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International Journal of Plant Biology & Research

Research Article

Evaluation of Some Defense Mechanisms in Crop Varieties Under Heavy Metal Stress

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Submitted: 08 January 2019

Accepted: 30 January 2019

Published: 01 February 2019

ISSN: 2333-6668

Keywords
- Antioxidant defense mechanisms; Crops; Heavy metals; Stress

Abstract

Today, heavy metal (HM) pollution is one of the most important ecological problems worldwide. Higher concentrations of HMs can lead to toxic effects in all organisms. Some HMs, such as Cd and Pb, although being non-essential and without physiological function, are very toxic even at very low concentrations, which cause some serious disruption in plant growth and productivity with a heavy losses in agricultural yield and crop production. Beside their negative impacts on plants, transfer of toxic elements to the food chain leads to several diseases which human beings faced. Therefore, to reduce the risk of contamination in human beings, HM tolerant varieties should be selected and use for phytoremediation purposes where necessary.In this study, the effects of HM stress onsome of those enzymatic and non-enzymatic antioxidant defense mechanisms, together with protein contents were investigated in two different crop varieties. The selected concentrations (0,150,300 µM) of single $PbCl_2$, $CdCl_2$ and their combinations ($PbCl_2$ + $CdCl_2$) were applied in hydrophonic solution to examine the changes of glutathione (GSH), protein and glutathione *S*-transferase (GST) activities in the roots and shoots of *Hordeum vulgare* cv. Erginel and *Triticum aestivum* cv. Bezostaya varieties.Results indicates that, both single and combined treatments cause a difference at some extend depending on the plant, plant parts and concentrations of HMs. Observation of high levels in examined parameters according to control values indicatesa general adaptability to stress conditions.In line with our results, barley variety were found to be more tolerant to HM stress by comparing to wheat andcan be used for remediation purposes at contaminated sites as a plant agent.

ABBREVIATIONS

HMs: Heavy Metals; GSH: Glutathione; GST: Glutathione *S*-Transferase; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; APX: Ascorbate Peroxidase; GR: Glutathione Reductase; GPOX: Glutathione Peroxidase; MT: Metallothionein; PC: Phytochelatin

INTRODUCTION

Heavy metals (HMs) are defined as metals having a specific density of more than 5g/cm^3, which affect the environment and living organisms adversely [1]. As reviewed by Tchounwou et al. [2], that metals like cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn) are essential nutrients that are required for various biochemical and physiological functions, although other metals such as aluminum (Al), antinomy (Sb), arsenic (As), barium (Ba), beryllium (Be), bismuth (Bi), cadmium (Cd), gallium (Ga), germanium (Ge), gold (Au), indium (In), lead (Pb), lithium (Li), mercury (Hg), nickel (Ni), platinum (Pt), silver (Ag), strontium (Sr), tellurium (Te), thallium (Tl), tin (Sn), titanium (Ti), vanadium (V), and uranium (U) have no established biological functions and are considered as non-essential metals.

HMs are increasing day by day, due to increased population and related household wastes, wrong agricultural applications or fast industrilization [3,4]. As they cannot be destroyed or recycled in the environment, they tend to accumulate in water, soil and air [5,6]. The problem begins for human being at this point, because they are transferable from contaminated plants and other food sources to humans [7].

Beyond permissible limits, HMs cause oxidative stress by formation of Reactive Oxygen Species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and some others [8]. These species are especially dangerous to lipids, carbohydrates, proteins and DNA in the cells of all living organisms [9]. Among HMs, Pb (lead) and Cd (cadmium) are regarded as the most toxic environmental pollutants [7], as they display the most profound mobility in the soil environment through many applications, like mining operations and ore outcrops, metallurgy and electroplating, chemical industries, petroleum refining, etc. Therefore, both elements cause major threat to the agricultural system and contamination affects the crops grown in the area [10].

It is clear that, HMs cause not only physiological, but also biochemical changes in plants above threshold levels [11]. Even the difficulty of making sharp discrimination between physiological and biochemical changes, leaf chlorosis, turgor loss, decrease in seed germination rate and dysfunctional photosynthetic apparatus can be associated symptoms with physiological changes as indicated by several authors [12-14]. On the other hand, increased ROS production, lipid peroxidation

(MDA levels), alterations in antioxidant systems, protein and enzyme synthesis can all be related to biochemical changes [15]. Both types of changes will definetely effect the regulation of plant metabolism and sometimes cause an irrepressible death [16]. However, plant organisms recruit some defense mechanisms to protect themselves against the dreadful effects of HMs through antioxidant enzymes (i.e. SOD, APX, GR, GST, etc.) and non-enzymatic antioxidants (i.e. selenium, tocopherol, ascorbate, GSH) [17].

Herein, the objectives of this study were to investigate the biochemical responses of crop varieties exposed to both Cd and Pb regimes, to study the relationship between oxidative stress and detoxification responses and to explore their tolerance ability under hydroponic systems.

MATERIALS AND METHODS

Plant materials

Plant seeds (*Triticum aestivum* L. cv. Bezostaya and *Hordeum vulgare* L. cv. Erginel) were obtained from Transitional Zone Agricultural Research Institute (Eskisehir, Turkey), as both were registered varieties [18] and free of additives. At the beginning of germination, seeds were surface sterilized in 1% (v/v) NaOCl for 10 min, washed and immersed in tap water for 2h and then in distilled water for a further 2h. Some of the specifications of those varieties were given in Table 1.

Seed germination

Germination was tested on wet Whatman (No. 42mm) filter paper, where twenty seeds were placed in each petri dish (Figure 1A). For all seeds, filter papers were moistened with 3 ml of dH_2O and were settled in the dark growth chamber (NUVE TK-600) at 22°C (±1°C) for 3 days. Later, seeds exposed to 16h photoperiod for 7 days. Germinated seeds were counted on 10th day after initiation of treatments [19]. Seeds were considered as germinated when the radicle touched the seed bed.

Plant growth

After germination, the seedlings were transferred to plastic beakers containing 250 ml of Hoagland solution (including 2mM $Ca(NO_3)_2$, 1mM $NH_4H_3PO_4$, 3mM KNO_3, 0.5mM $CuCl_2$, 50mM KCl, 25mM H_3BO_3, 2mM $ZnCl_2$, 0.5mM $(NH_4)_6MO_7O_{24}$, 1mM $MgSO_4$, 2mM $MnCl_2$ and 20mM Na_2Fe-EDTA) [20]. The solution was aerated continuously and replaced with fresh solution weekly (Figure 1B). Ten plants were arranged in a beaker and beakers were arranged in a randomized block design with Pb and Cd treatment applied in triplets. After 3 days in growth chamber, different single $PbCl_2$ and $CdCl_2$ (0, 150 and 300 µM) and combined $PbCl_2$ + $CdCl_2$ (150 + 150 and 300 + 300 µM) solutions were applied into the nutrient medium. Plants were exposed to a 16h photoperiod for further 7 days. Seedlings were harvested on 10th day after the application of treatments. Subsequently, roots and shoots were separated and pulverized with liquid N_2 for further analysis [19].

Preparation of plant cytosolic extract

Pulverized roots and shoots were extracted, in a ratio of 1:3 w/v, with 100 mM pH 7.0 phosphate buffer (including 0.05 mM DTE, 1 mM EDTA and 3.5 % (w/v) PVPP) at 4°C. The mixture was then homogenized for 4 × 30 s periods by Ultra-Turrax at 13500 rpm on ice. The crude homogenate was centrifuged at 15000 rpm for 30min. at 4°C. The pellet was discarded and the supernatant fraction was immediately subjected to protein determination and enzyme activity measurements [21].

Protein determination

The potein content was determined following the method of Lowry et al. [22], by using crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 to 0.5 ml of cytosol (from previous step) were taken into test tubes and were completed to a final volume of 0.5 ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1N Folin Phenol reagent was added to each test tube mixed immediately within 8 seconds by vortex and incubated 30 minutes at room temperature. The intensity of color developed in each tube was measured at 660 nm.

GST activity determination

Enzyme activity assays were conducted at 25°C by using a spectrophotometer equipped with thermoregulated cell holder. The GST activities with 1-chloro-2,4 dinitrobenzene (CDNB) as substrate were determined spectrophotometrically at 340 nm according to the method of Habig et al. [23]. The reactions were started with the addition of cytosolic fractions obtained from wheats or barleys and followed for 3 min. The activity was calculated from the slopes of initial reaction rates using the ε values of CDNB of 9.6 $mM^{-1}cm^{-1}$ [24].

GSH determination

After pulverization of roots and shoots, they were homogenized in a ratio of 1:4 w/v, with 5% (w/v) TCA by using

Table 1: Morphologic, agricultural and quality specifications of Erginel and Bezostaya varieties*.

Specifications	*Hordeum vulgare* L. cv. Erginel	*Triticum aestivum* L. cv. Bezostaya
Morphologic specifications	Six-rowed ear, white grain, 100-110 cm height	Spelt white ear, red grain, 95-105 cm height
Agricultural specifications	Winter and medium lodging resistant, Min- Max yield: 300-800 kg/daa	Winter and lodging resistant, Min- Max yield: 150-600 kg/daa
Quality specifications	Barley fodder, % of protein:11-13	Breadgrain, % of protein:12-15

*Tabulated from the information at Transitional Zone Agricultural Research Institute website [18]

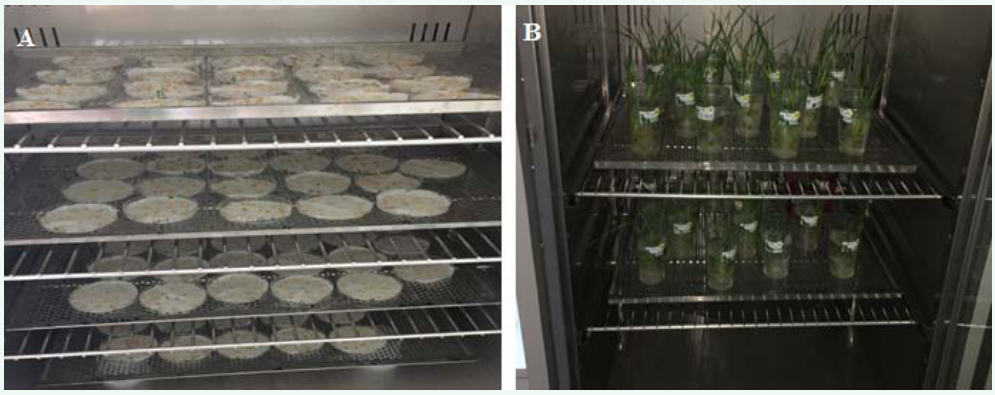

Figure 1 (A) Barley (1st and 2nd lines from the top) and wheat (3rd and 4thlines from the top) seeds at the beginning of the germination session, (B) Barley (1st line) and wheat (2nd line) seedlings during the growth session (For B, from left to right: Control (0 µM), Cd 150µM, Cd 300 µM, Pb 150 µM, Pb 300 µM, Comb 150 µM and Comb 300 µM).

UltraTurrax at 13500 rpm for 90 s at 4°C. The homogenate was centrifuged at 4°C, 12000 rpm for 15 min and the pH of the supernatant was adjusted to 4.0 - 5.0 with 1M NaOH. The content of GSH in crude extract was determined according to Ellman procedure [25]. The absorbance of the reaction mixture was read at 412 nm with the help of the standard curve calibrated by using reduced GSH.

For the results presented here, each application was replicated three times for three independent experiments.

RESULTS AND DISCUSSION

In the current study, the toxic impacts of different single and combined concentrations of Pb and Cd solutions were detected on *Hordeum vulgare* L. cv. Ergineland *Triticum aestivum* L. cv. Bezostaya varieties. With special attention to some biochemical parameters (such as GSH, protein contents and GST activities), obtained results were compared with the control samples both for shoots and roots. For more obvious comparison of HMs impact on tested parameters, values were expressed as a percentage of control values andthe absolute values for control samples from experiments are indicated in the legends of Figures 2-4.

Protein contents

Figure 2 shows the protein contents of all plant parts at the end of the exposure period with different single and combined Cd and Pb concentrations by comparing to control groups. The roots of both varieties have shown better protein levels according to the HM concentrations and the plant itself. In case of shoots, protein contents were found to be equal or higher to control samples for most of the concentrations tested (Figure 2).

HM treatments caused an increases of protein concentration in plant parts were sligthly dose dependent, except single Cd (150 and 300 µM) applications in Bezostaya roots. The highest protein concentrations were observed with 150 and 300µM of Pb + Cd treatment (128 and 135%, respectively) for Erginel root, for the same concentrations Bezostaya roots have 123 and 126% protein concentrations by comparing to the controls. Generally, single Cd applications were more effective than single Pb applications for applied concentrations on protein contents.

Protein degradation has been considered as an index of oxidative stress, because of enhanced level of protein oxidation and modification of cellular proteins, which is a common consequence of HM toxicity [26]. According to Patra et al. [27], proteins like metallothioneins (MTs) and phytochelatins (PCs) participate in detoxification against exess HMs. However, when they are overloaded, oxidative stress defense mechanisms recruited to overcome the existing metal toxicity.

Previous literature lists some decreasing or increasing contents of proteins in various plant organisms under different HM stresses [28-33]. In the current study, similar to findings of Chandra et al. [34], we observed increased protein contents in all plant parts for both varieties under HM treatments and also plants roots have been found to be higher protein contents than their corresponding shoots and control samples.

According to Shah and Dubey [35] HM stress has been shown to induce a variety of proteins resulting in an overall increase in protein content. It is also clear with our findings that, plants enhancing their protection capacity through encoding some

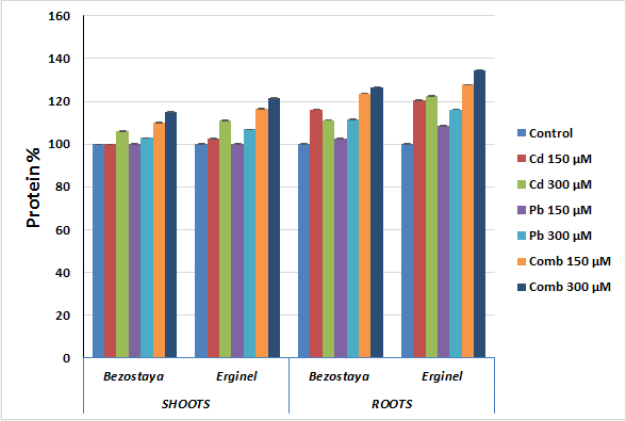

Figure 2 Effects of HM applications on protein contents in Bezostaya and Erginel varieties. The absolute values of protein contents for controls of Bezostaya and Erginel samples were measured as 4.45 and 5.62 mg/ml for shoots, 5.90 and 7.76 mg/ml for roots, respectively.

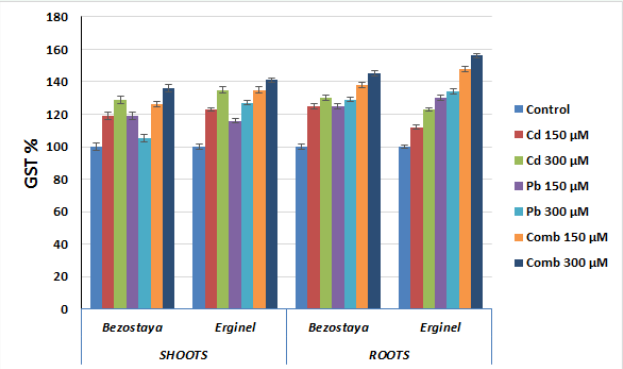

Figure 3 Effects of HM applications on GST activities in Bezostaya and Erginel varieties. The absolute values of GST activities for controls of Bezostaya and Erginel samples were measured as 262.90 and 280.00 nmol/min/mg for shoots, 337.60 and 417.50 nmol/min/mg for roots, respectively.

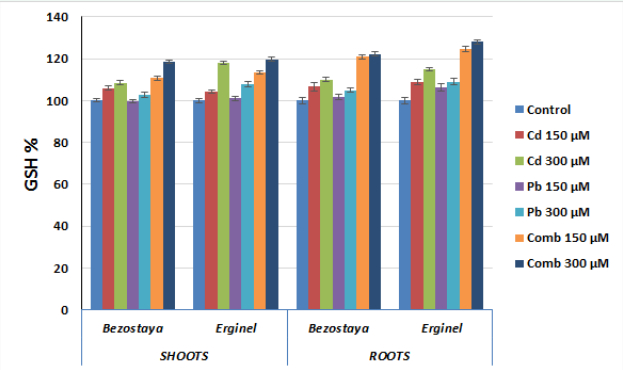

Figure 4 Effects of HM applications on GSH contents in Bezostaya and Erginel varieties. The absolute values of GSH contents for controls of Bezostaya and Erginel samples were calculated as 9.92 and 10.12 µg/ml for shoots, 16.05 and 19.00 µg/ml for roots, respectively.

proteins with defensive functions (like antioxidant enzymes involved in GSH and PC biosynthesis) to overcome HM stress.

GST activities

As shown in Figure 3, all HM treatments influenced the GST activities in plant varieties. Although, upon exposure to various HM concentrations, the extend of activity levels changes betweenplant parts and roots have shown better GST activity levels by comparing to their shoots.

Except single applications of Pb (150 and 300 µM) in Bezostaya shoots, all other treatments caused an increases of GST activities in adose dependent manner. While Erginel roots have shown the highest GST activities with 150 and 300µM combination treatments as 148 and 156% (respectively) of their controls, 138 and 145% activities were recorded in Bezostaya roots for the same concentrations. Comparison between single applications of Pb and Cd reveals that, single Cd treatments were more stimulatory on GST activitiesat least in the shoots of the plants.

As reviewed by Oztetik [36] in details, GSTs (EC 2.5.1.18) generally constitute a dimeric enzymes and catalyse the conjugation of the thiol group of the GSH to diverse electrophilic centres on lipophilic molecules with the formation of rather less active end products. However, they have other roles, like GSH dependent peroxidases counteracting oxidative stress [37], GSH dependent isomerases [38] or non-catalytically acting as flavonoid-binding proteins [39] and stress signalling proteins [40]. After their importance understood with regard to their role in detoxification (including HMs) and environmental safety, today the number of reports related to plant GSTs increased tremendously [41-43].

Beside other ways of attack, HMs mainly exert their effects on enzymes through the displacement of essential metal ions from specific binding sites [44,45]. Therefore, some reports announcing the decrese in activities of metallo enyzmes (such as CAT) when plants treated with Pb or Cd [17,46]. Conversely, the same authors reported an increase in the activities of superoxide dismutases (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and glutathione reductases (GR)) with metal treatments [17]. Although, by using different organisms and HMs (including Cd and Pb), increase in GST activities were also reported recently by several groups [47-50] and all coincide with our current results. On the other hand, recently relationships between Cd, GSH contents and GSTs in rice roots have been reported [51]. This finding is also confirming with our results, as in this study we observed high GSH contents (see GSH contents section) and GST activities in general with Cd treatments (but especially in Erginel shoots with 300µM Cd), suggesting that these two parameters of antioxidant defense system may be used as biomarkers of Cd induced stress.

GSH contents

In Figure 4, the results of individual treatments of the different single and combined concentrations of Cd and Pb on the GSH content of Bezostaya and Erginel roots and shoots are shown. In general, the roots of the plants slightly higher GSH levels, which is differing in range according to the concentrations of HM treatments and the plant itself. However, it seen that metals in the hydroponic environment influenced the GSH concentrations in the shoots of both species as well and GSH contents in HM treated samples were at least equal or higher than their respective controls (Figure 4).

All HM treatments caused an increases of GSH concentration in plant parts weredose dependent. While 125 and 128% of increases were observed in GSH contents of Erginel roots (with 150 and 300µM of Pb + Cd treatment, respectively), the contents of GSH were found to be 120 and 122% for Bezostaya roots for the same concentrations, compared to the controls. However, the increase in GSH concentration with the application of single 300µM Cd was marked for Erginel shoots (118%), as it shown a higher value than combined applications of Pb + Cd for 150µM and and also more effective than Pb itself for the same concentrations. Similarly, all single Cd applications were more effective than single Pb applications for tested concentrations.

A small molecule GSH (γ-L-glutamyl-L-cysteinyl-glycine) works for the sake of cell protection in many ways. It is not only take part in PC synthesis or ascorbate and tocopherol regeneration

reactions, but also involved in antioxidative defence in relation to redox capacity through glutathione peroxidase (GPOX) and glutathione reductase (GR) activities, acts as a source of amino acid for protein synthesis and have role as a co-substrate of GSTs in detoxification reactions [52]. Therefore, GSH protects plants from the deleterious effect of many stressors, including HMs.

According to Xiang et al. [53], elevation of GSH does not aways correlate with enhanced tolerance to HMs. However, there are several studies showing that the involvement of GSH in tolerance of plants to HM toxicity [54,55] available in the literature. For example, Freeman et al. [56], have reported that the increased GSH biosynthesis in *Thlaspi* showing tolerance to nickel (Ni). This and some other studies are in accordance with our results. In this study, we observed that the roots of the plants have shown moderately higher GSH levels by comparing to their corresponding shoots. This can be attributable to GSH's other responsibilities in shoots under HM concentrations to protect cells, like PC synthesis as it accompanied with a decrease in GSH pool.

CONCLUSION

The current study showed that biochemical mechanisms were affected differently with varying concentrations of Pb and Cd metals in plants examined. However, the high levels of GSH and protein contents and GST activities by comparing to control samples are the signs of general adaptability to stress conditions elicited by metals. Therefore, these parameters (activities of GST, levels of GSH and protein) can be used as a biomarkers of environmental quality assessments. Although, further studies can be designed to generate some transgenic plants with ability of regulating GSH or PC synthesis pathways or usage of different biomarkers to search for molecules with protection against HMs.

On the other hand, Erginel (barley) variety were found to be more tolerant to HM stress by comparing to Bezostaya (wheat) according to results. Therefore, tolerant variety can be used for remediation purposes in soil contaminated by HMs, such as phytoextraction, phytostabilization, etc.

ACKNOWLEDGEMENTS

Author thanks to journal for accepting this article free of cost for evaluation and possible publication.

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Cite this article

Öztetik E (2019) Evaluation of Some Defense Mechanisms in Crop Varieties Under Heavy Metal Stress. Int J Plant Biol Res 7(1): 1110.