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IN VITRO RELATIONSHIP BETWEEN CADMIUM STRESS AND THIOUREA IN TWO BARLEY GENOTYPES

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Cadmium (Cd) becomes very perilous pollutant for living things when its concentrations exceed certain threshold value. Barley (*Hordeum vulgare* L.) can accumulate high amount of Cd but the genotypic differences in barley for Cd uptake have not been clearly determined. The objective of this work was to determine the effects of three Cd levels (0, 500, 1000 and 1500 µM/L) and alleviation of its toxicity by 0 and 10 mM thiourea (TU) on relative fresh and dry weights, macro- and micronutrients and antioxidants activation for scavenging reactive oxygen species in two barley genotypes cultured *in vitro*. One month old *in vitro* cultured two barley genotypes were done under controlled conditions and TU was applied with and without increased doses of Cd. One month old calli were treated with Cd alone and in combination with of TU. After 15 days of growth, the plants were harvested and the data on relative fresh weight were collected and then the other data were taken according to the procedures. The increased doses of Cd significantly affected all the parameters and TU alleviates the Cd stress to some extent. The results revealed that high Cd toxicity is possibly associated with a decline in fresh and dry weight, macro and micronutrients and enhanced production of CAT and SOD but amelioration occurs by TU to some extent in both genotypes. However, Jau-83 proved more tolerant than Haider-91.
Keywords: Cadmium, thiourea, antioxidants, macronutrients, micronutrients, *in vitro* culture, barley

INTRODUCTION

In vitro techniques can be used for the propagation of cadmium (Cd) tolerant crop lines and *in vitro* technique is best for studying the whole mechanism of metal tolerance (Gonçalves *et al*., 2007; Chakarvarty and Srivastava, 1997; Ahmad *et al.*, 2013) especially tobacco, cuscuta, soya bean and sunflower have been used for understanding the mechanism of metal resistance (Bueno and Piqueras, 2002; Sobkowiak *et al*., 2004; Srivastava *et al*., 2004). The sunflower callus culture provides a suitable system for the evaluation of tolerance to heavy metals and detoxification (Azevedo *et al*., 2005). Some reportsare available about the effects of Cd on callus tissues on crops like tomato (Inouhe *et al*., 2000) and sugarcane (Fornazier *et al*., 2002).

Cadmium influence the uptake and distribution of macro- and micronutrients, Cd gets its entry into the plants in competition with Ca, Mg, K, Zn and Fe for the same transmembrane carriers (Korshunova *et al*., 1999; Connolly *et al*., 2002), thereby causes reduction in the concentration of these nutrients as increase in uptake of Cd happened (El-Beltagi *et al*., 2010).

To combatthe oxidative stress caused by Cd and other abiotic stresses, plants utilize antioxidant detoxifying machinery. Antioxidant defense system in plant cell is complex, compartmentalized and consists of both enzymatic and non-enzymatic mechanisms (Gill and Tuteja, 2011). CAT, SOD, AsPO and GPO increases in barley plant when treated with paraquat (Yonova *et al*., 2009).

Breeding of crop varieties holding promise against environmental stress is an expensive and long term venture. Therefore, emphasis has been placed on exploiting prompt and inexpensive means of obtaining satisfactory yield from stressed lands. One of pragmatic approaches is the exogenous use of stress-alleviating compounds either as seed or foliar application (Wahid *et al*., 2008; Asthir *et al.*, 2013). In this respect many nitrogenous compound, inorganic salts, natural and synthetic plant growth promoters such as kinetin and gibberellic acid, thiourea (TU) and nitrate, proline and betains (Osmotica) are well known in wild plants (Khan and Ungar, 2001). The use of TU to counteract the effect of Cdtoxicity as TU improve fresh and dry plant biomass (Siddiqui *et al*., 2006), mineral uptake plays an important role in lowering the damage by oxidative stress caused by salinity stress in wheat (Abdelkader *et al*., 2012) and in barley (Yonova *et al*., 2009). The main objective of the research is to elucidate the amelioration of Cdtoxicity by TU on two barley genotypes based on growth and physiological parameters at un-differentiated level (callus culture).

MATERIALS AND METHODS

After washing barley grains with detergent, running water and distilled water, dippedthe barley grains in 20% (v/v) sodium hypochlorite solution for 25 minutes and then in 0.01% mercuric chloride for 4 minutes. The seeds were rinsed with autoclaved distilled water aseptically in laminar flow cabinet to ensure complete removal of mercuric chloride. To initiate and establish callus, surface sterilized

mature embryos (grains) were used as source of explants, cultured in test tubes containing 10mL of LS medium (sucrose 30g/L, 2, 4-D 2.5 mg/L) (Linsmaier and Skoog, 1962) and solidified with agar. The culture was placed in growth room under constant temperature (25±2°C) and white florescent light.

Treatments i.e., Cd (control, 500, 1000 and 1500 µM/L) and thiourea (TU; control and10 mM) were applied to one month old calli. One month old callus tissues was placed in 100 mL Erlenmeyer flasks containing 40 mL liquid LS-medium, with the same concentration of 2,4-D and corresponding treatment and then placed on gyratory shaker, till the termination of experiment. Each treatment was replicated thrice and each replicate was consisted of ten flasks. After 15 days of treatment, calli were harvested and following parameters were studied.

Growth parameters:

***Relative growth rate (fresh)*:** Fresh weight of calli were determined with the help of analytical balance and relative fresh rate was calculated with the following formula

$$RGR\ (fresh) = ln\ (final\ wt) - ln\ (initial\ wt)$$

***Dry weight of callus*:** After washing with distilled water, callus was blotted with filter paper, kept for 3 days in an oven at 65°C and then weighed. The dry weights were taken in grams and mean values were calculated.

***Determination of macro and micronutrients*:** Dried and grinded material (0.5 g) of calli were digested in concentrated HNO_3 (5 mL) at 100 and 150°Cin digestion tubes and then made volume of the extracted up to 50 mL in the volumetric flask. Filtered the extract and used it for the determination of mineral nutrients concentrations. The dissolved amount of potassium and calcium were determined using flame photometer (Model PFP-7. Jenway, UK) and magnesium, manganese, and Cdwere determined with atomic absorption spectrophotometer (AAS; Model A Analyst 3000; Perkin Elmer, Norwalk).

***Determination of antioxidant enzymes*:** Prior to antioxidant activities, the protein contents were determined as described by Bradford (1976). The activities of CAT, and SOD were determined spectrophotometrically. Leaves were homogenized in a medium containing50 m*M* phosphate buffer with 7.0 pH and 1 mM dithiothreitol (DTT) as described by Dixit (2001).

***Catalase (CAT)*:** Catalase activity was assayed by measuring the conversion rate of hydrogen peroxide to water and oxygen molecules, following the method described by Chance and Maehly (1955). The activity was assayed in 3mL reaction solution comprising 50 mM phosphate buffer with 7.0 pH, 5.9 mM of H_2O_2 and 0.1 mL enzyme extract. The catalase activity was determined by decline in absorbance at 240 nm after every 20 sec due to consumption of H_2O_2. Absorbance change of 0.01 unit min^{-1} was defined as one unit catalase activity.

***Superoxide dismutase (SOD)*:** SOD activity was assayed by determining the inhibition rate of nitrobluetetrazolium (NBT) reduction with xanthine oxidase as a hydrogen peroxide generating agent adopting method by Giannopolitis and Ries (1977). The absorbance at 560 nm was measured using a UV-visible (IRMECO U2020) spectrophotometer. One unit SOD activity reflected enzyme quantity caused 50% photochemical inhibition of NBT.

***Statistical analysis*:** The experiments were designed in completely randomized with three replications of each treatment. Results were statistically evaluated using the software program COSTAT v 6.3 (CoHort software, Berkeley, California) at $p \leq 0.05$. Mean and standard errors were performed on Microsoft Excel Version-2007 and differences between individual means were tested using least significant differences (LSD) using Statistix 8.1.

RESULTS

***Relative fresh and dry weights*:** Data in Table 1 show that Cd imposition to culture medium had significant retarding influence on callus relative fresh and dry weights of barley genotypes and the interaction of all these factors is also significant for relative fresh weight but the interaction of genotypes × TU and genotypes × TU × Cd is non-significant for dry weight of callus. Genotype Jau-83 had higher fresh and dry biomass than Haider-91 at 10 mM TU concentration.

***Macronutrients*:** Metal toxicity considerably decreased accumulation of potassium and calcium in the calli of both barley genotypes. Difference in all genotypes for callus potassium and calcium accumulation was significant under normal or stress circumstances. Moreover, cv. Jau-83 at cellular level had considerably higher potassium and calcium concentration than Haider-91 (Table 2) but TU non-significantly affected genotypes in potassium content but significant difference was observed in calcium content.

***Micronutrients*:** Due to metal toxicity, significant decrease in content of magnesium and manganese was observed (Table 3) in calli of both genotypes, moreover, lesser content were observed in Haider-91 than that of Jau-83 but TU, non-significantly, enhances the content of both micronutrient in calli of both genotypes.

***Cadmium content*:** Callus Cd content was remarkably increased upon induction of Cd stress (Table 4). However, interaction between the genotype × TU was non-significant. Exogenous application of Cd substantially increased the endogenous concentrationof Cd. Under stress condition, endogenous level of Cd consistently increased with Cd application, being highest at 1500 µM/L of Cd whereas the application of TU showed decline in Cd accumulation.

Table 1. Means of relative fresh (LSD=0.062) and dry weight (LSD= ns) of calli of two barley genotypes under varying concentrations of Cd (0, 500, 1000 and 1500 µ*M*) along with 10 mM TU added in the culture medium

Treatments		Genotypes			
		Jau-83		Haider-91	
Cd (µM)	Thiourea(mM)	R f wt	Dry wt	R f wt	Dry wt
Control	Control(=0)	2.77±0.14	0.735±0.0037	2.34±0.0041	0.67±0.0025
500		1.87±0.014	0.261±0.011	1.08±0.0015	0.175±0.012
1000		1.21±0.0051	0.18±0.0018	0.75±0.0028	0.12±0.0035
1500		0.78±0.015	0.029±.0054	0.27±0.0049	0.017±0.0034
Control	10	2.97±0.034	0.89±0.016	2.79±0.0067	0.88±0.0019
500		2.36±0.0087	0.35±0.0013	1.69±0.011	0.26±0.0057
1000		1.48±0.043	0.189±0.0048	1.214±0.0017	0.14±0.0018
1500		1.08±0.0057	0.071±0.024	0.67±0.0051	0.038±0.0048

Table 2. Means of potassium (LSD=2.501) and calcium (LSD=6.268) concentrations in the calli of two barley genotypes under varying concentrations of Cd (0, 500, 1000 and 1500 µ*M*) along with 10 m*M* TU added in the culture medium

Treatments		Genotypes			
		Jau-83		Haider-91	
Cd (µM)	Thiourea(mM)	Potassium	Calcium	Potassium	Calcium
control	Control(=0)	33.81±0.151	5.68±0.55	32.22±0.196	7.17±0.31
500		31.52±0.057	2.25±0.23	29.6±0.152	2.34±0.25
1000		26.33±0.026	1.96±0.043	21.3±0.146	1.81±0.57
1500		24.56±0.014	1.71±0.59	18.6±0.134	1.61±0.01
Control	10	35.5±0.346	4.38±0.26	33.64±0.14	8.13±0.33
500		32.4±0.152	2.390.59	31.21±0.078	3.42±0.59
1000		30.9±0.037	2.25±0.21	25.43±0.142	2.65±0.26
1500		28.6±0.312	1.88±0.59	22.4±0.11	1.02±0.01

Table 3. Means of magnesium (LSD=5.264) and manganese(LSD=ns) concentrations in the calli of two barley genotypes under varying concentrations of Cd (0, 500, 1000 and 1500 µ*M*) along with 10 m*M* TU added in the culture medium

Treatments		Genotypes			
		Jau-83		Haider-91	
Cd (µM)	Thio (mM)	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}
Control	Control (=0)	18.34±0.012	6.58±0.44	16.26±0.025	7.17±1.11
500		18.26±0.026	3.63±0.56	16.07±0.02	2.33±0.24
1000		14.34±0.12	2.54±0.43	15.96±2.15	1.87±0.05
1500		17.92±0.27	1.74±0.39	15.83±2.43	1.023±0.81
Control	10	18.46±0.024	7.58±1.14	16.35±0.45	7.35±0.28
500		18.44±0.056	4.79±0.16	16.17±0.24	3.59±0.43
1000		15.45±0.46	3.12±0.43	15.96±0.21	2.48±0.16
1500		18.06±1.24	2.99±0.23	15.91±0.47	1.07±0.51

Table 4. Means concentration of Cd of callus of two barley genotypes with varying concentrations of Cd (0, 500 and 1500 µ*M*) and (0 and 10 m*M*) TU

Treatments		Genotypes	
Cd (µM)	**Thiourea(mM)**	**Jau-83**	**Haider-91**
Control	Control (=0)	0.0043±0.0274	0.0063±1.234
500		458±2.16	527±1.08
1000		975±2.15	1075±3.12
1500		1067±1.99	1146.6±0.27
Control	10	0.002±0.00012	0.0054±0.00014
500		434.3±2.87	503.6±1.67
1000		869±2.35	978±0.65
1500		1043±1.07	1127±1.64

***CAT and SOD*:** The SOD activity was found to be activated significantly along with the increase in metal concentration (Table 5). A clear trend of increase in SOD content was observed in both genotypes with and without TU application; however, the interaction of genotypes × TU was non-significant. Enhancement in the CAT activity was observed in both barley genotypes when exposed to metal stress. Upon TU application, increase in CAT activity was observed in stressed calli of both genotypes, but contrastingly decreased under control environment. However, at 1500 µM/L Cd toxicity, TU showed no change in CAT activity, may be due to this reason that TU combatsthe metal toxicity upto specific level.

DISCUSSION

The Cd uptake by plant increased proportionally by increasing Cd in soil. Some crops usually accumulate high Cd concentration such as durum wheat, flax and sunflower kernels, whereas corn, spring wheat and barley uptake less Cd (Smolders, 2001). In the present study, the accumulation of Cd in callus of barley was lower at 500 µM/L but higher at 1500 µM/L Cd. However, the Cdcontent of callus in both genotypes under the treatments of Cd+TU showed a greater decline than Cd alone. The present study revealed that the application of TU significantly decreased Cd uptake at cellular level that substantiates the earlier reports (Abdelkader *et al*., 2012; Perveen *et al*., 2011). Jau-83 had less accumulation or content ofCd than that of other genotype i.e., Haider-91 so considered as sensitive genotype. Calcium content in callus of both barley genotypes was decreased in present due to Cd application and more decline, was observed in Haider-91 than Jau-83. Cd-induced reduction of Ca content has been reported in earlier studies (Sandalio *et al*., 2001). Ca deficiency decreased the length, fresh and dry weight, and enhanced the toxicity of Cd in rice seedlings (Cho, 2012). In the present study, callus K content was decreased. Jau-83 exhibited more K content than Haider-91, which might be correlated with a less uptake of Cd in Jau-83 as compared to Haider-91; so the Jau-83 was considered as tolerant genotype. Similar decrease (~50%) was recorded in callus K of maize under 10 µM $CdCl_2$ exposure (Nocito *et al*., 2002), and up to 25% decrease in root and shoot of tomato plant at 100 µM of $CdCl_2$ (Lopez-Millan *et al.,* 2009) and up to 30% in maize (Hussain *et al*., 2012). The addition of TU into culture medium alone and with Cd, ameliorated the toxicity and showed increased content of Ca and Potassium than Cd alone. Abdelkader *et al*. (2012) reported that TU enhances the calcium and potassium uptake under drought stress condition in wheat. Cadmium affected the nutrient homeostasis. In the present study Mg and Mn contents of callus decreased by Cd (Table

Table 5. Means of CAT (LSD=3.779) and SOD (LSD= ns) of two barley genotypes under Cd stress (0, 500 and 1500 µ*M*) and thiourea (0 and 10 mM) added in the culture medium

Treatments		Genotypes			
		Jau-83		Haider-91	
Cd (µM)	Thiourea (mM)	CAT	SOD	CAT	SOD
Control	Control(=0)	0.35±0.04	3.23±0.21	0.34±1.21	3.18±0.35
500		0.367±0.15	3.28±1.13	0.35±0.65	3.28±0.57
1000		0.41±0.15	3.31±0.24	0.39±0.0014	3.29±1.13
1500		0.45±1.21	3.35±0.15	0.42±1.15	3.31±0.87
Control	10	0.38±0.68	3.35±0.87	0.35±0.29	3.26±0.47
500		0.35±1.35	3.31±0.001	0.36±1.26	3.28±1.14
1000		0.39±0.67	3.32±0.06	0.37±1.47	3.29±1.11
1500		0.46±1.14	3.34±1.21	0.43±1.54	3.29±0.16

3) and decline in Mg content was greater in the sensitive genotype (Haider-91) than the tolerant one (Jau-83). Chou *et al.* (2011) reported negative relationship between Cd and Mg uptake and found that roots and shoots of Mg deficient rice plant accumulated more Cd as compared to control. The present finding was justified with previous findings that Mn contents were reduced by Cd in rice (Chou *et al*., 2011), cucumber (Gonçalves *et al*., 2007) and tomato (Lopez-Millan *et al*., 2009; Zorrig *et al*., 2010). It is evident from the findings that Jau-83 had more magnesium and manganese content so considered tolerant than the sensitive Haider-91, which had less content of these micronutrients. TU addition increased the Mg content in callus of both genotypes that is similar to the findings of previous research on wheat under drought stress, TU increases the Mg content (Abdelkader *et al*., 2012). These findings are according to the previous research in barley plant where Cd reduces the Mg uptake (Ciećko *et al.,* 2005).

Exogenous application of Cd induced oxidative stress (Cho *et al.,* 2012). Antioxidant enzymatic system normally operatesin plant however their activity is increased during stress to resist the damage caused by reactive oxygen species produced during abiotic stress (Srivastava *et al*., 2011). Present study also showed an increase in SOD and CAT activities in callus under Cd toxicity. However, at low concentrations of Cd, no significant increase found in their content but higher concentrations of Cd showed significant increase. The present findings are in accordance with the previous research on Cd treated sugarcane callus that reported no change at lower Cd treatment but significant increase in CAT activity at higher concentrations (Fornazier *et al*., 2002). Genotype Jau-83 has more activity of antioxidants than the Haider-91 that is similar to the previous findings that tolerant one has more activity of antioxidants than the sensitive ones.

The precise mechanism of TU action is unclear yet, however, its action started either with the chelation (as TU has sulfur containing thiol group; owing to chelation of phytotoxic heavy metal ions) or storage in vacuole of Cd ions. Thus, mobilization of nutrients, occur that indirectly enhances the biomass and growth, moreover, reduces the oxidative stress.

***Conclusion*:** The present study revealed some novel biological properties of TU in improving growth at cellular level under Cd stress. Genotypic variation exists relating to differential accumulation of Cd in callus tissues.Cd reduced growth and nutrient uptake but exogenous application of TU ameliorated the effects of Cd stress in both barley genotypes by improving relative fresh weight and dry weight as well as reduction in oxidative stress observed. Cd-resistant genotype, Jau-83 performed better than Haider-91 under Cd stress in growth as well as for other attributes which helped plant in tolerating stress, and can be grown in marginally Cd-contaminated soils.

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