Hyoscyamine and scopolamine production in induced callus and hairy roots of *Hyoscyamus niger*

**ABSTRACT**

One of the biotechnological methods considered today to increase the drug content in medicinal herbs is hairy root induction by *Agrobacterium rhizogenes*-mediated transformation (ArMT). The study aimed at inducing hyoscyamine and scopolamine production in the callus and hairy roots of henbane (*Hyoscyamus niger*). To do this, seeds of wild *H. niger* growing in Hamadan province of Iran were collected and used for the experiments. *A. rhizogenes* strains were employed for hairy root induction. Furthermore, different concentrations of plant hormones auxin (NAA) and cytokinin (BAP) were applied for callus induction. ArMT of *H. niger* was confirmed by PCR amplification of rolB gene (780 bp) sequences. Upon results, induction of hairy root in *H. niger* was observed only when *A. rhizogenes* strains AR15834, A4 and D7 strains were used. Indeed, the highest percentage (63.33%) of hairy root induction was achieved by *A. rhizogenes* strain AR15834 applied to the leaf explants. However, root explants did not yield any hairy root and callus. Furthermore, the highest percentage (%100) of callus induction from *H. niger* leaf explants was observed in the solid MS medium containing 2 mg/L NAA; 2 mg/l NAA with 0.5 mg/l BAP; 2 mg/l NAA with 1 mg/l BAP; and 2 mg/l NAA with 2 mg/l BAP. Moreover, hyoscyamine and scopolamine contents were investigated by HPLC. Data indicated that dominant alkaloid both in hairy root and callus was hyoscyamine. Thus, hairy root induction by ArMT is being introduced for engineering *H. niger*, as a means for drug production enhancement.

**Key words:** Hairy root, callus, *Hyoscyamus niger*, hyoscyamine, scopolamine, *Agrobacterium rhizogenes*.

**Abbreviations:** *H. niger*, *Hyoscyamus niger*; *A. rhizogenes*, *Agrobacterium rhizogenes*; ArMT, *Agrobacterium rhizogenes*-mediated transformation; NAA, auxin; BAP, cytokinin.

**INTRODUCTION**

Henbane plant (*Hyoscyamus niger*) is a member of Asterales phylum, Solanaceae family and *Hyoscyamus* genus, with a chromosome number of 2n= 2x=28 (Lavania, 1986). Henbane is an herbage plant and its height reaches up to 80 cm. Its leaves are almost triangular and have relatively deep cuts. The length and the width of the leaves reach 20 and 7 cm, respectively.

This plant was previously anesthetized and used for painful muscle spasms caused by urinary tract disorder, asthma and hysteria and the most important active substances in this genus are alkaloids (Parr et al., 1990), mainly from the tropanian group. Some of tropanianalkaloids in henbane are hyoscyamine, atropine and scopolamine, which affect the central nervous system (Haas, 1995; Roddick et al., 1991). The plants of the Solanaceae family, such as henbane are commercial sources of these alkaloids that are synthesized in the roots of these plants, followed by the transfer and accumulation in leaves (Waller and Nowacki, 1977). Scopolamine is the most valuable tropane alkaloid, and since its global demand is
about 10 times higher than hyoscyamine, many efforts have been made to optimize its production (Hashimoto and Yamada, 1994). *Hyoscyamus niger*, *Hyoscyamus muticus* and *Hyoscyamus albus* are the most important sources for the production of scopolamine and hyoscyamine (Kosaku et al., 1993).

Massive and rapid production of some secondary metabolites is sometimes difficult or impossible with chemical methods. Organ cultures are relatively stable and secondary metabolites are produced in the cultivated organs in shorter periods than the original plant (Subroto et al., 1996). Absolutely, under controlled conditions, the secondary metabolites are not affected by environmental factors such as climate, pests, microbial diseases, and seasonal and geographical stresses.

Non-germination of live and healthy seeds, even under suitable environmental conditions such as water, light and oxygen is called dormancy or seed sleep (Hilhorst, 1995). Dormancy as a way of avoiding climatic stress is very important in preserving plant species. The length of the period of sleeping and the optimum conditions for seed germination depends on the genetic structure and the climate condition which the mother plant faces (Baskin and Baskin, 1999).

The callus is more or less an unstructured cell mass with a thin cell wall, and usually made up of parenchyma cells. The callus regeneration and seedling rooting ability depends on several factors including genotype, explants and its physiological conditions, culture medium and nutrient types, physical factors and environmental conditions such as light, temperature and pH, growth regulators and vitamins. In henbane plant, the type of explants and hormones play a decisive role in callus induction and plant regeneration from calluses (Basu and Chand, 1996). In general, the most important benefit of proliferation through tissue culture is the production of a large number of plants with the same genetic content and uniform quality in shorter time (Tripathi and Tripathi, 2003).

*Agrobacterium* (Rhizobiaceae) is well known as a gene transfer tool for plant cells (Berge et al., 1984). The host plants of Agrobacterium are often dicots, but monocots (Chawla 2000). These bacteria penetrate the wounded places of the host plants and cause the production of tumors of crown gall or hairy roots. These developmental responses are the result of a natural genetic engineering phenomenon which a DNA fragment is transferred from a tumor-inducing plasmid (plasmid Ti) or a root-inducing plasmid (plasmid Ri) from the agrobacterium to the plant cell. This transferred DNA fragment, called T-DNA, is integrated into the nucleus of the host plant. The TL-DNA region contains the genes rol (A, B, C and D). These genes play a major role in the production of hairy roots (Tzfira and Citovsky, 2008). Various factors including source and type of explant, culture medium, growth regulators, bacterial cell density, bacterial strain type and growth phase, infection method and time of co-culture are effective in transferring the intermediate gene of *Agrobacterium* (Swain et al., 2012).

The biggest advantage of hairy roots is the high production capacity of secondary metabolites in comparison with native plants. Rapid growth, reduced time, ease of storage, and ability to synthesize chemical compounds in hairy roots are among the benefits that have made them an important and permanent resource for the production of valuable secondary metabolites (Sevon and Oksman-Caldentey, 2002). Tropane alkaloids are mainly synthesized in the root and then transported to the air organs (Palazon et al., 1995). Due to the lack of efficiency of the undifferentiated systems such as cell or callus cultures in the production of tropane alkaloids (Palais et al., 2001; Gritothe and Drager, 2002), the attempt to produce scopolamine in biotechnological systems is mainly based on hairy root cultures (Palazon et al., 2008).

As a result of the medicinal importance of secondary metabolites of henbane, here, hyoscyamine and scopolamine productions were studied in henbane callus, as well as in henbane hairy roots induced by *Agrobacterium rhizogenes* in vitro.

**MATERIALS AND METHODS**

This research was performed in Biotechnology Laboratory of Agricultural Faculty, University of Bu-Ali Sina, Hamadan, Iran. Seeds of *H. niger* were collected from Hamadan province mountains. Chemicals and compounds of the culture were prepared from Merck Company (Germany) and PCR materials from Cinnagen Company (Tehran, Iran). PCR primers were purchased from Takapozist Company and the antibiotic cefotaxime from Jaber-Ebne-Hayyan Pharmaceutical Company (Tehran, Iran). *A. rhizogenes* strains AR15834, A4, AR318, LBA9402, A7, D7 and AR9534 were obtained from the National Institute of Genetic Engineering and Biotechnology of Iran. Data analysis was done using SPSS software version 16. MS medium (Murashige and Skoog, 1962) and ½ MS were used in this research.

**Seed germination**

Seeds were washed several times with water and then dried on paper. The sterilization of seeds was carried out under sterile conditions. The seeds were placed in 2.5% sodium hypochlorite for 4 min. Then, to remove the disinfectant solution, the seeds were washed three times with sterilized distilled water. Several treatments were applied for seed germination, including placing seeds (48 h) in distilled water, 30 s, 1, 5, 10 and 15 min in 98% sulfuric acid. In some treatments, gibberellic acid was added, seeds were immersed 7 days in gibberellic acid (240 mg/l) and...
then were cultured on a filter paper and gibberellic acid was added to it (240 mg/l).

Preparation of culture medium and A. rhizogenes suspension

The solid and liquid LB media were used for culturing the bacteria (Sambrook et al., 1989). To grow selectively of A. rhizogenes, 50 mg/l antibiotic rifampicin was added to the media.

A. rhizogenes was grown in liquid LB medium supplemented with antibiotic rifampicin at 28°C under dark conditions, at 110 rpm for 24 h. The concentration of bacterial suspension was determined using spectrophotometer (Lambda 45UV/Visibel) at OD600. An OD of 0.5 to 1.5 was considered for further experiments (Hongwei et al., 2006).

Preparation of plant explants and their inoculation with A. rhizogenes

Three-week old seedlings, containing 4 and 5 leaves from in vitro culture, were used for A. rhizogenes inoculations. The seedlings were divided to 0.5-1 cm explants (leaf with the petiole, stem, and root) under sterile conditions. Two or three wounds were created on explants to stimulate the bacterial pathogenesis system. Then the explants were transferred to a bacterial suspension, and the solution was shaken for 10 min. The explants were taken out of the bacterial suspension and transferred to solid MS media. The un-inoculated explants were transferred to a solid MS medium as control. Explants were incubated for 48 h in the growth room at 25 ± 2°C under dark conditions.

Transfer of inoculated explants to the culture medium containing antibiotic

To remove the bacteria, after co-culture, the explants were washed 3 times with sterilized distilled water and placed on a filter paper. Thereafter, explants were transferred on a solid ½ MS medium containing 500 mg/l antibiotic cefotaxime. Explants were kept at 25 ± 2°C with a light period of 16/8 h (light/darkness). For complete removal of bacteria in the next subcultures, lower concentrations (100-200-300 mg/l) of cefotaxime were used.

Hairy roots

After transferring of explants into the culture medium with cefotaxime, daily observations was performed to investigate the appearance of hairy roots and the health of explants inoculated with Agrobacterium. Upon appearance, the hairy roots (2.5-5 cm) were discarded and used for various experiments, including confirmation of transformation, the percentage of transgenic and the secondary metabolites. To ensure complete removal of the bacteria, the roots were transferred to a culture medium without cefotaxime.

Cultivation of hairy roots in liquid and semi-solid culture media

After subculture of roots for 6 to 8 weeks, 2.5 to 5 cm segments of root tips were cut and transferred to culture media containing 50 ml liquid ½ MS medium supplemented with 250 mg/l antibiotic cefotaxime. Also, the natural roots produced from control explants were placed in the same media. A number of hairy roots were transferred to ½MS semi-solid medium containing 250 mg/l antibiotic cefotaxime, 5.8 g agar per liter and 1% w/v active charcoal. The roots were incubated in the growth room on a shaker at a speed of 95 rpm, at 25 ± 2°C with a light period of 16/8 h (light/darkness) for further growth.

Preparation of culture medium and plant explants for callus induction

For callus induction in henbane, MS with different concentrations of hormones such as auxins (NAA) and cytokinin (BAP) were used according to (0 mg NAA with 0 mg BAP as control, 0 mg NAA with 1 mg BAP, 1 mg NAA with 0 mg BAP, 2 mg NAA with 0.5 mg BAP, 2 mg NAA with 1 mg BAP and 2 mg NAA with 2 mg BAP). Three-week old seedlings with 4 to 5 leaves were used for callus induction in vitro. The stems, leaves and roots of the seedlings were divided into 1-2 cm parts and transferred to callus induction medium containing different concentrations of auxin and cytokinin. Daily observation to investigate the appearance of callus and non-infection of explants was performed. After adequate and proper growth, calluses were used for various tests, including determining the percentage of callus induction and secondary metabolite production.

Extraction and determining the quantity and quality of genomic DNA

The genomic DNA was extracted using CTAB method according to Cai et al. (1997). The quantity of DNA was confirmed by spectrophotometry and gel electrophoresis.

Plasmid extraction from A. rhizogenes

A. plasmid of bacteria was extracted using alkaline lysis
Table 1: Sequence of specific primers for rolB gene proliferation.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences from 5’ to 3’</th>
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<tr>
<td>Forward primer</td>
<td>5’ATGGATCCAAAATTGCTATTCCTCCACGC3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’TTAGGCTTTTCATTCCGTTACTGCAG3’</td>
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**Confirmation of the presence of A. rhizogenes rolB gene in transgenic roots**

PCR with specific primers of rolB gene was applied to track the presence of A. rhizogenes rolB gene in transgenic roots (Table 1). PCR reaction solution in a final volume of 25 ml was consisted of 2 µl of Template DNA, 1 µl of Forward Primer (rolB), 1 µl of Reverse Primer (rolB), 1.5 µl of 10 mMdNTP mix, 2.5 µl of 50 mM MgCl₂, 2.5 µl of 10X PCR Buffer, 0.2 µl of TaqDNA polymerase and 143 µl of autoclaved distilled water. The temperature conditions included one cycle consisting of Initial denaturation at 94°C for 4 min, 34 cycles consisting denaturation at 94°C for one min, annealing at 60°C for one min and elongation at 72°C for 2 min and one cycle consisting final elongation at 72°C for 7 min, run in aBio-RAD T100 Thermocycler Amplified products were loaded on a 1% agarose gel with a voltage of 80 V and the bands by Gel doc (UV tec BTS-20-M) in ethidium bromide solution observed under ultraviolet light.

**RESULTS**

**Seed germination**

Seed germination was not observed when the seeds were disinfected after sterilization with distilled water. Seed germination percentages of 8, 24 and 29, using 1, 5 and 10 min of sulfuric acid treatment, respectively were observed. The results of the present study show that sulfuric acid use in a suitable time, and an appropriate concentration of gibberellic acid can lead to the germination of Henbane seeds. The use of gibberellic acid, together with sulfuric acid (10 min) showed 65% germination in H. niger seeds. The present study did not find any germination in low gibberellic acid concentrations. Immersion of seeds more than 7-days in gibberellic acid (240 mg/l) and culturing on filter paper in gibberellic acid (240 mg/l) resulted in 89.39% germination.

**Induction of hairy roots**

Stem, leaf and root explants from cultivated plants (Three-week old seedlings with 4 to 5 leaves) were prepared, and inoculated with 7 different strains of A. rhizogenes. The appearance of hairy roots from the wounds on the explants was monitored 14 days post-inoculation of A. rhizogenes. However, induction of hairy roots was observed only in three A. rhizogenes strains AR15834, A4 and D7.

Inoculation of leaf, stem and root explants by A. rhizogenesAR15834 strain showed that the hairy root appeared on leaf explants (ca. 63.33%) (Figure 1). Appearance of hairy roots on the stem explants using A. rhizogenesAR15834 strain was 15.33%. These stems were firstly induced to produce callus and hairy roots were formed on those calluses (Figure 2). A. rhizogenesA4 strain induced hairy roots only in leaf explants (17.67%). In the inoculation of leaf, stem and root explants with A. rhizogenesD7 strain, also induced hairy roots only in leaf explants (10.33%). However, after a while, these roots could not grow and were declined in the medium. In the LBA9402, A7, Ar9534 and Ar318 A. rhizogenes strains, it was not observed hairy roots in H. niger.

The results of the present study show that the type of explants and bacterial strains are very important for induction of hairy roots in the H. niger. Moreover, the leaf explants and A. rhizogenes AR15834 strain are better options for hairy root induction in H. niger.
**Figure 1:** The emergence of hairy roots from wounded sites (A) and end of petiole (B).

**Figure 2:** The conversion of stem into callus (A), and the appearance of hairy roots from callus 7 (B) and 14 (C) days after the appearance of the hairy roots obtained from the callus of stem.

**Growth of hairy roots in different mediums**

Initially, hairy roots were grown on solid ½ MS culture medium. Although the growth of hairy roots was rapid at the beginning, they showed a decreased growth after a short while, and the growth was stopped in some of the plant lines. To solve this problem, the hairy roots were transferred to 250 ml Erlen-Meyer flasks containing 50 ml of liquid ½ MS medium supplemented with 250 mg/L cefotaxime. The flasks were shaken at 90 rpm. It was observed that the growth of the hairy roots was much higher in the liquid medium (especially under the dark conditions) than the solid medium (Figure 3).

As a result of the small size and low density of hairy roots in the liquid medium, a weak growth was observed on shaker, but the solid medium did not show such a problem. Thus, the 1/2 MS medium was prepared, and 5.8 g agar with 0.1% active charcoal were added (Figure 4). The hairy roots in this medium showed a 3-fold growth every 21 days. Thus, the advantages of using this medium (Semi-solid ½ MS medium), as compared with liquid medium, are rapid proliferation in a short time, easy subculture and rescue of the healthy samples in the pollutions.

**Confirmation of rolB gene transfer to transgenic roots**

To confirm the transfer of the rolB gene into the plant genome, a portion of the gene was proliferated by the specific rolB gene primers (Table 1) using PCR reaction. PCR products of expected bands (ca. 780 bp) are shown in Figure 5. The DNA from non-transgenic roots as (negative control) and bacterial plasmid of A. rhizogenes as (Positive control) were used. The results indicated the presence of rolB gene in the transgenic hairy roots.

**Callus induction**

In the control medium (MS medium lacking any growth regulator) and the medium containing 1 mg/l BAP, callus induction was not observed in any of the stem, root and leaf explants. In the culture medium containing 1 mg/l NAA, callus induction was observed in leaf explants (ca. 10%) after 14 days of exposure, but no callus induction was observed in stem and root explants. In the medium containing 2 mg/l NAA, 2 mg/l NAA with 0.5 mg/l BAP, 2 mg/l NAA with 1 mg/l BAP, and 2 mg/l NAA with 2 mg/l BAP, callus inductions were observed in leaf explants (ca. 100%) and in stem explants (ca. 16-17%). However, root explants did not yield any callus.

**Morphological characteristics of the calluses**

All the calluses in the medium containing 2 mg/l NAA...
Figure 3: Cultivation of *H. niger* hairy roots in the liquid ½ MS medium.

Figure 4: The growth of *H. niger* hairy roots after 4 (A), 11 (B), 17 (C) and 21 (D) days on semi-solid ½ MS medium.


Supplemented with 0.5 mg/l BAP were transformed to leafy tissues. The color of calluses produced in all medium was yellowish green (Figure 6). The callus phenotypes were relatively soft and semi-compact. In none of the studied
medium, callus induction was observed in the root explants. The fresh weight average of calluses produced in leaf explants in 2 mg/l NAA with 0.5 mg/l BAP, 2 mg/l NAA with 1mg/l BAP, 2 mg/l NAA with 2 mg/l BAP and 2 mg/l NAA were 5.07, 4.11, 3.35 and 3.82 g (Figure 7), respectively, which is line with previous reports (Ibrahim et al., 2009) on explants of the leaf. The lowest fresh weight in leaf callus (1.57 g) was observed. The mean comparison of data was obtained by Duncan statistical procedure at P≤5% (Figure 7).

**Synthesis of scopolamine and Hyoscyamine alkaloids in H. niger hairy root and callus**

The amount of Hyoscyamine and Scopolamine alkaloids after extraction from hairy roots and callus was measured by HPLC. The results showed that the dominant alkaloid in hairy root and callus of *H. niger* was hyoscyamine. Moreover, the amount of hyoscyamine and scopolamine alkaloids in hairy root was higher than the callus. This finding is in line with previous reports (Hashimoto et al., 1986; Ibrahim et al., 2009; Kosaku et al., 1993).

**DISCUSSION**

Ehyai and khajehosseny (2012) observed 10% germination in cultivated seeds of Henbane plant (collected from khorasan province, Iran) on sterilized wet cotton with distilled water, and this is contrary to the result of the present study. This difference could be due to the genetic differences of the collected seeds. Those researchers also considered the possibility of seed dormancy in seeds with
less than 80% germination. Our result about the existence of dormancy in Henbane seeds supports the findings of Ehyai and khajehosseny (2012), Cuneyt et al. (2004) and Radosevich et al. (1997). Sharma et al. (2006) for 1 min using sulfuric acid, 6% germination, and Cuneyt et al. (2004) using sulfuric acid 1 and 3%, observed 26 and 26.5% germination, respectively in H. niger. According to Cuneyt et al. (2004), co-application of sulfuric acid and gibberellic acid (15 mg/l) had the highest germination rate in the H. niger seeds (64%). It is suggested that increasing the synthesis and release of gibberellic acid (GA3) in seeds cause the breakdown of stored starch and its conversion into useable materials to embryos and initiates the process of germination (Nadjaf et al., 2006).

The appearance of the hairy roots in wounded sites could be due to secretion and release of phenolic compounds, such as acetylsyringone, which absorbs the bacteria to the wound site and induced it to transmit T-DNA to the plant cell. Morphological assessments of the hairy roots showed novel characteristics such as rapid growth, abundant lateral branching and growth in medium deprived of hormone, which are characteristics of hairy roots, as compared with non-transgenic roots. In some reports, some of these characteristics of hairy roots are not observed, for example in Coffea arabica (Alpizar et al., 2008), possibly due to different expression of T-DNA genes encoding auxin and rolB in the plant genome.

Kosaku et al. (1993) and Zhang et al. (2003) reported the induction of hairy roots from the leaf explants of H. niger and H. muticus and this is similar to the results of the present study. A. rhizogenes AR15834 strain has been used to induce hairy roots in H. albus (Ataru et al., 2008). In our experiments, A. rhizogenes A4 strain induced hairy roots only in leaf explants (17.67%). This is in line with previous report (Zhang et al., 2003). Induction of hairy roots using A4 strain has been reported in H. muticus and H. albus (Mehar et al., 1998). It has been reported that inoculation of A. rhizogenes LBA9402 strain into leaf explant produced hairy root in Hyoscyamus sniger (Zhang et al., 2003).

Bacterial strains, species susceptibility, environmental conditions such as temperature, the presence of substances such as acetylsyringone, calcium and some enzymes in the culture medium can affect the appearance of hairy roots (Gururaj et al., 2006). Other various factors such as source and type of explants, culture medium, bacterial cell density and inoculum method, time of Co-culture and antibiotics of bacteria are effective in gene transfer with Agrobacterium (Swain et al., 2012). In accordance, induction of hairy roots, especially on the members of Solanaceae, has been facilitated using explants of leaf (Sevon and Oksman, 2002).

In line with the results of the present study, it is shown that in MS medium without growth regulators, callus induction was not observed in H. muticus, neither in leaf nor in root and hypocotyl explants (Ibrahim et al., 2009). They suggested that the addition of growth regulators to the culture medium stimulates the formation of callus on explants. Moreover, it seems that the type of explant and hormone play a critical role in callus induction (Basu and Chand, 1996).

Furthermore, addition of 2 mg/l NAA, 2 mg/l NAA with 0.5 mg/l BAP, 2 mg/l NAA with 1mg/l BAP and 2 mg/l of NAA with 2 mg/l BAP to culture media induced callus induction of H. muticus leaf explants (%100) (Ebrahim et al., 2009; Hashimoto et al., 1986). Moreover, Ibrahim et al. (2009) reported a 100% induction of callus on root explant, which contradict the results of the present study. This difference can be explained by differences between the two species of plants and their genetic difference. The results of the present study show that leaf explants are the best for callus induction in H. niger. The lowest fresh weight in leaf callus (1.57 g) was observed in the culture medium containing 1 mg/l NAA. This is contrary to the results of Ibrahim et al. (2009), who reported a fresh weight of callus of about 3.43 g.

**Conclusion**

In general, the results of the present study indicate the significance of the type of explant and strains of A. rhizogenes in the production of hairy roots in H. niger. Moreover, the type of hormone, culture medium and explant affects the production of calluses and secondary metabolites with medicinal properties, such as hyoscyamine and scopolamine. Thus, our results provide a good basis for Agrobacterium mediated gene transfer to different H. niger explants. Moreover, we provide information for enhancement of hyoscyamine production in H. niger with potential uses in biotechnology and pharmacology industry.

**REFERENCES**


