roots obtained were transgenic and contained root inducing *rol* B genes of Ri plasmid of *A. rhizogenes*.

The AM fungal spores of *Glomus microcarpum* germinated and infected the hairy roots of *Canavalia* sp. and formed runner hyphae. The pH 5.6 supported *Glomus* spore germination. Where as spores of *Acaulospora laevis* germinated best between pH 4 and 5 (Hepper, 1984), *Gigaspora* spp. at a pH from 4 to 6 and *Glomus* spp. between pH 6and 9 (Green *et al*., 1976). The initial stage, the pre penetration stage was observed on the 6^{th} day of mycorrhizal infection. The negative geotropism of transformed roots facilitates contact with mycorrhizal hyphae or spores of AM fungi (Becard and Fortin, 1988) and this has increased the chances of mycorrhizal infection in hairy roots.

Penetration of mycorrhizal fungi into the cortex tissue began on the 8^{th} day and showed a steady increase from the 10^{th} day onwards. Heavy ramification of AM fungal mycelia was observed in the stained roots. Vesicles and arbuscules were also found in the stained hairy roots (figure 5). Mycorrhizal colonization was observed from the 8^{th} day and reached a maximum of 60 % on the 20^{th} day of infection. The mycorrhized *Canavalia* sp. hairy roots were tested for its ability to use as mycorrhizal inoculum and to infect normal plant roots using pot culture experiment and showed a good result and the parameters, such as, increase in root biomass, mycorrhizal colonization percentage and presence of vesicles and arbuscules were observed.

Root biomass gradually increased from the 5^{th} day onwards from 0.540 g to a maximum of 5.768 g after 17^{th} day and the mycorrhizal colonization increased from 24 to 76 %. After 7 days of growth only mycelial filaments were found from stained roots and vesicles were found in the roots on the 9^{th} day, vesicles and arbuscules were found on 11^{th} day (Table 2). The vesicles are reserve organs of the fungus and they contain lipids. During stress situation (low supply of metabolites from the host plant), the fungus utilises these reserves and then the vesicles degenerate. This is mainly attributed to the property of stress tolerance of mycorrhiza infected plants. Arbuscules are highly branched haustoria-like fungal organs produced in the cortical cells and the plant-derived periarbuscular membrane surrounding them are regarded as the key sites of bi-directional nutrient exchange. Peri-arbuscular membrane, which is also a specialized structure formed at the site of arbuscular formation, consists of the membrane of the symbiotic partner (Gollotte *et al*., 1996) and facilitate the transport in both direction between the partners. Arbuscules are usually relatively short-lived and the hyphae are long lived (Smith and Dickson, 1991).

Table 2. Increase in root biomass in relation to days of growth.

Days of growth	Root biomass (fresh weight) in grams	Mycorrhizal colonization %	Presence or absence of mycorrhizal structures
5	0.540	Nil	Nil
7	1.431	24	Mycelial filaments
9	3.410	36	Vesicles
11	3.956	48	Vesicles and arbuscules
13	4.278	55	Vesicles and arbuscules
15	5.067	65	Vesicles and arbuscules
17	5.768	76	Vesicles and arbuscules

The presence of mycorrhizal colonization and the mycorrhizal structures in host root clearly indicated that the origin or the source of mycorrhizal fungi come only from the mycorrhized hairy roots which were used as inoculum. Further, this experiment clearly indicated the viability and infectivity of mycorrhized hairy root inoculum obtained using *Canavalia* sp. Fermenters can also be used for the large scale production of mycorrhizal inoculum using hairy roots. Jolicoeur *et al*., 1999, used an airlift bioreactor for the production of *Glomus intraradices* propagules and Chandran and Potty (2009) designed a simple bioreactor to grow mycorrhized hairy roots of *Ipomoea batatas* for mass production of *Glomus microcarpum* inoculum.

Mycorrhized *Canavalia* sp. hairy root bits can be used as a starter inoculum for large scale inoculum production by conventional methods and it can also be used to inoculate tissue cultured plantlets. AM fungi can reduce transplantation shock in tissue cultured plants. Fortuna *et al*., (1992) evaluated transplant shock tolerance by inoculation of *Glomus mosseae* and *G. coronatum* into micropropagated *Prunus cerasifera* and observed 100% survival of plants. In addition to this mycorrhized hairy root inoculum can also be used for establishing germplasm collection of AM fungi. In 1995, Diop established a bank of germplasms of AM monoxenically cultivated in association with isolated tomato or transformed carrot roots and the developing nations will benefit more from AM symbiosis using the monoxenic system, which is also cheaper than greenhouse culture (Diop *et al*., 1994) and can maintain mono specificity of mycorrhizal cultures.

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