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## Profiling of Anti-Oxidative Enzymes and Lipid Peroxidation in Leaves of Salt Tolerant and Salt Sensitive Maize Hybrids under NaCl and Cd Stress

(Pemprofilan Enzim Anti-Oksida dan Pengoksidaan Lipid dalam Daun Jagung Hibrid Toleransi Garam dan Sensitif Garam di bawah Tekanan NaCl dan Cd)

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### ABSTRACT

*Effects of NaCl salinity and cadmium on the anti-oxidative activity of enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and lipid peroxidation contents; malondialdehyde (MDA) were studied in two maize hybrids of different salt tolerance characteristics. An increase in the amount of lipid peroxidation indicated the oxidative stress induced by NaCl and Cd. The results also depicted that NaCl stress caused an increase in the activities of POD, SOD, CAT, APX and GR while cadmium stress increased the activities of POD, SOD and APX but showed no significant effect on CAT and GR in both the studied hybrids. The combined effect of salinity and cadmium on these parameters was higher than that of sole effect of either NaCl or Cd. It was also found that maize hybrid 26204 had better tolerance against both stresses with strong antioxidant system as compared to that of maize hybrid 8441. A comparison of the antioxidants and lipid peroxidation in two maize hybrids having varying level of NaCl and Cd stress tolerance corroborated the importance of reactive oxygen species (ROS) in defense against abiotic stresses.*

*Keywords: Antioxidant enzymes; cadmium; maize hybrid (Zea mays L.); NaCl; salinity*

### ABSTRAK

*Kesan kemasinan NaCl dan kadmium terhadap aktiviti anti-oksida enzim seperti superoksida dismutase (SOD), katalase (CAT), peroksidase (POD), peroksidase askorbate (APX), glutation reduktase (GR) dan kandungan lipid peroksidaan; malondialdehid (MDA) telah dikaji dalam dua ciri berbeza toleransi garam jagung hibrid. Peningkatan dalam jumlah pengoksidaan lipid menunjukkan tekanan oksida aruhan NaCl dan Cd. Keputusan juga menunjukkan bahawa tekanan NaCl menyebabkan peningkatan dalam aktiviti POD, SOD, CAT, APX dan GR manakala tekanan kadmium meningkatkan aktiviti POD, SOD dan APX tetapi tiada kesan ketara ditunjukkan ke atas CAT dan GR dalam kedua-dua hibrid yang dikaji. Kesan gabungan kemasinan dan kadmium ke atas parameter ini adalah lebih tinggi berbanding dengan kesan tunggal sama ada NaCl atau Cd. Juga didapati bahawa jagung hibrid 26204 mempunyai toleransi yang lebih baik berbanding dengan kedua-dua tekanan dengan sistem antioksidan yang kuat berbanding dengan jagung hibrid 8441. Perbandingan antioksidan dan pengoksidaan lipid dalam dua jagung hibrid yang mempunyai pelbagai tahap toleransi tekanan NaCl dan Cd menyokong kepentingan spesies oksigen reaktif (ROS) dalam pertahanan terhadap tekanan abiotik.*

*Kata kunci: Enzim antioksidan; jagung hibrid (Zea mays L.); kadmium; kemasinan; NaCl*

### INTRODUCTION

Amongst major abiotic stresses, salinity affects crop productivity about one quarter to one third in all agricultural lands across the globe. Increased saline water irrigation and increased use of uncultivable soils to meet the needs of the increasing global population causes more severe salinity problems (Munns 2002). A number of metabolic changes occurred in plants in response to salt stress. Osmotic stress and ion toxicity are the most illustrious along with generation of reactive oxygen species (ROS) that disturb the uptake and translocation of nutritional ions in the plant body (Misra & Dwivedi 2004; Mittler 2002). These kinds of dreadful effects can distract the physiology and biochemical functions occurring in

the plant cell, leading to the death of the plants (Xiong & Zhu 2002).

Cadmium (Cd) is usually considered as one of the highly toxic trace elements that mainly enter the environment from industrial waste and as a phosphate fertilizer contaminant (Kranterev et al. 2008). It alters the lipid composition and/or lipid peroxidation by causing membrane damage and disruption of the electron transport chain. For these disorders, induction of oxidative stress is the possible mechanism implicated (Kranterev et al. 2008; Smeets et al. 2005). Muhling and Lauchli (2003) observed that the effect of salinity becomes even more severe when plants were exposed to Cd stress; and it has been well-documented that dual menace (NaCl and Cd stress)

causes higher cell membrane permeability and enhanced the generation of oxygen radicals and  $H_2O_2$  in plants.

Salinity and heavy metal stresses induce oxidative stress to plants (Hernandez & Alamansa 2002; Schutzendubel et al. 2001) which leads to production of reactive oxygen species (ROS) in the cytoplasm, mitochondria and peroxisomes. These ROS can interrupt normal metabolism by causing damage to proteins, lipids and nucleic acids (Foyer & Noctor 2005; Turkan & Demiral 2009). ROS mainly comprises of single oxygen ( $O_2$ ) superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH^\cdot$ ). The balance between formation of ROS and their removal by the anti-oxidative scavenging systems determines the degree of damage to these molecules (Asada 1994).

In order to protect themselves against stress, plants have evolved complex antioxidant defense system includes enzymatic viz; SOD, APX, GR, CAT and non-enzymatic viz; ascorbate, glutathione and flavonoids (Hossain et al. 2007; Turkan & Demiral 2009). Superoxide dismutase (SOD) is one of the primary scavengers which catalyzes the dis-mutation of superoxide into  $H_2O_2$  and oxygen (Parida & Das 2005). Reaction of  $H_2O_2$  with various cellular targets, induces damage to DNA and proteins in addition to causing lipid peroxidation. By the activity of peroxidase and catalase, this  $H_2O_2$  was removed (Hossain et al. 2007). Ascorbate peroxidase (APX) by using the reducing power of ascorbate is the most important peroxidase, catalyzing the reduction of  $H_2O_2$  to water (Noctor & Foyer 1998). Glutathione reductase (GR) plays a critical role in catalyzing the final and rate-limiting reaction step of the Halliwell-Asada enzymatic pathway (Bray et al. 2000). Malondialdehyde (MDA) content is considered the general indicator of lipid peroxidation (Meloni et al. 2003; Wang & Zhou 2006).

In abiotic stress conditions, the response of maize anti-oxidative system has been studied under anoxia (Yan et al. 1996), low temperature (Iannelli et al. 1999), drought (Aroca et al. 2003) and aluminum toxicity (Boscolo et al. 2003). However, studies related to the maize anti-oxidative systems towards cadmium and salinity stress conditions are limited. Thus, the present study was conducted to evaluate the effects of cadmium and NaCl stress on lipid peroxidation and the activity of anti-oxidative enzymes in leaves of maize hybrids having different salt tolerance, which will not only help in cloning of new genes involved in salinity and tolerance of heavy metals and the development of transgenic and better breeding strategy, but also help in the development of accurate screening techniques eventually facilitating crop improvement in problematic soils.

## MATERIALS AND METHODS

### SEEDLING GROWTH AND TREATMENTS

The experiment was conducted at Zhejiang University, Hangzhou, China. Seeds of two maize hybrids 26204 and

8441 (salt tolerant and salt sensitive, based on previous screening studies) were germinated in moist quartz sand in a greenhouse. At two leaves stage (12 days old) healthy seedlings of uniform growth were specified and transplanted into 30 L tubs covered with a foamed plastic plate having uniform spaced holes. The composition of the basic nutrient media was (g/L): 48.2  $(NH_4)_2SO_4$ , 65.9  $MgSO_4$ , 15.9  $K_2SO_4$ , 18.5  $KNO_3$ , 59.9  $Ca(NO_3)_2$ , 24.8  $KH_2PO_4$ , 5 Fe citrate, 0.9  $MnCl_2 \cdot 4H_2O$ , 0.11  $ZnSO_4 \cdot 7H_2O$ , 0.04  $CuSO_4 \cdot 5H_2O$ , 2.9  $H_2BO_3$ , 0.01  $H_2MoO_4$  at  $pH 6.0 \pm 0.5$ . After two weeks of transplanting, Cd and NaCl stresses were generated by adding Cd and NaCl to the relative tubs in order to form the following four treatments i.e. control; 5  $\mu mol/L$  Cd; 100  $mmol/L$  NaCl; and 5  $\mu mol/L$  Cd+100  $mmol/L$  NaCl. The experiment was laid out in completely randomized design having three replications. The pH of each tub was maintained ( $pH 6.0 \pm 0.5$ ) on a daily basis using HCl or NaOH. The nutrient media in the pots was continuously aerated using electric aeration pumps and media changed at 5 days interval.

### ASSAY OF ANTIOXIDANT ENZYMES

For enzymatic analysis, the second fully expanded leaves of plant samples were collected after 15 days of stress. The 0.5 g fresh weight of leaves was homogenized in a pre-chilled mortar under ice-cold conditions in 5 mL of 50 mM cold sodium phosphate buffer ( $pH 7.8$ ). After centrifuging at  $13000 \times g$  for 30 min, the supernatants were collected and stored at  $4^\circ C$  for the measurements of various antioxidant enzymes.

By nitroblue tetrazolium (NBT) method, the activity of superoxide dismutase (SOD) was determined (Beauchamp & Fridovich 1971) by measuring photo reduction at 560 nm. The reaction mixture (3 mL) contained 50 mM sodium phosphate buffer ( $pH 7.8$ ), 75  $\mu M$  NBT, 13 mM methionine, 10  $\mu M$  EDTA, 2 mM riboflavin and enzyme extract (100  $\mu L$ ). Tubes were then placed under illumination of two 15 fluorescent lamps and reaction allowed for 10 min and stopped by switching off the light. Illuminated and non-illuminated reactions served as calibration standards without supernatant. Under experimental conditions, one unit of SOD was defined as the quantity of enzyme that produces 50% inhibition of NBT reduction.

According to the method of Putter (1974), guaiacol peroxidase (POD) activity was measured with some modification. The reaction mixture (3 mL) consisted of 100  $\mu L$  guaiacol, 100  $\mu L$  enzyme extract, 100  $\mu L$   $H_2O_2$  (300 mM) and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA ( $pH 7.0$ ). Increase in absorbance at 470 nm ( $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) by oxidation of guaiacol was measured. The activity of catalase (CAT) was determined according to Aebi (1984). The assay mixture (3.0 mL) consisted of 100  $\mu L$   $H_2O_2$  (300 mM), 100  $\mu L$  enzyme extract and 2.8 mL 50 mM phosphate buffer with 2 mM EDTA ( $pH 7.0$ ). By monitoring the decrease in the absorbance at 240 nm as a consequence of  $H_2O_2$  disappearance ( $\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ) the CAT activity was assayed.

Ascorbate peroxide (APX) activity was assayed following Nakano and Asada (1981). The reaction mixture consisted of 100  $\mu\text{L}$  ascorbate (7.5 mM), 100  $\mu\text{L}$  enzyme extract, 100  $\mu\text{L}$   $\text{H}_2\text{O}_2$  (300 mM) and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH7.0). By the change in absorbance at 290 nm ( $\epsilon=2.8 \text{ mMcm}^{-1}$ ) the oxidation of ascorbate was determined.

A method described by Garcia-Limones et al. (2002) was used to measure the activity of glutathione reductase (GR). The reaction mixture consisted of 100  $\mu\text{L}$  NADPH (2.4 mM), 100  $\mu\text{L}$  enzyme extract, 100  $\mu\text{L}$  oxidized glutathione (GSSG, 10 mM) and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH7.0). By its absorbance change at 340 nm ( $\epsilon=6.2 \text{ mMcm}^{-1}$ ) the oxidation of NADPH was determined.

#### LIPID PEROXIDATION

The lipid peroxidation level in plant tissues, expressed as malondialdehyde (MDA) was determined according to Hodges et al. (1999). Fresh samples (0.5 g) were homogenized in 4.0 mL of 1% trichloroacetic acid (TCA) solution and centrifuged at  $10000\times g$  for 10 min. The supernatant was added to 1 mL 0.5% (w/v) TBA made in 20% TCA. Mixture was heated for 30 min in boiling water and subsequently tubes are placed in an ice bath in order to stop the reaction. The samples were centrifuged at  $10000\times g$  for 10 min and absorbance of the supernatant read at 532 nm. Subtraction of absorbance at 600 nm was carried out in order to correct for non-specific turbidity. The lipid peroxidation level was expressed in terms of  $\text{nmol g}^{-1}$  fresh weight by using a molar extinction coefficient of  $0.155 \text{ mM cm}^{-1}$ .

#### STATISTICAL ANALYSIS

In this study, all reported values were means of three replicates. A two-way analysis of variance (ANOVA) was

performed using a statistical package, SPSS version 16.0 (SPSS, Chicago, IL). Standard Errors were used to determine the significant difference between different treatments tested.

#### RESULTS

In both maize hybrids, significant increase in SOD activity was observed under sole stress of Cd and NaCl when compared with control (Figure 1). In comparison with control and Cd treatment alone, the combined stress (NaCl+Cd) had a significantly higher SOD activity, but in both maize hybrids no significant difference was found between the combined stress (Cd+NaCl) and the NaCl treatment alone. However, hybrid 26204 showed a rapid increase of SOD activity as compared hybrid 8441 in NaCl alone and Cd+NaCl treatments.

NaCl and Cd stresses caused remarkable increase of POD activity in both maize hybrids (Figure 2). Higher POD activity was found by the combined stress (NaCl+Cd) than that of NaCl and Cd stress alone. In response to changes in POD activity, there was a diverse difference among both the maize hybrids to NaCl and Cd stresses. When plants were exposed to NaCl or Cd stress, salt tolerant maize hybrid 26204 showed more rapid increase in POD activity. Comparatively, maize hybrid 8441 considered as being sensitive to salinity had a rapid increase to Cd stress but show slower increase in POD activity when subjected to NaCl stress.

The activity of CAT in both maize hybrids is shown in Figure 3. The combined stress (NaCl+Cd) and NaCl treatment alone caused remarkable increase of CAT activity in both maize hybrids relative to control, but no significance difference was found between control and Cd treatment alone in both maize hybrids.

Cd and NaCl stresses caused faster increase of APX activity in hybrid 26204, but no significant improvement

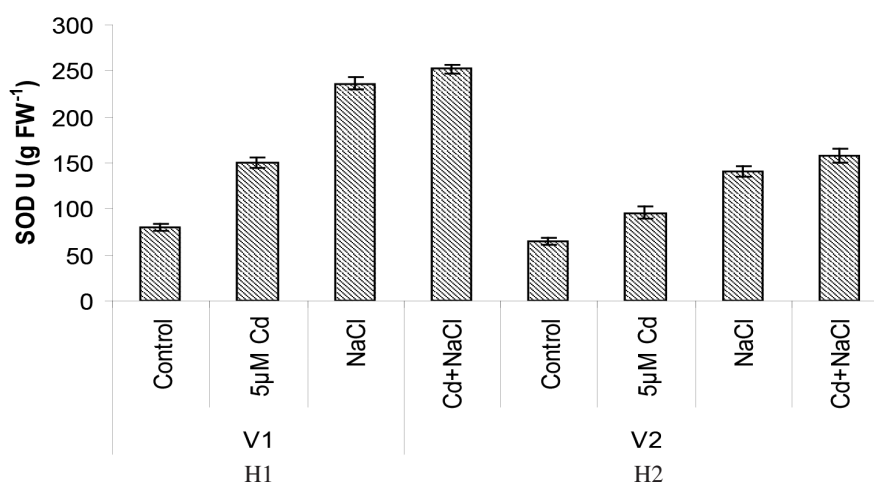


FIGURE 1. Effect of Cd and NaCl stress on SOD activity in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441). (Each bar is an average of 3 replicates $\pm$ S.E)

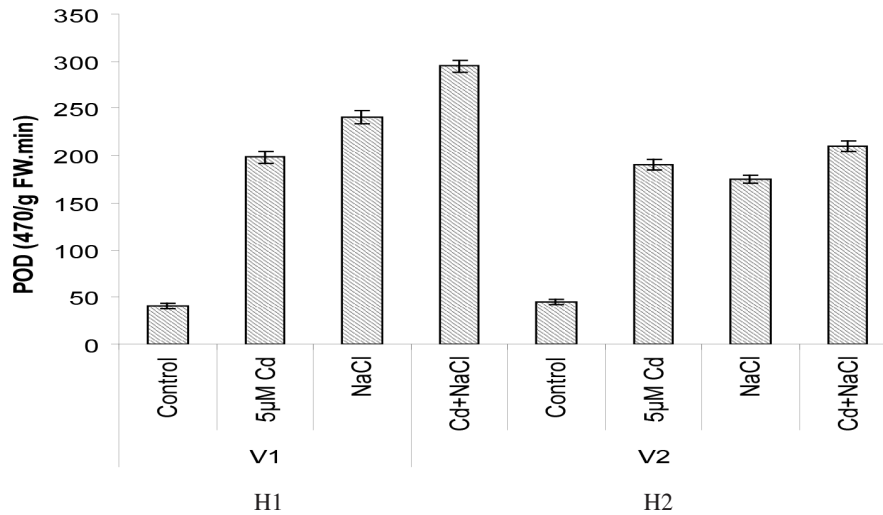


FIGURE 2. Effect of Cd and NaCl stress on POD activity in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441) (Each bar is an average of 3 replicates±S.E)

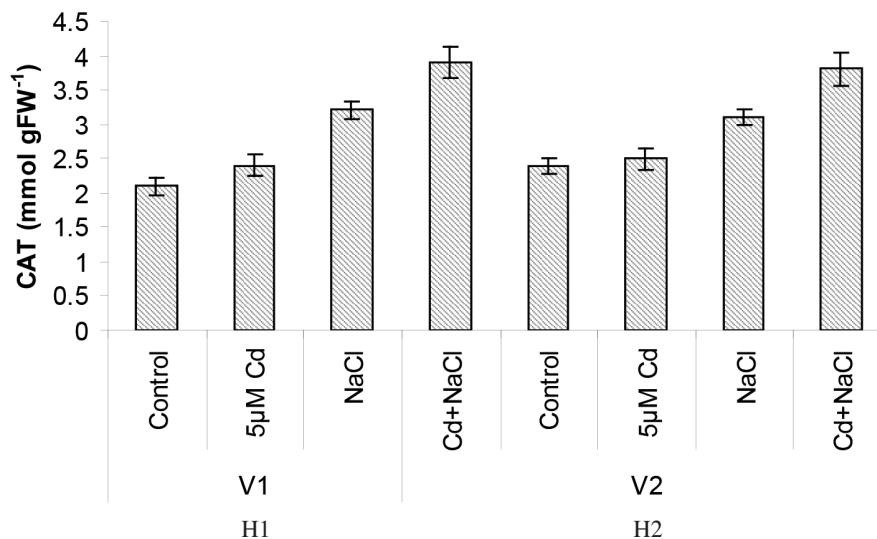


FIGURE 3. Effect of Cd and NaCl stress on CAT activity in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441) (Each bar is an average of 3 replicates±S.E)

was observed in hybrid 8441 as compared to control (Figure 5). Hybrid 26204 showed significantly higher APX activity than all other treatments. Under combined stress (NaCl+Cd), hybrid 8441 also displayed significantly higher APX activity than control, but no remarkable difference was found between combined stress and Cd or NaCl treatment alone.

GR activity in leaves of both maize hybrids is shown in Figure 6. In hybrid 26204, combined stress (NaCl+Cd) and NaCl treatment alone led to significant increase of GR activity relative to control but no significance difference was found between control and Cd treatment alone. In hybrid 8441, combined stress (NaCl+Cd) led to significant increase of GR activity relative to control but no remarkable difference was found between control and Cd or NaCl alone.

#### LIPID PEROXIDATION

In both maize hybrids, lipid peroxidation measured as MDA content, is given in Figure 4. In hybrid 26204 relative to control, combined stress (NaCl+Cd) caused significant increase of MDA contents whereas sole application of NaCl and Cd had no remarkable effect on MDA content as compared with control. In hybrid 8441, MDA content was significantly improved by all treatments.

#### DISCUSSION

Plants when exposed to biotic and abiotic stress exhibit, oxidative stress due to imbalances in the production of reactive oxygen species (ROS) and antioxidant defense at cellular level leading to severe theatrical physiological



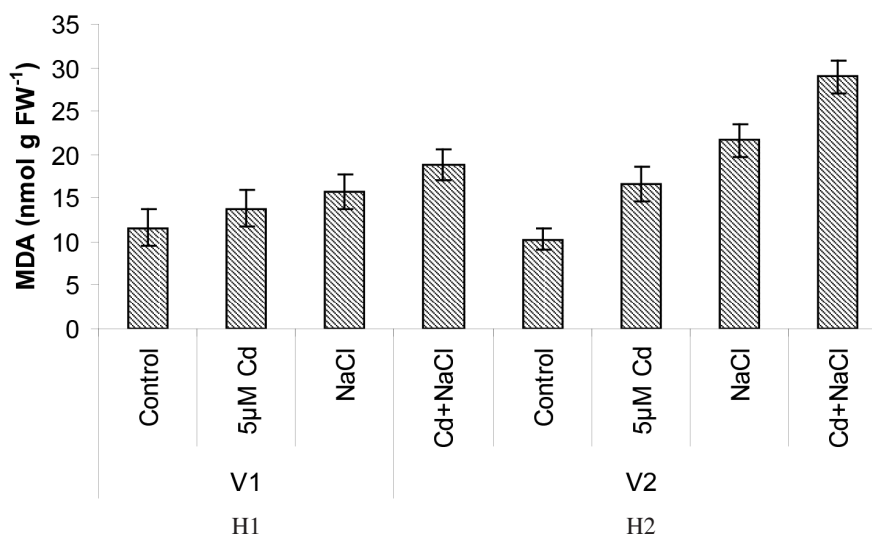


FIGURE 4. Effect of Cd and NaCl stress on MDA content in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441) (Each bar is an average of 3 replicates $\pm$ S.E)

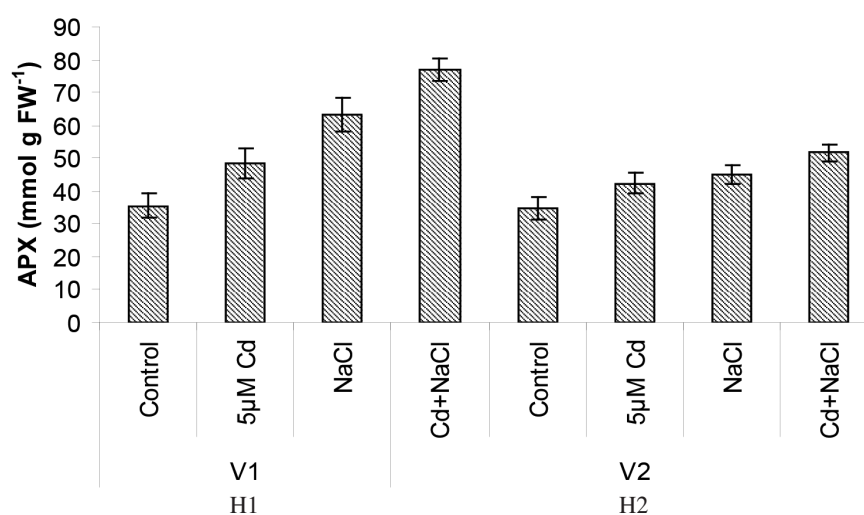


FIGURE 5. Effect of Cd and NaCl stress on APX activity in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441) (Each bar is an average of 3 replicates $\pm$ S.E)

challenges (Foyer & Noctor 2000). The production of ROS viewed as a cellular indicator of stress and secondary messengers is involved in different aspects of plant biology from expression of genes and translation to enzyme chemistry (Foyer & Noctor 2003). Plants make use of a complex antioxidant system to overcome the effects of induced salinity and heavy metals oxidative stress. In tolerant genotypes, higher activities of ROS-scavenging enzymes have been reported as compared to susceptible ones, suggesting that antioxidant system plays an important role in enhancing plant tolerance against acute stress conditions against environmental stress. Thus, genotypes respond differently to various stresses as a result of variations in their antioxidant systems (Mohammadkhani & Heidari 2007; Nawaz & Ashraf

2007). Under stress conditions, anti-oxidative enzymes protect the cell structures against ROS (Reddy et al. 2004). In the present study, the responses of SOD, POD, CAT, APX, GR enzymes activities and MDA content suggest that in maize plants oxidative stress is an important component of stress condition.

SOD is one of the major enzymes which play a key role in the mechanism of cellular defense against ROS. The relative amounts of  $O_2^-$  and  $H_2O_2$  modulated by its activity, are highly reactive and can cause severe damage to membrane, DNA and protein (Bowler et al. 1992). In the present study, Cd and NaCl stress in leaves of both maize hybrids increased SOD activity. However, in hybrid 26204, the increase in SOD activity was more prominent (salt-tolerant) relative to hybrid 8441 (salt-sensitive), implying

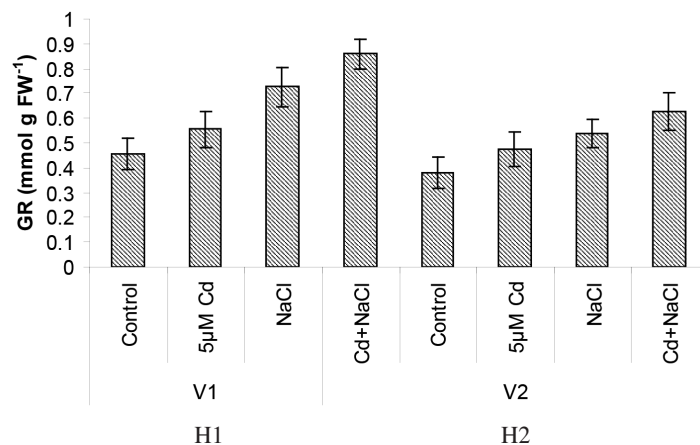


FIGURE 6. Effect of Cd and NaCl stress on GR activity in leaves of H1 (salt tolerant maize hybrid: 26204) and H2 (salt sensitive maize hybrid: 8441) (Each bar is an average of 3 replicates $\pm$ S.E)

that the salt-tolerant maize genotype has a better  $O_2^-$  radical scavenging ability. The increase in SOD activity has been reported previously for certain species of plants under salt stress (Hernandez et al. 2000; Shalata et al. 2001) or Cd stress (Qadir et al. 2004; Vitoria et al. 2001).

$H_2O_2$  is the product of SOD activity, which is toxic and in subsequent reactions must be eliminated by conversion to  $H_2O$ . In plants, intracellular levels of  $H_2O_2$  are regulated by a number of enzymes. Of these, CAT, POD and APX are considered the most important. Our data showed that in leaves of both maize hybrids, POD and APX activities were significantly high under sole application of Cd and NaCl as well as in Cd+NaCl treatment relative to control. It is important to note that Cd or salt-induced SOD activity in leaves of hybrid 26204 was accompanied by a greater increase in POD and APX activities compared to hybrid 8441, thus indicating that the  $H_2O_2$  scavenging mechanism was less effective in the latter hybrid. Thus, findings from the present study suggest that under salt stress POD and APX activities, coordinated with SOD activity, play major protective role in the  $O_2^-$  and  $H_2O_2$  scavenging process (Badawi et al. 2004; Liang et al. 2003) as also reported for barley plants under Cd stress (Wu et al. 2003).

Our results showed that CAT activity was significantly greater in leaves of both maize hybrids under NaCl alone and combine stress (NaCl+Cd) treatments than control plants. Cd stress resulted in no significant increase in CAT activities in both hybrids, similar to those reported by Hegedus et al. (2001). On the other hand, Shah et al. (2001) observed significant decline of CAT activity in wheat when exposed to Cd stress. Possible variations among species, stress intensity, growth stage and duration of exposure may be partially attributed to variations in CAT activity due to Cd stress. Differential responses of CAT activities due to Cd stress may show different targets of oxidative damage. Salt stress increased the activities of CAT in both maize hybrids, showing that accumulation of ROS might occur in response to salt stress. These findings are similar to that previously reported by Foyer and Noctor (2005).

In this study, Cd stress did not show significant increase of GR activity in maize hybrid 26204 but significant improvement was observed with NaCl alone and combined stress (NaCl+Cd) in hybrid 26204 when compared with that of control plants. On the other hand, sole application of NaCl and Cd have no effect on GR activity in hybrid 8441 and only combined stress (NaCl+Cd) significantly improved GR activity than control. Due to Cd stress the variation in GR activity may be attributed in part to possible variations among genotypes, stress intensity, growth stage and exposure duration. Previously the increase in SOD activity has been reported for various plant species under salt stress. Our results are in consensus with previously reported ones in which Cd stress in pea plants, did not show any major change in GR activities (Dixit et al. 2001; Sandalio et al. 2001). Under salt stress, the activities of GR increased, suggesting that the salt tolerance character is associated with increased GR activity in maize hybrid 26204 (Meloni et al. 2003; Sudhakar et al. 2001).

Lipid peroxidation caused by salinity, has usually been used as a sign of salt-induced oxidative damage in membranes (Hernandez & Almansa 2002). In the present study, we noted that MDA content was unaffected by Cd and salinity in maize hybrid 26204 leaves but increased significantly in leaves of hybrid 8441. In hybrid 26204, the lower level of lipid peroxidation suggested that tolerant plants were more protected from oxidative damage under Cd or salinity stress, and these results were in line with those of other researchers correlating lipid peroxidation to anti-oxidative system activity (Hernandez & Almansa 2002; Lin & Kao 2000; Shalata & Tal 1998). These researchers suggested that due to increase in anti-oxidative of enzyme activities, reduction in MDA content was observed which decreases the  $H_2O_2$  levels and membrane injury. In the present study the MDA concentration reduction in maize hybrid 26204 appears to be related to an increase in SOD activity as well as POD and APX activities, all of which facilitate reduction in  $H_2O_2$  concentration and therefore lipid peroxidation in leaves of this maize hybrid.

## CONCLUSION

Variations of antioxidant enzyme activities in response to salinity/ Cd stresses showed that maize hybrid 26204 showed tolerance to NaCl/Cd stress with better scavenging capacity of ROS. Activity of antioxidant enzymes especially POD, SOD and APX may help protect these plants oxidative damages. In the case of maize hybrid 8441, NaCl/Cd treatments perhaps caused more oxidative damage, indicating a less efficient protective mechanism. In addition, in maize hybrids lipid peroxidation level in leaves may be another important biochemical trait for salinity/ heavy metal tolerance.

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