

Original Article

Enhancement of Tropane Alkaloid Production among Several Clones and Explants Types of Hairy Root of *Atropa belladonna* L.

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Abstract

Agrobacterium rhizogenes (pRi), a causative agent of hairy root disease, effectively induces hairy root formation in a variety of plant species. In our study four bacterial strains AR15834, A4, 9435 and C318 and three explants types leaf, stems and roots, were examined. Hairy roots were induced from roots, stems and leaf explants. The highest transformation efficiency of 77% was achieved by using strain AR15834. The transgenic status of hairy roots was confirmed by polymerase chain reaction using *rolB* specific primers for the presence of *rolB* gene in the genomes of transformed roots. Six clones of hairy roots were established that differed in their morphology. The detacidni stluser taht 4–11 fold increasing in root biomass after 28 days compared with non-transformed seedling roots. The concentration of tropane alkaloids hyoscyamine and scopolamine obtained in transformed roots (4 mg/g dry weight) was 3-11 folds more than in non-transformed cultured roots (0.36 mg/g dry weight).

Key words: Hairy root, Bacterial strain, Explants type, Tropane alkaloid

Introduction

In vitro production of plant secondary metabolites can be possible through plant cell culture under controlled conditions. However, the major limitations of cell cultures are their instability during long-term culture and low product yields [6].

Agrobacterium rhizogenes causes hairy root disease of plants by infection to wound sites where the transfer of T-DNA from the bacteria to the plant cell occurs. Root induction is due to the integration and subsequent expression of a portion of bacterial DNA (T- DNA) from the bacterial Ri (Root inducing) plasmid in plant genome. Their fast growth, genetic and biosynthetic stability, low doubling time, ease of maintenance and having the ability to synthesize a range of chemical compounds that makes them a suitable system for *in vitro* production of secondary metabolites [18, 44]. Therefore great efforts have been focused on transformed hairy roots [27].

Atropa belladonna (Solanaceae) is one of the widely used medicinal plants in the world and contains tropane alkaloids, hyoscyamine, and scopolamine [45]. During the past two decades considerable efforts have been made to develop an economically feasible *in vitro* production of these compounds. Induced roots from infected *Atropa belladonna* with *A. rhizogenes* 15834 produced various amounts of

tropane alkaloids, and in most cases higher than in normal field-grown plants [1, 26, 41].

In order to increase the tropane alkaloid production, different explants and colon of bacterial strain were tested in this study. The growth rate and the alkaloid content of the 6 root lines obtained were compared.

Materials and Methods

Plant material

Seeds of *Atropa belladonna* were collected from the medicinal plant garden of Hamadan Iran. *A. belladonna* plantlets were obtained by *in vitro* proliferated plantlets derived of plantlets derived from sterilized seeds. Seeds were rinsed with running tap water for 1 h, soaked in 70% (v/v) ethanol for 30 s and then in 0.2% (w/v) sodium hypochlorite for 12 min, and finally thoroughly rinsed with sterilized water. They were cultured on a Murashing and Skoog (MS) solid medium [34] at pH 5.8 supplemented with 3% (w/v) sucrose. These cultures were maintained at 25°C with a daily 16 h photoperiod. Two to three week old plantlets with 4-6 cm height and 1 cm long roots and leaves were used for bacterial infection treatment. Intact roots from 4-week-old plantlets were excised and then used as control roots.

Bacterial strain and culture conditions

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Wild type *A. rhizogenes* AR15834, A4, 9435 and C318 obtained from Department of Molecular Physiology, Agricultural Biotechnology Research Institute of Karaj, Iran, were used for hairy root induction. The bacterium was maintained in Luria–Bertani medium (LB: contains 10 g/l Bacto-trypton, 5 g/l Bacto-yeast extract, and 10 g/l NaCl, adjusted to pH 7.0). A single bacterial colony was inoculated in 10 ml of LB liquid medium supplemented with 50 mg Rifamcin and the culture was placed on rotary shaker (120 rpm) in the dark at 28 °C for 16 h.

Induction and establishment of hairy root cultures
Different seedling parts including root, stem and leaf were isolated from in vitro grown plantlets and were used to infect. After 2 days, these explants were transferred to 1/2 MS medium containing 250 mg cefotaxime as to eliminate the residual *Agrobacterium*. All explants treated similarly except. Cefotaxime concentration was then halved each week from 250 to 50 mg/L, and finally cultures free of *A. rhizogenes* were transferred to 1/2MS [34] medium solidified with 0.2% Phytigel (Sigma). Hairy roots, which developed mainly from the cut surfaces of the explants. Developed roots segments in 4-5 cm were then transferred to 1/2 MS medium for further growth.

All the cultures were maintained in complete darkness at 25°C. No infected roots of roots of in vitro germinated seedlings were cultured similarly and served as controls. Six different hairy root lines were established, which each line representing the occurrence of an independent transformation event. These lines were maintained by subculture of 3–4 cm long pieces on 1/2 MS solid medium after 4 weeks and then were maintained on 1/2 MS liquid medium on a rotary shaker (80 rpm) in complete darkness.

Isolation of plasmid DNA

Plasmid DNA from *A. rhizogenes* strain LBA 9402 was extracted as described by Sambrook *et al.* (1989) by alkaline lyses method.

DNA analysis

DNA was extracted using the CTAB method Cai *et al* (1997) from each hairy root line as well as from control non-transformed roots (in vitro germinated seedling roots). Plasmid DNA from *A. rhizogenes* strain AR15834 was extracted as described by Sambrook *et al.* (1989) by alkaline lysis method. PCR primers were used for amplification of a 780 bp fragment of the *rolB* gene. The sequence of each primer was as follows (forward primer 5' ATGGATCCCAAATTGCTATTCCCCACGA-3 and reverse primer 5'-TTAGGCT TCTT TCATTCGGTTTACTGCAGC-3). The PCR reactions were carried out in a total 50 ml volume and consisted of 200 ng of DNA, 10 pm/ml primer, 200 Mm dNTP, 1 U of Tag DNA polymerase, 1 _ PCR buffer and 2 Mm MgCL₂. PCR conditions were 94°C

for 5 min (initial denaturation), 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 7 min.

Gel electrophoresis of amplified DNA

Amplified products were separated by electrophoresis on 0.9% agarose gel in 1× Tris-acetic acid buffer (TAE) detected by staining with ethidium bromide and visualizing under UV light using a Transilluminator.

Growth kinetics of hairy root lines

Hairy root of the line 1, because of their fast develop were grown on different liquid media to determine the medium for optimal growth. MS basal medium, modified MS medium (half MS) and B5 medium, were used. Growth was measured in terms of fresh weight and dry weight of the hairy roots at the end of 4 weeks. Three replicates were used for each observation.

Tropane alkaloid analysis

Harvested roots were from 2-4 weeks cultures. The 50 mg of powdered sample was weighed and subjected to the extraction of alkaloids. An appropriate volume of CHCl₃-MeOH-NH₄OH (15/5/1) (5ml extraction solvent 50 mg dry sample) was added to the weighed sample, sonicated for 15 min, and then kept at room temperature for 1 h [25]. After filtration, the residue was washed twice with 1ml of CHCl₃. The pooled filtrate was evaporated to dryness. To the residue, 5 ml of CHCl₃ and 2 ml of H₂SO₄ (1N) were added, then the solution was mixed well. The CHCl₃ phase was removed and the H₂SO₄ phase was adjusted to pH 10 with 28% NH₄OH in an ice-bath. From the solution, alkaloids were extracted once with 2ml and twice with 1ml of CHCl₃. The combined extracts were filtered after adding anhydrous Na₂SO₄ and then the residue was washed with 1 ml of CHCl₃. The combined filtrates were evaporated to dryness at 40°C.

HPLC analysis

HPLC analysis was carried out on a HPLC system (KNAUER; Berlin, Germany) equipped with a Eurospher C18 column (25 cm × 4.6 mm) and a UV detector. Elution was monitored at 215 nm. Isocratic elution with a mixture of triethylammonium phosphate buffer (30 mM, pH 6.2) and acetonitrile (75/25) at a flow rate of 1.0 ml/min was selected to achieve maximum separation and sensitivity. Hyoscyamine and scopolamine hydrobromide were obtained from Sigma-Aldrich. The calibration graphs for standard samples were constructed by plotting the peak area of the alkaloids against their concentrations.

Results and Discussion

Induction of hairy roots

An effective system for hairy root induction from *Atropa belladonna* development was developed. with *A. rhizogenes* (Fig 1. a). Hairy roots appeared from deeply wounded sites after 10–20 days of inoculation from leaf explants with AR15834, A4, 9435 and C318 strains of *A. rhizogenes* (Fig1. b). These roots were fast growing, highly branching, and showed hormone autotrophy (Fig 1. c). However, AR15834, A4, 9435 and C318 strains showed different level of transformation frequency and AR15834 proved to be more competent than A4, 9435 and C318 strains. The highest transformation frequency was observed in

strain AR15834 with 77% (Fig 2). Factors including *Agrobacterium* strains, age and differentiation status of plant tissue, co-culture time, and activation factor, i.e. acetosyringone are often considered in order to increase frequency of transformation [30]. The different infectivity of *A. rhizogenes* might be due to different host susceptibility of the strains used [14]. Hairy roots induced by Ri T-DNA of *A. rhizogenes* are widely used for production of important pharmaceuticals from diverse medicinal plants [3, 29].

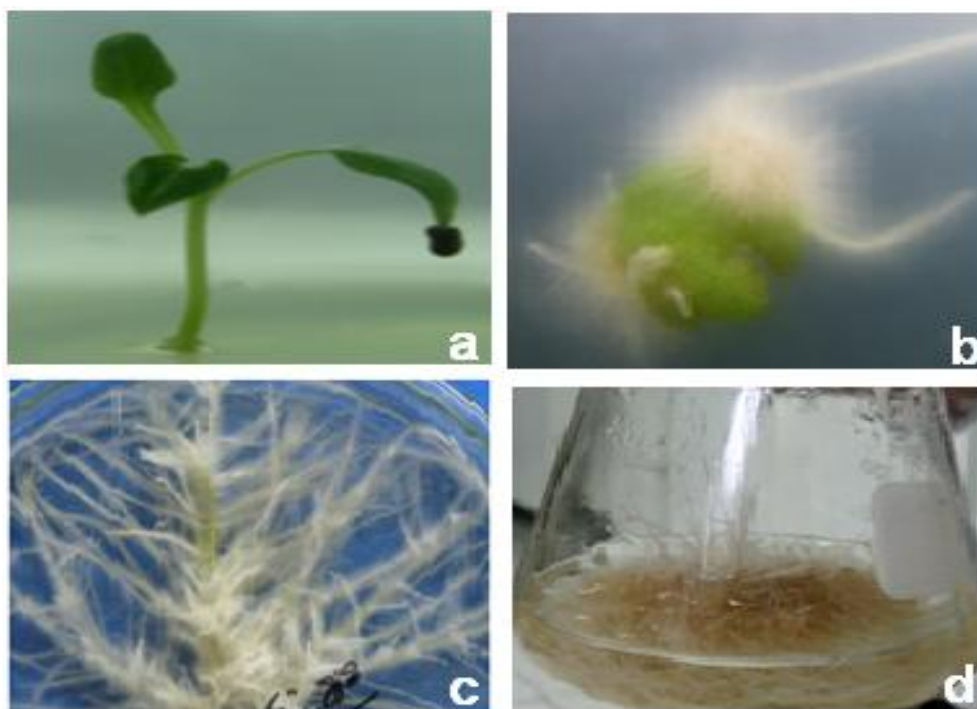


Fig 1. Hairy Root Induction of *Atropa belladonna*

(a) Sterile seedlings of *Atropa belladonna*. (b) Infected leaves were cultured on 1/2 MS solid medium at 25 °C in the dark for 15 d. (c) The selected hairy root clones were cultured on 1/2 MS solid medium at 25°C in the dark for 20 d. (d) The typical hairy roots were cultured in 1/2 MS liquid medium at 25 °C in the dark for 21 d.

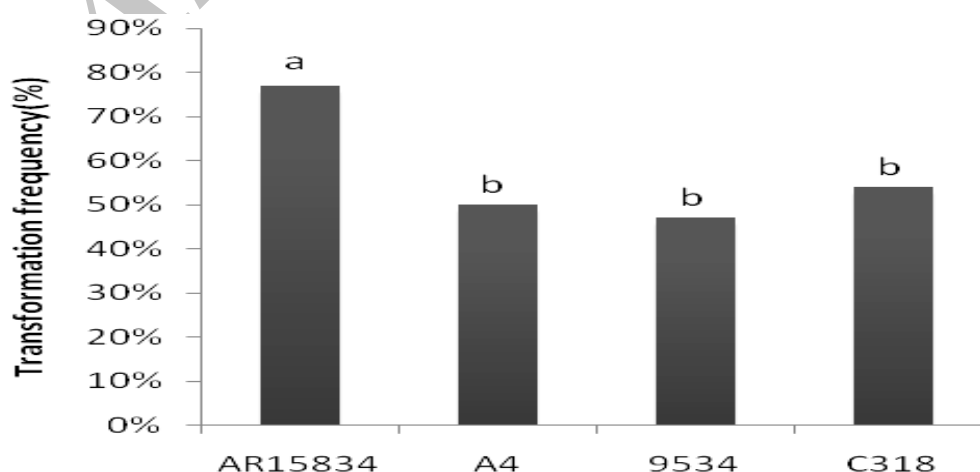


Fig 2. Hairy root induction from *A. belladonna* and *A. rhizogenes* tested were AR15834, A4, 9435 and C318. Data were means of three replicates, each with 10 explants.

Different types of explants may have diverse competence to *A. rhizogenes* infection. In this study, leaf, stem and root section were used as explants to determine which type of explants is most suitable for *A. rhizogenes* strain AR15834-mediated transformation in *Atropa belladonna*. As shown in Fig. 3, transformation frequency changed with explants types. The highest transformation frequency 74% was obtained when the leaf sections were used as explant. The transformation frequency obtained with leaf sections as explants was significantly

different (Fisher's Least Significant Difference (LSD) test; $P < 0.05$) to all other types of explants tested. The imperative role of explants in determining the plant genotype-bacterial strain compatibility has received substantial research attention over the years [4, 19, 23, 46]. The cloned nature of individual hairy root lines has made it mandatory to screen and select the best performer among a wider, independently generated, heterogeneous background as reported in earlier analogous studies [11].

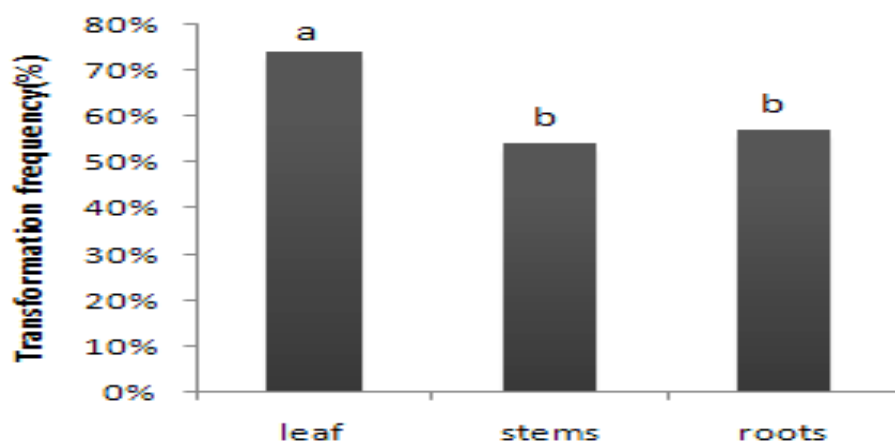


Fig 3. Selection of the most suitable explant for *A. rhizogenes* mediated transformation of *A. belladonna*. Means of transformation frequencies were compared using a FLSD test ($P < 0.05$) and column bars with the same letter are not significantly different. The experiment was performed in independent triplicate and each experiment contained about 10 samples.

About 20 root lines were established by using *A. rhizogenes* strain AR15834. As shown in Fig. 6, we identified six independent lines exhibiting rapid growth capability. Among them, three lines, C1, C3 and C5 established from the leaf explants.

Confirmation of transgenic status of hairy roots
The presence of the *rolB* gene in the hairy root lines was tested by PCR amplification of the DNA using

rolB forward and reverse primers. *A. rhizogenes* (colony PCR) served as the positive control and DNA from the non-transformed seedlings roots served as the negative control. All transformants showed presence of the 780 bp *rolB* amplified product (Fig. 4) and no *rolB* gene activity was found in control tissue.

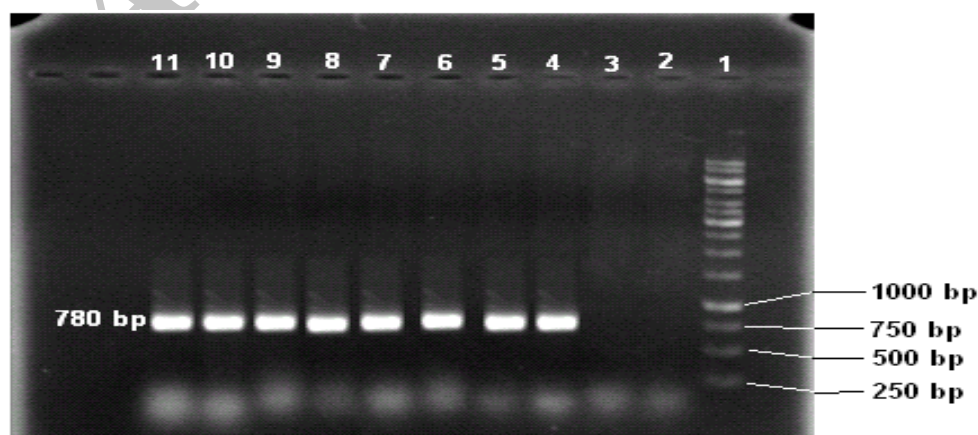


Fig 4. PCR amplification of a 780 bp fragment of the *rolB* gene using hairy root derived DNA. Lane 1; molecular weight marker (1000 bp ladder), lanes 2 & 3; DNA from non-transformed roots (negative control), lanes 4 & 5; *Agrobacterium rhizogenes* DNA (positive control), lane 6; line C₁, lane 7; line C₂, lane 8; line C₃, lane 9; line C₄, lane 10; line C₅ and lane 11; line C₆.

Growth of transformed roots in culture and Growth rates of hairy root lines

Growth of transformed root line C1 in different liquid media MS, 1/2 MS and B5 each supplemented with 30 g/l sucrose, showed that root growth was optimal in 1/2 MS medium lacking growth regulators (Fig 5), resulting in maximum biomass and hence this medium was used for maintenance of the hairy root lines. The composition of culture medium, especially the sucrose concentration, is known to influence the growth of transformed roots [18, 36]. Growth of the hairy root lines in terms of fresh or dry weight

increases showed in Table 1. Lines C1, C5 and C3 all of which were obtained from leaf explants, showed fast growth and a large number of lateral roots. Line C6, which was obtained from root showed slower growth, fewer lateral roots (Fig. 1d). The biomass accumulated at the end of 4 weeks was about 3-11 times the weight of initial inoculums in the different hairy root lines (Table 1). Line C1 showed highest biomass accumulation at the end of 4 weeks (5.4 g fresh weight), whereas line C6 showed the least (1.3 g). As previously reported for a few other hairy root systems [24, 33, 39, 47].

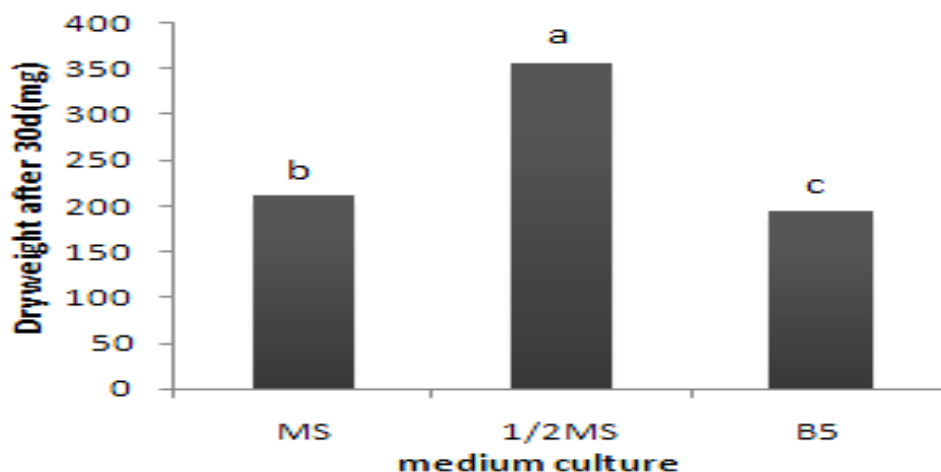


Fig 5. Growth of transformed roots of line 3 of *A. belladonna* in liquid media after 28 d of culture Values followed by different letters are significantly different ($P < 0.05$ FLSD test).

Rapid elongation is a characteristic phenotypic feature of transformed roots, and this rapid elongation growth and lateral branching are responsible for the enhanced biomass accumulation of transformed roots (Fig. 6). Difference in growth rate of hairy roots of same and different species was realized earlier [32]. Above results also showed the similar trend. The

differences of morphological traits and growth capacity between transformed roots (Fig 6) might be related to the effect of the *rolB*, the varied levels of their expression among the root lines could alter the endogenous auxin/cytokinin ratio or the sensitivity to the previous hormonal balance of each genotype [17].

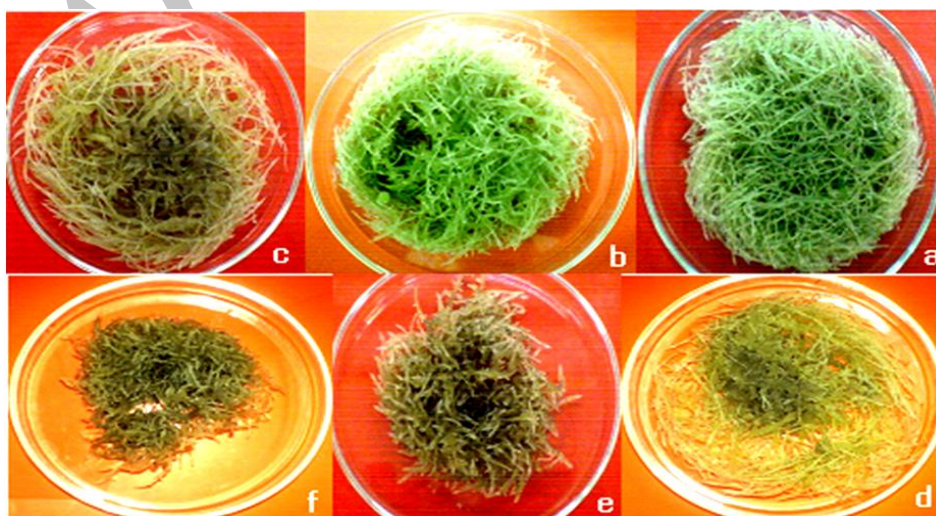


Fig 6. Different lines of hairy root production and growth rate during one month.
a) C1, b) C5, c) C3, d) C2, e) C4, f) C6

Alkaloid production

HPLC was used to assess hairy root clones of *Atropa belladonna* for the production tropane alkaloid (hyoscyamine and scopolamine) in dry roots. The results indicated that the production stabilizes at the

30-day period. The content tropane alkaloid (hyoscyamine and scopolamine) increase increased and reached to maximum level (3.99 mg/g) at 30 days of culture.

Table 1 The growth rate (root fresh or dry weight/inoculum fresh dry weight) of six hairy root lines obtained from infection with *A. rhizogenes* AR15834 (C1, C2, C3, C4, C5 and C6) and Hyoscyamine and scopolamine content of hairy root lines on liquid 1/2MS medium

Line	Fresh weight after 4 weeks (mg)	Dry weight after 4 weeks (mg)	Hyoscyamine content (mg/g dry weight)	Scopolamine content (mg/g dry weight)
Control	5.3	6.25	0.30	0.06
Line C1	47	71	3.31	0.68
Line C2	22	32	1.2	0.34
Line C3	29.5	48	1.88	0.61
Line C4	18	26.5	0.82	0.31
Line C5	43	60	3.02	0.57
Line C6	13	21	0.73	0.20

Differences in the amounts of accumulated tropane alkaloid (hyoscyamine and scopolamine) content were observed (Table 1).

The amount of hyoscyamine and scopolamine were 3.99 mg/g and 3.59 mg/g respectively in transformed C1 and C5 clone which was approximately 11 times higher than control root (Table 1). In term of yield maximum 3.99 mg/g (C1) of dry weight tropane alkaloid (hyoscyamine and scopolamine) produced after 30 days of culture as compare to 0.36 mg/g of control plant. Hairy root of *Atropa belladonna* grow faster (10 times) than normal root in standardized liquid culture condition and accumulate the higher (11-fold) amount of tropane alkaloid (hyoscyamine and scopolamine). This higher alkaloid production, fast-growing hairy root of *Atropa belladonna* offer exciting possibility for large-scale production of larger biomass by bioreactors and stable high production of tropane alkaloid (hyoscyamine and scopolamine) and other metabolites for pharmaceutical use. Clones are different in their tropane alkaloid (hyoscyamine and scopolamine) (Table 1).

Similar variations in the metabolite profiles of transformed clones were also reported in potato [40] and tobacco [10]. These differences may be attributed to the secondary variation in the T-DNA insertion, copy number, size and location of integration of T-DNA of Ri-plasmid into the plant genome [13] as also noted in horseradish hairy roots [16].

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