Pharmacognosy Magazine [Phcog Mag.]
Vol 5, Issue 20, Oct-Dec, 2009 Page 336-342
(An Official Publication of Pharmacognosy Network Worldwide)
Received: Jun 24, 2009
Modified: Jul 8, 2009
Accepted: Jul 8, 2009

PHCOG MAG.: Research Article

Agrobacterium rhizogenes Mediated Genetic transformation of *Abrus precatorius* L.

Karwasara Vijai Singh and Dixit VK*

Biotechnology Research Laboratory, Department of Pharmaceutical Sciences, Dr. H.S. Gour University, Sagar-470003 (M.P.) India Phone: +91-7582264582; Fax: +91-7582264236;

**** Author for Correspondence:** Email: dixitvk2011@rediffmail.com*

ABSTRACT

Abrus precatorius L. known as Indian liquorice is a common deciduous vine containing sweet principle compound known as glycyrrhizin. Hence it can be used as a very good substitute for Liquorice. Genetic transformation has proved to be an effective way to enhance secondary metabolites in plant cell cultures. The transformation of *Abrus precatorius* L. mediated by *Agrobacterium rhizogenes* was studied using three bacterial strains i.e. MTCC 532, MTCCC 2364 and NCIM 5140. Putative hairy roots were obtained after the transformation. The effects of bacterial strains, bacterial concentration, acetosyringone and co-cultivation pH on transformation of Abrus were investigated. Co-cultivation with Strain MTCC 532 for 2 days with 100 μmol L^{-1} acetosyringone at pH 6.5 provided the optimal conditions under which transformation frequency approached 84%.

KEYWORDS: *Abrus precatorius; Agrobacterium rhizogenes; Genetic transformation; Hairy roots.*

INTRODUCTION

Abrus precatorius L. is a slender shrubby woody vine that climbs shrubs and low trees and is ubiquitous in temperate and subtropical areas all over India. It is locally known as "Gumchi" and belongs to the Fabaceae family. Several triterpenoid saponins, flavanol glycosides, isoflavaquinones, abrine, pentosans, anthocyanins, etc have been isolated and studied pharmacologically. Diverse activities have been reported in different parts of *Abrus precatorius* due to presence of a broad range of chemical constituents like abrine, abraline, abrussic-acid, abruquinone, anthocyanins, campesterol, cycloartenol, delphinidin, gallic-acid, glycyrrhizin, hypaphorine, trigonelline etc. (1–7). Preparations of Abrus have been used for centuries as folk remedies for cancer, contraceptive, convulsion, cough, diarrhea, diuretic, gonorrhea, jaundice, rheumatism, trachoma, and as vermifuge. It is also used as sweetener, aphrodisiac, abortifacient etc. (8–11).

One of the most prominent secondary compounds of *Abrus precatorius* is glycyrrhizin which occurs in leaves and roots. Glycyrrhizin is the principal component of liquorice (12, 13) which is used as natural sweetener, antiulcerative and anti-inflammatory agent (14, 15). Furthermore glycyrrhizin exhibit anti-hepatotoxic (16), anti-HIV (17, 18), antitumor (19) and immunomodulatory activity (20). Production of glycyrrhizin in cell culture of liquorice has not been successful and thus its production from alternative sources has assumed significant (21, 22). It is worthwhile to undertake the genetic transformation of such an important and varied activity bearing plant like *Abrus precatorius*. Hairy roots have been induced in many dicotyledonous plants by transformation with *A. rhizogenes* strains (23, 24). Hairy root cultures resulting from *Agrobacterium rhizogenes* transfection of plant material produce the same secondary metabolites as those usually synthesized in intact plant with similar or higher yields (25). However, sometimes hairy root culture have been

shown to accumulate the metabolites which are known to accumulate in the aerial parts of intact plant. e.g. *Lawsonia inermis* (lawsone) and *Artimisia annua* (artimisinin). This feature, together with genetic and biochemical stability and rapid growth with high lateral branching in simple media without phytohormones, makes them especially suitable for biochemical studies which is not achievable with intact plant roots (26). However, the profiles of secondary metabolites depend on the genetic make up of the hairy roots (27). The production of secondary metabolites by hairy root culture has been studied for many plants (28, 29). The transformed roots provide great potential as a production system of plant metabolites (30, 31).

Agrobacterium rhizogenes strains contain a single copy of a large Ri T-DNA which is transferred to wounded plant cells and becomes stably integrated into the host genome (32). Hairy roots are able to regenerate whole viable plants with high genetic stability. Like most members of the Leguminosae (Fabaceae) family, *A. precatorius* is very difficult to transform with *Agrobacterium rhizogenes*. The transformation of Abrus mediated by *A. rhizogenes* has not yet been reported. In this study, we describe the Ri plasmid-mediated transformation of *Abrus* and optimized the effects of several major physical and chemical factors, on *Abrus* transformation viz. effect of *Agrobacterial* strain and concentration, infection time, acetosyringone, co-cultivation days, effect of pH of pre and co-cultivation medium.

MATERIALS AND METHODS

Plant materials

Seeds of *Abrus precatorius* were collected from medicinal plant garden, Department of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar, M.P. The seeds were surface sterilized by 70% ethanol for 1–2 min, then with sodium hypochlorite (4% v/v) for 10–12 min and then rinsed 7–8 times with sterilized double distilled water to remove all traces of chemical sterilant. Seed germination was tested by putting few seeds on absorbent cotton moistened on half-strength Murashige and Skoog medium (1/2 MS) (33) and few seeds were placed on absorbent cotton dampened with sterile distilled water. Cultures were maintained at 25±2 °C under a 12-h photoperiod regime with white fluorescent light. For transformation experiments, petiole, leaves and epicotyl were obtained from aseptically germinated young *Abrus* plantlet.

Agrobacterium

Agrobacterial strains MTCC 532 and MTCC 2364 were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. Bacterial strain NCIM 5140 was obtained from National Collection of Industrial Microorganisms, Pune. *A. rhizogenes* strains MTCC 532 and MTCC 2364 belong to the agropine type and harbor the Ri plasmid with two parts of T-DNA (left T-DNA and right T-DNA). For the transformation experiments, MTCC 532 strain was inoculated into liquid YEB medium (sucrose 5g L^{-1}, beef extract 1 g L^{-1}, yeast extract 1 g L^{-1}, peptone 5 g L^{-1}) and the MTCC 2364 strain was inoculated into liquid xanthomonas medium (galactose 20 g L^{-1}, yeast extract10 g L^{-1}, calcium carbonate 20 g L^{-1}) and shaken for 48 h at 28°C. NCIM 5140 strain was inoculated on nutrient agar plates having following composition: beef extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1} sodium chloride 5 g L^{-1} and shaken for 48 h at 26°C. The cultures were then centrifuged at 1500×g and resuspended in liquid MS medium containing 100 μmol L^{-1} acetosyringone.

Transformation

Abrus explants were pre-cultivated on MS medium solidified with 0.8% (w/v) agar at a varying pH range (4.5, 5.5, 6.5, 7.5) and concentrations of acetosyringone (0, 50, 100, 150, 200 μmol L^{-1}) for 2 days. The explants were then transferred to the Agrobacterium suspensions containing 100 μmol L^{-1} acetosyringone and incubated for definite time and then blotted dry on sterilized filter paper. The explants were returned to the same pre-cultivation medium for co-cultivation. The explants were transferred to media (MS medium containing 500 mg L^{-1} cefotaxime) after every four days until hairy root formation observed. The numbers of explants with roots (a minimum 22 days after infection) were recorded. The standard condition maintained were 45 min infection and 2 days pre- and co-cultivation at pH 6.5 using a bacterial concentration of 1.0 (OD_{600}). To determine the optimum transformation conditions, one factor of the standard conditions was changed each time and the effects on hairy root formation observed. The *Agrobacterium rhizogenes* MTCC532, MTCC2364 and NCIM 5140 strain were streaked and subcultured for 48 hr in the dark at 26±1°C on recommended solidified agar medium.

PCR analysis

Total genomic DNA was isolated using the AuPrep Kit (Life Technologies Pvt. Ld., India). Integration of the TL DNA (*rol* gene) and TR DNA (*ags* gene) regions of the pRi plasmid was confirmed by PCR analysis of the genes located in this region. All primers were obtained from Microsynth (Switzerland). Presence of *ags* gene was confirmed by using 5'-CGGAAATTGTGGCTCGTTGTGGAC-3'

and 5'-AATCGTTCAGAGAGCGTCC GAAGTT-3' as primers. Amplification by PCR was performed under following conditions: initial denaturation at 94°C for 4 min, annealing at 58°C for 1 min and extension at 72°C for 1 min for 35 cycles, with a final extension at 72°C for 5 min. The amplicons were analyzed by electrophoresis on 1% agarose gel (w/v).

Statistical analysis

The data on hairy root formation are the mean of three independent experiments with a minimum of 20 explants per treatment. All roots that survived in the selection medium for 20 days were designated as hairy roots. The data among different treatment were tested by Graph Pad InStat 3 software run on XP professional Intel Pentium IV processor.

RESULTS

Abrus seed germination

Seeds germinated on both 1/2 MS agar medium as well as on damp absorbent cotton with or without fluorescent light after 4 days. The percentage of germinated seeds on the half strength MS medium after 10 days was much higher than that of sterile distilled water damp absorbent cotton (Table I).

Table I: The effect of media composition on the germination frequency of *A. precatorius* seeds (25 seeds per test).

Medium composition	Light/Dark Treatment	% seed germination
Absorbent cotton+ Sterile DW	12/12 light/dark	70±10%
Absorbent cotton+ ½ MS liq. media	12/12 light/dark	95±04%
½ MS media with 1.0% agar	12/12 light/dark	48*±12%

*Seedling germination associated with callus formation
DW (Distilled Water) and MS (Murashige and Skoog)

Agrobacterium and Abrus transformation optimization protocol

Three strains of *A. rhizogenes*, MTCC 532, MTCC 2364 and NCIM 5140 were tested for their transformation efficiency (Fig. 1A). MTCC 532 exhibited maximum transformation efficiency (65%) and was the best amongst the tested agrobacterial strains. Transformation efficiency of 29.5% and 20% was recorded with MTCC 2364 and NCIM5140 respectively suggesting low transformation by these strains. Among the various explants taken from the aseptically germinated Abrus plantlet (Fig 2A), the leaf explant shows maximum transformation efficiency while it was least with petiole (Fig 1B). *A. rhizogenes* MTCC 532 induced hairy root has been shown in Fig 2B. Fig. 3A shows the effect of infection time on the frequency of hairy root formation. The transformation frequency markedly increased with increase in infection time from 15 to 45 min. At 15 min. the transformation frequency was 26.5 % which was increased to nearly three fold i.e. 67.5% at 45 min. The hairy root formation frequency increased two-folds when the bacterial concentration was raised from 0.5 to 1.0 (OD600). It however, slowly decreased with further increase in agrobacterial concentrations (Fig. 3B).The hairy root frequency was increased progressively with increasing co-cultivation time from 2 to 4 days (Fig. 3C). At longer co-cultivation period, problem of increased bacterial cell densities in the selection medium was encountered. Therefore, co-cultivation was restricted to 2 or 3 days and this was found to be sufficient for transformation. The concentration of acetosyringone has profound effect on hairy root frequency, which was most favorable at about 100 μ mol L^{-1} (Fig. 3D). The pH of co-cultivation medium had a strong influence on hairy root induction and the most favorable pH was 6.5, higher pH inhibited growth (Fig. 3E). Effect of mode of explant wounding was also studied which showed a better transformation with manual wounding as compared to the high speed vortexing. Hairy root production under the optimized conditions determined as above was performed: Bacterial strain MTCC 532, co-cultivation for 2 days, 100 $\mu molL^{-1}$ acetosyringone, and a pH 6.5 in the co-cultivation medium yielded the hairy root production with 84.4% frequency.

Confirmation of transformation

Root clones each from MTCC 532 and MTCC 2364 were randomly selected for opine assay. All roots analysed show the presence of opine as a dark spot on paper electrophoretogram, which indicate that the roots were transformed. For PCR amplification of the *ags* gene, roots were excised from rooting explants that had been infected for one month. All roots were found to harbor the *ags* gene which shows their transformed nature (Fig. 4).

DISCUSSION

The germination of *Abrus precatorius* seeds in MS medium was similar to MS medium solidified by agar and damp absorbent cotton, and 12/12 light/dark cycle strongly promoted this process. The maximum seed germination was found on absorbent cotton soaked with half strength MS medium while the seed germination frequency was lower on the distilled water damp absorbed cotton due to the nutrient constraint. Unexpectedly seed germination percentage was lowest on the MS solidified medium due to frequent seed callus development instead of seedling

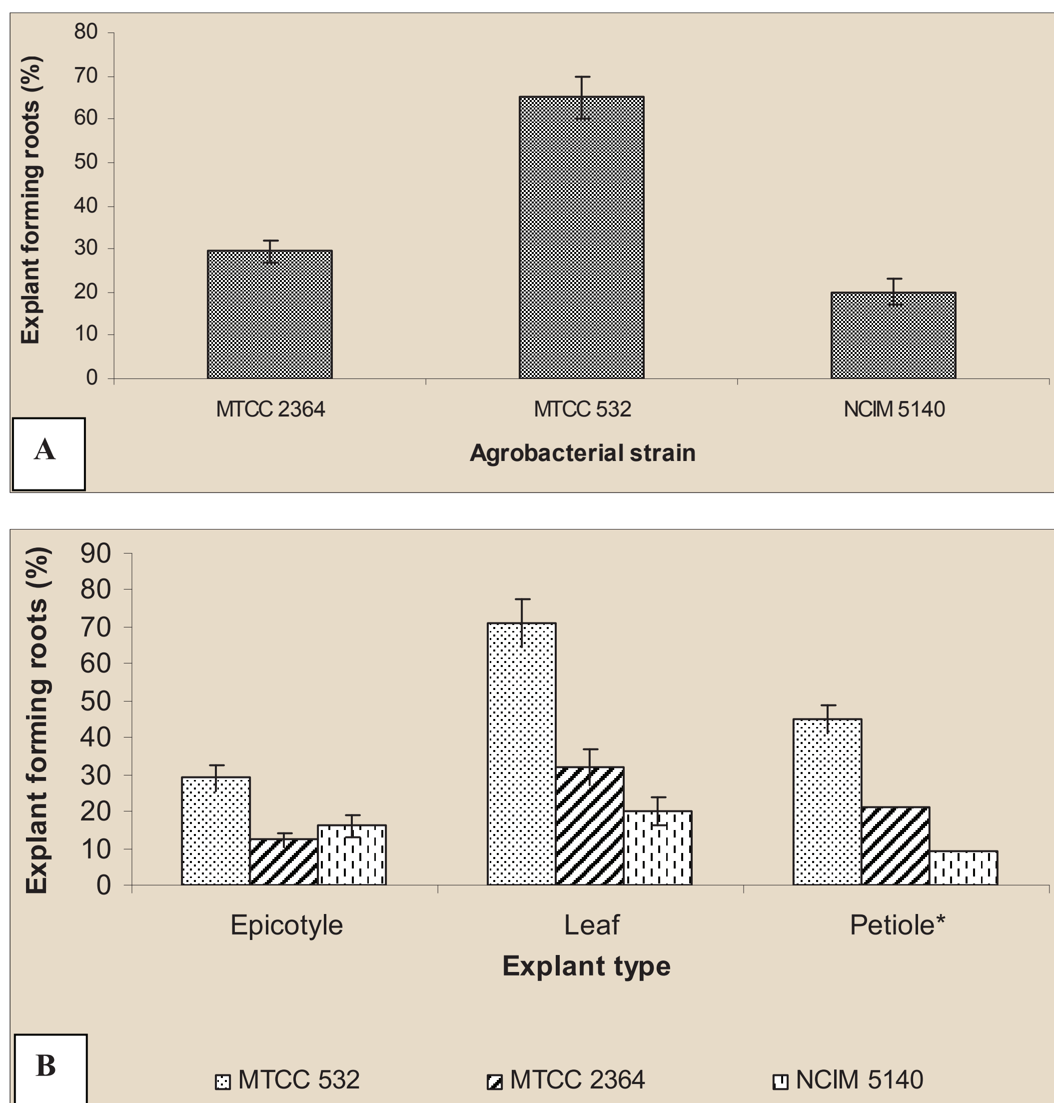

Figure 1: (A) Efficiency of transformation to hairy roots by *A. rhizogenes* strains under standard conditions. (B) Effect of different explants on the transformation efficiency by selected agrobacterial strain. Each value represents the mean of three different experiments.
*No hairy root induction in petiole explant there was only callus induction.

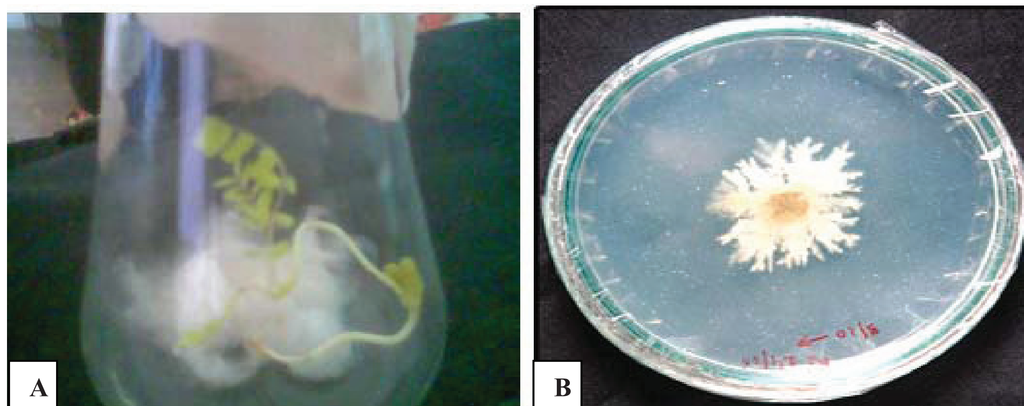

Figure 2: (A) Aseptically germinated *Abrus precatorius* plantlet (B) MTCC 532 induced hairy roots of *Abrus precatorius* (12 days after agrobacterial infection)

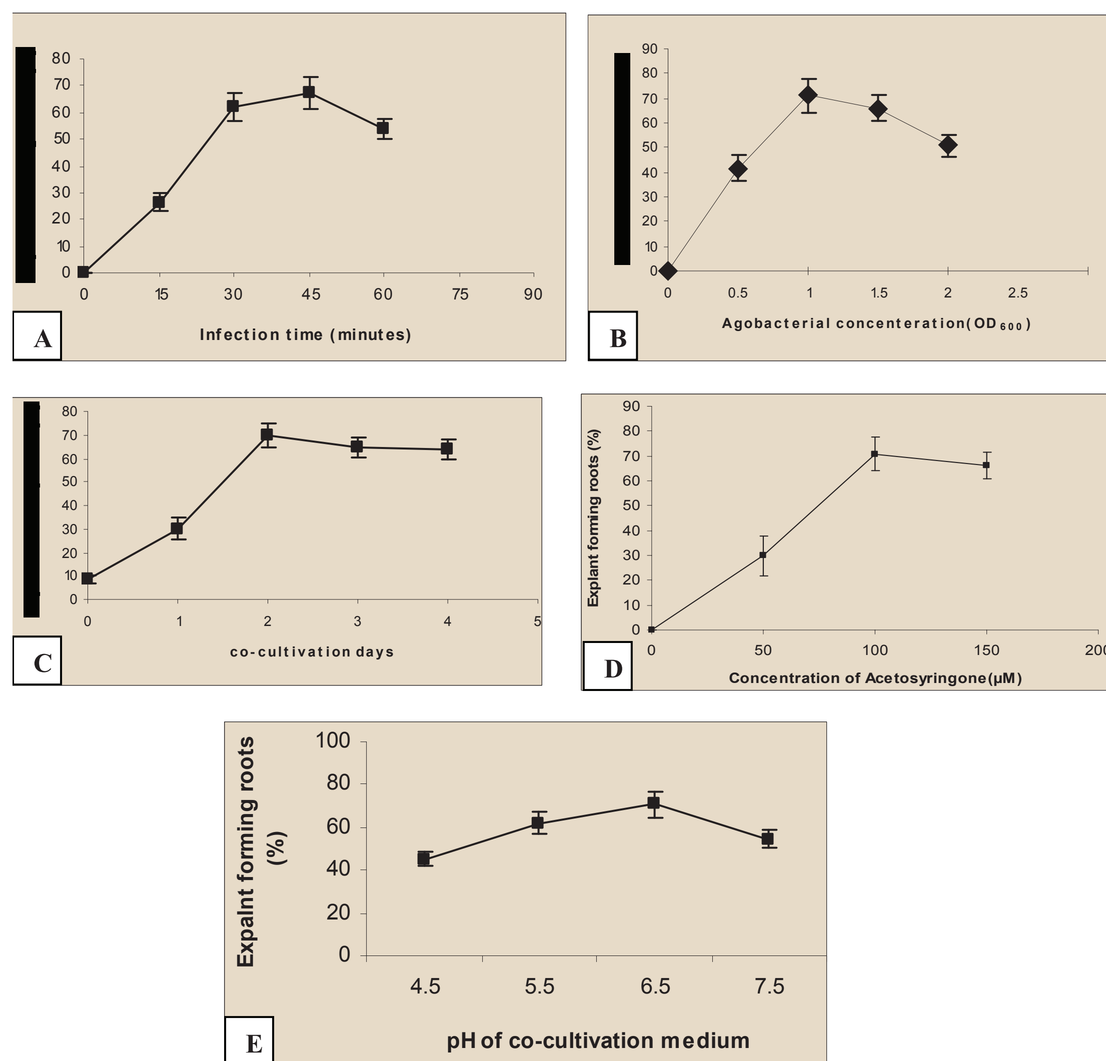

Figure 3: Effects of infection period (A), bacterial concentration (B), co-cultivation period (C) and acetosyringone concentration (D) on *Abrus precatorius* hairy root formation. All root-inducing conditions were standardized in experiments except for the variables investigated. Each value represents the mean of three different experiments.

germination. The genetic transformation mediated by *Agrobacterium* is affected by various factors like explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules etc. Different strains of *A. rhizogenes* vary in their transforming ability (34, 35). This was confirmed in our study that the MTCC 532 strain had greater hairy root transformation capacity than the MTCC 2364 in Abrus transformation. The plasmids contained by these strains carry genes of Ri T_L-DNA which direct the synthesis of substances that induce the cell to differentiate the root formation under the influence of endogenous auxins (36, 37). The *tms* loci in the Ri T_R-DNA also synthesis auxin which induce root formation. Variations in plasmid of strains may be responsible for the variability of transformation efficiency (38). Bacterial concentration plays an important role in the production of transformed roots. Suboptimal agrobacterial concentrations result in low availability of bacteria for transforming the plant cells while high concentrations decreased their potential by competitive. The results show the optimum bacterial concentration was 1.0 (OD_{600}), which was similar to the effect of bacterial concentration on hairy root formation in soybean transformation described by Kumar et al. 1991. Therefore it may have the same mechanism for the transformation.

Co-cultivation is very important step in the transformation process. Bacteria attachment, T-DNA transfer and integration occurs during this stage (39,40). These processes can be accelerated by supplementing some ingredients in the co-cultivation medium or

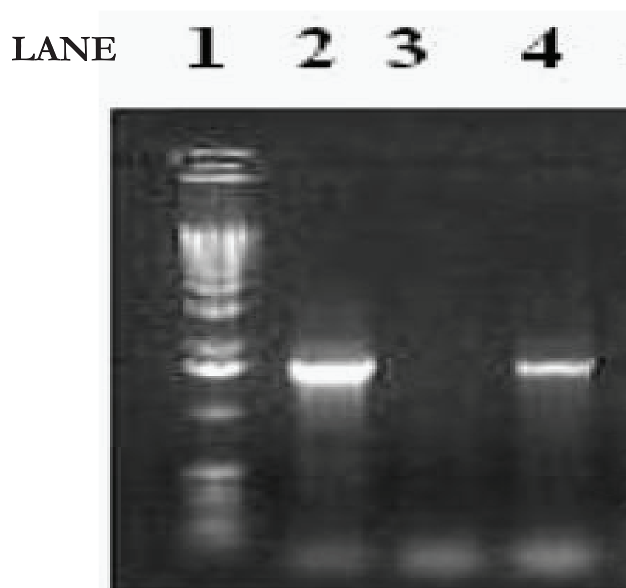

Figure 4: PCR analysis of Ri- transformed culture
Lane 1: 500 bp Ladder (Marker)
Lane 2. Positive control-II (pRi–MTCC532),
Lane 3. Negative control (*A. precatorius* leaf),
Lane 4. Transformed hairy root culture.

prolonging co-cultivation time so that these processes can be completed effectively. Our results indicated that, when the co-cultivation time was too short then these transformation processes could not be completed, while long co-cultivation time could negatively affect transformation by reducing bacterial affinity to the plant cell or by competitive inhibition. Some recalcitrant plant species can be transformed by inducing the *vir* genes of the bacteria by signal molecules or it can be achieved in vitro by co-cultivating agrobacterium with wounded tissue (41). Acetosyringone or related compounds functions as signal molecules, which have been reported to improve the agrobacterium-mediated transformation in several plant species (42). In our work, exogenous acetosyringone at 50 to 100 µmol L^{-1} was must for the hairy root formation, which according to the finding of Cervera et al. 1998 (43). The transformation frequency was not significantly increased with application of higher concentration of (>100µmol L^{-1}) acetosyringone. We suggest that, in Abrus transformation, presence of acetosyringone in relatively lesser concentration is obligatory for signaling but at high concentration the molecule function is not increased suggesting optimization of response at lower concentration.

Studies indicate that low medium (e.g. Nutrient broth medium and Xanthomonas medium) pH for activating bacteria promotes activation of some *vir* genes. Like VirD2, which are effectively activated under acidic pH, optimum being pH 5.1–5.2 (44). When pH of medium is decreased from 7.2 to 5.8, the hairy root formation frequency markedly increased which is in agreement with the finding of Yu et al. 2001(45). Hairy root formation frequency increased when pre- and co-cultivation medium pH was increased from 4.5 to 6.5, but decreased when pH was higher than 6.5. This is in accordance with the findings of Aida and Shibata 1995(46) the underlying mechanism of which is not clear. The transformation of roots induced from *Abrus* leaves by *A. rhizogenes* was obtained with relative ease and it was demonstrated that the transformation frequency could approach 84.4% under optimal conditions. *Abrus* could become an important research plant with special reference to genetic transformation and the secondary metabolite production by virtue of its wide-ranging list of chemical constituents present in its different parts.

ACKNOWLEDGEMENT

One of the authors wishes (Vijai Singh Karwasara) to acknowledge All India Council for Technical Education, New Delhi for providing the scholarship for the research work.

REFERENCES

1. Hooper D. *Abrus Precatorius*: A Chem. Examination of the Leaves and Roots. *Pharm. J. Trans.* **24** (3): 937–938 (1894).
2. Inglette G.E. and May J.F. Tropical plants with unusual taste properties. *Econ Bot* **22**: 326 (1968).
3. Lupi A., Monache F.D. and Marini B.B. Abruquinones: new isoflavanquinoes. *Gazzatta Chimica Italian.* **109**: 9–12 (1979).
4. Akinloye B.A. and Adalumo L.A. *Abrus precatorius* leaves a source of glycyrrhizin. *Nigerian J. Pharm.* **12**: 405 (1981).
5. Kuo S.C, Chen S.C, Chen L.H, Wu J.B, Wang J.P and Teng C.M. Potent antiplatelet, anti-inflammatory and antiallergic isoflavanquinones from the roots of Abrus precatorius. *Planta Medica.* **61**(4): 307–12 (1995).
6. Saxena V.K and Sharma D.N. A new isoflavone from the roots of *Abrus Precatorius*. *Fitoterapia* **70**: 328–29 (1999).
7. Yadav R.N. and Reddy V.M. A new biologically active flavonol glycoside from the seeds of *Abrus precatorius* Linn. *J Asian Nat Prod Res.* **4**(2): 103 (2002).
8. Shoemaker J. V. Jequirity It's Use in Diseases of the Skin. *The Lancet* **2**: 185–187 (1884).
9. Ratnasooriya W. D., Amarasekera A. S., Perera N. S. D. and Premakumara G. A. S. Sperm antimotility properties of a seed extract of *Abrus precatorius*. *Journal of Ethno pharmacology.* **33**(1-2): 85–90 (1991).
10. Nwodo O. F. C. Studies on *Abrus precatorius* seeds: Uterotonic activity of seed oil. *J Ethnopharmacol.* **31**(3): 391–394 (1991).
11. Molgaard P., Nielsen S. B., Rasmussen D. E., Drummond R. B., Makaza N. and Andreassen J. Anthelmintic screening of Zimbabwean plants traditionally used against schistosomiasis *J Ethnopharmacol.* **74**: 257–64 (2001).
12. Jackinovich W., Moon C., Choi Y.H. and Kinghorn A. D. Evaluation of Plant Extracts for Sweetness Using the Mongolian Gerbil. *J. Nat. Prod.* **53**(1): 190–95 (1990).
13. Parrotta J. A. *Healing plants of Peninsular India.* (CAB International, Wallingford, UK. 2001) 944.

14. Akamatsu H., Komura J., Asada Y. and Niwa Y. Mechanisms of Anti-inflammatory action of Glycyrrhizin: Effect of Neutrophil functions including reactive oxygen species generation. *Planta Medica.* **57**(2): 119 (1991).
15. Toivonen L. and Rosenqvist H. Establishment and growth characteristics of *Glycyrrhiza glabra* hairy root cultures. *Plant Cell Tissue Organ Cult*. **41**: 249–58 (1995).
16. Agarwal M.K., Iqbal M. and Athar M. Inhibitory effect of 18?-glycyrrhetinic acid on 12-O-tetradecanoyl phorbol-13-acetate- induced cutaneous oxidative stress and tumor promotion in mice. *Redox Rep*. **10**: 151–57 (2005).
17. Ito M., Nakashima H., Baba M., Pauwels R., De Clercq E., Shigeta S. and Yamamoto N. Inhibitory effect of glycyrrhizin on the *In Vitro* infectivity and cytopathic activity of the HIV virus. *Antiviral Research* **7**: 127 (1987).
18. Hirabayashi K., Iwata S., Matsumot H., Mori T., Shibata S., Baba M., Ito M., Shigta S., Nakashima H. and Yamamoto N. Antiviral activities of glycyrrhizin and its modified compounds against Human Immunodeficiency Virus Type I(HIV-1) and Herpes Simplex Virus Type-1(HSV-1). *In vitro chemical Pharmal bull.* **39**(1): 11 (1991).
19. Matsui S., Matsumoto H., Sonoda Y., Ando K., Aizu-Yokota E., Sato T. and Kasahara T. Glycyrrhizin and related compounds downregulate production of inflammatory chemokines IL-8 and eotaxin 1 in a human lung fibroblast cell line. *Int Immunopharmacol* **15**: 1633–1644 (2004).
20. Yoshida T., Tsuda Y., Takeuchi D., Kobayashi M., Pollard R.B. and Suzuki F. Glycyrrhizin inhibits neutrophil- associated generation of alternatively acitivated macrophages. *Cytokine* **33**(6): 317 (2006).
21. Hayashi H., Fufui H. and Tabata M. Examination of triterpenoids produced by callus and suspension culture of *Glycyrrhiza glabra*. *Plant Cell Rep.* **7**: 508–511 (1988).
22. Arias-Castro C., Scragg A.H. and Rodriguez-Mendiola M.The effect of culture conditions on the accumulation of formononetin by suspension culture of *Glycyrrhizia glabra*. *Plant Cell Tissue Organ Cult*. **34**: 63–70 (1993).
23. Binns A.N. and Thomashow M.F. Cell biology of *Agrobacterium* infection and transformation of plants. *Annu. Rev. Microbiol.* **42**: 575–606 (1988).
24. Costamtino P., Capone L., Cardarelli M., De Paolis A, Mauro M.L. and Trovato M. Bacterial plant oncogenes: the rol genes saga. *Genetics* **94**: 203–11 (1994).
25. Giri A. and Narasu M. L. Transgenic hairy roots: recent trends and applications. *Biotechnol Advances*. **18**: 1–22 (2000).
26. Shih Y.H., Chia H.H and Shih N.C. Innovative strategies for operation of mist trickling reactors for enhanced hairy root proliferation and secondary metabolite productivity. *Enzyme Microb. Technol.* **35**: 22–32 (2004).
27. Aoki T., Matsomoto H., Asako Y., Matsunaga Y. and Shimomura K. Variation of alkaloid productivity among several clones of hairy roots and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834. *Plant Cell Rep.* **16**: 282–86 (1997).
28. Bhagyalakshmi N. R., Thimmaraju R. and Narayan M.S. Various hexoses and di-hexoses differently influence growth, morphology and pigment synthesis in transformed root cultures of red beet (*Beta vulgaris*). *Plant Cell Tissue Organ Cult*. **78**: 183–195 (2004).
29. Simomura K., Sudo H., Saga H. and Kamada H. Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. *Plant Cell Rep.* **10**: 282–85 (1991).
30. Parr A.J. and Hamill J.D. Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants.*Phytochemistry.* **26**: 3241–3245 (1987).
31. Bourgaud F., Bouque V., Gontier E. and Guckert A. Hairy root cultures for the production of secondary metabolites. *AgBiotech News Information*. **9**(9): 205–08 (1997).
32. Chilton M.D., Tepfer D.A., Petit A., David C., Casse-Delbart F. and Tempe J.*Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. *Nature*. **295**: 432–34 (1982).
33. Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology Plant*. **15**: 473–97 (1962).
34. Kumar V., Jones B. and Davey M. R. Transformation by *Agrobacterium rhizogenes* and regeneration of transgenic shoots of the wild soybean *Glycine argyrea*. *Plant Cell Reports*. **10**,135–38 (1991).
35. Giri A., Banerjee S., Ahuja P.S. and Giri C.C. Production of hairy roots in *Aconitum heterophyllum* wall. Using *Agrobacterium rhizogenes*. *In Vitro Cell Dev Biol Plant*. **33**, 280–84 (1997).
36. Nguyen C., Bourgaud F., Forlot P. and Giri A. Establishment of hairy root cultures of Psoralea species. *Plant Cell Reports* **11**: 424–27 (1992).
37. Ooms G., Twell D., Bossen M.E., Hoge J.H.C. and Burrell M.M. Development regulation of Ri T-DNA gene expression in roots, shoots and tubers of transformed potato (*Solanum tuberosum* cv. Desiree). *Plant Molecular Biology*. **6**: 321–30 (1986).
38. Capone L., Cardarelli M. and Trovato M. Upstream no-coding region which confers polar expression to Ri plasmid root inducing rol B. *Mol Gen Genet*. **216**: 239–44 (1989).
39. Sunilkumar G., Vijayachandra K. and Veluthambi K. Preincubation of cut tobacco leaf explants promotes Agrobacterium-mediated transformation by increasing *vir* gene Induction. *Plant Sci.* **141**: 51–58 (1999).
40. Su J., Duan R.Q., Hu C.Q., Li Y.P. and Wang F. Regeneration and Agrobacterium-mediated transformation for Chinese cabbage. *Fujian J Agricultural Sci.* **17**(4): 241–43 (2002).
41. Satchel S.E., Messens E., Van Montagu M. and Zambryski P. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium rhizogenes*. *Nature*. **318**: 624–29 (1985).
42. Hu Z.B. and Alfermann A.W. Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. *Phytochemistry*. **32**: 699–703 (1993).
43. Cervera M., Pina J.A., Juarez J., Navarro L. and Pena L. *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. *Plant Cell Rep*. **18**: 271–78 (1998).
44. Jacq B., Lesobre O., Sangwan R.S. and Sangwan-Norreel B.S.Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. *Plant Cell Rep*. **12**: 621–624 (1993).
45. Yu S.H., Liu C.F., Li L. and Pan R.C. Factors affecting genetic transformation of *Pueraria lobata* by *Agrobacterium rhizogenes*. *Chin J Appl Environ Biol.* **7**(5): 474–77 (2001).
46. Aida R. and Shibata M. Agrobacterium-mediated transformation of Torenia (*Torenia fournieri*). *Breeding Sci*. **45**(1): 71–74 (1995).