CATRINA (2013), 8 (1): 75 -85

Ameliorating Role of Nitric Oxide in Germinating Mung Bean Seeds (*Vigna Radiata*) Under Lead Stress

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ABSTRACT

Protective role of nitric oxide (NO) on seed germination and growth was studied in mung bean seeds (*Vigna radiata*) subjected to lead (Pb) stress. Germination percentage was increased up to 50 µM sodium nitroprusside (SNP) and then steady declined. Under lead stress, and absence of SNP, percentage of germination, fresh biomass, succulent value and the activity of α-amylase and protease as well as H-ATPase were significantly decreased. This was accompanied with a significant increase of hydrogen peroxide and malondialdhyde accumulation, catalase and guiacol peroxidase activities. Furthermore, total phenolic compounds as well as some individual phenolic acids were increased in a dose-dependent manner of lead. The protein pattern showed appearance of stress protein with M. M. of 110, 97 and 21 KDa at various lead stresses. Moreover, supplementation of SNP, as source of NO, increased fresh biomass, succulent values and enhanced the activities of H-ATPase, α- amylase, protease, catalase and guiacol peroxidase in lead stressed germinating bean seeds, whereas reduced the lead-induced increase in lead, hydrogen peroxide, malondialdhyde and phenolics accumulation. A new stress protein with M.M. 65 KDa was appeared in all lead stressed seedlings in presence of SNP. Taken together these results suggest that SNP could increase antioxidant capability in mung bean seeds leading to an improvement in the plasma membrane integrity and enhancement of the activities of hydrolytic enzymes and hence, an ameliorating role against the inhibitory impact of this toxicant on the germination and growth of mung bean seeds under lead stress.

Key words: α -Amylase, Antioxidant Enzymes, H-Atpase, Lead, Mung Bean, Phenolic Compounds, Protease, SNP.

INTRODUCTION

Lead is one of the hazardous heavy metal pollutants of the environment that originates from various sources like mining and smelting of lead-ores, burning of coal, effluents from storage battery industries, automobiles exhausts, metal plating and finishing operations, fertilizers, pesticides and from additives in pigments and gasoline. Increasing lead levels in soil environment inhibits germination of seeds and exerts a wide range of adverse effects on growth and metabolism of plants (Jaja and Odoemena, 2004; Odjagba and Fasidi, 2006).

One of the phytotoxic effect of lead appears to be induction of oxidative stress in growing plant parts due to enhanced production of reactive oxygen species (ROS) resulting in an unbalanced cellular red ox status (Dubey, 2003). The damage of the cell membrane system is another primary event in heavy metal toxic action in plants. Astolfi *et al*., 2003 concluded that the disturbance of membrane integrity is thought to be an effect of a complex interaction between heavy metals and functional groups of membranes leading to reduce the activity of H^+-ATPase associated with plasma membrane.

Also, (Hu *et al*., 2007) have reported that during germination of various seeds, several hydrolytic enzymes are synthesized and secreted to mobilize reserves, among these enzymes, α-amylase and protease synthesized *de novo* in the aleuronic layer, which more sensitive to various heavy metal stress. It has been shown that there are several defence mechanisms developed against the inhibitory effect of heavy metals (singh *et al*., 2008).

Enzymic and non-enzymic antioxidants are considered as ROS scavengers for shifting off the oxidative processes (Hu *et al*., 2007). Phenolic compounds are plant secondary metabolites that constitute one of the most common and wide spread groups of substances in plants. Many authors (Lattanzio and Ruggiero, 2003; and Michalak, 2006) have stated that plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. Also, phenolic compounds have been described as electron donating agents (hydrogen donor) to oxidant free radicals (Jung *et al.*, 2002), moreover it has been suggested that phenolics may act as biomarkers of metal exposure (Bialonska *et al*., 2007). The induction of specific proteins bands induces the resistance to several environmental stresses (Neumann *et al.*, 1994; Wollgiehn and Neumann, 1999; Wang and Yang, 2005).

It has been reported that nitric oxide (NO) is a bioactive molecule and participates in the whole life of plant, such as growth, development, programmed cell death, and abiotic and biotic stresses as pro-oxidant as well as anti-oxidant (de pinto *et al*., 2002; Pagnussat *et al*., 2002; He *et al* ., 2004; Delledonne, 2005 and de pinto *et al*., 2006). Zhao *et al.*, 2004 and Laspina *et al*., 2005 stated that exogenous NO can alleviate oxidative stress due to salt, herbicides, and heavy metals, mainly by modulating the enzymes participating in antioxidant metabolism or by scavenging ROS, directly.

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The aim of this study was an attempt to address how exogenous application of NO can ameliorate the inhibitory effect of lead stress on mung bean seed germination and growth as well as providing evidences of the involvement of NO in alleviating oxidative stress.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Mung bean seeds (*Vigna radiata*) were obtained from the Agricultural Research Center, Giza, Egypt. They were surface sterilized with 2.5% sodium hypochlorite for 5 min, rinsed with distilled water, then soaked for 24h at 25˚C in aerated distilled water. Sodium nitroprusside (SNP) was used as NO donor and lead chloride (Pb Cl_2) as a source of lead (Pb). To investigate the effect of SNP on germination, a preliminary experiment was conducted where twenty soaked seeds were transferred to petri dishes (15 cm diameter) containing filter paper moistened with 20 ml of 0,25, 50,75 and 100 µM SNP, covered by lid, and incubated at 25±2°C for 3 days.

The germination percentage was calculated as a standard of radical emergence and the specified concentration of SNP at which the germination percentage dropped to about half was at 50 µM. The effect of different concentrations of lead alone or with SNP on the seed germination was evaluated by the same previously mentioned method. The soaked seeds were placed in petri dishes containing filter paper moistened with 20 ml of one of the following treatment solutions: (1) distilled water alone (control); (2) 50 µM SNP; (3) 30 ppm lead; (4) 30 ppm lead + 50 µM SNP; (5) 70 ppm lead; (6) 70 ppm lead + 50 µM SNP; (7) 100 ppm lead; and (8) 100 ppm lead + 50 µM SNP. In all there were eight treatments including the control with three replicates each.

The experiment was laid out in a completely randomized design in an environmentally controlled growth chamber under a 16h photoperiod at an irradiance of about 20 µmol m^{-2} s^{-1} (cool white fluorescent tubes) and 28/21 ± 2ºC light/dark temperature for 6 days.

Germination rate and fresh biomass

Germination rate was estimated by counting germinated seeds, as standard of radical emergence, daily for 6 days. The germination percentage was calculated as a standard of radical emergence and the results were expressed as percentage over control. Seven-days old germinated seeds, from each treatment, were taken for determination of fresh and dry biomasses. The separated cotyledons were either oven-dried at 65°C to a constant weight for total lead content determination or flash-frozen in liquid nitrogen and stored at - 80°C for further analyses.

Estimation of hydrogen peroxide content and lipid peroxidation

Hydrogen peroxide content was determined according to the method of (Velikova *et al*., 2000). Lipid peroxidation was monitored by spectro-photometric determination of malondialdhyde (MDA) using thiobarbituric acid (TBA) as described by (Valentovič *et al*., 2006).

Determination of total phenolic compounds

Total phenolic contents in the cotyledons untreated and Pb-treated mung bean seedlings were determined using the modified Folin-Ciocalteau reagent (McDonald *et al*., 2001). Water extract of each sample (0.5ml) was mixed with Folin Ciocaltean reagent (5ml, 1:10 diluted with distilled water) then aqueous 1M Na_2CO_3 (4 ml) was added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetrically by measuring the absorbance at 765 nm. Total phenol value was expressed as mg Gallic acid equivalents g^{-1} d. m. All samples were analyzed in three replications.

HPLC determination of individual phenolic compounds

Individual phenolic compounds in treated and untreated mung bean cotyledons were analyzed by HPLC by direct injection of 20 µl of the filtered methanol-extract (Marquez-Garcia *et al*., 2009). The separation and quantitative estimation were carried out using a HPLC system (Perkin Elmer series 200 LC and UV/VIS detector 200 LC, USA) equipped with a 5 µm column (Spheri-5 RP-18, 220 × 4.6 mm, Brownlee).

The mobile phase consisted of the following linear gradient: 5% methanol: 95% water (pH 2.6) and 80% methanol: 20% water (pH 2.2). The flow rate was 1 ml/min and the UV detector was set at 290 nm for the integration of peak areas after calibration with the external standard.

Enzyme assay

α- amylase activity (EC 3.2.1.1) was extracted and assayed following the method of (Swain and Dekker, 1966). One unit of α- amylase was defined as the amount of enzyme that produces 1µmol of glucose 30 min^{-1} at 37°C. The activity of neutral protease (EC 3.4.21.19) was assayed after (Cliffe and Law, 1982) where one unit of protease activity was defined as the amount of the enzyme required to produce an absorbance change of 0.1 30 min^{-1}. Under conditions of the assay, H^+-ATPase activity (E.C. 3.6.3.6) was assayed according to (Zenoff *et al*., 1994) by following the change in the pH value of the incubation medium, due to proton extrusion, during 30 min. For estimation of catalase (CAT, E.C. 1.11.1.6) and guiagol peroxidase (GPX, E.C.1.11.1.7) activities,
frozen plant tissues were homogenized in ice-cold 0.1 M potassium phosphate buffer (pH6.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 15,000 g for 20 min at 4°C and the supernatant was used for enzyme assays.

CAT activity was determined according to (Rios-Gonzalez *et al*., 2002) where the decomposition of H_2O_2 was followed at 240 nm (1 EU = 1 µmol H_2O_2 decomposed 1 min^{-1}). GPX activity was determined according to Urbanek *et al*., 1991, where the increase in absorbance was recorded at 470 nm for 1 min, expressed as µmol H_2O_2 min^{-1} g^{-1} d. m.

Protein extraction and gel electrophoresis

For SDS-PAGE, cotyledon tissues of each treatment were ground to powder under liquid nitrogen and melted in ice-cold extraction buffer (50 m M NaH_2PO_4 pH 7 ; 10 m M EDTA pH 8 ; 10 m M β -mercaptoethanol; 0.2% Triton X-100) per g of tissue, followed by centrifugation at 14,000 rpm, at 4°C for 15 min. The supernatant was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein quantification was done according to the method given by Bradford (1976). Electrophoresis was performed on 12.5% SDS-gel after (Laemmli, 1970) using vertical gel electro-phoresis apparatus (Model EC175, E-C Apparatus Corporation, St. Petersburg, USA). Gels were stained in 0.5% Coomassie Brilliant Blue R 250 in ethanol and 10% 3-chloroacetic acid.

Determination of lead

Oven-dried cotyledons were used for determination of lead concentration by the wet digestion methods using Flame atomizer (Humphries, 1956).

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as means ± standard error (SE). The data of all experiments were analyzed using the least significant differences (LSD) at level of P≤0.05 as described by (Steel and Torrie, 1980).

RESULTS

Increasing exogenous application of SNP levels stimulate mung bean seed germination up to 50µM and then steady declined (Fig 1). In absence of SNP, there was a significant decrease in germination percentage (Fig 2), fresh biomass and succulent value of mung bean seeds with increasing lead levels (Table 1), and this was accompanied with a significant increase in the lead accumulation. Under the prevailing experimental conditions, the increasing of lead accumulation was associated by increasing MDA concentrations (Fig 3B) (indicator of enhancement of lipooxygenase activity) which revealing the disturbance of plasma membranes in the cotyledons of germinating mung bean seeds.

On the other hand, addition of 50µM SNP could improve the plasma membranes integrity from the damage effect of lead increasing the activity of membrane -bounded H-ATPase enzyme (Table 2).

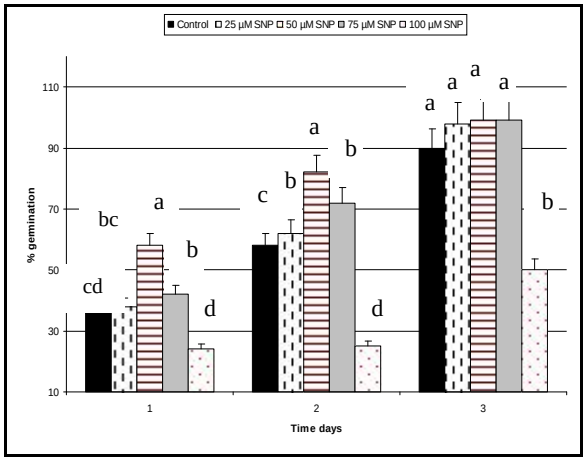

Figure (1): Changes in the germination percentage of mung bean seeds germinated at different SNP concentrations . Values are the means of 10 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Table (1): Effect of SNP (50 µM) on the lead-induced changes in fresh and dry biomass (g germinating seed ^{1}), succulent and lead-accumulation (g germinating seed ^{1}) in the cotyledons of mung bean germinating seeds exposed to Pb (30, 70 and 100 ppm, as lead chloride). Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Pb conc. (ppm)	Fresh biomass (g germinating seed $^{-1}$)		Dry biomass (g germinating seed $^{-1}$)		Succulent		Pb accumulation (µmol g - 1 DM)	
	-SNP	+ SNP	-SNP	+ SNP	-SNP	+ SNP	-SNP	+ SNP
0	5.34^{a} ±0.375	6.67^{a} ±0.468	0. 62^{c} ±0.043	0.39^{c} ±0.027	8.61^{a} ±0.604	17.10^{a} ±1.199	0.00^{d} ±0.000	0.00^{d} ±0.000
30	5.00^{a} ±0.351	6.28^{ab} ±0.440	0. 67^{b} ± 0.047	0.41^{c} ±0.029	7.46^{a} ± 0.523	15.31^{a} ± 1.074	92.5^{c} ± 6.488	16.7^{c} ± 1.171
70	3.46^{b} ± 0.243	5.66^{bc} ± 0.397	0. 80^{a} ± 0.056	0.59^{b} ± 0.041	4.33^{b} ± 0.304	9.59^{b} ± 0.673	1286.6^{b} ±90.237	959.1^{b} ï67.267
100	3.04^{b} ±0.213	4.89^{c} ±0.343	0.97^{a} ±0.068	0.68^{a} ±0.048	3.13^{c} ± 0.220	7.19^{b} ±0.504	4190.2^{a} ±293.884	3363.4^{a} ï 235.896

The result in (Table 2) clearly demonstrate that an increase in lead concentrations resulted in a significant decrease in α-amylase and protease activities of mung bean cotyledons which may led to a decrease in the row materials of the structural components (soluble sugars and amino acids) and hence suppress the seed germination . In contrast, supplementation of SNP with lead medium resulted in a marked increase of fresh matter, succulent value and the activity of both amylase and protease enzymes.

Exposure of mung bean seeds to various lead concentrations alone resulted in a significant accumulation of H_2O_2 and MDA (Fig 3A and B), revelling an enhancement of oxidative damage of plasma membrane integrity and decrease water absorption process. In addition, lead exposure, in absence of SNP, resulted in a significant increased of GPX and CAT activities (Fig 4A and B) Furthermore, addition of SNP with lead medium caused a marked increase the activity of CAT and GPX, comparing to absence of SNP

These observations were accompanied with a great decrease of lead and H_2O_2 as well as MDA content (Table 1, Fig 3 A and B). Increasing lead concentrations in absence of SNP resulted in a significant increase of total phenolics in mung bean cotyledons (Fig 3C). Furthermore, there was a marked increase of total tested individual phenolic components, particularly Gallic, chlorogenic and ferulic acids. At the same time, there was a marked decrease of cinnamic and coumaric acid (Table 3 and Fig 5) indicating the rapid biosynthesis of these phenolics, starting from phenylalanine via cinnamic and coumaric acid under lead stress.

Table (2): Effect of SNP (50 µM) on the lead-induced changes in α -amylase, protease (Unit mg ^{1} protein) activities in the cotyledons of mung bean germinating seeds exposed to Pb(30, 70 and 100 ppm, as lead chloride). Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Pb conc. (ppm)	*α -amylase (unit mg^{1} protein)*		*Protease (unit mg^{1} protein)*		*H^{+}-ATPase (Δ pH) (pH of incubation medium 30 min^{-1})*	
	-SNP	**+ SNP**	**-SNP**	**+ SNP**	**-SNP**	**+SNP**
0	78.43^{a} ±5.501	80.07^{a} ±5.616	1.43^{a} ±0.100	1.85^{a} ±0.136	7.00^{a} ± 0.491	6.5^{a} ±0.456
30	75.54^{a} ±5.298	84.48^{a} ±5.925	0.86^{a} ±0.060	1.86^{a} ±0.13	5.4^{b} ±0.379	5.5^{b} ± 0.386
70	61.98^{b} ±4.347	73.91^{b} ±5.184	0.54^{b} ±0.038	0.78^{b} ±0.055	5.7^{b} ±0.400	5.7^{b} ±0.400
100	39.81^{c} ±2.792	57.31^{c} ±4.019	0.41^{b} ±0.029	0.55^{c} ±0.039	6.3^{b} ±0.442	5.9^{b} ±0.414

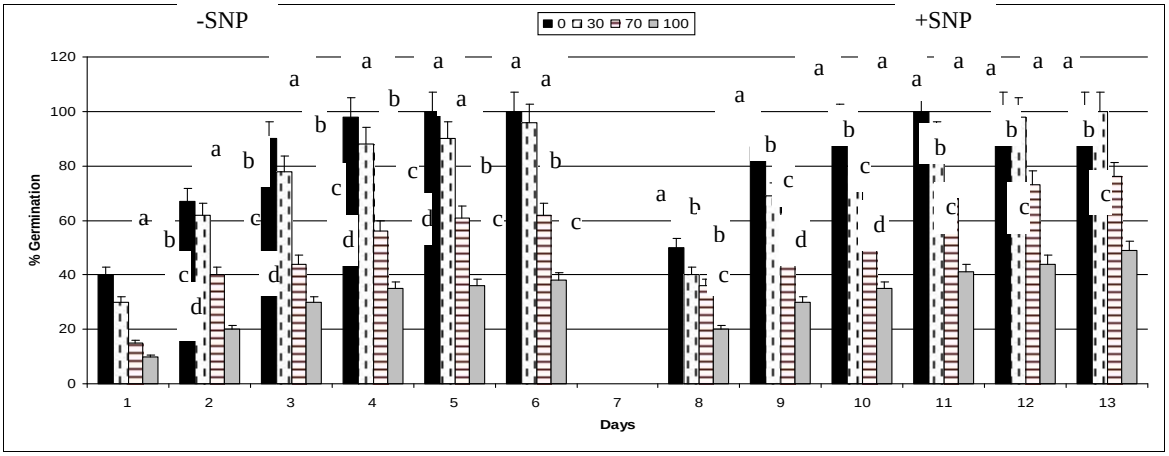

Figure (2): Changes in the germination percentage of mung bean seeds germinated at different Pb concentrations in presence or absence of 50 µM SNP. Values are the means of 10 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

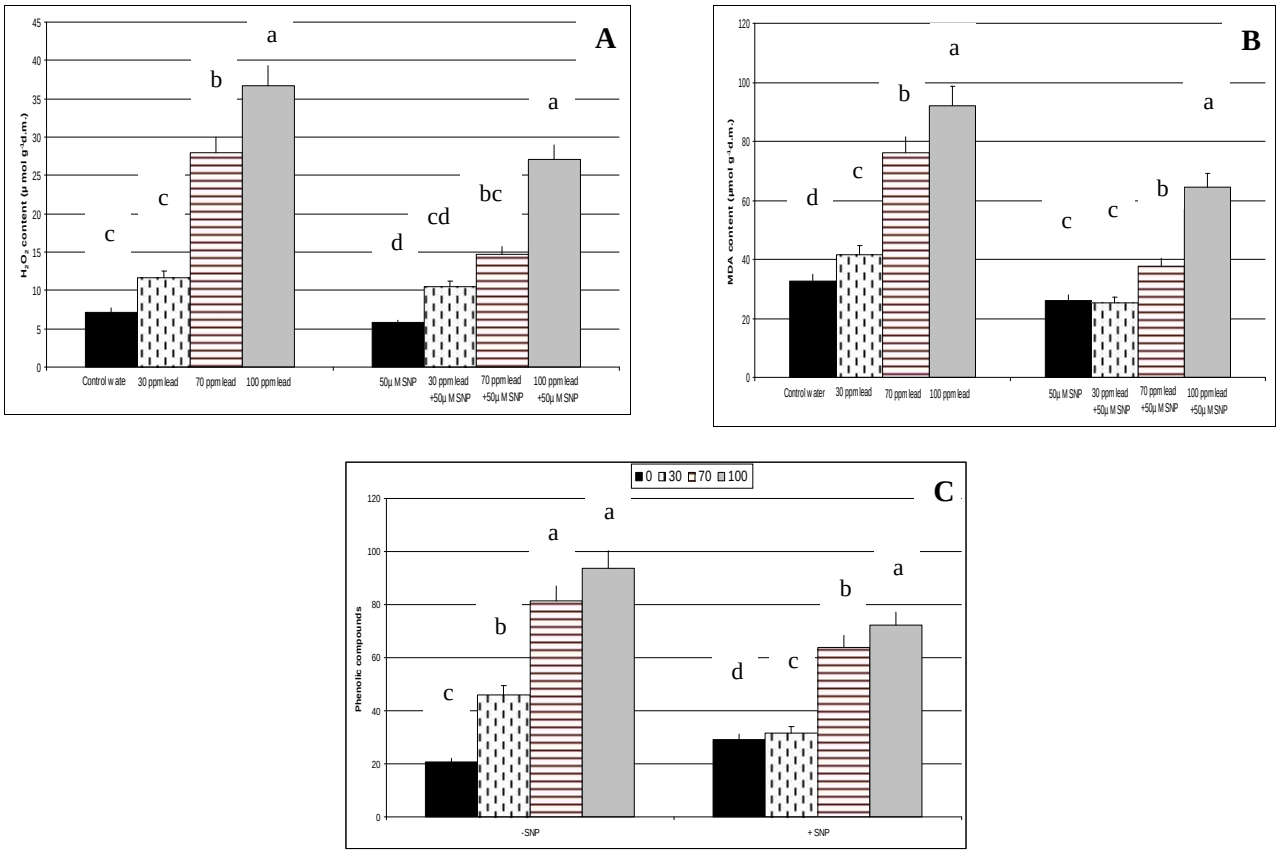

Figure (3): Effect of SNP (50 µM) on the Pb-induced (**A**) H_2O_2 content (**B**) lipid peroxidation, and (**C**) changes in total phenolic compounds in the cotyledons of mung bean germinating seeds, exposed to Pb (30, 70 and 100 ppm, as lead chloride). Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

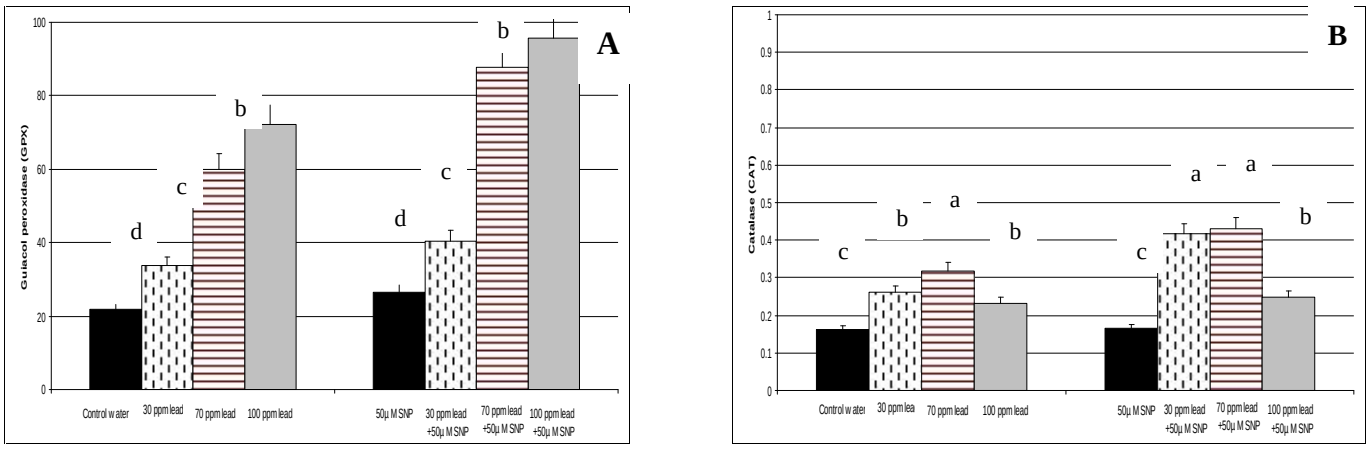

Figure (4): Effect of SNP (50 µM) on the change in activities of (A) GPX and (B) CAT in the cotyledons of mung bean germinating seeds, exposed to Pb (30, 70 and 100 µM, as lead chloride). Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

It is well noted that increasing lead concentration resulted in appearance of dark brown color in cotyledons as well as decrease of germination percentage, this may reveal toenhancement of chlorogenic and ferulic acid accumulation.Conversely, adding of 50 µM of SNP with various lead concentrations resulted in a marked decrease of total phenolic and individual compounds, comparing to absence of SNP. These observations accompanied with a great decline of H_2O_2 accumulation (Fig 3A) indicating the role of NO and phenolic components in ameliorating the oxidative stress caused by lead.

Increase in the lead concentrations in presence and absence of SNP brought about drastic changes in the polypeptide patterns in the cotyledons of the germinating mung bean seeds (Table 4 and Fig 6). There were three stress proteins with molecular mass of 110 , 97 and 21 KDa were appeared in presence of 30 µM lead alone or in the presence of SNP (lane, 4), while the stress protein of 110 KDa was disappeared in presence of 70 µM lead (lane, 5). At 100µM lead alone or in the presence of SNP these stress proteins were completely disappeared (lane 7, 8). A polypeptide stress protein with molecular mass 65 KDa was appeared in presence of SNP (lanes 2,4,6,8,). In addition, 74 KDa polypeptide stress protein was appeared only with high levels of lead and absence of SNP. On the other hand, polypeptide with 81 KDa (lane 1) was completely disappeared in all treatments.

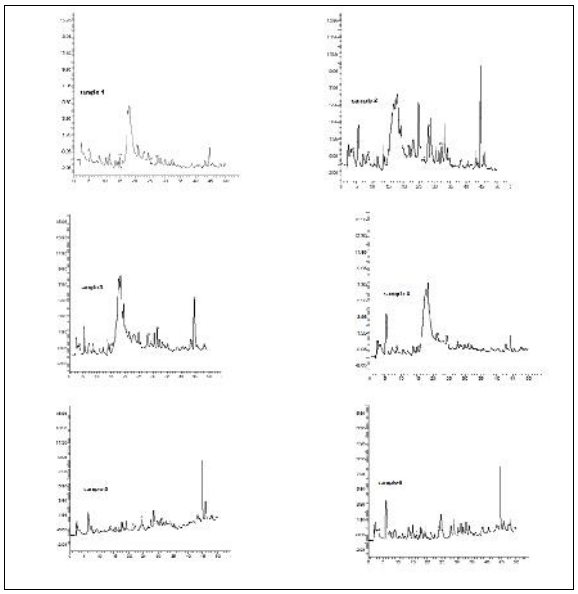

Figure (5): HPLC chromatograms of phenolic compounds in the cotyledons of mung bean germinating seeds treated with different concentrations of Pb (30, 70 and 100 ppm) in absence or presence of 50 µM SNP. Sample (1) control; Sample (2) 50 µM SNP; Sample (3) 30 ppm Pb; Sample (4) 30ppm Pb +SNP; Sample (5) 100 ppm Pb; Sample (6) 100 ppm Pb +SNP

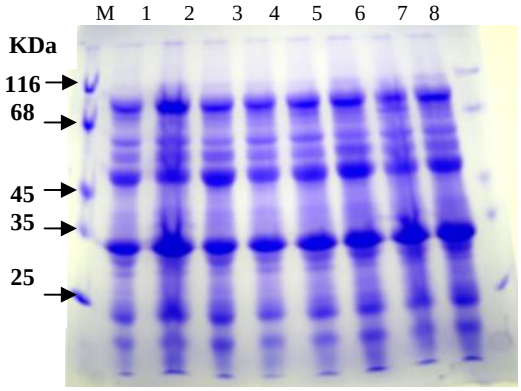

Figure (6): Changes in polypeptide patterns in the cotyledons of mung bean germinating seeds treated with different concentrations of Pb (30,70 and 100 ppm) in absence or presence of 50 µM SNP. M, size marker; (1) control; (2) 50 µM SNP; (3) 30ppm Pb;(4) 30ppm Pb + SNP; (5) 70 ppm Pb ; (6)70 ppm Pb + SNP; (7) 100 ppm Pb; (8) 100 ppm Pb + SNP. Fifteen micrograms of protein were loaded in each lane and the gel was stained with CBB.

DISCUSSION

Stimulation of mung bean seed germination in presence of 50 µM of SNP were consistent with other studies reported for several plant species including lupin (Kopyra and Gwozdz, 2003), Arabidopsis (Bethke *et al*., 2007), warm seasons grases (Sarath *et al*., 2006), wheat (Hu *et al*., 2007), lentil (Erol *et al*., 2008) and rice (Habib *et al*., 2010). The enhancement of mung bean seed germination may be related to the inducing effect of NO on the synthesis of gibberellins, as endogenous auxins, (Correa-Aragunde *et al*., 2004 and Singh *et al*., 2007) and ethylene which plays an important role in the breakdown of seed dormancy(Shri *et al*., 2009; Shaibur and Kawai, 2010) Similarly, several studies have showed that increased lead concentrations caused a reduction of plant growth in a number of plants (Dubey, 2003; Astolfi *et al*., 2003; Kalaji and Loboda, 2007; Shaibur and Kawai, 2010).

The impaired of mung bean seed germination may be, in part, due to disturbance of plasma membrane integrity and hence the decrease in water absorption as well as the inhibition of several biochemical processes. It is showed that increasing lead levels resulted in a significant decrease of H^+-ATPase activity (as indicated by high pH of incubation medium) revealing the disturbance of plasma membrane integrity (Table 2). (Dubey, 2003) found that increasing lead concentrations resulted in a disorder of plasma membrane of growing rice plants. In this connection, (Astolfi *et al*., 2003) have reported this observation in Avena sativa roots. (Meharg, 1993) concluded that the damage of plasma membrane may relate to oxidation the cross-linking of protein thiol, changes of the composition and fluidity of membrane lipids via increasing lipooxygenase activity. Recently, (Cui *et al*., 2010) reported that application of 100µM SNP adjusted the activity of H^+- ATPase in tomato plants.

The depression of α-amylase and protease activities due to other heavy metals like chromium and nickel has been reported (Ashraf *et al.*, 2011). Consequently, increase in the dry biomass (Table 1) may be due to the accumulation of more un-hydrolized storage material, and hence decrease the germination of mung bean seeds. In contrast, addition of SNP to lead medium resulted in a marked increase of fresh matter, succulent value and the activity of both amylase and protease enzymes revealing that SNP improves- to some extent- the germination of mung bean seeds due to its counteracting the inhibitory effect of lead. (Zhang *et al*., 2005) reported that NO plays an important role in α-amylase and protease induction in wheat seeds during early germination.

In addition, (Hu *et al*., 2007) stated that NO has an inductive effect on α-amylase under copper stress. It is shown from the current study that, supplementation of SNP greatly decreased the lead accumulation and which associated with a marked increase of α-amylase and protease activities in mung bean cotyledons (Table 2). Many authors (Loureiro *et al.*, 2006; Hu *et al*., 2007 and Gupta *et al*., 2009) have reported that lead promotes the formation of reactive oxygen species in plants leading to oxidative stress.

Exposure of mung bean seeds to various lead concentrations alone resulted in a significant accumulation of H_2O_2 and MDA (Fig 3A and B) revealing an enhancement of oxidative damage of plasma membrane integrity and decrease water absorption process.

In addition, lead exposure, in absence of SNP, resulted in a significant increased of GPX and CAT activities (Fig 4) indicating the development of a defense strategy mechanism to overcome lead- induced oxidative stress These observations are in agreement with those reported for some hyper-accumulator fern species (Srivastava *et al*., 2005), Indian mustard (Khan *et al*., 2009), rice (Singh *et al*., 2009) and tall fescue (Jin *et al.*, 2010). In contrast, addition of SNP with lead medium markedly increased the activity of CAT and GPX, comparing to absence of SNP (Fig 4).These observations were accompanied with a great decrease of lead and H_2O_2 as well as MDA content (Table 1, Fig 3), indicating that SNP ameliorate the lead-induced toxicity in germinating mung bean via suppressing lead uptake and hence decreasing the generation of ROS and oxidative stress. (Singh *et al*., 2009 and Jin *et al*., 2010) reported that NO (as SNP) resulted in a marked decrease of ROS generation and preserves membrane integrity by inhibiting lipid peroxidation (Sakihama *et al*., 2006 and Michalak, 2006) stated that the increase of the synthesis of several phenolic compounds in plant tissues under different environmental stress was clearly observed. (Lea, 1990) stated that oxidation of chlorogenic acid by polyphenol oxidase resulting in yellow and brown pigments

.Also, Van Sumere *et al*.,1989 reported that presence of ferulic acid at 10 - 4 M resulted in a marked decrease of germination of Raphanus sativus . It has been reported that phenolics may act as a reducing agents and singlet oxygen quenchers prevent in the evolution of ROS (Grace, 2005 and Bidel *et al*., 2007) In addition, (Sroka and Cisowski, 2003 and Ramma *et al*., 2005) revealed that gallic acid was responsible for the antioxidant activity of black tea and suggested that these seemed to be a good correlation among gallic acid content and antioxidant activity.

It is noted in the present study that, there was an increase of gallic acid accumulation (Table 3) with an increase of the activity of both antioxidant enzymes GPX and CAT (Fig. 4), therefore, indicating the role of accumulated phenolics such as gallic acid for alleviating the oxidative damage by scavenging the generated ROS.

There are two possibilities to explain this ameliorating mechanisms : a) The bioactive molecule (NO) act as direct acceptor of the generated oxygen radicals i.e. direct antioxidant. (Jin *et al*., 2010) reported that NO reacting with O_2^- and generating peroxynitrite ion ($ONOO^-$) which is unstable product protonated and decomposed to a nitrate anion and a proton or it can react with hydrogen peroxide to yield a nitrite anion and oxygen and b) in presence of NO, the phenolics were enhanced to donate their protons to reduce the free oxygen radicals. (Rezazadeh *et al.*, 2012) concluded that phenolics act as antioxidant for singlet oxygen as H-donor.

Table (3): Effect of SNP (50 µM) on the lead-induced changes in total individual phenolics in the cotyledons of mung bean germinating seeds exposed to Pb (30, 70 and 100 µM, as lead chloride). Colum 1 (Gallic acid), Colum 2 Chlorogenic acid, Colum 3 Protocatechuic acid, Colum 4 Ferulic acid, Colum 5 Cinnamic acid, Colum 6 Coumaric acid, Colum 7 Vanillic acid, Colum 8 Sinapic acid .

Pb conc. (ppm)	Total individual phenolics (µg g^{-1} d.m.)							
	-SNP							
	gallic	**Chlor**	**Prot.**	**Fer.**	**Cinn.**	**Cou.**	**Van.**	**Sina.**
0	13668	365	225	491	16553	19816	119	286
30	39056	3276	5012	1784	3619	1695	6376	6008
100	104909	4413	8132	2934	818	467	17315	36776
	+ SNP							
	Gallic	**Chlor**	**Prot.**	**Fer.**	**Cinn.**	**Cou.**	**Van.**	**Sina.**
0	14352	757	2326	1356	7181	4823	163	2381
30	28352	1856	4153	1356	4572	3321	708	2686
100	95990	3705	7332	2525	1176	1125	7476	5686

Table (4): Occurrence of protein bands and their molecular masses (MM) as revealed by SDS-PAGE of total protein in the cotyledons of mung bean germinating seeds treated with different concentrations of Pb (30, 70 and 100 ppm) in absence or presence of 50 µM SNP.

MM of bands (kDa)	Control (H_2O)	50 µM SNP	30 ppm Lead	30 ppm Lead+ SNP	70 ppm Lead	70 ppm Lead + SNP	100 ppm Lead	100 ppm Lead+ SNP
110	-	+	+	+	-	+	-	-
97	-	+	+	+	+	+	-	-
88	+	+	+	+	+	+	+	+
81	+	-	-	-	-	-	-	-
74	_	-	-	-	+	+	+	+
65	_	+	-	+	-	+	-	+
62	+	+	+	+	+	+	+	+
57	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+
43	+	+	+	+	-	-	-	-
31	+	+	+	+	+	+	-	-
28	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
21	-	+	+	+	+	+	-	-
18	+	+	+	+	-	-	-	-

The variations of appearance stress proteins were shown in several plants subjected to heavy metal stress (Ahsan *et al.*, 2010). (Latif *et al*., 2010) reported that the induction of a new polypeptide of 78.61 kDa in radish plant exposed to Ni stress. Frydman,(2001) and (Huang and Xu, 2008) reported that the appearance of stress proteins may reveal their roles in preventing aggregation and assisting refolding of non-native proteins under both normal and stress conditions. In addition, under the prevailing experimental condition, the disappearance of some protein bands could be due to lowered protein synthesis and/or the depletion of reserve proteins to overcome stress.

It is concluded from this study that the drastic effect of lead on the mung bean seed germination and growth may relate to enhancement of ROS generation and oxidative stress as shown by increasing H_2O_2 and MDA content in lead treated cotyledons. This resulted in disturbance of plasma membrane integrity decrease of water absorption as well as decline of hydrolytic enzymes activities. Addition of SNP may play an ameliorating role as a direct antioxidant or induces phenolic compounds to reduce the inhibitory effect of ROS as H- donating agent

REFERENCES

AHSAN, N., D. LEE, K. KIM, I. ALAM, S. LEE, K. LEE, H. LEE., AND B. LEE. 2010. Analysis of arsenic stress-induced differentially expressed proteins in rice leaves by two-dimensional gel electrophoresis coupled with mass spectrometry. Chemosphere **78**: 224 - 231.

ASHRAF, M.Y., R. SADIQ, M. HUSSAIN, M. ASHRAF, AND M.S.A. AHMAD . 2011. TOXIC EFFECT OF nickel (Ni) on growth and metabolism in germinating seeds of sunflower (*Helianthus annuus L.*) BiolTrace Element Res (Online publication date 15 Jan).

ASTOLFI, S., S. ZUCHI, A. CHIANI, AND C. PASSERA . 2003. In vivo and in vitro effects of cadmium on H+ATPase activity of plasma membrane vesicles from oat (*Avena sativa L.*) roots . J . Plant PhysioL **160**: 387 - 393.

BETHKE, P.C., I.G. LIBOUREL, N. AOYAMA, Y.Y. CHUNG, D.W. STILL, AND R.L .JONES. 2007. The Arabidopsis aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. Plant Physiol **143**: 1173 - 1788.

BIALONSKA, D., A.M .ZOBEL, M. KURAS, T. TYKARSKA, K. SAWICKA - KAPUSTA. 2007. Phenolic compounds and cell structure in bilberry leaves affected by emissions from a Zn-Pb smelter. Water Air and Soil Pollutant **181:** 123 - 133.

BIDEL, L.P.R., S. MEYER, G. YOULAS, Y. CADOT, AND Z.G. CEROVIC. 2007. Responses of epidermal phenolic compounds to light acclimation. In vivo qualitative and quantitative assessment using chlorophyll Fluorescence excitation spectra in leaves of three woody species . J. Photochem. Photobiol **88**: 163 - 179.

BRADFORD, M . M. 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **72**: 248 - 254.

CLIFFE , A.J., AND B.A. LAW. 1982. A new method for the detection of microbial proteolytic enzyme in milk. J. Dairy Res **49**: 209 - 219 .

CORREA-ARAGUNDE, N., M. GRAZIANO, AND L .LAMATTINA . 2004. Nitric oxide plays a central role in determining lateral root development in tomato. Planta **218** (6): 900 - 905.

CUI, X.M., Y.K. ZHANG, X.B. WU, AND C.S. LIU. 2010. The investigation of the alleviated effect of copper toxicity by exogenous nitric oxide in tomato plants. Plant Soil and Environment **56**: 274 - 281.

DE PINTO, M.C., A. PARADISO, P. LEONETTI. 2006. Hydrogen peroxide, nitric oxide and cytosolic ascorbate peroxidase at the crossroad between defense and cell death. Plant J **48**: 784 - 795.

DE PINTO, M.C., F .TOMMASI, AND L. DE GARA. 2002. Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco bright yellow 2 cells. Plant Physiol **130**: 698 - 708.

DELLEDONNE, M . 2005. NO news is good news for plants. Curr Opin Plant Biol **8**: 390 - 396.

DUBEY, S.V.R.S. 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Science **164:** 645 - 655.

EROL, Ç.S., I. ÎSMAILOGLU, G.C.Ö.M. ÜNAL, AND S. YENTÜR'. 2008 . The effects of nitric oxide on some early germination parameters and mitotic activity in Lentil (Lens Culunaris Medik.). Journal of Applied Biological Sciences **2** (3): 01 - 07.

FRYDMAN, J. 2001. Folding of newly translated proteins in vivo: the role of molecular chaperons. Annual Review of Biochemistry **70**: 603 - 647.

GRACE, S.C. 2005. Phenolics as antioxidants. In Antioxidants, and Reactive Oxygen Species in Plants Smirnoff N [ed]. Blackwell Scientific Publishers Oxford, U.K. 141 - 168.

GUPTA, M., P. SHARMA, N.B. SARIN, A.K. SINHA . 2009. Differential response of arsenic stress in two varieties of *Brassica juncea* L. Chemosphere **74**: 1201 - 1208.

HABIB, N., M. ASHRAF, AND M.S.A . AHMAD. 2010 . Enhancement in seed germinability of rice (*Oryza Sativa* L.) by pre-sowing seed treatment with nitric oxide (NO) under salt stress. Pakistan Journal of Botany **42** (6): 4071 - 4078.

HE, Y.K. , R.H .TANG, Y. HAO, R.D. STEVENS, C.W. COOK, S.M. AHN, L. JING, Z. YANG , L. CHEN , F. GUO, F. FIORANI, R.B. JACKSON, N.M. CRAWFORD, AND Z.M. PEI . 2004. Nitric oxide represses the Arabidopsis floral transition science **305**: 1968 - 1971.

HU, K.D., L.Y. HU, Y.H. LI, F.Q. ZHANG, AND H. ZHANG . 2007. Protective roles of nitric oxide on germination and antioxidant metabolism in wheat seeds under copper stress. Plant Growth Regulation **53**: 173 - 183.

HUANG, B., AND C. XU. 2008. Identification and characterization of proteins associated with plant tolerance to heat stress. Journal of Integrative Plant Biology **50**: 1230 - 1237.

HUMPHRIES, E.C. 1956. Mineral composition and ash analysis in : Modern Methods Of Plant Analysis Peach K and Tracey M V eds Springer Verlag Berlin Gottingen Heidlberg 468 - 502.

JAJA, E.T. , AND C.S.I. ODOEMENA . 2004 . Effect of Pb, Cu and Fe compounds on the germination and early seedling growth of tomato varieties. Journal of Applied Science and Environment Management **8** (2): 51 - 53.

JIN, J.W., Y.F. XU, AND Y.F. HUANG . 2010. Protective effect of nitric oxide against arsenic-induced oxidative damage in tall fescue leaves. African Journal Biotechnology **11:** 1619 - 1627.

JUNG, C., V. MAEDER , F. FUNK , F. FREY , H . SHEHER , AND I. FROSSARD. 2002. Release of phenols from *Lupinus albus L*. roots exposed to Cu and their possible role in Cu detoxification. Plant and Soil **252**: 301 - 312.

KALAJI, H.M., AND T. LOBODA. 2007. Photosystem II of barley seedlings under cadmium and lead stress. Plant Soil Environ . **53** (12): 511 - 516.

KHAN, I., A. AHMAD, AND M. IQBAL. 2009. Modulation of antioxidant defense system for arsenic detoxi-fication in Indian mustard. Ecotoxicol Environ Saf **72**: 626 - 634.

KOPYRA, M., AND E.A. GWOZDZ. 2003.Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of Lupinus luteus. Plant Physiol Biochem **41**:1011 - 1017.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680 - 685.

LASPINA, N.V., M .D. GROPPA , M. L. TOMARO, AND M.P .BENAVIDES. 2005. Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. Plant Science **169**: 323 - 330.

LATIF, H.H. 2010 The influence of nickel sulphate on some physiological aspects of two cultivars of *Raphanus sativus* L. Archives of biological Sciences Belgrade **62**: 685 - 693.

LATTANZIO, V., AND P. RUGGIERO. 2003. Biochimica *Agraria Scarponi* L. [ed.]. Patron Editore Bologna 631.

LEA, A.G.H. 1990. Bitterness and astringency: the procyanidins of fermented apple ciders. In: Roussef R.L. [ed.]. Bitterness in Food and Beverages Elsevier New York. 123 - 143.

LOUREIRO, S., C. SANTOS, G. PINTO, A. COSTA, M. MONTEIRO, A.J.A NOGUEIRA, AND A.M.V.M. SOARES. 2006. Toxicity assessment of two soils from Jales Mine (Portugal) using plants growth and biochemical parameters. Archives of Environmental Contamination Toxicology **50**: 182 - 190.

MARQUEZ-GARCIA, B., M.N. FERNANDEZ, AND F. CORDOBA. 2009. Phenolics composition in Erica sp. Differentially exposed to metal pollution in the Iberian Southwestern Pyritic Belt. Bioresource Technology **100**: 446 - 451.

MCDONALD, S., PD. PRENZLER, M. ANTOLOVICH, K. ROBARDS, (2001) Phenolic content and antioxidant activity of olive extracts. Food Chem **73**: 73 - 84.

MEHARG, A. A. 1993. The role of plasma lemma in metal tolerance in angiosperms. Physiol Plantarum **88**: 191 - 198.

MICHALAK, A. 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies **15**: 523 - 530.

NEUMANN, D., O. LICHTENBERGER, K. TSCHIERSCH, AND L. NOVER. 1994. Heat- shock proteins induce heavy-metal tolerance in higher plants. Planta **194**: 360 - 367.

ODJAGBA, V.J., AND I.O. FASIDI. 2006. Effects of heavy metals on some proximate composition of Eichhornia crassipes. Journal of Applied Science and Environment Management **10** (1): 83 - 87.

PAGNUSSAT, G.C., M. SIMONTACCHI, S. PUNTARULO, AND L. LAMATTINA. 2002. Nitric oxide is required for root organogenesis. Plant Physiology **129**: 954 - 956.

RAMMA, A.L., T. BAHORUN, A. CROZIER , V. ZBARSKY, K.P. DATLA , D. T. DEXTER, AND O.I. ARUOMA. 2005. Characterization of the antioxidant functions of flavonoids and proanthocyanidins in Mauritian black teas. Food Res. Int. **38**: 357 - 367.

REZAZADEH, A., A. GHASEMNEZHAD , M. BARANI , AND T. TELMADARREHEI. 2012. Effect of salinity on phenolic composition and antioxidant activity of Artichoke (*Cynara scolymus L.*) leaves. Res. J. Med. Plant 1819 - 3455.

RIOS-GONZALEZ, K., L. ERDEI, AND S. HERMAN. 2002. The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. Plant Science **162**: 923 - 930.

SAKIHAMA, Y.M.F., S.C. COHE, H. GRACE, AND H. YAMASAKI. 2006. Plant phenolic antioxidant and prooxidant activities :phenolic induced oxidative damage mediated by metals in plants. Toxicology **177**: 67 - 80.

SARATH, G., P.C . BETHKE, R. JONES, L.M. BAIRD, G. HOU, AND R.B. MITCHELL. 2006. Nitric oxide accelerates seed germination in warm-season grasses. Planta **223** (6): 1154 - 1164.

SHAIBUR, M.R., AND S. KAWAI. 2010. Effect of Arsenic on Nutritional Composition of Japanese Mustard Spinach: An Ill Effect of Arsenic on Nutritional Quality of a Green Leafy Vegetable. Nature and Science **8** (8): 186 - 194.

SHRI, M., S. KUMAR , D. CHAKRABARTY, P.K. TRIVEDI, S. MALLICK, P. MISRA, D. SHUKLA, S. MISHRA, S. SRIVASTAVA, R.D. TRIPATHI, AND R. TULI. 2009. Effect of arsenic on growth, oxidative stress, and antioxidant system in rice seedlings. Ecotoxicology and Environmental Safety **72**: 1102 - 1110.

SINGH, H.P. , D.R . BATISH , G . KAUR, K. ARORA , AND R.K. KOHLO. 2008. Nitric oxide (as sodium nitroprusside) supplementation ameliorates Cd toxicity in hydroponically grown wheat roots Environmental and Experimental botany **63**: 158 - 167.

SINGH, H.P., D.R. BATISH, R.K. KOHLO, AND K. ARORA. 2007. Arsenic- induced root growth inhibition in mung bean (Phaseolus aureus Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regulator **53**: 65 - 73.

SINGH, H.P., S . KAUR , D.R. BATISH , V. P. SHARMA, N SHARMA, AND R.K. KOHLI. 2009 Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of Oryza sativa (rice). Nitric Oxide **20**: 289 - 297.

SRIVASTAVA, M., L.Q. MA, N. SINGH, AND S. SINGH .2005. Antioxidant responses of hyperaccumulator and sensitive fern species to arsenic. Journal of Experimental Botany **56**: 1335 - 1342.

SROKA, Z., AND W. CISOWSKI. 2003. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. Food Chem. Toxicol **41**:753 - 758.

STEEL, R.C., AND J.H. TORRIE. 1980. Principles and procedures of statistics, (2nd ed.) Mc Graw Hill, New York.

SWAIN, R.R., AND E.E. DEKKER. 1966. Seed germination studies. I Purification and properties of an α -amylase from the cotyledons of germinating peas. Biochimica Biophysica Acta **122**: 75 - 86.

URBANEK, H., E. KUZNIAK-GEBAROWSKA, AND K. HERKA.1991. Elicitation of defense responses in bean leaves by Botrytis cinerea polygal-acturonase. Acta Phys. Plant **13**: 43 - 50.

VALENTOVIČ, P., M. LUXOVA, L. KOLAROVIC, AND O. GASPARIKOVA. 2006. Effect of osmotic stress on compatible solutes content, membrane stability and water relations in two maize cultivars. Plant Soil Environment **52**: 186 - 191.

VAN SUMERE, C.F. 1989. Methods in Plant Biochemistry, Vol. 1 Plant Phenolics, Dey, P.M. and Harborne, J.B. [Eds.]. Academic Press,London. 29.

VELIKOVA, V., I. YORDANOV, AND A. EDREVA . 2000. Oxidative stress and some antioxidant system in acid rain treated bean plants: Protective role of exogenous polyamines. Plant Science **151**: 59 - 66.

WANG, Y.S., AND Z.M .YANG. 2005. Nitric oxide reduces aluminum toxicity by preventing oxidative stress in the roots of Cassia tora *L Plant Cell Physiology* **46**: 1915 - 1923.

WOLLGIEHN, R., AND D. NEUMANN. 1999. Metal stress response and tolerance of cultured cells from Silene vulgaris and *Lycopersicon peruvianum*: role of heat stress proteins. Journal of Plant Physiology **154**: 547 - 553.

ZENOFF, A.M., M. HILAL, M. GALO, AND H. MORENO . 1994. Changes in roots lipid composition and inhibition of the extrusion of proton during salt stress in two genotypes of soybean resistant or susceptible to stress. Plant Cell Physiol **35**: 729 - 735.

ZHANG, H. , W.B .SHEN, W. ZHANG, AND L.L. XU .2005. A rapid response of b-amylase to nitric oxide but not gibberellins in wheat seeds during the early stage of germination. Planta **220**: 708 - 716.

ZHAO, L. ,F. ZHANG , AND J. GUO .2004. Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. Plant Physiol **134**: 849 - 857.

Received August 5, 2013
Accepted April 9, 2013

دور اكسيد النيتريك فى معالجة نبات اللوبيا النامى تحت تاثير عنصر الرصاص

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الملخص العربـــــى

هذا البحث يدرس مدى امكانية معالجة تاثير عنصر **الرصاص** على نبات اللوبيا باستخدام اكسيد النيتريك (محلول نيتروبروسيد الصوديوم ٥٠ ميكرومول) ذلك فى مرحلة الانبات الاولى لنبات اللوبيا حيث لايتعدى ذلك ثلاثة ايام وذلك فى ظل الظروف الطبيعية **فى المعمل. وقد اوضحت النتائج نقصا ملحوظا فى نسبة الانبات و الوزن الطازج والمحتوى المائى وكذلك نشاط** انزيمى الاميليز والبروتييز المسئولين عن قدرة النبات على الانبات وانزيم الاتيبيز وكان ذلك مصحوب بالزيادة الواضحة فى كل من كمية الهيدروجين بيراوكسيد والمالون دايالدهيد وانزيم الكتاليزوالجواياكولبيراكسييز بالاضافة الى الزيادة الموجودة فى المركبات الفينولية كليا او الزيادة فى الاحماض الفينولية المفردة ويوجد تغير ملحوظ فى الظهور او الاختفاء للبندات البروتينية الموجودة فى مختلف تركيزات الرصاص المدروسة فى هذا البحث.
عند اضافة اكسيد النيتريك يوجد تحسن واضح وملحوظ فى الوزن الطازج والمحتوى المائى وكذلك كل الانزيمات المدروسة فى هذا البحث وايضا التراكم الواضح فى كلا من الهيدروجين بيراوكسيد والمالونداالدهيد والمركبات الفينولية وظهور بروتينات جديدة كنوع من انواع الحماية فى وجود اكسيد النيتريك.