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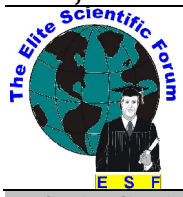

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RESEARCH ARTICLE

Screening of Cadmium Tolerance in Sugarcane Using Antioxidative Enzymes as a Selection Criteria

Syed Hammad Raza*, Fahad Shafiq and Maleeha Tahir
Department of Botany, Government College University, Faisalabad, Pakistan

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***Corresponding Author:**
hamad_shah@yahoo.com

ABSTRACT

Impact of cadmium stress on sugarcane (*Saccharum officinarum* L.) callus was investigated to screen the tolerant and sensitive varieties. Fresh, young and disease free leaves of four sugarcane varieties (HSF-240, HSF-242, CPF-246 and CPF-247) were used as explants to induce callus on MS medium. Callus (25-Day old) was subjected to various cadmium chloride levels (control, 0.1, 0.5 and 1 mM) and data for growth was recorded after 7 and 15 days however enzyme activities were recorded after 10 days, respectively. Results clearly highlighted the detrimental effects of cadmium stress on all the four varieties. Morphologically all the varieties showed red patches, and a significant alteration in fresh and dry weights were recorded after 7^{th} and 15^{th} days of cadmium exposure. HSF-240 showed 11% and 16% increase in fresh and dry weight after 7^{th} and 15^{th} days respectively as compared to other varieties. Furthermore, total protein, CAT and POD activities were positively triggered under Cd stress especially in HSF-240. Na^+ and K^+ concentration varied under cadmium treatment in a way that HSF 240 had 11% more values of K^+ than HSF-242, while HSF-242 showed 11% higher values than other two varieties (CPF-246 and CPF-247). Based on the findings, HSF-240 was Cd-tolerant while HSF-242, CPF-246, and CPF-247 were found susceptible to cadmium.

INTRODUCTION

One of the major problems in focus for agricultural lands around the world is heavy metal pollution and toxicity. Heavy metals alter mineral uptake, seed germination, growth, physiological functioning, and inhibit growth of plants (Epstein and Bloom, 2005). Heavy metals accumulation in soil is due to the use of waste water and the estimated use of waste water is about 20 million hectares in 50 countries (Khan et al., 2011).

Cadmium is a major industrial pollutant associated with heavy road traffic, smelting of zinc, phosphate fertilizers application in agriculture (Grant, 2011). Cadmium induce variations at genetic, physiological and biochemical levels (Azevedo et al., 2005) and generate severe effects on plant systems (Grant and Sheppard, 2008). At elevated concentrations cadmium toxicity resulted in a decline of fresh and dry mass in chickpea, mungbean and soybean (Balestrasse et al., 2001). Not only this, cadmium metal inhibits photosynthesis (Vassilev et al., 2005), degrades chlorophyll, inhibits chlorophyll biosynthesis and reduces carotenoid contents (Pandey et al., 2007).

Furthermore, cadmium stress results in the enhanced production of ROS that damage cellular compartments (Cuypers et al., 2012). Plants are equipped with defensive mechanism comprising up of enzymatic (peroxidases, catalases and superoxide dismutases) and non-enzymatic (ascorbate glutathione, tocopherols) antioxidants, which counteract excessive ROS generated through various biotic and abiotic stresses conditions. Higher activities of antioxidant enzymes aid in oxidative stress tolerance (Hernandez, 2009).

Sugarcane (*Saccharum officinarum* L.) is an important commercial crop in many countries of the globe. The preliminary points of sugarcane production are tropical South Asia and Southeast Asia (Sharpe and Peter, 1998). This tall perennial grass is widely distributed in Malaysia, India, China, Micronesia and Polynesia and its byproducts are very significant economically (Moore and Maretzki, 1996). Modern sugarcane cultivars are very much of importance due to high sucrose concentration with thick stalks, rare flowering and have low fibers (Ming et al., 2001). Recent investigations emphasized that high biomass producing ability of sugarcane can direct its use for phyto-extraction (Yadav

et al., 2011). In addition, like many other crops and vegetables, sugarcane cultivation is exposed to heavy metal hazards worldwide.

In this scenario there is a requirement for selection of tolerant sugarcane genotypes that will perform better under cadmium contaminated environment. New modern biotechnological approaches like tissue culture (Abbas et al., 2012) can be used to screen metal tolerant plant varieties at very early stages based upon their growth, biochemical and antioxidant profiles. Therefore in the present investigation different sugarcane varieties at callus stage were subjected to various cadmium levels in pursue to identify Cd tolerant and sensitive varieties on the basis of growth, morphology, activities of antioxidant enzymes (CAT and POD) and ion uptake.

MATERIALS AND METHODS

Investigation to identify Cd tolerant sugarcane varieties was carried out at Environmental Botany Lab, GC University, Faisalabad. For this purpose, an experiment was conducted using completely randomized design with four approved genotypes of *Saccharum officinarum* L. viz. HSF-240, HSF-242, CPF-246 and CPF-247 under four levels of cadmium stress with three replicates for each experimental unit.

Callus induction, regeneration and shoot multiplication

Callus induction was the first step to obtain experimental plant material which was performed at Biotechnology Department of Ayub Agricultural Research Institute to obtain callus of all the selected sugarcane varieties. Disease free explants from young premature leaves were cultured in test tubes containing MS (Murashige and Skoog, 1962) basal medium supplied with all the necessary ingredients (10 mL of iron, 10 ml vitamin, 30 g sucrose, 2-4, Dichlorophenoxyacetic acid 3 mL, 100 mL macro nutrients, and 1 mL micro nutrients with 1.5 g agar gel, pH 5.7) for 20 days at 16-25°C in incubation room in dark under controlled conditions. This period was followed by light exposure (2500- 3000 lux) for 10 days in growth room.

Callus regeneration media included cocktail of vitamins, iron, sucrose, casein hydrolysate, kinetin, micro nutrients, macro nutrients and agar gel (Sigma-Aldrich). Regeneration was induced depending on the capacity of sugarcane variety. Briefly, iron (10 ml), vitamin (10 ml), sucrose (30 g), casein hydrolysate (0.48 g), macro & micro nutrients (4.30 g), kinetin (5 ml) and gel (1.5 g) was used in 1 L distilled water. Regeneration was induced within 20-25 days with a temperature range from 16-25ºC. After this shoot induction was achieved in the shoot induction media having all the necessary ingredients as mentioned previously. Sub-shoot induction was achieved with the same procedure used for shoot induction.

Cadmium stress treatment

Cadmium stress was applied using cadmium chloride (Sigma-Aldrich) at control, 0.1, 0.5 and 1 mM concentrations respectively in the growing medium during sub-shoot induction phase.

Growth analysis

Callus growth of all the four varieties was studied after 7^{th} and 15^{th} days of stress induction (DSI). Moreover morphological changes in the callus of all the four varieties were also recorded.

Enzyme extraction

Extraction for antioxidant enzymes was carried out after 15 DSI. For this purpose, 0.25 g of fresh callus material was homogenized in 2.5 ml chilled potassium phosphate buffer (100 mM, pH 7.8) under ice cold conditions. After centrifugation (Hemle Labortechnik, Z216 mk, Germany) at 15000 *g* for 20 min at 4°C, the supernatant was separated and kept at -80°C for enzyme analysis.

Determination of antioxidant enzymes

The activity of CAT was monitored at 240 nm as described by Chandlee and Scandalios (1984) with slight modifications (Raza et al., 2007). The activity of POD was determined at 470 nm as described by Chance and Maehly (1955) with the aid of (UV-VIS 4000 Spectrophotometer, Shimadzu, Japan). Catalase and peroxidase activities were expressed as unit mg^{-1} protein. Furthermore total soluble protein was estimated by following the method of Bradford (1976). The OD for total soluble protein was recorded at 595 nm by using (UV-VIS 4000 Spectrophotometer, Shimadzu, Japan) and the protein content of the samples were estimated from a standard curve prepared from BSA.

Ion analysis

For ion analysis, 0.01 g dried callus material was digested using 65% ultra pure nitric acid as described by Netondo et al. (2004). The final volume of the digested samples was made 50 ml with distilled water and the analysis of Na^+ and K^+ ions was carried out by flame photometer (Jenway PFP-7, UK). The amount of Na^+ and K^+ ions was calculated from standard curve and expressed in mg/g of dry weight basis.

Statistical analysis

Data for all the variables was subjected to ANOVA using Duncan's Multiple Range (DMR) test at ($P \leq 0.05$) for growth, ions and ($P \leq 0.01$) for analysis of antioxidant enzymes respectively.

RESULTS

Growth characters

Cadmium contamination in the growing medium significantly ($P \leq 0.05$) retarded callus growth in all sugarcane varieties investigated (Table 1). After 7 and

Table 1: F values from ANOVA along with their probability level for growth characters and ion analysis of callus from all the four sugarcane varieties under cadmium stress

	Fresh weight (g)		Dry weight (g)	Na^+	K^+	Na^+/K^+
	07 days	15 days	(15 days)			Ratio
Control						
HSF-240	0.397^{b}±0.01	0.703^{a}±0.04	0.073^{c}±0.01	14.43^{c}±0.71	94.17^{b}±2.03	0.153^{d}±000
HSF-242	0.443^{a}±0.01	0.510^{bc}±0.01	0.057^{d}±0.01	18.47^{bc}±0.78	35.68^{bc}±2.18	0.520^{c}±0.02
CPF-246	0.320^{b}±0.01	0.607^{b}±0.02	0.033^{e}±0.00	28.36^{b}±0.64	19.99^{d}±2.26	1.458^{a}±0.18
CPF-247	0.310^{b}±0.01	0.527^{bc}±0.02	0.137^{b}±0.01	33.83^{a}±0.78	21.90^{d}±0.86	1.552^{a}±0.09
0.1 mM Cadmium chloride level						
HSF-240	0.407^{a}±0.01	0.803^{a}±0.02	0.217^{a}±0.02	22.80^{a}±0.88	82.29^{b}±1.52	0.277^{d}±0.00
HSF-242	0.283^{c}±0.03	0.283^{d}±0.01	0.050^{d}±0.01	23.13^{a}±1.29	96.13^{b}±1.01	0.241^{d}±0.01
CPF-246	0.310^{b}±0.01	0.700^{ab}±0.01	0.153^{b}±0.01	19.95^{bc}±1.34	24.11^{d}±1.60	0.833^{bc}±0.06
CPF-247	0.257^{c}±0.01	0.333^{d}±0.01	0.087^{cd}±0.01	18.35^{bc}±0.75	19.82^{d}±0.19	0.926^{bc}±0.03
0.5 mM Cadmium chloride level						
HSF-240	0.403^{a}±0.02	0.413^{cd}±0.02	0.127^{b}±0.01	20.60^{bc}±1.34	74.79^{ab}±2.08	0.276^{bc}±0.01
HSF-242	0.387^{b}±0.00	0.380^{d}±0.01	0.093^{bc}±0.01	20.03^{bc}±1.33	129.03^{a}±3.34	0.155^{c}±0.00
CPF-246	0.310^{b}±0.02	0.473^{c}±0.029	0.080^{bc}±0.01	20.27^{bc}±1.41	21.02^{d}±1.60	0.966^{ab}±0.02
CPF-247	0.247^{c}±0.01	0.393^{cd}±0.01	0.043^{de}±0.00	22.14^{bc}±1.25	22.50^{d}±1.60	0.993^{ab}±0.08
1 mM Cadmium chloride level						
HSF-240	0.457^{a}±0.00	0.320^{cd}±0.01	0.110^{b}±0.01	23.73^{ab}±1.39	105.1^{b}±2.51	0.225^{d}±0.00
HSF-242	0.307^{b}±0.01	0.411^{c}±0.02	0.057^{d}±0.00	26.94^{ab}±0.31	138.38^{a}±4.50	0.195^{d}±0.00
CPF-246	0.310^{b}±0.01	0.223^{d}±0.01	0.063^{d}±0.01	16.96^{c}±0.98	19.75^{d}±1.79	0.867^{bc}±0.05
CPF-247	0.153^{c}±0.01	0.233^{d}±0.01	0.069^{d}±0.00	23.93^{ab}±1.81	17.67^{d}±1.64	1.377^{b}±0.15
ANOVA						
Var	11.85***	130.99***	15.44***	6.56***	115.09***	19.79***
Cd	68.14***	62.21***	21.17***	9.86***	1589.35***	197.53***
Var × Cd	8.88***	35.40***	12.35***	23.53***	131.62***	6.63***

*** Significant, ns non- significant, Var varieties, Cd levels of cadmium stress

15 days of Cd exposure to callus, maximum growth characters were recorded in control (without cadmium) while lowest values for fresh and dry weights were recorded in 1 mM $CdCl_2$. Sugarcane callus treated with 1 mM exhibited 42.1 and 33.9% reduction in fresh weight after 07 and 15 days while the decline was 51.1% in dry weight after 15 days cadmium (1 mM $CdCl_2$) exposure ($P \leq 0.05$). Above all HSF-240 was found significantly superior in terms of growth among the four varieties investigated. In contrast, CPF-247 was susceptible as it showed 19.0% and 95% reduction in its fresh weight after both successive harvests ($P \leq 0.05$).

Ion analysis

Elevated Na^+ concentrations were found in calli treated with 1 mM $CdCl_2$ (16.9% more values than control). Among varieties investigated, HSF-240 exhibited higher values of Na^+ but least accumulation of K^+ (Table 1). Overall, HSF-240 had 22.7, 30.5 and 38.8% less values for potassium ions than HSF-242, CPF-246 and CPF-247 respectively ($P \leq 0.05$). Increasing cadmium concentrations resulted in reduction of K^+ uptake by sugarcane varieties. Highest K^+ uptake was found in 0.1 mM $CdCl_2$ treated level while control calli had significant 77.1% more values than extreme level of Cd stress. As a result Na^+/K^+ ratio shifted among the four varieties as well as among Cd treated levels. Overall HSF-240 variety exhibited greater of this ratio (Table-1).

Antioxidant enzyme activities

Catalase activity varied significantly ($P \leq 0.01$) among the varieties due to cadmium contamination in the growth medium. Presence of low cadmium concentration (0.1 mM $CdCl_2$) in the growth medium increased the activity of CAT enzyme in almost all the varieties compared with control (Fig 1-a). However, high Cd concentrations had an inhibitory effect on CAT activity. Nonetheless, HSF-240 was the only variety in which CAT activity increased linearly with the magnitude of cadmium contamination. Peroxidase activity in the HSF-240 variety exhibited similarity to CAT showing a linear increase (Fig 1-b). To some extent, HSF-242 and CPF-247 exhibited similar trend in POD enzyme activity, however CPF-247 did not exhibit prominent POD activity. Therefore variations in total soluble protein concentration were evident in all the varieties as well as in response to stress (Fig 1-c).

DISCUSSION

Present study focused on sugarcane response/magnitude of antioxidative defense system in order to identify

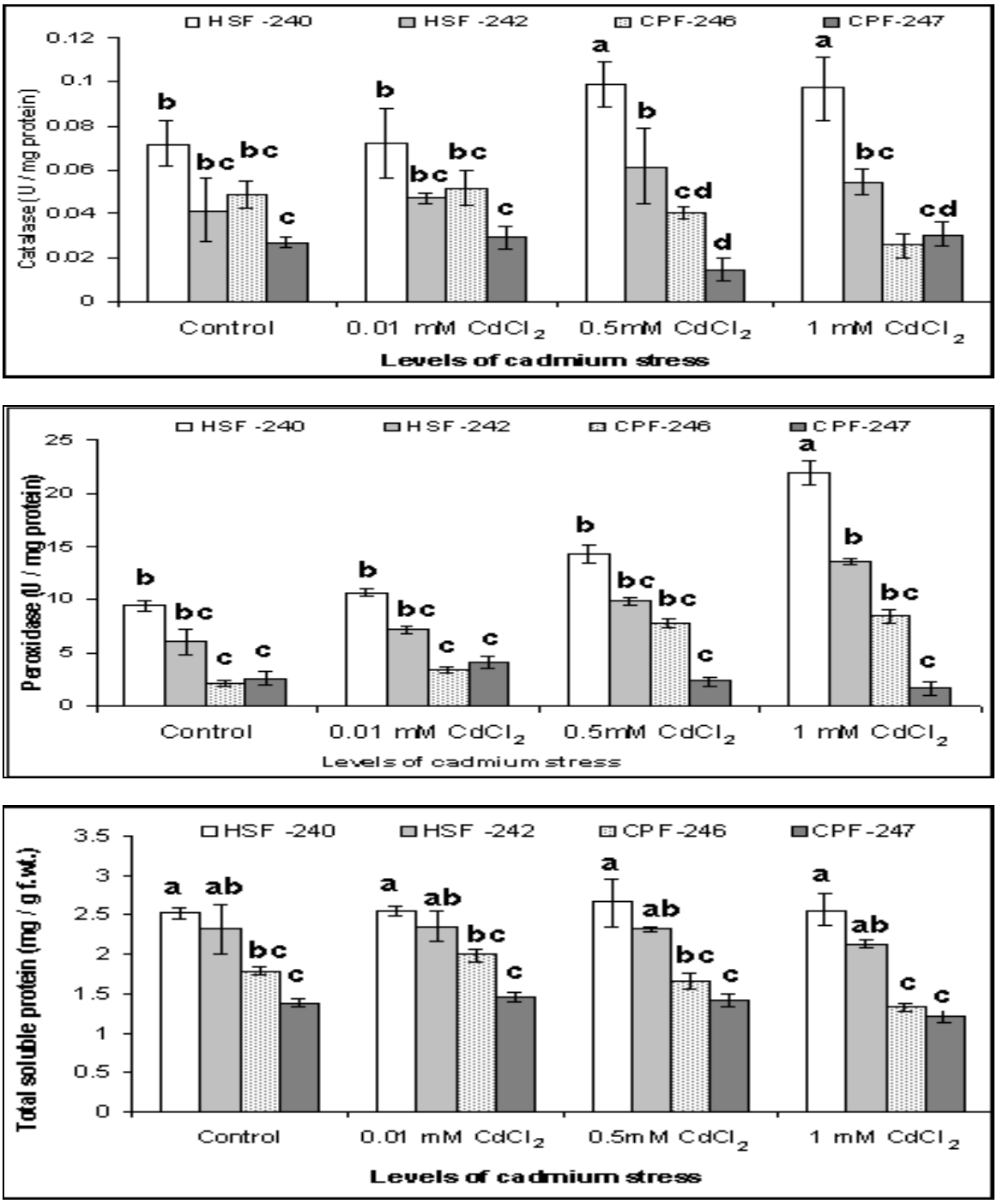

Fig. 1 a-c: Antioxidant enzyme activities of callus from all the four sugarcane varieties under various regimes of cadmium stress

better variety under cadmium stress. In the present study, a significant decrease in fresh and dry weight of callus was recorded with reddish brown patches and necrotic spots under Cd toxicity. Similar to our findings, Cd stress induced through $CdCl_2$ in pea plant resulted in reddish brown patches and discoloration of leaves (Sandalio et al., 2001). Toxicity generated by cadmium can lead to altered physiological processes in plant species. This castration in metabolism can therefore induce the formation of reactive oxygen species (Schützendübel and Polle, 2002). Such variations in metabolism cause changes in the growth of

plants species. In addition, elevated cadmium levels leads to senescence and chlorosis in plants. Cadmium imparts inhibitory effect on growth of sugarcane. Our results showed maximum discoloration and reddish brown patches in callus at 1 mM cadmium. CPF-247 showed more sensitivity to Cd stress as red brown patches appeared earlier in this variety. These results mimic with those reported by Foranzier et al. (2002). In our findings, growth inhibition was detected at 1 mM level of cadmium stress. Lower levels of cadmium did not impart such inhibitory effects as observed in 0.01 mM and 0.5 mM cadmium chloride levels. In fact, low concentration of cadmium cause increase in growth rate depending on variety.

In the present study catalase activity increased in HSF-240, while this did not happen for other three varieties as high Cd concentration rather inhibited catalase activity in the other three varieties (HSF-242, CPF-246 and CPF-257). In contrast an increase in POD activity was recorded in response to Cd toxicity. The increase in CAT and POD activities in HSF-240 increased due to oxidative stress match with the earlier findings of Foranzier et al. (2002). Inhibited CAT activity in callus of *Leucaena leucocephalla* was reported under chromium, nickel and zinc toxicity (Rout et al., 1990). Antioxidant response and total soluble protein varied among varieties.

Overall uptake of sodium ions increased while that of potassium was curtailed due to cadmium stress. Especially in response to elevated cadmium levels, HSF-240 exhibited significantly higher (2-3 folds) increase in uptake of Na^+ ions while K^+ uptake was significantly reduced. Similar decline in potassium ions is reported by Fardous et al. (2010) and Kurtyka et al. (2007). In contrast in HSF-242, K^+ uptake increased due to cadmium inclining levels.

Improved fresh and dry weight in HSF-240 despite of high Na^+ and low K^+ accumulation in the presence of cadmium is attributed to the role of antioxidant enzymatic defense (CAT and POD) that was simultaneously up-regulated in this variety. On the other hand, improvement in potassium status of HSF-242 under Cd toxicity did not contribute significantly to its growth and visual characters that also signify the role of antioxidative defense. In addition, sensitivity of the other two remaining varieties can be correlated with these findings.

Conclusions

Growth characters of all the sugarcane varieties investigated were found susceptible to cadmium stress particularly at high concentrations however it varied among varieties. From the results it was concluded that CAT and POD activity was enhanced due to Cd stress (0.1 mM, 0.5 mM and 1 mM) respectively. HSF-240 showed more CAT and POD activity than CPF-246 and CPF-247 that contributed toward cadmium tolerance in this variety. The results also showed that HSF-240 and HSF-242 were more tolerant as compared to CPF-246 and CPF-247. In addition, potassium uptake solely does not contributed to cadmium stress tolerance while the magnitude of antioxidative enzyme activities played a significant role in this scenario.

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