Biol Res 46: 79-85, 2013

Physiological behavior of bean (*phaseolus vulgaris l.*) Seedlings under metal stress

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ABSTRACT

The effects of nickel, cobalt, chromium and zinc on the content of vitamins A, E and C, malondialdehyde (MDA), chlorophyll and carotenoids were investigated in bean seedlings (*Phaseolus vulgaris* L.) grown in Hoagland solution Control and heavy metal-treated plants were grown for ten days in Hoagland solution. Vitamin A, E, and C content were measured in primary leaves by high performance liquid chromatographic (HPLC). MDA, chlorophyll and carotenoids were measured in leaves by spectrophotometer. In heavy metal treated plants, the levels of MDA, vitamins A, E and C and carotenoids significantly increased, while chlorophyll content decreased in leaves of seedlings. The results indicate that heavy metals caused an oxidative stress in bean plants. The strongest effect on vitamins A, E and C, MDA, chlorophyll and carotenoids was found in plants exposed to nickel, followed by the sequence cobalt > chromium> zinc.

Key Words: Heavy metal, vitamins A, E and C, MDA, chlorophyll and carotenoids

INTRODUCTION

Heavy metals released from industrial units, metallurgical operations and mining activities pose a threat to the environment. The retention of high concentrations of heavy metals in the environment exerts toxic effects on fauna and flora (Mishra and Tripathi, 2008). Some of these heavy metals, such as As, Cd, Hg, Pb or Se, are not essential, since they do not perform any known physiological function in plants. Others, such as Co, Cu, Fe, Mn, Mo, Ni and Zn, are essential elements required for normal growth and metabolism of plants. These latter elements can easily lead to poisoning when their concentration rises to supra-optimal values. Heavy metal phytotoxicity may result from alterations of numerous physiological processes caused at the cellular/molecular level such as inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting essential elements and disrupting membrane integrity (Rascio and Navarri-Izzo, 2011).

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). Tolerant plants have evolved different antioxidative mechanisms involving enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), or small metabolites such as ascorbic acid (ASA), phenolics and carotenoids to prevent and counteract the increase in and effects of ROS (Kelman et al., 2009). A second group of antioxidants includes several vitamins and nutritional trace minerals that can reduce oxidative stress by directly scavenging free radicals and by interfering with free radical-producing mechanisms. For example, vitamin A (retinyl esters and all-trans retinols) and E (α-tocopherol) preserve against the development of oxidative damage (Sies and Stahl, 1995). There are many studies on the antioxidant properties of plants exposed to various stress factors (Havaux and Kloppstech, 2001). However, studies related to the effect of heavy metal-generated stress on vitamin levels of plants are limited. Some studies showed that lead and mercury caused an increase in ascorbic acid and α-tocopherol levels in two *Oryza sativa* cultivars (Mishra and Choudhuri, 1999), and mercury exposure of *Bacopa monnieri* increased the ascorbic acid levels in this plant (Sarita et al., 1996). Demirevska-Kepova et al. (2006) reported that the content of oxidized ascorbate increased during Cd exposure in *Hordeum vulgare* plants.

An excess of metals has deleterious effects on the content and functionality of the photosynthetic pigments (Broadley et al. 2007). This can be caused by the inhibition of pigment synthesis (Prasad and Prasad 1987), the formation of metal-substituted chlorophylls of reduced functionality (Küpper et al. 1996), or direct oxidative damage to the pigments (Oláh et al. 2010). The amount of chlorophyll was reduced in *Triticum aestivum* L. (Gajewska et al., 2006) exposed to Ni, and in *Phaseolus vulgaris* L. cv. Anupama (Chatterjee et al. 2006) exposed to Co.

The aim of the present study was to investigate the effects of four heavy cations, namely nickel, cobalt, chromium and zinc, on the content of vitamins A, E and C, chlorophyll, carotenoids and MDA in bean *(Phaseolus vulgaris* L.) seedlings.

MATERIALS AND METHODS

In this study, 7-day old bean seedlings (*Phaseolus vulgaris* L.) were used. Stock solutions of nickel ($NiCl_2.6H_2O$), cobalt ($CoCl_2.6H_2O$), chromium ($CrCl_3.6H_2O$) and zinc ($ZnCl_2.H_2O$) were prepared at concentrations of 0.1, 0.3, 0.5 mM Ni; 0.5, 0.7 and 1.0 mM Co; 0.5, 0.7 and 1mM Cr and 1.5, 2.0 and 2.5 mM Zn. The recovery rates of standards were determined as 96.8% for vitamin A, 96.7 % for vitamin E and 95.5% for vitamin C. Separation times, using a flow rate of 1 ml/min, were 3.2 min for vitamin A, 3.6 for vitamin C and 5.6 min for vitamin E.

The bean seeds were surface sterilized in $HgCl_2$ for 2 min, washed in distilled water and germinated between wet paper

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Received: November 11, 2012. In Revised form: February 2, 2013. Accepted: February 22, 2013

towels at 25 ºC in the dark for 3 days. Subsequently plants cultivated hydroponically in a growth chamber at a light intensity of 4500 photon/sec/m^2 (16 h light/ 8 h). During this period day/night temperatures were 25 ºC. After 7 days, plants were transferred to Hoagland solution containing 0 mM (control) and various amounts of nickel, cobalt, chromium or zinc. After 10 days of heavy metal treatment, seedlings were used for vitamin A, E and C, chlorophyll, carotenoids and MDA determinations.

The extraction of vitamin A and E was done according to Catignani (1983) and Miller et al. (1984). Leaf tissues were homogenized in ethanol and the homogenate was centrifuged at 4500xg for 5 min. The supernatant was treated with n-hexane. Vitamin A and E were extracted twice in hexane phase and the collected extract was dried in liquid nitrogen. The dried extract was solubilized in 0.5 ml methanol for HPLC. Injections were made in duplicate for each sample. The quantification was according to (Catignani, 1983; Miller et al., 1984) utilizing absorption spectra of 326 and 296 nm for vitamin A and E, respectively. HPLC separations were accomplished at room temperature with a Perkin-Elmer liquid chromatography system (Series 1100) consisting of a sample injection valve (Cotati 7125) with a 20 µl sample loop, an ultra-violet (UV) spectrophotometric detector (Cecil 68174), an integrator (HP 3395) and a Techsphere ODS-2 packed (5 µm particle and 80 ºA pore size) column (250 x 4.6 ID) with a methanol: acetonitrile: chloroform (47: 42: 11, v/v) mobile phase at 1 ml min^{-1} flow rate. The extraction of vitamin C was done according to Cerhata et al. (1994) Leaf tissues were homogenized in perchloric acid and volume was adjusted to 1 ml by adding ddH_2O. The mixture was centrifuged at 4500 x for 5 min at 4 ºC. The supernatant was filtered as above and the vitamin C level was determined using the method of Tavazzi et al. (1992) by HPLC, utilizing a column (250 x 4.6 ID) packed with Li-60 reversed-phase material (10 µm particle size) with mobile phase (3.7 mM phosphate buffer, pH 4.0) at 1 ml min^{-1} flow rate.

The level of lipid peroxidation was measured in terms of MDA content, a product of lipid peroxidation. Leaf samples (0.5 g) were homogenized in 10 ml of 0.1% TCA. The homogenate was centrifuged at 15,000g for 5 min. To as 1.0 ml aliquot of the supernatant 4.0 mL of 0.5% TBA in 20% TCA was added. The mixture was heated at 95 ^{0}C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000g for 10 min, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The Following formula should be added after the MDA equivalent was calculated as follows (Heath and Packer 1968).

$$\text{MDA (nmol/mL FW)} = [(A532-A600)/155,000] \times 10^6$$

For the purpose of identifying the amount of photosynthetic pigments, about 1 g of fresh leaf tissue was obtained from the seedlings and extracted. The absorbance of these extracts was measured separately at 645 nm and 633 nm wavelengths in a blind manner. To determine absorbance, quartz tubes with a volume of 1 cm^3 were utilized. Using the absorbance values, chlorophyll a+b and carotenoid content were calculated as mg/g FW (Witham et al. 1971).

All experiments were repeated three times. Statistical analysis was performed using the SPSS (version 10.0) program. In order to detect the significance of differences ($p<0.01$ or $p<0.05$) of variables, a multiple comparison (LSD) test was performed. All values are expressed as mean ± 1 SE.

RESULTS

Figs 1-7 summarize the results for the effects of selected heavy metals (Ni, Co, Cr, and Zn) on vitamins, chlorophyll, carotenoids and MDA in primary leaves of bean seedlings. Significant increases of the content of vitamins, chlorophyll, carotenoids and MDA ($p<0.05$ or $p<0.01$) were detected after ten day exposure to the heavy metals.

In nickel-treated seedlings the amount of vitamin was significantly greater than in control seedlings ($p<0.05$). Vitamin A content in the primary leaves increased noticeably at 0.1, 0.3 and 0.5 mM nickel concentrations (Fig. 1A). Vitamin A content in leaves increased by 14.1%, 15.8% and 18.4%, respectively, compared to control seedlings ($p<0.05$). Nickel caused the increase of vitamin E between 12-18% ($p<0.05$) (Fig. 1B). These seedlings had significantly greater vitamin C content than control seedlings (Fig. 1C). Vitamin C content in seedlings increased noticeably at 0.1, 0.3 and 0.5 mM nickel concentrations. With 0.1, 0.3 and 0.5 mM nickel concentrations, vitamin C contents were increased a dose-dependent manner (7.5%, 13.6%, and 19.7%).

In cobalt treated seedlings vitamin content was significantly more effective than in control seedlings ($p<0.01$). The vitamin content of seedlings increased with increasing concentration of this metal (Fig. 2A, 2B, 2C). In seedlings treated with 0.5, 0.7 and 1 mM cobalt, vitamin A content increased by 12.3%, 14.9%, and 17.5%, respectively, compared to control plants ($p<0.01$). In seedlings treated with 0.5, 0.7 and 1 mM cobalt, vitamin E content increased by 10.2%, 13.1%, and 17.1%, respectively, compared to control plants ($p<0.01$) (Fig 2B). Vitamin C content of seedlings was increased by 4.6%, 10.3% and 14.6% compared to controls (Fig 2C).

Vitamin A, E, and C content of the seedling increased with increasing concentration of chromium (Fig. 3A, 3B, 3C). Chromium caused the increase of vitamin A between 10.5-16.7% ($p<0.05$) (Fig. 3A). In chromium-treated seedlings the vitamin E content was significantly greater than in control seedlings ($p<0.01$) (Fig 3B). In seedlings treated with 0.5, 0.7 and 1.0 mM chromium, vitamin C content increased by 7.51%, 12.2%, and 15%, respectively, compared to control plants ($p<0.01$) (Fig 3C).

The increase of zinc concentration in seedlings caused significant vitamin accumulation. In seedlings treated with 1.5, 2.0 and 2.5 mM zinc; vitamin A content increased by 9.6%, 12.7%, and 14.1%, respectively, compared to control plants ($p<0.01$) (Fig 4A). These seedlings had significantly greater vitamin E content than control seedlings (Fig. 4B). Vitamin E content in seedlings increased noticeably (5.9%, 9.1% and 12.2%) at 1.5, 2.0 and 2.5 mM zinc concentrations. The increase of zinc concentration in primary leaves caused significant vitamin C accumulation. In primary leaves treated with 1.5, 2.0 and 2.5 mM zinc, vitamin C content increased by 4.2%, 8.9%, and 13.1%, respectively, compared to control plants ($p<0.01$) (Fig. 4C).

The effect of heavy metals on MDA content is presented in Figure 5. Significant increases in MDA content in *bean* plants were observed in the experiments. MDA content increased linearly with increased heavy metal levels in the solution. The MDA content was increased by 47.2%, 55.4%, and 66.3% at 0.1,

0.3 and 0.5 mM Ni, 41.8%, 49.1% and 53.6% at 0.5, 0.7 and 1.0 mM Co, 27.2%, 31.8% and 42.7% at 0.5, 0.7 and 1.0 mM Cr, 20%, 26.3% and 30.9% at 1.5, 2.0,2.5 mM Zn, respectively

The chlorophyll content in the plants was significantly affected by heavy metal treatment (Fig. 6). For example, chlorophyll contents in leaves decreased by 25.6%, 23.1%, 21.2% and 19.3% at 0.5 mM Ni, 1 mM Co, 1 mM Cr and 2.5 mM Zn, respectively, compared to the control seedlings ($p<0.05$).

Carotenoids decreased significantly with increasing concentrations of heavy metals (Fig. 7). For example, carotenoids increased by 15%, 23.1% and 27.3% at at 0.1, 0.3 and 0.5 mM Ni, 8%, 13.5% and 17% at 1.5, 2.0 and 2.5 mM Zn respectively.

DISCUSSION

Many abiotic stresses, including exposures to heavy metals, can cause damage to plant cells either directly or indirectly through the burst of ROS. Plant cells are able to respond to elevated levels of ROS by activating their antioxidant defense system (Dazy et al., 2008). It is well known that exposure of plants to heavy metals induces the generation of active oxygen species (AOS), which are harmful to plants (Zenk, 1996). The injury of plant cells caused by heavy metals is, to a great extent, related to the destruction of the balance between the generation and detoxification of AOS. Plants possess the protective mechanisms to scavenge the toxic AOS, but the ability to balance between the generation and detoxification of AOS varies greatly among different plant species (Pang et al., 2003). Heavy metal treatment could enhance the activities of antioxidant vitamins. Plants contain a wide range of vitamins that are essential not only for human metabolism but also for plants, because of their redox chemistry and role as cofactors, and some of them also have strong antioxidant potential. The antioxidant vitamins that have been the focus of most attention in plants are carotenoids (pro-vitamin A), ascorbate (vitamin C) and tocochromanols (vitamin E, including both tocopherols and tocotrienols) (Asensi-Fabado and Munné- Bosch 2010). We

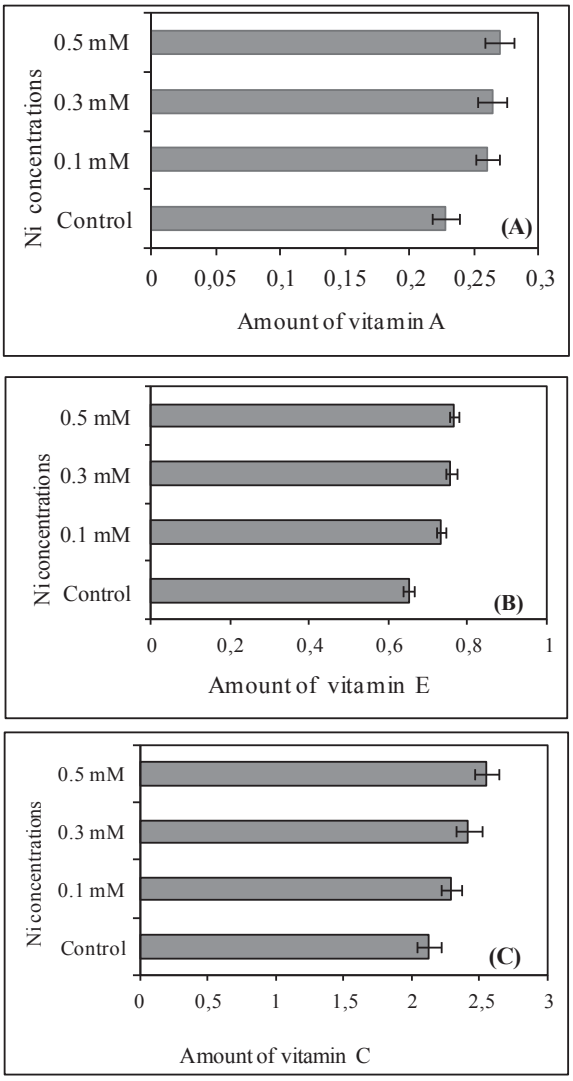

Figure 1. (A) Vitamin A [μg/g], **(B)** Vitamin E [μg/g fw] and **(C)** Vitamin C [μg/g] content in the primary leaves of bean seedlings applying different concentrations of nickel. Error bars indicate ± 1 SE.

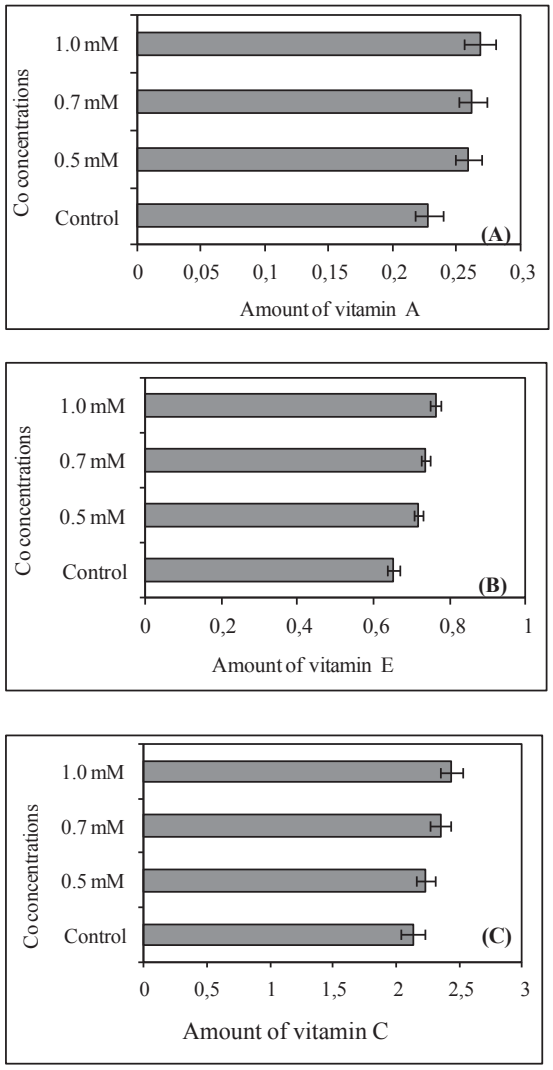

Figure 2. (A) Vitamin A [μg/g], **(B)** Vitamin E [μg/g fw] and **(C)** Vitamin C [μg/g] content in the primary leaves of bean seedlings applying different concentrations of cobalt. Error bars indicate ± 1 SE.

found that all four metals caused a significant ($p<0.01$, $p<0.05$) increase in vitamins A, E and C.

It was determined that all four metals caused a significant ($p<0.01$, $p<0.05$) increase in vitamin E. Increasing levels of α-tocopherol has been found in *Arabidopsis thaliana* under Cu and Cd stress (Collin et al., 2008). Yusuf et al. (2010) reported that the content of total tocopherol increased during salt, heavy metal and osmotic stress in *Brassica juncea* plants. Vitamin E includes tocopherols, one of the most powerful antioxidants, and tocotrienols (Schneider, 2005). Tocopherols have been suggested to play a major role in maintenance and protection of the photosynthetic machinery. There is clearly a correlation between the degree of stress and tocopherol concentration (Munné-Bosch and Alegre, 2002a). Gajewska and Sklodowska (2007) and Collin et al. (2008) have suggested that increased tocopherol content confers enhanced tolerance to plants against drought and heavy metal (Ni, Cu, Cd) stress. Tocopherols are able to quench physically or scavenge chemically $^1O_2$ (Krieger-Liszkay and Trebst, 2006). Fryer (1992) suggested that the changes in α-tocopherol during plant responses to environmental stress are characterized by two phases. In the first phase, there is an increase in tocopherol synthesis, which is followed by a second phase of net tocopherol loss. Initial enhanced tocopherol levels contribute to protection by reducing by ROS levels and inhibiting lipid peroxidation, thus avoiding oxidative damage. When the stress is too severe, tocopherol degradation exceeds its synthesis and levels decrease (phase II). Consequently, lipid peroxidation increases and cell death occurs if α-tocopherol deficiency cannot be compensated for by another mechanism of protection. In stress-tolerant plants, only the first phase is apparent (unless stress is too severe), while only the second phase is usually observed in stress-sensitive plants (Munné-Bosch, 2005).

In the present study, exposure to heavy metal (Ni, Co, Cr, Zn) level in the growth medium resulted in increased vitamin C in bean plants. The exposure of *Bacopa monnieri*

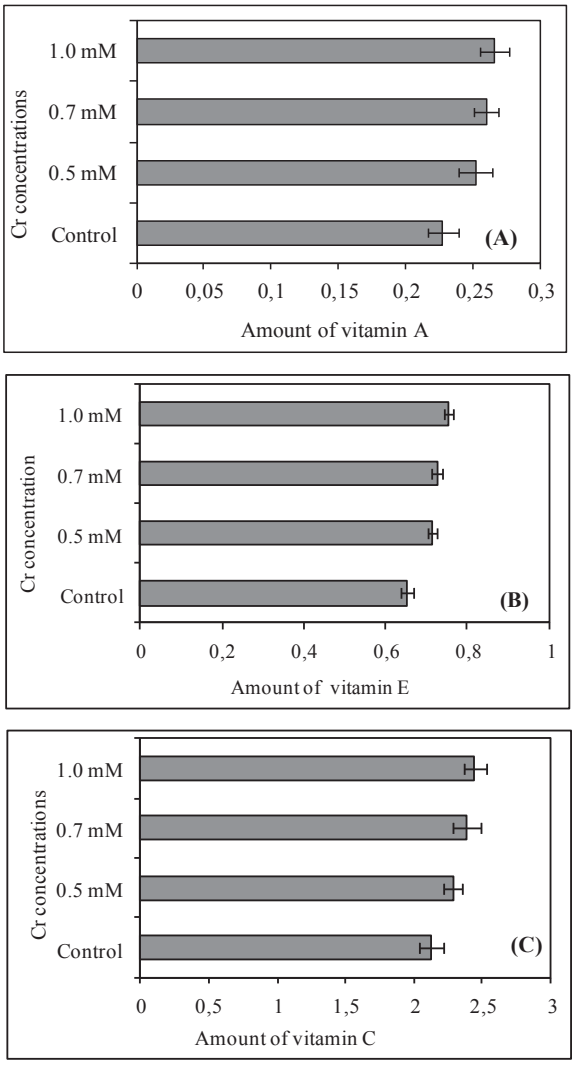

Figure 3. (A) Vitamin A [μg/g], **(B)** Vitamin E [μg/g fw)] and **(C)** Vitamin C [μg/g] content in the primary leaves of bean seedlings applying different concentrations of chromium. Error bars indicate ± 1 SE.

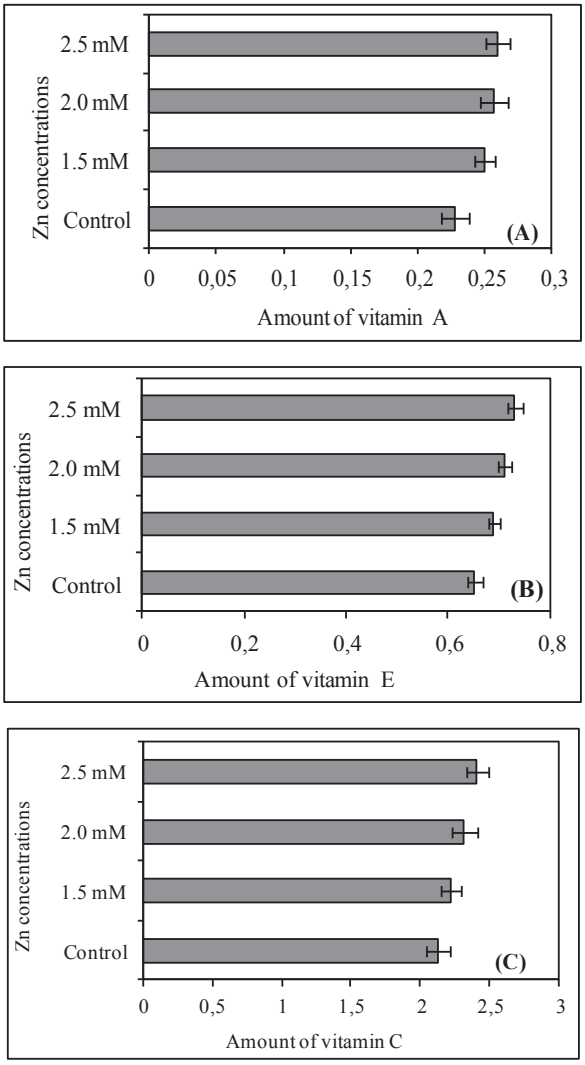

Figure 4. (A) Vitamin A [μg/g], **(B)** Vitamin E [μg/g fw] and **(C)** Vitamin C [μg/g] content in the primary leaves of bean seedlings applying different concentrations of zinc. Error bars indicate ± 1 SE.

to various concentrations of mercury for 14 days caused an increase in ascorbic acid levels (Sarita et al., 1996). ASA is the most abundant antioxidant in plants and plays a role in responding to oxidative stress (Chao et al., 2010). Besides, it participates in a variety of processes, including photosynthesis, photoprotection, the cell cycle, cell-wall growth and cell expansion, synthesis of ethylene, gibberellins, anthocyanins, and hydroxyproline. ASA is found in the thylakoid lumen and stroma of chloroplasts (Asada, 1999), α-tocopherol and β-carotene are found in the lipid matrix, and associated with protein domains in the tylakoid membrane (Munné-Bosch and Alegre, 2002a), which provides a protective effect of ASA on α-tocopherol and β-carotene oxidation, likely by scavenging and/or preventing the formation of OH^-. Exposure to heavy metal (Ni, Co, Cr, Zn) levels in the growth medium resulted in increased vitamin A of bean plants (Fig 1A, 2A, 3A, 4A). Vitamin A (retinol) is the most effective naturally occurring quencher of singlet oxygen; it is a radical scavenger and an effective chain-breaking antioxidant (Alpsoy et al., 2009). Vitamin E is a potent lipid-soluble antioxidant, with the ability to quench free radicals directly, and functions as a membrane stabilizer (Clarke et al., 2008). Carotenoids, ubiquinols, selenium (Se), copper (Cu) and flavonoids are also included in this group of nutritional antioxidants (Surai, 2003).

The level of MDA content has been considered as an indicator of oxidative stress. MDA is the decomposition product of polyunsaturated fatty acids of biomembranes and its increase shows that plants are under high-level antioxidant stress. Cell membrane stability has been used freqently to discriminate stress tolerant and sensitive cultivars of many crops (Hou et al., 2007). In the experiments, MDA concentration increased linearly with increased heavy metal (Ni, Co, Cr, Zn) levels in the solution. Similar results were obtained with *S. polyrrhiza* L. (Upadhyay, 2010) and *Ceratophyllum demersum* L. (Devi and Prasad, 1998). Thus increased MDA content shows the 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).

Inhibition of photosynthetic pigment biosynthesis is one of the primary events in plants during heavy metal stress and decreases in photosynthetic pigment content have also been reported in many plants under heavy metal stress (Cenkci et al., 2010). It was suggested that heavy metals could interfere with chlorophyll biosynthesis either through the direct inhibition of enzymatic steps or through the substitution of the central Mg ion (Cenkci et al., 2010; Pourraut et al., 2011). Carotenoids serve as antioxidants against free radicals and photochemical damage (Sengar et al., 2008). Thus less effect on carotenoids might represent its supportive role against oxidative stress.

CONCLUSIONS

In the present study, exposure to heavy metals affected different parameters of bean: vitamin, MDA, chlorophyll and carotenoid content. Exposure of ten-day-old bean seedlings to nickel, cobalt, chromium and zinc increased vitamin, MDA and carotenoid contents. Chlorophyll content decreased with heavy metal (Ni, Co, Cr, Zn) treatment in comparison to the control.

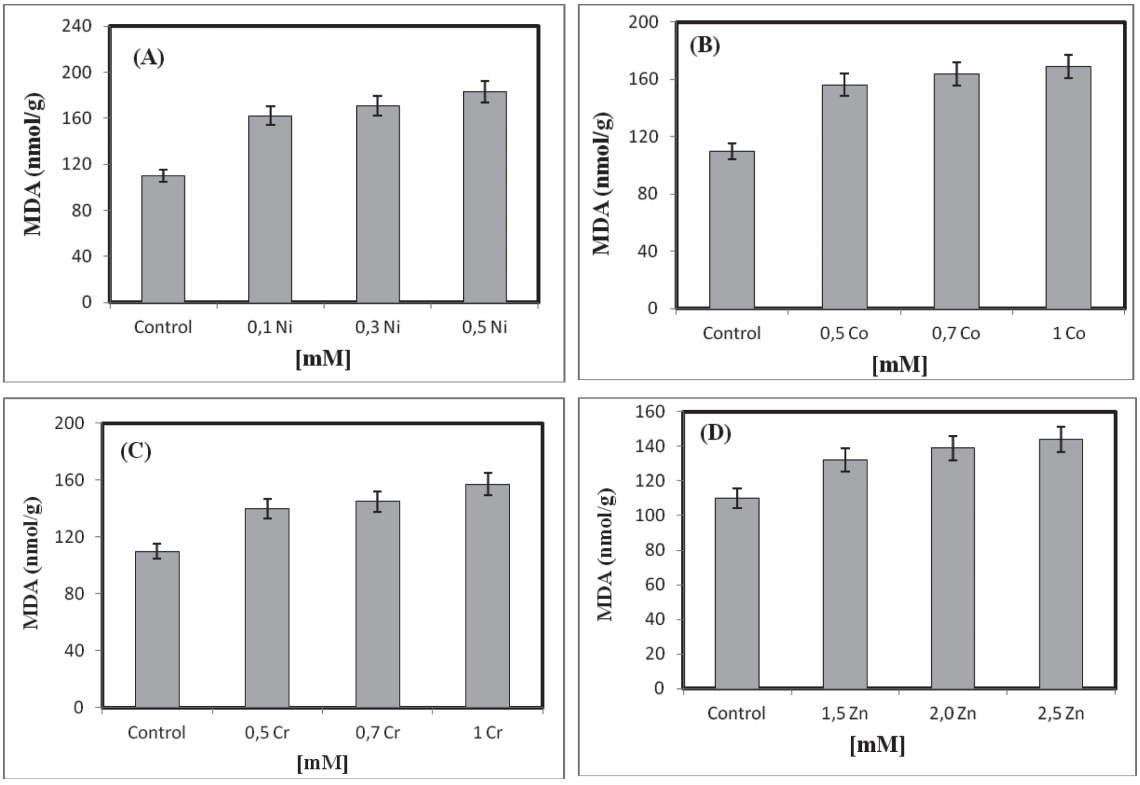

Figure 5. MDA content [nmol g^{-1} fw] in the leaves of bean seedlings at various concentrations of heavy metals. **(A)** Nickel, **(B)** Cobalt, **(C)** Chromium, **(D)** Zinc. Error bars indicate ± 1 SE.

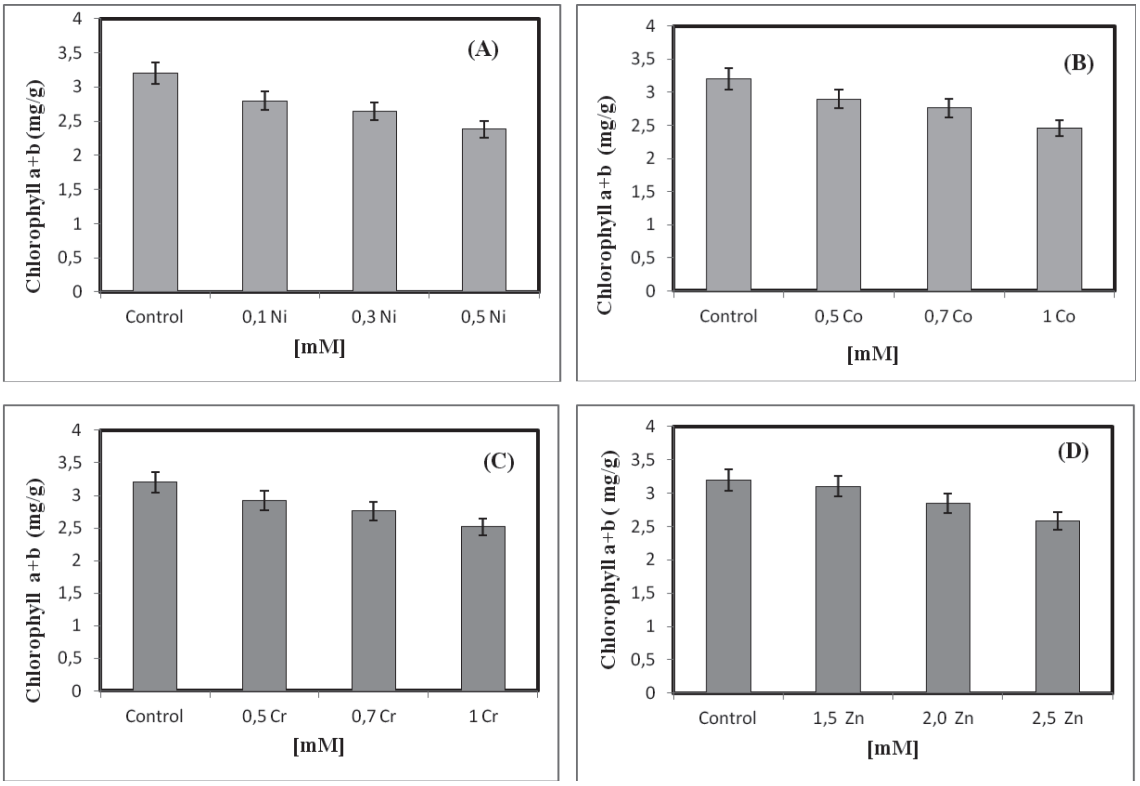

Figure 6. Chlorophyll (a+b) content [µmol/l fw] in the leaves of bean seedlings at various concentrations of heavy metals. **(A)** Nickel, **(B)** Cobalt, **(C)** Chromium, **(D)** Zinc. Error bars indicate ± 1 SE.

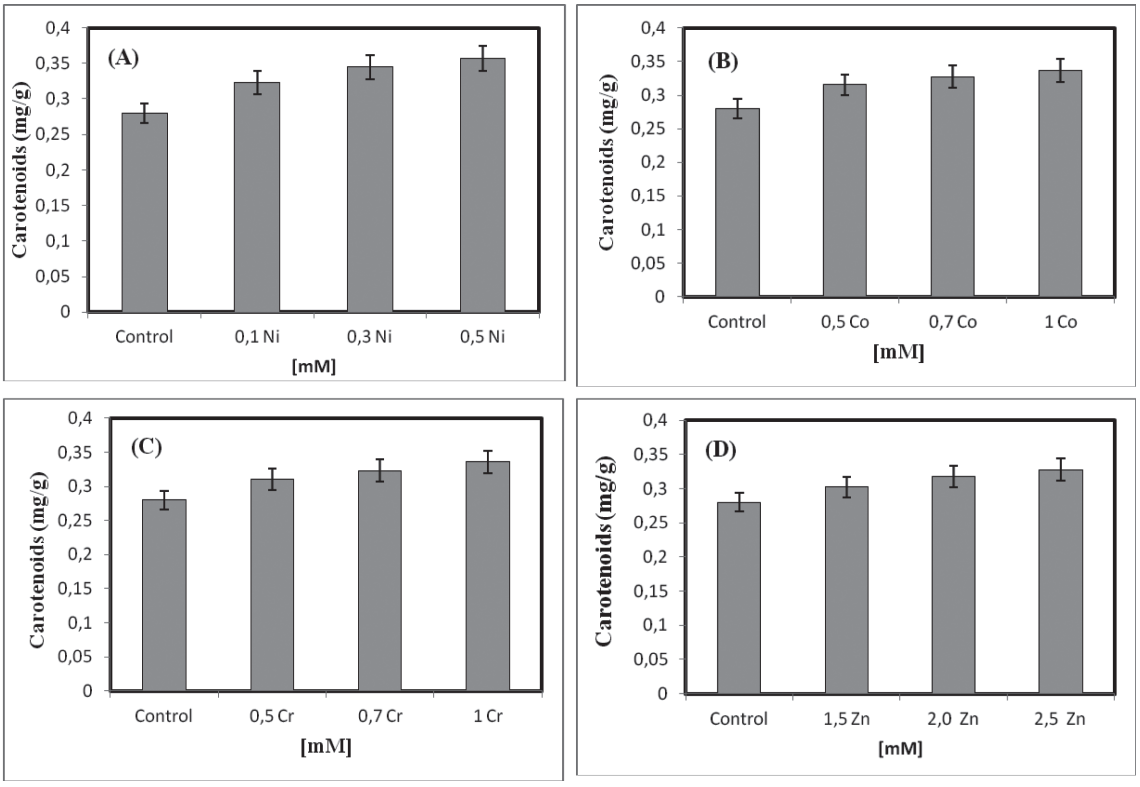

Figure 7. Carotenoid content [µmol g^{-1} fw] in the leaves bean seedlings at various concentrations of heavy metals. **(A)** Nickel, **(B)** Cobalt, **(C)** Chromium, **(D)** Zinc. Error bars indicate ± 1 SE.

The greatest effects were produced in plants exposed to nickel, followed by the sequence cobalt> chromium>zinc.

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