www.bti.org.in
ISSN 0974-1453
Research Article

INDUCTION AND GROWTH OF HAIRY ROOTS IN BRASSICA JUNCEA L.CZERN (INDIAN MUSTARD)

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ABSTRACT

Plant model system capable of accumulating a wide range of organic and inorganic contaminants is of considerable interest and potential candidate for phytoremediation studies with*in vitro* generation of hairy roots. *Brassica juncea* L. czern belonging to *Brassicaceae* family was used to induce hairy roots with the infection of *Agrobacterium rhizogenes* belongs to *Rhizobiaceace* family. Two to three weeks old sterile seedlings were used for infection with two wild type strains MTCC 2364 and MTCC 532 cultured in YMB medium. After four weeks of inoculation, hairy roots were induced from the wounded portions of sterile seedlings and the culture was maintained in MS basal medium devoid of phytohormones with the addition of cefotoxime antibiotic to prevent any cross contamination. *Agrobacterium rhizogenes* mediated transformation and hairy root induction was confirmed by PCR amplification of DNA from hairy roots.

Keywords: *Agrobacterium rhizogenes, Brassica juncea,* Hairy roots, PCR.

INTRODUCTION

Agrobacterium rhizogenes (formerly *Phytomonas rhizogenes*) was first identified more than 70 year ago (Riker *et al*., 1930; Hildebrand *et al*., 1934) as the causative agent of the plant disease known as hairy-root syndrome or root-mat disease. Transformed plant roots, also called hairy roots, are adventitious roots that are caused by infection with the soil pathogen *Agrobacterium rhizogenes* and the development of these roots is the result of natural genetic engineering in which a specific region of bacterial DNA contained in the Ri (Root inducing) plasmid is transferred from the bacterial cell to the plant cell and this fragment of DNA (T-DNA) is integrated

into the plant genome and expressed (Tepfer, 1984 and Zambryski, 1992).

Most plants materials, such as hypocotyls, leaf, stem, cotyledon, protoplast, storage root, or tuber, can be used to induce hairy roots (Mugnier, 1988; Han *et al*., 1993; Drewes *et al.,* 1995; Giri *et al*., 2001; Krolicka *et al*., 2001; Azlan *et al*., 2002). However, for different species, the proper explants material may vary and the age of material is most critical, with juvenile material being optimal.

To induce hairy root, explants are separately wounded and co-cultivated or inoculated with *A. rhizogenes*. The application of hairy roots to phytore mediation has been suggested by various authors (Metzger *et al.,* 1992; Pletsch *et al.,* 1999), mainly because of their biochemical resemblance to the roots of the plant from which they have derived. Furthermore, growth and maintenance of such cultures is a cheap and straight forward procedure which allows the studies to be carried out without the interference of the micro biota.

Hairy roots are also amenable to genetic transformation, making gene transfer and characterization possible in a system that may pose minimum health or environmental concerns. Another advantage of using hairy roots for studying phytoremediation is their ability to produce large quantities of exudates which are composed of enzymes and some metal chelating compounds that may detoxify or sequester harmful organic and inorganic contaminants (Bais *et al.,* 2006; Doty, 2008). Hairy roots have been used to assess the potential of several plant species to remove contaminants from the environment. Plants can be regenerated from hairy root cultures either spontaneously (directly from roots) or by transferring roots to hormone-containing medium.

The advantage of Ri plasmid based gene transfer is that spontaneous shoot regeneration is obtained avoiding the callus phase and somaclonal variations. Ri plasmid-based gene transfer also has a higher rate of transformation and regeneration of transgenic plants; transgenic plants can be obtained without a selection agent thereby avoiding the use of chemicals that inhibit shoot regeneration; high rate of co-transfer of genes on binary vector can occur without selection.

Further, *Agrobacterium tumefaciens* mediated transformation results in high frequency of escapes whereas *Agrobacterium rhizogenes* mediated transformation consistently yields only transformed cells that can be obtained after several cycles of root tip cultures. These hairy roots can be maintained as organ cultures for a long time and subsequent shoot regeneration can be obtained without any cytological abnormality. (Giri and Narasu, 2000)

Among different biological systems used in investigating remediation processes, hairy root cultures are considered a remarkably valuable research tool (Zhou *et al*., 2011). Hairy roots develop as the consequence of the interaction between *Agrobacterium rhizogenes* - a Gram negative soil bacterium-and the host plant. Hairy roots are fine fibrous structures that are formed on plant tissues infected by *Agrobacterium rhizogenes*, a soil bacterium responsible for the root mat disease

(Georgiev *et al.,* 2007; Veena and Taylor, 2007). After infecting the cells, *A. rhizogenes* stably transfers several of its genes to the plant genome resulting in physiologic changes in the host cell leading to enhanced growth in hormone-free media (Srivastava and Srivastava, 2007). The observed changes in root physiology and morphology are associated with the transfer of a cluster of genes from the *A. rhizogenes* large Ri (root-inducing) plasmid into the plant genome. The symptoms observed with *A. rhizogenes* infection may suggest that the transformed cells have been rendered more sensitive to auxin without altering the production of these plant hormones (McAfee *et al.,* 1993; Srivastava and Srivastava, 2007).

MATERIALS AND METHODS

In vitro culture

Indian mustard *(Brassica juncea*), seeds were purchased from Tamil Nadu Agriculture University (TNAU), Coimbatore, India and Two wild type strains of *A. rhizogenes*, MTCC 2364 and MTCC 532 were obtained from Microbial Type Culture Collection Centre, (IMTECH) Chandigarh, India.

Indian mustard (*Brassica juncea* L. czern) seeds were used as a source for initiating *in vitro* cultures for generating explants to form hairy roots by *A. rhizogenes* infection. The seeds were sterilized with 70% ethanol for 40 s followed by 0.1 % mercuric chloride for 5 min. The sterilized seeds were subsequently washed five times with sterile double distilled water and aseptically inoculated for germination on MS basal medium, supplemented and solidified with 3% sucrose and 0.8% agar respectively at pH 5.5-5.8. Two to three weeks old sterile seedlings were used for infection by *A. rhizogenes*.

Preparation and culture of *A. rhizogenes*.

Two wild type strains of *A. rhizogenes,* MTCC 2364 and MTCC 532 were cultured by inoculating a conical flask containing approximately 20 ml of YMB medium with *A. rhizogenes* strains at 25^0C for 24 h at 120 rpm in a orbital shaker.

The bacterial suspension of 24 h old culture of *A. rhizogenes* was transferred to sterile centrifuge tubes and centrifuged at 4000- 5000 rpm for 5-10 min and the resultant cell suspension or cell biomass of the bacteria was resuspended in liquid MS medium supplemented with 3% sucrose at a density of $\times 10^8$ cell and the optical density of bacterial suspensions were between 0.6-0.8 at 600 nm and this culture of *A. rhizogenes* at exponential growth phase was used for transformation studies.

A manual wound was made on the stems of *in vitro* grown seedlings of Indian mustard with a fine needle attached to a syringe containing culture of *A. rhizogenes* and inoculated 1-2 drops onto the wounded portion of the tissue. Excess liquid was removed as this might be lead to excessive growth of *Agrobacterium* on the surface of the medium. The infected plants were carefully placed in the same medium for co-cultivation for another 2-3 weeks without disturbing the media even without adding antibiotic to avoid contamination.

Induction of hairy roots

The average co-cultivation duration was about two or three days. After that, the explants were transferred to a solid medium containing an antibiotic to eliminate the

bacteria. Cefotaxime (250-500 mg L^{-1}) was often used to eliminate the bacteria. The explants were then transferred onto a solid hormone-free medium in the dark at 20-25°C, and the first roots appeared after a few weeks (usually 1 to 4). The roots were then transferred to Erlenmeyer flasks containing liquid phytohormone-free MS basal medium. The typical transformed root phenotype is a highly branched root covered with a mass of tiny root hairs and these cultures do not require phytohormones. Concerning the growth rate, the average doubling time of hairy root lines was around 2-3 days. (Bensaddek *et al., 2008)*

Growth and maintenance of hairy roots

All the explants were pre-cultured for two days on MS basal medium. The explants were co-cultivated with bacterial suspension for 30 min. After three days, they were transferred to MS medium supplemented with cefotaxime 400mg/l. cefotaxime concentration was then halved each week from 400mg/l to 50mg/l, and finally cultures free of *Agrobacterium rhizogenes* were transferred to B5 (Gamborg *et al*., 1968) medium solidified with 0.2% Phytagel (Sigma). Hairy roots, which arose mainly from the cut surfaces of the explants, were separated, when they attained a length of 4-5 cm. All the cultures were maintained in complete darkness at 25 ± 2°C. Excised roots of in vitro germinated seedlings were cultured similarly and served as controls. (Dhakulkar *et al.*, 2005)

Isolation of genomic DNA from hairy roots

The genomic DNA was isolated from hairy root cultures of *Brassica juncea* L. for transformation confirmation as described by Pich and Schubert (1993) and stored at -20^{0}C for polymerase chain reaction.

PCR

For amplification of the ORF-13 coding sequence of TL DNA with a 498 bp domain, the primers used were (+) 5’ CAG CTT CTA AAT GTG GAG GCC and (-) 5’ CCT TGC CGA TTG CCA GTA TGG C. For amplification of *mas* 1’ sequence of TR DNA with a 970 bp domain, the primers used were (+) 5’ CGG TCT AAA TGA AAC CGG CAA ACG and (-)5’ GGC AGA TGT CTA TCG CTC GCA CTC C.

For amplification of *vir* B10 coding sequence with a 644 bp domain, the primers used were (+)5’ CAA TCC CGA TCA AGT CGT GCT C and (-) 5’ AGA CGC CAA CCT CGT GAA ACC G. DNA amplification was performed on an eppendorf thermal cycler in 25µl reaction mixture containing 25 ng template, 2.5 µl 10 x Taq DNA polymerase buffer 3-Tris (hydroxymethyl) methylamine propane sulfonic acid pH 8.8, 15 mM $MgCl_2$, 500 mm KCl, 0.1 % gelatin).100 µM each of dNTPs, 0.2 µM primer and 0.5 U Taq polymerase (Bangalore Genei Pvt. Ltd). Following an initial denaturation step at 94^{0}C for 2 min, the amplification programme was 30 cycles of 30s at 94^{o}C, 30 s at 56^{o}C and 1 min at 72^{o}C. Amplification products were separated by electrophoresis on 1.5 % agarose gel in 1x TBE buffer, stained with ethidium bromide and visualized under UV transilluminator.

RESULT AND DISCUSSION

In vitro cultivated *Brassica juncea* L. czern (Indian mustard) seedlings were used

as starting (explants) material to induce hairy roots by *A. rhizogenes* infection. The hairy roots of *Brassica juncea*, due to their highly branched nature, have a large surface area in comparison with normal roots, rapid growth in simple hormone-free medium under defined aseptic conditions.

Induction of hairy roots by infection

Both wild type *A. rhizogenes* strains MTCC 2364 and MTCC 532 were capable of inducing hairy roots, A4 strain MTCC 532 induced highly branched profuse root system from the wounded portions of in *vitro* grown seedlings of *Brassica juncea* within 1- 4 weeks of inoculation period. The hairy roots derived from MTCC532 were isolated and cultured on solid MS basal medium supplemented with cefotaxime antibiotic at 500 mg/l and roots grew plagiotropically with extensive branching while non -transformed (control) roots failed to grow on MS medium devoid of phytohormones.

PCR amplification

PCR amplification of hairy roots DNA with primers specific for ORF 13 and mas 1' sequences indicated the expected fragment sizes of 495 and 985 bp, respectively. The control plant did not show any amplification with primers which indicated the integration of TL and TR DNA regions of *A. rhizogenes* with the genome of *Brassica juncea* L. czern hairy roots.

PCR analysis using primers specific to vir B10, a non-transformed region of Ri-plasmid showed amplification only in the positive control (plasmid DNA) and not in DNA isolated from the hairy roots, eliminating the possibility of any contamination with *A. rhizogenes* (Fig.1 & Fig.2).

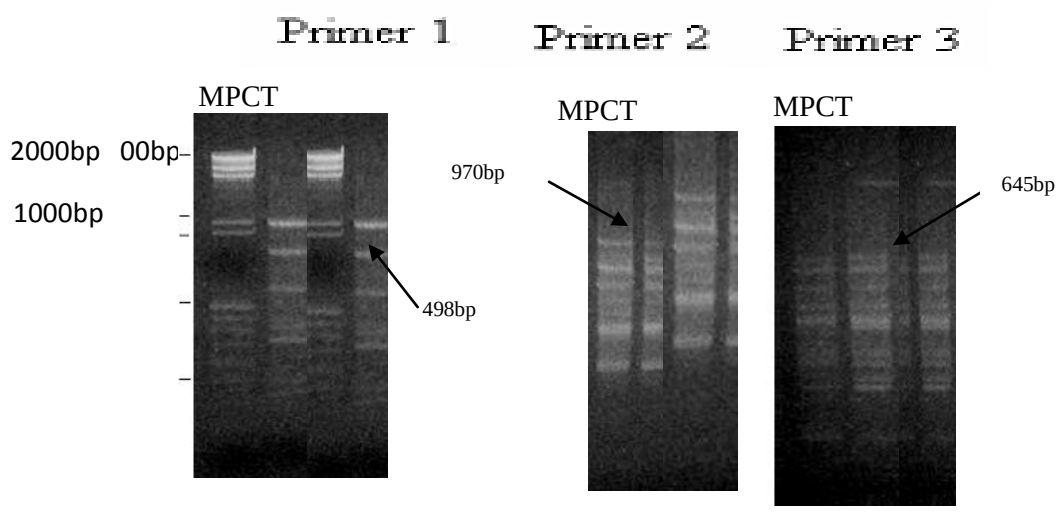

Fig. 1. PCR analysis of *B. juncea* hairy roots. M- 100 bp DNA ladder; P-positive control (Ri plasmid DNA) C- Control / Non transformed roots; T- transformed roots, arrows indicated expected fragments.

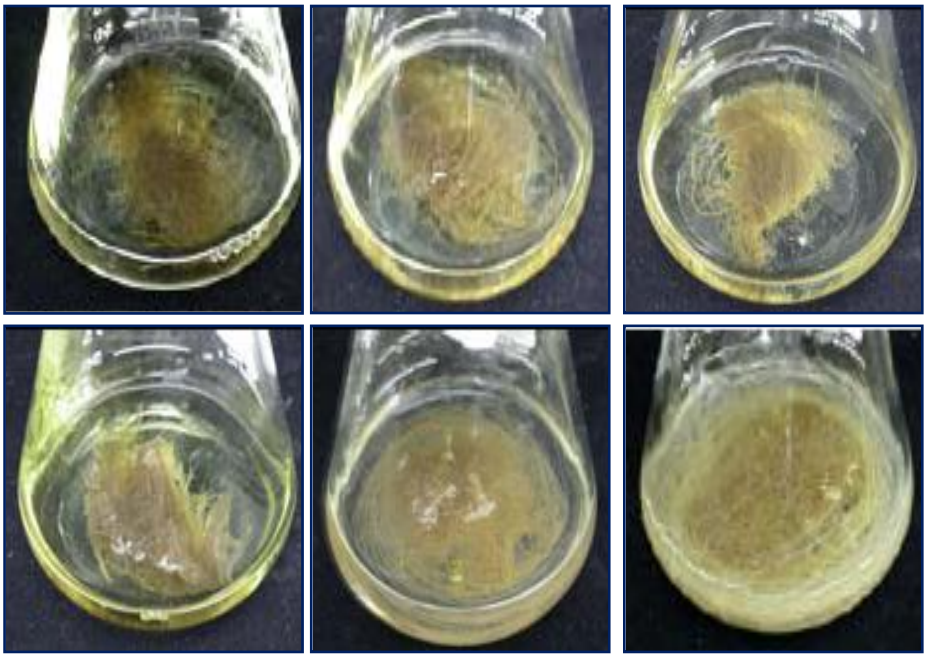

Fig. 2. Hairy Roots of *Brassica Juncea* L.Czern (Indian Mustard) cultured in MS Medium devoid of phytohormones.

CONCLUSION

In vitro plant cultures are commonly used as tools for conducting basic laboratory studies of phytoremediation. *In vitro* root culture is having slow growth rate and low biomass and also due to some notable disadvantages, it is not suitable for phytoremediation studies to be carried out. The generation of hairy root cultures and their use as a laboratory root model for the *in vitro* study for the removal of organic and inorganic contaminants is of particular importance for studying the interaction of contaminants with this plant organ but the *in vitro* cultures of transformed roots (hairy roots) have the properties of fast growth and extensive root proliferation by *A. rhizogenes*, generally considered as an undesirable characteristic, may find good utility for phytoremediation as roots for their larger penetrating ability to retrieve the pollutants from aqueous solution, autotrophy in phytohormone and genotype and phenotype stability and the transformed roots (hairy roots), due to its fast growth, a high degree of lateral branching, the profusion of root hairs and the absence of geotropism remarkably considered as valuable research tool in modern plant and eco technology platform and hairy root cultures of *Brassica juncea* L. czern could be useful in rhizo filtration studies due to its natural ability to accumulate metals in wide range, fast growing and high biomass capacity. These transformed roots could be cultured under axenic conditions and are easily established and propagated *in vitro* as they are

characterized by fast growth, exhibit genetic and biochemical stability and have pattern of metabolites similar to that of the original plant.

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