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Uptake of Heavy Metals, and Antioxidative Enzymes in *Brassica juncea* L. Seedlings as Affected by Zn in Binary Combinations with Other Heavy Metals

Rupinder Kaur • Renu Bhardwaj • Ashwani K. Thukral*

Department of Botanical and Environmental Sciences, Guru Nanak Dev University Amritsar, 143005, India

Corresponding author: * **akthukral@rediffmail.com**

ABSTRACT

The present study attempts to understand the uptake of heavy metals and stress tolerance in *Brassica juncea* L. seedlings under the effect of Zn in binary combinations with Cr, Ni, Co and Cu through the production of antioxidative enzymes - superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). It was observed that the order of uptake of heavy metals by the seedling in single metal solutions was Zn > Cu > Co > Cr > Ni. Zn in binary combination with other heavy metals, mutually decreased the uptake of each other, the maximum decrease being 66.1% in the uptake of Cr in (Cr100+Zn100) binary solution. All the metals, whether applied singly or in combinations, significantly increased the activities of antioxidative enzymes, except for CAT. Zn was the most effective metal in increasing the activities of antioxidative enzymes. At 100 mg l^{-1}, it increased the activities of GR, GPX and APX by 101%, 64%, and 42% respectively, whereas maximum SOD activity (16 mM UA mg^{-1} protein) was induced by 100 mg l^{-1} Cr. Of all the binary combinations, Zn+Co and Zn+Ni were most effective in increasing the activities of GPX and GR, respectively, whereas Zn+Cu and Zn+Cr increased the activities of APX and SOD, respectively. Binary interaction models revealed that Cr, Ni, Co and Cu act antagonistic to Zn to increase the activity of GR, whereas for GPX, APX and SOD, these metals in binary combinations with Zn, were mutually antagonistic, thereby causing a negative interactive effect. Cr, Co and Cu were mutually antagonistic to Zn for catalase activity, whereas interaction between Ni and Zn was synergistic for this enzyme.

Keywords: chromium, cobalt, copper, metal interactions, nickel

Abbreviations: ANOVA, analysis of variance; **APX**, ascorbate peroxidase; **CAT**, catalase; **EDTA**, ethylenediaminetetraacetic acid; **GDHP**, guaiacol dehydrogenation product; **GPX**, guaiacol peroxidase; **GR**, glutathione reductase; **GSSG**, glutathione disulphide; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NBT**, nitroblue tetrazolium chloride; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase

INTRODUCTION

The advent of technology in the late 20th century ushered in an era of industrial revolution with the accompanying undesirable generation of waste products on a vast scale. Industrial wastes impregnated with heavy metals, besides being detrimental to the health of humans and animals, extensively damage living and natural resources in the environment. Heavy metals are nondegradable pollutants that undergo bioaccumulation along higher trophic levels of the food chain, thereby accentuating their toxic effects. Phytoremediation is a technology for the abatement of pollution that embraces unique property of plants for the uptake and accumulation of heavy metals to decontaminate polluted soils and waters. The hypertolerance of plants to metals is a key characteristic required for hyperaccumulation of metals, and the tolerance capacity of plants depends on an interrelated network of physiological and molecular mechanisms (Khan *et al*. 2009). Plants exposed to heavy metals evoke a vast array of defense mechanisms, such as immobilization, exclusion, chelation and compartmentalization of heavy metal ions, expression of stress proteins and activation of ethylene response to stress (Cobbett 2000). Much of the injury to the plants caused by stress exposure is associated with oxidative damage at the cellular level. The generation of reactive oxygen species (ROS) is implicated as a stress response (Dietz *et al*. 1999; Mollar *et al.* 2007). Heavy metal stress in plants results in the production of ROS such as O_2, H_2O_2 and $^{\cdot}OH$. ROS are also generated in plant cells during normal metabolic processes, where their production is well regulated, but metal toxicity enhances the formation of ROS up to 30-fold (Mittler 2004), leading to disturbances in the metabolic pathway and macromolecule damage (Hegedus *et al.* 2001). However, plants are surfeited with various mechanisms to combat excessive ROS formation. The deleterious effects resulting from cellular oxidative stress may be alleviated by the antioxidative defence machinery of the plant (Halliwell 1987) comprising of enzymatic and non-enzymatic free radical scavengers. Enzymatic scavengers include SOD, APX, GPX, GR, dehydroascorbate reductase and monodehydroascorbate reductase. The non-enzymatic antioxidants include ascorbic acid, α-tocopherol, glutathione, carotenoids and polyamines, etc. (Wang *et al*. 2004; Narang *at el.* 2008a). The antioxidative enzyme defence layer is largely provided by specific enzymes including SODs as a family of metalloenzymes, catalyzing the dismutation of the superoxide anion to H_2O_2 and molecular oxygen (Alsher *et al*. 2002). A fine regulation of H_2O_2 is achieved by the enzymes and metabolites of the ascorbate glutathione cycle that are crucial for determining the steady state levels of O_2 and H_2O_2 (Narang *et al.* 2008b).

Hyperaccumulator plants used for phytoremedation have acclimated themselves well against homeostatic disturbances and cellular damages by evoking antioxidative enzyme induction as a general adaptive response to toxic effects of heavy metals (Van and Clijsters 1998). The extent of such tolerance and the degree of adaptation is highly variable in which the efficiency and capacity of the detoxi-

Received: 26 February, 2009. Accepted: 9 December, 2009.

Original Research Paper

fication mechanism play an important role. Several studies have been carried out on oxidative stress and defense mechanism in plants under stress (Hall 2002; Lin *et al.* 2004; Liu *et al.* 2007). However, most of the studies on the impact of heavy metals on the antioxidative defence mechanism of higher plants have focused on the effects of individual metals, generally neglecting multiple metal stresses, thus leaving a dearth of information on the possible interactive effects arising due to natural association among various metals co-existing in contaminated soils. It is notable that very often industrial wastes are impregnated with a variety of heavy metals in potentially toxic concentrations (Wallace 1989; Siedlecka 1999; Prasad 2002) that may have synergistic, additive or antagonistic effects in plants. Zinc, a group II-B transition element is considered to be one of the most essential micronutrients for plants. Zn plays an important role in several cellular functions such as photosynthetic carbon metabolism, gene expression and chromatin structure (Marschner 1995; Cakmak and Braun 2001). It is an important constituent of various vital enzymes playing catalytic, co-catalytic or structural roles, as well as being a structural stabilizer for proteins, membrane and DNA binding proteins (Vallee and Auld 1990). However, beyond a certain threshold value, it is toxic at higher concentrations. Moreover, Zn in combination with other heavy metals may exhibit different interactive effects on the physiology and metabolism of plants. Therefore, the effect of metal mixtures on a model plant system needs to be investigated critically, as the phytotoxic and interactive aspects of metal mixtures are complex processes (Taylor *et al.* 1990). Exploration of biochemical and molecular mechanisms by which plants tolerate multiple metal stresses leads to a thorough understanding of the plasticity of metabolic pathways and their limits of functioning, which is essential from a practical standpoint of optimizing phytoremediation by improving the cellular defence mechanism to combat heavy metal stress of hyperaccumulator plants (Aravind and Prasad 2005). In view of this, the present investigation explores the interactive effects of Zn and other heavy metals on the antioxidative metabolism of *B. juncea*, in order to understand the biochemical strategies adopted by this plant against oxidative stress and that would further improve its phytoremediation potential.

MATERIALS AND METHODS

Plant growth conditions

Certified seeds of *B. juncea* L. cv 'PBR-91' used in the present investigation were procured from Punjab Agriculture University, Ludhiana, India. This cultivar was chosen from among the various commercial varieties of *B. juncea*, *viz*., PBR-97, PBR-91, Laha-101 and Pusa Agrani, since it shows overall stability for most of the important yield contributing characters under the prevailing conditions in the area of study healthy seeds, selected by flotation method, were surface sterilized with 0.01% $HgCl_2$ and were allowed to germinate in Petri dishes lined with Whatman #1 filter paper containing solutions of heavy metals (Cr, Ni, Co and Cu) in different concentrations, singly or in binary combinations with Zn. For heavy metal uptake studies, the concentrations of heavy metals used were: 0, 25, 50 and 100 mg l^{-1}, both for single metal treatments and binary combinations. At concentrations higher than this, the seedlings did not survive in solutions containing Cr, Ni, Co and Cu. Further, it was observed (Tables 2-6) that maximum uptake of metals occurs at 100 mg l^{-1} concentrations for both Zn and other heavy metals in single metal solutions, and that Zn in binary combinations at this concentration decreases the uptake of other heavy metals and ameliorates the growth of seedlings. Therefore, for study on antioxidative enzymes following treatments were defined:

(i) Single metal treatments – 0 and 100 mg l^{-1} of each metal (Cr, Ni, Co, Cu and Zn).

(ii) Binary treatments – Zn treatments in binary combinations with other metals, all at 100 mg l^{-1}.

Surface-sterilized seeds were germinated on Whatman No. 1 filter paper, lined inside 9 cm diameter sterilized Petri plates containing aqueous solutions of heavy metals either single or in binary mixtures. Solutions were prepared using AR grade, K_2CrO_4, $NiSO_4 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$. All chemicals were procured from Sigma-Aldrich. Seedlings grown in double distilled water served as the controls. The Petri dishes were kept in a growth chamber maintained at 25 ± 0.5°C, 16:8 h dark-light photoperiod (1700 Lux), for a 7-day growth period.

Metal uptake analysis

7-day old seedlings were harvested, thoroughly washed with distilled water and dried at 80°C for 24 h. The dried samples were then digested using digestion mixture (H_2SO_4: HNO_3: $HClO_4$ in a 1: 5: 1 ratio) (Allen *et al*. 1976). The concentration of metals in the digests was determined using an atomic absorption spectrophotometer (Model AA 6200, Shimadzu, Japan).

Antioxidative enzymes assays

The seedlings (1 g fw) were homogenized in 3 ml of 100 mM potassium phosphate buffer at pH 7.0 containing 1% insoluble polyvinylpyrrolidone (Polyclar-AT, Sigma-Aldrich) in an ice chilled pestle and mortar. The homogenates were centrifuged at 15,000×*g* for 20 min at 5°C and the supernatants were collected and used for assessing the activities of antioxidative enzymes (catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD) and the protein content (Lowry 1951). Chemicals used in enzyme assays were procured from Sigma-Aldrich, Qualigens, Loba chemi, Sd-fine-chem and Central drug house, India.

Guaiacol peroxidase (EC 1.11.1.7)

The activity of peroxidase was estimated according to the method given by Putter (1974). The reaction mixture comprising 3 ml phosphate buffer, 50 µl guaiacol solution, 100 µl enzyme sample and 30 µl H_2O_2 solution was taken in the test cuvette. The rate of formation of GDHP was followed spectrophotometrically at 436 nm. Enzyme activity was calculated using extinction coefficient of 25 mM^{-1} cm^{-1}.

Catalase (EC 1.11.1.6)

Catalase activity was determined as per the method of Aebi (1974). The reaction mixture was prepared using 1.5 ml potassium buffer (100 mM, pH 7.0), 1.2 ml H_2O_2 (150 mM) and 300 µl of enzyme extract. The decrease in absorption per minute was recorded spectrophotometerically at 240 nm. Enzyme activity was determined using an extinction coefficient of 6.93×10^{-3} mM^{-1} cm^{-1}.

Glutathione reductase (EC 1.6.4.2)

Glutathione reductase activity was measured using the method proposed by Carlberg and Mannervik (1975). The reaction mixture consisted of 1.8 ml of phosphate buffer, 300 µl of EDTA, 300 µl of NADPH, 300 µl of glutathione disulphide (GSSG) and 300 µl of enzyme extract. GR activity was determined by the oxidation of NADPH represented by decrease in absorbance per minute at 340 nm. Enzyme activity was determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and was calculated as the amount of enzyme required to oxidize 1 µmol of NADPH min^{-1} g^{-1} tissue.

Ascorbate peroxidase (EC 1.11.1.11)

The activity of ascorbate peroxidase was estimated according to the method proposed by Nakano and Asada (1981) following a decrease in absorbance at 290 nm in a reaction mixture containing 1.5 ml K-phosphate buffer (100 mM, pH 7.0), 300 µl ascorbate (5 mM), 600 µl H_2O_2 (0.5 mM) and 600 µl of enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}, and was calculated as the amount of enzyme required to oxidize 1 µmol of ascorbate min^{-1} g^{-1} tissue.

Superoxide dismutase (EC 1.15.1.1)

The activity of SOD was estimated according to Kono (1978) by monitoring its potential to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are produced by the autooxidation of hydroxylamine hydrochloride. The reduction of NBT was followed by the increase in absorbance at 540 nm in reaction mixture constituting 1.3 ml Na-carbonate buffer (50 mM, pH 10.0), 500 µl NBT (96 µM) and 100 µl Triton X-100 (0.6%). The reaction was initiated by the addition of 100 µl hydroxylamine-HCl (20 mM, pH 6.0) and 2 min later 70 µl of en-zyme extract was added and the enzyme activity was determined as the concentration of SOD required to inhibit the reduction of NBT by 50%.

Statistical analysis

The data were analyzed for mean, standard deviation, standard error and coefficient of variation. Comparison among different treatments was done using Student's '*t*' test, and one-way and two-way ANOVA (Sokal and Rholf 1981; Bailey 1995). Honestly statistical difference (HSD) values were obtained using Tukey's multiple comparison test. Self coded software developed in MS-Excel was used. The binary interaction model developed using multiple regression technique was:

$$Y = a + b_1X_1 + b_2X_2 + b_3X_1X_2$$

where, Y is the studied parameter, X_1 and X_2 are metals in binary combinations, b_1 and b_2 are partial regression coefficients due to the effects of X_1 and X_2 respectively, and b_3 is the partial regression coefficient due to interaction between X_1 and X_2. Unitless β-regression coefficients, β_1, β_2 and β_3 were computed to determine the relative effects of independent variables, X_1,X_2 and interaction between X_1 and X_2. Metal interaction was interpreted as described in **Table 1**.

RESULTS

Heavy metal uptake

Metal uptake analysis of seedlings (**Tables 2, 3**) grown in single metal treatments revealed that Zn showed maximum uptake of 0.531 mg g^{-1}, whereas Ni was found to be the metal least accumulated (0.135 mg g^{-1}). In binary combinations of Zn with other metals, both the metals mutually decreased the uptake of each other. Lowest Zn uptake by the seedlings was observed in Zn (100 mg l^{-1})+Cr (100 mg l^{-1}) combination, where Cr (100 mg l^{-1}) caused maximum inhibition (79.6%), followed by (Zn 100+Ni 100) combination where Ni (100 mg l^{-1}) caused (70.2%) inhibition, with respect to Zn (100 mg l^{-1}) treatment (**Tables 4, 5**). In turn, Zn (100 mg l^{-1}) in binary combinations with other metals (100 mg l^{-1}) exerted maximum inhibitory effect on the uptake of Cr (66.1%) followed by Cu (60.4%) with respect to the treatments where these metals were applied individually. Multiple regression analysis of metal uptake revealed that in binary combinations of Zn with other heavy metals, the uptake of Zn increased with increase in Zn concentration but decreased with increase in concentration of Cr, Co and Cu (**Table 6**). The interaction between two metals decreased the uptake of Zn in all the cases. Similarly when the uptake of other heavy metals (Cr, Ni Co and Cu) was studied, there was an increase in the uptake of heavy metals in response to their concentrations in binary combinations, but the interactive effect of Zn with other metals decreased their uptake. It was observed that supplementation of Zn to other heavy metal solutions decreased the uptake of heavy metals and ameliorates the seedling characteristics. The data obtained from the enzyme assays of single metal stressed seedlings showed that there was significant enhancement of antioxidative enzymes activity except for catalase (**Table 7**). Zn was observed to be the most effective metal in increasing the activities of antioxidative enzymes. Maximum increase in the activities of GR, GPX and APX (8.2, 54.07 and 7.96 mM UA mg^{-1} protein respectively), were observed in the seedlings treated with Zn 100 mg l^{-1} alone, whereas maximum activity of SOD (48.87 mM UAmg^{-1}protein) was observed in the seedlings grown in Cr (100 mg l^{-1} treatment).

The binary combinations of metals also increased the activities of antioxidative enzymes (**Fig. 1**). It was observed that (Zn+Co) combination increased the GPX activity by 74% as compared to the control where seedlings are grown in a medium devoid of any metal. (Zn+Ni) proved to be most effective in increasing the activity of GR (8.8 mM UA mg^{-1} protein). Maximum APX activity (8.53 mM UA mg^{-1} protein) was observed in the seedlings treated with (Zn+Cu), whereas maximum SOD (16 mM UA mg^{-1} protein) activity was observed in (Zn+Cr) stressed seedlings. One-way ANOVA showed that the activities of GR, GPX, APX and SOD were significantly different from each other in various binary metal combinations of Zn with other metals (**Table 8**).

Binary interaction models (**Table 9**) for the relative activities of different antioxidative enzymes as a function of two metals in binary combinations derived using multiple regression analysis revealed that there are significant correlations between various enzymatic activities and binary metal treatments. For GR, the partial and β regression coefficients for Zn in all the binary treatments were negative, whereas for Ni, Co, Cu and Cr, these were positive. The overall interactive effect of binary treatments on GR activity was found to be positive, thereby showing that Ni, Co, Cu and Cr are antagonistic to Zn for the activity of GR. The partial and β regression coefficients due to all the metals for GPX, APX and SOD were observed to be positive, showing thereby that these metal ions increased the activities of these enzymes but the interactive effects of Zn with other metals (Ni, Co, Cu and Cr) on the activity of these enzymes were negative, implying that these metals in binary combinations were mutually antagonistic and thus reduced the activities of GPX, APX and SOD.

DISCUSSION

In nature the plants occurring at industrially polluted and metalliferous sites are exposed to multiple heavy metal stress (Krupa *et al* 2002). Higher plants are not very often used in such experiments because of the high degree of complexity, although algal models have been extensively

Table 1 Binary metal interaction in terms of β regression coefficients.

Variables			Interaction
X_1	X_2	X_1 X_2	
β-regression coefficients			
β_1	β_2	β_3	
+	+	+	Synergistic (S)
-	-	-	Synergistic (S)
+	+	-	Antagonistic (A)
-	-	+	Antagonistic (A)
+	-	+	Mixed: X_1 antagonistic to X_2, but X_2 synergistic to X_1 (M)
+	-	-	Mixed: X_1 synergistic to X_2, but X_2 antagonistic to X_1 (M)
+/-	+/-	0	Additive (0)

Table 2 Metal uptake (Mean ± SE) by the seedlings of *B. juncea* grown in Petri plates containing binary combinations of Zn with other heavy metals.

Metal treatments (mg l^{-1})	**Cr+Zn**			
	Cr uptake (mg g^{-1} dw)			
	Zn (0)	**Zn (25)**	**Zn (50)**	**Zn (100)**
Cr (25)	0.100 ± 0.02	0.083 ± 0.01	0.076 ± 0.01	0.060 ± 0.007
Cr (50)	0.119 ± 0.01	0.066 ± 0.01	0.049 ± 0.004	0.060 ± 0.007
Cr (100)	0.180 ± 0.04	0.072 ± 0.02	0.051 ± 0.004	0.061 ± 0.011
	Zn uptake (mg g^{-1} dw)			
	Cr (0)	**Cr (25)**	**Cr (50)**	**Cr (100)**
Zn (25)	0.221 ± 0.05	0.147 ± 0.02	0.130 ± 0.003	0.113 ± 0.04
Zn (50)	0.316 ± 0.08	0.180 ± 0.084	0.175 ± 0.032	0.153 ± 0.025
Zn (100)	0.531 ± 0.11	0.217 ± 0.07	0.202 ± 0.05	0.108 ± 0.021
	Ni+Zn			
	Ni uptake (mg g^{-1} dw)			
	Zn (0)	**Zn (25)**	**Zn (50)**	**Zn (100)**
Ni (25)	0.093 ± 0.026	0.088 ± 0.01	0.085 ± 0.02	0.100 ± 0.02
Ni (50)	0.135 ± 0.045	0.061 ± 0.02	0.064 ± 0.001	0.089 ± 0.005
Ni (100)	0.135 ± 0.022	0.092 ± 0.04	0.057 ± 0.01	0.074 ± 0.002
	Zn uptake (mg g^{-1} dw)			
	Ni (0)	**Ni (25)**	**Ni (50)**	**Ni (100)**
Zn (25)	0.221 ± 0.05	0.204 ± 0.03	0.128 ± 0.004	0.116 ± 0.009
Zn (50)	0.316 ± 0.08	0.172 ± 0.05	0.189 ± 0.08	0.156 ± 0.04
Zn (100)	0.531 ± 0.11	0.396 ± 0.07	0.25 ± 0.09	0.158 ± 0.05
	Co+Zn			
	Co uptake (mg g^{-1} dw)			
	Zn (0)	**Zn (25)**	**Zn (50)**	**Zn (100)**
Co (25)	0.110 ± 0.016	0.103 ± 0.001	0.076 ± 0.001	0.084 ± 0.03
Co (50)	0.158 ± 0.12	0.093 ± 0.004	0.087 ± 0.02	0.102 ± 0.01
Co (100)	0.224 ± 0.06	0.185 ± 0.07	0.109 ± 0.005	0.187 ± 0.09
	Zn uptake (mg g^{-1} dw)			
	Co (0)	**Co (25)**	**Co (50)**	**Co (100)**
Zn (25)	0.221 ± 0.05	0.234 ± 0.02	0.134 ± 0.01	0.129 ± 0.03
Zn (50)	0.316 ± 0.08	0.140 ± 0.03	0.239 ± 0.02	0.187 ± 0.005
Zn (100)	0.531 ± 0.11	0.368 ± 0.06	0.271 ± 0.07	0.257 ± 0.02
	Cu+Zn			
	Cu uptake (mg g^{-1} dw)			
	Zn (0)	**Zn (25)**	**Zn (50)**	**Zn (100)**
Cu (25)	0.081 ± 0.02	0.065 ± 0.01	0.068 ± 0.004	0.056 ± 0.001
Cu (50)	0.168 ± 0.006	0.094 ± 0.013	0.077 ± 0.029	0.062 ± 0.01
Cu (100)	0.235 ± 0.008	0.114 ± 0.02	0.068 ± 0.02	0.093 ± 0.02
	Zn uptake (mg g^{-1} dw)			
	Cu (0)	**Cu (25)**	**Cu (50)**	**Cu (100)**
Zn (25)	0.221 ± 0.05	0.260 ± 0.07	0.144 ± 0.09	0.130 ± 0.005
Zn (50)	0.316 ± 0.08	0.241 ± 0.01	0.121 ± 0.04	0.143 ± 0.02
Zn (100)	0.531 ± 0.11	0.267 ± 0.06	0.290 ± 0.04	0.276 ± 0.09

employed to examine the interactions among heavy metals (Taylor 1989; Visviki and Rachlin 1991; Rachlin and Grosso 1993). The present study revealed that the uptake of an ion is mutually inhibited by the uptake of Zn in the binary combinations. The study finds support from Symeonidis and Karataglis (1992) who reported decreased Pb uptake in the presence of Zn in *Holcus lanatus*. Wu and Zhang (2002) also found that increasing Zn application could alleviate Cd toxicity stress in barley by improving growth and reducing membrane damage. It is generally conceded that the uptake of nutrients in plants is metabolically regulated (Salisbury and Ross 1992). The mutual inhibition of ion uptake may be attributed to competitive interactions during ion uptake as is generally observed for a group of closely related anions or cations. Further, the interaction of dissolved metals with biological cell membranes influences the transportation, chemistry, bioaccumulation, and relative toxicity of metals. Metal ions react with various surface functional groups such as sulphydryl, amino, carboxyl, hydroxide, oxide, etc. thereby complicating the development of a general relationship between the aqueous chemistry of metals, their interaction among themselves and subsequently their toxicological properties (Dirilgen 2001).

Uptake and accumulation of heavy metals in plants is often accompanied by the activation of numerous intracellular changes, some of which are directly involved to the contribution of metal tolerance capacity of plant, especially of hyperaccumulators (Hall 2002). Overproduction of ROS is the most common consequence of heavy metal stress, and to maintain the homeostasis under stress conditions, the balance between generation degradation of ROS is necessary. To mitigate and to repair the oxidative damage caused by the enhanced production of ROS, a strong antioxidative system in plants is induced (Schutzendubel and Polle 2002). Cao *et al.* (2005) on the basis of molecular, physiological and genetic approaches demonstrated that the elevation in antioxidative enzymes was the result of enhanced expression of DET 2 gene which enhanced the resistance to oxidative stress in A*rabidopsis thaliana*. The response of antioxidative enzymes to metal stress, remains controversial and shows variation among plant species and among different tissues (Mazhoudi *et al.* 1997), and also varies with metal concentration (Chaoui and El Feriani 2005). Narang *et al*. (2008) observed an enhancement in antioxidative enzymes in response to mercury accumulation in *Eichhornia crassipes.* In the present investigation, it was expected that the exposure of mustard plants to high metal stress could elevate the levels of antioxidative enzymes. Comparing the activity of 5 anti-oxidative enzymes, it was evident that the uptake of heavy metals induced a strong antioxidative response in *B. juncea* seedlings by increasing the activities of all the antioxidative enzymes except for catalase. Our re-

Table 3 Two-way ANOVA for metal uptake in *B. juncea* seedlings grown in Petri dishes containing binary combinations of Zn with other heavy metals.

Source of variation	df	SS	MSS	F-ratio	SS	MSS	F-ratio
				Zn+Cr			
		Zn uptake (mg g^{-1} dw) (Cr+Zn)			**Cr uptake (mg g^{-1} dw) (Zn+Cr)**		
Zn	3	0.167	0.056	30.50***	0.021	0.007	28.66***
Metal	2	0.068	0.034	18.61***	0.001	0.001	2.92
Zn x Metal	6	0.048	0.008	4.35*	0.007	0.001	4.64*
Error	12	0.022	0.002		0.003	$2.4x10^{-4}$	
Total	23	0.304			0.032		
				Zn+Ni			
		Zn uptake (mg g^{-1} dw) (Ni+Zn)			**Ni uptake (mg g^{-1} dw) (Zn+Ni)**		
Zn	3	0.125	0.042	13.72***	0.010	0.003	5.56*
Metal	2	0.160	0.080	26.46***	3.3×10^{-05}	1.7×10^{-5}	0.029
Zn x Metal	6	0.034	0.006	1.881	0.005	0.001	1.403
Error	12	0.036	0.003		0.007	0.001	
Total	23	0.355			0.021		
				Zn+Co			
		Zn uptake (mg g^{-1} dw) (Co+Zn)			**Co uptake (mg g^{-1} dw) (Zn+Co)**		
Zn	3	0.097	0.032	15.23***	0.016	0.005	3.46*
Metal	2	0.138	0.069	32.395***	0.031	0.015	9.782**
Zn x Metal	6	0.052	0.009	4.094*	0.006	0.001	0.607
Error	12	0.026	0.002		0.019	0.002	
Total	23	0.313			0.072		
				Zn+Cu			
		Zn uptake (mg g^{-1} dw) (Cu+Zn)			**Cu uptake (mg g^{-1} dw) (Zn+Cu)**		
Zn	3	0.121	0.040	14.97***	0.033	0.011	36.12***
Metal	2	0.112	0.056	20.724***	0.014	0.007	23.499***
Zn x Metal	6	0.050	0.008	3.090*	0.014	0.002	7.403***
Error	12	0.032	0.003		0.004	$3.1x10^{-4}$	
Total	23	0.315			0.065		

Significant at*** $p \leq 0.001$, ** $p \leq 0.01$ *$p \leq 0.05$

Table 4 Percentage change in uptake of metals in *B. juncea* seedlings grown in binary combinations of Zn (100 mg l^{-1}) with other heavy metals (100 mg l^{-1}) with respect to single metal treatments.

Treatments		Uptake in single metal solution (100 mg g^{-1}) (Mean ± SE)	Uptake in binary combination (mg g^{-1}) (Mean ± SE)	% change in uptake in binary combination
Uptake of Zn				
Zn+Cr	Zn	0.531 ± 0.06	0.108 ± 0.02***	-79.6
Zn+Ni	Zn	0.531 ± 0.06	0.158 ± 0.05**	-70.2
Zn+Co	Zn	0.531 ± 0.06	0.257 ± 0.03**	-51.8
Zn+Cu	Zn	0.531 ± 0.06	0.276 ± 0.09*	-48.0
Uptake of other metals				
Zn+Cr	Cr	0.180 ± 0.04	0.061 ± 0.01**	-66.1
Zn+Ni	Ni	0.135 ± 0.022	0.074 ± 0.002**	-45.2
Zn+Co	Co	0.224 ± 0.059	0.187 ± 0.09	-16.9
Zn+Cu	Cu	0.235 ± 0.008	0.093* ± 0.019***	-60.4

Significant at ***$p \leq 0.001$ **$p \leq 0.01$ *$p \leq 0.05$ using '*t*' test

Table 5 One-way ANOVA and Tukey's multiple comparison test for metal uptake in *B. juncea* seedlings grown in single heavy metal solutions of Cr, Ni, Co, Cu and Zn, each at 100 mg l^{-1}.

Source of variation	SS	df	MS	F-ratio	HSD
Between groups	0.20	4	0.05	29.45**	0.16
Within groups	0.01	5	0.002		
Total	0.20	9			

Significant at **$p \leq 0.01$

sults find support from Wang *et al.* (2004) who reported that in *B. juncea* seedlings treated with Cu^{2+} ions there is increase in POX, APX and SOD activities, whereas the CAT activity decreased. It is also reported that in many plant species, excessive uptake of heavy metals such as Ni, Cd and Pb induce a strong increase in POX activity (Mazhoudi *et al.* 1997; Baccouch *et al.* 2001). Since SOD plays a pivotal role, as it is the first line of defence against ROS (Hassan and Scandalios 1990), it catalyses the conversion of superoxide anion to O_2 and H_2O_2. An increase in SOD activity in response to heavy metal stress has been reported earlier (Schutzendubel *et al.* 2001). Khan *et al.* (2009) observed increase in SOD activity under As-stressed *B. juncea* seedlings. Moreover, Mobin and Khan (2006) also reported pronounced increase in SOD activity in *B. juncea* seedlings grown in Cd treatments. Increase in the activity of SOD under Zn, Co, Cu and Ni stress as observed in the present study may be due to the increased availability of these metal ions that act as prosthetic groups in SOD. Moreover this increased activity of SOD may be due to the induction of genes of SOD by superoxide mediated signal transduction (Alavarez *et al.* 1997), which causes *de novo* synthesis of enzyme proteins (Verma and Dubey 2003), thereby causing more superoxide generation. H_2O_2, the product of SOD activity, is also toxic to cells and has to be further detoxified by CAT and the ascorbate-glutathione cycle. In the present study, no significant change in the catalase activity showed that CAT did not involve in the active reduction of H_2O_2 irrespective of high metal stress. Similar pattern of CAT activity has been reported earlier in response to various abiotic stresses (Kocis *et al.* 2002).

Ascorbate glutathione cycle in chloroplast is the main component of defence system for scavenging H_2O_2 that ultimately converts H_2O_2 to H_2O and O_2. This cycle mainly involves APX, POX and GR. The results of the present study showed increase in APX activity which is coherent with the findings of Israr *et al.* (2006) and Yoshimura *et al.* (2000). Since APX and peroxidase eliminate H_2O_2 by converting ascorbate to dehydroascorbate, the increased production of dehydroascorbate is recycled back to the ascor-

Table 6 Multiple regression interaction models for metal uptake (Y) in the seedlings of *B. juncea* grown in Petri dishes containing binary combinations (X_1+X_2) of Zn (X_1) with other heavy metals (X_2).

Treatments	Multiple regression equations with interaction	Correlation coefficient r	β regression coefficients			Interaction type
			β_1	β_2	Interaction β_3	
Zn uptake in binary combinations						
Zn+Cr	$Y(Zn) = 0.12 + 2.9E\text{-}03\ X_1 - 1.9E\text{-}04\ X_2 - 2.7E\text{-}05\ X_1X_2$	0.8224**	0.83	-0.07	-0.69	M
Zn+Ni	$Y(Zn) = 0.10 + 3.8E\text{-}03\ X_1 + 7.1E\text{-}05\ X_2 - 3.6E\text{-}05\ X_1X_2$	0.9488***	1.02	0.02	-0.86	A
Zn+Co	$Y(Zn) = 0.12 + 3.4E\text{-}03\ X_1 - 2.2E\text{-}04\ X_2 - 2.2E\text{-}05\ X_1X_2$	0.8841**	0.96	-0.07	-0.56	M
Zn+Cu	$Y(Zn) = 0.17 + 2.6E\text{-}03\ X_1 - 1.0E\text{-}03\ X_2 - 1.0E\text{-}05\ X_1X_2$	0.8365**	0.74	-0.35	-0.27	M
Metal uptake in binary combinations						
Zn+Cr	$Y(Cr) = 0.08 + 5.4E\text{-}04\ X_1 - 1.3E\text{-}04\ X_2 - 7.8E\text{-}06\ X_1X_2$	0.6888*	0.48	-0.13	-0.62	M
Zn+Ni	$Y(Ni) = 0.08 + 3.4E\text{-}04\ X_1 + 2.1E\text{-}04\ X_2 - 8.2E\text{-}06\ X_1X_2$	0.5597	0.44	0.33	-0.95	0
Zn+Co	$Y(Co) = 0.07 + 1.2E\text{-}03\ X_1 - 3.1E\text{-}04\ X_2 - 9.4E\text{-}07\ X_1X_2$	0.8104*	0.78	-0.24	-0.06	M
Zn+Cu	$Y(Cu) = 0.06 + 1.3E\text{-}03\ X_1 - 6.9E\text{-}05\ X_2 - 1.2E\text{-}05\ X_1X_2$	0.8027*	0.81	-0.05	-0.69	M

Significant at***p ≤0.001, ** p≤0.01, * p≤0.05

Table 7 Specific activities of antioxidative enzymes in the seedlings of *B. juncea* grown in binary combinations of Zn (X_1, mg l^{-1}) and other metals (X_2, mg l^{-1}), and HSD values using Tukey's multiple comparison test.

Metal treatments	Enzyme activity (mMoleUA/mg protein) Mean±SD				
	Catalase	Glutathione reductase	Guaiacol peroxidase	Ascorbate peroxidase	Superoxide dimutase
Zn+Cr					
Zn0+Cr0	4.23 ± 1.17	4.07 ± 0.75	32.9 ± 5.78	5.6 ± 0.85	10.67 ± 1.46
Zn100+Cr0	3.83 ± 0.91	8.2 ± 0.7	54.1 ± 4.07	7.96 ± 0.5	14.8 ± 1.49
Zn0+Cr100	2.96 ± 0.3	6.76 ± 1.09	47.3 ± 4.55	6.5 ± 0.7	15.23 ± 1.36
Zn100+Cr100	2.7 ± 0.62	7.5 ± 0.7	52.7 ± 3.17	7.56 ± 0.58	16 ± 0.7
Zn+Ni					
Zn0+Ni0	4.23 ± 1.17	4.07 ± 0.75	32.9 ± 5.78	5.6 ± 0.85	10.67 ± 1.46
Zn100+Ni0	3.83 ± 0.91	8.2 ± 0.7	54.1 ± 4.07	7.96 ± 0.5	14.8 ± 1.49
Zn0+Ni100	3 ± 0.2	7.53 ± 0.65	47.7 ± 5.12	7.36 ± 0.85	14.8 ± 0.36
Zn100+Ni100	2.5 ± 0.62	8.8 ± 0.26	54 ± 3.22	7.43 ± 1.06	15.6 ± 0.45
Zn+Co					
Zn0+Co0	4.23 ± 1.17	4.07 ± 0.75	32.9 ± 5.78	5.6 ± 0.85	10.67 ± 1.46
Zn100+Co0	3.83 ± 0.91	8.2 ± 0.7	54.1 ± 4.07	7.96 ± 0.5	14.8 ± 1.49
Zn0+Co100	3.16 ± 0.65	7.73 ± 0.76	48.9 ± 5.85	7.03 ± 1.42	13.6 ± 0.6
Zn100+Co100	2.96 ± 0.57	7.67 ± 0.8	57.5 ± 3.07	7.43 ± 1.08	15.1 ± 0.96
Zn+Cu					
Zn0+Cu0	4.23 ± 1.17	4.07 ± 0.75	32.9 ± 5.78	5.6 ± 0.85	10.67 ± 1.46
Zn100+Cu0	3.83 ± 0.91	8.2 ± 0.7	54.1 ± 4.07	7.96 ± 0.5	14.8 ± 1.49
Zn0+Cu100	2.96 ± 0.66	7.67 ± 0.8	44.4 ± 4.58	7.6 ± 0.79	15.1 ± 1.15
Zn100+Cu100	2.73 ± 0.58	8.1 ± 0.36	47.3 ± 3.59	8.53 ± 0.65	15.43 ± 0.93

bate with the help of GR, thereby catalyzing this last rate limiting step of glutathione cycle. Increased activity of GR under metal stress has been reported by many workers in agreement with our findings. Prasad *et al.* (1999) observed increase up to 158% in shoot GR activity in *B. juncea* seedlings raised under Zn toxicity and suggest that *B. juncea* seedlings try to counteract high concentration of oxygen species produced under zinc toxicity through a coordinated increase in the activities of enzymes involved in their detoxification In our studies it was observed that in general, the combinations of Zn with other metals further increase the activities of SOD, GPX, APX and GR.

CONCLUSION

The present study established that Zn in binary combinations with Cr, Ni, Co and Cu mutually decreased the uptake of each other. Supplementation of media containing these heavy metals with Zn helps in overcoming heavy metal toxicity due to antagonistic interactions between the two. The oxidative stress induced in *B. juncea* seedlings by heavy metals (single or in binary combination) is tolerated through the hyperactivity of antioxidative enzymes. The activities of SOD, GPX, APX and GR were enhanced under multiple heavy metal stress which alleviates the toxicity of heavy metal mixtures and helps in the establishment of its seedlings at multielement contaminated sites. Zn in binary combinations with other metals (Cr, Ni, Co and Cu) have mutually antagonistic effects to each other for the activities of APX, GPX and SOD. The interaction of Zn with other heavy metals for GR is a mixed interaction, Zn being synergistic to the heavy metals, but the other metals antagonistic to it. The interactions between Zn, and Cr, Co and Cu were antagonistic for catalase, but Zn-Ni interaction was synergistic.

ACKNOWLEDGEMENTS

Thanks are due to the Head of Department of Botanical & Environmental Sciences, Guru Nanak Dev University, Amritsar, for providing research facilities. Financial assistance from Ministry of Environment and Forests, Govt. of India is duly acknowledged.

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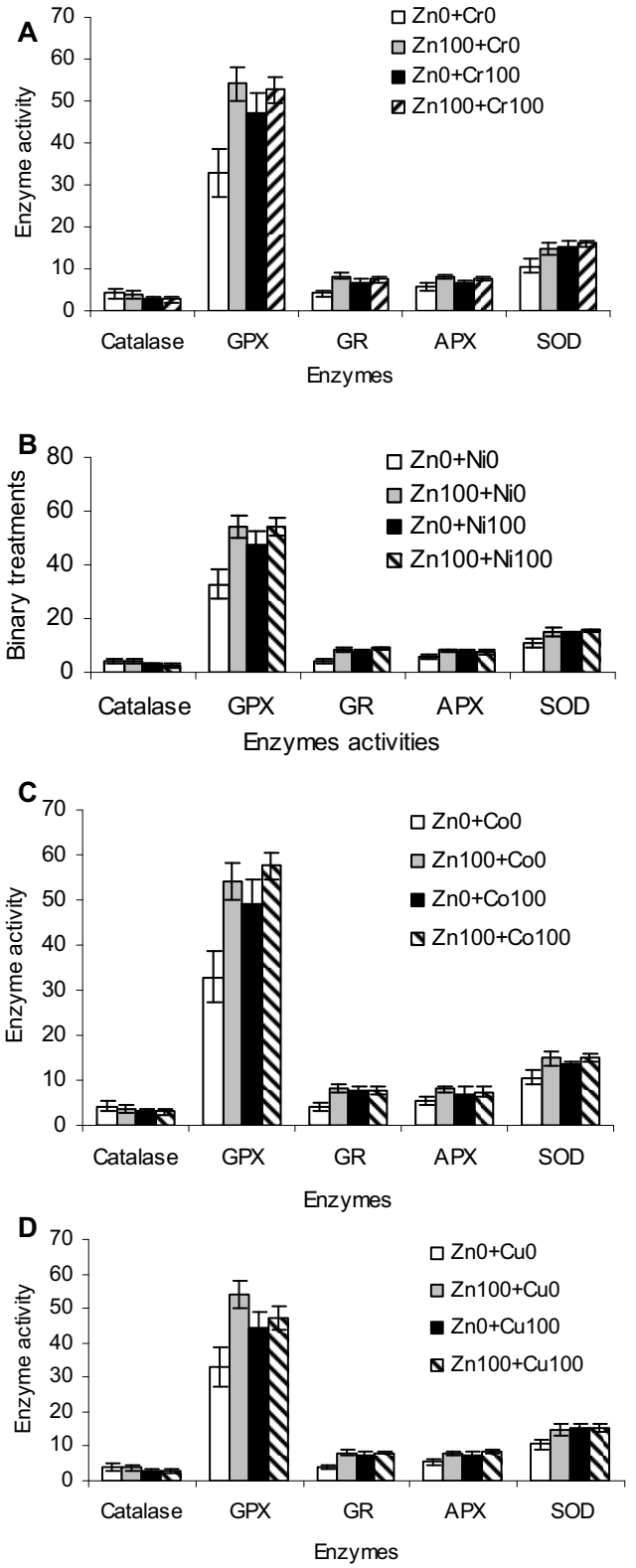

Fig. 1 Specific activities of antioxidative enzymes in the seedlings of *B. juncea* grown in binary combinations of Zn with other heavy metals. (A) Zn+Cr; (B) Zn+Ni; (C) Zn+Co; (D) Zn+Cu.

Table 8 One way ANOVA and Tukey's multiple comparison test for specific activities of antioxidative enzymes in the seedlings of *B. juncea* grown in binary combinations of Zn (100 mg l^{-1}) and other metals (100 mg l^{-1}).

Source of variation	df	SS	MS	F-ratio	HSD
Catalase					
Zn+Cr					
Between groups	3	4.67	1.56	2.33	2.14
Within groups	8	5.34	0.67		
Total	11	10			
Zn+Ni					
Between groups	3	5.56	1.85	2.83	2.12
Within groups	8	5.23	0.65		
Total	11	10.8			
Zn+Co					
Between groups	3	3.1	1.03	1.41	2.24
Within groups	8	5.87	0.73		
Total	11	8.97			
Zn+Cu					
Between groups	3	3.1	1.03	1.41	2.24
Within groups	8	5.87	0.73		
Total	11	8.97			
Glutathione reductase					
Zn+Cr					
Between groups	3	29.4	9.81	14.29**	2.17
Within groups	8	5.49	0.69		
Total	11	34.9			
Zn+Ni					
Between groups	3	40.4	13.5	34.86***	1.63
Within groups	8	3.09	0.39		
Total	11	43.5			
Zn+Co					
Between groups	3	33	11	19.38***	1.97
Within groups	8	4.54	0.57		
Total	11	37.5			
Zn+Cu					
Between groups	3	35.1	11.7	25.62***	1.77
Within groups	8	3.65	0.46		
Total	11	38.7			
Guaiacol peroxidase					
Zn+Cr					
Between groups	3	843	281	13.92**	11.8
Within groups	8	162	20.2		
Total	11	1005			
Zn+Ni					
Between groups	3	894	298	13.77**	12.2
Within groups	8	173	21.6		
Total	11	1067			
Zn+Co					
Between groups	3	1068	356	15.2**	12.7
Within groups	8	187	23.4		
Total	11	1255			
Zn+Cu					
Between groups	3	702	234	11.16**	12
Within groups	8	168	21		
Total	11	870			
Ascorbate peroxidase					
Zn+Cr					
Between groups	3	10.3	3.43	7.55*	1.76
Within groups	8	3.63	0.45		
Total	11	13.9			
Zn+Ni					
Between groups	3	9.55	3.18	4.46*	2.21
Within groups	8	5.7	0.71		
Total	11	15.2			
Zn+Co					
Between groups	3	9.25	3.08	2.95	2.67
Within groups	8	8.34	1.04		
Total	11	17.6			
Zn+Cu					
Between groups	3	14.6	4.88	9.59**	1.87
Within groups	8	4.07	0.51		
Total	11	18.7			

Table 8 (Cont.)

Source of variation	df	SS	MS	F-ratio	HSD
	Superoxide dimutase				
			Zn+Cr		
Between groups	3	51.4	17.1	10.26**	3.38
Within groups	8	13.4	1.67		
Total	11	64.8			
			Zn+Ni		
Between groups	3	44.8	14.9	12.74**	2.83
Within groups	8	9.39	1.17		
Total	11	54.2			
			Zn+Co		
Between groups	3	36.9	12.3	8.703**	3.11
Within groups	8	11.3	1.41		
Total	11	48.1			
			Zn+Cu		
Between groups	3	45	15	9.174**	3.35
Within groups	8	13.1	1.64		
Total	11	58.1			

Significant at *** $p \leq 0.001$, ** $p \leq 0.01$, *$p \leq 0.05$

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Table 9 Multiple regression binary interaction model for the activities of different antioxidative enzymes in the seedlings of *B. juncea* grown in water cultures containing binary combinations of Zn and other metals.

Treatments	Multiple regression equation with interaction	β regression coefficients			Nature of interaction
		β_1	β_2	Interaction β3	
Catalase					
Zn+Cr	$Y = 4.23 - 0.004\ X_1 - 0.013\ X_2 - 1.4E\text{-}05\ X_1X_2$	-0.321	-1.019	0.097	A
Zn+Ni	$Y = 4.23 - 0.004\ X_1 - 0.012\ X_2 - 1.0E\text{-}05\ X_1X_2$	-0.295	-0.906	-0.064	S
Zn+Co	$Y = 4.23 - 0.004\ X_1 - 0.011\ X_2 + 2.0E\text{-}05\ X_1X_2$	-0.392	-1.049	0.170	A
Zn+Cu	$Y = 4.23 - 0.004\ X_1 - 0.013\ X_2 + 1.7E\text{-}05\ X_1X_2$	-0.325	-1.033	0.120	A
Glutathione reductase					
Zn+Cr	$Y = 4.07 - 0.039\ X_1 + 0.027\ X_2 + 4.6E\text{-}04\ X_1X_2$	-0.677	0.470	0.698	M
Zn+Ni	$Y = 4.07 - 0.039\ X_1 + 0.035\ X_2 + 5.1E\text{-}04\ X_1X_2$	-0.579	0.518	0.666	M
Zn+Co	$Y = 4.07 - 0.039\ X_1 + 0.037\ X_2 + 3.8E\text{-}04\ X_1X_2$	-0.624	0.590	0.532	M
Zn+Cu	$Y = 4.07 - 0.039\ X_1 + 0.036\ X_2 + 4.3E\text{-}04\ X_1X_2$	-0.607	0.565	0.584	M
Guaiacol peroxidase					
Zn+Cr	$Y = 32.9 + 0.218\ X_1 + 0.144\ X_2 - 1.6E\text{-}03\ X_1X_2$	1.279	0.845	-0.833	A
Zn+Ni	$Y = 32.9 + 0.218\ X_1 + 0.148\ X_2 - 1.5E\text{-}03\ X_1X_2$	1.244	0.843	-0.764	A
Zn+Co	$Y = 32.9 + 0.218\ X_1 + 0.160\ X_2 - 1.3E\text{-}03\ X_1X_2$	1.143	0.838	-0.597	A
Zn+Cu	$Y = 32.9 + 0.218\ X_1 + 0.115\ X_2 - 1.9E\text{-}03\ X_1X_2$	1.390	0.736	-1.044	A
Ascorbate peroxidase					
Zn+Cr	$Y = 5.6 + 0.024\ X_1 + 0.009\ X_2 - 1.3E\text{-}04\ X_1X_2$	1.278	0.487	-0.610	A
Zn+Ni	$Y = 5.6 + 0.024\ X_1 + 0.018\ X_2 - 2.3E\text{-}04\ X_1X_2$	1.326	0.989	-1.115	A
Zn+Co	$Y = 5.6 + 0.024\ X_1 + 0.014\ X_2 - 2.0E\text{-}04\ X_1X_2$	1.348	0.817	-0.969	A
Zn+Cu	$Y = 5.6 + 0.024\ X_1 + 0.020\ X_2 - 1.4E\text{-}04\ X_1X_2$	1.070	0.906	-0.561	A
Superoxide dimutase					
Zn+Cr	$Y = 10.7 + 0.041\ X_1 + 0.046\ X_2 - 3.4E\text{-}04\ X_1X_2$	0.999	1.102	-0.704	A
Zn+Ni	$Y = 10.7 + 0.041\ X_1 + 0.041\ X_2 - 3.3E\text{-}04\ X_1X_2$	1.069	1.069	-0.747	A
Zn+Co	$Y = 10.7 + 0.041\ X_1 + 0.029\ X_2 - 2.6E\text{-}04\ X_1X_2$	1.180	0.838	-0.651	A
Zn+Cu	$Y = 10.7 + 0.041\ X_1 + 0.044\ X_2 - 3.8E\text{-}04\ X_1X_2$	1.067	1.144	-0.851	A