

Effect of arginine pre-treatment on nickel accumulation and alleviation of the oxidative stress in *Hyoscyamus niger*

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Abstract

Nickel is essential for many plant species to complete their life cycle but higher concentrations of this metal are toxic and may severely interfere with many physiological and biochemical processes of plants. In recent researches, either sodium nitroprusside (as nitric oxide donor) or exogenous polyamines were used to counteract the effect of heavy metal stress. In this research hyoscyamus plants were pre-treated with 10 and 20 μM Arg (as a precursor of NO or polyamines) and then plants were treated with 50 and 100 μM Ni. Elevated hydrogen peroxide content and lipoxygenase (LOX) activity showed that Ni induced oxidative damages. Antioxidant enzyme activity also increased in those plants which were under Ni stress. In plants, which were pretreated with Arg, the concentration of Ni increased in shoot, while the H_2O_2 content and the activity of LOX, catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) enzymes decreased. It is shown for the first time that pre-treatment of hyoscyamus plant with Arg increased plant capability to accumulate Ni in its aboveground organs by 1.5-2 times and reduced its toxic effects indicated by measurement of oxidative parameters. It might be true because Arginine induced alleviation of Ni toxicity and accelerated transport by chelating of Ni in plants directly or through production of polyamines.

Keywords: Antioxidant enzymes, heavy metals, hydrogen peroxide, polyamines

1. Introduction:

Heavy metal pollution is a worldwide problem with serious environmental consequences. Amongst heavy metals, nickel (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 (Seregin and Kozhevnikova, 2006) but higher concentrations of this metal are toxic and may severely interfere with many

physiological and biochemical processes of plants (Seregin and Kozhevnikova, 2006). If the plants taken up larger amounts of nickel, the common symptoms of Ni toxicity are chlorosis, weak plant growth, yield depression and may even be accompanied by reduced nutrient uptake and disorders in plant metabolism (Yang, 1996). Ni excess in the environment also decreases chlorophyll content and growth in metal

sensitive plants (Boominathan and Doran, 2002). Many abiotic environmental stresses including salinity, drought stress, temperature extremes, and metal toxicity disrupt the redox homeostasis of cells and exert a wide range of adverse effects on plant growth and metabolism (Sharma and Dubey, 2007; Nasibi and Kalantari, 2009). These stressful conditions induce overproduction of reactive oxygen species (ROS). ROS are known to damage cellular membranes by inducing lipid peroxidation and can damage DNA, proteins and chlorophyll (Mittova *et al.*, 2000). The lifetime of active oxygen species within the cellular environment is determined by the antioxidant system, which provides crucial protection against oxidative damage. The antioxidative system comprises numerous enzymes such as super oxide dismutase (SOD), CAT, APX and GPX and non-enzymatic antioxidants with low molecular weight including ascorbic acid and reduced glutathione (GSH) (Sharma and Dubey, 2007). It has been suggested that Ni-induced phytotoxicity, is associated with Ni-stimulated oxidative stress in certain plant species (Boominathan and Doran, 2002). Since Ni is a redox-inactive metal, it cannot directly generate ROS. Membrane peroxidation, however, has been observed in various plants subject to Ni toxicity (Boominathan and Doran, 2002; Gajewska and Sklodowska, 2005). In addition, enzymes related to antioxidant defense systems have also been shown to be stimulated in response to Ni-induced oxidative stress (Gajewska and Sklodowska, 2005).

In previous studies, the specific role of some amino acids in the plant responses to abiotic stress have been reported. For example it has been reported that accumulation of proline caused prevention of metal-induced lipid peroxidation (Mehta and Gaur 1999) or complex of histidine (His) with Ni, caused accumulation of Ni in shoots of Hyper accumulator plants (Shanti *et al.* 2006). In addition, upon exposure to metals, plants often synthesize a set of diverse metabolites that accumulate to concentrations in the millimolar 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 (Sharma and Dietz, 2006). 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 (Liu *et al.* 2006). In previous research, sodium nitroprusside (SNP) as a NO donor or exogenous polyamines was used to counteract the effect of heavy metal (Wang, 2007; Hsu and Kao, 2004; Tewari *et al.*, 2008). However not any data are available on the effect of exogenous arginine as a precursor of these compounds in the possible anti oxidative responses of plants against Ni stress. The aim of this work is designed to study the effects of arginine pre-treatment on alleviation of oxidative damages induced by Ni stress. We also examined the effects of Arg on Ni accumulation in *Hyoscyamus* plants. Comparing these responses can be useful for understanding the physiological and biochemical mechanisms of Arginine in those plants which have to cope with heavy metal stress.

2. Materials and Methods

2.1. Plant material

Henban plants (*Hyoscyamus niger*) were grown from seeds in plastic pots contains sand and compost until the seeds were germinated. The seedlings were irrigated with water once a day and half-strength Hogland solution once a week. After four weeks of growth, the seedlings were transferred to bottles containing Hoagland solution aerated with air pump and were pretreated with 10 or 20 μM Arginine (Arg was added to nutrient solution). These concentrations of Arg were optimized in preliminary experiment. After 24 h, plants were subjected to Ni stress. For this purpose 3 seedlings were placed in aerated bottle containing distilled water served as a control and 50 or 100 μM Ni solutions. After 24 h of Ni stress, the shoot and root of plants were harvested and

immediately frozen in liquid nitrogen and stored at -80 °C for future analysis.

2.2. Chlorophyll content

The amount of photosynthetic pigment, (chlorophyll a, b, total and carotenoids), were determined according to the method of Lichtenthaler *et al.* (1987). The pigment extract was measured against a blank of 80% (V/V) acetone at wavelengths of 646.8 and 663.2 nm for chlorophyll assays.

2.3. Hydrogen peroxide content

Hydrogen peroxide levels were determined according to Alexieva *et al.* (2001). Fresh leaves (0.5 g) were homogenized in ice bath with 5 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $12,000 \times g$ for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. 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 prepared with known concentration of H₂O₂.

2.4. Proline determination

Determination of free proline content performed according to Bates *et al.* (1973).

2.5. Enzyme extraction and activity determination

Five hundred mg leaves were homogenized in an ice cold mortar using 50 mM potassium phosphate buffer (pH= 7.0) containing 1mM EDTA, 1% (w/v) soluble PVP, and 1mM PMSF. After centrifugation ($20000 \times g$, 20 min) the supernatant was used for determination of LOX, CAT, GPX and APX activities.

2.6. Lipoxygenase activity

The LOX activity was estimated according to the method of Dodere *et al.* (1992). For measurement of LOX activity 50 µl of enzyme extraction was added

absorbance was recorded at 234 nm and the activity was expressed as unit per mg protein in the leaves (extinction coefficient of $25000\text{M}^{-1}\text{cm}^{-1}$).

2.7. Catalase activity (EC 1.11.1.6)

The CAT activity was assayed by measuring the initial rate of H₂O₂ disappearance at 240 nm using the extinction coefficient of $40\text{mM}^{-1}\text{cm}^{-1}$ for H₂O₂ (Velikova *et al.*, 2000)

2.8. Guaiacol peroxidase (EC1.11.1.7)

The GPX activity was determined using the method of Plewa *et al.* (1999) following the formation of tetraguaiacol by measuring the absorbance at 470 nm and using an extinction coefficient of $25.5\text{mM}^{-1}\text{cm}^{-1}$.

2.9. Ascorbate peroxidase (EC 1.11.1.11)

The APX activity was determined spectrophotometrically according to the oxidation of ASA. The reaction solution contained 50 mM potassium phosphate buffer (pH =7.0), 0.5 mM ascorbate, 0.1 mM H₂O₂ and 150 µl enzyme extract. H₂O₂-dependent oxidation of ASA was followed by measuring the decrease in absorbance within 1min at 290 (extinction coefficient of $2.8\text{mM}^{-1}\text{cm}^{-1}$) (Nakano & Asada, 1981).

2.10. Total soluble proteins

Protein content was determined according to the method of Bradford (1976) using Bovine serum albumin as standard.

2.11. Determination of Ni concentration in leaves of plants

Nickel content in leaves was determined by atomic absorption spectrometry (SpektrAA 300, Varian, Mulgrave, Australia) following wet digestion of oven dried tissue in HNO₃ solution at 140 °C.

2.12. Statistical analysis

The experiments were performed in a randomized order. Data were expressed as mean of three replicates with standard error. Statistical 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.

3. Results

3.1. Chlorophyll content

Results showed that chlorophyll a and total chlorophyll decreased in plants, which were under 100 μM nickel while Ni stress had no effects on chlorophyll b content. Arginine pre-treatment (10 μM) increased the chlorophyll content in Ni stressed plants (Table 1).

Table 1: Effect of Arg pre-treatments (10 and 20 μM) on photosynthetic pigments in *Hyoscyamus niger* leaves under control and Ni stress (50 and 100 μM) condition. Data are means \pm SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p < 0.05$ as significant.

Treatments	Chlorophyll a (mg gfw ⁻¹)	Chlorophyll b (mg gfw ⁻¹)	Total Chlorophyll (mg gfw ⁻¹)
Control	88.5021 abcd	40.2071 b	128.7092 bcd
10 μM Arg	96.12797 abc	32.06897 bc	128.1969 bcd
20 μM Arg	106.019 ab	39.4051 b	145.4241 abc
50 μM Ni	80.33937 bcd	39.1971 b	119.5365 cde
50 μM Ni+10 μM Arg	111.0541 a	54.7634 a	165.8175 abc
50 μM Ni+20 μM Arg	98.74033 ab	40.97307 b	139.7134 abc
100 μM Ni	67.1349 d	30.9924 bc	98.1273 de
100 μM Ni+10 μM Arg	108.9297 a	51.27473 a	160.2045 abc
100 μM Ni+20 μM Arg	69.98387 cd	25.9962 c	95.98007 e

3.2. H_2O_2 accumulation and LOX activity

Ni stress caused an increase in H_2O_2 accumulation in leaves of plants (Figure 1-A). Arg pre-treatment had no significant effect in decreasing of H_2O_2 in control condition. However, in those plants which were under Ni stress, 10 and 20 μM Arg pre-treatments decreased the amount of hydrogen peroxide. Lipoxygenase is an oxidative enzyme that induces lipid peroxidation. The activity of this enzyme increased significantly in Ni stressed plants (Figure 1-B). Arg pre-treatment decreased the activity of this enzyme in those plants which were under Ni stress. However pre-treatment of plants with 10 μM Arg was more effective when compared with those plants which were pretreated with 20 μM Arg.

3.3. Proline content

The amounts of proline increased significantly under Ni stress. Treatment of plants with Arg decreased the proline content in control and 100 μM Ni stressed plants while Arg pre-treatment had no effects on proline content of plants which were under 50 μM Ni stress (Figure 2).

3.4. Antioxidant enzyme activities

The effect of Ni stress on CAT, GPX and APX in *Hyoscyamus* leaves, either with or without Arg pre-treatment was assayed. As it is shown in (Figure 3-A, B and C) the activity of CAT, GPX and APX were higher in stressed plants than those of the control groups, which may be a reflection of the oxidative stress induced by Ni stress. Pre-treatment of plants with Arg decreased the activity of CAT and APX enzymes in control, and stress conditions however Arg pre-treatment decreased the GPX in 100 μM Ni stress and had no effects on GPX in control and 50 μM Ni stress condition.

3.5. Ni concentration in leaves of plants

Determination of Ni content by atomic absorption showed that the Ni concentration increased in leaves

of plants under Ni stress and the Arg pre-treatment increased the Ni content in stressed plants.

4. Discussion

Nickel is toxic at high plant-available concentrations and can have detrimental effects on plant growth and metabolism (Seregin and Kozhevnikova, 2006). Our results showed that Ni toxicity in *hyoscyamus* plant is strongly correlated with higher oxidative damage in shoot, as demonstrated by decrease in chlorophyll content, elevation of hydrogen peroxide and LOX activity, which induced lipid peroxidation. Uptake of Ni increased with increasing Ni concentration in the growth medium as revealed by the shoot Ni content. The increase in hydrogen peroxide content and LOX activity with increasing Ni levels suggests that Ni induces oxidative stress in growing *hyoscyamus* plants. The elevated CAT, GPX and APX activities in shoot treated with high Ni concentration may be a defense response to Ni toxicity. Enhanced the activity of this enzymes in stress condition are available 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 (Sharma and Dubey, 2007). In the present study, application of Arg, significantly decreased the amount of H_2O_2 and LOX activity (Figure 1) which is very important for stress tolerance. In this investigation Ni toxicity stimulates the production of proline which is anti-an oxidative metabolite. Arg pre-treatment had no significant effects on the amount of this compound and even in some cases Arg pre-treatment decreased the amount of proline in control and stressed plants (Figure 2). It appears that the Arg pre-treatment induced the PAs pathways and so proline content decreased significantly in these plants. Pre-treatment of *hyoscyamus* plants with Arg Also decreased the activity of CAT, GPX and APX under stress condition when compared with non-pretreated plants (Figure 3).

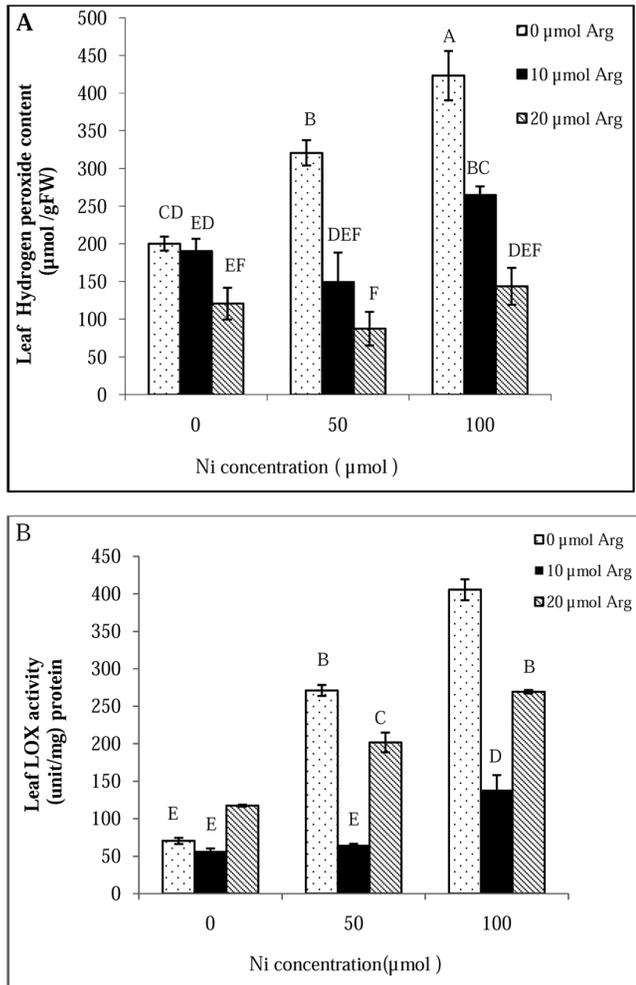


Figure 1. Effect of Arg pre-treatment on H_2O_2 content (A) content and LOX activity (B) in *hyoscyamus* leaves under control and Ni stress condition. Data are means \pm SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p < 0.05$ as significant

In previous study, it has been shown that Arg pre-treatment decreased the activity of CAT and GPX in tomato plants under drought stress (Nasibi *et al.*, 2011). 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 endproduct will be ornithine or nitric oxide respectively. Ornithine is a precursor for polyamines or proline biosynthesis (Liu *et al.*, 2006). In previous studies, the effects of nitric oxide, proline and polyamines in the protection of plants against heavy metal stress were reported. For example, oxidative

stress induced by excessive Cu was effectively alleviated in tomato plant by application of SNP. Cui *et al.*, (2010) reported that exogenous NO was able to improve shoot height and fresh weight of tomato plants subjected to Cu stress. By determining the activities of SOD, peroxide dismutase (POD), APX and CAT, they concluded that NO exerted its protective effect through the activation of some antioxidative enzymes, not by preventing Cu uptake and transport. In other studies, it was reported that the function of NO alleviation of oxidative damage could well be the induction of various ROS-scavenging enzyme activities (Hsu and Kao, 2004). Wang *et al.* (2007) reported that exogenous application of spermidine (Spd) and spermine (Spm) prevented lipid peroxidation, reduced the excessive accumulation of Cu, maintained the balance of nutrient elements, and effectively alleviated the toxicity of Cu in *Nymphoides peltatum* leaves. Therefore, it seems that in our study protective effects of Arg may be related to polyamines, NO production or proline biosynthesis. In this study, the measurement of Ni concentration in shoot showed that when plants were pretreated with Arg the concentration of Ni increased in the shoot. Therefore, it seems that Arg had the chelator role for the translocation of Ni from root to shoot. In many cases the effects of some amino acids such as histidine, serine, proline and methionine in protection of plants against heavy metal stress has been reported (Shanti *et al.*, 2006). For example *Alyssum montanum*, when supplied with exogenous histidine, transported more Ni to the shoot as a result of enhanced Ni flux into the xylem and showed tolerance to applied Ni concentrations (Kerkeb and Krämer, 2003). The Ni hyper-accumulation trait in *Alyssum* species (*Brassicaceae*) has been demonstrated to be specifically linked to the ability for free histidine production (Krämer *et al.*, 1996). Genetically increased capacity for metal-induced Cys synthesis was shown to support survival of *Arabidopsis* under acute Cd stress (Dominguez-Solis *et al.*, 2004). In this connection, the capability of plant to accumulate stress-induced polyamine is of

great interest; polyamines manifest a multifunction defensive role against various abiotic factors, heavy metal in particular (Kuznetsov *et al.*, 2006). Therefore, their properties, such as an easy chelation of heavy metal, Ni in particular (Koutensky *et al.*, 1995), attract the attention of researchers.

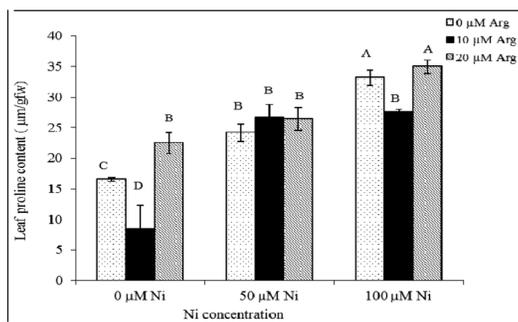


Figure 2. Effect of Arg pre-treatment on proline content in leaves of *hyoscyamus* under control and Ni stress condition. The significant of different between treatments was determined by one-way ANOVA taking $p < 0.05$ as significant.

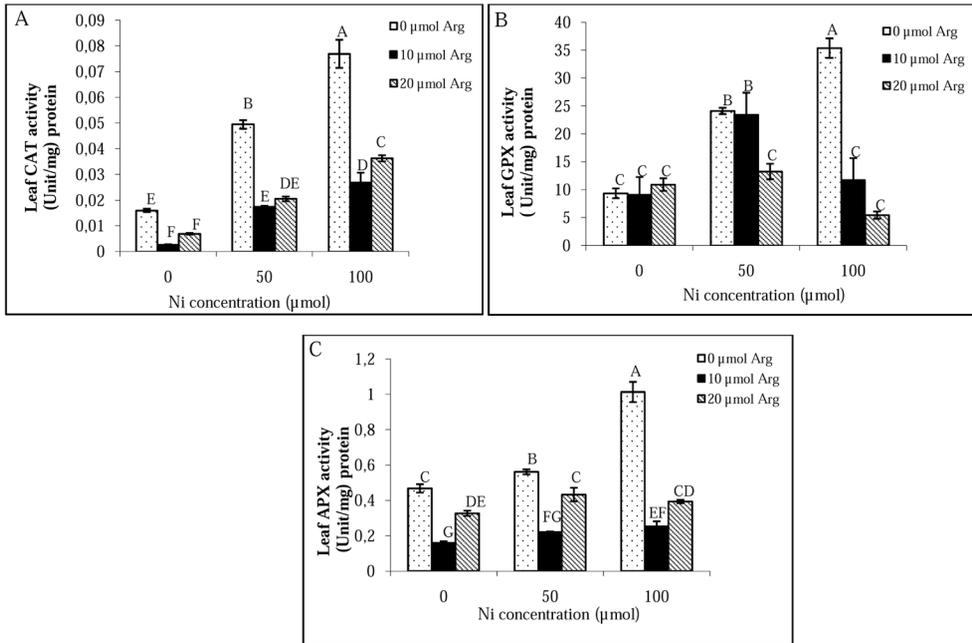


Figure 3. Effect of Arg pre-treatment on CAT (A), GPX (B) and APX (C) activity in tomato plant leaves under control and Ni stress condition. Data are means± SE of three replicates. The significant of different between treatments was determined by one-way ANOVA taking $p < 0.05$ as significant.

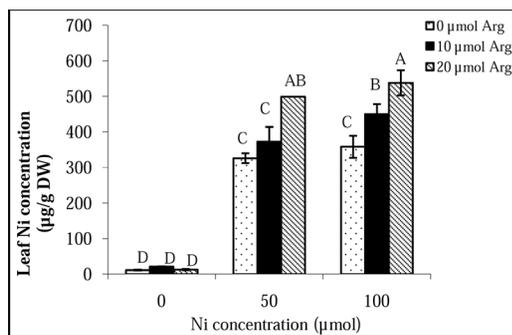


Figure 4. Effect of Arg pre-treatment on Ni content in leaves of *hyoscyamus niger* under control and Ni stress condition. The significant of different between treatments was determined by one-way ANOVA taking $p < 0.05$ as significant.

Conclusions

In conclusion it is shown for the first time that pre-treatment of hoscycamus plant with Arg increased their capability of Ni accumulation in aboveground organs by 1.5-2 times and reduced its toxic effects on chlorophyll content and oxidative parameters. It might be that arginine induced weakening Ni toxicity and its accelerated transport in plants (chelation) directly on through production of polyamines or NO.

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