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Regeneration of transformed plants of Aconitum violaceum from Agrobacterium rhizogenes mediated hairy root cultures

Janhvi Mishra Rawat[*1], Balwant Rawat[2], Shweta Pandey[2], Jigisha Anand1, Nishant Rai[1], Mr Purushottam Das[3]

[1]Department of Biotechnology, Graphic Era Deemed University, Dehradun, Uttarakhand, India
[2]School of Agriculture, Graphic Era Hill University, Dehradun, Uttarakhand, India
[3]Assistant Professor, Department of Computer Science and Engineering, Graphic Era Hill University, Dehradun

ABSTRACT

Shoot tip explants of Aconitum violaceum Jacq. a highly important medicinal plant of alpine Himalaya, were used to initiate Agrobacterium rhizogenes mediated hairy roots. These hairy roots (4 lines) were used as explants which produced adventitious shoots when cultured on MS basal medium supplemented with various concentration of 6-benzyladenine (BAP) and α-naphthaleneacetic acid (NAA), following a callus phase. Results showed that 10 µM 6-benzyladenine and 5µM α-naphthaleneacetic acid were the best combination in terms of adventitious shoot formation (frequency of up to 79.66 %) through callus. Plants derived from hairy roots showed high-volume rooting. All the transformed plants showed similar morphology; however, they showed increase in leaf numbers and leaf size. Leaves of transformed plants also showed leaf wrinkling. Comparatively, higher amount of aconitine content was recorded in transformed plants in comparison to non-transformed plants. Hairy root induction is quite tough in A. violaceum, therefore, development of transformed plants of A. violaceum using in vitro secondary metabolite production potential may deliver an essential alternative against exploitation of this endangered plant species.

Keywords: Aconitine, Aconitum violaceum, Agrobacterium rhizogenes, genetic transformation

INTRODUCTION

Abbreviations

MS	Murashige and Skoog's medium
BA	6-Benzyladenine
NAA	α-Naphthaleneacetic acid
PCR	Polymerase Chain Reaction
PGSs	Plant Growth Substances

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Introduction

Aconitum violaceum Jacq. is well known for its medicinal and pharmaceutical importance. It belongs to Himalayan range (3000 to 4500 amsl) of India. Extract of the underground parts of plant is useful in treatment of high fever, rheumatism, renal pain, wounds, allergy, cuts, disorder of gall bladder, etc. (Ameri 1998; Bhattarai et al. 2010; Rawat et al. 2013a).

In view of the high demand of this species there has been unregulated collection from the natural population, and also not seriously considered for commercial cultivation. These factors have led to considerable reduction of *A. violaceum* under natural conditions, and consequently listed under threatened plant species of Indian Hmalayan Region (CAMP 2003). Considering the current status of the plant there is an urgent need to develop scaling-up multiplication methods to get the desired phytomolecules to fulfil the industrial (pharmaceutical) demand.

Successful propagation protocols were reported in *A. violaceum* using tissue culture methods (Rawat et al. 2013a, b), but hairy root culture in this plant is still not done. Although few attempts were done to establish the hairy root induction in the Aconitum species using *Agrobacterium rhizogenes*; however, those attempts have been unsuccessful (Mugnier, 1988; Giri et al. 1997). It is also observed that induction of hairy root cultures in Aconitum is quite difficult due to the presence of high amount of phenolics.

The current research finding is first report of hairy root induction and successful regeneration of transformed plants of *A. violaceum* from callus, which were regenerated through *A. rhizogenes* genertaed hairy root cultures. The results of the study may be helpful to enhance the production of secondary metabolites, specially, root-derived phytomolecules and will open the ways towards metabolic engineering.

Materials and methods

Plant material and culture media

Leaf explants excised from *in vitro* cultures of *A.violaceum* were used for inferring of hairy roots. This *in vitro* grown plant of *A. violaceum* served as control plant. The basal MS medium (Murashige and Skoog; 1962) was used to initiate culture. The pH was adjusted to 5.8; and cultures were maintained under CFL light (cool-white fluorescent lamps) for 16 h photoperiod at 25±1 ºC.

Bacterial strain and co-cultivation

A. rhizogenes strain A-4 was used for co-cultivation to initiate the culture from leaf explants. *A. rhizogenes* were grown in YMB medium for 48 h in rotary shaker at 28 ± 2ºC (Hooykass et al. 1977). Before inoculation leaf explants were wounded with the help of sterilized needle and dipped in bacterial culture for 10 mins. After 10 mins. these leaf explants were rinsed thrice with autoclaved double distilled water and placed onto solid MS basal medium supplemented with 250 mg/l cefotaxime and co-cultivated. Explants were shifted onto 250 mg/l cefotaxime containg half-strength solid MS basal medium. After 7 to 14 days hairy roots were formed which were sliced into 1cm long explants and followed by culturing on half strength liquid MS medium. These hairy roots were sub-cultured after every 4-week intervals in PGSs free liquid Murashige and Skoog (1962)

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medium. Each line was maintained properly. Four hairy root lines (HR1, HR2, HR5 and HR6) were used for plant regeneration.

Regeneration of transformed plants

Hairy root cultures were used as explant to induce adventitious shoots. Approx. 1 cm long explant was cultured on MS media containing 0.0-10.0 µM 6-benzyladenine (BA) and 0.0-5.0 µM α-naphthaleneacetic acid (NAA) in combination. Ten replicates were prepared for each experiment. After 4 weeks explant produced callus and subsequently adventitious shoots were observed. These adventitious shoots were further transferred on to half strength MS basal medium for root initiation. After that the well rooted plants were acclimatize in soil pots and further transferred to green house. Growth parameters and phytochemical constituents were also recorded in regenerated plants and control plant after 9 months.

Conformation of transformed nature through PCR analysis

Transformed nature of hairy roots and its regenerated plants was affirmed through the Ri T-DNA detection. Fresh tissue was used for DNA extraction following the protocol Doyel and Doyel (1987) with minor modifications. Isolated DNA was resuspended in 100 µl TE (1mM). Quality and quantity of DNA was monitored by both gel electrophoresis and spectrometric measurements. The transformation of hairy roots and regenerated plants was confirmed through the primer (*rol B* gene 5'-TCGTAACTATCCAACTCACATCAC and 5'-CTTATGACAAACTCATAGATAAAGGTT-3') amplification. Using Polymerase chain reactions (PCRs). For amplification of the gene sequence, denaturation step at 94ºC was maintained for 5 mins, annealing maintained for 60 sec at 52ºC and extension at 72ºC for 60 secs., finally extension was done at 72ºC for 10 min. in a Thermocycler with 40 cycles (Biometra; Germany).

Southern blot analysis was followed using the method of Rawat et al. (2013). Isolated DNA (20 µg) was used for gel electrophoresis. DNA was digested with SalI and electrophoresed. Further, blotting was done using a Hybond N+ membrane (Amersham Biosciences). DNA isolated from *A. rhizogenes* strain A4 was also digested and further transferred to the above-mentioned membrane. Primers 5'-CATCGCACTGGCTATGTCG-3' and 5'-ATGGCATTCAATAACGACCGTAC-3' was used for amplification and identified as probe which was labelled with [α-^{32}P] dCTP. Prehybridization and hybridization were done at 65 °C using 6x SSC (saline sodium citrate) and 0.5% SDS (sodium dodecyl sulphate). At the end, picture was taken after washing of the membranes using 0.2x SSC and 0.1% SDS at temperature of 65 °C for 30 min .

Morphological parameters assessment

Morphological and physiological parameters were used to analysed the performance of the transformed plants in comparison with control plant. Leaf area meter (LI-3000A; LICOR, USA) was used to record leaf average area (cm2). Plant height (cm), leaf number and root yield (fresh and dry weight basis) were also recorded to compare the growth of the plants.

Secondary metabolite analysis

Secondary metabolites were extracted from powdered sample which were extracted with ammoniacal ether (ether containing 5% v/v, ammonia solution). Elutes of the sample were dried in

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vacuo (30°C) and dissolved in methenol for HPLC analysis.

Results and Discussion

Hairy roots were induced using *Agrobacterium rhizogenes* strain A4 in *A. violaceum* (Fig. 1 a, b & c). Various combinations of NAA and BA as well as supplementary vitamin source were used for regeneration of transformed shoots through hairy roots following callus phase (Table 1). MS medium supplemented with 10 µM BA and 5µM NAA was best in adventitious shoot formation. Since, each transformation event was observed distinct from the other 4 different hairy root lines (HR1, HR2, HR5 and HR6), selection of A4 origin was done for regeneration studies. Amongst these, 3 regenerants (HR1, HR5 and HR6) showed callus formation within 3-4 weeks of subculturing (Fig. 1 d). After 4 weeks these callus turned greenish in colour and gave rise adventitious shoots (Fig 1 e & f). Regeneration of adventitious shoots from callus induced by hairy roots has been reported in *Catharanthus roseus* (Choi et al. 2004). Approx. 80% frequency of shoot regeneration was reported after 8 weeks of culture (Fig 1 g; Table 2). Different hairy root lines showed notable difference in the frequency of adventitious shoot formation (Table 2). It showed the effect of genotype on the level of competence for the formation of adventitious shoot. Normal growing roots in same culture conditions were taken as control. Control roots did not showed any growth. A total of 15 transformed plants were selected from 25 plants and transferred to green house. Morphological data related with plant height and leaf characteristics were recorded. It was recoeded that transformed plant prolific rooting and bigger leaf size in comparison to control (Table 3). Similar results had also been reported in another Himalayan plant i.e. *Picrorhiza kurrooa* (Rawat et al. 2013c).

PCR analysis affirmed *rol B* genes presence in hairy roots and its derived plant. The study further confirmed that TL-DNA was conserved stably in the plant genome (Fig. 1i). Number of copies of the rol B gene was confirmed with Southern blot analysis.

Physiological evaluation has also been done for transformed plants. CO_2 and water vapour exchange parameters indicated that transformed plants showed better photosynthetic actitvity. Measurements for photosynthetic parameters in control plant was 2.67 CO_2 µmol /m^2/s, whereas in transformed plants it was 4.78 CO_2 µmol/m^2/s (Table 3). Transpiration rate also increased in transformed plants. Over all bettere morpholocical and physiological response given by transformed plants (Table 3).

The quantitative analysis revealed that transformed plants accumulated higher amount of aconitine in comparison to the control (roots of field grown plant of same age). All the samples examined, higher concentration of aconitine (1.29 % on dry weight basis) was recorded in hairy roots (line HR 5; further used to initiate callus and adventitious shoots) and transformed plants. In the untransformed samples aconitine concentration was low (control; 0.74 % on DW basis; Table 3).

On the basis of the results, it can be concluded that the study is very helpful to validate the genetic transformation in plants at genetic / molecular level. The results of the study not only help in consorving biodiversity indirectly, but also help to produce sufficient secondary metabolites to fulfil the requirements/need of the industries working on plant based pharmaceuticals.

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Table 1 Various plant growth substances used for callus and shoot regeneration from hairy roots of Aconitum violaceum on MS medium

Plant growth substances (μM)		Results on half strength MS	Results on full strength MS
BA	NAA		
0.0	0.0	NR	NR
2.5	0.5	NR	NR
5.0	2.5	NR	NR
10.0	5.0	++++ OR	+ OR
0.0	0.5	NR	NR
2.5	2.5	NR	NR
5.0	5.0	NR	NR
10.0	0.0	NR	NR
0.0	2.5	NR	NR
2.5	5.0	++ OR	+ OR
5.0	0.0	NR	NR
10.0	0.5	NR	NR
0.0	5.0	NR	NR
2.5	0.0	NR	NR
5.0	0.5	+ OR	NR
10.0	2.5	NR	NR

+ 20% shoot regeneration; NR, No response; OR, Organogenesis

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Table 2 Frequency (%) of adventitious shoot formation on root explants of Aconitum violaceum and the mean number of adventitious shoot per explant on MS medium

Plant growth substances (µM)		Hairy root lines							
BA	NAA	HR1		HR2		HR5		HR6	
		F	N	F	N	F	N	F	N
0.0	0.5	0	0	0	0	0	0	0	0
2.5	5.0	5.3±0.9	2.1±1.9	0	0	8.0±1.9	2.1±1.3	40±8.4	5.1±2.1
5.0	0.5	0	0	0	0	15.2±3.5	4.2±1.5	9.2±2.5	1.2±0.4
10.0	5.0	0	0	0	0	18.3±5.2	2.6±0.9	79.6±13.9	12.3±3.3

Hairy root lines, HR1, HR2, HR5 and HR6
F Percentage frequency (±SD) of adventitious shoot formation on hairy root explants
N Mean number (±SD) of adventitious shoots per explant/callus

Table 3 Differences in morphological, physiological and phytochamical parameters between transformed and non-transformed plants of Aconitum violaceum

Phenotype	**Parameters**									
	Morphological					**Physiological**				**Phytochemical**
	Plant height (cm)	**Leaf area (cm^2)**	**Number of leaves**	**Root yield (g)**		**Photosynthesis (CO^2 µmol/m^2/s)**	**Transpiration (mmol/m^2/s)**	**WUEx 100**	**Ci/Ca**	**Aconitine (% on dry weight basis)**
				Fresh weight	**Dry weight**					
Non transformed (normal) plants	5.6(±1.4)	1.86 (±0.3)	5.6±0.9	4.6±1.1	1.6±0.6	2.67±1.1	1.34±0.1	0.518±0.3	0.478±0.3	0.741±0.6
Transformed plants	6.5(±1.7)	2.83 (±0.3)	8.3±2.3	6.7±1.3	2.8±1.2	4.78±1.4	2.13±0.9	0.549±0.6	0.673±0.4	1.291±0.6*

values are mean of three replicates followed by standard error (±SE)
Data of HR5 regenerated plants have been taken to represent as HR5 hairy root line showed maximum frequency of adventitious shoot formation.

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Figure captions

Fig. 1 Generation of *Agrobacterium rhizogenes* mediated transformed plants in *Aconitum violaceum*; **a.** Wounded Aconitum violaceum leaf Half MS medium **b.** Root induction and subcultured on half MS medium **c & d.** Swelling of roots and callus formation on hairy root explants **e.** Adventitious shoot formation on callus **f.** well established transformed plant **g.** difference in roots of control plant 'C' and regenerated plant 'T' **i.** PCR analysis confirming the presence of TDNA in hairy root lines, where, A-HR1; B- HR2; C- HR5; D- HR6; E- negative control and M- weight marker