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Original article

Effect of elicitation on antioxidant activity and production of tropane alkaloids in *Hyoscyamus reticulatus* hairy root cultures

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Abstract

Background and objectives: *Hyoscyamus reticulatus* contains two distinguished tropane alkaloids, hyoscyamine and scopolamine and both of the compounds possess potential acute or chronic toxicity. In the present study, a simple and efficient transformation system was established for *in vitro* hairy roots induction in *Hyoscyamus reticulatus*. **Methods**: Effect of different factors including *Agrobacterium rhizogenes* strains (A7, 15834, A13 and D7), various explant types (cotyledon, hypocotyl, two weeks old leaf, four weeks old leaf, two weeks old internode and four weeks old internode), two inoculation methods (immersion and injection) and four types of culture media (MS, ½ MS, ¼ MS and B5) on hairy roots induction efficiency in *Hyoscyamus reticulatus* were tested. In the second part of the experiments, elicitations with different concentrations of colchicine (0, 0.01, 0.03 and 0.05% w/v) and different UV-B exposure time (0, 3, 6 and 9 min) were used to analyze hyoscyamine and scopolamine production. Transgenic status of hairy roots was confirmed by PCR using specific primers of the *rolB* gene. The total antioxidant activity was evaluated by DPPH) method. **Results**: Induction of hairy roots in *H. reticulatus* was affected by bacterial strain and explant type. A7 strain and cotyledon explants were detected as the best strain and explants for induction of hairy root in *H. reticulatus*. Hairy roots growth was significantly affected by medium type. The highest fresh weight was produced in MS and B5 medium. Fresh and dry weight of hairy root reached 1.44 and 0.134 mg at 0.05 percentage of colchicine after 48 h, respectively but in UV-B treatment fresh weight was decreased. In addition, antioxidant activity of hairy root samples treated with colchicine and UV-B increased to 27% (0.05 colchicine) and 26% (UV-B 9 min), respectively compared to the antioxidant activity level in non-transgenic roots (12%) and transgenic roots (18%). The highest amount of hyoscyamine and scopolamine (0.58% and 1.9 %) found in elicited hairy root cultures was 3.2 and 5.1 folds higher than the non-transformed roots (0.18% and 0.37%), respectively. B5 and MS medium were detected as the best appropriate medium for growth of *H. reticulatus* hairy roots. Antioxidant activity in elicited hairy roots with elicitors increased in comparison to the antioxidant activity level in transgenic and non-transgenic hairy roots. **Conclusion**: Hairy root lines developed and elicited in this study can be used to investigate the production of pharmaceutically important metabolites of *H. reticulatus.*

Keywords: antioxidant activity, colchicine, hairy root, *Hyoscyamus reticulatus*, tropane alkaloids

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Introduction

Hyoscyamus reticulatus (Solanaceae), is an important medicinal plant distributed in China, Afghanistan, India, Japan, Korea, south west Asia, North Africa and throughout Europe [1]. *H. reticulatus* contains two distinguished tropane alkaloids, hyoscyamine and scopolamine and both of the compounds possess potential acute or chronic toxicity [2-4]. Tropane alkaloids are mostly synthesized in the young root cells and then transported to the aerial parts of the plant [5]. Hairy root cultures can be used as an alternative production system for production of these alkaloids [6]. During the past two decades considerable efforts have been made to develop an economically feasible *in vitro* production of tropane alkaloids. Unfortunately, cell cultures of different Solanaceous species have shown low tropane alkaloid production, mainly due to the lack of differentiation [6, 7]. Root cultures can produce hyoscyamine and scopolamine at higher levels than cell cultures or even parent roots, since these alkaloids are synthesized specifically in plant roots [8,9].

Agrobacterium rhizogenes is a Gram negative bacterium which induces hairy roots in plants. It transfers a DNA segment from plasmid into the plant genome. Genetic transformation by Ri T-DNA of *A. rhizogenes* has been found as an effective indirect way for accumulating high levels of secondary metabolites in plant cells [10,11]. These roots are preferred for their genetic and biochemical stability, high growth rate, hormone-autotrophy, lateral branching, relatively low-cost culture requirements and multi-enzyme biosynthetic potential in comparison to the parent plants [12,13]. So far, hairy root cultures of many dicotyledonous and monocotyledonous plants have been established and found to accumulate the same metabolites as natural roots [14]. Furthermore, transgenic root systems have a tremendous potential for introducing additional genes along with the T-DNA of *A. rhizogenes* for alteration of metabolic pathways and production of useful metabolites or compounds of interest [12].

Several factors affect the rate of *A. rhizogenes* mediated transformation [15]. The strain and age of *A. rhizogenes*, type of explants and media composition are some of the effective factors in induction of hairy root cultures [10]. Many studies have shown that various plant tissues have different responses to transformation with different strains of *A. rhizogenes* [16-23].

Different concentrations of salts in culture media have a major role in hairy root growth and induction of secondary metabolites production. It is revealed that favored ratio of NH_4/NO_3 and sugar concentration in the medium are necessary for hairy root growth and production of biomass [24]. Another main factor that contributes to achieving hairy root induction is the type of explant used. Several studies on hairy roots in medicinal plant species have used various explants such as hypocotyls, cotyledons, leaves and mesophyll protoplasts.

To increase productive capacity of *in vitro* plant culture systems, many strategies have been used. The use of abiotic and biotic elicitors is one of the acceptable strategies to increase the productivity of induced hairy roots. Elicitors can stimulate accumulation of antimicrobial phytoalexins and different types of defense responses in plants [25]. Moreover, elicitors improve the release of the metabolites in the medium.

To the best of our knowledge, no investigation has been reported about evaluation of the hairy roots induction, growth and tropane alkaloid production in *H. reticulatus* hairy roots. Therefore, this study was conducted to determine the best condition for hairy root formation and for increasing tropane alkaloids production. Hairy root culture of *H. reticulatus* was exposed to colchicine to evaluate its effect on some morphological and biochemical characteristics of hairy roots.

Experimental

Plant material

The seeds of *Hyoscyamus reticulatus* (lattice henbane) were collected from the foothills surrounding areas of Naqhadeh, Iran and identified at the West Azarbaijan Agricultural and Natural resources Research Center of West Azarbaijan, Urmia, Iran by Mrs. Mahnaz Heidary. The voucher specimens were deposited at Natural Resources Research Center of West Azarbaijan Herbarium. Dormancy of seeds was broken by immersing them in 100 ppm GA_3 solution for 24 h. Seeds of *H. reticulatus* were surface-sterilized with 70% (v/v) ethanol for 1 min and 50% (v/v) sodium hypochlorite solution for 10 min, followed by three rinses with sterile distilled water. They were cultured on semi solid hormone-free Murashige and Skoog media [26] containing 3% sucrose and 0.7 % plant agar (Duchefa, Netherlands). Cultures were maintained in a growth chamber at 25 ± 2 ℃ and 16/8 h (light/dark) photoperiod with a photon flux density of 60 µmol/m^2s. The pH of the medium was adjusted to 5.8 with KOH (1 N) or HCl (0.1 N) prior to autoclaving at 121 ℃ for 15 min.

Bacterial strains

Single clone of four *A. rhizogenes* strains (A7, 15834, A13 and D7) provided by bank of microbes at the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran, were grown for 24 h at 28 ℃ with shaking (180 rpm) in liquid Luria-Bertani (LB) medium containing rifampicin (50 mg/L) (Sigma, USA). The *A. rhizogenes* cells were collected by centrifugation at 4000 rpm for 10 min and resuspended in liquid inoculation medium (MS salts and vitamins containing 50 gr/L sucrose, pH= 5.5).

Establishment of hairy root cultures

The leaves of *H. reticulatus* were taken from plants grown under *in vitro* condition at different explants age (one, two or four weeks old). Excised leaves were dipped into four *A. rhizogenes* strains suspension culture for 1 min, then were blotted dry on sterile filter paper, and incubated on MS media at 25 ℃ in the dark. After 2 days of co-cultivation, inoculated explants were transferred to a MS hormone-free medium containing 200 mg/L cefotaxime. Different explants including cotyledon, hypocotyl, two weeks old leaf and internode, four weeks old leaf and internode were tested. Numerous hairy roots were distinguished emerging from the wound sites of leaf explants within 2 weeks after inoculation.

Inoculation methods

Leaves obtained from cotyledon explants were infected by bacterial strain A7 and the effects of two inoculation methods (immersion and injection) were tested. In the immersion method, explants were immersed into a beaker containing the bacterial suspension for 1 min after wounding. The injection method was carried out by A7 suspension which was injected in different parts of cotyledon explants using a 0.5 mL insulin syringes (Exel, USA).

Effect of MS and B5 salt strength on hairy root production and growth

The hairy roots were separated from the explant tissue and subcultured in the dark at 25 ℃ on agar-solidified MS medium. Selected hairy roots line were initiated by transferring fresh hairy roots equivalent to 1 g into 250 mL Erlenmeyer flask containing 30 mL of liquid MS medium for more hairy roots growth. Isolated hairy root lines were transferred to various cultivation medium including; MS, 1/2 MS, 1/4 MS and B5 [27] to evaluate basal salts concentrations on growth and antioxidant activity of hairy root cultures. The cultures were incubated on a gyratory shaker at 180 rpm under standard cool white fluorescent tubes and 16 h light/8 h dark photoperiod and 26 ℃. The hairy roots were harvested after 21 days

to examine the dry weight and antioxidant activity.

Preparation of elicitors and elicitation

Hairy roots of *H. reticulatus* were exposed to the elicitor for 48 h in exponential phase (15 days old cultures). Filter sterilized (0.22-µm pore size) colchicine (Duchefa, Netherlands) at concentrations of 0, 0.01, 0.03 and 0.05% w/v, was added to the culture medium (B5 + 3% sucrose, pH 5.7). After 48 h colchicine treatment, hairy roots were transferred to fresh B5 medium and incubated at 26 °C in the dark for 3 weeks.

To assess the effects of UV-B radiation (20 W, HITAGH 1) on tropane alkaloids production and antioxidant activity, 2 gram of hairy roots from 18 days old samples was exposed to UV-B light for 2 weeks and 3 times per week for 3, 6, 9 min. Treated hairy roots were transferred to fresh B5 medium and incubated at 28 °C in the dark for 21 days.

Dry weight and alkaloids content was measured based on Hashimoto method [6]. Antioxidant activity analyses were performed by DPPH method [29].

PCR analysis of hairy roots

Total genomic DNA based on CTAB method [28] from each of the hairy root lines (0.5 g FW), as well as from control roots (non-transformed) were extracted. *Rol* B gene specific primers for the amplification of the 780 bp fragment were:

Forward primer:
5'ATGGATCCCAAATTGCTATTCCCCCACGA3'
Reverse primer:
5'TTAGGCTTCTTTCATTCGGTTTACTGCAGC 3'

After initial denaturation at 95 ^{o}C for 5 min, 30 cycles of amplification were performed, 94 °C for 1 min, 53 °C for 1.2 min and 72 °C for 1.3 min and a final extension at 72 °C for 10 min. The PCR amplification products were analyzed by electrophoresis in 1% agarose gels [22].

Alkaloid extraction and GC/MS analysis of hyoscyamine and scopolamine

Freeze dried adventitious roots were ground into fine powder and the powdered roots of *H. reticulatus* were extracted with methanol in a Soxhlet apparatus for 130 min. Methanol was evaporated to dryness under vacuum at a 50 °C. The pH of the residue was adjusted to 3 with 2 N H_2SO_4 and then filtered. The filtrate was extracted with 20 mL of chloroform for 3 times. The alkaloids were extracted 4 times with 50 mL of chloroform from the alkali aqueous solution (pH= 10) using ammonia. The chloroform was evaporated to dryness. Sodium sulfate was added to remove water and the residue was dissolved in 5 mL of dichloromethane. One µL of extract was directly injected into the GC/MS.

GC-MS analyses were performed with a Shimadzu GCMS-QP5050A spectrometer with DB-1 columns (60 m length, internal diameter 0.25 mm and layer thickness 0.25 µm). Injector temperature was set at 290 °C. Helium was the carrier gas, at a flow rate of 0.8 mL/min. One µL of diluted samples (1/10 in methanol v/v) were injected in the split/splitless (10:1 split) mode. The identification of alkaloids was based on the comparison of their GC retention time and the mass spectra (MS) data with their standards substances (HYO. HCl and SCO. HBr, Merck, USA).

Statistical analysis

The experiments were based on a completely randomized design (CRD) with 3 replications per treatment. Analysis of variance (ANOVA) based on CRD was performed on the data with the General Linear Model Procedure using SAS 9.1 software and the means were compared using Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

Results and Discussion

Due to the fact that various plants can show discriminative susceptibility to a given

Agrobacterium rhizogenes strain, various strains for hairy roots induction were tested. All A7, 15834, A13 and D7 strains of *A. rhizogenes* used in this study were able to produce hairy roots at the explants site infection after 12 days of inoculation (table 1). The highest infection frequency (76.22%) was found in A7 strain in one week old explants. The lowest infection frequency (14%) was obtained in the D7 strain in four weeks old leaves. Different strains of *A. rhizogenes* produce different types of opine [30,31]. Besides, the explant age is also an important factor that influences further hairy root growth and even production of transgenic roots [31]. Influence of *A. rhizogenes* strain on hairy root induction frequency has been presented earlier in some plant species [18,19,33]. Also, the different percentages of rooted explants could be explained possibly by differential expression of T-DNA genes present in the explants, variable copy numbers of T-DNA inserts and positional integration effects of the T-DNA in the host genome [15].

Table 1. Influence of *Agrobacterium* strain and explants age on transformation frequency (%)

	Transformation Frequency		
***Agrobacterium* strain**	**One week old**	**Two weeks old**	**Four weeks old**
A7	76.22	68.6	62.21
15834	72.33	65.33	29
A13	26	23.33	13.66
D7	16	16	14

Among the different explants tested (cotyledon, hypocotyl, two weeks old leaf, two weeks old internode, four weeks old leaf, four weeks old internode), maximum transformation frequency was documented in cotyledon explants (77.66%) whereas the minimum transformation frequency (34%) was recorded in four weeks old internode segments which was comparatively lower than other explants (figures 1 and 2). The results showed that all explant types used in this experiment showed the ability of hairy roots production.

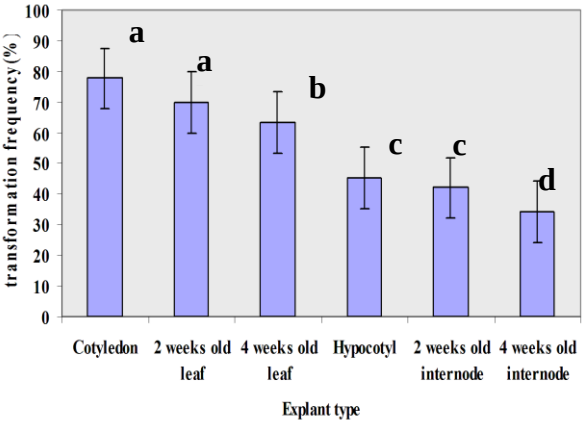

Figure 1. Influence of explants type on transformation frequency in *Hyoscyamus reticulatus*; different letters indicate significant differences at $p \leq 0.05$.

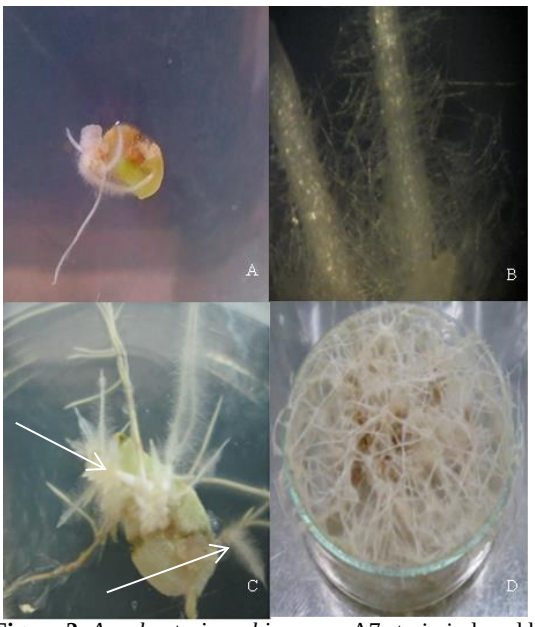

Figure 2. *Agrobacterium rhizogenes* A7 strain induced hairy roots in cotyledon explants of *Hyoscyamus reticulatus.* A) Hairy root appearance; B) Hairy root appearances in Cotyledons; C) Arrows indicate the hairy root induced on different parts of the explant; D) Hairy roots of *Hyoscyamus reticulatus* growing on hormone-free B5 medium.

However, transformation frequency of hairy roots induction and growth significantly depended on the applied explants type. Juvenility and nature of explant influenced the *Agrobacterium* mediated

transformation process [34,35]. The high percentage of transformation in cotyledon might be due to the higher sensitivity of these explants to bacteria compared with other explants types. This sensitivity depended on the physiological state of tissues [36].

Growth of plant cells and production of secondary metabolites in cell cultures depend on the concentration and interaction of nutrients present in cultivation medium [37]. Medium type's ½ MS, ¼ MS, MS and B5 displayed significant effect on both fresh and dry weight of *H. reticulatus* hairy roots (figure 3).

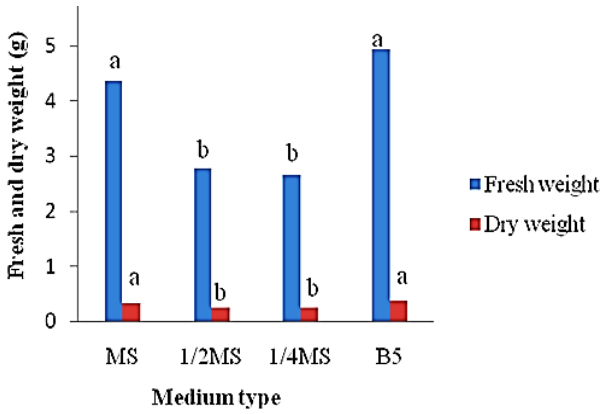

Figure 3. Influence of medium strength and type on *Hyoscyamus reticulatus* hairy root biomass; different letters indicate significant differences at $p \leq 0.05$

Both the fresh and dry mass of hairy roots grown in B5 medium were significantly higher than other medium. B5 medium was the best medium among the four media studied, in which the dry mass of hairy roots obtained 0.32 g per flask after 21 days. The results showed that the media color was changed by media compounds to yellow (figure 4).

Hairy root cultures are used to synthesize stable amounts of secondary metabolites but the main compounds are poorly released into the medium and their store in the roots can be restricted by feedback inhibition. Media manipulations have been reported to aid the release of metabolites. Betacyanin released from hairy roots of *Beta vulgaris* was achieved by oxygen starvation [38].

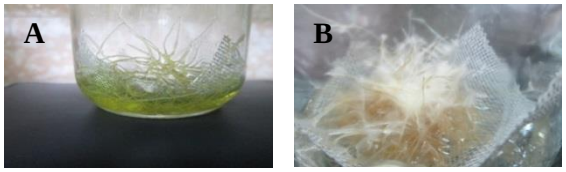

Figure 4. A) Released metabolites into the medium and changed media color to yellow B) Hairy root culture grown on B5 medium

As the medium strength decreased to half strength, solasodine level in *Solanum mauritianum* hairy roots was lowered but ajmaline and ajmalicine levels in *Pauvolfia micrantha* were increased [39,40]. An increase of ammonium concentration in the culture medium resulted in lowering the growth rate while an increase of the nitrate concentration had a deleterious effect on the alkaloid biosynthesis and accumulation. The highest biomass and alkaloid yields were obtained with reduced levels of both nitrogen sources. The results obtained by Sivakumar *et al*. [41] in ginseng hairy roots suggested that mineral elements are an important regulatory factor of growth and biomass.

PCR method is used for detecting T-DNA sequences in putative transformed hairy roots [42]. The *rolB* gene is absolutely essential for the induction of hairy roots [21]. To confirm the integration of T-DNA from the *A. rhizogenes* Ri plasmid into the hairy root genomic DNA, DNA extracted from the hairy roots was subjected to PCR analysis with specific *rolB* gene primers. 1% agarose gel electrophoresis of PCR products revealed that all the hairy root lines contained *rolB* gene (780 bp bands) which was a part of *A. rhizogenes* T-DNA. No such amplicon was detected in the nontransformed root (negative control) sample. *Agrobacterium rhizogenes* Ri plasmid was served as positive control (figure 5).

Figure 6 shows the effect of elicitor treatments on

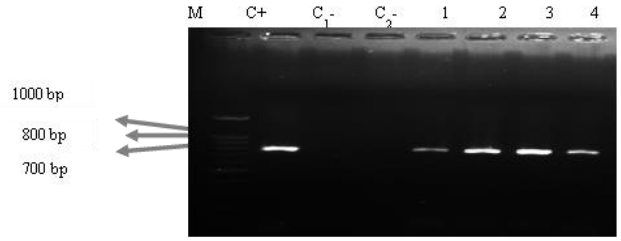

Figure 5. 1% Agarose gel electrophoresis of *rolB* gene PCR products. The *rol*B gene size is around 780 bp. M; Size DNA Ladder (1 Kb); C+; *A. rhizogenes* Ri plasmid as positive control. C_1- and C_2- : nontransformed root as negative control. Lane, 1 to 4: *H. reticulatus* hairy roots.

dry and fresh weight of adventitious roots of *H. reticulatus*. The results indicated that the highest level of fresh and dry weight (1.44 and 0.134 mg, respectively) was obtained with 0.05% concentration of colchicine after 48 h (p<0.05) (figure 6-A). Adventitious root fresh weight significantly increased with increasing the elicitor concentration and inoculation time.

ANOVA results showed that hairy roots biomass was affected by UV-B stress. High fresh weight was obtained in control hairy roots and with increasing exposer to UV-B radiation. Fresh weight decreased and the lowest level of fresh weight was observed in 9 min treatments. The results indicated that the lowest level of fresh weight (1.8 g) appeared with elicitation at 9 min (p<0.05) (figure 6-B). Growth of all hairy roots in culture media supplemented with different amounts of elicitors was normal, healthy and similar in appearance.

Antioxidant activity of hairy roots was estimated by the DPPH assay. In colchicine treatments, the maximum and minimum antioxidant activities were detected in 0.05% and non-transgenic roots respectively. In addition, antioxidant activity in elicited hairy roots with colchicine was increased to 27% (0.05 colchicine), in comparison to the antioxidant activity level in non-transgenic root (12%) and transgenic hairy roots (18%) (figure 7-A). Moreover, significant increases were noted in antioxidant activity of the roots that were exposed to UV-B light. Antioxidant activity in hairy root samples treated with UV-B radiation was increased to 26% after 9 min exposer to UV-B light in comparison to the non-transgenic roots (11.66%) and transgenic root (19%) (figure 7-B).

Colchicine disrupts mitosis by binding to tubulin, the protein subunit of microtubules, inhibiting the formation of microtubules and the polar migration of chromosomes. The result is a cell with double chromosome number [43]. Probably the increase of fresh and dry mass was due to an increase in the hairy root growth change in quantity and quality of plants secondary metabolites.

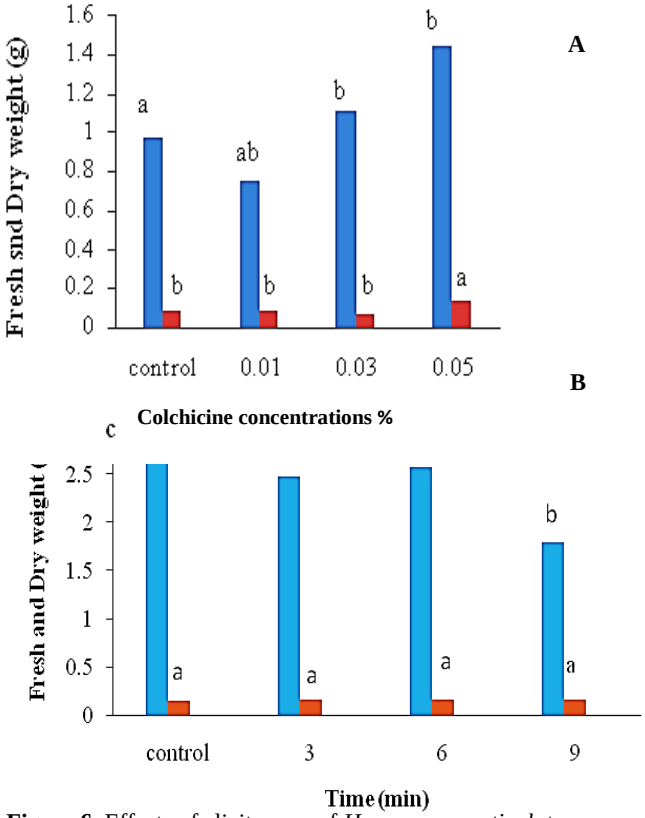

Figure 6. Effects of elicitors on of *Hyoscyamus reticulatus* hairy root fresh weight. A; colchicine, B; UV-B; different letters indicate significant differences at $p \leq 0.05$

Many other researchers have reported that the increase of ploidy often causes anatomical and structural changes [44,45]. Exposure of roots to

UV radiation results in multiple responses including increased synthesis of UV-screening pigments, as well as the reinforcement of the antioxidant system and other defense mechanisms. Many plants are capable of avoiding UV radiation by accumulating UV-filtering flavonoids and other secondary metabolites. For instance epidermal flavonoids are enhanced in response to increased UV-B [46]. High doses of UV-B and UV-C radiation affect growth, development, photosynthesis, and other important processes in plants negatively, leading to overproduction of reactive oxygen species (ROS) and development of oxidative stress, acting negatively on macromolecules, decrease cell viability and cell death [47].

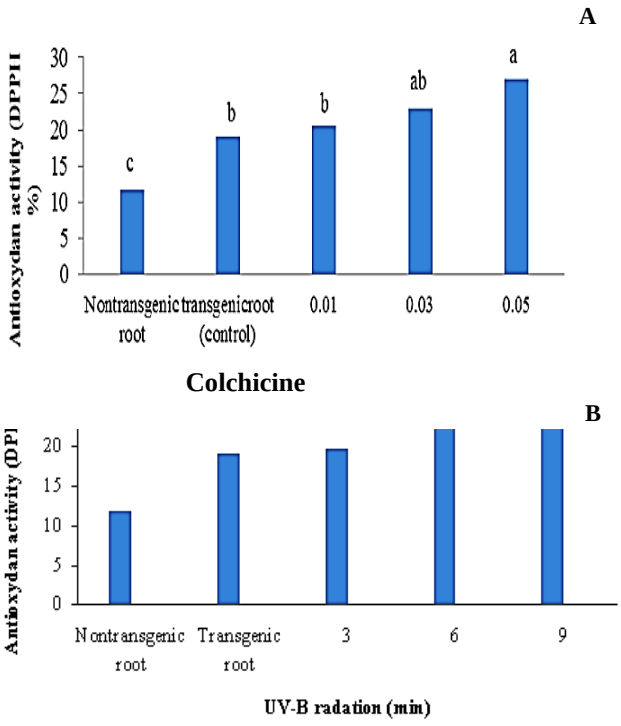

Figure 7. Influence of different elicitors on *Hyoscyamus reticulatus* hairy root antioxidant activity. A; colchicine, B; UV-B; different letters indicate significant differences at $p \leq 0.05$

To the best of our knowledge, colchicine application for increasing tropane alkaloids production is being reported for the first time. In the present report, GC/MS analysis showed that tropane alkaloid content significantly increased in induced hairy roots compared to non-transgenic roots. Hyoscyamine and scopolamine content in transgenic hairy roots was 0.48 and 2.8 % that was 3 and 8 folds more than control roots (0.18 and 0.37 %). But in elicited hairy roots with different concentrations of colchicine, hyoscyamine content was increased to 0.58%, but scopolamine content decreased to 1.9 %. The level of scopolamine production in hairy root samples treated with colchicine was reduced to 2.3 fold in comparison to the level of scopolamine production in control hairy roots (table 2).

Table 2. Influence of elicitation on tropane alkaloids content (%) in *Hyoscyamus reticulatus* hairy roots

	Tropane alkaloids content	
	Hyoscyamine (%)	Scopolamine (%)
Non transgenic root	0.18	0.37
transgenic root	0.48	2.8
Colchicine	0.58	1.9
UV-B radiation	0.33	0.68

GC/MS analysis showed that hyoscyamine and scopolamine content in transgenic hairy roots elicited by UV-B-light were affected, both hyoscyamine (0.33%) and scopolamine (0.68 %.) content was decreased in comparison to transgenic hairy roots although it was higher than non-transgenic roots (table 2).

In conclusion, although the physiological effects of colchicine or UV-B are not generally predictable, and the responses are often species-specific, depending on the concentration and exposure time, doubling the chromosome number of a plant increases the number of genes and thus changes enzymatic activity and isozyme diversity. This can affect the biosynthetic pathways of secondary metabolites. Induction of artificial autotetraploids in many medicinal plants has often increased quantities of secondary metabolites and also altered them in a qualitative manner [48-50].

Induction, production and elicitation of Hairy roots by various strains of *A. rhizogenes* have received a lot of engrossment from biotechnologists for the increasing production of secondary metabolites. Genetic stability, growth in hormone frees media and tends to produce high levels of secondary metabolites are characteristic of hairy roots. Considering the results of the present study, it could be concluded that the use of appropriate explants, bacterial strain and media type can improve the induction, formation and growth of hairy root cultures of *H. reticulatus*. Finally, hairy root techniques may be considered as a convenient system for production of hyoscyamine and scopolamine in hairy root cultures of *H. reticulatus***.**

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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