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Full Length Article

In vitro Study of Interactive Effect of Cadmium and Salicylic Acid on Growth and Biochemical Parameters in Tetra and Hexaploid Wheat

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Abstract

In vitro cultures of plants offer a useful tool to study the adaptive mechanisms of plants in the presence of high metal concentrations. Callus cultures of tetraploid (Durum-97) and hexaploid (Shafaq-06) wheat genotypes exposed to different cadmium (Cd) concentrations (0, 100, 400, 800 and 1200 µM) and its alleviation by salicylic acid (0, 0.5 mM) were assayed for growth, antioxidant responses and Cd accumulation characters. Cadmium hampered the growth and notably enhanced antioxidant activity in all Cd regimes. We provided evidence that the two genotypes hexaploid and tetraploid are characterized by different response to cellular homeostasis and detoxification to Cd in callus culture. In particular, the high tolerance in hexploid genotype (Shafaq-06) is associated with relatively higher activity of antioxidants and Cd accumulation behaviour as indicated by Cd contents, bioconcentration factor (BCF) and stress tolerance index (STI). However, the follow-up treatment (0.5 mM) with salicylic acid (SA) mitigated the Cd generated stress and significantly ($P \leq 0.05$) improved the aforesaid parameters.

Keywords: Antioxidant; Cadmium; Teraploid; Hexaploid; Salicylic acid

Introduction

Unprecedented bioaccumulation of heavy metals in the environment has become a dilemma for all the living organisms including plants. This phytotoxcity is problem of paramount importance for ecological, nutritional and environmental reasons and has serious implications in agriculture as it has acquired new dimensions with the advent of the industrial era (Nagajyoti *et al*., 2010; Iqbal *et al*., 2015). Cadmium is a highly toxic metal which has no known biological function (Chen *et al*., 2007; 2014). Moreover, beside its primary effects, it may also provoke secondary effects that in turn may activate in convergence (Prasad and Hagemeyer, 1999), such as photosynthesis and respiration decay or interference with mineral nutrition (Jiang *et al*., 2004; Dong *et al*., 2006) and numerous serious morphological, metabolic, and physiological anomalies.

Cadmium is a non-redox metal and unable to participate in Fenton-type reactions but causes oxidative stress via overproduction of reactive oxygen species (ROS), disturbing the equilibrium between prooxidant and antioxidant homeostasis (Garnier *et al*., 2006) and increasing lipid peroxidation. This condition implicates the causation of multiple deteriorative disorders such as, oxidation of protein and lipids, ion leakage, oxidative DNA attack, redox imbalance, and denature of cell structure and membrane, ultimately resulting in the activation of programmed cell death (PCD) pathways (Rascio and Navari-Izzo, 2011; Sharma *et al*., 2012). Plants are equipped with a repertoire of mechanisms to counteract heavy metal toxicity and in response, activation of antioxidant defense mechanism is pursued (Emamverdian *et al*., 2015). The enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) are involved in the detoxification of O_2 and H_2O_2, thereby preventing the formation of OH radicals (Gill and Tuteja, 2010).

Alleviation of Cd toxicity by rebalancing the impaired prooxidant and antioxidant ratio through supplementation has gained a wide interest. Owing to considerable evidence of the injurious effects of Cd on plants, it was hypothesized that SA can assuage the toxic effects of Cd on wheat. Salicylic acid is considered as endogenous phytohormone of phenolic nature that can modulate plant responses to a wide range of abiotic or oxidative stresses or acts as an endogenous regulator that potentially affects the growth and productivity (Sakhabutdinova *et al*., 2003).

Tissue culture techniques offer valuable tool for studying, testing, improving and selecting plant material for tolerance to stress factors and cellular stress response (Azevedo *et al*., 2005; Shekhawat *et al*., 2010; Ahmad *et al*., 2013). This evaluation of the behaviour of *in vitro* cell cultures exposed to Cd toxicity may contribute to the knowledge that these cells may used in genetic improvement programs to withstand Cd toxicity.

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Tetraploid *Triticum durum* Desf. is generally considered the hardiest of all wheats and has been regarded as an ancestral relation to modern day hexaploid bread wheat (Elias and Manthey, 2005). It seems that the amount of work done to study the effects of Cd toxicity on wheat callus tissue has not been significant. So the present study was focused to investigate different aspects related to the genotypic variations in hexaploid and tetraploid wheat calli under Cd toxicity. Meanwhile, for an in-depth understanding of the harmfulness of heavy metal as well as the mechanisms for protection and tolerance provided by SA which have been shown to vary in intensity in wheat genotypes at ploidy level.

Materials and Methods

Tetraploid (Durum-97) and hexaploid (Shafaq-06) wheat genotypes were provided by Nuclear Institute of Agriculture and Biology (NIAB) Faisalabad, Pakistan. For the induction of callus sterilized grains were cultured aseptically in test tubes containing 10 mL of MS medium (Murashige and Skoog, 1962) (solidified with agar) supplemented with (5 mg L^{-1}) 2, 4-dichlorophenoxy acetic acid (2, 4-D). Incubation temperature was maintained at 25ºC with white florescent light. To study the effects of Cd toxicity alone and Cd along with SA (control, 100, 400, 800 and 1200 µM) levels of Cd (as $CdCl_2$) and two levels (control, 0.5 mM) of salicylic acid ($C_7H_6O_3$) were applied to one month old calli. Callus tissues were cultured in 100 mL Erlenmeyer (Pyrex) flasks, containing 40 mL liquid MS medium, with the same concentration of 2, 4-D as above mentioned and corresponding treatments 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.

Callus Fresh Weight/Relative Growth Rate (RGR-fresh) (g)

Fresh weight of calli was determined on analytical balance and relative fresh weight of callus growth was calculated as:

$$\ln(\text{Final fresh weight}) - \ln(\text{Initial fresh weight})$$

Where ln denotes the natural logarithm.

Callus Dry Weight (g)

After determination of fresh weight, calli were placed in oven at 65ºC for a week and the dry weight was determined.

Callus Bioconcentration Factor (BCF)

The callus BCF was calculated as follows:

$$\text{BCF} = \frac{\text{Cd accumulation in callus (mg/kg)}}{\text{Cd concentration in the medium (mg/kg)}}$$

Callus Stress Tolerance Index (STI)

The callus STI was calculated as follows:

$$\text{STI} = \frac{\text{Average dry weight of callus (treated)}}{\text{Average dry weight of callus (control)}}$$

Determination of Cd Contents

The dried ground material (0.5 g) of calli was digested in concentrated HNO_3 (5 mL) at 100 in digestion tubes and volume of the extract was maintained up to 50 mL. Filtered extract was used for estimation of Cd contents with atomic absorption spectrophotometer.

Antioxidant Enzyme Activity Assay

Fresh samples of calli (0.5 g) were homogenized in 100 mM phosphate buffer (pH 7) containing 0.1 mM EDTA and the supernatant was used for the following antioxidant enzymes activity assay.

Superoxide Dismutase (SOD)

Superoxide dismutase activity was appraised following Giannopolitis and Ries (1977) with minor modifications. The photoreduction was measured by recording an increase in absorbance at 560 nm using UV-visible spectrophotometer (IRMECO U2020). One unit of SOD activity reflected the amount required to cause 50% inhibition of the NBT at 560 nm.

Catalase (CAT) and Peroxidase (POD)

The activities of CAT and POD enzymes were determined according to Chance and Maehly (1955) with minor modifications. For CAT, changes in absorbance of the solution every 20s were monitored at 240 nm and were expressed as µmol of H_2O_2 decomposed per min per mg of protein. Whilst for POD, changes in absorbance of the reaction solution were recorded at 470 nm after every 20 seconds. One unit of POD activity was equivalent to change in absorbance per mg of protein.

Malanodialdehyde (MDA) and Hydrogen Peroxide (H_2O_2)

The level of lipid peroxidation in terms of concentration of thiobarbituric acid reactive substance (TBARS) was determined following the method of Yagi (1982). The absorbance was read at 532 nm. The absorption coefficient was calculated at 155 mmol cm^{-1} and expressed as nmol/MDA/g fresh weight.

$$\text{MDA level (nmol)} = (\text{A532nm} - \text{A600NM})/1.56\text{x}10^{5}$$

Hydrogen peroxide (H_2O_2) contents were determined according to Velikova *et al*. (2000) and absorbance was recorded at 390 nm.

Statistical Analysis

An analysis of variance (ANOVA) was used to analyse the data for all parameters using a computer software COSTAT (Cohort Software Berkeley, California). The least significant differences (LSD) between means were calculated using Duncan's New Multiple Range test ($P \leq 0.05$).

Results

The growth of calli grew and proliferated with the course of time on Cd treatments (control, 100, 400, 800 and 1200 µM) were compared and revealed significant ($P<0.05$) reductions in the calli relative growth rate (RGR) and dry weight exhibiting that Cd in culture medium inhibited the callus growth as compared to control (Fig. 1). Compared to the control RGR, reduction of 60% and 56% at 1200 µM Cd was observed in Durum-97 and Shafaq-06, respectively and greater extent of suppression was recorded in Durum-97 as compared to Shafaq-06.

Cadmium accumulation by the callus of two wheat genotypes significantly ($P<0.05$) accelerated by the imposition of Cd stress in the medium (Table 1). However, Durum-97 accumulated more Cd contents as compared to Shafaq-06. On the other hand SA application with the elevating Cd stress declined the callus Cd accumulation in both genotypes. To assess the capability of calli to grow in the presence of a given concentration of Cd, the stress tolerance index (STI) was calculated. Plants with STI greater than 0.6 are considered tolerant (Lux *et al*., 2004). As presented in Table 2 Shafaq-06 showed a good tolerance to exposed Cd up to 800 µM and showed values equal/higher than 0.6. On the other hand, Durum-97 showed a lower tolerance index from 400 µM ($P<0.05$). Upon application of SA, both genotypes showed a significant extent of improvement in STI to Cd toxicity.

The evaluation of the ability of plants to concentrate Cd from external solutions in their tissues is determined by bioconcentration factor (BCF). In Fig. 1, calli of both the genotypes showed higher values at 100 µM whilst showing a decreasing trend along with the increasing Cd concentration in the medium and differences recoded were significant ($P<0.05$). Lower values of BCF of Shafaq-06 calli were recorded as compared to Durum-97. By the application of SA values of BCF showed suppression by the calli of both genotypes.

Malanodialdehyde (MDA) is an index of lipid peroxidation. Treatments and genotypes differed significantly ($P<0.05$) for this parameter. Its level in Durum-97 and Shafaq-06 increased by 146% and 127%, respectively at 1200 µM in comparison with control and SA treatments efficiently reduced the stimulatory effect of Cd stress on status of lipid peroxidation (Fig. 3).

Superoxide dismutase (SOD) activity revealed a significant increase in both the genotypes. However, in Durum-97 SOD activity peaked at 400 µM whilst in Shafaq-06 it peaked at 800 µM. Declined SOD activity at 1200 µM was still higher than the control. The increase in SOD activity induced by the excessive heavy metal was alleviated by SA application. The POD activity increased gradually with Cd concentrations, but changes were not always significant and its activity elevated to 54% and 50% in Durum-97 and Shafaq-06, respectively under Cd toxicity. Salicylic acid induced a declining effect on enzyme activity upon Cd exposure. Catalase (CAT) is an important enzyme to eliminate H_2O_2 and O^{-2}. The CAT activity showed an enhancement under Cd stress in SA free calli in both the genotypes (Fig. 2). The activity was significantly ($P \leq 0.05$) higher at the elevated concentrations of Cd and peaked at highest concentrations of Cd. However, minimal activity of CAT was observed in SA treated calli upon heavy metal exposure.

Fig. 3 reveals significant ($P \leq 0.05$) enhancement in H_2O_2 contents that is manifestation of oxidative stress under SA free conditions, H_2O_2 contents increased by 210% in Durum-97 and 206% in Shafaq-06 at 1200 µM, which under SA treatment decreased to about 127% and 134%, respectively.

Discussion

In vitro study conducted to appraise the Cd accumulation turned out a cascade of events. Inhibition of growth exhibited by both genotypes showed differential genotypic responses suggesting that Durum-97 had higher callus Cd accumulation that caused inhibition in RGR and dry weight and resulted in callus growth inhibition. Similar results have been demonstrated in the calli of sunflower (Azevedo *et al*., 2005), *Sesbania drummondii* (Israr *et al*., 2006) and safflower (Namjooyan *et al*., 2012). Callus STI decreased with the elevating Cd concentrations in the culture medium. Though the accumulation pattern was very similar among genotypes, yet Shafaq-06 exhibited high STI as compared to the other. The superior ability of Shafaq-06 to better cope with Cd toxicity as compared to Durum-97 was elicited at the highest Cd level. At the highest Cd level, lower BCF value of Shafaq-06 might be ascribed to the maintenance ability of metal influx and efflux matrix whereas marked callus growth reduction in Durum-97 attributed to cell membrane damage. Differential genotypic behaviour under Cd stress had been reported in *Populus nigra* clones (Iori *et al*., 2012).

Cytotoxic ROS production is inevitable consequence of Cd-induced oxidative stress. Reactive oxygen species (ROS) accumulation as a result of various environmental stresses is a major cause of loss crop productivity worldwide (Mittler, 2002). It has been well established that SOD, POD activity, H_2O_2 and MDA contents are important parameters for estimating what extent plants suffer from adverse stress (Wu *et al*., 2003). Cadmium stress enhanced MDA and H_2O_2 accumulation in calli suggesting that this increase

Table 1: Mean values of H_2O_2, CAT, MDA, SOD and POD with Cd alone as well as Cd in combination with salicylic acid of tetraploid (Durum-97) and hexaploid (Shafaq-06) calli with standard errors

		Cadmium+					Cadmium+Salicylic acid (0.5 mM)				
Treatment Cadmium	Genotypes	H_2O_2	CAT	MDA	SOD	POD	H_2O_2	CAT	MDA	SOD	POD
Control	Durum-97	0.484±0.01m	21.66±0.3i	37.92±0.8k	28.27±0.9j	15.73±0.7g	0.409±0.09o	19.64±0.3l	41.01±0.8j	32.66±1.3j	16.82±0.3h
	Shafaq-06	0.438±0.01n	23.95±0.4i	35.33±0.1l	26.70±1.0k	17.53±0.3gh	0.375±0.01p	23.49±0.3i	39.49±0.4jk	30.15±0.9ij	17.91±0.4gh
Cd 100	Durum-97	0.968±0.04e	28.25±0.4f	58.83±1.0g	41.85±1.5de	17.69±0.4gh	0.728±0.03k	20.66±0.6kl	50.95±0.3h	30.23±0.5i	17.85±0.8gh
µM	Shafaq-06	0.916±0.02h	26.22±0.1h	50.48±1.1h	45.65±1.3c	19.89±0.3ef	0.675±0.02l	21.65±1.2jk	44.17±0.6i	35.52±0.4g	18.85±1.3g
Cd 400	Durum-97	1.01±0.01gh	31.64±0.8cd	74.02±0.8c	50.50±0.2b	24.82±1.2ef	0.841±0.03i	24.78±1.0i	52.24±1.16g	39.83±0.4e	19.89±0.6f
µM	Shafaq-06	1.18±0.03d	32..61±0.3c	69.74±1.0e	48.92±0.8b	23.79±0.9e	0.795±0.01j	26.19±0.3gh	62.36±0.3f	41.81±0.5de	20.69±0.7ef
Cd 800	Durum-97	1.22±0.04c	32.48±0.3c	80.88±0.2b	44.76±1.5c	29.45±0.5bd	0.909±0.05g	27.93±0.3b	72.82±0.9d	39.99±1.0ef	25.91±0.5d
µM	Shafaq-06	1.31±0.03b	27.80±0.6b	62.09±1.0e	55.07±1.4a	31.92±0.3b	0.892±0.02h	29.84±1.0ef	59.89±0.3g	42.56±0.8d	27.63±0.2cd
Cd1200	Durum-97	1.39±0.02a	37.07±0.1a	93.51±2.6a	48.49±1.2fg	30.26±0.3b	0.998±0.04h	29.25±0.3f	80.25±0.5b	28.32±0.5jk	29.85±0.7b
µM	Shafaq-06	1.28±0.02b	38.30±0.6a	80.23±0.4b	39.08±0.6ef	34.80±0.6a	1.019±0.06f	31.63±0.9cd	69.15±0.7e	38.43±0.4h	30.13±0.3b

Table 2: Mean values of Cd contents, RGR, Dry weight, BCF and STI with Cd alone as well as Cd in combination with salicylic acid of tetraploid (Durum-97) and hexaploid (Shafaq-06) calli with standard errors. ND: Not detectable

		Cadmium+					Cadmium+Salicylic acid (0.5 mM)				
Treatment Cadmium	Genotypes	Cd	RGR callus	Dry weight callus	BCF	STI	Cd	RGR callus	Dry weight callus	BCF	STI
Control	Durum-97	ND	0.50 ± 0.01b	0.274±0.008c	--	--	ND	0.490±0.001b	0.23±0.05a	--	--
	Shafaq-06		0.547 ±0.02a	0.276±0.04c				0.50±0.01b	0.269±0.01b		
Cd 100	Durum-97	138.66±0.8l	0.462±0.003	0.232±0.004f	1.34±0.003a	0.795±0.005	120.29±0.5m	0.521±0.01d	0.261±0.02d	1.16±0.004b	0.835±0.02b
µM	Shafaq-06	143.50 ±0.8k	g0.504±0.01e	0.246±0.05e	1.1±0.004b	b0.854±0.02d	109.35 ±0.3n	0.53±0.04d	0.271±0.006cd	0.9±0.003c	0.914±0.01a
Cd 400	Durum-97	203.99±0.4h	0.313 ±0.01i	0.129±0.01g	0.78±0.04d	0.662±0.01f	165.31±0.3j	0.422±0.006g	0.233±0.01f	0.69±0.02d	0.719±0.01e
µM	Shafaq-06	190.01±0.5i	0.372±0.007h	k 0.193±.02g	0.71±0.005de	0.703±0.008e	171.77±1.2j	0.416±0.01g	0.229±0.024f	0.65±0.07e	0.873±0.09c
Cd 800	Durum-97	253.32±0.5e	0.289±0.006	0.104±0.003j	0.73±0.01de	0.517±0.02h	219.66±0.2g	0.335±0.02i	0.174±0.04h	0.62±0.004f	0.607±0.01g
µM	Shafaq-06	232.45±0.5f	k0.324±0.003j	0.147±.002i	0.69±0.02ef	0.604±0.02g	205.67±0.5h	0.344±0.02i	0.188±0.009g	0.54±0.008g	0.614±0.02g
Cd1200	Durum-97	336.98±1.8a	0.203±0.01o	0.034±0.009	0.64±0.05fg	0.314±0.02k	295.99±0.3c	0.206±0.005	0.096±0.003l	0.56±0.003g	0.346±0.02j
µM	Shafaq-06	320.98±0.8b	0.238±0.003n	m .081±.01l	0.57±0.008gh	0.389±0.08j	291.07±0.3d	m0.293±0.01l	0.115±0.003k	0.44±0.001i	k0.459±0.04i

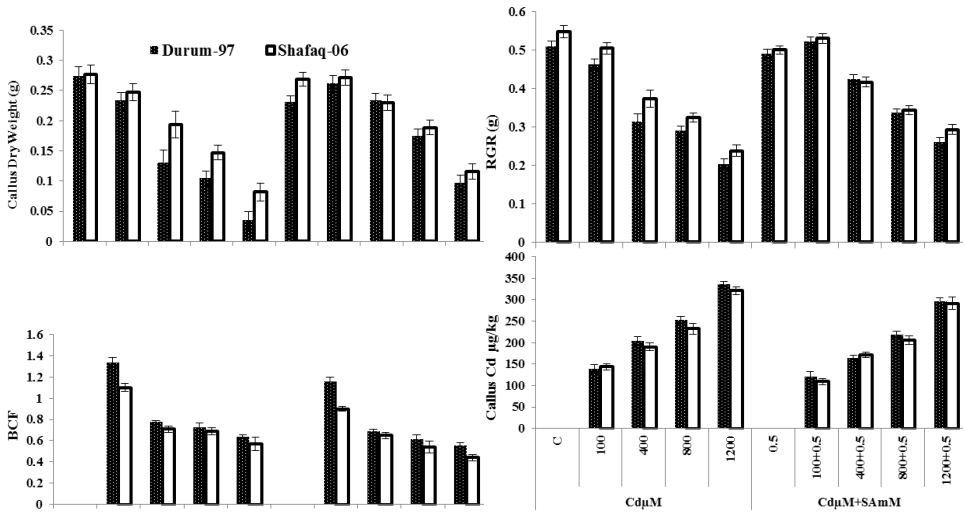

Fig. 1: Effect of increasing concentrations of Cd and combined effect of Cd and SA on calli (a) dry weight (b) RGR (c) Bioconcentration factor. (d) Cd contents ($P \leq 0.05$)

results in oxidative stress that in turns hampered the calli growth due to greater damage to cell membrane system. A marked increase in MDA status in Durum-86 as compared to other genotype might be attributed to the greater Cd induced injury in membranes and production of more ROS. On the contrary, Shafaq-06 showed low MDA contents signifying that reduced MDA concentration was due to efficient antioxidant enzyme activity (SOD, CAT, POD), that decreased H_2O_2 levels and conferred membrane stability. Similar findings have been reported previously in tomato (Shalata *et al*., 2001) and maize (Neto *et al*., 2006). However, the pragmatic approach of exogenous application of SA might induced the Cd tolerance and resulted in lesser MDA contents that could be attributed to the modifications in compartmentalization and modulation of redox balance through antioxidant activities. The decline in SOD activity at 1200 µM might indicate its impaired functionality under higher concentrations of Cd as previously reported in

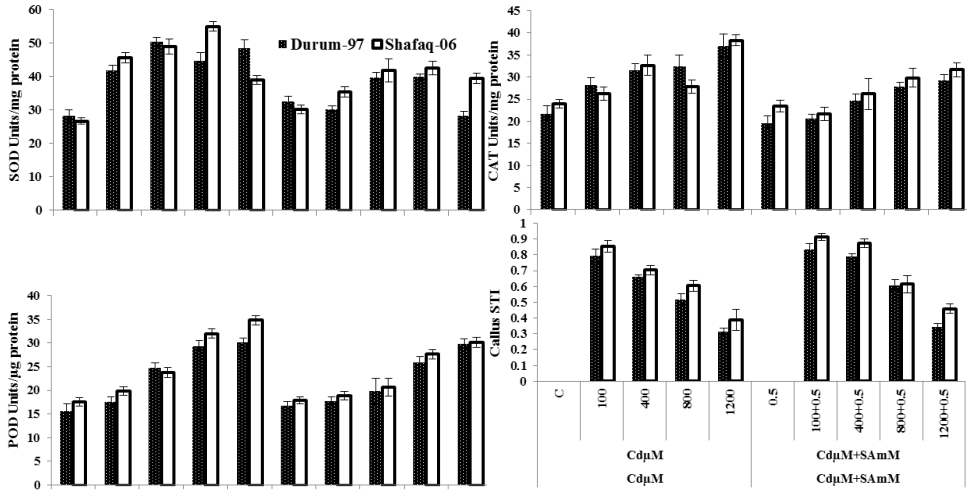

Fig. 2: Effect of increasing concentrations of Cd and combined effect of Cd and SA on calli (a) SOD (b) CAT (c) POD (d) STI. (P≤0.05)

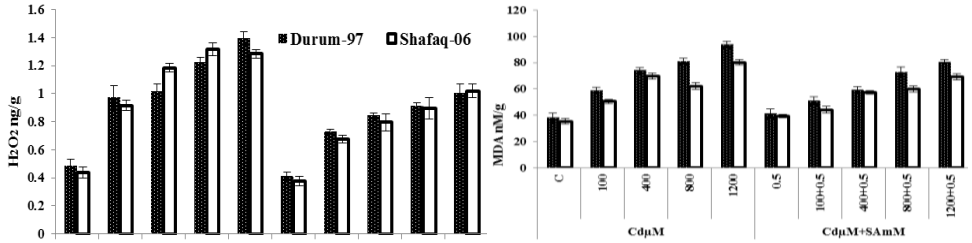

Fig. 3: Effect of increasing concentrations of Cd and combined effect of Cd and SA on callus (a) H_2O_2 (b) MDA. (P≤0.05)

Alyssum (Schickler and Caspi, 1999). The activity of SOD observed to be greater in Shafaq-06 as compared to Durum-97 suggesting that the enhanced response provided a better protection against ROS (Takemura *et al.,* 2000; Zhang *et al*., 2005). It may also suggest on the ground of the results in the present study that Cd induced a reduction in the activity of SOD at higher levels. Thus this reduction might be accompanied by the accumulation of H_2O_2 in different compartments which may cause the inactivation of this enzyme (Vitoria *et al.,* 2001). Similar trends in the SOD activity under Cd stress had been observed in *Sesbania drummondi* callus (Israr *et al*., 2006), *B. juncea* callus (Shekhawat *et al*., 2010) and the adapted calli of sunflower and safflower (Gallego *et al*., 2002; Namjooyan *et al*., 2012).

Catalase (CAT) is another integral component of antioxidant system and its role in protecting the organism from damage caused by ROS is indispensable. It protects SOD against inactivation caused by the higher accumulation of H_2O_2 (Willekens *et al*., 1994; Fridovich, 1995). The progressed CAT activity with the increasing Cd levels in the culture medium had been observed in agreement with that of *Leucaena leucocephalla* callus (Rout *et al*., 1999) and *Trema orientalis* callus (Samantaray *et al*., 1999).

Peroxidase (POD) is one of the principle enzymes that play key role in the elimination of ROS. Increased activity of POD is now assigned as a biomarker of heavy metal stress; moreover, lignin biosynthesis takes place with the contribution of POD activity that builds up a physical barrier against lethal heavy metal toxicity (Hegedus *et al*., 2001). Salicylic acid application with the elevating Cd stress depressed the activities of CAT and POD revealing that this may be the manifestation of the beneficial effect of SA during earlier growth period, which prevented cumulative damage development response to Cd. Moreover, SA may activate Cd tolerance mechanisms different from Cd distribution and antioxidant defense. One mechanism may be the avoidance of damage and includes any mechanisms of Cd binding resulting in lowered plasmatic free Cd.

Conclusion

The studies led to conclusion that the positive SA effect on Cd-challenged calli of tetra and hexaploid wheat could have many hypothetical explanations. On the basis of antioxidant profile and STI, BCF, MDA levels, we can safely conclude that hexalopid wheat (Shafaq-06) performs better upon exposure to heavy metal stress and SA prevented cumulative damage development in response to endogenous Cd accumulation. This suggests that SA could be used as a potential growth regulator to improve the plant growth under Cd stress.

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