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Original Article

Effects of Silicon and AgNO3 Elicitors on Biochemical Traits and Antioxidant Enzymes Activity of Henbane (*Hyoscyamus reticulatus* L.) Hairy Roots

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Abstract

Lattice henbane *(Hyoscyamus reticulatus* L.) is an herbaceous, biennial plant belonging to Solanaceae family. *H. reticulatus* hairy roots were established from two-week-old leaves infected by A7 strain of *Agrobacterium rhizogenes* on solid Murashige and Skoog (MS) medium. In this study, abiotic elicitors including; Sodium silicate (Na_2SiO_3) with different concentrations (0, 1, 5 and 7 mM) and silver nitrate ($AgNO_3$) concentrations (0, 0.5, 1 and 2 mM) were added to hairy roots culture media. The results showed that, Na_2SiO_3 and $AgNO_3$ significantly affected hairy roots fresh weight after 24h. Also, the highest hairy root fresh weight was observed in the control, and with broadening elicitor concentrations, fresh weight was decreased in both treated hairy roots with $AgNO_3$ and Na_2SiO_3 but the effect of exposure duration was not significant. Biochemical analysis showed that total antioxidant activity (TAA), total phenol (TP), catalase (CAT), ascorbate peroxidase (APX) and Guaiacol peroxidase (GPX) activities were enhanced in elicitated hairy roots compared to non elicitated hairy roots. The highest CAT, APX and GPX activities were observed in hairy roots treated with 7mM Na_2SiO_3 and 2mM $AgNO_3$. Our results suggest that, Na_2SiO_3 and $AgNO_3$ can stimulate the antioxidant defense systems and protect the plants from subsequent stresses.

Keywords: *Agrobacterium rhizogenes*, *Hyoscyamus*, Abiotic stresses, Total antioxidant
Abbreviations: APX, ascorbate peroxidase, $AgNO_3$, silver nitrate; CAT, catalase; GPX, Guaiacol peroxidase; MS, Murashige and Skoog; Na_2SiO_3, Sodium silicate; TAA, total Antioxidant activity; TP, total phenol

Introduction

Lattice henbane (*Hyoscyamus reticulatus* L.) is biennial plant, belonging to the Solanaceae family and is native to arid and semi-arid regions of south-west Asia, Iran, and Turkey [1]. The major bioactive component of Lattice henbane is Hyoscyamine and scopolamine, which are medicinally important tropane alkaloids [2]. *Hyoscyamus* drug also exhibits a number of pharmacological effects, including spasmolytica, mydriatica, spasmolytica, analgetica, antiperspirants, asthma, Parkinson's disease, and motion sickness [3]. Insertion of T-DNA segments of Ri plasmid from the pathogenic soil bacterium, *Agrobacterium rhizogenes*, into the genome of plant induce hairy root initiation in inoculated explants. The advantage of hairy roots is that they often show rapid growth, increasing production of metabolites, genetic stability and multiply promptly in liquid media without plant growth regulators. Hairy root Cultures cause important changes on some secondary metabolites production under *in vitro* condition. T-DNA of Ri plasmids comprises the genes encoding oncoproteins *rolA*, *rolB*, *rol*C and *rol*D which play significant role in hairy root formation with the aim of genetic manipulation of secondary pathways [4-6]. The effect of the *rolB* is

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similar to the stress induction in plants and plays a significant role in plant defensive reactions. Reactive oxygen species (ROS) are produced in all cell types and serve as important cellular signals for both intra- and inter-cellular communications. In fact, ROS are key signals regulating growth and development and coordinating responses to abiotic and biotic stress [7].

In order to enhance secondary metabolites production in plant tissue cultures, many conventional scenario have been applied. Biotic elicitors (derived from a pathogen or plant) and abiotic elicitors (physical, mineral and chemical compounds) are compounds that trigger the increased production of secondary metabolites in plant cells [8, 9]. Silicon (Si) is the most abundant mineral element in the soil and various beneficial effects of Si have been identified in plants. The previous studies have shown that the addition of Si to the tissue culture medium decreases oxidative damage and oxidative phenolic browning and enhances antioxidant enzymes, secondary metabolites production, and tolerance to both biotic and abiotic stresses [10, 11]. Si application also increases plasma membrane H^+-ATPase activity, which may be related to the silicon-mediated reduce in oxidative damage to proteins under stress [10, 11]. Heavy metals, usually promote accumulation of ROS in plant cells, which consequently stimulated overexpression of specific antioxidant enzymes and increased tolerance to a wide range of abiotic stresses in transgenic plant [12].

The $AgNO_3$ plays a significant role in somatic embryogenesis, shoot formation, and efficient root formation, which are the prerequisites for successful genetic transformation. The addition of $AgNO_3$ to culture media considerably improved the regeneration of both dicot and monocot plant tissue cultures [13].

To the best of our knowledge, there are not enough data about the effects of Na_2SiO_3 and $AgNO_3$ on antioxidant compounds and antioxidant enzymes of transformed Lattice henbane hairy roots. Therefore, the objective of this research was to study the effects of Na_2SiO_3 and $AgNO_3$ on biomass, antioxidant capacity, Total phenol, and antioxidant enzyme activities of Lattice henbane hairy roots.

Material and Methods

Seed Culture

All experiments were done in horticulture departments of Urmia University during 2014-2015. Seeds of henbane (*H. reticulatus*) were collected from the foothills surrounding areas of Piranshahr, Iran. In order to break their physiological dormancy, the seeds were treated by immersion in 500 mg L^{-1} gibberellic acid (GA3) solution for 24 h in darkness at room temperature (25°C) and washed three times with sterile distilled water. The seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 50% (v/v) NaOCl solution for 10 min, followed by three rinses with sterile distilled water. Seeds were cultured in MS medium ([14] containing 30 gL^{-1} sucrose and 7 gL^{-1} plant agar (Dutchefa-Netherlands). The pH of the medium was adjusted to 5.8 with KOH (1 N) or HCl (0.1 N) prior to autoclaving at 121°C for 15 min. The seedlings were grown under white fluorescent light (60 µmol m^{-2} s^{-1}) at a photoperiod of 16/8 h (light/dark) and temperature of 25±2 °C.

Bacterial Strains

The A7 strain of *A. Rhizogenes* was used in the present study. A7 strain was obtained from the National Institute for Genetic Engineering and Biotechnology Microbial Bank, Tehran, Iran (NIGEB). Single clone of the bacterial strain was grown in Luria-Bertani (LB) medium [15] containing 50 mgL^{-1} rifampicin, at 28 °C for 48 h on a rotary shaker (180 rpm) in dark condition. The bacteria suspension was collected from the liquid medium by centrifuging (5000 rpm) for 10 min and bacterial pellet were resuspended in MS liquid medium (*Agrobacterium* Infection Medium) containing 50 mgL^{-1} sucrose, pH=5.5. Before using bacterial suspension for inoculation, its concentration was adjusted at Abs_{600}= 0.4–0.5.

Hairy Root Initiation and Establishment

Approximately 2 weeks after seed germination, cotyledon explants gently were scraped and infected with the *A. rhizogenes* A7 strain for 1 min by immersion method. Then transferred to MS hormone-free solid medium which is supplemented with 30 gL^{-1} sucrose, pH=5.8, 7 gL^{-1} plant Agar (Duchefa- Netherlands), 0.1 $g.L^{-1}$ myo-inositol (Duchefa, Netherlands). These cultures were maintained at 25 ºC with a daily 16h artificial light and 8h dark period. After 48h co-cultivation, infected explants were washed with sterile distilled water and cefotaxime (Fig. 1a). The explants were transferred to MS solid medium fortified with Cefotaxime (200 $mg.L^{-1}$). The hairy roots emerging

from the inoculated sites were maintained on the same respective media (complete darkness under foil) and the antibiotic concentration was gradually reduced (Fig. 1b,c).

Optimization of Hairy Root Growth in Liquid Media

After cultures had been cleared of bacteria, high growing hairy roots in the MS solid medium were selected and transferred to 250 mL Erlenmeyer flasks containing 30 mL of MS liquid media with 3% sucrose on a rotary shaker (180 rpm) in dark at 28 ± 1°C. Subcultures at 7 days interval were made in the same media (Fig 1d,e).

Treatment of Na_2SiO_3 and $AgNO_3$ at Various Concentrations

Approximately, 100 mg hairy roots were transferred in 250 mL Erlenmeyer flasks containing 30 mL of MS medium, supplemented with 3% sucrose. The Na_2SiO_3 and $AgNO_3$ elicitors separately were filter-sterilized (0.22 µm) and different concentrations of them were added to 7-day-old hairy root cultures for 24h. Thus Na_2SiO_3 was used at 0, 1, 5 and 7 mM and $AgNO_3$ was used at 0, 0.5, 1, 1.5 and 2 mM. Stock solutions were prepared by dissolving the elicitors in distilled water and adjusting the pH to 5.5. Then hairy roots were washed with sterile distilled water and were transferred to liquid MS medium and were kept in the same conditions experienced before. After 24h, the hairy roots were taken out and washed with sterile distilled water and biomass (fresh and dry weight) was measured.

Total Phenolics Content (TP)

Total phenolic compounds were evaluated according to the Folin-Ciocalteu method ([16]. A total of 0.2–0.4 g of each sample was weighed into a 10 mL flask and filled up with distilled water. The extracts were centrifuged (5, 000 rpm) for 5 min. Two hundred µL supernatant were mixed with 800 µL of Na_2CO_3 solution and 1 mL of Folin reagent. The samples were kept for 120 min at room temperature before the absorbance was measured at 765 nm. Gallic acid was used as a standard and the results were expressed as mg Gallic acid equivalents (GAE) per 100 g FW.

Total Antioxidant Activity (TAA)

For the determination of 2, 2-dipheynl-1-picrylhydrazyl (DPPH) free radical scavenging activity, samples were extracted with methanol. Then, they were centrifuged (Sigma 3K30, Germany) at 15,000×g for 10 min. The supernatants were concentrated under reduced pressure at 40 °C. The dried extracts were dissolved in methanol. The total antioxidant activity was evaluated by DPPH method [17]. The absorbance of samples was measured by spectrophotometer at 515 nm. Methanol was used as an experimental control. The percent of inhibition was calculated according to the following equation:

%inhibition of DPPH=(Abs control-Abs sample)/Abs control×100; Abs control is the absorbance of DPPH solution without the extract.

Antioxidant Enzyme Measurements

The enzymes were extracted by homogenizing 5 g fresh tissue in 3 mL extraction buffer (Tris-HCl 50 mM (pH=7) containing 3 mM $MgCl_2$ and 1 mM EDTA). The homogenate was centrifuged at 5, 000 g for 20 min and the supernatant was used for enzyme assay [18].

Ascorbate Peroxidase (APX)

The Ascorbate peroxidase (EC 1.11.1.7) activity was assayed according to the following method of Nakano and Asada [19]. The reaction mixture contained 50 mM potassium phosphate, pH 7.0, 1 mM AsA, 10 mM/L H_2O_2 and 0.1 mL enzyme extract. The total reaction volume was 1 mL. The rate of change in absorbance at 290 nm was measured, and the level of enzyme activity was expressed as the difference in absorbance.

Catalase (CAT)

The Catalase (EC 1.11.1.6) assay was done according to the following method of Aebi [20]. Briefly, 20 µL of 100-fold diluted tissue supernatant were added to 980 µL of the assay mixture comprising 900 µL of 10 mM/L of H_2O_2, 50 µL of Tris HCl buffer (pH 8.0) and 30 µL of distilled water. The rate of decomposition of H_2O_2 was read by spectrophotometer at 240 nm. One unit of CAT activity is expressed as that amount of enzyme which breaks down 1 µmol of H_2O_2/min.

Guaiacol Peroxidase (GPX)

GPX activity was assayed according to Upadhyaya *et al.* [21] Method. The reaction mixture contained 100 mM potassium phosphate, pH 6.5, 15 mM guaiacol, 10 mM/L H_2O_2 and 60 µL Enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of guaiacol was determined by

the increase in A_{470} (£=26.6 mM^{-1} cm^{-1}). One unit of GPX activity was defined as the amount of enzyme that produced 1 µmol min^{-1} oxidized guaiacol under the above assay conditions.

PCR Analysis

Genomic DNAs from transformed hairy roots and non–transformed roots (0.5 g FW) were isolated by the CTAB method [22]. PCR analysis was used to investigate the presence of *rolB* gene in hairy roots. The *rolB* gene specific primers for the amplification of the 780 bp fragment were F: 5'ATGGATCCCAAATTGCTATTCCCCCACGA-3' and R: 5'-TTAGGCTT CTTTC ATT CGGTTTACTGCAGC-3'. PCR was performed in total volumes of 25 µL of reaction mixture containing: 1 µL of each primer (10 µmol/L), 2.5 µL of 10X PCR buffer (Mg^{2+} plus), 10 µmol/L dNTPs, 1 unit of *Taq* polymerase with 25 ng of genomic DNA as template. The PCR reactions were as follows: initial denaturation for 5 min at 94 ^{o}C, followed by 39 cycles consisted of denaturation for 1 min at 94 ^{o}C, annealing for 80s at 53 ^{o}C, extension for 80s at 72 ^{o}C and further extension for 10 min at 72 ^{o}C. Amplified DNAs were visualized by 1% (w/v) agarose gel electrophoresis.

Statistical Analysis

The two separate experiments were performed based on the randomized completely design with 3 replicates per treatment. The significant differences among means of data were determined by Duncan's Multiple Range Test using SAS software (9.3). Differences at $p \leq 0.05$ were considered as significant.

Results

Effect of Na_2SiO_3 and $AgNO_3$ on Fresh Weight and Dry Weight

The results illustrated that Na_2SiO_3 and $AgNO_3$ hairy root fresh weight were significantly affected after 24h ($p \leq 0.05$) (Figs. 2a, 2b). In low concentration of Si (1mM), hairy root growth was increased (8.5 gr), but significant difference was not recorded from non-treated hairy roots. But with increasing Si concentration, hairy root growth was prevented.

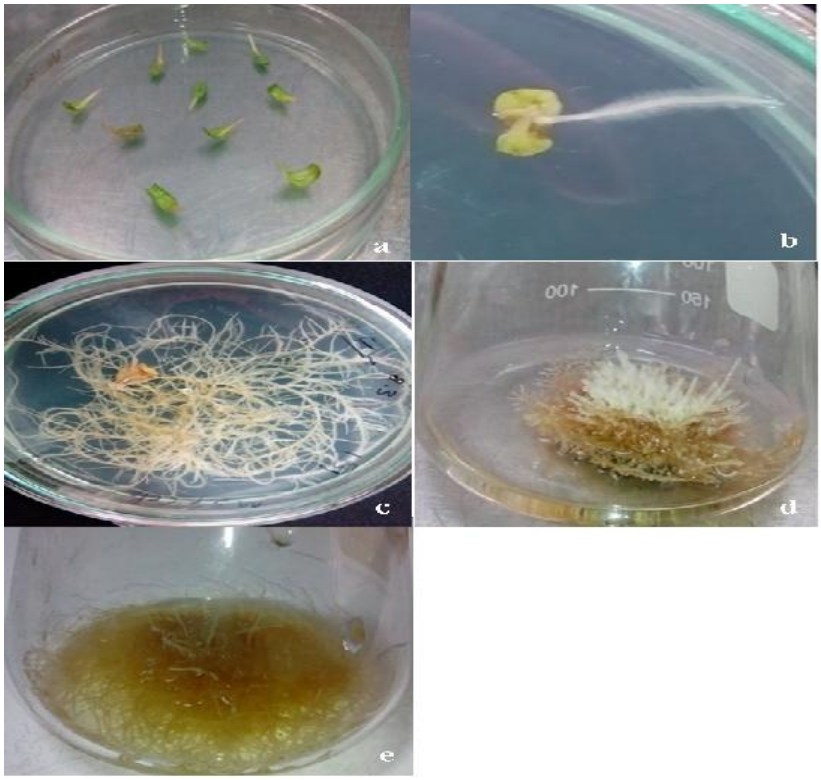

Fig. 1 A7 induced hairy roots in cotyledon explants of *Hyoscyamus reticulatus* L.. a) two-week-old leaves, b) Hairy root appearance, c) Hairy roots growth in Hormone-free solid MS media 4 weeks after induction, d) Hairy roots growth on hormone-free liquid MS media, e) Hairy roots growth in MS media 24 h after elicitation.

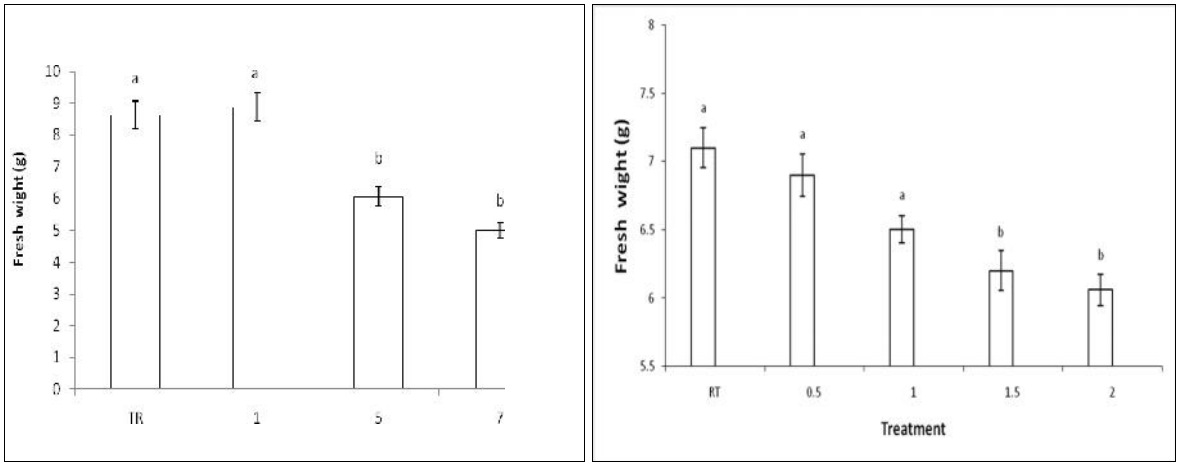

Fig. 2 Effects of Si (a) and $AgNO_3$ (b) on *Hyoscyamus reticulatus* L. hairy root fresh weight after 24h of treatment. TR—transformed hairy roots. Different letters on top of bars indicate significant differences at $P \leq 0.05$.

Hence, the lowest fresh weight was obtained in 7mM Na_2SiO_3 compared to non-treated or control roots (Fig. 2a). Also in $AgNO_3$ treatments, the highest hairy root fresh weight (7 gr) was observed in the control and fresh weight significantly decreased in high concentration of Ag (Fig. 2b). No significant difference in hairy root dry weight was observed after 24h.

Effect of Na_2SiO_3 and $AgNO_3$ on Total Phenolic Content

The current study revealed that TP significantly increased after 24h ($p \leq 0.05$). The highest TP of elicited hairy roots was observed in 7 mM Na_2SiO_3 (Fig. 3a). According to the $AgNO_3$ elicitor, the highest content of TP was observed in 2mM $AgNO_3$ (Fig. 3b). Effect of Na_2SiO_3 and $AgNO_3$ on Total Antioxidant Activity (TAA)

Plant cells are equipped with antioxidant defense systems such as enzymatic and nonenzymatic antioxidants. ANOVA results showed that Na_2SiO_3 and $AgNO_3$ significantly affected TAA of hairy roots after 24h ($p \leq 0.05$). TAA was significantly enhanced using Na_2SiO_3 (7 mM) concentration (Fig. 4a). As shown in Fig. 4b, the highest TAA of elicited hairy roots was observed in $AgNO_3$ (2mM) compared to none elicitated (control) hairy roots. It seems that Si has more effect on the increasing of TAA than $AgNO_3$.

Effect of Na_2SiO_3 and $AgNO_3$ on The Activity of CAT, APX, and GPX

The results indicated that, the activity of antioxidant enzymes were significantly altered by Na_2SiO_3 and $AgNO_3$ treatments ($p \leq 0.05$). The pattern of CAT activity in hairy root was significantly different between treated and control samples. The hairy roots treated with 7mM Na_2SiO_3 and 2mM $AgNO_3$ had the highest CAT activity at the 24h (Figs. 5a, 5b).

According to the results, the APX activity significantly increased after 24h. As a result, the both of Na_2SiO_3 and $AgNO_3$ treatments significantly affected hairy root APX activity ($p \leq 0.05$). The highest APX activity was observed in hairy roots treated with 7mM Na_2SiO_3 and 2mM $AgNO_3$ (Figs. 6a, b).

The activity of GPX in the hairy roots treated with Na_2SiO_3 and $AgNO_3$ treatments were significantly ($p \leq 0.05$) increased compared to control. The highest GPX activity was observed in hairy roots treated with 7 mM Na_2SiO_3 after 24h (Fig. 6c), while 2mM $AgNO_3$ was showed the highest activity of GPX after 24h (Fig. 6d).

Molecular Analysis

In this study, the integration T-DNA of *A. rhizogenes* Ri plasmid into *H. Reticulatus* genome was confirmed by PCR analysis using primers specific to *rol*B. The electrophoresis of hairy root genomic DNA revealed the presence of expected 780 bp band of *rol*B gene, but no such amplicon was observed in the untransformed root sample (negative control). *A. rhizogenes* plasmid was used as positive control (Fig.7).

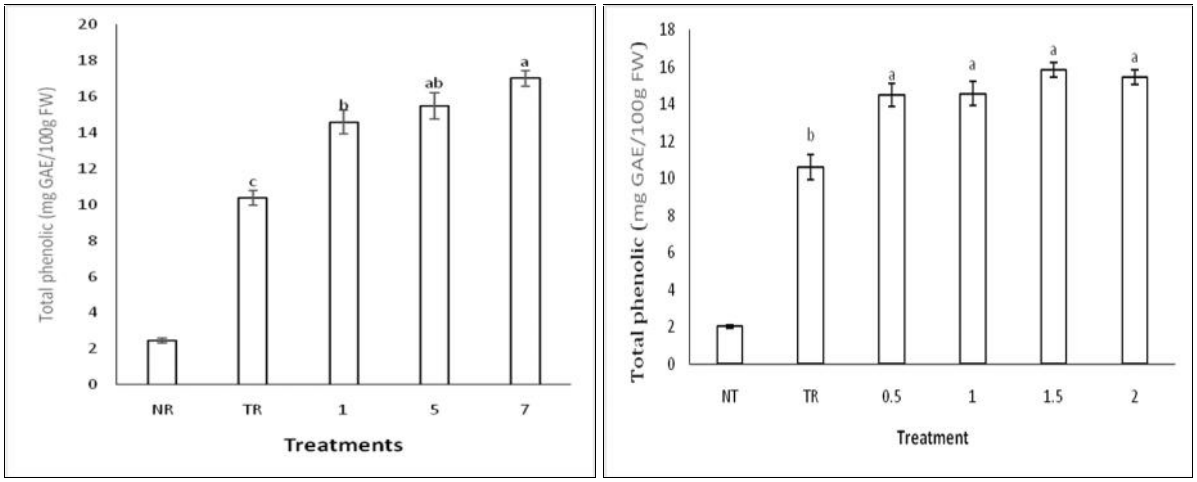

Fig. 3 Effects of Si (a) and $AgNO_3$ (b) on TP of *Hyoscyamus reticulatus* L. hairy roots after 24h of treatment. NR-non-transformed root; TR-transformed hairy roots. Different letters on top of bars indicate significant differences at P≤0.05.

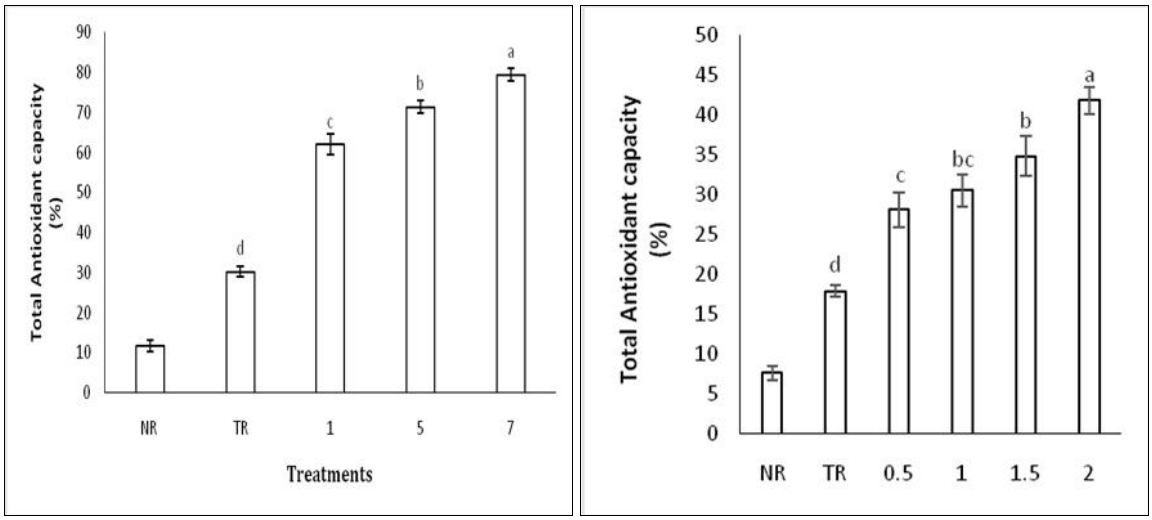

Fig. 4 Effects of Si (a) and $AgNO_3$ (b) on the TAA of *Hyoscyamus reticulatus* L. hairy roots after 24h of treatment. NR-non-transformed root; TR-transformed hairy roots. Different letters on top of bars indicate significant differences at P≤0.05.

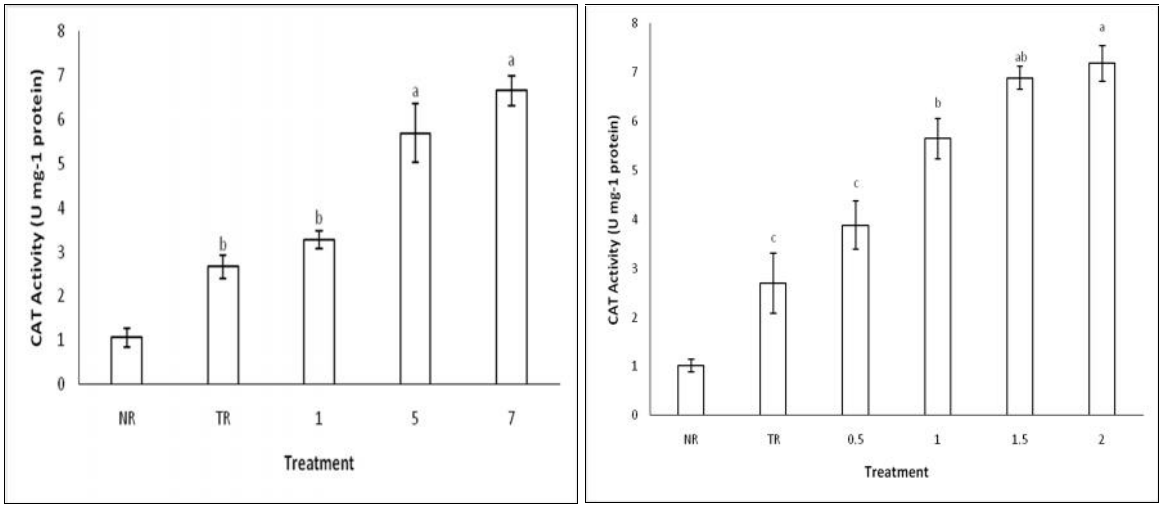

Fig. 5 Effects of Si (a) and $AgNO_3$ (b) on the CAT enzyme activity of *Hyoscyamus reticulatus* L. hairy roots after 24h of treatment. NR-non-transformed root; TR-transformed hairy roots. Different letters on top of bars indicate significant differences at P≤0.05.

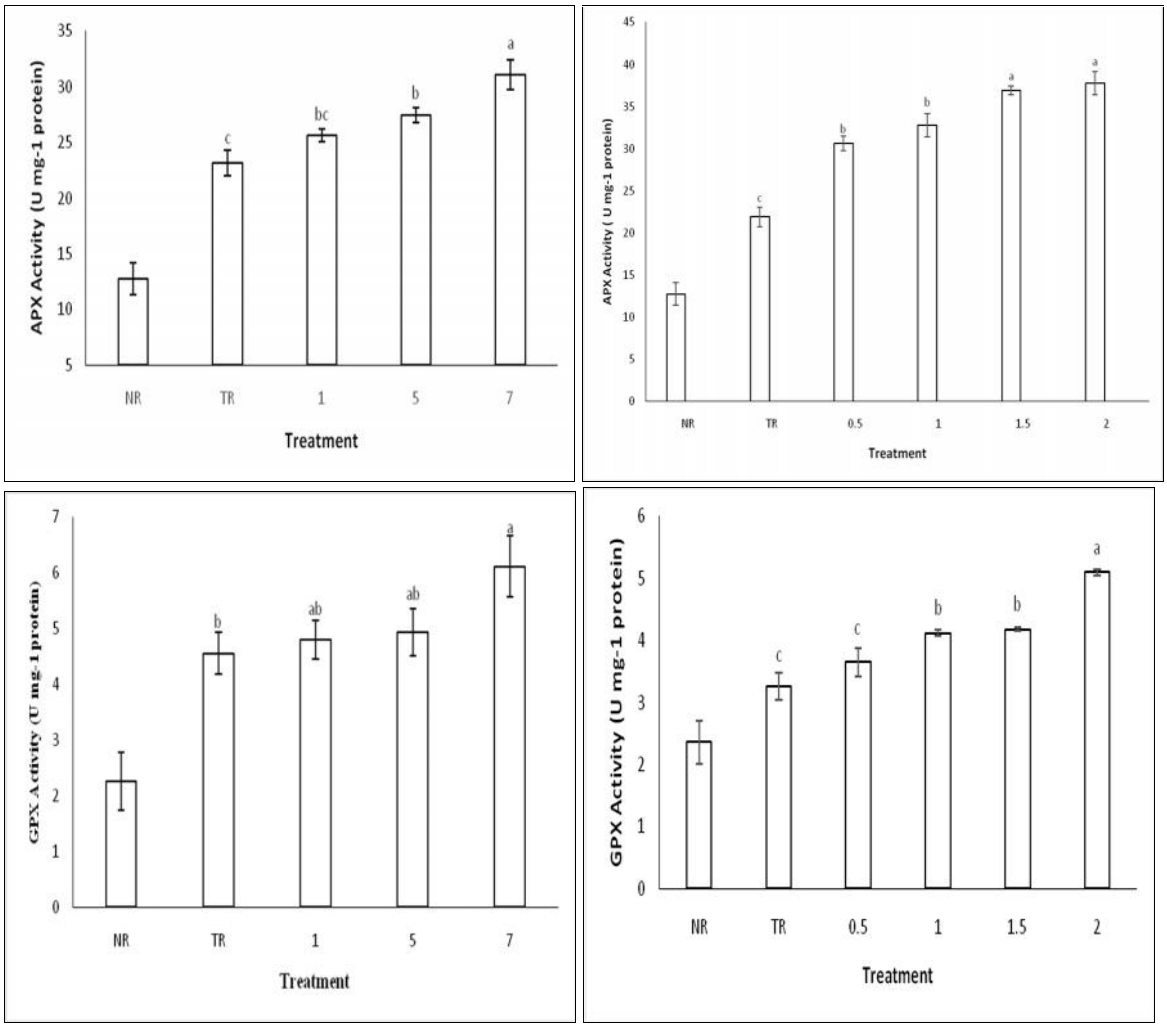

Fig. 6 Effects of Si (a, c) and $AgNO_3$ (b, d) on APX and GPOX enzymes activity of *Hyoscyamus reticulatus* L. hairy roots after 24h of treatment. NR-non-transformed root; TR-transformed hairy roots. Different letters on top of bars indicate significant differences at P≤0.05.

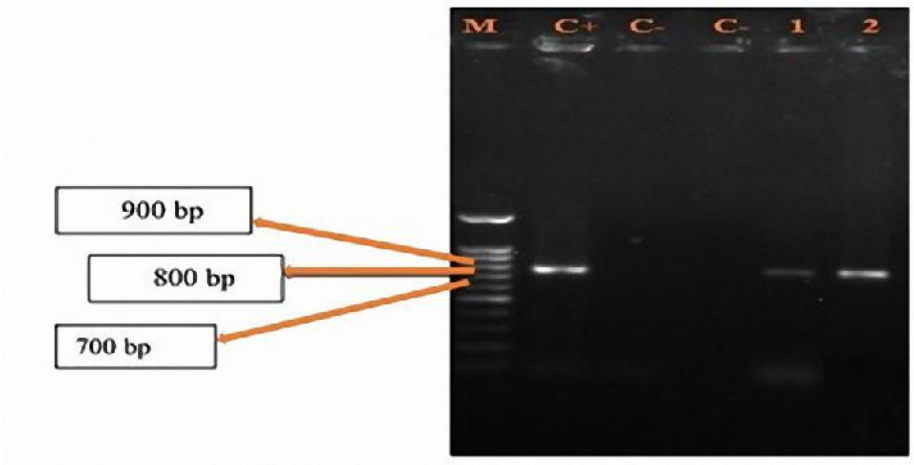

Fig. 7 PCR amplification of *rol*B gene in *Hyoscyamus reticulatus* L. hairy roots. Lane M: Molecular size marker (1 kb ladder Fermentase); lanes 1–2: Transgenic hairy roots induced on two-week-old leaf explants infected by A. rhizogenes A7 strain; lane C–: Non transformed roots as negative control; lane C+: *A. rhizogenes* A7 strain as a positive contro

Discussion

Elicitors play a significant duty in plant cells intracellular signal transduction systems and promote plant defense reaction and synthesis of phytoalexin. It has been demonstrated that the exogenous treatment of elicitors, enhance the production of secondary metabolites in plant cells [23-25]. The inhibitory effect of elicitors on growth rate has been reported by several authors [24, 26].

The mode of action of $AgNO_3$ in plant tissue culture is assumed to be associated with the ethylene production and action, which competes with the ethylene for binding sites on its receptor [27]. So, the results indicated that treatment with $AgNO_3$ cause increase in hairy roots TAA. Also, it has been revealed that use of silicon cause decrease in the malon di aldehyde (MDA) concentration, on the other hand increased antioxidant capacity in plant under stress and reduced membrane lipid peroxidation [10]. In fact, the Si is a key component involved in stress resistance mechanisms of plants [10].

Phenolic compounds are the important components of antioxidants. The elicitors activate phenylalanine ammonia lyase (PAL) as the key enzyme in phenolics, jasmonate and salicylic acid biosynthesis, the latter considered as the main activator of local and systemic acquired resistance [23, 28]. The effect of Si on phenolic compounds has been previously observed by Dragisic Maksimovic *et al.* [23]who reported the application of Si induced a significant increase in the levels of phenolic compounds. Since, the elicitors of Na_2SiO_3 and $AgNO_3$ increased TP of hairy roots, it can be demonstrated that Na_2SiO_3 and $AgNO_3$, in addition to activation of PAL, has a role in establishing the systemic resistance network by affecting on enzymes activity. Our results on the effect of Na_2SiO_3 on TAA are in agreement with Liang *et al*., [10] who reported the decreasing of oxidative damage by silicon is related to its involvement on the antioxidant systems.

The effect of Na_2SiO_3 and $AgNO_3$ on enhancing the activity of CAT, APX and GPX after 24h indicates the major role of these minerals in establishing resistance networks in hairy roots of henbane. Therefore, it can be concluded that Na_2SiO_3 and $AgNO_3$ may play a direct or indirect role in the systemic resistance network. Many reports have demonstrated the effect of $AgNO_3$ on plant tissue culture [27]. It has been previously observed that heavy metals increased CAT activity to decrease the oxidative stress [29]. Our results on the effect of Si on CAT, APX and GPX activity are in agreement with previous studies [10]. Sivanesan & Jeong [11] demonstrated that the addition of Si on MS medium containing 2iP and IAA enhanced the activity of antioxidant enzymes such as APX, and CAT in *Ajuga multiflora* Bunge. Similarly, Song *et al.* [30] has been shown that the application of Si in Zn-stressed plants effectively reduced the MDA and H_2O_2 levels, while increased CAT and APX activities. It has been demonstrated that Si inhibited the ROS and promoted the antioxidant enzyme activities such as APX and GPX in the leaves of the regenerated shoots of *Ornithogalum dubium* Hout. [31].

The expression of *rol*B gene induces resistance to abiotic stresses such as salt, low and high temperatures, excessive light [32]. Previous studies showed that the *rolB* gene has a more pronounced effect on growth and secondary metabolism [5]. It has been recently shown that *rolB* gene stimulates defensive systems [32]. This peculiarity of *rolB* may be important for moderate activation of the ROS-detoxifying enzymes in hairy roots [33].

Conclusion

In conclusion, the results of this study indicate that 7 mM Na_2SiO_3 and 2mM $AgNO_3$ can be used for increasing production of important stress tolerance metabolites such as antioxidants and phenolic compounds as well as secondary metabolites in hairy roots cultures. These abiotic elicitors can be considered as new powerful elicitors to enhance the yield of secondary metabolites in plant cells and organs by biotechnology techniques. In regard to less effect of Na_2SiO_3 on environment compared to $AgNO_3$, and also more increase in fresh weight, it can be concluded that Na_2SiO_3 could be more effective than $AgNO_3$. Therefore, it is possible to evaluate elicitor's effects on secondary metabolite production in *H. reticulatus* hairy roots.

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