SODIUM NITROPRUSSIDE STIMULATED PRODUCTION OF TROPANE ALKALOIDS AND ANTIOXIDANT ENZYMES ACTIVITY IN HAIRY ROOT CULTURE OF *HYOSCYAMUS RETICULATUS* L.

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Hyoscyamus reticulatus L. is a herbaceous biennial belonging to the solanaceae family. Hyoscyamine and scopolamine as main tropane alkaloids accumulated in henbane are widely used in medicine to treat diseases such as parkinson's or to calm schizoid patients. Hairy roots media manipulation which uses elicitors to activate defense mechanisms is one of the main strategies for inducing secondary metabolism as well as increasing the production of valuable metabolites. Cotyledon-derived hairy root cultures were transformed by *Agrobacterium rhizogenes*. Sodium nitroprusside (SNP), a nitric oxide donor), was used in various concentrations (0, 50, 100, 200 and 300 µM) and exposure times (24 and 48 h). Treatment with SNP led to a significant reduction in fresh and dry weight of hairy roots, compared to control cultures. ANOVA results showed that elicitation of hairy root cultures with SNP at different concentrations and exposure times significantly affected the activity of as antioxidant enzymes such as catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). The highest hyoscyamine and scopolamine production (about 1.2-fold and 1.5-fold increases over the control) was observed at 50 and 100 µM SNP at 48 and 24 hours of exposure time, respectively. This is the first report of SNP elicitation effects on the production of tropane alkaloids in hairy root cultures.

Keywords: Elicitation – hairy root – *Hyoscyamus reticulatus* L. – nitric oxide – tropane alkaloids

INTRODUCTION

Hyoscyamine and scopolamine as tropane alkaloids are heterocyclic amines found in a number of solanaceae families such as *Mandragora*, *Scopolia, Atropa*, *Hyoscyamus,* and *Duboisia* as secondary metabolites. These alkaloids act on the parasympathetic system. They are also used in pharmacotherapy as antispasmodic, sedative and mydriatic agents [34]. Secondary metabolites are stored in cells in small quantities and are produced mainly in specialized cells at a certain stage of the plant's life cycle [12, 32]. Various biotechnological techniques can be employed in order to produce these compounds. However, low yields, low culture repeatability, and lack of stability are among limiting factors in undifferentiated cultures, including callus and cell suspension culture. Today, new techniques such as hairy root cultures with the aim of

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eliminating these defects are used [38]. The induction and growth of hairy roots are the most suitable systems used for the production of valuable metabolites. Under laboratory conditions, transformation of the plant by *Agrobacterium rhizogenes* results in the production of hairy root system with a high growth rate, the ability to synthesize important metabolites, and even unknown metabolites [1]. Other features like biochemical and genetical stability as well as the ability to grow in hormone free medium encourage their use in biotechnology [8]. Due to the low production of secondary metabolites in plant organs, different approaches such as using different stimulus and stimulation biosynthetic pathway to increase the production of metabolites in hairy root cultures are adopted [8]. Since the synthesis of plant secondary metabolites is a usual response of plants to environmental stresses, their synthesis can be stimulated by biotic and abiotic elicitors [37]. In other words, the elicitation is a way to mimic the stress effect that causes physiological responses in the synthesis and accumulation of secondary metabolites in hairy root cultures [3].

Sodium nitroprusside (SNP) is a combined donor of nitric oxide (NO) that is highly sensitive to light when it is in solution form, and its decomposition is accelerated by oxygen and high temperature. NO is a free radical gas that has relatively a long half-life (compared to other free radicals). It has a half-life of 3–5 seconds in biological systems [3]. NO has a complex biological function in plants. Toxicity of some stresses is also inhibited by NO, therefore it can also act as a cell protectant. Dual function of NO as an oxidant or antioxidant is dependent on the concentration and position of NO in cells [4, 35]. Recent studies have shown that NO can act as a signal transduction molecule phenomenon in plants and interfere in different pathophysiological, developmental and physiological processes, such as seed germination, stomatal closure, response to pathogens and root development [13, 31]. On the other hand, as a mediator, NO can participate in regulating plant growth process and metabolism of oxygen free radicals (ROS), and it has been shown by several studies that NO is, involved in transition of message and response to biotic and abiotic stresses [9]. Previous studies indicated that NO also participates in the production of secondary metabolites, such as ginseng saponins [21] and taxanes [39], in plant cell and tissue cultures.

To the best of our knowledge, no previous study has investigated the effects of NO as abiotic elicitor on the enhancement of the production of tropane alkaloids. The aim of this study was to evaluate the antioxidant activity, hairy roost growth and production of hyoscyamine and scopolamine elicitated by NO of different concentrations and exposure times in hairy roots culture of *H. reticulatus* L.

MATERIALS AND METHODS

Plant materials

The seeds of lattice henbane were obtained from Pakan Bazr Company, Esfahan, Iran. Seeds were successively surface sterilized in 70% (v/v) ethanol for one min and in 50% (v/v) NaOCl for 10 min. Then, they were washed three times with sterile dis-

tilled water. Next, seeds were cultured in MS medium [26] supplemented with 3% (w/v) sucrose, 7 g/L plant agar and 0.1 g/L myo-inositol. The pH of the medium was adjusted to 5.8 before adding agar. Culture medium was autoclaved at 121 °C for 20 min. Cultures were kept in artificial light (fluorescent tube giving light intensity 3500 lux) for 16 h followed by keeping them in the dark for 8 h at 25 °C.

Agrobacterium rhizogenes *cultures and hairy roots induction*

Agrobacterium rhizogenes A7 strain was grown on solid LB medium [7], supplemented with 50 mg/L rifampicin and incubated at 28 °C for 48 h. A portion of one-week-old cotyledon *in vitro* seedling was used for infection. The infected cotyledon fragments were placed on the MS medium under similar culture conditions. After 2 days, cotyledon fragments were placed on MS medium supplemented with 200 mg/L filter-sterilized cefotaxime. After 10 days, the hairy roots were induced at the wound sites. They were sub-cultured every 10 days. Hairy roots at every stage of the sub-culture were cultured on fresh MS medium with reduced concentrations of cefotaxime (100–50 mg/L) until bacteria were completely eradicated. Then, the cultures were transferred to 250 ml Erlenmeyer flasks which contained 30 ml hormone free liquid MS media, and were grown at 25 °C on a rotary shaker (110–120 rpm) in darkness and sub-cultured every 10 days.

Polymerase chain reaction analysis

The presence of *Agrobacterium rhizogenes rol*–B gene in *H. reticulatus* hairy root was evaluated through polymerase chain reaction. PCR amplification of DNA was performed in hairy roots and untransformed root using specific primers including forward 5′-ATGGATCCCAAATTGCTATTCCCCCACGA- 3′ and reverse 5′-TTAGGCTTCTTTCATTCGGTTTACTGCAGC- 3′. The PCR was performed under the following conditions: 94 °C for 5 min, 35 cycles of three steps containing denaturation 94 °C for 50 s, annealing at 60 °C for 1 min and 72 °C for 1 min and 10 s and 72 °C for 10 min for final extension using a thermocycler. The amplicons were analyzed by electrophoresis on 1% (w/v) agarose gel with 1 kb DNA ladder.

Elicitation experiments

A stock solution of SNP (Sigma–Aldrich, St. Louis, MO, USA) was prepared in distilled water and was sterilized by filtration (0.22 m sterile PES filters, Millipore, Billerica, MA, USA). To investigate the influence of SNP (NO donor), different concentrations of this elicitor (0, 50, 100, 200 and 300 μM) were added to MS culture medium. Hairy roots culture treated with SNP for 24 and 48 h was placed in elicitor-free liquid MS culture medium supplemented with 3% (w/v) sucrose and 0.1 g/L

myo-inositol to grow and produce tropane alkaloids. Hairy roots were harvested after ten days, and then they were air-dried and milled in order to extract alkaloid.

Alkaloid extraction

Tropane alkaloid of hairy roots was extracted by the method of Kamada et al. [22]. Five hundred mg of powdered plant material from hairy roots was homogenized in $CHCl_3$, MeOH and NH_4OH (25%) (15:5:1). After that, it was sonicated for 30 min and the material was kept at room temperature for 1 h under dark condition. The homogenate was filtered through paper filter and was washed twice with 1 ml $CHCl_3$. Then, the solvent was evaporated and dried using a rotary evaporator. 5 ml $CHCl_3$ and 2 ml of 1 N H_2SO_4 were added and mixed thoroughly. $CHCl_3$ fraction was removed and the solvent was adjusted to pH 10 with 28% NH_4OH on ice. Alkaloids were extracted once with 2 ml, and twice with 1 ml of $CHCl_3$. After the addition of anhydrous Na_2SO_4 was added, the solvent was filtered through filter paper. Then the residue was washed with 1 ml $CHCl_3$. At the final stage, the powder was dissolved in 500 µl MeOH following evaporation. The obtained extract was evaluated by GC-MS method.

GC-MS analysis

GC-MS analysis was carried out with a Hewlett–Packard HP 7890A GC (Palo Alto, CA, USA) equipped with a split/splitless injector and 5975C mass selective detector system. Chromatographic separation was carried out in a HP-5 capillary column (30 m × 0.25 mm, 0.25 µm in film thickness). The MS was operated in the EI mode (70 eV). The GC-MS interface, ion source, and quadrupole temperatures were set at 280 °C, 230 °C, and 150 °C respectively.

Preparation of enzyme extract

All enzymes analyzed in hairy roots were extracted in 50 mM Tris-HCl (pH 7.5) extraction buffer. 0.5 g roots were ground in 2.5 ml extraction buffer for 5 min using a pestle and mortar on ice, followed by centrifugation at 12,000 rpm and 4 °C for 20 min. Then, the supernatant was used as the crude extract to assay antioxidant enzyme activity.

Enzyme assay

Catalase (CAT – EC 1.11.1.6) activity was assayed by measuring the consumption of H_2O_2 at 240 nm [2]. The reaction mixture contained 2.5 mM of 50 mM potassium

phosphate buffer (pH = 7) and 20 μl of 3% hydrogen peroxide and 20 μl enzyme extract.

Peroxidase (POD – EC 1.11.1.7) activity was determined by measuring increases in absorbance at 470 nm due to the oxidation of guaiacol [24]. The reaction mixture contained 0.81 ml of 50 mM potassium phosphate buffer (pH = 6.6), 90 μl of 1% guaiacol, 20 μl of enzyme extract and 0.3% of hydrogen peroxide.

Ascorbate peroxidase (APX – EC 1.11.1.11) activity was determined by measuring the reduction in absorbance at 290 nm due to the oxidation of ascorbate [27]. The reaction mixture contained 2 ml of 50 mM potassium phosphate buffer (pH = 7), 0.2 ml of 50 μM ascorbate, 0.1 ml enzyme extract and 0.2 ml of 3% hydrogen peroxide.

Statistical analysis

The experiment was conducted as a factorial based one on a completely randomized design with three replications. Analysis of variance (ANOVA) of the results was performed using one-way ANOVA. The means were compared using Duncan's multiple range test at 99% confidence level ($P \leq 0.01$) using SAS 9.1 software.

RESULTS

PCR analysis

The presence of *rol–B* gene in the genomic DNA of putative transgenic hairy roots and non-transgenic roots were confirmed by PCR using specific primers yielded fragments of 780 bp. No amplification was observed in the control roots as negative control with the primers. All transformed hairy roots showed the presence of 780 bp *rol*–B product amplicons (Fig. 1).
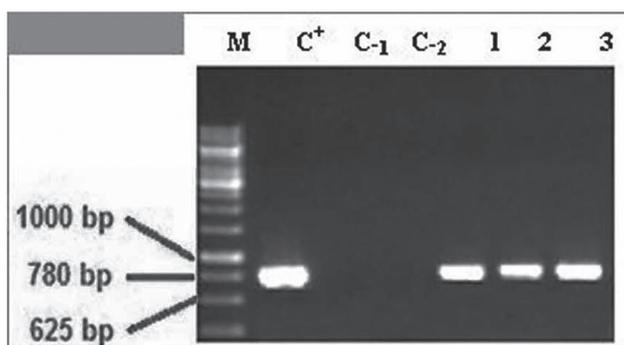

Fig. 1. Polymerase Chain Reaction analysis to determine the presence of *rol*–B gene in transgenic roots *H. reticulatus*. Lane M: Marker (1 kbp), Lane C+: Strain A7 bacteria Agrobacterium as positive control, Lane C–: Non-transgenic roots *Hyoscyamus reticulatus* L. as negative control, Induced hairy root at cotyledon explants by *A7 strain of A. rhizogenes*

Effect of NO on hairy roots growth

Analysis of variance (ANOVA) showed that the growth of *H. reticulatus* hairy roots are significantly ($P \leq 0.01$) affected by different exposure times and NO concentrations. The results showed that treatment with NO led to a significant reduction in fresh and dry weight of hairy roots compared to control cultures (Fig. 2a, b). The lowest hairy roots fresh and dry weights were found in the medium supplemented with 300 µM NO for 48 h (9 and 0.47 g, respectively) compared to fresh and dry weights of control hairy roots (12.75 and 0.65 g, respectively).
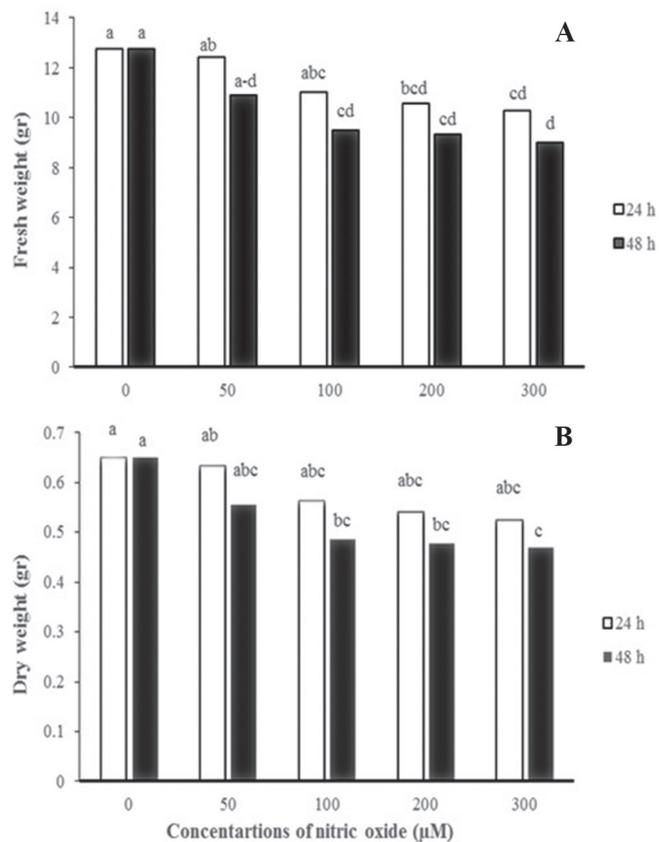

Fig. 2. The effects of different concentrations of NO and exposure time on (A) fresh and (B) dry weight of *H. reticulatus* hairy root. Different letters indicated significant differences ($P < 0.01$) given by Duncan test. White bars for 24 h and black bars for 48 h exposure time

Effect of NO on tropane alkaloids production

The GC-MS results revealed that NO significantly affected hyoscyamine and scopolamine production in hairy root cultures when applied at different concentrations and exposure times. The highest hyoscyamine accumulation was observed in the cultures exposed to 50 µM NO for 48 h (23.66% vs. 19.75% in the control cultures, about 1.2-fold increase) (Fig. 3). Treatment with the highest concentration of 300 µM NO for 24 h led to a reduction in hyoscyamine accumulation. The highest scopolamine production (23.56%) was observed in 100 µM NO at 24 h of exposure time (about 1.5-fold increase over the control) while the lowest scopolamine was observed in control hairy roots (15.1%).
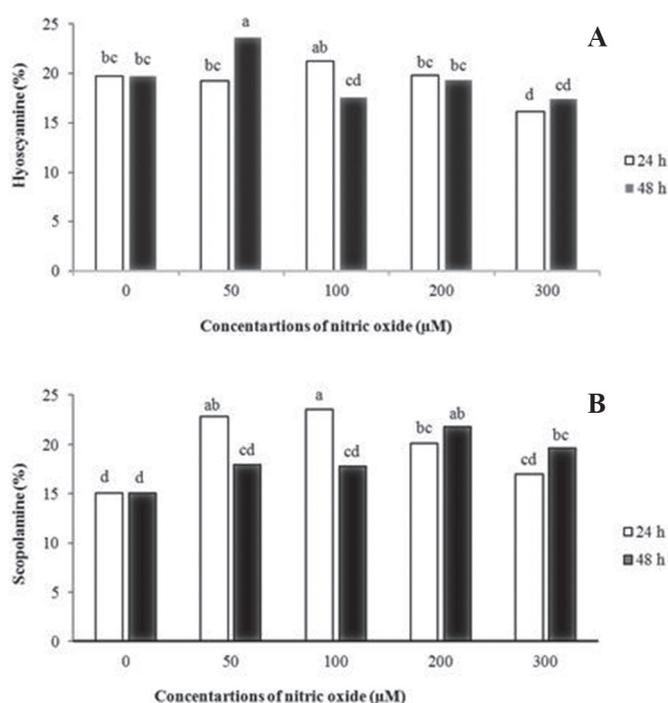

Fig. 3. The effects of different concentrations of NO and exposure time on (A) hyoscyamine and (B) scopolamine percentage of *H. reticulatus* hairy root. Different letters indicated significant differences ($P < 0.01$) given by Duncan test. White bars for 24 h and black bars for 48 h exposure time

The effect of NO on antioxidant enzymes activity of H. reticulatus *hairy roots culture*

The results revealed that the elicitation of hairy root cultures with NO at different concentrations and exposure times significantly affected POD and APX activity ($P \leq 0.01$) and CAT activity ($P \leq 0.05$) (Table 1). The highest CAT and POD activities

were detected in hairy root cultures exposed to 100 and 50 μM NO for 24 h, respectively, and the highest APX activity was observed in 300 μM NO at 48 h of exposure time.

Table 1

Variance analysis of the concentration and exposure time with NO on antioxidant enzymes activity of *Hyoscyamus reticulatus* hairy root

Treatments	df	Means of squares (MS)		
		CAT	POD	APX
Elicitor concentration (a)	4	1.86**	9.72**	4.83**
Treatment time (b)	1	0/93^{ns}	1/68^{ns}	0/02^{ns}
Reaction (a×b)	4	0/10*	2/57**	0/43**
Error	20	0/55	1/17	0/15
Coefficient of variation (%)		19/76	19/10	14/81

ns – non-significant; *significant at $P \leq 0.05$; **$P \leq 0.01$, respectively.

DISCUSSION

Our results demonstrated that by increasing the concentration and duration of NO treatment, *H. reticulatus* hairy root growth gradually declined. NO applied in relatively high concentrations disrupts the normal metabolism of plants by damaging membranes, proteins and nucleic acids in the cells. The changes in the potential of photosynthetic electron transport prevent the development of root and shoot form damaging DNA and cell death [18]. It is known that the induction of programmed cell death (PCD) can be determined by the interaction of NO and a variety of reactive oxygen such as superoxide anion and hydrogen peroxide [10]. Reduced growth and increased cell death under the influence of different concentrations of SNP were reported by Fadzillah et al. [16].

On the other hand, it has been reported that NO released from SNP stimulates the expression of genes encoding phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) in *Glycine max* [10]. Also, the transcriptional induction of PAL by NO in tobacco cell cultures have been reported [14]. PAL is a key enzyme of phenylpropanoid pathway. Actually, this enzyme intermediates between primary and secondary metabolism in the plants. Hence, it seems that phenylalanine pathway stimulated by NO can influence the production of tropane alkaloids, which is one of the side branches on the biosynthetic pathway of tropane alkaloids [14].

The results showed that the production of scopolamine at 24 h of NO exposure time was high in low concentrations, and vice versa. The external application of NO in plants or cell cultures leads to the suppression of the activity of some enzymes such as aconitase [9]. NO and transition metals such as iron can be combined to form the iron-nitrosyl complex. This process alters the structure and function of proteins. For example, NO radicals lead to the activation of guanylate cyclase and prevent acu-

nitase from working [19, 20]. Thus, the inhibition of cytosolic aconitase may be a factor acting as an iron regulator protein which increases the intracellular Fe^{2+} [39]. Therefore, it is expected that the stimulation of hairy roots by NO can provide result the production of the needed iron to carry out the enzymatic reaction, thereby leading to an increase in scopolamine production in low concentrations. Therefore, it seems that in most concentrations of NO, iron reacts with hydrogen peroxide thereby leading to the Fenton-type reaction [5]. Under normal growth conditions, there is a balance between the production and purification of free radicals in plant cells. By comparison, through induction stress, this equilibrium is similar to the increase in free radicals or the decrease in the amount or activity of scavengers in the cell [17].

Reactive oxygen species (ROS) compounds can react with proteins, lipids, carbohydrates and nucleic acids resulting in protein degradation, deactivation of enzymes and damage to membranes and decomposition of polysaccharides [25]. Plant cells posses a range of protective mechanisms to counteract the destructive effects of the active oxygen species. These mechanisms enable them to prevent the damage of basic cellular structures by collecting ROS. Cellular defense mechanisms include non-enzymatic antioxidants (such as ascorbate, glutathione, tocopherol and carotenoid) and antioxidant enzymes (such as superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase) [15]. NO acts as a secondary messenger in plants. One of the most important effects of NO is to act as an effective oxidant and antioxidant [40].

The ability of NO to protect against oxidative stress involves three ways:

1. Reaction with free radicals in the lipid to stop lipid oxidation;

2. The absorption of anion superoxide and conversion into proxy nitrite that is toxic to plants but can be neutralized by ascorbate;

3. Activating antioxidant enzymes of superoxide dismutase (SOD), CAT and peroxidase (POD).

The results of the present study indicate that the activity of antioxidant enzymes, with the exception of ascorbate peroxidase, increased at lower NO concentrations and decreased at higher concentrations. Perhaps one reason for a rise in the activity of antioxidant enzymes at low concentrations is that NO itself can eliminate the toxicity of active oxygen species through reaction with radical superoxide (O_2^-) to produce peroxynitrite ions ($ONOO^-$). In the physiological pH range, proxynitrite ion can receive proton to be decomposed into an nitrate anion and proton, or to react with hydrogen peroxide (H_2O_2) to produce nitrogen and oxygen anions [15 and 19]. As mentioned previously, the use of foreign NO in plants or in cell cultures leads to the prevention of some enzyme activities such as acunitase [9]. Blocking these enzymes reduces energy consumption, which can lead result in the reduction of the electron flow through the mitochondrial chain. This subsequently reduces the production of active oxygen species [30]. The dual functions of NO as a strong oxidizing agent or antioxidant are dependent on its concentration [9]. Therefore, it appears that at high concentrations, proximity nitrite reacts with the thiol group of proteins and unsaturated loop radicals to the membrane fatty acid which causes serious damage in the cell structure [15]. In another study, reduced activity of some enzymes such as superoxide dismutase, peroxidase and ascorbate peroxidase at high concentrations of NO

has been reported. The researchers attributed it to nitrosative stress in these concentrations [6, 15 and 29]. According to the findings, the activity of the ascorbate peroxidase enzyme increased as its concentrations went up. NO removes superoxide anion from the cell by converting that into hydrogen peroxide using the superoxide dismutase enzyme. On the other hand, by the induction of antioxidant enzymes, such as ascorbate peroxidase, it reduces the toxicity of hydrogen peroxide [23].

The results of this study revealed that the application of SNP as an abiotic elicitor was an effective method for the enhancement of tropane alkaloids. According to the findings, exposing the hairy roots culture of *H. reticulatus* to 50 µM NO for 48 h and 100 µM NO for 24 h was the best treatment for the elicitation of hyoscyamine and scopolamine, respectively. This study is the first report on NO application on hairy roots culture for the production tropane alkaloids. Based on our findings and similar ones in the literature on the positive effect of NO on the enhancement of secondary metabolite production, it seems that the application of NO as abiotic elicitors could be an effective strategy for increasing the productivity of pharmaceutical compounds in medicinal plants.

ACKNOWLEDGMENT

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