

The effects of Co^{2+} and Zn^{2+} on the contents of protein, abscisic acid, proline and chlorophyll in bean (*Phaseolus vulgaris* cv. Strike) seedlings

Fikriye Kirbag Zengin

Department of Biology, Faculty of Science, Firat University, Elazig 23119, Turkey

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Abstract: 17-day-old bean plants (*Phaseolus vulgaris* cv. Strike) were used to analyze the effects of Co^{2+} and Zn^{2+} on the time course of proline, total protein, chlorophyll and abscisic acid (ABA) levels in leaves. Controls, Co^{2+} and Zn^{2+} -treated plants were grown for 8 days in Hoagland solution. Samples were taken at 2 day intervals. Proline, chlorophyll (a+b) and total protein contents of 17 day old primary leaves were determined by a spectrophotometer. ABA contents in roots and leaves of the seedlings were measured by high-pressure liquid chromatography. The presence of Zn^{2+} and Co^{2+} significantly increased the ABA contents in roots and leaves ($p < 0.05$ or $p < 0.01$). The increase of the abscisic acid content in the leaves was related to the content of the roots. This was further substantiated by enhanced accumulation of proline in the leaves of seedlings exposed to zinc and cobalt. The contents of chlorophyll (a+b) and total protein decreased with the concentration of both metals ($p < 0.05$ or $p < 0.01$). Cobalt proved to be comparatively more toxic than zinc.

Key words: Abscisic acid, Chlorophyll, Heavy metal, Proline, Protein.

Introduction

The heavy metals, spread in the environment through industrial activities, fertilizers and pesticides in agriculture, urban wastes, volcanic activities, mining, smelting and the disposal of sewage sludge, threaten all living beings, especially plants. Ions such as Zn^{2+} and Cu^{2+} at appropriate concentrations are required for structural and catalytic components of proteins and enzymes, and as cofactors essential for normal growth and development of plants (Moustakas *et al.*, 1994). Excess accumulation of these micronutrients and other heavy metals such as Cd^{2+} , Pb^{2+} and Ni^{2+} in plants imposes stress causing physiological constraints leading to decreased vigour and plant growth (Ouzounidou, 1993).

Proline accumulation, accepted as an indicator of environmental stress. Heavy metals lead to proline accumulation (Alia-Saradhi, 1991). Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization, and (d) hydrolysis of proteins (Charest and Phan, 1990).

The phytohormone abscisic acid (ABA) regulates physiologically important stress and developmental responses during the life cycle of plants (Kuhn and Schroeder, 2003). During vegetative growth, ABA mediates adaptive responses to various environmental stresses like salts, drought (Leung and Girauda, 1998). It is reported that the abscisic acid (ABA) content increased in plants which are exposed to copper and nickel pollution (Monni *et al.*, 2001).

Heavy metals are known to interfere with chlorophyll synthesis either through the direct inhibition of an enzymatic step or through the induced deficiency of an essential nutrient (Van Assche and Clijsters, 1990). In *Triticum aestivum* cv.

Vergina grown on Cu-enriched soil (Lanaras *et al.*, 1993) and in *Brassica oleracea* var. Botrytis cv. Maghi exposed to Cu^{2+} , Co^{2+} and Cr^{2+} (Chatterjee and Chatterjee, 2000) the amount of chlorophyll reportedly decreased. The growth of mung bean seedlings that were exposed to 5 mM cobalt was prevented and chlorosis occurred (Liu *et al.*, 2000).

In this study, the effects of Zn^{2+} and Co^{2+} on the content of chlorophyll (a+b), chlorophyll a/b ratio and, proline, protein and abscisic acid in bean seedling leaves were investigated with respect to concentration of the heavy metal.

Experimental procedures: In this study, 9 day old bean seedlings (*Phaseolus vulgaris* cv. Strike) were used. The stock solutions of zinc ($\text{ZnCl}_2 \cdot \text{H}_2\text{O}$) were prepared at a concentration of 1.5, 2.0, 2.5 mM and the other stock solutions had concentration of 0.5, 0.7 and 1.0 mM for cobalt ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$). Seeds of bean (*Phaseolus vulgaris* cv. Strike) were surface sterilized in 10^{-3} M HgCl_2 for 2 min (Sresty and Madhava Rao, 1999), washed in distilled water and germinated between wet paper towels at 25 °C in the dark for 3 days. Subsequently plants cultivated hydroponically in a growth chamber at a light intensity of 4500 lux (16 hr light/ 8 hr). During this period day/night temperatures were maintained at 25°C. After 9 days, plants were transferred to Hoagland solutions containing 0 (control), $\text{ZnCl}_2 \cdot \text{H}_2\text{O}$ (1.5, 2.0 and 2.5 mM) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5, 0.7, 1.0 mM). After 8 days of heavy metal treatment, the primary leaves were harvested and used for pigment, proline and ABA analyses. Proline was extracted and its concentration determined by the method of Bates *et al.* (1973). Leaf tissues were homogenized in sulphosalicylic acid, and the homogenate was centrifuged at 3000 rpm for 20 min. The supernatant was treated with acetic acid and acid-ninhydrin, boiled for 1 hr, and then absorbance at 520 nm was determined. Proteins of fresh tissue of seedling's primary leaves were extracted according to

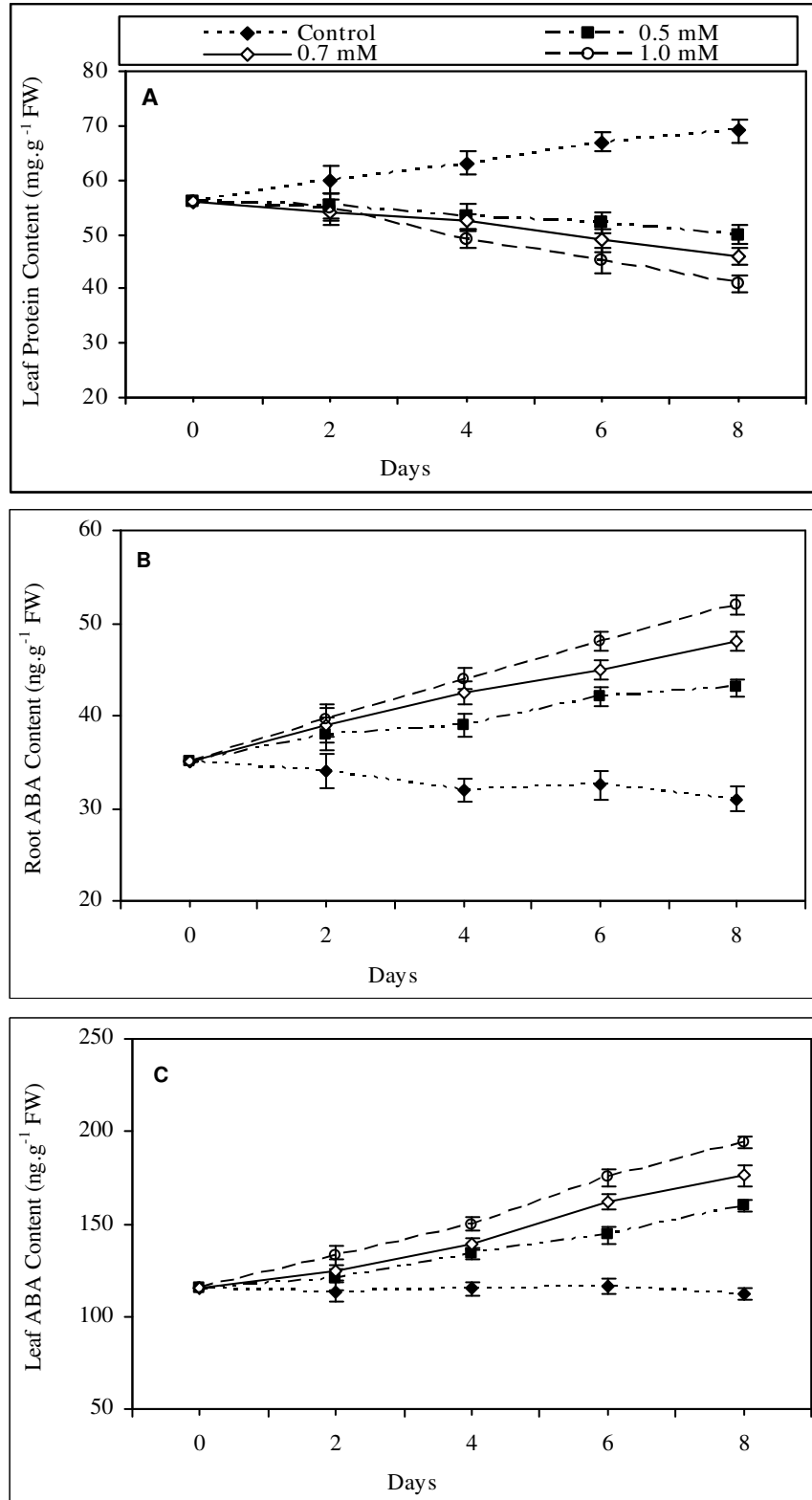


Fig. 1: Effect of Co on protein and abscisic acid, chlorophyll, proline and chlorophyll ratio. **A:** Total protein (mg.g⁻¹ FW) contents, **B:** Root, **C:** Leaf ABA [ng.g⁻¹ (F.W.)] contents in the primary leaves of bean seedlings grown in different concentrations of cobalt (CoCl₂.6H₂O). Error bars indicate ± SE.

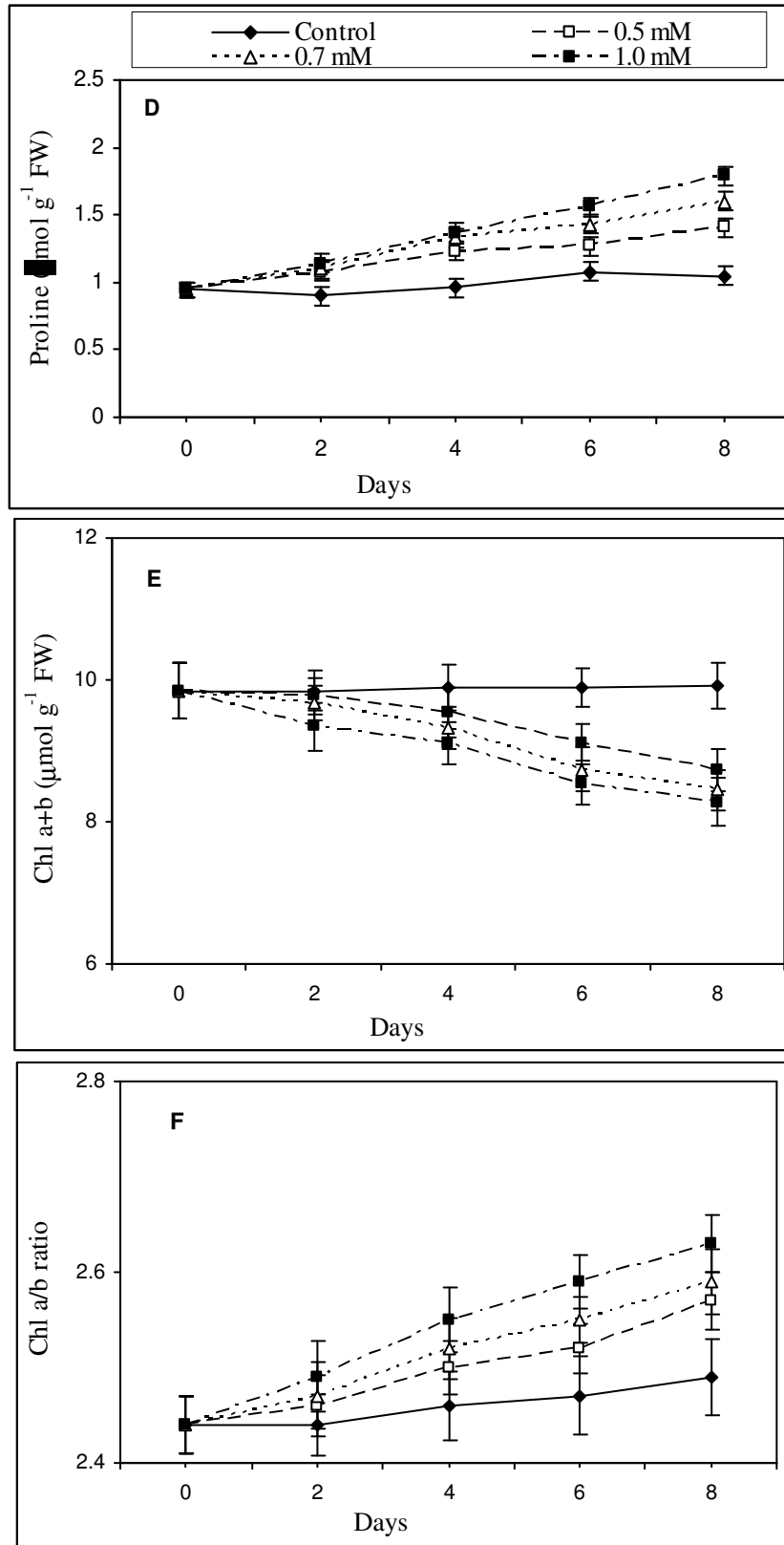


Fig. 1: D: Proline contents ($\mu\text{mol g}^{-1}\text{FW}$), E: Chlorophyll a+b contents ($\mu\text{mol l}^{-1}\text{FW}$), F: Chlorophyll a/b ratio in the primary leaves of bean seedlings grown in different concentrations of cobalt ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$). Error bars indicate \pm SE.

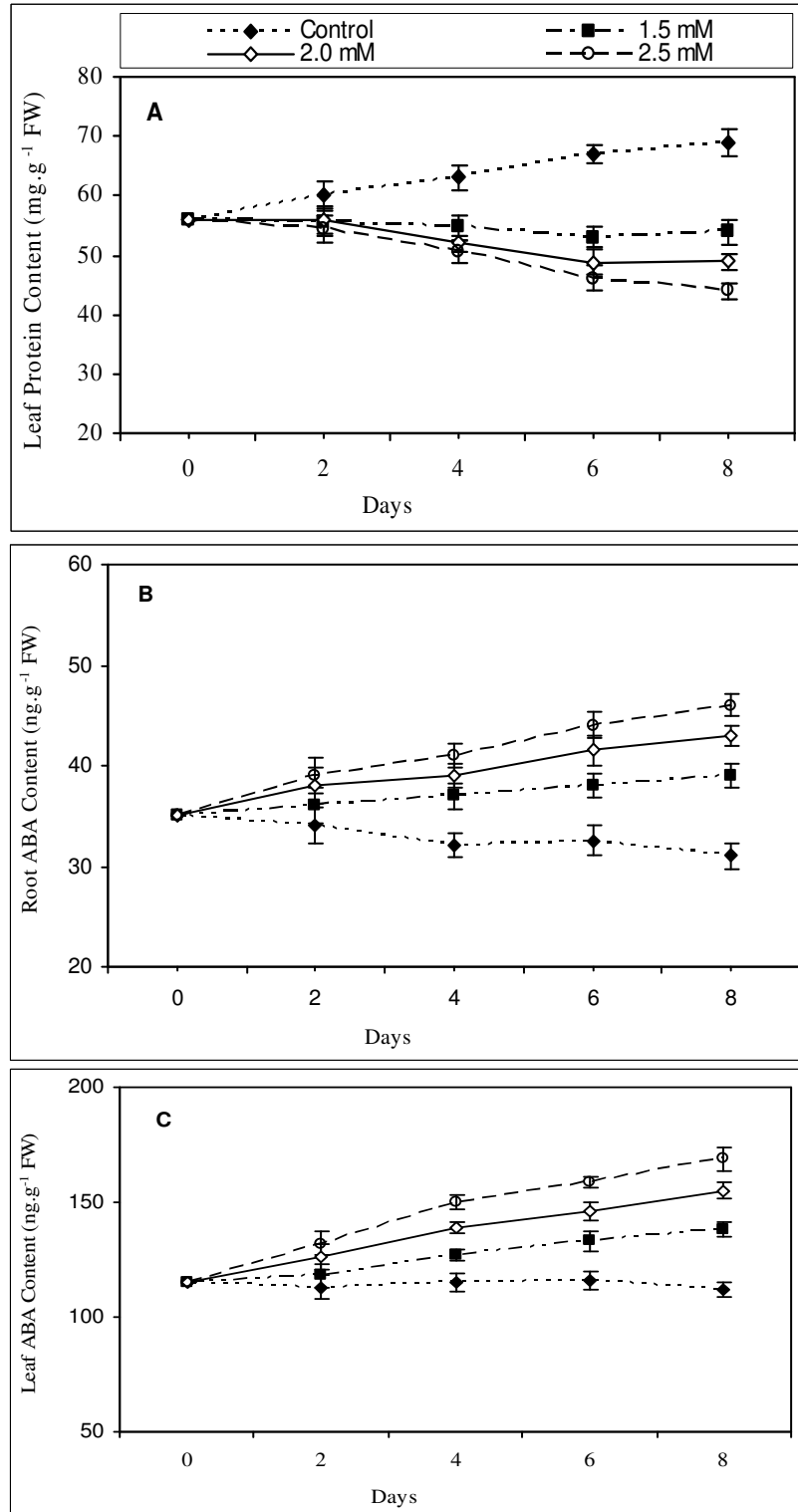


Fig. 2: Effect of zinc on protein and abscisic acid, chlorophyll, proline and chlorophyll ratio. **A:** Total protein (mg.g⁻¹ FW) contents, **B:** Root, **C:** Leaf ABA [ng.g⁻¹ (F.W.)] contents in the primary leaves of bean seedlings grown in different concentrations of zinc (ZnCl₂.H₂O). Error bars indicate \pm SE.

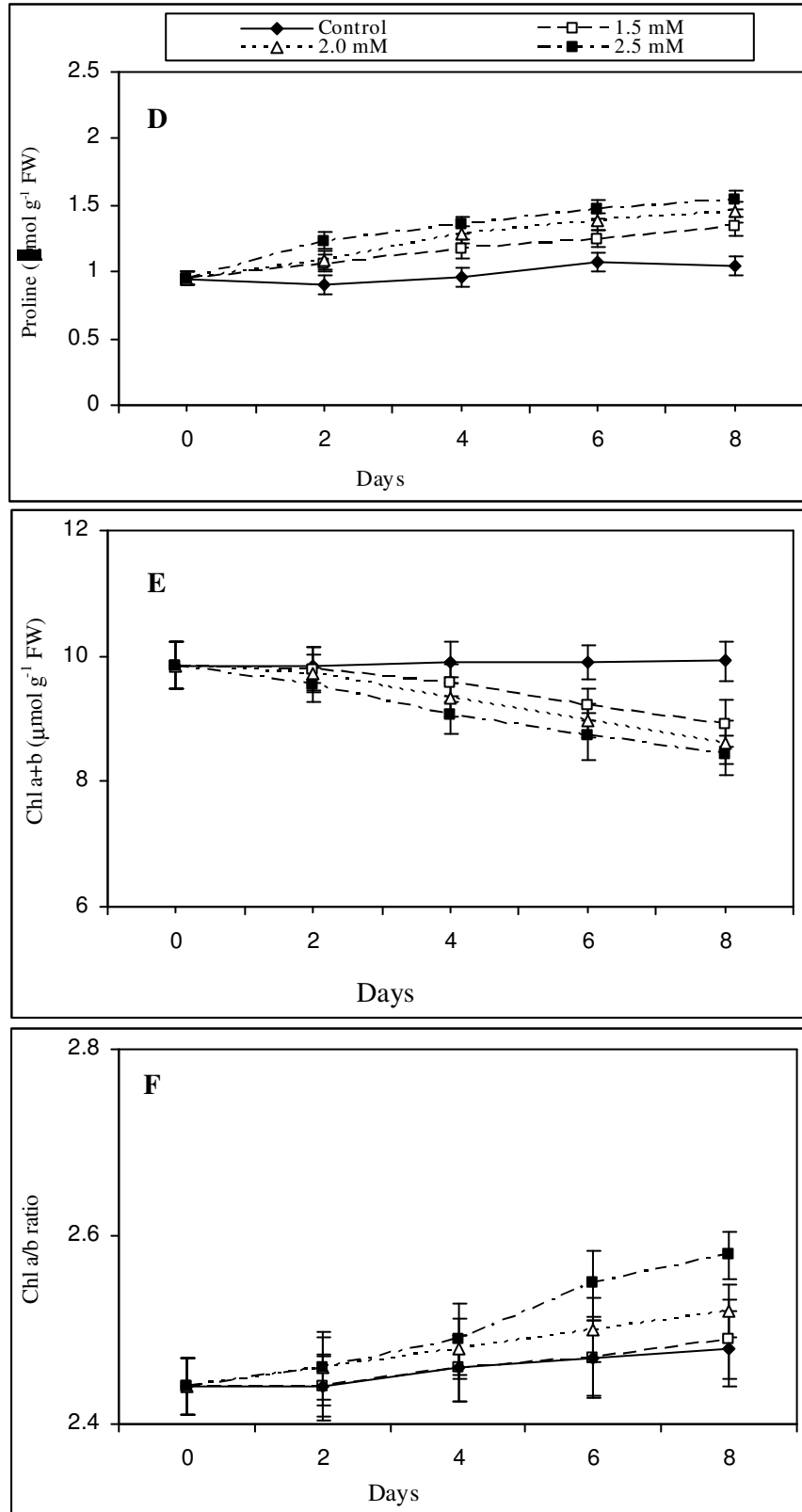


Fig. 2: D: Proline contents ($\mu\text{mol g}^{-1}\text{FW}$), **E:** Chlorophyll a+b contents ($\mu\text{mol l}^{-1}\text{FW}$), **F:** Chlorophyll a/b ratio in the primary leaves of bean seedlings grown up different concentrations of zinc ($\text{ZnCl}_2 \cdot \text{H}_2\text{O}$). Error bars indicate \pm SE.

the method of Larson and Beevers (1965). The protein content in the extracts was determined according to Lowry *et al.* (1951). For determining the protein content a CE-5502 Scanning double beam UV spectrophotometer, 750 nm wave length and a quartz tube of 1 ml volume was used. Chlorophyll was determined according to Graan and Ort (1984). Abscisic acid (ABA) extraction of the primary roots and leaves of bean seedlings grown in the heavy metal solutions was performed according to Cabot *et al.* (1986). Dried extract was solubilized in 2 ml methylene chloride for HPLC. Injections were made in duplicate for each sample. The quantification was according to absorption maximum of 254 nm for ABA. HPLC separations were accomplished at room temperature with a Perkin-Elmer liquid chromatography system (Series 1100) consist of a sample injection valve (Cotati 7125) with a 20 μ l sample loop, an ultra-violet (UV) spectrophotometric detector (Cecil 68174), an integrator (HP 3395) and a Hi-Chrom ODS-1 packed (5 μ m particle) column (250 x 4.6 ID) with a methanol mobile phase at 1.5 ml min⁻¹ flow rate. Data presented are the results from four separate analyses with 20 seedlings in each. Statistical analysis was performed based on 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 \pm SEs.

Results

Figs. 1-2 summarize the results of the effects of selected heavy metals (Zn and Co) on protein and abscisic acid, chlorophyll (a+b), proline contents and chlorophyll (a/b) ratios in primary leaves of the bean seedlings. Chlorophyll (a+b) and total protein contents ($p < 0.05$ or $p < 0.01$) declined progressively with increasing concentrations of heavy metals (Zn and Co) in comparison with controls. The chlorophyll a/b ratio increased slightly with increasing heavy metal concentrations. Significant increases of the contents of proline ($p < 0.05$ or $p < 0.01$) and abscisic acid ($p < 0.05$ or $p < 0.01$) were detected after eight-day exposure to the heavy metals.

Cobalt: In cobalt-treated plants (0.5, 0.7 and 1.0 mM) the in leaves total protein was significantly affected at the 8 day harvest. These plants had significantly lower total protein contents than control plants (Fig. 1 A). Exposure of 8 day old plants of cobalt concentrations (0.5, 0.7 and 1.0 mM) led to a significant ($p < 0.01$) reduction of the total protein contents of 27.5%, 33.3% and 40.6% in primary leaves of the seedlings as compared to the control seedlings. Abscisic acid contents of the primary leaves increased with increasing concentration of this metal (Fig. 1 C). In leaves treated with 0.5, 0.7 and 1.0 mM cobalt, abscisic acid content increased by 42.8%, 57.1%, and 73.2%, respectively, compared to the control plants ($p < 0.05$). ABA in the in root of cobalt treated seedlings (0.5, 0.7, 1.0 mM) increased in the 2, 4, 6 and 8 days harvest ($p < 0.05$) (Fig. 1 B). The effects of cobalt (0.5 and 1.0 mM) on abscisic acid 38.7-67.7% compared to the control plants ($p < 0.05$). Proline contents in control leaves remained almost unchanged up to the 8 days harvest. It is clear from Fig. 1 D that accumulation of

proline induced by $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was evident at 2 days after treatment. In seedlings treated with 0.5, 0.7 and 1.0 mM cobalt, proline content increased by 34.3%, 52.4%, and 75.2%, respectively, compared to the control seedlings ($p < 0.01$). The chlorophyll (a+b) contents of primary leaves decreased with increasing concentration of this metal (Fig.1 E). The chlorophyll (a+b) contents of the primary leaves decreased noticeably at 0.5, 0.7 and 1.0 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ concentrations of 8 day harvest. The chlorophyll (a+b) contents in leaves were decreased by 12.1%, 14.8% and 16.5%, respectively, compared to the control seedlings ($p < 0.01$). Chlorophyll (a/b) ratio increased between 3.2-5.6% ($p < 0.05$).

Zinc: The protein contents in the primary leaves decreased noticeably at 1.5, 2.0 and 2.5 mM Zn concentrations (Fig. 2 A). The protein contents in leaves were decreased by 21.9%, 29.0% and 36.2%, respectively, compared to the control seedlings ($p < 0.05$).

From 2nd day to the end of the experiment, a progressive increase of the leaf ABA content was found in zinc treated (1.5, 2.0 and 2.5 mM) plants. The root contents of ABA zinc treated plants increased an almost 50.9% increase on the 8th day harvest. In the leaves Zn treatment (2.5 mM) led to increases of ABA levels 2, 4, 6 and 8 days harvests ($p < 0.05$). In 2.5 mM zinc treated roots the abscisic acid contents were 39, 41, 44, 46 ng.g⁻¹ fresh weight from the 2, 4, 6 and 8 days harvests, respectively (Fig. 2 B). The effects of zinc (1.5 and 2.0 mM) on abscisic acid 21-33.3% compared to the control plants ($p < 0.05$). Leaves of Zn treated plants showed higher abscisic acid contents than roots. The content of the stress indicator amino acid proline increased with the increase in the concentration of zinc (Fig.2 D). The proline content in the seedlings increased between 27.6% - 46.6% ($p < 0.01$). The chlorophyll (a+b) contents in the primary leaves decreased noticeably at 0.5, 0.7 and 1.0 mM $\text{ZnCl}_2 \cdot \text{H}_2\text{O}$ concentration of 8 day harvest (Fig. 2 E). The chlorophyll (a+b) contents in leaves were decreased by 10.1%, 13.1% and 15.1%, respectively, compared to the control seedlings ($p < 0.05$). The chlorophyll a/b ratio increased between 0.4-3.6% ($p < 0.05$) (Fig. 2 F).

Discussion

Each of these heavy metals (Zn, Co) at three concentrations decreased the seedlings chlorophyll and protein content; increased the proline and ABA content. Extension of the exposure time to heavy metals and increasing concentration decreased the chlorophyll and protein content; increased the proline and ABA content.

One of the major effects of the heavy metals on plants is reported to be a decrease in the protein content by hindering protein synthesis. Lead, zinc and cadmium in *Hordeum vulgare* plant (Stiborova *et al.*, 1986a), copper and lead in *Zea mays* (Stiborova *et al.*, 1986b), lead and cadmium in *Lemna minor* (Mohan and Hosetti, 1997) have been reported to decrease protein contents.

Chloride salts of cobalt and zinc also affect abscisic acid (ABA) contents and at high metal concentrations, there is a

large increase of ABA in the seedlings roots and leaves. In general ABA levels increase more in leaves than in roots. Studies performed by Talanova *et al.* (2000) showed that the ABA content increased in the seedling of *Cucumis sativus* when lead and cadmium were applied at 1, 4 and 7 days. It was pointed out that the content of ABA in the roots and leaves increased with stoma resistance and the water content of leaf decreased at the *Phaseolus vulgaris* L. cv Contender plants which were kept in cadmium for 144 hr (Poschenrieder *et al.* 1989). ABA plays a major role in the adaptation to abiotic environmental stresses (Jensen *et al.*, 1996). Plants are able to employ several strategies for survival when exposed to heavy metals. Heavy metal resistance can be based on either avoidance or tolerance mechanisms. Plants can be protected externally against metals they can tolerate high tissue concentrations through specific physiological mechanisms (Baker, 1987). One of these mechanisms is hindering the transmission of the heavy metals from roots to shoot. Heavy metals were highest in the root. Significant amounts were also seen in the cell walls. The root epidermis served as a barrier to transport of lead to aboveground tissues (Weis and Weis, 2004).

Accumulation of free proline in response to heavy metal exposure seems to be wide- spread among plants. The functional significance of this accumulation would lie in its contribution to water balance maintenance (Costa and Morel, 1994). These authors also suggested that proline-mediated alleviation of water deficit stress could substantially contribute to cadmium tolerance of the plant. Proline increases the stress tolerance of the plants through such functions as osmoregulation, the protection of enzymes against denaturation, and the stabilization of protein synthesis (Kuznetsov and Shevyakova, 1997).

Determination of the chlorophyll content of plants is often carried out to assess the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic plant productivity (Parekh *et al.* 1990). In *Vetiveria zizanioides* L., at Pb /Zn treatment the chlorophyll content decreased (Pang *et al.*, 2003). The chlorophyll content may be due to the inhibition of chlorophyll biosynthesis (Stobart *et al.*, 1985; Stiborová *et al.*, 1986) or accelerated degradation of chlorophyll (Luna *et al.*, 1994). Chlorophyll a/b ratio, which is used as a stress indicator, increased slightly with increasing metal treatments, which was also seen in *Empetrum nigrum* leaves near the Cu-Ni smelter in the field (Monni *et al.*, 2000). The chlorophyll a/b ratio has been reported to increase due to environmental stress (Delfine *et al.*, 1999).

In the present study, the exposure of heavy metal significantly affected different parameters of bean such as proline, chlorophyll (a+b) contents, chlorophyll (a/b) ratio, total protein and abscisic acid (ABA) contents. Exposure bean seedlings to cobalt and zinc led to decrease in chlorophyll (a+b) contents. The observed increase in chlorophyll (a/b) ratio in heavy metal treated seedlings. At the same time, metal-treated

seedlings showed significantly higher ABA than control. The increase in cobalt and zinc concentration in bean seedlings caused significantly proline accumulation. Cobalt was determined to be the most inhibitory metal on these parameters.

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Correspondence to :

Dr. Fikriye Kirbag Zengin

Department of Biology, Faculty of Science

Firat University, Elazig 23119, Turkey

E-mail: fzenjin@firat.edu.tr

Tel.: +90 424-237-0000/Ext.6600

Fax: +90 424-237-0062