Kinetin and/or calcium affect growth of *Phaseolus vulgaris* L. plant grown under heavy metals stress

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Abstract

Heavy metals stress is one of the major abiotic stresses that cause environmental pollution and hence indefinite hazards to living organisms in recent decades. The current study was conducted with the goal in the mind to clarify the ameliorative role of seed presoaking in kinetin (30 ppm) and/or calcium chloride (40 mM) in counteracting the deteriorative effect of foliar application of nickel (2.5 mM) and/or lead (0.5mM) on the growth and some metabolic activities of *Phaseolus vulgaris* L. plant. Nickel and/or lead treatments significantly reduced the leaves area/plant, total pigments, soluble and insoluble sugars, amino-nitrogen, catalase enzyme, total nitrogen, potassium, calcium and magnesium contents. On the other hand, proline, amino-N, MDA, total phenols, SOD, POX, nickel and lead contents were significantly increased in response to heavy metal treatments. On contrary, seed presoaking in kinetin and calcium chloride alone or in combination significantly increased the leaves area/plant, total pigments, soluble and insoluble sugars, amino-nitrogen, catalase enzyme, total nitrogen, potassium, calcium and magnesium contents. Meanwhile, proline, MDA, total phenols, SOD, POX, nickel and lead contents were significantly decreased by kinetin and calcium chloride treatments. Kinetin and/or calcium chloride were found to be the most effective in enhancing the plant tolerance towards nickel and/or lead toxicity.

Key words: Antioxidant enzymes; Calcium; Kinetin; Lead; Nickel; *Phaseolus vulgaris*.

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1. Introduction

Heavy metals stress is a major factor that limits agricultural productivity. Excessive concentration of heavy metals is known to cause deleterious effects on many physiological processes of plants such as photosynthesis, mineral nutrition, and the water relationships (Ali *et al*., 2013 and Li *et al*., 2013). In addition to the direct effects on plants, heavy metals adversely affect plants indirectly by producing an excess reactive oxygen species (ROS) (Thounaojam *et al*., 2012 and Li *et al*., 2013). Aldoobie and Beltagi (2013) reported that, Leaf area of *Phaseolus vulgaris* was significantly decreased in response to lead, and nickel, treatments. Leaf area per plant was also declined in bean plants under the individual treatments of both Ni and Al (Al-Qurainy, 2009). While, Ratushnyak *et al. (*2012) did not detect any statistically significant variations in leaf length and width of *Pisum sativum* in response to lead treatment.

Photosynthesis is one of the most sensitive processes to lead. The reduction in leaf growth led to decreased photosynthetic pigments, changed chloroplast structure, and declined enzyme activities for CO_2 assimilation (Parys *et al*., 1998). Heavy metals applications reduced the chlorophyll content of *Phaseolus vulgaris* L. seedlings. Also, it caused an oxidative stress in bean plants (Zengin, 2013). The strongest influence on chlorophyll was found in plants in response to nickel, followed by the sequence cobalt > chromium> zinc. Nickel and cobalt of chick pea plant induced physiological disorders like reduction in leaf chlorophyll (Khan and Khan, 2010). Also, Gajewska *et al*. (2006) registered a reduction in the amount of chlorophyll in *Triticum aestivum* L. when exposed to Ni, while, the total chlorophyll content and relative content proportion of chlorophyll a and b were decreased by lead, through inhibition of chlorophyll biosynthesis (Ernst *et al*., 2000).

The total soluble sugars contents in *Phaseolus vulgaris* Nebraska plants increased under treated with lead, chromium, nickel and cadmium. Moreover, the contents of polysaccharides were reduced under all heavy metals stress, and then reduced by liming treatment except for lead-treated plants (Aldoobie and Beltagi, 2013).

Soluble protein contents of *Zygophyllum xanthoxylon* were significantly decreased at lower Ni concentration (50 mg/kg), but when exposed to high Ni doses (150, 450 and 900 mg/kg), it was in- creased as compared with control (Yan Lu *et al*., 2010). Proline accumulation is not only an indicator of environmental stress, but considered also as an important protective against heavy metal stress (Debnath and Bisen, 2008). Accumulation of these amino acids in plants subject to Ni

stress has been well documented (Gajewska and Skłodowska, 2008). The cumulative capacity of proline is a manifestation of the self-protection ability of plants exposed to heavy metals. Application of the highest Ni concentration increased proline content in leaves and roots of *Zygophyllum xanthoxylon* plants (Yan Lu *et al*., 2010).

Singh (2002) reported that, the treatment of nickel strongly inhibited nitrate reductase activity in two strains of *Bradyrhizobium sp*. (RA5 and M05), *Cajanuscajan* and *Vignaradiata* plants. Carbonic anhydrase (CA) catalyzes the inter-conversion of CO_2 to HCO^{3-}, and its activity is regulated by the density of photon flux density, CO_2 concentration, availability of Zn (Tiwari *et al*., 2005) and the genes expression encoding CA protein (Kim *et al*,. 1994). Fariduddin *et al*. (2013) recorded a significant reduction in the activity of CA in both the cultivars (Rocket and Jumbo) grown in the presence of NaCl and/or Cu compared with untreated plants. Yan *et al*. (2010) found that, SOD activity in roots and leaves of *Zygophyllum xanthoxylon* plantsincreased significantly when treated with Ni over control.

Cadmium application in *Lemna minor* and *Amaranthus lividus* resulted in a decline in CAT activity due to inhibition of enzyme synthesis or change in assembly of enzyme subunits (Mac Rae and Ferguson, 1985). Peroxidase activity induced at low Ni concentration and reduced at the highest Ni concentration compared with untreated plants (Yan Lu *et al*., 2010).The higher peroxidase activities contribute to the heavy metal tolerance by mitigating the reactive oxygen species (ROS) damage (Chiang *et al*., 2006).

The levels of MDA in *Phaseolus vulgaris* significantly increased linearly with increased heavy metal (Ni, Co, Cr, Zn) levels in the solution (Zengin, 2013). Similar results were recorded with *S. polyrrhiza* (Upadhyay, 2010). MDA was also significantly accumulated under nickel stress in the leaves and roots of *Zygophyllum xanthoxylon* plants over control (Yan *et al*., 2010).

The addition of nickel to soil with normal background level markedly increased the Ni content in plants. On the other hand the application of higher dose of Ni (600 mg kg-1) in soil increased shoot Ni concentration up to 62 mg kg^{-1} (Rathor *et al*., 2014). Aldoobie *et al*. (2013) reported that, heavy metals were absorbed from the soil solution, and then accumulated in the tissues of common bean plants in variable concentrations. Where, the highest in accumulation were in the order: Pb> Cr > Cd > Zn > Ni. Lead reduced the uptake and transport of nutrients in plants, by blocking the entry or binding of the ions to ion-carriers making them unavailable for uptake and transport from roots to leaves (Xiong, 1997).

Plant hormones are chemical messengers that respond to environmental signals and regulate normal growth and developmental changes (Sabir *et al.,* 2013).The application of exogenous hormones, as well as the introduction of gene (s) of their biosynthetic pathways, seems to initiate various biochemical pathways that enhance plant tolerance against various abiotic stresses (Gangwar *et al*., 2014). Cytokinns act at the cellular level by inducing some genes expression, stimulate mitosis and chloroplast development but also on the organ level by releasing buds from apical dominance or by inhibiting shoot and root growth (Yaronskay *et al.,* 2007). Kinetin isan essential component of plant cells, affected the uptake and accumulation of nutrient elements (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}) in maize leaves (Barciszewski *et al*., 2000). Application of 10 μM of kinetin For *Pisum sativum* seedlings enhanced Mn tolerance and also increases seedlings' growth by improving ammonium assimilation and the antioxidant defence system (Gangwar *et al*., 2010).

$CaCl_2$ is a crucial regulator of growth and development in plants. The supply of Ca^{2+} (as $CaSO_4$) to cadmium exposed plants improved the content of chlorophylls, in comparison to control (Bhat *et al*., 2014). Moreover, calcium play an important role in plant cell elongation and division and structure as well as permeability of cell membranes, carbohydrate translocation and nitrogen metabolism (White, 2000).

2. Material and Methods

Pure strain of *Phaseolus vulgaris* L. (common bean) seeds obtained from Agriculture Research Center, Giza, Egypt. Seeds were sterilized with 0.01 % mercuric chloride for 5 min, and then thoroughly washed with distilled water. The seeds were then divided into 4 equal groups. Before sowing, each group was soaked for 6 hours in 30 ppm kinetin, 40 mM calcium chloride either each alone or in combination and one group soaked, for the same time in water to serve as a control. Ten seeds were sown in plastic bags containing mixed soil (sand: clay, 1:2 v/v) and irrigated with water holding capacity. Super phosphate and urea fertilizers were added to the soil during first week of cultivation. Each group was divided into four sub-groups, the first sub-group was sprayed with tap water, while the other three subgroups were foliar application of heavy metals in the form of nickel chloride solution, lead acetate solution and the mixture of them performed after twenty days from sowing.

Samples of plants were collected at 45 days old for determination of leaf area and some fresh samples were stored at -20 ˚C after grounding under liquid nitrogen for biochemical analyses.

2.1.Estimation of Photosynthetic pigments

The method of Arnon (1949) was used in chlorophyll extraction and the concentration of the pigment fractions were calculated as µg/ ml using the following equations:

$$\text{Chlorophyll } (a) = 10.3 \times \text{O.D } 633 - 0.918 \times \text{OD } 644 = \mu g/ml.$$

$$\text{Chlorophyll } (b) = 19.7 \times \text{O.D } 644 - 3.87 \times \text{OD } 633 = \mu g/ml.$$

Then the fractions were calculated as µg/ g dry weight of the differently treated plant waves.

2.2.Estimation of carbohydrate content

Soluble sugar was extracted from air –dried leaf tissue with 80% ethanol. (Homme *et al*., 1992). The soluble sugars were determined by the anthrone sulfuric acid method described by Whistler *et al*. (1962). Polysaccharide content was determined in the dry residue left after extraction of soluble sugars. Total carbohydrates content was calculated as the sum of the amounts of soluble sugars and polysaccharides in the same sample. All data were calculated as mg 100 g^{-1} DW of leaves.

2.3. Estimation of protein-N

Proteins were estimated by the method of Bradford (1976). Absorbance was recorded photometrically at 595 nm (Beckman 640 D, USA) using bovine serum albumin as a standard.

2.4. Estimation of Amino nitrogen (Amino-N)

Amino-N was extracted as described by (Yemm and Willis, 1956) and then determined colourimetrically according to the method of (Muting and Kaiser, 1963).Glycine solution was diluted to 20 and 10 mg / 100 ml for standard curve.

2.5. Estimation of proline content

Free proline was extracted and determined in fresh leaves according to the method described by Bates *et al*. (1973). Proline concentration was determined and calculated as mg 100 g^{-1} DW of leaves.

2.6. Estimation of lipid peroxidation (LPO)

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) contents using the method of (Hodges *et al*., 1999). The MDA content was calculated using its absorption coefficient of 155 n $mole^{-1}cm^{-1}$ and expressed as n mole (MDA) g^{-1} fresh weight.

2.7. Estimation of total phenols

Total phenols were determined in leaves according to the method described by (Malik and Singh, 1980) using the Folin- Ciocalteau reagent. The absorbance was read at 650 nm was measured against a reagent blank. A standard curve was prepared using different concentrations of catechol. From the standard curve the concentration of phenols in the test sample was estimated and expressed as mg phenols/100 g material.

2.8.Estimation of carbonic anhydrase (CA) activity

The activity of carbonic anhydrase in the leaves was measured by the method described by Dwivedi and Randhava (1974). The activity of the enzymes was calculated by putting the values in the formula;

$$CA = (V \times 22 \times N) / W \qquad (\text{mol } (CO_2) \text{ Kg-1 (leaf FM) s-1})$$

V = Difference in volume (cm^3 of HCL used in control and test sample during titration)

22 = Equivalent weight of CO_2

N = Normality of HCL

W = fresh mass of tissue used.

2.9. Estimation of Nitrate reductase (NR) activity

The activity of nitrate reductase (NR) was measured following the method laid down by (Jaworski, 1971). The nitrate reductase activity was expressed on fresh weight basis as *n* mole NO_2 g^{-1} (FM) S^{-1}.

2.10. Determination of Antioxidant Enzymes Activities

Sample preparation was as described by Mukherjee and Choudhury (1983). SOD activity (EC 1.15.1.1) was measured in accordance with the method of Dhindsa *et al.* (1981) by determining its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme that caused half the maximum inhibitions of NBT reduce to blue formazan at 560 nm under the experimental conditions. Catalase (CAT; EC 1.11.1.6) activity was assayed in a reaction mixture (3 ml) composed of phosphate buffer (50 mM, pH 7.0), 30% (w/v) H_2O_2 and 0.5 ml enzyme extract (Aebi, 1983). Catalase activity was estimated by the decrease of absorbance at 240 nm using a Spectronic 601 UV spectrophotometer as a consequence of H_2O_2 consumption and was expressed in accordance with Havir and Mellate (1987) as μM H_2O_2oxidised g-1 fresh weight (FW) min-1. Peroxidase (POD; EC 1.11.1.7) activity was

determined using guaiacol. The reaction mixture (3 ml) composed of 10 mM KH_2PO_4– K_2HPO_4 (pH 7.0), 10 mM H_2O_2, 20 mM guaiacol and 0.5 ml crude extract (Malik and Singh, 1980). The increase in absorbance as a result of dehydrogenation of guaiacol was monitored at 470 nm (Klapheck *et al.,* 1990) using a spectronic 601 UV spectrophotometer. Enzyme activity was expressed as the change in the optical density g^{-1} FW min^{-1}.

2.11. Estimation of total-N

The total nitrogen was determined by the conventional semimicro-modification of Kjeldahl method of Chibnall *et al*. (1943) and Pirie (1955). Using the micro-Kjeldahl as adopted by Pregi (1945). The sample was determined by titration against a standard sulphuric acid (0.0143 N), using bromocresol green and methyl red (3:2 v/v) as indicator till a faint red end point was obtained. The titration figures were converted into mg nitrogen using:

1 ml of 0.0143 N $H_2SO_4 \equiv$ 0.28 mg Nitrogen

2.12. Estimation of elements

According to the wet ashing method, plant materials were dried in an oven at 80 °C till constant weight. The dried matter digested according to the method of Chapman and Pratt (1978). Potassium and magnesium were estimated by the flame emission technique as adopted by Ranganna (1977). Calcium**,** nickel and lead were determined simultaneously by ICP spectroscopies according to the method of Saltanapour (1985) Data were calculated as ppm.

2.13. Statistical analysis

The data were statistically on complete randomized design system of the differently treated groups and a comparison among means was carried out by computer programming method (Costat- Display ANOVA). Values in the tables indicate mean values of four independent determinations. The least significant difference (L.S.D) was used to test the difference between treatments; ≤ 0.05 was considered statistically.

3. Results and Discussion

3.1.Total leaf area, photosynthetic pigments, soluble and insoluble sugars

The data in Table (1) showed that, heavy metals; nickel and lead alone or in combination generally reduced the total leaf area / plant of *Phaseolus vulgaris* plant. The maximum reduction achieved with combination treatments and was estimated by 57.72%.Application of kinetin and

calcium chloride by seeds presoaking induced a non-significant increment in leaves area /plant, this increment was evaluated by 107.38% for kinetin and 101.80% for calcium chloride. The maximum value in area per plant was gained by applying kinetin plus calcium chloride treatment and estimated by 112.58% as compared with the control plants. The contents of photosynthetic pigments, total chlorophylls and total pigments were significantly decreased by heavy metals treatment than the control. The highest inhibitory effects of the heavy metals on total chlorophylls and total pigments were recorded by the combined treatment and were calculated by 47.82% and 52.79%, respectively. Seed presoaking in kinetin and/ or calcium chloride significantly alleviated this inhibitory effect and induced a significant stimulatory effect for the biosynthesis of the total chlorophylls and total pigments. The maximum biosynthesis of total chlorophylls and total pigments was attributed to the combination of kinetin with calcium chloride treatment and were evaluated by 132.45% and 131.13% respectively. The contents of total soluble sugars and insoluble sugars significantly decreased by applying heavy metals compared with those of non-stressed plants. The maximum deteriorative effect of the examined heavy metals was attributed to the nickel single treatment and was evaluated by 82.62% and 58% for total soluble and insoluble sugars respectively. Contradictory, kinetin and/ or calcium chloride treatments generally stimulated a high significant increment in the carbohydrate contents in the shoots of bean plants. The maximum soluble sugars content was achieved in plants subjected to nickel stress after seed presoaking in kinetin plus calcium chloride solution and calculated by161.86%. Meanwhile, the maximum values of insoluble sugars contents obtained by kinetin plus calcium chloride seed presoaking combined treatment and were estimated by 127.67% (Table 1).

The toxicity generated by nickel and/ or lead retarded the growth in terms of leaves area/plant of *Phaseolus vulgaris* plant, especially when nickel was used in association with lead, could come either from a decrease in the sink root effect or from an inhibition in the leaves due to starch degradation into sucrose and then of the transport of this later to the root (Rathor *et al*., 2014). In this aspect, Ouzounidou *et al.* (1998) suggested that the inhibitory action of heavy metals on leaf area seems principally to be due to chromosomal aberrations and abnormal cell divisions and may also be correlated with the metal-induced inhibition of photosynthetic process and respiration in the shoot system and protein synthesis in the root, or due to the reduction in cell proliferation and growth (Maria and Tadeusz, 2005). The stressful effect of nickel that was more

pronounced than that of lead may be attributed to the influence of anionic radicals in metallic salts (lead acetate) to reduce metal toxicity on plants growth (Stevens *et al.*, 2003).

In contrary, the follow-up treatment of the stressed plants with kinetin and/or calcium chloride ameliorated the harmful effect of nickel and/or lead and induced a non-significant increase in leaves area/plant. In this concern plant growth regulators could have enhanced the resistance of plants against heavy metal stress or decreased the physiological and metabolic adverse effects of the heavy metal (Tassi*et al.*, 2008; Lequeux*et al.*, 2010). Calcium chloride recovers the Pb^{2+} induced reduction in biomass accumulation of mung bean seedlings (Singh *et al.*, 1994), where Ca^{2+} channels play major roles in the initiation of a large number of signal transduction processes in higher plant cells, including bud formation, terminal growth, gas exchange regulation, regulation of growth and development (Hepler and Wayne, 1985).

The foliar application of the used heavy metals; nickel and lead alone or in combination significantly reduced the photosynthetic pigments in bean leaves. These results are in harmony with that of Smirnoval *et al.* (2006) who stated that, in sensitive plants, high concentration of these metals inhibits enzymes involved in photosynthetic reaction. The deleterious effect of lead on the photosynthetic pigments biosynthesis may be due to the direct inhibition of enzymatic steps or through the substitution of the central Mg ion from the tetrapyrol ring of chlorophyll molecule by lead which is one of the primary events in plants during heavy metal stress leading to denaturing of the pigments as evident from the significant reduction in leaf contents of chlorophyll (Cenkci *et al.*, 2010 and Pourraut *et al.*, 2011). The plant with high lead concentration fastens the production of ROS, causing lipid membrane injury that ultimately leads to damage of chlorophyll and photosynthetic processes and suppresses the overall growth of the plant (Najeeb *et al.*, 2014). Lead is also known to affect photosynthesis by inhibiting activity of carboxylating enzymes (Stiborova *et al.*, 1987). High level of Pb also causes inhibition of enzyme activities (Sinha *et al.*, 1988a, b), water imbalance, and alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005).In the current study, the decrease in chlorophyll content under metal stress is accompanied by a corresponding increase in proline content which led to the postulation that the available nitrogen might be directed to the synthesis of proline instead of chlorophyll (Da La Rosa-Ibarra and Maiti, 1995).

On contrary, the applications of kinetin and/or calcium chloride found to counteract the

deteriorative effect of heavy metals on the photosynthetic pigment content. In the present study, kinetin plus calcium chloride treatment induced the maximum increment in chlorophyll a and total pigments. The degradation of cytokinin may be one of the prime reasons through which stressors cause toxicity and that exogenous application of cytokinin protects plants against stress (Vodnik *et al.,* 1999). Addition of $CaCl_2$ to stressed plants improved the content of chlorophyll. A strong interaction between calcium and cell wall constituents may be important in providing sufficient calcium to the plasma membrane to maintain its integrity (Kumar *et al.,* 2006).

It is also evident from these results, the total soluble sugars and polysaccharides, significantly decreased under nickel and/or lead treatment. This may be due to lowered synthesis or diversion of the metabolites to other synthesis processes (Aldoobie, and Beltagi 2013). On the other hand, kinetin and/or calcium chloride treatments generally caused a significant increase in the all estimated carbohydrate fractions. This induced enhancement in growth represented by increasing leaf area and consequently increased the fresh and dry matter presumably as a result of larger surface area available for anabolic activities. These stimulations were further corroborated by the significantly higher carbohydrates contents (Al-Whaibi *et al*., 2012).

3.2.Total protein, amino nitrogen, proline, malondialdehyde and total phenols contents

The foliar application of nickel and/or lead significantly reduced the production of the total protein content (Table 2). The maximum inhibition was evaluated by 38.48% in case the combination of nickel and lead treated plants. Whereas; the metals stress significantly enhanced the production of the amino-N and proline contents of common beans. The maximum inductions were observed in plants sprayed with nickel plus lead and were estimated by 113.87% and 162.66% respectively. Kinetin and /or calcium chloride significantly increased the production of the protein nitrogen. The maximum increment obtained by kinetin treatment and it was calculated by 185.22%. Meanwhile, kinetin and calcium chloride treatments resulted in a high significant increment in amino-N concomitant with a significant decrease in proline content. The maximum values of amino-N were 166.03%, while the low values of proline contents were about 74.05% at treated with $CaCl_2$. Malondialdehyde (MDA) and total phenols accumulated in beans shoot in response to the heavy metals stress. The highest inductions in MDA and total phenols were obtained in nickel plus lead treatment and were 248.24 % and 125.48% respectively. Meanwhile, malondialdehyde (MDA) and total phenols severely decreased by kinetin and/or calcium chloride

treatments. The reduction values registered in plants treated with kinetin plus calcium chloride and was calculated by 59.30% and % 68.08, respectively.

Proteins are important constituents of the cell that are easily damaged in environmental stress condition (Wu *et al.*, 2010). Hence, any change in these compounds can be considered as an important indicator of oxidative stress in plants (Aldoobie, and Beltagi, 2013). The changes in insoluble protein content with different metal treatments might reflect different levels of antioxidant defense. The toxic metals reduce enzyme activity and the synthesis of protein (Lin and Kao, 2006; Maheshwari and Dubey, 2007). This toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure, or from displacement of an essential element, resulting in deficiency effects (Capuana, 2011). The detected reduction in protein content may be due to the decrease in protein synthesis and/or to the increase in its decomposition (Dietz *et al.*, 1999).

The presence of nickel and/or lead significantly lead to the accumulation of amino-N and proline. However, application of kinetin and calcium chloride resulted, in a high significant increase in amino-N simultaneously with a reduction in proline contents.

Proline is another important component of the defence system of the plants to counter stress by acting as osmoprotectant (Hartzendorf and Rolletschek, 2001). It acts as a source of carbon and nitrogen for rapid recovery from the stress and acts also as membrane stabilizer and ROS scavenger (Jain *et al*., 2001). The synthesis of proline is a gene-regulated process that involves the activation of genes of its biosynthesis and degradation (Sumithra and Reddy, 2004). The significant increase in ammonia-N which is expressed by the increasing amounts amino nitrogen and protein content in shoot tissue is a biomarker of stress resistance is due to the fact that, ammonia is considered to be the unit of nitrogen metabolism from which different amino acids are produced, these being further incorporated into protein synthesis (Ibraheem, 1999).

Another biomarker for oxidative stress is lipid peroxidation (MDA), since the free radical collects electron from lipid molecules present inside the cell membrane, which eventually causes lipid peroxidation (Wadhwa *et al.*, 2012; Flora *et al.*, 2012). MDA is the decomposition product of polyunsaturated fatty acids of biomembranes and its increase shows that plants are under high-level antioxidant stress. The data of this work indicated that, the total phenols and lipid peroxidation level as indicated by accumulated MDA increased significantly in response to Ni

and/or Pb foliar application. The maximum induction in plants treated with the combination of nickel and lead. This increased MDA content shows generality of oxidative stress and this may be one of the potential mechanisms by which toxicity due to heavy metals is manifested in plant tissues (Gupta *et al.,* 2009). Contradictory to the heavy metal effect, a marked significant decrease in MDA and total phenols was obtained as a response to kinetin and/or calcium chloride treatments. A good parallelism was observed in the present investigation in trend of response of proline content, SOD, POX, MDA and total phenol towards nickel and/or lead stress with or without kinetin and /or calcium chloride presoaking treatments.

3.3.Carbonic anhydrase, nitrate reductase, and antioxidant enzymes.

Carbonic anhydrase and nitrate reductase activities were highly significantly decreased in the stressed plants (Table 3). The maximum reductions were observed in plants sprayed with nickel and lead combination were 58.4 % and 12.81%, respectively. In contrary, application of kinetin and/or calcium chloride by seed presoaking caused a high significant increase in both enzymes content. Both kinetin single treatment and kinetin + calcium chloride treatment stimulated the maximum activity of carbonic anhydrase, which was 148%, while; only kinetin + calcium chloride treatment induced a sharp maximum activation of nitrate reductase evaluated by 300%.

In this investigation the reduction in CA may be interpreted as a result of the decrease in photosynthetic rate mediated by the stress as a result of the closure of stomata, thereby decreasing CO_2 supply (Hayat *et al.,* 2008) as well as the decrease of the internal CO_2 concentration and consequently a decrease in the activity of CA, because its activity, to a large extent, is regulated by the CO_2 concentration (Tiwari *et al.,* 2005). this stressful effect may also be attributed to the metal role in altering the structure of the chloroplast and thylakoid membrane due to metal interference with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of thylakoid membranes and fluidity of the plasma membrane, thereby reducing the internal CO_2 concentration and the uptake of Zn which is responsible for the expression of genes encoding CA protein (Bernel *et al.,* 2004; Yruela, 2005 and Azmat and Riaz, 2012). The decrease in the NR activity in the stressed plant may be considered a biochemical adaptation to conserve energy by stopping nitrate assimilation. This inhibition may be due to a reduced supply of NADH (Gengenbach, *et al.,* 1993) disorganization of chloroplast (Rebechini and Hanzely, 1974).

In contrast, seed presoaking in kinetin and/or calcium chloride induced a high significant increase in carbonic anhydrase and nitrate reductase contents. The reason that seems most appropriate to explain that, kinetin elevated the activity of nitrate reductase and corrected the stress mediated damage to the plasma membrane. The membrane correction/stabilization could have facilitated the increased uptake of nutrients including that of nitrate, which act as an inducer of NR (Campbell, 1999).The exogenously supplied $CaC1_2$ also alleviates the inhibition of NRA as calcium is considered as an important nutrient ion and is known to increase the membrane permeability and, thus, greater availability of other ions to the site of the enzyme action is possible, which may cause an indirect regulation of the enzyme activity in the presence of $CaC1_2$ in addition to its role as secondary messenger for signal transduction (Kumar *et al.,* 1993 and Bhat *et al*., 1996). The application of kinetin in the present work also found to enhance the chlorophyll content and neutralize the negative effect of the stress on CA.

Nickel and lead separately or in combination, on the other side, raised the activities of the oxidizing enzymes superoxide dismutase (SOD) and peroxidase (POX). Meanwhile, these treatments a significantly decreased catalase (CAT) enzyme activity. Nickel plus lead treatment induced the high activities for superoxide dismutase and peroxidase were 270.27% and 123.17% respectively, while the inhibition value in catalase activity was calculated by 25%. Kinetin plus calcium chloride treatment reversed the trend inducing a high significant decrease in the activities of superoxide dismutase and peroxidase enzymes were 57.57%, 31.27% respectively, accompanied with a significant increase in the activity of catalase enzyme calculated by 250 %.

In this concern, the high values of SOD and POX in response to nickel and/or lead stress may be a result of the production of stress-inducible genes which are directly related to protect against the different stresses. They include the enzymes responsible for the synthesis of various osmoprotectants like late embryogenesis abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes (Debnath *et al.,* 2011).

The oxidative stress was an indirect effect of metal toxicity leading to ROS production which increased tissue level of SOD, APX and GR (John *et al.,* 2007). Moreover, heavy metals induce oxidative stress leading to programmed cell death (PCD), which is initiated and propagated through the generation of ROS (Jacobson, 1996). In addition, the redox-active (Cu, Fe) and non-redox-active (Cd, Ni, As) metals may catalyze, directly or indirectly, the formation of free radicals (FR) and reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide

(H_2O_2) and hydroxyl radicals (OH^-), which generate oxidative stress and cause cell damage by inducing lipid peroxidation, protein oxidation, enzyme inhibition and DNA damage (Sharma and Dietz, 2009). In contrast, kinetin and/or calcium chloride treatments in the present work significantly raised CAT activity and reduced the activities of both SOD and POX. These findings may be a result of kinetin and/or calcium chloride role in elevating the level of antioxidant system, at least in part, increasing the tolerance of the plant to the incident stress, thus protected the photosynthetic machinery (Hayat *et al*., 2008).

3.4.Nitrogen, potassium, calcium, magnesium, nickel and lead ions contents

The data in Table 4 showed that, heavy metals stress with or without kinetin and /or calcium chloride generally induced an inhibitory effect on the production of total -N content calculated by 70.89 % as compared with untreated plants. In contrast, seed presoaking in kinetin and /or calcium chloride significantly induced a stimulatory effect on the nitrogen content. Application of kinetin alone improved the nitrogenous compounds production attaining a maximum value of total-N as calculated by 111.59%.

The content of potassium in *Phaseolus vulgaris* shoot significantly diminished by nickel and/or lead foliar application to 70.27% of the control plants. In contrary the combination of kinetin and calcium chloride enhanced the accumulation of potassium ions by 131.97% over the control plants. In addition, Ca^{++} and Mg^{++} ions accumulation was also dropped by the heavy metals stress achieving the high inhibition recorded at nickel + lead application evaluated by 25.02% and 78.63% respectively. Whereas, Kinetin and calcium chloride combined treatment significantly improved Ca^{++} and Mg^{++} accumulation to 200% and 117.83%, respectively.

On the other hand, bean shoots content of Ni^{++} ions reached a maximum value in response to nickel foliar application as nickel chloride solution and this value was estimated by 133.33%. In contrast, the low Ni^{++} content was obtained by kinetin + calcium chloride treatment by seeds presoaking to 28.33% of the control value. The same attitude was observed with lead ions; where the maximum accumulation achieved in response to lead foliar application as lead acetate solution and it was calculated by 157.14%, whereas, seed presoaking in kinetin and calcium chloride combined treatment alleviated the stressful effect of lead to the level that lead content could not be detected (Table 4).

The uptake of N^{++}, K^+, $Mg^{++}$and Ca^{++} ions significantly reduced by the foliar application with the heavy metals; nickel and lead as compared with the control plants. The ionic mechanism

of lead toxicity occurs mainly due to the ability of lead metal ions to replace other bivalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations like Na^{+}, which ultimately disturbs the biological metabolism of the cell (Flora *et al.*, 2012). Seed presoaking in kinetin and/or calcium chloride overcame this stressful effect of nickel and lead, A possible reasoning for this increment might be involved in the detoxification and thus tolerance to heavy metal stress (Hall, 2002).The main detoxifying strategy of plants contaminated by heavy metals is the production of phytochelatins (PCs) (Mendoza-Cozatl *et al.*, 2010). The presence of some cations in the soil solution such as Ca^{2+} and Mg^{2+} compete with cations of heavy metals efficiently and prevent it from adhering with plasma of plant cells and subsequently their accumulation decrease (Kiekens, 1983). It has been proposed that the effects of plant hormone on growth and development are mediated by hormone induced shifts in cytoplasmic Ca^{+2} levels with Ca^{+2} acting as an intracellular messenger conveying information about the nature of a particular stimulus or stress impinging on the cell to target proteins that guide the cellular response (Johannes *et al.*, 1991). Thus, Ca^{2+} plays a pivotal role in signal transduction by communicating signal perception at a localized receptor to other parts of the cell, where the effectors of the cellular responses are located (Harper *et al.*, 1991).

Conclusion

The present wok demonstrated that, the presoaking of *Phaseolus vulgaris* seeds in 30 ppm kinetin, and or 40 mM calcium for 6 hrs, especially in combination treatment, helps in mitigating the toxic effect of heavy metals stresses on growth criteria, metabolites and mineral content.

Table 1: Effect of kinetin and/or calcium chloride on leaves area, chlorophyll a + chlorophyll b, total pigments, total soluble sugars and polysaccharides of *Phaseolus vulgaris* L. plant grown under nickel and/or lead stress.

Treatment	Leaves area (cm^2) / plant	Chl. a + Chl. b µg/ g dry weight	Total pigments µg/ g dry weight	Total soluble sugars mg 100 g^{-1} DW	Poly saccharides mg 100 g^{-1} DW
Control	23.84^{abc}	192.6٢^{d}	266.73 ^{d}	1034.66 ^{k}	1712.76 ^{g}
Ni	18.02^{abc}	128.34 ^{k}	180.49 ^{k}	854.86 ^{o}	993.52 ^{m}
Pb	20.00^{abc}	141.40^{ij}	196.16 ^{j}	859.43 ^{o}	1453.71 ^{j}
Ni + Pb	13.76 ^{c}	90.94 ^{l}	139.19 ^{l}	955.43 ^{l}	1267.81 ^{l}
Kin.	25.60^{ab}	219.73 ^{b}	*301.38* ^{b}	1307.43 ^{d}	1948.95 ^{d}
Kin + Ni	19.13^{abc}	171.07 ^{f}	237.71^{fg}	1069.71 ^{j}	1962.66 ^{d}
Kin. + Pb	21.23^{abc}	159.42 ^{g}	230.97 ^{g}	874.67 ^{n}	1810.28 ^{f}
Kin. + (Ni+Pb)	17.91^{abc}	138.98 ^{j}	202.33^{ij}	914.28 ^{m}	1557.33 ^{i}
$CaCl_2$	24.27^{abc}	204.32 ^{c}	283.72 ^{c}	1214.47 ^{f}	2090.66 ^{b}
$CaCl_2$ + Ni	18.87^{abc}	147.08 ^{h}	207.41 ^{hi}	1171.81 ^{g}	2017.52 ^{c}
$CaCl_2$ + Pb	20.25^{abc}	146.06 ^{h}	212.33 ^{h}	1264.76 ^{e}	1865.14 ^{e}
$CaCl_2$ +(Ni + Pb)	15.33^{bc}	127.81 ^{k}	184.38 ^{k}	1104.76 ^{i}	1412.57 ^{k}
Kin. + $CaCl_2$	26.84 ^{a}	255.13 ^{a}	349.76 ^{a}	1674.66 ^{a}	1950.47 ^{d}
Kin. + $CaCl_2$ + Ni	20.05^{abc}	171.97 ^{f}	244.42 ^{f}	1408.00 ^{c}	2186.66 ^{a}
Kin. + $CaCl_2$ + Pb	22.10^{abc}	180.50 ^{e}	255.28 ^{e}	1123.05 ^{h}	1595.43 ^{h}
Kin. + $CaCl_2$ +(Ni+Pb)	18.46^{abc}	144.65 ^{hi}	213.29 ^{h}	1609.14 ^{b}	1814.85 ^{f}
L.S.D at 0.05	10.84	3.110	6.855	13.260	20.324

Table 2: Effect of kinetin and/or calcium chloride on total protein, amino nitrogen, proline, malondialdehyde and total phenols contents of *Phaseolus vulgaris* L. plant grown under nickel and/or lead stress.

Treatment	Protein-N mg 100 g^{-1} DW	Amino-N mg 100 g^{-1} DW	Proline mg 100 g^{-1} DW	Lipid -peroxidation MDA(µg/gFW)	Total phenols (mg/100g DW)
Control	861.80 ^{l}	70.67 ^{k}	11.06 ^{i}	1.99 ^{e}	117.63 ^{e}
Ni	492.39^{n}	77.40 ^{j}	14.54 ^{b}	2.36 ^{c}	130.89 ^{b}
Pb	666.23^{m}	79.80 ^{i}	13.84 ^{d}	2.32 ^{c}	127.10 ^{bc}
Ni + Pb	331.59 ^{o}	80.47 ^{i}	17.99 ^{a}	4.94 ^{a}	147.60 ^{a}
Kin.	1596.26 ^{a}	103.80 ^{e}	10.36 ^{j}	1.30 ^{h}	87.15 ^{h}
Kin + Ni	1292.05 ^{g}	113.53 ^{c}	13.08 ^{e}	2.25^{cd}	117.11 ^{e}
Kin. + Pb	1435.46 ^{e}	115.4^{b}	11.68 ^{g}	1.73 ^{f}	98.34 ^{g}
Kin. + (Ni+Pb)	970.45 ^{k}	117.33 ^{a}	14.00 ^{c}	2.75 ^{b}	123.66 ^{cd}
$CaCl_2$	1461.54 ^{d}	93.00 ^{g}	8.19 ^{l}	1.24 ^{h}	82.32 ^{i}
$CaCl_2$ + Ni	992.18 ^{j}	109.47 ^{d}	11.67^{g}	2.03 ^{e}	108.50 ^{f}
$CaCl_2$ + Pb	1144.29 ^{i}	102.00 ^{f}	11.46 ^{h}	1.66 ^{fg}	90.07 ^{h}
$CaCl_2$ +(Ni + Pb)	1435.46 ^{e}	116.00 ^{ab}	12.57 ^{f}	2.40 ^{c}	120.04 ^{de}
Kin. + $CaCl_2$	1539.77 ^{b}	91.13 ^{h}	9.73 ^{k}	1.18 ^{h}	80.08 ^{i}
Kin. + $CaCl_2$ + Ni	1257.28 ^{h}	110.40 ^{d}	11.37 ^{h}	2.12 ^{de}	91.28 ^{h}
Kin. + $CaCl_2$ + Pb	1335.51 ^{f}	112.20 ^{c}	11.09 ^{i}	1.53 ^{g}	90.37 ^{h}
Kin. + $CaCl_2$ +(Ni+Pb)	1483.27 ^{c}	113.33 ^{c}	11.65 ^{g}	2.04 ^{e}	106.78 ^{f}
L.S.D at 0.05	21.657	1.400	0.143	0.172	4.597

Table 3: Effect of kinetin and/or calcium chloride on carbonic anhydrase, nitrate reductase, superoxide dismutase, catalase and peroxidase activities of *Phaseolus vulgaris* L. plant grown under nickel and/or lead stress.

Treatment	Carbonic anhydrase µ.mol/l	Nitrate reductase n.moleNO_2/g/sFM	Superoxide dismutase (unit/g protein)	Catalase x 10^2 UMH_2O_2 oxidized /g FW	Peroxidase (Change in optical density/g FW/min)
Control	68.75 ^{gh}	25.38 ^{g}	0.370 ^{i}	8.16 ^{d}	2.59 ^{c}
Ni	43.45^{ij}	4.23 ^{k}	0.909 ^{b}	4.76^{hi}	3.18 ^{a}
Pb	46.75^{i}	4.23 ^{k}	0.833 ^{c}	3.40^{j}	2.82 ^{b}
Ni + Pb	40.15 ^{j}	3.25 ^{k}	1.000 ^{a}	2.04 ^{k}	3.19 ^{a}
Kin.	101.75^{a}	50.76 ^{b}	0.238 ^{m}	17.00 ^{b}	1.34 ^{i}
Kin + Ni	70.95 ^{fg}	25.38 ^{g}	0.328 ^{j}	6.80 ^{e}	2.17 ^{e}
Kin. + Pb	79.75 ^{d}	29.61 ^{f}	0.286 ^{k}	6.12 ^{f}	1.58 ^{h}
Kin. + (Ni+Pb)	68.75 ^{gh}	12.69 ^{j}	0.500 ^{e}	4.42 ^{i}	2.30 ^{d}
$CaCl_2$	92.22 ^{b}	46.53 ^{c}	0.250 ^{lm}	13.60 ^{c}	1.43 ^{i}
$CaCl_2$ + Ni	68.75 ^{gh}	16.92 ^{i}	0.476 ^{f}	6.12 ^{f}	2.33 ^{d}
$CaCl_2$ + Pb	75.35 ^{e}	21.15 ^{h}	0.400 ^{h}	5.44 ^{g}	2.53 ^{c}
$CaCl_2$ +(Ni + Pb)	65.45 ^{h}	12.69 ^{j}	0.714 ^{d}	3.40 ^{j}	2.57 ^{c}
Kin. + $CaCl_2$	101.75 ^{a}	76.14 ^{a}	0.213 ^{n}	20.40 ^{a}	0.81 ^{j}
Kin. + $CaCl_2$ + Ni	74.25 ^{ef}	33.84 ^{e}	0.303 ^{k}	8.16 ^{d}	1.59 ^{h}
Kin. + $CaCl_2$ + Pb	87.45 ^{c}	42.30 ^{d}	0.256 ^{l}	6.80 ^{e}	1.94 ^{g}
Kin+$CaCl_2$+(Ni+Pb)	71.50 ^{efg}	21.15 ^{h}	0.454 ^{g}	5.10 ^{gh}	2.07 ^{f}
L.S.D at 0.05	3.884	2.034	0.0177	0.491	0.089

Table 4: Effect of kinetin and/or calcium chloride on nitrogen, potassium, calcium, nickel and lead ions contents (mg/g DW) of *Phaseolus vulgaris* L. plant grown under nickel and/or lead stress (ppm).

Treatment	N^{++}	K^{+}	Mg^{++}	Ca^{++}	Ni^{++}	Pb^{+++}
Control	3710.00 ^{c}	14.70 ^{f}	12.73 ^{e}	16.03 ^{h}	0.60 ^{bc}	0.07 ^{e}
Ni	3060.00 ^{j}	10.50 ^{n}	10.51 ^{l}	6.01 ^{l}	0.80 ^{a}	0.10^{b}
Pb	3120.00 ^{h}	11.30 ^{l}	10.32 ^{m}	8.02 ^{k}	0.62 ^{b}	0.11 ^{a}
Ni + Pb	2630.00 ^{n}	10.33 ^{o}	10.01 ^{n}	4.01 ^{m}	0.61 ^{bc}	0.11^{a}
Kin.	4140.00 ^{a}	16.80 ^{b}	14.31 ^{b}	26.05 ^{c}	0.18 ^{g}	-^{h}
Kin + Ni	3330.00 ^{f}	12.40 ^{j}	11.87 ^{h}	12.02 ^{i}	0.42 ^{de}	-^{h}
Kin. + Pb	3500.00 ^{d}	12.50 ^{i}	12.27 ^{g}	20.04 ^{f}	0.36 ^{ef}	0.09^{c}
Kin. + (Ni+Pb)	3010.00 ^{k}	11.30 ^{l}	11.07 ^{j}	10.02 ^{j}	0.35 ^{ef}	0.06^{f}
$CaCl_2$	3720.00 ^{c}	16.40 ^{c}	13.55 ^{c}	28.06 ^{b}	0.19 ^{g}	-^{h}
$CaCl_2$ + Ni	3090.00 ^{i}	11.10	10.88 ^{k}	24.05 ^{d}	0.54 ^{c}	-^{h}
$CaCl_2$ + Pb	3150.00 ^{g}	12.70 ^{h}	10.56 ^{l}	20.00^{f}	0.44 ^{d}	0.08^{d}
$CaCl_2$ +(Ni + Pb)	2790.00 ^{m}	11.70 ^{k}	11.14 ^{j}	18.04 ^{g}	0.46 ^{d}	0.06^{f}
Kin. + $CaCl_2$	3850.00 ^{b}	19.40 ^{a}	15.00 ^{a}	32.06 ^{a}	0.17 ^{g}	-
Kin. + $CaCl_2$ + Ni	3120.00^{h}	16.20 ^{d}	13.35 ^{d}	22.04^{e}	0.33 ^{f}	-
Kin. + $CaCl_2$ + Pb	3410.00 ^{e}	15.10 ^{e}	12.61 ^{f}	24.05 ^{d}	0.22 ^{g}	0.06^{f}
Kin. + $CaCl_2$ +(Ni+Pb)	2910.00 ^{l}	14.40 ^{g}	11.42 ^{i}	16.03^{h}	0.24 ^{g}	0.02^{g}
L.S.D at 0.05	13.543	0.098	0.119	0.090	0.072	0.009

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الملخص العربى

تأثير الكينتين وكلوريد الكالسيوم على نمو نبات الفاصوليا النامى تحت اجهاد العناصر الثقيلة

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يعد الإجهاد بالعناصر الثقيلة أحد الإجهادات غير الحيوية الرئيسية التي تسبب تلوثا بيئيا، ومن ثم مخاطر غير محدودة للكائنات الحية فى العقود الأخيرة. أجريت الدراسة الحالية بهدف توضيح الدور التحفيزى لنقع البذور في الكينيتين (٣٠ جزء في المليون)

أو كلوريد الكالسيوم (٤٠ ملم) أو الأثنين معا في مواجهة التأثير الضار للنيكل (٢,٥ ملم) و الرصاص(٠,٥ملم)على النمو وبعض الأنشطة الأيضية لنبات الفاصوليا .وأوضحت النتائج أن المعاملة بكل من النيكل أو الرصاص أدت الى انخفاض معنوى فى مساحة الأوراق / النبات،والصبغات الكلية والسكريات الذائبة وغير الذائبة، والبروتين الكلي والنيتروجين الكلى ، وإنزيم الكاتاليز، ، ومحتوى كل من البوتاسيوم والكالسيوم والمغنيسيوم .ومن ناحية أخرى، لوحظ زيادة معنوية فى كل من محتوى البرولين والنيتروجين الامينى ، فوق أكسدة الدهون، الفينول الكلي، السوبر أوكسيد ديزميوتيز، البيروكسيديز، النيكل والرصاص نتيجة للمعاملة بالعناصر الثقيلة. وعلى النقيض من ذلك، فقد أدى نقع البذور في كل من الكينيتين وكلوريد الكالسيوم منفردا أو كلاهما معا إلى زيادة معنوية في مساحة الأوراق، والصبغات الكلية السكريات الذائبة وغير الذائبة، والنيتروجين الامينى، وإنزيم الكاتاليز، والنيتروجين الكلى ومحتوى كل من البوتاسيوم والكالسيوم والمغنيسيوم .وفي الوقت نفسه، انخفض محتوى كل من البرولين، فوق أكسدة الدهون، الفينول الكلي، السوبر أوكسيد ديزميوتيز، البيروكسيديز، النيكل و الرصاص بشكل معنوى نتيجة للمعاملة بكل من الكينيتين وكلوريد الكالسيوم. وكانت المعالجة بكل من الكينيتين وكلوريد الكالسيوم منفردا أو بصفة مزدوجة خاصة فعالة في تعزيز قدرة النبات على تحمل التاثي الضار لكل من النيكل و الرصاص.