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Research Article

INDUCTION OF HAIRY ROOTS IN *ATROPA KOMAROVII* USING *AGROBACTERIUM RHIZOGENES*

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ABSTRACT
The species *Atropa komarovii* was infected by the soil gram-negative bacterium Agrobacterium rhizo genes that were resulted in the neoplastic disease with the formation of hairy roots at the site of the infection. The utility of hairy root culture producing valuable phytochemical could be improved by repartitioning more of the desired phytochemicals into the spent culture. In the present study, hairy roots were induced directly from the wounds of leaves excised from 15 day old aseptic all grown seedlings of *Atropa komarovii* in MS solid medium using *Agrobacterium rhgizogenes* strain ATCC15834. Approximately 70% of leaves demonstrated positive reaction to the bacterium. Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium. Amplification of the rolB gene region, were accomplished using Polymerase Chain Reaction (PCR). The results confirmed the integration of T-DNA fragment of Ri plasmid from Agrobacterium rhizo genes to plant genome. The hairy roots were observed within three weeks and subculture on MS medium without phytohormones with 500mgl^{-1} cefotaxime in dark. Ri plasmid present in the bacterium capable for inducing hairy roots which can grow as root organs in phytohormone-free medium. Autonomous proliferation of induced roots is the result of genes expression in the T-DNA of the Ri plasmid integrated into the host plants.

Keywords: *Agrobacterium rhizogenes, Hairy Root, Atropa komarovii, rolB*

INTRODUCTION
The genus *Atropa* consists of four species distributed in the Mediterranean region, South Euorope and Asia. Atropa belladonna L. has long been used as a reputed drug in Europe and is still regarded as one of the few indispensable drugs of the plant origin (Parvaz *et al.,* 2006). It is a perennial herbaceous plant and is the most important commercial source of the pharmaceutical tropane alkaloids in the family of Solanaceae which is endemic of Turkmenistan. However, the species belonging to the genus are found in central Asia, *Atropa komarovii,* (Kopet-daghmountains) is regarded to be more or less equivalent to Atropa belladonna in terms of chemical constituents. The drugs scopolamine and hyoscyamine extracted from the plant act as stimulant to the sympathetic nervous system and are employed as antidote to opium. The plant is of interest due to its production of bioactive tropane alkaloids like *Atropa belladonna* including scopolamine and hyoscamine, which are wildly used as antagonists of acetylcholine in both the autonomic and central nervous system (Liu *et al.,* 2009). Because the regeneration of fertile plants from tissue culture is much easier for Atropa than that of other tropane alkaloid-producing plants (e.g. *Hyoscyamus niger*), the species of Atropa have been used for the production of somatic hybrids and transgenic plants with herbicide resistance or with improved alkaloid composition (Suzuki *et al.,* 1999). Gram - negative soil bacteria *Agrobacterium tumefaciens* and Agrobacterium rhizo genes are two species wildly used to transform higher plants. Agrobacterium rhizo genes causes hairy root disease in plant via genetic transformation in a manner similar to that of crown gall disease caused by *A. tumefaciens*, both systems rely on a similar transformation mechanism. Hairy root cultures are also attractive experiment system, as they are long – term aseptic root clones genetically stable with growth rate comparable to those of the fastest-growing cell suspension cultures (Sudha *et al.,* 2013). Many dicotyledonous plants are susceptible to A.rhizogenes and plants have been regenerated from hairy root cultures in a wide range of

specie (Christey, 2001). However Agrobacterium plant transformation is not completely understood and highly efficient transformation event remain elusive. Bacterial cell walls or cellulose fibril generation may be important in initial bacterial binding to plant cell before T-DNA transfer. The bacteria recognize signal molecules exuded by susceptible wounded plant cells and become attached to it (Wu *et al.,* 2012). Hairy roots are developed by infecting plant leaf or stem tissue with *Agrobacterium rhizogenes* transfer genes that encode hormone biosynthesis in plants. Among them Agrobacterium rhizo genes is one of the most widely studied (Park *et al.,* 2009). The rhizogenic strains contain a single copy of large Ri plasmid its induces the development of hairy roots when RiT-DNA is integrated in to a nuclear genome of the plant cell and encode genes that direct the synthesis of auxin (Indole -3 acetic acid) or increase sensitivity of the transformed cell to auxin. The endogenous production of auxin and or an increase in auxin sensitivity can lead to the formation of hairy roots at the site of infection (Li *et al.,* 2003). *Agrobacterium rhizogenes* carries on its T-DNA four rol- (root – loci) genes, which are main determinants for the development of hairy roots. The four rol – genes are termed rolA, rolB, rolC and rolD, respectively (Christensen and Muller, 2009). Following agrobacterial infection these genes are transferred into plant genome. The product of rolB is a protein that possesses tyrosine phosphatase activity so rolB act as a strong inhibitor of cell growth and rolC encode a glucosidase that hydrolyses the plant cell wall and releases the oligosaccharides involved in plant development in the plant cell cultures. Maintenance of hairy root growth and abundant flowering of transformed plants contribute to rolD. All of the rol genes induce secondary metabolite production and rolB is apparently the most powerful inducer of secondary metabolism (Nikravesh *et al.,* 2012). The combination of rolA, rolB, rolC loci is sufficient for producing the hairy root phenotype and the capacity of rolA, rolB and rolC genes to induce roots with faster growth rates than normal roots is equivalent to that of the whole T_L-DNA (Christencen and Muller R, 2009). Hairy roots are highly branching on hormone-free culture medium, show rapid growth, plagiotropic root growth and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Zhao *et al.,* 2012). They abilities are investigation of gene function and the study of root biology and culture systems revealed the pharmacological activities in addition to the enhanced production of bioactive molecules and the possibilities for utilization of artificial polyploidization for improving germplasm and breeding (He *et al.,* 2011). In the present study, *Atropa komarovii* leaf disks were infected with an *A. rhizogenes* ATCC15834 strain and hairy roots obtained from the leave and some leave were wounded without infected for controls then the growth rate of these hairy roots were compared with that of the normal roots used as control.

MATERIALS AND METHODS

Plant Material

The mature seeds of the species *Atropa komarovii* were collected from the Gorgan Province in Ali-abad road, Guzlugurben in 2500 m height. The fruits were matured and dispersal seeds had not occurred and dormancy of seeds was broken by scratched.

Seed Sterilization and Germination

The mature seeds were washed with running tap water and excess moisture was removed with filter paper. Surfaces of *Atropa komarovii* seeds were sterilized with 70%(v/v) ethanol for two min, rinsed briefly in sterile water, then soaked in 20% (v/v) sodium hypochlorite solution (NaOCl) for 15 min, rinsed with sterile distilled water four times, and were blot-dried inside a laminar hood. The sterilized seeds were inoculated on MS media (Murashige and Skoog, 1962) for one week in the darkness and then they were transferred into controlled condition in a growth chamber set at $25+1^0$C to the 16 h light/ 8 h dark photoperiod. After two weeks, seeds were germinated and grew.

Growth of Agrobacterium Rhizogenes

We used Agrobacterium rhizo genes ATCC15834 for the induction of hairy root in *Atropa komarovii*. The bacterium was maintained on LB (Luria-Bertani) agar medium containing (Tryptone 1.25gl^{-1}, 15% Yeast extract ,1% Nacl, pH 7), prior to inoculation, a loopful of bacterial cultures were inoculated on LB liquid medium contain 50mgl^{-1}rifampicine and cultured for 24 h at 28 ^{o}C in the darkness on a rotary

shaker at 100 rpm and 1ml from suspension of bacteria were transferred to the 30 ml LB liquid medium and maintained on a rotary shaker for 24 h, after which the culture broths were collected and optical density was measured at 600 nm (OD= 0.7) using a spectrophotometer.

Induction of Hairy Roots

For transformation four week - old micropropagated shoots, leaves were cut about 3cm ^{2} length pieces and small cut was made perpendicular to the mid rib into the center of the squared leaf. Each explant was immersed in bacterial suspension for 15 min. Then, they were placed in sterilized filter paper in petridish to remove excess of moisture in surface of explants and wounds were made gently on the leaves without bacterial treatment served as controls.

Co – Cultivation and Hairy Root Induction

After infection the leaves were transferred into MS basal medium for 24 h in the darkness for co-cultivation. When co- cultivation period was over, the leaves (segments) were washed with MS basal medium with cefotaxime (500 mg l^{-1}) to remove over the growth of A.rhizogenes on the surface of the explant. After washing, the explants were transferred into hormone – free medium containing MS salt and vitamins, 30gl^{-1}sucrose, 500 mg l^{-1}cefotaxim and medium solidified with 8gl^{-1}phytagar for hairy root induction.

Hairy Root Culturing

After 20 days of inoculation hairy roots, which arose mainly from the cut surfaces and leaf midribs explants and controls. The roots were separated when they attained a length of four to five cm. They were transferred into hormone – free MS basal medium containing 30 gl^{-1} sucrose, and supplemented with cefotaxime 500 mg l^{-1}. Cefotaxime concentration was then halved each three weeks form 500 mg l^{-1} to 250 mg l^{-1}.

Isolation of Genomic Dna

Total DNA was isolated from hairy roots and normal roots (control) of Genomic DNA was extracted from hairy roots and normal roots (control) of Atropakomarovii using the cetyl –trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1980). Fresh hairy root tissues 0.4g were harvested, frozen in liquid nitrogen and ground into fine powder. The frozen powder was transferred into two mlmicrocentrifuge tube and homogenized in 0.8 ml of CTAB extraction Buffer (2% CTAB,100mMTris-HCl (p^{H} 8), 20 Mm EDTA,1.4 NaCl, 0.1ml protein as K) and incubated at 60°C for 1 hour and add 0.8 ml chloroform/isoamylalcohol (24:1) solution. The supernatant was transferred into a new tube and 0.06 ml isopropanol was added and centrifuged for 15 min at 1400 g. The plate was dried by leaving tube open for 25min and then responded in 50 µl TE (10mMTris –HCl, P^{H} 7 and 1mM EDTA, P^{H} 8).

PCR Analysis of Hairy Roots

Integration of the rolB gene into the plant genome was confirmed by PCR analysis. PCR amplification of DNA from untransformed root and hairy root with specific primers for the rolB gene were forward 5' ATGGATCCCAAATTGCTATTCCCCACGA 3' and reverse – 5' TTAGGCTTCTTTCATTCGGTTTACTGCAGC- 3' respectively according to (Martins *et al.*, 2003). The PCR assay was carried out in 20 µl reaction mixture containing H_2O 14.7 ml, PCR buffer 20µl, dNTP 0.5µl, 50 ng of genomic DNA, 1u of Taq DNA polymerase, 10pmoles primers, $Mgcl_2$2mM. The PCR was carried out by amplifying an initial denaturation 94 ^{0}C for 1.0 min, 1.0 min annealing at 57^{0}C and 1.0 min extraction at 72^{o}C with a final extension of 72^{0}C for 10 min using a Thermocycler. The amplicons were analyzed by electrophorizing on 1.4 % (W/V) agarose gel along with 1Kbp DNA marker follow by Ethidium bromide staining DNA from untransformed root cultures that used as negative controls.

RESULTS AND DISCUSSION

Results

Several parameters of the agro-infection method were adjusted. The leaves were harvested from five week-old plantlets and the incubation time was in 15-20 minutes. The co-culture was accomplished in two days and at this situation the best result was obtained. The transformation experiments with other species of Atropa carried out several times but there is not yet any report about transformation in *Atropa*

komarovii. The five to six young leaves of *Atropa komarovii* plantlet were infected by *A. rhizogenes* strain ATCC15834. The results demonstrated high sensitivity to this strain for the sake of transformed roots that could emerge from 70 percent of leaves. After co-cultivation, explant tissues were transferred into agar-solidified MS medium containing 500 mgl^{-1} cefotaxime, to eliminate *A. rhizogenes*. The visible roots were formed 10-12 days after infection at the site of bacterial inoculation of leaf disc. The hairy roots were formed on the midrib of the infected leaves and roots formation was observed in control explants. The transformed roots, which had lots of lateral branching, grew so rapidly on hormone – free MS basal medium and negative geotropic. After three weeks hairy roots and control were excised from the necrotic explant tissues and subculture on fresh agar – solidified medium containing 500 mgl^{-1} cefotaxime in darkness these data are shown in Figure 1.

PCR Analysis

The presence of rol B gene in genomic DNA of putative transgenic and non- transgenic (control) regenerated hairy roots was confirmed by PCR using specific primers yielded fragments of 500bp.The transgenic DNA of hairy roots was used as template. No amplification was observed in control hairy roots (negative control) with the primers (Figure 2). All transformants hairy roots showed the presence of diagnostic 500bp rolB product amplification.

Discussion

Various bacterial species have the ability to transfer genes to the higher plants (Kim *et al.,* 2010). Agrobacterium rhizo genes, a gram-negative soil bacterium, is one of the most widely studied bacteria that infects the plant cell and leads to the formation of hairy roots (Guillon *et al.,* 2006). The results presented in this paper indicated that *A.rhizogenes* considers as the one that promotes rooting on leaves, although different responses to infection might be occurred. The first step in the infection process is the host/pathogen interaction. Several factors contribute to establish this relationship. Sugar and phenolic compounds released from the plants and membrane – binding proteins could be different according to plant genotypes, therefore they produce different responses (Winans, 1999). There are some reports that suggest the successful use of *A.rhizogenes* harboring binary vectors with desired gene constructs for plant genetic transformation and different strains of *A. rhizogenes* have differentially influenced hairy root induction (Christey *et al.,* 2001). Agrobacterium rhizogenes strain ATCC15834 is one of the most common strains used for the induction of hairy roots and shows a great ability in inducing hairy roots in another Atropa species. In the recent years, there are many researches on other species of Atropa in the world because they were the source of many pharmaceutical tropane alkaloids in the family Solanaceae. However nowadays, development in plant cell cultures, tissue cultures and genetic engineering, had made the manipulation of metabolic pathways so feasible to succeed in establishing a hairy root culture system for a certain plant species. Several essential conditions should be taken into consideration. These conditions include the bacterial strain of *A. rhizogenes*, an appropriate explant, proper antibiotic to eliminate redundant bacteria after co-cultivation, and a suitable culture medium (Hu and Du, 2006). Especially the type of explant is very important to induction hairy roots (Lin *et al.,* 2003). Approximately 70% of leaves of *Atropa komarovii* responded to hairy root induction. The response of hairy root proliferation on hormone –free MS solid media was similar to that reported by Liu et al 2010 for Atropa belladonna and Hirano *et al.,* (2003) was reported the grow of transformation roots in Atropa belladonna. The proliferating roots were subcultured every three weeks. During the subculture some of the hairy root growth was arrested and they turned into brown and some of them died. Most of the roots grew rapidly with multiple branching and negative geotropism in the MS solid medium hormone-free. The roots were subcultured every three weeks. Zhao and Foster (2012) induced hairy roots of *Nicotiana tabacum* in the recent years. There are several studies on the induction of hairy roots in Solanaceae genera, for the presence of secondary metabolites in this family. There are at least three possible means of taking advantage of A.rhizogenes for transformation plants. One of which, is to place the rol genes under an inducible gene expression system and have them expressed only during the transformation process. Another option is to use co-transformation, where two separate T-DNA contained within a single bacterium is transferred into plant genomes. By now the hypothesis of a direct auxin release from auxin-

glucoside esters by the action of rolB products has been abandoned (Maurel *et al.*, 1994). Changes in the auxin sensitivity of the rolB in transformed tobacco cells seem to be a consequence of an altered permeability of the membrane auxin binding protein (Filippini *et al.*, 1994).

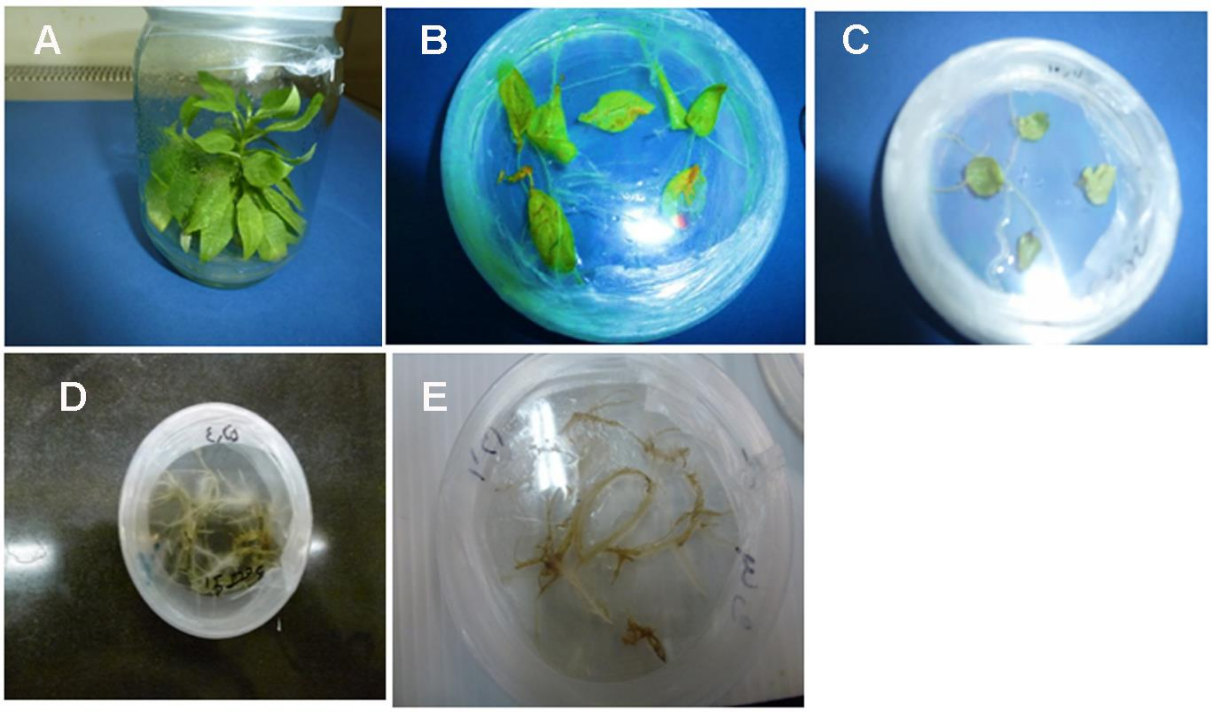

Figure 1: Establishment of hairy root cultures of *Atropa komarovii*. A: plantlet of *Atropa komarovii* in MS medium; B: transformed roots emerged from wounded sites with Agrobacterium rhizogenes, 14 days after infection; C: roots emerged from leave segments free of bacteria; D: hairy roots culture in MS medium without plant growth regulator; E: non transformed roots in MS medium without plant regulator

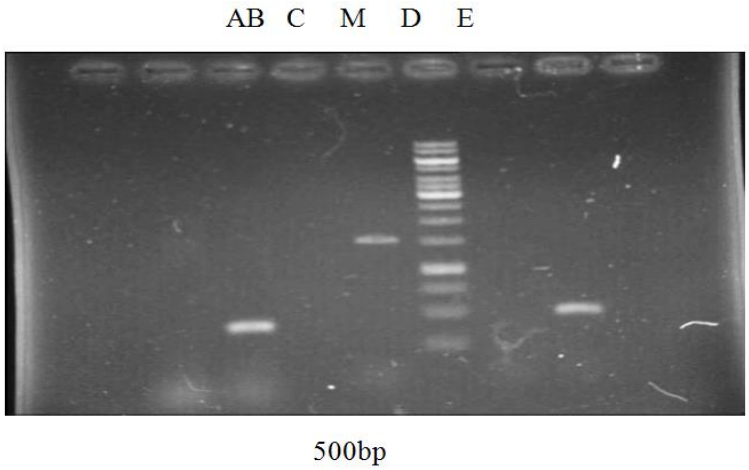

Figure 2: PCR analysis of hairy root culture of *Atropa komarovii* transformed *Agrobacterium rhizogenes* ATCC 15834. lane M –Marker (1kbp): lane A - genomic DNA of hairy root culture showing amplified fragment of rolB (500bp): lane B- genomic DNA from normal root culture (negative control): lane C- PCR(positive control): lane D - PCR (negative control): lane E- genomic DNA of hairy root culture showing amplified fragment of rolB (500bp)

Capone and co-workers 1994 identified some regulatory domains of the rolB promoter region, responding to endogenous plant regulatory proteins whose presence and /or concentration can differ in different cell types, and might be in different genetic backgrounds.
The presence and expression of rolB is necessary but not sufficient to induce rhizogenesis in transformed cells, because a high rooting potential should be associated with the presence of pre-committed cells, which potentiality could be amplified by rolB. In *Gmelina arborea* used cotyledons for induction of hairy roots with *Agrobacterium rhizogenes* ATCC15834 and transgenic nature of the hairy roots was confirmed by PCR using rolB specific primers and mostly resemble in our study (Dhakulkar and Bhargava, 2005). Hairy roots were induced in *Atropa belladonna* leaf disk with *Agrobacterium rhizogenes* 15834 and PCR analysis showed that rolB and rolC are transferred in infected roots and it's partly resembled in our study. In many transgenic hairy roots in medicinal plants not only rolB but also rolC was detected because many transformation process with different types of strains from *Agrobaccterium rhizogenes* for evaluation of the secondary metabolites.

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