Pak. J. Bot., 43(3): 1521-1525, 2011.

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF THE LEAVES OF *VERBASCUM WIEDEMANNIANUM* FISCH. & MEY. TO CADMIUM

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Abstract

The effect of cadmium (Cd) on the root and seedling lengths, chlorophyll a, b and proline content, lipid peroxidation and peroxidase activity of *Verbascum wiedemannianum* has been examined. Plants grown in Van Waes-Deberg culture medium were treated with 0.00 (control), 0.01 mM and 0.025 mM cadmium root and shoot growth was remarkably decreased against 0.01 and 0.025 mM Cd as compared to control group. Malondialdehyde (MDA) content, which is an index of lipid peroxidation, and guaiacol peroxidase activity (POD) increased approximately as twice as control group in leaves of plants treated with 0.025 mM Cd. Although chlorophyll a and b contents were decreased in response to the increasing of Cd concentration but statistically significant differences were not found in this respect. However, proline content was significantly increased in response to increasing Cd.

Introductıon

Metals in the environment may present a more insidious problem than organic chemicals because they cannot be degraded to innocuous products, such as carbon dioxide and water. Because metals are transported very well by the atmosphere, many urban areas have been loaded with considerable amounts of toxic metals from point and non-point sources due to human activity (Fargašová, 1998; Mishra & Choudhury, 1999). It is a known fact that the widespread accumulation of metals in the environment is increasingly becoming a problem for organisms of every kind. Metals are continuously released into the biosphere by volcanoes, natural weathering of rocks, and by industrial activities such as mining, the combustion of fossil fuels and the release of sewage. Therefore, they present a risk for primary and secondary consumers and ultimately humans (Munzuroglu & Geckil, 2002).

Cadmium (Cd) is of particular concern to human health as it can be readily absorbed by roots and be concentrated by many cereals, potatoes, vegetables, and fruits. Elevated levels of Cd generally inhibit seed germination, cell growth as well as whole plant growth, nutrient uptake, distribution and photosynthesis. The photosynthetic process has been reported to be very sensitive to Cd. Experiments have shown the effect of Cd on stomatal function, on chlorophyll biosynthesis, on electron transport, and on the Calvin cycle as well as on the ultrastructure of chloroplasts (Catak *et al.*, 2000; Oncel *et al.*, 2000; Zhang *et al.*, 2003; Iqbal & Shazia, 2003; Jeliazkova & Cracker, 2003; Gür *et al.*, 2004; Wang & Zhou, 2005; Farooqi *et al*., 2009). Cd has been found to generate free radicals that may damage plant tissues. Reactive oxygen species (ROS) are a main part of free radicals. They can lead to oxidative stress. ROS cause lipid peroxidation, membrane damage and inactivation of enzymes (Sanitá di Toppi & Gabrielli, 1999; Skórzyńska-Polit *et al.*, 2003/2004; Zhang *et al*., 2003; Monterio *et al*., 2009). In cellular level, lipid peroxidation is the most significant damage caused by ROS. The MDA (product of lipid peroxidation) level is regarded as a biochemical marker for injury mediated by ROS (Palma *et al*., 2002; Verma & Dubey, 2003; Sinha *et al*., 2005; Monterio *et al*., 2009).

Many environmental stresses have been reported to increase the level of proline in plants, such as heavy metals, temperature and drought (Saradhi & Saradhi, 1991, Rai *et al.*, 2004; Claussen, 2005). Hayat *et al.*, (2007) determined that proline level increased the physiological drought stress generated by cadmium. Also the similar results were found by Dhir *et al.*, (2004) and the levels of proline of leaves of *Brassica juncea* increased with increasing Cd concentration.

Peroxidases are known to play a significant role in oxidative stress conditions and it has been shown that peroxidase activity can be used as a potential biomarker for sublethal metal toxicity in plants (Radotic *et al.*, 2000; Sinha *et al*., 2005). Since peroxidase activity is related to ROS formation, toxic heavy metals cause stimulation in activity of peroxidase (Stoeva *et al*., 2005; Ganesh *et al*., 2008).

Verbascum wiedemannianum is an endemic and medicinal plant distributed in Turkey. This species is different from the other *Verbascum* species with its red-dark purple flowers and it has been clasified as a threatened species (Ekim *et al.*, 2000). It was known that the various parts of *V. wiedemannianum* (leaves, stem, flowers etc.) had antioxidative and antimicrobial activity (Tepe *et al.*, 2006). This endemic species has useful properties. This study is aimed to determine biochemical changes on leaves of *V. wiedemannianum* after exposure to Cd which is one of the most toxic heavy metals. In our study, the effect of cadmium (Cd) root length and seedling growth, lipid peroxidation, GPOD (guaiacol peroxidase) activity, contents of proline, chlorophyll a and b leaves of *V. wiedemannianum* have been examined. It was determined that how this parameter changed due to concentration of the heavy metal.

Materıals and Methods

The seeds of *V. wiedemannianum* were collected from Çorum- Turkey. Seeds were air dried and stored at room temperature. They were sterilized in a 1.5% NaOCl for 30 min., then washed three times with sterilized ddH_2O and sown in 9.5cm Petri dishes on ½ strength Van Waes-Deberg culture media (Van Waes & Debergh, 1986) supplemented with 10 g L^{-1} sucrose, 7% agar and with or without 0.00; 0.01; 0.025 mM Cd. Metal solutions were added to the medium after autoclaving in sterilised condition. Cd concentration was determined as a result of pre-treatment studies. Plant growth was completely inhibited by using >0.025 mM Cd.

Cultures were incubated in a growth chamber at 20 ± 2ºC under full dark condition. Germinated seeds (with 3-5 mm radicle) on control medium were transferred to media containing no metal and Cd at different concentrations. The Petri dishes were incubated in 16:8 h (light: dark) photoperiod and at 20 ± 2ºC. Length of root and seedling and parameters of physiological and biochemical were measured after 20 days from incubation. Fresh and dried weight of leaves and roots of 25 seedlings was measured.

Determination of lipid peroxidation: The level of lipid peroxidation products was measured by a procedure based on the method of Heath & Packer (1968). Two-hundred mg leaf material was placed in liquid nitrogen and then homogenized in 2 ml 10% Trichloroacetic acid containing 0.25% Thiobarbituric acid, using glass powder and pre-chilled mortar and pestle. This mixture was heated at 95ºC for 30 min., then quickly cooled in an ice-bath and centrifuged at 15000 g for 15 min. The absorbance of supernatant was determined at 532 nm. The concentration of lipid peroxides together with the oxidatively modified proteins of plants was quantified and expressed as total TBARS (thiobarbituric acid reactive substances) in terms of nmol g^{-1}fresh weight, using an extinction coefficient of 155 mM^{-1} cm^{-1}. TBARS is an index of lipid peroxidation.

Guaiacol peroxidase activity: Leaves (0.4 g) were placed into liquid nitrogen and then homogenized in 4 ml 50 mM K-phosphate buffer (pH 7.0) containing 1 mM Ethylene diamine tetraacetic acid (EDTA) and 0.1%(w/v) insoluble Polyvinyl polypyrrolidone (PVPP), using glass powder and prechilled mortar and pestle. The exract was filtered through muslin cloth and centrifuged at 15000 g for 20 min +4 ºC. It was stored at -20 ºC and used for the assay of Guaiacol peroxidase.

Guaiacol peroxidase (EC 1.11.1.7) was measured by following the change of absorption at 70 nm due to its oxidation (extinction coefficient 25.5 mM^{-1} cm^{-1}). The activity was assayed for one minute in a reaction solution (3 ml) composed of 100 mM K-phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 50 µl diluted (1:20) enzyme extract. Enzyme specific activity was expressed as mol of H_2O_2 reduced per min and mg protein (Polle *et al.,* 1994).

Estimation of protein: Protein content in leaves was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as the standard protein.

Proline content: Proline contents of leaves was determined according to Claussen (2005). One g leaf sample was ground in mortar after addition of a small amount of glass powder and 10 mL of a 3% (w/v) aqueous Sulfosalicylic acid solution. The homogenate was filtered through two layers of glass-fibre filter. Glacial acetic acid and ninhiydrin reagent (1 mL each) were added to 1 mL of the filtrate. The closed test tubes with the reaction mixture were kept in a boiling water bath of room temperature for minutes. The absorbance was recorded at 546 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (µmol proline g^{-1} FW).

Chlorophyll determination: Chlorophyll content in leaves of treated with Cd and control plants were extracted in 80% acetone and estimated by method of Arnon (1949).

Statistical analysis: Results were based on at least eight replicates from two independent experiments. Data of root and leaves lengths, dry and fresh weights of the roots and the leaves were the mean of fifteen. All data were subjected to one-way analysis of variance (ANOVA). The significance of differences between treatments was statistically evaluated by standard deviation. Asteriks were used to identify the levels of significance in the difference between control and treatments (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical analysis were performed by SPSS 10.0 version software (1999).

Results and Dıscussıon

The effects of cadmium on the seedlings and roots of *V. wiedemannianum* are given in Table 1. Our results also showed that both root and shoot elongations, fresh and dried weights decreased significantly with increasing Cd. Cadmium showed a significant negative effect on root and shoot elongations. Because cadmium has been considered as an extremely toxic heavy metal (Das *et al*., 1997) excessive application of Cd usually lead to growth inhibition, both in hyperaccumulators and non-hyperaccumulators. The toxic effect at various levels of Cd on root and shoot elongations was observed by other workers (Ewais, 1997; Astolfi *et al.*, 2004; Wang & Zhou, 2005; Demirevska- Kepova *et al.*, 2006; Liu *et al*., 2007; Kabir *et al.*, 2008). Our findings regarding the effect of Cd on physiological parameters are in agrement with the previous results.

Chlorophyll a and b contents were decreased under Cd treatments, however these results were not statistically significant (Table 2, Fig. 1). It has been reported in some studies chlorophyll a and b contens have been decreased due to increased Cd concentrations. Although low Cd concentrations have no significant effect on chlorophyll contents, it was remarkably decreased at high Cd concentrations (Ewais, 1997; Astolfi *et al*., 2004; Monterio *et al*., 2009; Qui *et al*., 2008). Observed decrease in chlorophyll content in response to Cd supply may be due to disorders in chlorophyll biosynthesis as well as to speeding up ageing of the photosynthetic apparatus (Astolfi *et al*., 2004).

Heavy metals can cause damages to plant cells either directly or indirectly through the production of reactive oxygen species (ROS). The most distinctive indication of oxidative stress is lipid peroxidation. Under heavy metal stress, H_2O_2 and $O_2^{\cdot-}$, via the Haber-Weiss reaction, are converted into highly reactive $OH^{\cdot}$ radical and this causes lipid peroxidation (Apel & Hirt 2004). In cellular level, lipid peroxidation is the most significant damage caused by ROS. The peroxidation of cell membranes severely affects its integrity. The MDA (product of lipid peroxidation) level is regarded as a biochemical marker for injury mediated by ROS (Palma *et al*., 2002; Verma & Dubey, 2003; Sinha *et al.*, 2005; Skórzyńska-Polit & Krupa, 2006; Zhang *et al*., 2007; Monterio *et al*., 2009). In our study lipid peroxidation significantly increased at all concentrations in leaves. The content of MDA in the leaves of plants exposed 0.025 mM Cd concentration increased approximately two fold than control group. Various studies showed that the applications of heavy metals increased with the MDA content in plant tissues (Chaoui *et al*., 1997; Sinha *et al.*, 2005; Tamas *et al*., 2006; Uysal *et al*., 2009).

Plant cells are protected against ROS by their antioxidant defence systems. Peroxidases are known to play a significance role in oxidative stress conditions and it has been shown that peroxidase activity can be used as a potential biomarker for sublethal metal toxicity in plants (Radotic *et al*., 2000; Sinha *et al.*, 2005). Peroxidases work independently in different parts of the plants to break up H_2O_2. The increasing of GPOD activity indicates cellular defense mechanism against ROS. GPOD appeared to play a more important role in the antioxidant response once the stress became severe (Qiu *et al.*, 2008; Gratão *et al*., 2008). In *V. wiedemannianum* leaves GPOD activity increased approximately three fold than control in 0.025 mM Cd concentration. Similar results were obtained from other studies. For example, GPOD activity increased in spruce needles (Radotic *et al*., 2000), in rice seedlings (Shah *et al.*, 2001) and in the leaves of *A. hypogaea* (Dinakar *et al.*, 2008). Since peroxidase activity is related to ROS formation, toxic heavy metals cause stimulation in activity of peroxidase (Stoeva *et al*., 2005; Ganesh *et al.*, 2008).

Various environmental stresses such as heavy metals, drought and temperature have been caused to increase the level of proline (Claussen, 2005; Hayat *et al*., 2007). The level of proline increased approximately two fold than control in

0.025mM Cd concentration in the present study. Hayat *et al.*, (2007) determined that proline level was increased with the physiological drought stress generated by cadmium. It has been reported that proline protected cells from Hg^{2+}induced oxidative stress by scavenging reactive oxygen species (Wang *et al*., 2009). The same mechanism probably holds true for Cd treatments. Proline played a role in increasing the cadmium absorption and alleviating cadmium toxicity by detoxifying ROS (Xu *et al*., 2009).

Cadmium usually causes osmotic stress very similar to drought stress at high concentrations. As a result of this, free amino acid concentration in vacuoles has been increased to adjust intracellular osmotic pressure in plants exposed to cadmium stress. The observed increase in proline content due to increased Cd concentration probably for increasing intracellular osmotic pressure (Claussen, 2005).

In conclusion, Cd causes oxidative stress in the leaves of *V. wiedemannianum* is remarkable. As a result of excess oxidative stress ROS formation was observed and ROS was primarily increased lipid peroxidation by damaged membrane lipids (Gülen *et al.*, 2008). The increasing of GPOD activity was also indicated the activity of antioxidative defense mechanism against ROS in leaves of *V. wiedemannianum*.

Table 1. Shoot and root elongation and dry weights in response to cadmium treatment. Asterisk indicates that the mean value is significantly different between treatments and controls (± Standard deviation) (* *p*<0.05, **p<0.01, ***p<0.001).

Cd (mM)	Shoot elongation (mm)	Root elongation (mm)	Shoot FW (g)	Shoot DW(g)	Root FW(g)	Root DW(g)
0.00	30.18±5.17	57.54±11.7	0.53±0.21	0.036±0.008	0.33±0.08	0.025±0.005
0.01	19.45±5.8*	28.54±11.2*	0.3±0. 03*	0.027±0.005	0.21±0.05*	0.016±0.003*
0.025	10.9±2.5*	14.54±4.2*	0.22±0. 08**	0.019±0.006**	0.1±0.02***	0.008±0.002***

Table 2. Lipid peroxidation, GPOD (guaiacol peroxidase) activity, proline, chlorophyll a and b conten in leaves of *V. wiedemannianum* in response to cadmium treatment. Asterisk indicates that the mean value is significantly different between treatments and controls (± Standard deviation) (* p<0.05, **p<0.01, ***p<0.001).

Cd (mM)	Lipid peroxidation (MDA)	GPOD	Proline	Chl a	Chl b
0.00	58.48 ± 26.7	0.28 ± 0.065	1262 ± 165	0.74 ± 0.73	1.01 ± 1.4
0.01	84.9 ± 8.39*	0.49 ± 0.11*	1810 ± 325*	0.6 ± 0.34	0.53 ± 0.64
0.025	101.46 ± 8.02**	0.61 ± 0.09***	2390 ± 291**	0.35 ± 0.004	0.11 ± 0.001

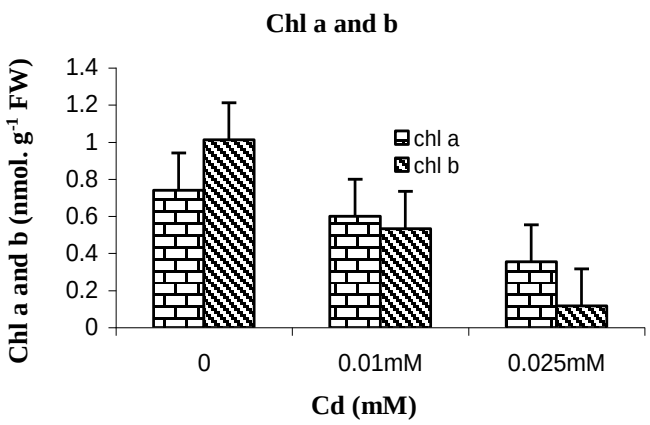

Fig. 1. Chlorophyll a and b contents in response to cadmium treatment. Vertical bars indicate ± SE.

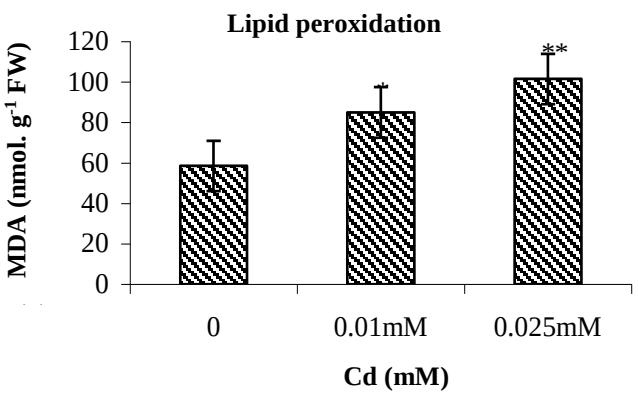

Fig. 2. Lipid peroxidation in response to cadmium treatment. (MDA content was calculated as nmol.g^{-1} FW).Vertical bars indicate ± SE. Asterisks indicate that mean values are significantly different between treatments and control (*p<0.05, **p<0.01).

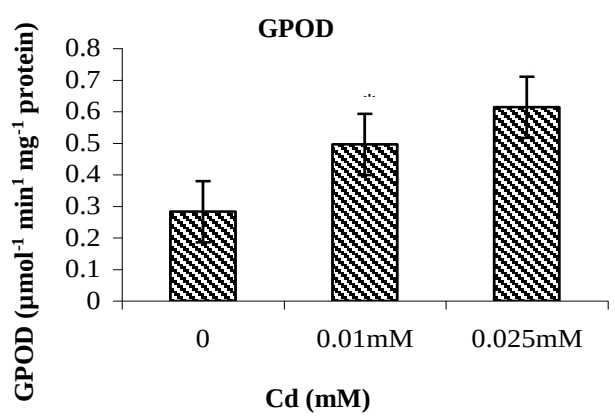

Fig. 3. GPOD (guaiacol peroxidase) activity in response to cadmium treatment. Vertical bars indicate ± SE. Asterisks indicate that mean values are significantly different between treatments and control (*p<0.05, ***p<0.01).

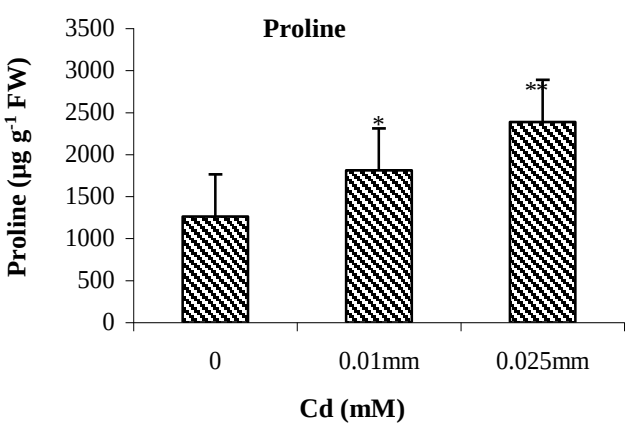

Fig. 4. Proline content in response to cadium treatment. Vertical bars indicate ± SE. Asterisks indicate that mean values are significantly different between treatments and control (*p<0.05, p<0.01).

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(Received for publication 20 January 2010)