Plant Knowledge Journal
ISSN: 2200-5390
EISSN: 2200-5404
3(1):31-38 (2014)

Southern Cross Publishing Group
Australia

Effects of copper, nickel and zinc on biochemical parameters and metal accumulation in gouan, *Aeluropus littoralis*

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Abstract

To study the effects of three heavy metals (Cu, Zn and Ni) on *Aeluropus littoralis*, a halophytic plant, a research was carried out in greenhouse. The study was laid out in a randomized complete block design with 5 replicates. Two different concentrations (50 µM and 100 µM) for each metal were used. Some cellular oxidative biomarkers such as activity of three main antioxidant enzymes (superoxide dismutase (SOD), guaiacol peroxidase (GPx) and catalase (CAT)), as well as accumulation of proline, phenolic compounds, photosynthetic pigments and soluble protein content were investigated. The results showed that the proline and phenolic compounds accumulated with increasing heavy metal concentrations, while total chlorophyll content decreased in all treatments. Heavy metals contents in shoots increased with the increasing of heavy metals concentration without any important morphological changes. In all treatments SOD activity increased, but GPx activity decreased while CAT activity and soluble protein only decreased in Ni and Cu treatments, respectively. The results showed that chlorophyll a was more sensitive to Zn and Ni, but chlorophyll b was more sensitive to Cu. In addition, it was revealed that each heavy metal has unique biotoxicities or biofunctions and affects the analyzed parameters plant in particular way. Consequently, *A. littoralis* heavy metal tolerance depends on the species and concentration of metal.

Keywords: enzyme activities; heavy metals; photosynthetic pigments; proline; stress.
Abbreviations: CAT_catalase; DHAR_dehydroascorbate reductase; GPx_Guaiacol peroxidase; GR_glutathione reductase; MDHAR_monodehydroascorbate reductase; ROS_reactive oxygen species; SOD_ superoxide dismutase.

Introduction

In the last decades, abiotic stresses have been one of the main topics that draw global attention, because they can limit plant growth, development and productivity (Liu et al., 2007). Problems arise when not only the heavy metal concentration due to natural processes (such as weathering and erosion) and anthropogenic activities are on increase but also, their non-biodegradability promotes their accumulation in the environment for long periods of time. On the other hand, high mobilization of these metals leads to the entrance of them into food chains and endanger the organism health. Previous studies indicated that heavy metals are toxic in high concentrations in both groups (essential and non-essential for plant) (Goyer, 1997). The most important reasons for metal toxicity according to their concentrations and properties are oxidative stress (Cheng et al., 2006), disruption of the function of pigments, photosynthesis enzymes and electron transport (Choudhury and Panda, 2004), alteration of membrane permeability due to lipid peroxidation (Choudhury and Panda, 2004), changes in protein activity and structure owing to high affinity of heavy metals for carboxyl, thioyl and histidyl groups present in catalytic and transport sites (Sharma and Dietz, 2006) and interfering with signal transduction pathways (Galanis et al., 2009). In general, plants, similarly to other organisms, use different multitude of physiological and biochemical ways at both intracellular and intercellular levels to protect against stress conditions. The main plant mechanisms of tolerance to stress consists of chelation by ligands, detoxification by deposition to cell wall, compartmentalization and sequestration of metals in organelles such as vacuoles, expression of proteins and response mechanisms related to stress (Cobbett, 2000; Pavlíková et al., 2007). Oxygen-containing free radicals (ROS) are more toxic and reactive in comparison with molecular oxygen because they contain one or more single electron. Reactive oxygen species are constant risk for organisms with aerobic metabolism, including the super oxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), which are inevitably generated via a number of metabolic pathways (Kanazawa *et al.,* 2000). These molecules by production another radical, leading to oxidative damage to cell. In plants, under normal conditions, there is a balance between various pathways producing ROS

in aerobic metabolism (such as electron transfer chains in photosynthesis and respiration and so on) and the scavenging mechanisms but under heavy metals stress, the production of ROS surpasses its scavenging. In addition, plants are equipped with antioxidative defense systems to eliminate or reduce the oxidative damage (Larson, 1988). The plant antioxidant network consist of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APx), Guaiacol peroxidase (GPx), catalase (CAT), glutathione reductase (GR), MDHAR and DHAR and non-enzymatic antioxidative molecules such as ascorbate, glutathione, α-tocopherol, flavonoids, carotenoids, cysteine, proline and phenolic compounds (Singh and Sinha, 2005; Michalak, 2006). *Aeluropus littoralis* is a C4 perennial graminaceous halophyte and salt excreter that can thrive in saline habitats and survive up to 1,100 mM NaCl (Bodla et al., 1995). The small genome of this plant (2n=2X=14 ≈ 342 Mb) and tolerance to salt, heat and drought predisposes it for genetic research (Zouari et al., 2007). But, unfortunately up to now, few studies have done on how heavy metals affect its antioxidative defense system. The effects of four heavy metals, Cd, Co, Pb and Ag on *A. littoralis* were previously studied and revealed that this plant can tolerate various concentrations of these heavy metals (Rastgoo and Alemzadeh, 2011). The aim of the present study was to elucidate the relationship between metal toxicity and plant tolerance to three different metal treatments (Cu, Zn and Ni) in two concentrations (50 and 100 µM) with changes in activity or content of enzymatic and non-enzymatic oxidative stress biomarkers (SOD, CAT, POD (peroxidase) activities, POD isozymes, soluble proteins, phenolic compounds, proline, and photosynthetic pigments) in *A. littoralis* plants in a pot experiment under controlled conditions. The finding of relationship between antioxidative defense capacity and heavy metals and Zn/Ni/Cu accumulation in *A. littoralis* and provides an important plant material for understanding the mechanisms of Zn/Ni/Cu accumulation and for phytoremediation of the heavy metal contaminated areas.

Results and Discussion

Heavy metals accumulation in A. littoralis shoots

Data indicated that heavy metals content in shoots increased with increase of its concentrations (Fig 1). Under Cu and Ni treatments, the concentrations of these metals in shoots did not differ significantly between the two levels used (50 µM and 100 µM), but in the case of Zn this difference was significant (Fig 1). In addition, the plants showed no visible symptoms of metal-induced toxicity when were grown at both heavy metals levels (data not shown). In case of Cu and Ni, the absorption rate of heavy metal at low concentration was higher than at high concentration, but in case of Zn, the rate of absorption was the same regardless of concentration. The results from this study show that *A. littoralis* uses different mechanisms of uptake, transport and accumulation for Cu, Ni, and Zn. It is possible there are different carriers for various heavy metals uptake. One heavy metal-detoxification strategy is to reduce heavy metal uptake (salt et al., 2000) and it may indicate that in this plant, the high concentrations of Cu and Ni are more toxic than Zn.

Protein content changed by heavy metals treatment

In oxidative stress, total soluble proteins is usually studied as an index of metabolic changes, because, under stress conditions, ROS cause serious damage by interaction with cellular components such as proteins, nucleic acids and lipids (Sabatini et al., 2009). Increase in total soluble proteins under Zn and Ni stresses can be considered as a plant tolerance mechanism, or in other words, the synthesis of stress proteins that participate in cellular detoxification was induced under stress conditions (Sabatini et al., 2009). Protein accumulation in leaves was also seen in other plants treated with heavy metals (Heiss et al., 2003). Induction of total soluble proteins under heavy metals stress indicates that this organism is adapted to produce specific proteins during metal stress conditions. Plant cells have developed defense mechanisms against heavy metals, which are highly toxic compounds and these proteins can confer heavy metal tolerance on these organisms. It was suggested that these induced proteins might be important to repair proteins damaged by heavy metals (Hall, 2002).

In contrast, under the Cu treatments soluble protein content decreased steadily (Fig 2). It has been shown that some heavy metals accumulation is accompanied by a decline in transcription rate (Haag-Kerwer et al., 1999).Decline in protein content under metal stress can be related to the inhibition of protein synthesis or increase in protein degradation (Wang et al., 2009). The most interesting result is the finding that in this plant, protein content changed significantly under the high concentrations of heavy metals. Although the low concentrations of these metals affected the protein content, but it was not significant (Fig 2). It may indicate that this plant has a moderate tolerance to these heavy metals.

Effect of heavy metals on enzyme activities

Considerable changes in enzymes activities were observed that depended on the heavy metal species and concentration (Table 1). SOD and GPx activities increased and decreased in all treatments, respectively. The activity of CAT was changed in a metal-dependent manner. Cu and Zn in both concentrations resulted in a noticeable increase in CAT activity, but its activity decreased under Ni stress. The induction of SOD activity may be explained in three ways: (i) the sensitivity of genes encode this enzyme to environmental stresses (ii) up-regulation of this enzyme due to increase in ROS production under stress conditions (Ramalho et al., 1998) or (iii) increase in enzyme synthesis under all heavy metals stresses (Chongpraditnum et al., 1992). Also, Pandey and Sharma (2002) demonstrated that heavy metals inhibit Fe uptake, so, the peroxidase activity may be decreased because peroxidase is a Fe porphyrin enzymes. Other reasons for decrease in POD activity can be the substitution of heavy metal instead of essential ions in the enzyme structure or because of increase in ROS accumulation and their effects on plant signal transduction pathways (Schützendübel and Polle, 2002). Induction of enzyme detoxifying the H_2O_2 (CAT) is a circumstantial evidence of plant general strategy required for ROS scavenging in cells for the maintenance of their redox state under stress conditions. Since some earlier studies have stated that peroxidase and catalase enzymes have different affinity for peroxide hydrogen, plants use either from the two pathways (SOD/CAT and ascorbate-glutathione) for H_2O_2 detoxification (Mittler, 2002). It seems that in *A. littoralis* the primary pathway is more active towards decomposing the H_2O_2 under Zn and Cu treatment. But, under the Ni treatment we observed a different trend. In other words, while under 100 µM Ni, we observed an approximately 3.5 fold increase in SOD activity, the decrease in the activities of the two remaining antioxidative enzymes may indicate that CAT plays only a mainor role in detoxifying H_2O_2 under this

Table 1. Effect of different heavy metals on enzyme activity in A. littoralis leaves. Means in the same column followed by the same letters are not significantly different (α=0.05), according to Duncan's test.

Treatment	superoxide dismutase (U/mg protein)	Guaiacol peroxidase (U/mg protein)	catalase (U/mg protein)
Zn (µM)			
0	0.611[a]	0.321[a]	0.438[b]
50	0.858[c]	0.303[ab]	0.483[c]
100	1.238[f]	0.266[d]	0.62[d]
Cu (µM)			
0	0.611[a]	0.321[a]	0.438[b]
50	0.746[b]	0.298[abc]	0.512[c]
100	1.108[e]	0.288[bcd]	0.678[e]
Ni (µM)			
0	0.611[a]	0.321[a]	0.438[b]
50	0.896[d]	0.325[a]	0.381[a]
100	2.178[g]	0.274[cd]	0.345[a]

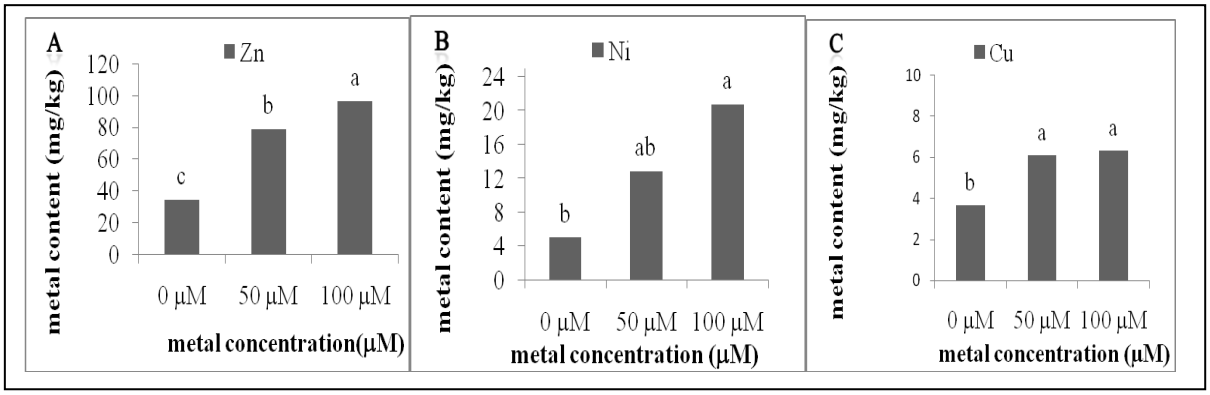

Fig 1. Heavy metals content in *A. littoralis* leaves. **A)** The concentration of Zn in leaves. **B)** The concentration of Ni in leaves. **C)** The concentration of Cu in leaves. Similar letters denote values not significantly different at α=0.05, according to Duncan's test.

treatment. Up to now, many instances of decline in CAT activity under various heavy metals have been reported, and some of them due to CAT inhibition by $O_2^{\bullet}$ (Salin, 1988), the effect of Fe deficiency on enzyme synthesis (Pandey and Sharma, 2002), or the induction of enzyme decomposition by some proteases, activated under stress condition (Distefano et al., 1999). In summary, in present study, such variation in antioxidative enzymes activities under different metals stresses may be due to direct targets of heavy metals.

Accumulation of proline in the leaves of plants exposed to heavy metals

As the results show, proline content increased significantly with increasing concentration in all treatments (Fig 3). Proline accumulation is one of the common reactions of plants to abiotic stresses, reported from algae to angiosperms (Sharma and Dietz, 2006). Among different environmental stresses, heavy metals are the strongest inducer for proline accumulation (Sharma and Dietz, 2006). It has been reported that heavy metals induce proline accumulation in the sequence of Cu >Cd >Zn in *Silene vulgaris* (Schat et al., 1997), but our data showed that the accumulation of proline in *A. littoralis* is in the sequence of Zn > Cu > Ni (Fig 3). It means that, the effect of metals on proline accumulation depend on the plant species. A accumulation of proline by plants under stress condition occurs because of increase in its *de novo* synthesis, or decline of its degradation under stress condition (Kishor et al., 2005), and decreased the mitochondrial electron transport pathway (Saradhi et al., 1995). The unique properties of proline that cause it to act as antioxidant are its ability as osmolyte in osmoprotective processes (Hartzendorf and Rolletschek, 2001), regulation of cytosolic acidity and membrane and protein stabilization (Kishor et al., 2005), the preservation of enzyme structure and activity (Kishor et al., 2005), scavenging singlet oxygen and hydroxyl radicals (Matysik et al., 2002), inercellular storing of nitrogen and carbon during plant's recovery (Kishor et al., 2005), metal chelation (Sharma and Dietz, 2006), electron sink (Sharma and Dietz, 2006), and restriction of metal uptake and transport by transpiration due to stomatal closure (Sharma and Dietz, 2006). Proline is suggested to quench ROS and reactive nitrogen species and to relative the oxidative burden from the glutathione system (Siripornadulsil et al., 2002). This may facilitate phytochelatin synthesis and enhance metal tolerance.

Change of pigments content in response to heavy metals treatment

Previous studies have proved that chlorophyll content may reflect plant's sensitivity to stress conditions. In general, the chlorophyll a and b content decreased with the increasing metals concentrations for all metal treatments, compared with their respective controls (Table 2). The decrease observed in Cu treatment (100 µM) indicated that the chlorophyll is more sensitive to Cu than Zn and Ni. It has been previously reported that chlorophyll destruction induced by copper in plants (MacFarlance and Burchett, 2001, Singh and Agrawal, 2007). Furthermore, the data analysis showed that the sum of chlorophyll a and b content (Chl a+b) was influenced by the higher metal concentrations and there was a decreasing trend in total chlorophyll content with increasing metal concentrations in all treatments (Table 2). The chlorophyll

Table 2. Photosynthetic pigments (chlorophyll a, b and carotenoids) content in different treatments. Values represent mean ± Standard Error (n = 5). Means in the same column followed by the same letters denote values not significantly different (α=0.05), according to Duncan's test.

Treatment	Chlorophyll a (mg/g Fw)	Chlorophyll b (mg/g Fw)	Total chlorophyll (a+b)	Chlorophyll a/b ratio	Carotenoids (µg/g FW)	Chl (a+b)/ carotenoids
Zn (µM)						
0	0.995^{a}	0.639^{a}	1.634^{a}	1.557^{b}	93.8^{abc}	0.01759^{b}
50	0.803^{b}	0.541^{b}	1.344^{c}	1.485^{c}	83.6^{abc}	0.01629^{c}
100	0.741^{de}	0.478^{cd}	1.219^{e}	1.551^{b}	62.8^{c}	0.01984^{a}
Cu (µM)						
0	0.995^{a}	0.639^{a}	1.634^{a}	1.557^{b}	93.8^{abc}	0.01759^{b}
50	0.864^{c}	0.532^{b}	1.396^{b}	1.624^{a}	111.5^{a}	0.01254^{d}
100	0.717^{e}	0.461^{d}	1.178^{f}	1.555^{b}	70.9^{bc}	0.01666^{bc}
Ni (µM)						
0	0.995^{a}	0.639^{a}	1.634^{a}	1.557^{b}	93.8^{abc}	0.01759^{b}
50	0.802^{b}	0.579^{b}	1.381^{b}	1.385^{d}	107.1^{a}	0.013021^{d}
100	0.748^{d}	0.492^{c}	1.24^{d}	1.521^{bc}	92.6^{ab}	0.0135^{d}

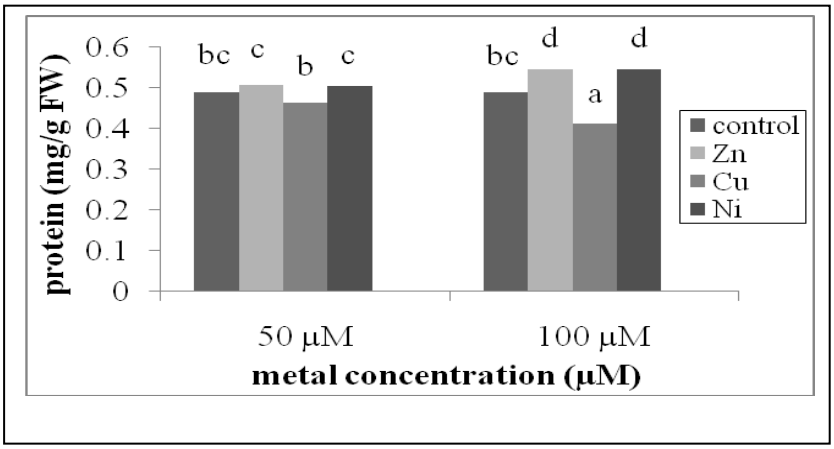

Fig 2. Effect of different heavy metals on protein content in *A. littoralis* leaves. Similar letters denote values not significantly different at α=0.05, according to Duncan's test.

ratio (a/b) did not change significantly in all high concentration treatments (100 µM), but under low concentrations (50 µM) it decreased in Zn and Ni, and increased in Cu treatment in comparison with control. It is means that chlorophyll a is more sensitive to Zn and Ni, but chlorophyll b is more sensitive to Cu. Also, the results of this research indicated that carotenoids in this species are most sensitive to Zn and then followed by Cu and Ni. The carotenoids increased under low concentrations of Cu and Ni, but under high concentration of Zn in decreased (table 2). In this study, progressively metal-dependent reduction of chlorophyll concentration with increasing concentrations of metals in growth medium could be well explained by (i) replacing Mg^{+2} in chlorophyll by the other ions such as Ni^{+2}, Cu^{+2} or Zn^{+2}, and reduction in electron transfer rates (Küpper et al., 1996; 1998); (ii) degrading the chlorophyll by induction of chloroplast membranes peroxidation and cooxidation of chlorophyll by ROS (Marschner and Cakmak 1986); (iii) reducing the efficiency of enzymes acting in its biosynthesis (Ouzounidou, 1995) by metal binding to the -SH groups (Singh, 1995) or decreasing the availability and absorbing another essential mineral nutrients, such as Fe, Zn, and Mn (Hou et al., 2007). Carotenoids are one of the most important part of plant energy system, that-as an antioxidant-have a defensive role against the photooxidative damage, by quenching singlet state of chlorophyll and ROS and inhibiting the lipid peroxidation under stress conditions (Hou et al., 2007).

The content of phenolic compounds under heavy metals treatment

Treatments of *A. littoralis* planets with heavy metals resulted in a metal-dependent accumulation of phenolic compounds in the leaves (Fig 4). Also, comparison between heavy metals effects revealed that phenolic compounds content increased in the sequence of treatments: Cu>Zn>Ni. Accumulation of the phenolic compounds under heavy metals stress has been reported previously (Üstün et al., 2000). Díaz et al. (2001) reported that under heavy metals stress, these components accumulate because of their functions as intermediates in lignin biosynthesis, Preserving the plant cells by building physical a barrier. Induction of phenolic accumulation observed in this study, proves the antioxidative properities of these components in plant response to oxidative stress. The metal chelating properties of these components under salt stress may infer their functions: These compounds are able to bind heavy metals, such as Fe and C, to their carboxyl and hydroxyl groups (Jung et al., 2003). In addition, they expand their role in stress defense as radical scavengers (Kováčik et al., 2009), increase activity of enzymes involved in phenolics synthesis (Michalak, 2006), decrease the ROS synthesis by the prevention of reactions superoxide production (Fenton

Table 3. Correlation coefficients between the nine indexes measured. * and ** indicated to Significant at *P*= 0.05 and *P*= 0.01, respectively.

	Protein	SOD	GPx	CAT	Proline	Chlorophyll a	Chlorophyll b	Carotenoids	phenolics
Protein	1	0.46	-0.27	-0.52	0.03	-0.05	0.07	-0.04	-0.17
SOD		1	-0.69	-0.24	0.54	-0.61	-0.58	-0.22	0.37
GPx			1	-0.41	-0.84*	0.67	0.86**	0.63	-0.79*
CAT				1	0.58	-0.36	-0.55	-0.69	0.75*
Proline					1	-0.93**	-0.96**	-0.70	0.96**
Chlorophyll a						1	0.90**	0.51	-0.84*
Chlorophyll b							1	0.57	-0.94**
Carotenoid								1	-0.66
Phenol									1

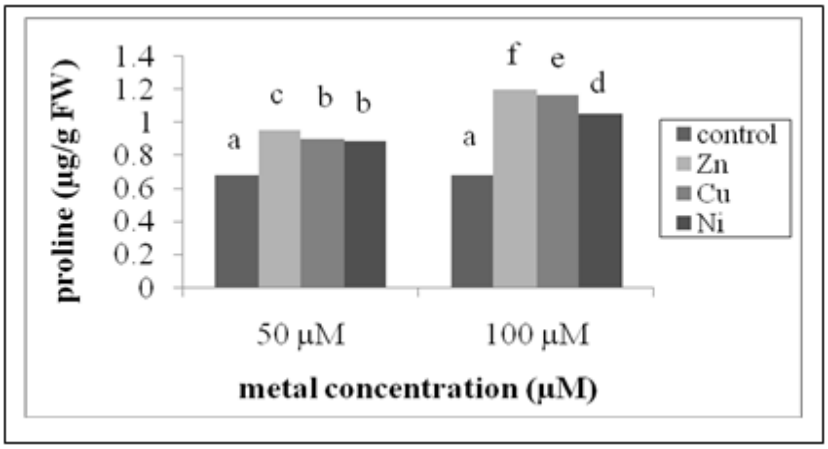

Fig 3. Effect of different heavy metals on proline content in *A. littoralis* leaves. Similar letters denote values not significantly different at α=0.05, according to Duncan's test.

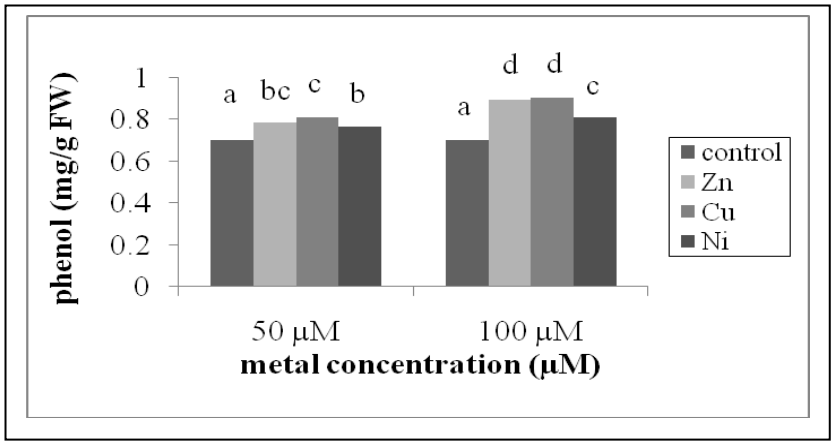

Fig 4. Effect of different heavy metals on phenolics content in *A. littoralis* leaves. Similar letters denote values not significantly different at p<0.05, according to Duncan's test.

reactions), inhibit the lipid peroxidation, membrane stabilization, and prevent the ROS transportation (Michalak, 2006). The results indicate a significant and positive correlation between proline and phenolic compounds (r = 0.96) (Table 3). We suggest that the joint amount of proline and phenolic compounds can be a suitable biomarker for zinc and copper stress. Also, significant and negative correlation were found between proline and Chl a, Chl b, and GPOX. On the other hand significant and positive correlation coefficient was found between phenol and CAT (r = 0.75) (Table 3).

Materials and Methods

Cultivation of plants and experimental design

Aeluropus littoralis seeds were obtained from Pakan Seed Research Centre, Isfahan Province, Iran. The seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 20 min and then rinsed three times with distilled water. The seeds were sown in pots filled with mixture of sand/grit (1/1) under greenhouse conditions (with 25:16º C day:night

temperature, 16 h light/ 8 h dark photoperiod) and irrigated with 100 ml of MS nutrient solution every three days. After one month, uniform plants were selected and separately subjected to modified MS solutions containing of $CuSO_4$, $ZnSO_4$, and $NiSO_4$, each at two different concentrations (50 µM and 100 µM) for seven consecutive days. The experiment was carried out in a randomized complete block design with 5 replicates. After one month exposure to heavy metals, the plants were removed and thoroughly rinsed with deionized water and used for proline, chlorophyll, and carotenoids measurements. For other biochemical analyses, leaves were detached and immediately frozen in liquid nitrogen and kept at -80º C

Measurement of heavy metal content

Heavy metal content was measured as previously described by Chapman and Pratt (1961). Shoots of *A. littoralis* was washed by tap water and dried in an oven at 65º C for 72 h and then weighted. After drying, one g of each sample was placed into porcelain crucible and heated in a furnace. The furnace temperature was slowly increased from room temperature to 550º C in 1 h. Samples were ashed for 3 h. The residue was dissolved in 5 ml HCl (2 N) and the total volume was adjusted to 50 ml by adding distilled water. The metal content was then analyzed by atomic absorption spectroscopy.

Protein measurement

Proteins content were quantified by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. Extraction buffer (pH 7.0) was obtained by dissolving of 6.07 g Tris and 0.5 g polyvinylpolypyrrolidone (PVP) in 500 ml water. Detached leaves were ground in liquid nitrogen in a cold pestle and mortar and 0.5 g powder was used in 1 ml extraction buffer under ice-cold conditions. The samples were centrifuged at 10000 g for 20 min at 4º C. After centrifuge, supernatant was used to measure total protein content. Then, 100 mg coomassie brillant blue G 250 was dissolved in 50 ml methanol (96%) and 100 ml of *ortho*-phosphoric acid (85 %) and then is filled up to 200 ml with deionised water. For protein assay, 50 µl sample solution, 50 µl extraction buffer and 5 ml assay reagent were mixed. The mixture was vortexed for 2 min. After 5 min, absorbance was measured spectrophotometrically at 595 nm.

Determination of superoxide dismutase activity

SOD activity was determined by inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by superoxide radicals (Dhindsa et al., 1981). The assay mixture contained 50 mM phosphate buffer (pH 7), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA and 20 µL of enzyme extract then were brought to a final volume of 3 ml and finally 4 µM riboflavin was added. The test tubes were shaken immediately. The reaction was started by keeping the tubes under 90 mmol m^{-2} s^{-1} for 10 min. The absorbance was recorded at 560 nm. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium to farmazan under the assay condition. The specific enzyme activity was expressed as units per mg of protein.

Determination of guaiacol peroxidase activity

GPx activity was measured by a spectrophotometric method described by Polle et al. (1994). Three ml mixture contained of 100 mM phosphate buffer (pH 7), 10 mM H_2O_2, 20 mM guaiacol and 50 µl protein extract. GPx activity was determined by the change in absorbance at 470 nm for 5 min (extinction coefficient of 26.6 mM^{-1} cm^{-1} per 1 min was used.). Increase in the absorbance is due to the polymerization of guaiacol to tetraguaiacol.

Determination of catalase activity

CAT activity was determined spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm (Aebi, 1984). One ml reaction volume composed of potassium phosphate buffer 50 mM (pH 7), 30 % (m/v) H_2O_2 and protein extract. The enzyme activity was expressed as units (mmol of H_2O_2 metabolized per minute) per milligram of total protein (extinction coefficient= 43.6 mM^{-1} cm^{-1}).

Determination of proline content

Around 0.5 g of leaf tissue was ground with 10 ml of 3% (w/v) sulphosalicylic acid. The homogenate was centrifuged at 3000 g for 5 min. Ninhydrin reagent was produced by dissolve 1.25 g ninhydrin in 20 ml 6 M phosphoric acid and 30 ml glacial acetic acid. Then, 2 ml ninhydrin reagent added to an equal volume of supernatant and glacial acetic acid. The tubes incubated for 1 hour in 100º C and reaction terminated by cooling them on ice. The reaction vortexed after adding 4 ml of toluene. Toluene absorbance (upper phase) determined spectrophotometrically at 520 nm (toluene was used as a blank.). Lastly, proline concentration was calculated according to standard curve.

Determination of pigments

The extraction of chlorophyll was carried out by the method of Lichtenthaler and Wellburn (1983). Around 0.2 g of leaf tissue was ground with 4 ml of acetone (80%). Homogenate was centrifuged for 5 min at 3000 g. The absorbance of the supernatant was measured photometrically at 663, 646 and 470 nm for chlorophyll a, b and carotenoids, respectively. Acetone 80% was used as blank. The concentrations of total chlorophyll (a+b) and carotenoid calculated according to Lichtenthaler and Wellburn (1983).

Determination of phenolic compounds

Phenolics content was measured as described by Campbell and Ellis (1992). Around 0.5 g of leaf tissue was ground in 1 ml methanol 50% and kept in 80° C for 90 min and centrifuged for 5 min at 3000 g. Then, deionized water added to 50 µl of supernatant to reach the volume to 1 ml. After that, 1.5 ml sodium carbonate (20%) and 0.5 ml of Folin-Ciocalteu was added to the reaction. The spectrophotometric absorbance was measured after 20 min shaking, at 725 nm. Standard curve was constructed using serial dilutions of caffeic acid.

All data were analyzed by analysis of variance (ANOVA) procedures using MSTATC software package. Treatment means were separated by Duncan's multiple range tests (α=0.05).

Conclusions

The complied data demonstrate *A. littoralis* has tolerance to Ni, Zn and Cu stress and accumulate these heavy metals in shoot, although, the high concentrations of Cu and Ni are more toxic than Zn. Since the various heavy metals have specific chemical properties and induce distinct responses of adaptation and damage development, it is not surprising accumulating metal ions display a variety of function such as antioxidant defense and proline accumulation. We also found that there are differences between these metals in terms of their accumulation by *A. littoralis*. In addition, the results provide evidence that enzymatic and non-enzymatic biomarkers of oxidative stress can be sensitive indicators of heavy metal stress.

Acknowledgments

The authors wish to acknowledge the financial support of Iran National Science Foundation (INSF).

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