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Review Article

Influence of Different Strains of *Agrobacterium rhizogenes* on Induction of Hairy Roots in *Ocimum tenuiflorum* L. - A Review

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A B S T R A C T

Agrobacterium rhizogenes is a Gram negative soil bacterium that produces hairy root disease in dicotyledonous plant. *A.rhizogenes* induces the formation of proliferative multi-branched adventitious roots at the site of infection; so-called 'hairy roots' (Chilton *et al.*,) The chemical constituents of *Ocimum tenuiflorum* are highly complex. It contains many nutrients such as beta carotene, vitamin C, A, and minerals like zinc, iron, calcium. It contains many biologically active compounds such as euginol, urosolic acid, carvacrol, linalool, limatrol, caryophyllene and estragol. The present work has been carried out with a view to establish the protocol for *Agrobacterium rhizogenes* mediated hairy root induction *Ocimum tenuiflorum* L, an important medicinal plant, using two different strains of *Agrobacterium rhizogenes* MTCC 2364 and MTCC 532. Aseptic explants for transformation were obtained by inoculating shoot tips of *O.tenuiflorum* on MS media supplemented with BAP- 2.0 mg/l, $AdSO_4$- 50mg/l and Citric acid- 1.0 mg/l. Both strains were evaluated for their transformation efficiency in shoot tips, nodes and leaves of *O.tenuiflorum.* MTCC 2364 was maintained at 28˚C for 48 hours in YEP medium and MTCC 532 strain was grown in YEN medium at 25˚C for 24 hours, Acclimatization of bacterial culture in MS medium for 4 hours was found effective for transformation. The explants were treated for different co-culture periods. The percentage of hairy root induction varied with infection period.

Keywords

Influence of different strains, *Agrobacterium rhizogenes*, *Ocimum tenuiflorum*, Hairy roots

Introduction

A.rhizogenes mediated transformation

Agrobacterium rhizogenes mediated hairy root production is a valuable tool for studies on the biosynthesis of secondary metabolites and for exploitation in metabolic engineering. Hairy roots are characterized by rapid growth and extensive branching in growth regulator-free medium. In general, they exhibit genetic stability and, in certain cases, they have the capability of synthesizing secondary metabolites normally present in roots and organs of the species of origin. *Agrobacterium rhizogenes* inserts T-DNA from the Ri plasmid into the genomes of the host plants root cells, having the *rol* genes involved in root initiation and development.

Medicinal plant and its value

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants use were instinctive, as is the case with animals (Stojanoski). In view of the fact that at the time there was not sufficient information either concerning the reasons for

the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants' usage gradually abandoned the empiric framework and became founded on explicatory facts. Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis (Kelly). Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again.

Medicinal plants have been identified and used throughout human history. Plants make many chemical compounds that are for biological functions, including defense against insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10% of the total (Tapsell L.C.) Chemical compounds in plants mediate their effect on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus, herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to have beneficial pharmacology, but also gives them the same potentials conventional pharmaceutical drugs to cause harmful side effects (Lai and Roy, 2004).

Agrobacterium rhizogene

Schmulling *et al.*, (1989) observed Chimeric genes containing the 8-glucuronidase reporter gene under the control of the *rol* A, B, and C promoters of *Agrobacterium rhizogenes*are expressed in a regulated manner in transgenic plants. The intergenic region separating the *rol B* and C genes represents a bidirectional promoter. This bidirectional promoter regulates transcription for both genes in a similar fashion in aerial organs of the plants, but in a distinct way in roots. Moreover, both *rol* B and C promoter activities differ from those characteristic of the *rol* A promoter. Thus, promoters of bacterial origin show differential expression in transgenic plants, and regulation of *rol* gene expression plays a role in the biological effects caused by the *rol A*, B, and C genes.

Brevert and Morel (1990) reported auxin regulates the promoter of the root inducing *rol* B gene of *Agrobacterium rhizogenes*1855 by using the beta glucuronidase (GUS) reporter system in transgenic tobacco plants. A 20-100 fold increase of GUS activity was reported by auxin in *rol* B GUS transformed mesophyll protoplasts. The spatial pattern of *rol* B –GUS expression could be strongly modified by the addition of exogenous auxin.

Shunan *et al.,* (1997) reported hairy root production in *Gingko biloba* Land concluded that hairy roots exhibit rapid growth, hereditary stability and high secondary metabolite synthesis.

Taskin *et al.,* (1999) reported hairy root transformation in *Rubia tinctorum* following infection of cotyledons with four different strains of *Agrobacterium rhizogenes*15834, 2628, 9365, R1000. Strains 15834, R1000, 9365 produced hairy roots in infected explants and the percentage of anthroquinone in roots was found to be different. Strain 2628 failed to produce hairy roots and induced callus formation on the wounded surface of cotyledons.

Moyano *et al.,* (1999) observed that, due to enhanced demand for plant products, plants have been indiscriminately harvested, cut for their products. This has been appropriately balanced by growing new plants. There are other parameters like environmental factors, climatic conditions, etc. which control the content of a particular compound in the plant,

so there is an upmost need to facilitate the hairy root cultures, through which we get plants with desired level of compounds, which are not influenced by any physical, chemical or biological stress

Karthikeyan *et al.,* (2006) reported hairy root induction from hypocotyls explants excised from seven day grown seedlings of groundnut using *Agrobacterium rhizogenes* 15834.The hairy roots were thin, slender and highly branched. The cefotaxime concentration of 250mg/L was found to be most suitable for hairy root production in groundnut.

Lonkova *et al.,* (2008) induced hairy root cultures from leaf explants of *Limnum tauricum* by infection with *Agrobacterium rhizogenes* strains ATCC 15834 and TR 105. Acetosyringone in cultivate medium was used to increase the frequency of hairy root induction. Transformed nature of tissue was confirmed by the production of opines. The highest amount of lignin was found in transformed line induced by strain ATCC 15834.

Mahesh *et al.,* (2011) reported hairy root induction on leaf and petiole explant *Taraxacum officinale* after infection with *Agrobacterium rhizogenes strains* A4 and ATCC 15834. A4 strain exhibited highest induction of hairy roots. Hairy roots developed from leaf tissues produced more biomass than non transformed roots. The induced root cultures accumulated higher levels of Sesquiterpene lactones.

Jafari and Samadi (2012) established hairy roots in different explants of *Linum mucronatum*, an anti-cancer agent producing plant, via a mikimopine type strain of *Agrobacterium rhizogenes*, A13. The percentage of hairy root induction varied with explants, and nodes were found to be highly susceptible to *A. rhizogenes* infection with the highest (60%) rate of hairy root induction. Four different Murashige and Skoog (MS)-based liquid culture media were used for well establishment of hairy roots. Hairy root growth medium D (HRGM-D) containing hormone-free MS basal medium with an extra one day pre-incubation period at 35°C was found to be more efficient for profuse growth (fresh weight; 8500 mg per 25 ml culture medium) of hairy roots. Hairy root system presented in this study may offer a suitable platform for optimization and production of satisfactory level of secondary metabolite.

Stefanache *et al.,* (2013) reported that, Among the other alternatives for plants products, plant cell culture have been widely studied for the production as well as for understanding the basic mechanism involved in a synthesis of a a particular compound. Cell culture grown under controlled condition on a define medium are free from environment fluctuation. The growth cycle of a cell in culture is fast as compared to the plants. Recently, cell cultures have been widely used in genetic engineering of plants, which have opened up new frontier to understand and produce valuable plant products in desirable quantity. Several genes of interest have been incorporated to then plant genome for better yield of the products. However major limitation of the cell culture is their instability, during long-term cultures and low product yields.

Fu *et al.,* (2014) reported production of cholinergic acid and its derivatives in hairy root cultures of *Stevia rebaudiana* following infection of leaves with *Agrobacterium rhizogenes* ATCC C58C1, which were further verified by PCR detection of *rol B* and *rol*C genes. HPLC –MS and HPLC analysis revealed cholorogenic acid and 4,5-dicaffeoylquinic acid with a total yield of 223.40 mg/100ml.

Vyas *et al.,* (2014) studied the development of a rapid and efficient *Agrobacterium rhizogenes* mediated transformation system for *Ocimum tenuiflorum* L., a traditional Indian medicinal plant that occurs in red and green forma. The plant is a repertoire of several pharmaceutically and nutraceutically important metabolites. Three different types of explants i.e. leaves, hypocotyls and excised shoots, obtained from shoot cultures of *in vitro* germinated red and green forma plants were transformed using *Agrobacterium rhizogenes* strain ATCC 15834.

Establishment of aseptic culture

Many scientists have done the micro propagation of *ocimum tenuiflorum* successfully, to establish the aseptic culture using MS medium supplemented with different harmones.

Banu and Bari (2007) by using nodal segment for *in vitro* production in MS medium added with 0.30 mg/l kinetin and 0.1% $HgCl_2$ (w/v) as surface sterilant, got the best result for shoot initiation.

Shilpa *et al.,* (2009) outlined a procedure for plant regeneration and antimicrobial screening of this medicinal herb, *Ocimum tenuiflorum,* through *in vitro* culture of nodal segments with auxillary buds. MS medium supplemented with 2.0 mg/l BAP and 1.13 mg/l IAA was found to be most effective for inducing bud breaking and growth, and in initiating multiple shoot proliferation at rate of 39 micro shoots per nodal explants after 30 days of culture by repeating sub culturing, a high- frequency multiplication rate was established for production of elite lines of *Ocimum tenuiflorum*. MS medium supplemented with 2.0 mg/l IBA was found to be best for rooting. *In vitro* and *in vivo* grown leaf extract in different solvent system were screened for potential antimicrobial activity against medicinally important bacterial and fungal strain by agar well diffusion method.

Saha *et al.,* (2010) observed that shootlets were regenerated from nodal explants of *Opium sanctum* through axillary shoot proliferation. The induction of multiple shoots from nodal segments was found to be highest in MS medium supplemented with 1.5mg/ lt IBA + 0.5mg/l KN. For rooting, different concentration of IBA, NAA and IAA were used and highest rooting percentage (97.66%) was recorded on MS medium with 0.1mg/lt IAA. The rooted plantlets were hardened and successfully established in soil.

Hairy root induction by *Agrobacterium rhizogenes*

Agrobacterium rhizogenes is a gram negative soil bacterium that induces hairy root disease in plants characterized by root proliferation at the site of infection (Meyer et al., 2000).

Chilton *et al*., (1982) reported that neoplastic hairy root induction by *Agrobacterium rhizogenes* involves the transfer of Ri plasmid DNA to the plant genome of *Nicotiana glauca*.

Spano *et al*., in 1982 reported the region of virulence plasmid of agropine type *Agrobacterium rhizogenes*1855 (pRI-1855) which was transferred to cells of *Daucuscarota* upon infection. T- DNA has been identified by means of Southern blot hybridization with the T-DNA of the mannopine type *A. rhizogenes* 8196.

Constantino *et al.,* (1984) reported that the T-DNA of *Agrobacterium rhizogenes* is transmitted through meiosis to the progeny of hairy root plants by regenerating plants from tobacco hairy root callus cultures. All

progeny plants of F1 generation contained the same full length T-DNA as the parents.

Schmulling and Schell in 1989 found that combined expression of the *rol* A, B and C loci of *Agrobacterium rhizogenes* establishes hairy root syndrome. However, when expressed separately, they provoked distinct developmental abnormalities characteristic for each *rol* gene. These results indicated that the different *rol* genes have different targets or qualitatively different effect on the same target.

Brevert *et al*., (1990) reported that auxin regulates the promoter of the root inducing *rol B* gene of *Agrobacterium rhizogenes* 1855 by using the beta glucuronidase (GUS) reporter system in transgenic tobacco plants. A 20-100 fold increase of GUS activity was reported by auxin in *rol B* GUS transformed mesophyll protoplasts. The spatial pattern of *rol B* –GUS expression could be strongly modified by the addition of exogenous auxin.

Uchida (1993) reported an efficient method of genetic transformation of *Hyoscyamus niger* by infecting sterile seedlings with *Agrobacterium rhizogenes* strain 15834. After subculturing the explants on hormone free ½ MS agar medium supplemented with antibiotics (Carbeniciiln and Vancomycin) to remove the bacteria, axenic hairy roots were maintained on B5 agar medium without antibiotics. Shunan *et al*., in 1997 reported hairy root production in *Gingko biloba* (L) and concluded that hairy roots exhibit rapid growth, hereditary stability and high secondary metabolite synthesis. Three different strains R1000, 15834 and A4 were used for genetic transformation of leaves, buds and stems of *Gingko biloba* (L). The explants were immersed in bacterial suspension and then cultured on ½ MS agar medium containing 0.5 gm/l ampicillin culture was kept at 26± 1 ˚C. Hairy roots were observed in each type of explants chosen for the experiment.

Tashkin *et al*., in 1999 reported *Agrobacterium rhizogenes* mediated genetic transformation in *Rubia tincotrum* (L). Cotyledons were used as explants, inoculated with *Agrobacterium rhizogenes* strains of 15834, 2628, R1000 and 9365. These strains were grown on NB (Nutrient Broth) medium at 28°C for 2 days. The inoculated cotyledons were co-cultivated with *A. rhizogenes* strains for 2 days at 25°C with a 16 hour photoperiod. After cocultivation, explants were transferred to semi-solid MS basal medium solidified with 0.8% agar, and contained 3% sucrose, 0.4 g/l Augmentin to kill the bacteria at pH 5.7 at a density of 10 explants per plate and cultured at 25°C. Forty days after co-cultivation, hairy roots were weighed out and transferred to 50 ml of MS liquid medium pH 5.7 containing 3% sucrose, and shaked in an orbital shaker at 120 revolutions/min at 25°C in the dark. The roots were then sub-cultured onto the same medium every 4 weeks. After 4 months in liquid culture, hairy roots from each explant were weighed out and the mean weight for each treatment was calculated. Hairy roots were initiated on the cut surface of the cotyledon explants from four populations, 10-12 days after co-cultivation with strain 15834. Hairy root formation occurred at the highest frequency of 75%. The strain R1000 induced hairy roots at a frequency of 50%. The untreated control cotyledon explants failed to elicit any hairy root response or substantial callus. It was found that strains 15834, R1000 and 9365 induced hairy roots on the explants whereas strain 2628 was ineffective to induce hairy roots.

Pawar and Maheswari in 2004 reported hairy root induction in *Withnia somnifera* and *Solanum surratanse* following infection with *Agrobacterium rhizogenes* MTCC 532 and

MTCC 2364.They tested various media like LB, YEN, NB, YEP, Tryptone broth and tricalcium phosphate broth to check optimal growth medium for the two strains. YEP medium was found to be most suitable medium for growth and maintenance of both the strains.48 hour old bacterial culture was found most suitable for infection. Culture was centrifuged at 3000 rpm for 5 minutes and re-suspended in 5 ml MS basal liquid medium. All the explants were dipped in co cultivation medium and incubated at 37 ˚C for 2 days. After 2 days explants were washed with liquid MS medium supplemented with 1% mannitol and 250 mg/l cefotaxime. Washed explants were inoculated on MS medium containing 250mg/l cefotaxime. Cultures were kept at 25± 1˚C Induced Hairy roots from leaves, hypocotyls, midribs and stems were established on hormone free MS basal medium. The growth rate of transformed roots was found to be 10 folds higher than the control.

Bhargava *et al*., (2005) reported hairy root production in *Gmeliana arborea* Roxb., an important medicinal plant. It is valued for its medicinal properties. Wild type *Agrobacterium rhizogenes* ATCC 15834 (harboringpRi 15834) was used for hairy root induction. Different seedling explants like root, stem, leaf, hypocotyl, cotyledons, cotyledonary nodal segments and embryo axis were isolated from *in vitro* grown seedlings. The explants were co-cultivated with bacterial suspension for 30 min. 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. Six different hairy root lines were established. These lines were maintained by subculture of 3-4 cm long pieces on B5 solid medium for four weeks. The hairy root cultures were also maintained in B5 liquid medium on a rotary shaker (80 rpm) in complete darkness. Genomic DNA was extracted using CTAB method from each of the hairy root lines as well as from control non transformed roots. PCR primers specific for the amplification of the 780 base pairs fragment of the *rol* B gene were used. Cotyledons isolated after 5 days from in vitro grown seeds showed maximum (32%) root induction. The presence of *rol* B gene in the hairy root lines was detected by PCR analysis. All transformants showed the presence of diagnostic 780 bp*rol B* product amplification.

Bonhomme *et al*., (2005) reported *Agrobacterium rhizogenes* mediated genetic transformation in *Papaver somniferum* L. They used two strains of *Agrobacterium rhizogenes* (15834 and LBA 9402) to compare their ability to induce hairy roots on wounded hypocotyls of *Papavar somniferum* L. Five weeks after infection LBA 9402 strain showed transformation efficiency of 80% compared to 64% transformation efficiency of strain 15834 in hormone free MS liquid medium. The total alkaloid content was also found higher in transformed roots compared to untransformed roots.

Giri *et al*., (2005) reported influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artesmisnin production in *Artemesia annua*. A4, 15834, 9365, 9340 were evaluated for hairy root production in shoot tips of the plant. Hairy root line induced by strain 9365 was found to contain the highest amount of artesmisnin (0.23%).

Chang *et al*., in 2005 reported *Agrobacterium* mediated genetic transformation in *Gynostemma pentaphyllum* (Thunb). using ATCC 15834 strain. Leaf discs were used as explants. Bacterial strain was cultured and

maintained in YEB medium. The bacterial pellet after centrifugation was suspended in 40 ml hormone free MS liquid medium, supplemented with 20µM acetosyringone. The inoculated leaves were transferred to MS solid medium and kept at 25˚C in dark. It was found that hairy root initiation begun 2 weeks post infection.

Izabela *et al*., (2006) reported *Agrobacterium rhizogenes* mediated transformation in *Salvia offcinalis* L. Shoots of *Salvia officinalis,* a medicinally important plant, were infected with *Agrobacterium rhizogenes* strains ATCC 15834 and A4 which led to the induction of hairy roots in 57% and 37% of the explants, respectively. Seven lines of hairy roots were established in WP liquid medium under light and dark conditions. The transformed nature of the root lines was confirmed by polymerase chain reaction using *rol B* and *rol*C specific primers. Transformed root cultures of *Salvia officinalis* showed variations in biomass and rosmarinic acid production depending on the bacterial strain used for transformation and the root line analyzed. Both parameters (growth and rosmarinic acid content) of ATCC 15834 induced lines were significantly higher than the A4-induced lines. The maximum accumulation of rosmarinic acid (about 45 mg of dry weight) was achieved by hairy root line 1 (HR-1) at the end of the culture period (50 days). The level was significantly higher than that found in untransformed root culture (19 mg of dry weight).

Karthikeyan *et al.,* (2006) reported hairy root induction from hypocotyls explants excised from seven day grown seedlings of groundnut using *Agrobacterium rhizogenes* 15834.The bacterial culture was established and maintained in YEB medium at 27± 2 ˚C. The hairy roots were thin, slender and highly branched. The cefotaxime concentration of 250 mg/l was found to be most suitable for removal of excess of bacteria from culture and hairy root production in groundnut.

Satdive *et al.,* in 2006 had reported hairy root induction in *Azadirachta indica* (Juss rapid *Agrobacterium rhizogenes* strain LBA 9402 was grown on YMB (Yeast Mannitol Broth) medium with 50 mg/l rifampicin at 28˚C for 48 hours. Three week old germinated seedlings were infected by puncturing with a sterile needle and smearing the bacteria on the wounded surface. Co- cultivation was carried out for 48 hours followed by inoculating the explants on MS hormone free medium supplemented with 3% sucrose, cefotaxime 500mg/l and solidified with agar 0.8% agar (w/v). After 3 weeks, 55-60% of seedlings showed initiation hairy roots from the wounded surfaces.

Mehrotra *et al*., in 2008 used *Agrobacterium rhizogenes* strain K599 to infect leaf explants of *G.glabra.* The strain was cultured and maintained on semi solid YMB medium. All the infected as well as controlled explants were cultured on semi solid MS basal media. To eliminate excess of bacterial growth, explants were transferred to hormone free fresh medium supplemented with 1mg/ml concentration of antibiotics, sporidex and ampicillin respectively. Maximum transformation frequency of 47% was obtained in 3 weeks old explants after 25 days of incubation on MS basal solid medium. NB and B5 media composition showed 20% transformation frequency after 28 and 38 days respectively. It was also found that leaf explants of 2 and 5 weeks old culture were not responsive to bacterial infection in context of hairy root induction.

Lonkova *et al*; in 2009 induced hairy root cultures from leaf explants of *Lignum tauricum* by infection with *Agrobacterium rhizogenes* strains ATCC 15834 and TR 105. Acetosyringone in cultivation medium was used to increase the frequency of hairy root

induction. Transformed nature of tissue was confirmed by the production of opines. The highest amount of lignin was found in transformed line induced by strain ATCC 15834.

Karwasara and Dixit in 2009 reported transformation of *Abrusprecatorius* L. using 3 different strains of *Agrobacterium rhizogenes*, MTCC 532, MTCC 2364 and NCIM 5140. Co-cultivation with strain MTCC 532 for 2 days with 100µM/l acetosyringone at pH 6.5 provided the optimal conditions under which transformation frequency approached 84%.

Gangopadhyay *et al*., in 2010 reported hairy root induction in *Plumbago indica* by infecting leaves and stems of in vitro grown *Plumbago indica*, using three different strains of *Agrobacterium rhizogenes*, (ATCC 15834, A4 and LBA 9402). Bacterial strains were cultured and maintained on YEB medium at 27 ± 1°C for 48 hours. MS liquid medium was chosen as co cultivation medium. Explants were dipped in co-cultivation medium for 24 hours followed by 3 times washing with MS liquid supplemented with 250mg/l cefotaxime and distilled water. The explants were then inoculated in MS basal hormone free medium. The first appearance of hairy roots, transformation frequency and plumbagin accumulation was found to be maximum in hairy roots induced in leaf explants infected with *Agrobacterium rhizogenes* ATCC 15834 as compared with other two strains. Genetic transformation was confirmed by PCR analysis of *rol B* gene.

Jeong and Sivaensen in 2010 developed an efficient protocol for adventitious and hairy root cultures of *Plumbago zeylanica.* Adventitious roots were initiated from leaf and stem explants cultured on MS medium with different concentrations and combinations of auxins. The highest number of roots was obtained when the explants were cultured on MS medium with 1.0 mg/l IBA and 0.5 mg/l NAA. Hairy root culture of *Plumbago zeylanica* was obtained by infecting leaf explants cultured *in vitro* with *Agrobacterium rhizogenes* MTCC 532. The highest frequency of explants transformation was about 93% when explants were co-cultivated with the bacterium for 3 days. The developed culture exhibited fast growth and high lateral branching on growth regulator free MS medium containing 250 mg/l carbenicilin. The root cultures obtained were inoculated into B5, MS and SH media supplemented with different carbon sources with or without auxins and were placed on a rotary shaker 80 rpm for 35 days under dark or light conditions. Of the three media tested, MS medium sustained better root growth than others and sucrose proved to be the best carbon source. The biomass in hairy root culture was higher than in non transformed root culture.

Lee *et al*., in 2011 tested five different *Agrobacterium rhizogenes* strains to investigate the ability for transformation and production of secondary metabolites in *Rubia akane Nakai*. The culture of five *Agrobacterium rhizogenes* strains (13333, 15834, R1000, R1200 and R1601) was initiated from glycerol stock and grown overnight at 28˚C with shaking (180 rpm) in liquid Luria-Bertani medium, to mid-log phase (OD 600 = 0.5). Leaves of *Rubia akane* were taken from plants grown *in vitro* and were cut at the ends into sections 7 X 7 mm. Excised leaves were dipped into five *Agrobacterium rhizogenes* strains (13333, 15834, R1000, R1200 and R1601), cultured in liquid inoculation medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25˚C on agar-solidified MS medium. After 2 days of co-cultivation, the explant tissues were transferred to a hormone-free medium

containing MS salts and vitamins, 30 g/l sucrose, 500 mg/l cefotaxime, and 8 g/l agar. Numerous hairy roots were observed emerging from the wound sites of explants within 2 weeks. The hairy roots were separated from the explant tissue and subcultured in the dark at 25˚C on agar-solidified MS medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (0.5 g D.W./l) were transferred to 30 ml of MS liquid medium, containing 30 g/l sucrose, in 100 ml flasks. Root cultures were maintained at 25˚C on a gyratory shaker (100 rev/min). All the strains of *A. rhizogenes* (13333, 15834, R1000, R1200 and R1601) used in this study were able to produce hairy roots. The strain R1601 performed the highest infection frequency (85.6 %) along with the highest number of hairy roots (5.3) per explant and root length (1.6 cm). The growth and anthraquinone (alizarin and purpurin) production in each hairy root of R. akane from infection by five different *A. rhizogenes* was investigated. Hairy roots were dried at -80˚C for at least 48 hr using freeze dryer. Dried samples were ground into a fine powder using a mortar and pestle. Samples (0.5 g) were extracted twice with 10 ml methanol at 50˚C in a water bath for 1 hour. After centrifugation, the supernatant was concentrated under reduced pressure and the residue dissolved in 1 ml methanol. The quantitative analysis of alizarin and purpurin produced in the hairy root cultures was done by Beckman Coulter DU 700 series UV/Vis spectrophotometers. The absorbance of alizarin and purpurin were detected at 572 and 516 nm, respectively. The highest growth (11.1g dry wt/l) and production of alizarin (4.3 mg/g D.W.) and purpurin (4.9 mg/g D.W.) was found in R1601.

Zhang *et al*., in 2010 reported hairy root induction in leaves of *Gentian amacrophylla* using *Agrobacterium rhizogenes* strain R1000. Explants were incubated in MS liquid medium containing *Agrobacterium rhizogenes* R1000 for 2 hours with consistent shaking between 100- 110 rpm. After the co culture period, explants were washed 2-3 times with autoclaved distilled water and inoculated on hormone free MS agar medium supplemented with 500mg/l cefotaxime. After 10 days of culture explants were transferred to MS basal hormone free medium (0.8% w/v agar) and kept at 24 ˚C with 16 hour photoperiod. Transformation frequency of 18.3% was achieved.

Mahesh and Jayachandran in 2011 reported *Agrobacterium* mediated transformation in *Taraxacum officinale* using leaves and petioles as explants. *Agrobacterium rhizogenes* strains A4 and ATCC 15834 were used for infection. The highest frequency of hairy root induction was observed in leaf explants after infection with A4 strain. It was also found that transformed root cultures accumulated higher levels terpenoids than non transformed and wild plant roots.

Rizkita and Kusuma in 2011 reported that *in vitro* production of gossypol was enhanced by hairy root culture. Experiment was conducted to evaluate the effect of temperature and humidity on growth and gossypol production of *Gossypiumhirsutum* hairy root cultures. Hairy root cultures were established by infecting cotyledons with *Agrobacterium rhizogenes*, strain ATCC 15834. The sterile explants (4 days old cotyledons) were inoculated with suspension of *Agrobacterium rhizogenes*, strain ATCC 15834 (OD 600 = 0.56), and grown on the solid LS medium for 3-4 days at different incubation condition, i.e. the conditioned box (stable 28-29°C, Relative humidity 54-61%), the room condition (2730°C, Relative humidity 37-60%, highly fluctuated), and the incubator (24°C,Relative humidity 48-78%). Explants were cultured at different

temperature and humidity. Hairy roots emerged at day 7. The root size was small with few lateral branching, slow growth rate. Gossypol production pattern from both cultures was similar, and the gossypol was released into the medium. In the medium, the highest gossypol content was achieved at day 16 for both cultures. Gossypol samples for analysis were obtained by extracting 1 g dry weight of root tissue and 10 ml of culture medium. Quantitative and qualitative analysis was conducted with HPLC. The result showed that environmental condition affected both, growth and gossypol production in hairy root culture of *Gossypium hirsutum.*

Bhansali *et al.*, in 2013 reported genetic transformation in *Ecliptaalba (L.) Hassk* by *Agrobacterium rhizogenes* MTCC 532 strain. Explants from healthy growing roots were inoculated with *Agrobacterium rhizogenes* strain MTCC 532. Inoculation of the bacteria in yeast extract broth (YEB) culture medium was used to obtain suspension and this suspension was left standing at 250 rpm for 16 hr at 25˚C in an orbital shaker under constant stirring.. The explants were cocultivated in 25 ml liquid MS media containing bacterial at 200 rpm in a shaker under dark. The root explants were then blotted on sterile tissue paper after incubation period of 3 hr and were placed on the growing medium containing cefotaxime (250 mg/l).Half of inoculated explants were incubated in light (16 hr light/8 hr dark) and the other half in dark at 24 ± 2°C. *Invitro* grown roots without *A.rhizogenes* treatment were also planted under each condition. The hairy roots of *E. alba* obtained were white, slender, highly branched with several lateral branches. Hairy root cultures were identified both by morphological as well as genetic markers. The important morphological markers included rapid growth, lateral branching and plagiotropism (negatively geotropic). The genetic marker included *rol* B. Bacterial gene transfer was confirmed using this marker. The *rol B* gene was detected from the DNA of hairy roots using PCR analysis. A minimum of 780 base pair fragment could be detected in PCR amplification.

Pandey and Krishnaswamy in 2014 reported establishment of hairy root culture and production of secondary metabolites in Coleus (*Coleus forskohlii*). Production of hairy roots through transformation and in vitro culture was attempted in Coleus using MTCC 2364 strain of *Agrobacterium rhizogenes*. Nodal stem part and mature leaves were used as explants. The nodal stem part responded with huge amount of root production. The transformation frequency was up to 30% for nodes, the best co-cultivation time was of 48 hours. After four weeks of culture, tips of hairy roots were cut down and inoculated on to the MS and B5 liquid and semi solid medium without any growth regulators and with cefotaxime 400 mg/L. The cultures were maintained at 25°C in dark and the roots in liquid medium were maintained on rotary shaker at 100 rpm. The cultured tips showed axillary growth within three weeks of culture and these actively grown root tips were again sub cultured at regular interval of three weeks. The concentration of cefotaxime was reduced in every subculture to 200 mg/L. The induced hairy roots were sub cultured on MS and B5 liquid medium for further proliferation. The proliferation of hairy roots was seen on B5 liquid medium. The confirmation of transformation of plant was done by polymerase chain reaction (PCR) with *rol* A gene primer. It showed 400 bp band. High performance liquid chromatography (HPLC) analysis revealed good amount of forskolin accumulation in hairy roots.

Vyas and Mukhopadhyay in 2014 reported that rapid and efficient *Agrobacterium*

rhizogenes mediated transformation system for *Ocimum tenuiflorum* L., a traditional Indian medicinal plant that occurs in red and green forma, was developed. The plant is a repertoire of several pharmaceutically and nutraceutically important metabolites. Three different types of explants i.e. leaves, hypocotyls and excised shoots, obtained from shoot cultures of *in vitro* germinated red and green forma plants were transformed using *Agrobacterium rhizogenes* strain ATCC 15834. The transformation efficiency was equal between similar explants of both forma. Transformation efficiency was best in leaves of 4 days while excised shoots and hypocotyls had 6 and 8 days respectively. Transformation frequency of green forma leaves was the highest (70.6%) among all explants. Excised shoots of green forma plants exhibited better transformation (58.3%) than the red forma excised shoots (42.59%). Red forma hypocotyl explants displayed marginally better (26.27%) transformation frequency than green hypocotyl explants (21.14%). Transformation with hairy root was confirmed by the presence of *rol*C gene through PCR amplification and Southern hybridization.

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