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Original Article

Effects of Arginine Pretreatments on Oxidative Stress Damages and Alkaloid Content in Roots of *Hyoscyamus niger* under Nickel Stress

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Abstract

Heavy metal pollution is a worldwide problem with serious environmental consequences. The objective of the present experiment was to investigate whether arginine as nitric oxide precursor and or polyamines substrate can decrease the destructive effects of oxidative stress induced by nickel contamination in *Hyoscyamus niger* plant. In this study the effects of arginine pretreatment on alkaloid content of *Hyoscyamus* plant under heavy metal stress were investigated. In this research, four weeks seedlings were pretreated with 10 or 20 µmol arginine and then subjected to 50 or 100 µmol Ni solutions. Results showed that hydrogen peroxide content, lipoxigenase, catalase and guaiacol peroxidase activity increased in those plants which were under Ni stress, while ascorbate peroxidase activity did not change. Alkaloids content decreased in Ni stressed plants. Arginine pretreatment decreased the amounts of hydrogen peroxide and activity of these enzymes in stressed plants when compared with non-pretreated plants. Proline content also increased in Ni-stressed plants while arginine pretreatment decreased the proline content. Pretreatment of plants with arginine increased the amounts of total alkaloids in plants which were under Ni stress. In this study, it seems that protective effects of arginine were related to either polyamines or indirect synthesis of NO from polyamines.

Key words: Antioxidant enzyme, Heavy metal, Nitric oxide, Polyamines, Proline

Introduction

Heavy metal contamination has become a wide spread problem world over. Metal pollutions continuously increasing and the main cause are the anthropogenic activities which interfere with the environment activities and makes condition hazardous for living organism. Excess concentrations of some heavy metals such as Cd (II), Cr (IV), Ni (II) and Zn (II) in soils have caused disruption of natural aquatic and terrestrial ecosystems [1]. Toxic effects of Ni on plant growth and photosynthesis have been reported in higher plants as well as in algae [2]. Symptoms of Ni phytotoxicity are including decrease of seed germination, reduction of root growth, induction of leaf chlorosis and reduction of biomass [3]. Increasing evidence suggests that Ni toxicity in plants is also associated with oxidative stress [4-6]. Excessive Ni leads to significant increases in the concentration of reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions, and hydrogen peroxide [6-8]. Since Ni is not a redox-active metal, it cannot directly generate these reactive oxygen species. However exposure of plants to Ni at low concentrations has been shown to increase the activities of super oxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), and guaiacol peroxidase (GPX) in order to enhance the activation of other antioxidant defenses and hence lead to the removal or scavenging of [9].
L-arginine is one of the most functionally diverse amino acids in living cells. In addition to serving as a constituent of proteins, arginine is a precursor for biosynthesis of polyamines, agmatine and proline as well as the cell signaling molecules glutamine and nitric oxide (NO) [10,11]. Two of the most intensive pathways of arginine metabolism are

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those catalyzed by arginase (ARG) and nitric oxide synthase (NOS). Arginase hydrolyzes arginine to urea and ornithine, the latter is a precursor for polyamine and proline synthesis and the NOS pathway products are NO and citrulline [11]. NO can also mediate plant growth regulators and ROS metabolism and increasingly evident have shown which it is involved in signal transduction and responses to biotic and abiotic stress such as drought, low and high temperatures, UV and ozone exposure, heavy metal, herbicides, cold, and salt stress [12-13]. It has been reported that exogenous NO reduces the destruction of these stress on plants [14]. NO has been reported to counteract the toxicity of ROS generated by cadmium [15,16], excessive copper [17,18], salt stress [19,20] and drought stress [21]. Several authors also described alleviation of the symptoms of Pb and Cd stress in plants by exogenous NO [16,22]. Polyamines (PAs) modulate several biological processes in plants, including cell division, differentiation, and senescence [23], and it has been suggested that they participate in cellular defense against oxidative damage through the inhibition of lipid peroxidation [24] and scavenge of free radicals [25]. Research has shown that PAs applied exogenously to plants confer some protective effects against heavy metal stress such as copper [26] and Cd [15] stress. In addition, Upon exposure to metals, plants often synthesize a set of diverse metabolites that accumulate to concentrations in the milimolar range, particularly specific amino acids, such as proline and histidine, peptides such as glutathione and phytochelatins (PC), and the amines spermine, spermidine, putrescine, nicotianamine, and mugineic acids [27]. Some of the studies on the effects of arginine in plants have focused on its role in mobilizing arginine as a nitrogen source during post-germinative growth [28,29] and its endogenous metabolism as polyamine precursor. The objective of the present experiment was to investigate whether arginine as NO precursor and or polyamines substrate is involved in regulation of ROS metabolism in *Hyoscyamus niger* and to elucidate the physiological mechanisms of exogenous arginine in increasing tolerance of Hyoscyamus plant to Ni stress and alkaloid production.

Material and Methods

Plant material

Henban plants (*Hyoscyamus niger* L.) were grown from seeds in plastic pots containing sand and compost, until the seeds were germinated. The seedlings were irrigated with water once a day and half-strength Hoglands nutrient solution [30] once a week. After four weeks, the seedlings were transferred to bottles containing Hoaglands nutrient solution aerated with air pump and were pretreated with 10 or 20 µmol arginine (Arg was added to nutrient solution). After 24 h, plants were subjected to Ni stress. For this purpose, three seedlings were placed in aerated bottle containing distilled water (control) and 50 or 100 µmol Ni solutions. After 48 h of treatment with Ni solution, the shoots and roots of the treated plants were harvested and immediately were frozen in liquid nitrogen and stored at -80 ^{o}C for further analysis.

Hydrogen peroxide content

Hydrogen peroxide content was measured spectrophotometrically after reaction with potassium iodide (KI) according to the method of Alexieva *et al.* [31]. Leaf tissues (500 mg) were homogenized in ice bath with 5 mL 0.1% TCA in distilled water (w/v). The homogenate was centrifuged at 12000×g for 15 min. The reaction mixture consisted of 0.5 mL of supernatant, 0.5 mL of 100 mM K-phosphate buffer (pH= 7.0) and 2 mL reagent (1M KI in fresh double-distilled water). The blank probe consisted of 0.1% TCA in the absence of leaf extract. The reaction was carried out for 1h in darkness and absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve ($Y=0.0013X+0.0356$, $R^2=0.998$) prepared with known concentration of H_2O_2.

Proline determination

Determination of free proline content performed according to Bates *et al.* [32]. Leaf samples (0.5 g) from each plant were homogenized in 3% (w/v) sulphosalycylic acid and the homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, resulting mixture was heated at 100 °C for 1 h in water bath. Reaction was then stopped by using ice bath. The mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline

concentration was determined using calibration curve (Y=0.0093X+0.0022, R^2=0.983).

Enzyme extraction and activity determination

Five hundred milligrams leaves were homogenized in cool 50 mmol potassium phosphate buffer (pH=7.0) containing 1% (w/v) soluble polyvinylpyrrolidone (PVP), 1mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) with the addition of 10 mM ascorbic acid in the case of the APX assay. All of the procedures were done at 4 °C. The homogenate was centrifuged at 20000×g for 20 min and the supernatant was used for assay of the activity of enzymes.

Lipoxygenase activity (LOX)

Lipoxygenase activity was estimated according to the method of Doderer *et al.* [33]. For measurement of LOX activity, the substrate solution was prepared by adding 35 µL linoleic acid to 5 mL distilled water containing 50 µL Tween-20 .The solution was kept at pH 9.0 by adding 2 ml 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by adding 2ml 0.2 M HCl, 0.1M phosphate buffer (pH=6.5) was added to make a total volume of 100 mL. LOX activity was determined spectrophotometrically by adding 50 µL of enzyme to 2.95 mL substrate. Solution absorbance was recorded at 234 nm and the activity was expressed as unit per mg protein in the leaves (extinction coefficient of 25000M^{-1} Cm^{-1}).

Catalase activity (CAT) (EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm using the extinction coefficient of 40 mM^{-1} Cm^{-1} for H_2O_2 [24].

Guaiacol peroxidase (GPX) (EC1.11.1.7)

The GPX activity was determined using the method of Plewa *et al.* [34] following the formation of tetraguaiacol by measuring the absorbance at 470 nm and using an extinction coefficient of 25.5 mM^{-1} Cm^{-1}.

Ascorbate peroxidase (APX) (EC 1.11.1.11)

Ascorbate peroxidase was determined spectrophotometrically according to the oxidation of ascorbic acid. The reaction solution contained 50 mmol potassium phosphate buffer (pH=7.0), 0.5mmol ascorbate, 0.1 mmol H_2O_2 and 150 mL enzyme extract. H_2O_2-dependent oxidation of ascorbic acid was followed by measuring the decrease in absorbance within 1min at 290 nm (extinction coefficient of 2.8 mM^{-1} Cm^{-1}) [35].

Total soluble proteins

Protein content was determined according to the method of Bradford [36] using Bovine serum albumin as standard (Y=0.0147X-0.0846 R^2=0.992).

Determination of Ni concentration in roots: Nickel content in leaves was determined by atomic absorption spectrometry (Spect rAA 300, *Varian*, Mulgrave, Australia) following wet digestion of oven dried tissues in HNO_3 solution at 140 °C.

Extraction of total alkaloids

Alkaloids were extracted using 1 g (FW) of plant material in 50mL of 96% ethanol overnight at room temperature. The extracts were filtered through Whatman filter paper No.1 and dried with rotary evaporator. The dry residues were dissolved in 5% (V/V) sulfuric acid, and then were washed and clarified with diethyl ether: sulfuric acid 5% (1:1 v/v). Colorless aqueous phases were collected, and the pH values were adjusted to 10 with 1ml 10 N NaOH and were extracted with three times with volumes 30, 40 and 40 mL of chloroform. The chloroformic phases were collected, evaporated and the residues were dissolved in 500µL of pure methanol and solution absorbance was recorded at 258 nm. The calibration curve (Y=0.00065X+0.0178 R^2=0.987) was made with standard scopolamine hydrobromide (Sigma Chemical Co.) and Alkaloids were quantified as mg g^{-1} FW [37].

Statistical analysis: Data are means± SE of three replicates. Statistic assays were carried out by one-way ANOVA using Duncan test to evaluate whether the means were significantly different, taking $p< 0.05$ as significant.

Results

Lipoxygenase activity

Lipoxygenase is an oxidative enzyme that contributes to oxidation of polyunsaturated fatty acids. The activity of this enzyme under Ni stress increased about 4 fold when compared with control (Fig. 1).

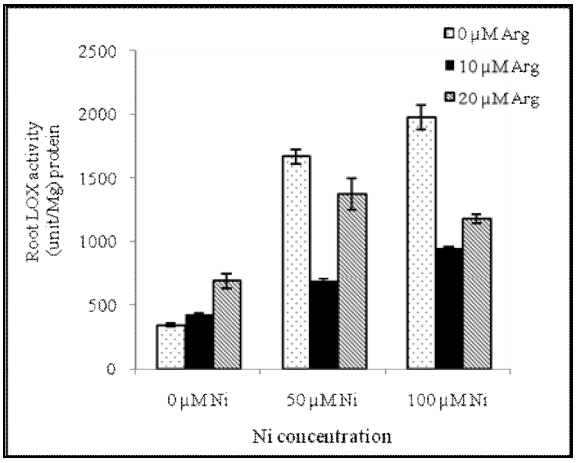

Fig. 1 Effect of Arg pretreatment on LOX activity in roots of *Hyoscyamus niger* under control and Ni stress condition. Data are means± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

Activity of this enzyme decreased in those plants which were pretreated with arginine (Arg) and then treated with Ni. However, 10 µmol Arg was more effective than 20 µmol Arg pretreatment.

H_2O_2 content and antioxidant enzyme activities

In this study Ni stress increased the H_2O_2 content (Fig. 2-A). In those plants which were under Ni stress, Arg pretreatment decreased the content of hydrogen peroxide. However, in control plants when were pretreated with 10µmol Arg the amount of H_2O_2 decreased while in 20 µmol Arg pretreated plants H_2O_2 increased when compared with non-Arg pretreated plants. Change in specific activity of antioxidant enzymes is the consequence of oxidative stress. The effect of Ni stress on CAT, GPX and APX in roots of Henban plant, either with or without Arg pretreatment was assayed. As it is shown in (Fig.s. 2-B, C and D), the activity of CAT and GPX were higher than those of the control groups which may be a reflection of the anti-oxidative role of these enzymes against the Ni stress.

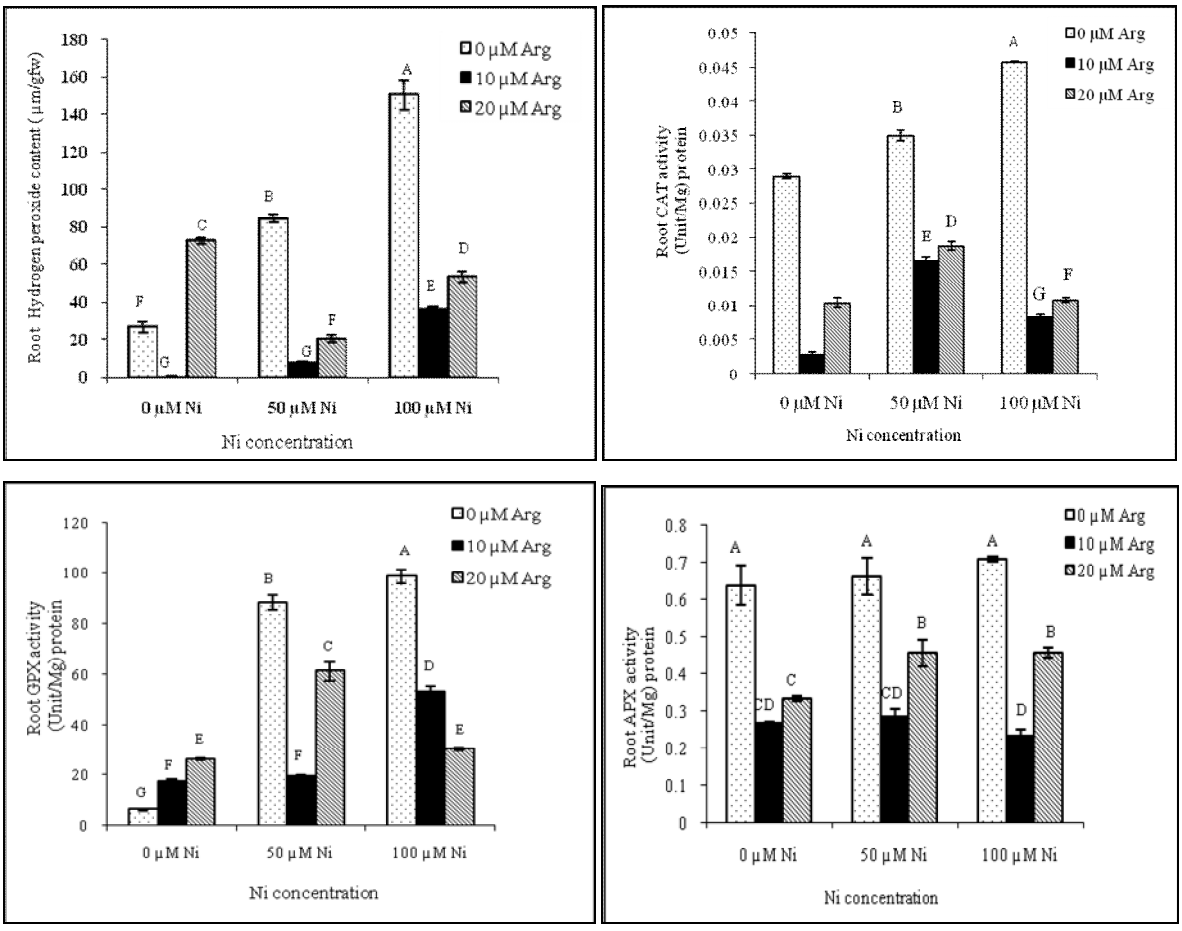

Fig. 2 Effect of Arg pretreatment on H_2O_2 content (A), CAT (B), GPX (C) and APX (D) activity in *Hyoscyamus niger* roots under control and Ni stress condition. Data are means± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

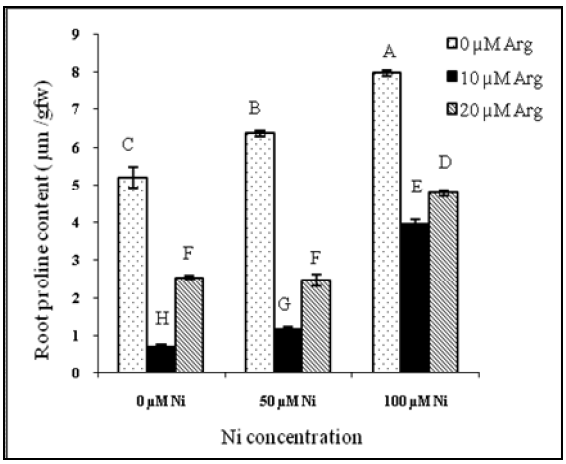

Fig. 3 Effect of Arg pretreatment on proline content in *Hyoscyamus niger* roots under control and Ni stress condition. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

The activity of APX did not change under Ni stress when compared with control plants. Arginine pretreatment decreased the activity of CAT, GPX and APX in stressed plants when compared with non- pretreated plants.

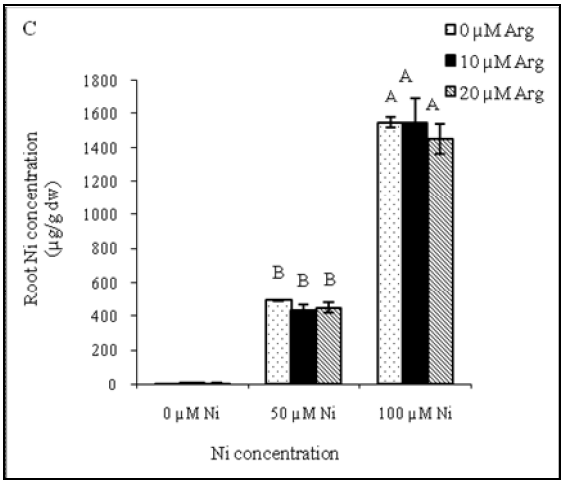

Fig. 4 Effect of Arg pretreatment on Ni content in roots of *Hyoscyamus niger* under control and Ni stress condition. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

Proline content

The amounts of proline increased significantly under Ni stress. Treatment of plants with 10 or 20 µmol Arg decreased the free proline content in control and stress condition (Fig. 3).

Ni concentration in roots of plants

Determination of Ni content by atomic absorption showed that the Ni concentration increased in roots of plants under Ni stress and the Arg pretreatment had no effect on the Ni content in control and stressed plants (Fig. 4).

Total alkaloid content: Total alkaloid content decreased in 50 and 100µmol Ni stress in comparison with control plants. Arginine pretreatment had no effects on alkaloids content in control condition but in those plants which were under Ni stress, pretreatment with 20µmol Arg increased the alkaloids content (Fig. 5).

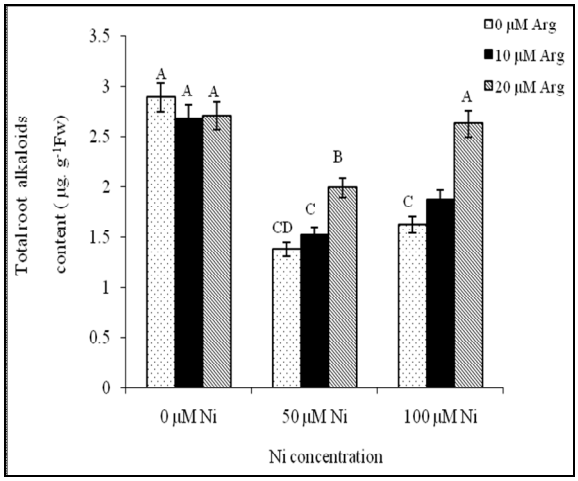

Fig. 5 Effect of Arg pretreatment on total alkaloids content in roots of *Hyoscyamus niger* under control and Ni stress condition. The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.

Discussion

Heavy metal pollution is a worldwide problem with serious environmental consequences. Amongst heavy metals, Ni is an essential micronutrient for plant growth. It is a constituent of the enzyme urease and in small quantities is essential for many plant species to complete their life cycle [3] but higher concentrations of this metal are toxic and may severely interfere with many physiological and biochemical processes of plants [3]. Our results show that Ni toxicity in Henban plant is strongly correlated with higher oxidative damage in root, as demonstrated by elevated hydrogen peroxide and LOX activity, which induced lipid peroxidation**.** Uptake of Ni increased with increasing Ni concentration in the growth medium revealed by the root Ni content**.** The increase in hydrogen peroxide content and LOX activity with increasing

Ni levels suggests that Ni induces oxidative stress in henban plants. The elevated CAT and GPX activities in root treated with high Ni concentration may be a defense response to Ni toxicity while the APX activity did not changed in plants under Ni stress when compared with control plants. Enhancement of the activity of CAT and GPX enzymes in stress condition are required for H_2O_2 scavenging, but the enhanced activity of these enzymes is apparently not sufficient to maintain a cellular redox balance under Ni toxicity. This finding is consistent with reports on elevated root and shoots antioxidant enzymes activities in several plant species grown under toxic levels of metals [4, 38]. In the present study, application of Arg, significantly decreased the amount of H_2O_2 and LOX activity (Fig.1 and Fig. 2-A). This effect is very important for plant stress tolerance**.** Pretreatment of henban plants with Arg also decreased the activity of CAT, GPX and APX under stress condition when compared with non-pretreated plants (Fig. 2-B to D). These finding were in agreement with the results of previous study, which showed that Arg pretreatment decreased the activity of CAT and GPX in tomato plants under drought stress [39]. However in some researches it has been reported that NO increased the activity of CAT, POD and APX in iron deficiency [40] and in drought stress condition in tomato plant [21]. In contrast to our result, which obtained in tomato plants under drought stress the APX activity increased and therefore it is possible that APX play key role in H_2O_2 detoxification [21], in this research APX had no significant effects on H_2O_2 detoxification and the Arg pretreatment decreased the activity of this enzyme. In this investigation Ni toxicity stimulated the production of the proline and the Arg pretreatment decreased the amount of this compound in control and stressed plants (Fig.. 3). Arginine is the main amino acid in plants and two main pathways of its metabolism have been reported which are catalyzed by either arginase or nitric oxide synthase so that the end product will be ornithine or nitric oxide respectively. Ornithine is a precursor for polyamines or proline biosynthesis [11]. In many studies, the effects of exogenous nitric oxide, polyamines and proline in the protection of plants against heavy metal stress were reported. For example, oxidative stress of tomato induced by excessive Cu was effectively alleviated by application of sodium nitroprusside (SNP) [17]. It was shown that the function of NO on alleviation of oxidative damage was the induction of various ROS-scavenging enzyme activities [15-16]. Reaction of NO with $O_2^{\bullet-}$ produces peroxynitrite ($ONOO^-$), which is considered to be a highly toxic product. However, $ONOO^-$ can be protonated and decomposed to a nitrate anion and a proton or it can react with hydrogen peroxide to yield a nitrite anion and oxygen [40]. So reduction of H_2O_2 under Arg pretreatment may be related to NO production from Arg directly or indirectly through polyamines. Wang *et al.* [26] also reported that exogenous application of spermidine (Spd) and spermin (Spm) prevented lipid peroxidation, reduced the excessive accumulation of Cu, maintained the balance of nutrient elements, and effectively alleviated the toxicity of Cu to *Nymphoides peltatum* leaves. In another cases the effects of some amino acids such as histidine, serin, proline and methionine in protection of plants against heavy metal stress has been reported [27].

In this research, Arg was used as a substrate of NO, polyamines and or proline, to study the role of this compound in some physiological parameters under Ni stress. The amelioration of the Ni stress on hyoscyamus plants was observed when Arg was used, which could be considered that these effects may be related to either NO or polyamines production because proline content decreased in Arg pretreated plants (Fig. 3). There are some reports that showed endogenous arginine produced polyamines in stress condition for example in wheat leaves, cadmium enhanced both ADC and ODC activities, which are the key enzymes of polyamines biosynthesis [41]. Lin and Kao have reported that under Cu^{2+} treatment, ODC seemed to be the main enzyme responsible for Put biosynthesis [42]. In previous study on tomato plants, our findings showed that the Arg or Arg+LNAME (inhibitor of NOS enzyme) in the same manner decreased lipid peroxidation under drought stress [39]. Therefore it seems that under this situation, protective effects of Arg are related to either polyamines or indirect synthesis of NO from polyamines which has been reported previously [43]. In conclusion it is shown for the first time that pretreated of henban plant with Arg ameliorate the oxidative damages induced by Ni toxicity. It might be that arginine alleviated Ni toxicity directly on through production of

polyamines. Arginine also is the precursor of tropan alkaloids in plants. In this experiment Ni toxicity decrease the alkaloid content of plant while the pretreatment of plant with Arg increased the alkaloids content. So Arg pretreatment had dual role in this situation: positive effects on alleviation of oxidative stress and increment of the alkaloids content.

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